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

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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

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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

Chapter 1

Introduction and Literature Review

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

This thesis concerns the mechanism of androgen receptor regulation of *TRPM8* gene transcription. TRPM8 expression is elevated in the early stages of prostate cancer, decreased in the advanced stages of the prostate cancer and not detectable in the hormone refractory prostate cancer. This indicates that TRPM8 may play a role in the early progression of prostate cancer and itself may be a potential diagnostic marker for the progression of prostate cancer. In this study, putative androgen-responsive elements on the promoter of *TRPM8* have been investigated. In this introduction, the current knowledge on the following topics has been explored: prostate cancer, androgen receptor signalling pathways and prostate cancer, androgen-responsive element, experimental approaches for the search for the androgen-responsive element, TRPM8, TRPM8 and prostate cancer and androgen receptor regulation of TRPM8 expression. A hypothesis of the mechanism of androgen receptor regulation of *TRPM8* transcription has been proposed and the aims of the study in this thesis have been outlined.

1.2 Prostate cancer

1.2.1 Incidence and mortality of prostate cancer

Prostate cancer is now the second leading cause of male death in the developed countries, such as in Australia, New Zealand, North America and North West Europe (Chong and Rashid 2005;William K. Oh 2003). In Australia, there were 13,526 men diagnosed with prostate cancer in 2003 and an estimated 18,700 new cases in 2006. Deaths caused by prostate cancer have been increasing through the years: there were 2,837 in 2003, 2,761 in 2004 and 2,946 in 2005 (Registries 2007).

1.2.2 Risk factors for prostate cancer

The risk factors for prostate cancer include age, genetic inheritance, life style and nutritional factors. Aging can be defined as a progressive non-reversible decline of the human body's resistance to stress, damage and disease (Minelli et al. 2009). In this regard, aging can be deemed as a significant risk factor for prostate cancer (Abate-Shen and Shen 2000;Minelli et al. 2009;Tuohimaa 2009). Although prostatic intraepithelial neoplasia (PIN, refer to section 1.2.3.1 for definition) can be detected in men in their early twenties, clinically detectable prostate cancer is not normally significant until the seventh decade (Abate-Shen and Shen 2000). The underlying mechanism may have involved various age-related changes, such as age-related

decrease in the ratio of androgens to estrogens and age-related reduction of telomere length (Abate-Shen and Shen 2000;De Marzo et al. 2003;Gonzalgo and Isaacs 2003). It is relatively common that morphological changes related to prostate cancer initiation, such as precancerous lesions (up to 1 in 3 men), appears in early life. However, the progression to prostate cancer in later life as a consequence of these early morphological changes is more limited in the population (up to 1 in 9 men) (Abate-Shen and Shen 2000).

It is estimated that genetic predisposition accounts for 43% of early-onset ($\leq$ 55 years old) prostate cancer and is partially related to more than 40% of prostate cancer cases (Campbell et al. 2007;Carter et al. 1992;Gonzalgo and Isaacs 2003). However, the known eight candidate prostate cancer susceptibility genes, RNase L/HPC1, ELAC2/HPC2, SR-A/MSR1, BRCA2, CHEK2, OGG1, MIC1 and PON1, have only accounted for a small proportion of prostate cancer cases (Campbell et al. 2007). It is possible that the known susceptibility gene number will increase.

Among lifestyles, diet has played a very significant role in the difference in the incidence of prostate cancer between men in western countries and Asia. Several food components, such as lycopenes from tomatoes, selenium and vitamin E, have been found to significantly reduce the risk of prostate cancer (Damber and Aus 2008). Some evidence shows that vitamin D also has a protective effect against

prostate cancer. Three out of fourteen studies show a positive correlation between total fat intake and prostate cancer risk (Bianchini et al. 2002).

1.2.3 Pathology of prostate cancer

1.2.3.1 Prostatic intraepithelial neoplasia

Prostatic intraepithelial neoplasia (PIN) (Fig 1.1) consists of benign prostatic ducts lined by cytologically atypical cells (Abate-Shen and Shen 2000;Campbell et al. 2007). PIN is classified into low grade PIN and high grade PIN. There is evidence that high-grade PIN may be a premalignant condition (Epstein and Herawi 2006;Prange et al. 2001;Zlotta and Schulman 1999).

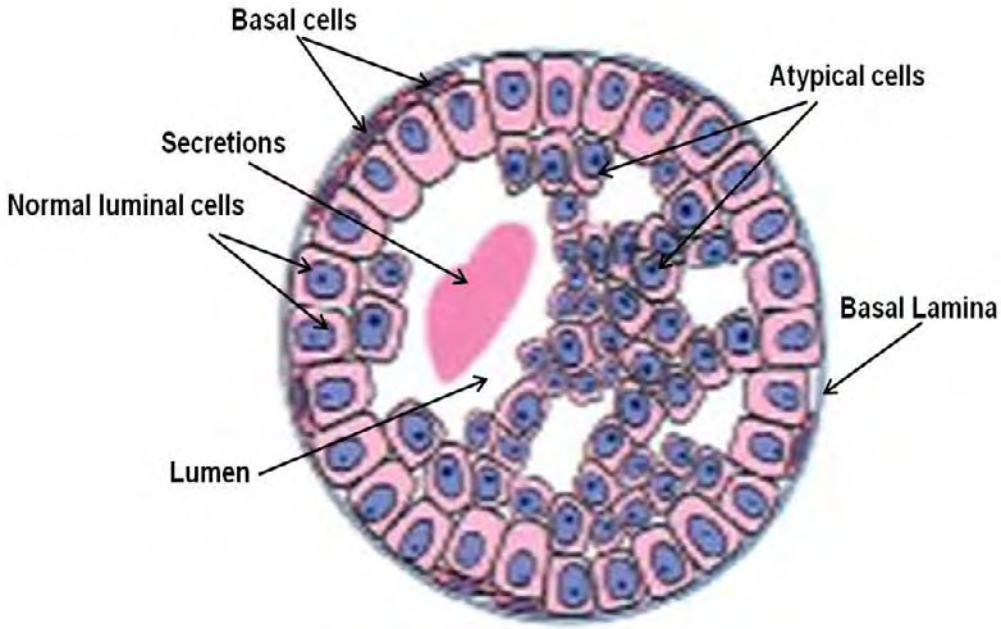


Figure 1. 1 Schematic illustration of Prostatic intraepithelial neoplasia

This is an illustration of a benign human prostatic duct which shows reduced basal cells and the presence of cytologically-atypical cells. The Basal Lamina, Basal cells, normal Luminal cells and lumen form a human prostatic duct. (Adapted from (Abate-Shen and Shen 2000))

1.2.3.2 Prostate adenocarcinoma

More than 95 per cent of prostate cancers are adenocarcinomas (Stamey 1992;William Oh 2003), which derive from prostate epithelial cells (William Oh 2003). Adenocarcinoma is characterized by disruption of the basal cell layer and invasion of the stroma (Stamey 1992). The majority of organ-confined prostate tumours are located peripherally (Epstein et al. 1994) with the other cases located mainly in the transition zone. The initial spread of peripherally-located tumours tends to go through perineural space invasion. Further spread of the tumour normally leads to seminal vesicle invasion with rare spread to the rectum. When progressing to metastatic prostate cancer, lymph node and bone are the most frequent sites of prostate cancer metastases. Lung, bladder, liver, and adrenal gland are the next most common sites that can be detected by autopsy for prostate cancer metastases (Saitoh et al. 1984).

1.2.4 Clinical stages of prostate cancer

To date, there are two main clinical staging systems: tumour, node, metastases (TNM) and Whitmore-Jewett classification systems. Here the TNM classification system will be introduced. The currently used TNM system was adopted in 1992 and modified in 1997(Schroder et al. 1992). This system includes 4 T stages (T1-T4),

one N+ stage and one M+ stage (Figure 1.2). The T1 stage, which has 3 sub-stages: T1a-T1c, classifies tumours that are small, unapparent, and unable to be detected by digital rectal exam (DRE). These tumours may be found by chance during surgery. The tumours of stage T2 (T2a-T2c) are prostate confined. They are normally larger in size so that they can be detected by DRE or biopsy. The tumours staged at T3 (T3a-T3c) have spread outside the prostate capsule to nearby tissues such as seminal vesicle(s). When at stage T4 (T4a-T4c), the tumours have spread further to the organs nearby, such as the bladder. The N stage (N0-N3), describes tumours spreading to regional lymph nodes. Metastasis to organs such as bone and brain is described by the M stage (M0-M1c).

1.2.5 Treatment of prostate cancer

The current treatments for prostate cancer include radical prostatectomy, cryotherapy, radiation therapy, hormonal therapy and chemotherapy. Radical prostatectomy is effective, or even curative, only in organ-confined prostate cancer (early primary tumour, T1-T2b, stage)(William Oh 2003). For patients with locally advanced (T3-T4) tumours, radical prostatectomy is not recommende (William Oh 2003). Radiation therapy only has a favorable prognosis in patients with an early primary tumour (T1 or T2a stage with PSA<10ng/ml and Gleason grade <6) (William Oh 2003). It is also often used for the palliation of bone metastases, especially for those

hormone-refractory bone metastases (William Oh 2003). Hormonal therapy is the most effective treatment for metastatic tumours (T4, N and M stages). However, after initial suppression of the cancer by hormonal therapy, a more advanced hormone-refractory cancer usually developes which is currently incurable. Although chemotherapy can be used as adjunctive therapy with other therapies, there are no effective chemotherapeutic drugs for prostate cancer yet (Stamey 1992). In conclusion, prostate cancer is only curable when it is organ-confined, and incurable in the advanced stage, especially in the hormone-refractory stage. Therefore novel approaches, particularly those for hormone-refractory cancer, are urgently needed.

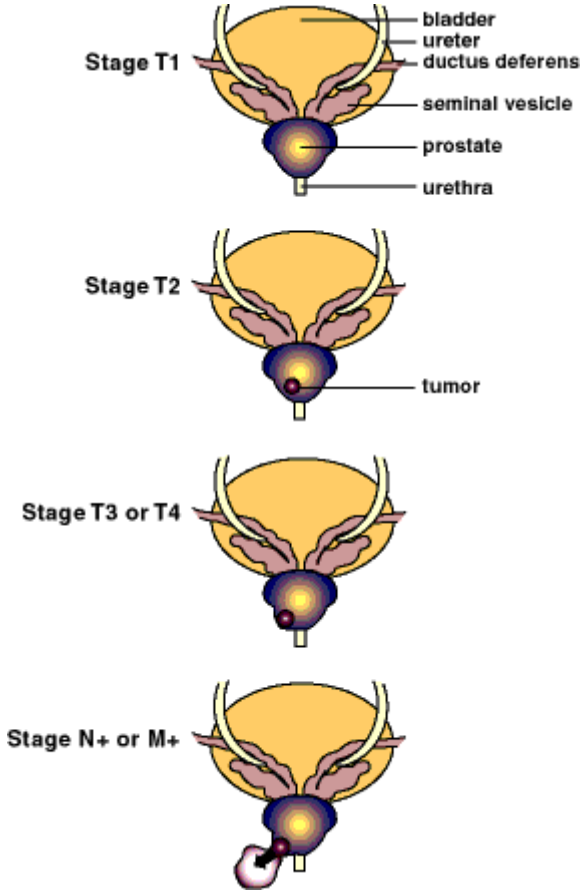


Figure 1. 2 Schematic TNM stages of prostate cancer

Here T = primary tumour, N= lymph nodes, M= distant metastases

(Taken from www.phoenix5.org)

1.3 Androgen regulation and prostate cancer

Androgens are the main male sex hormones. The principal circulating androgens are testosterone and its more potent metabolite 5α-dihydrotestosterone (DHT). They are essential for the development of a male phenotype, male sexual maturity and male reproductive function. Low levels of androgens, e.g. testosterone, are also present in females and can be converted to the female sex hormone, estradiol, by the enzyme aromatase. Apart from their effects on the reproductive system, androgens also have wide effects on other tissues such as skin, muscle, bone and brain (Heemers et al. 2006;Li and Al-Azzawi 2009). Testosterone and DHT exert these functions by interacting with the androgen receptor (AR) through its signalling pathways. AR belongs to the nuclear hormone receptor superfamily which also includes estrogen receptor, progesterone receptor and glucocorticoid receptor (Robinson-Rechavi et al. 2001). Other than the functions mentioned above, there is compelling evidence showing that AR is expressed in all stages of prostate cancer and has been involved in every stage of prostate cancer including prostate cancer carcinogenesis, early stage of prostate cancer, advanced stage of prostate cancer and hormone-refractory prostate cancer (Ramsay and Leung 2009;Robinson-Rechavi et al. 2001). In the following sections of this chapter, the AR signalling pathways and the involvement of aberrant regulation of AR signalling in the development of hormone-refractory prostate cancer will be discussed.

1.3.1 Androgen receptor signalling

AR exerts its functions through two categories of AR signalling pathways: a genomic signalling pathway and a non-genomic signalling pathway. They will be described below. But first, in order to elucidate the role of AR and its signalling pathways, it is important to understand the structure and function of AR.

1.3.1.1 Androgen receptor structure

The AR protein, comprising 919 amino acids, is coded by the human AR gene which is located on chromosome X at Xq11.2-q12 (gene structure see Fig 1.3A top panel) (Bennett et al. 2010;Li and Al-Azzawi 2009). There are two AR isoforms: AR-A (87 kDa) and AR-B (110 kDa) (Li and Al-Azzawi 2009). The AR protein consists of three functional domains, an N-terminal regulatory domain, a DNA–binding domain, a carboxyl terminal ligand-binding domain, and a short hinge region (H) (see Fig 1.3 A lower panel or B top panel) (Li and Al-Azzawi 2009). The N-terminal regulatory domain exerts AR's transcriptional activity. Through it AR interacts with co-regulators and members of the transcription machinery. The DNA–binding domain is responsible for DNA recognition and binding through its first zinc finger motif. The second zinc finger of the DNA–binding domain mediates dimerization between AR monomers and also helps to stabilize the DNA binding (Fig 1.3 B). The short hinge

region is important for AR nuclear localization, phosphorylation, acetylation and degradation. The ligand-binding domain exerts AR's ligand binding, eg. binding to DHT.

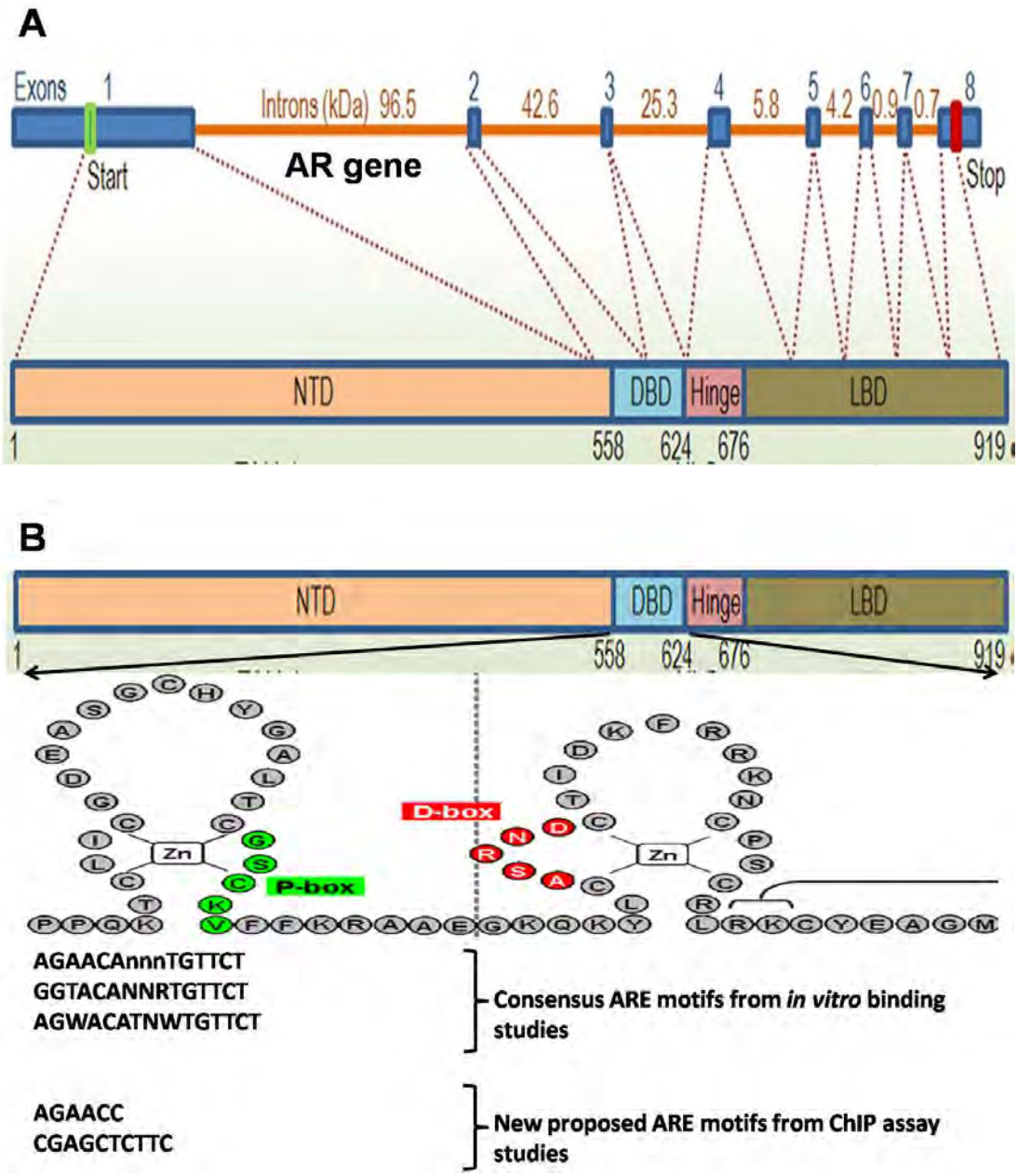


Figure 1. 3 Schematic illustration of the structures of the AR gene, AR protein, the two zinc fingers and the sequence of the consensus ARE motifs

A. The structure of the AR gene, protein and the alignment between gene exons and the domains on the protein (adapted from (Bennett et al. 2010)). **B.** the structures of two zinc fingers in the DNA–binding domain and the sequences of the commonly used consensus ARE motifs (adapted from (Bennett et al. 2010;Claessens et al. 2008)).

1.3.1.2 Genomic signalling pathway

The AR genomic signalling pathway results in either initiation or inhibition of AR-regulated gene transcription (Fig 1.4) (Keller et al. 1996;Lin et al. 2009;Moehren et al. 2004;Nantermet et al. 2004).

In the cytoplasm, mature ARs are bound to multi-protein chaperone complexes, such as heat shock proteins (Bennett et al. 2010;Loy et al. 2003). The binding of androgen leads to an AR conformational change at helices 3, 4 and 12 within the ligand-binding domain (Bennett et al. 2010;Loy et al. 2003). This conformational change results in a series of events: the dissociation of AR from the heat shock proteins; interaction with AR co-regulators, such as ARA70; phosphorylation of AR serine residues by kinases, such as mitogen-activated protein kinases; AR dimerization and rapid nuclear transportation (Bennett et al. 2010;Cutress et al. 2008;He et al. 2002;Loy et al. 2003;Rahman et al. 2004;Schaufele et al. 2005).

In the nucleus, the activated AR dimmer binds to a specific DNA sequence (androgen-responsive element) in the promoter of a target gene followed by the recruitment of transcription coregulators and members of the basal transcription machinery or general transcription machinery (GTF) (Bennett et al. 2010;He et al.

2002;Powell et al. 2004). This leads to the transcriptional initiation or repression of the genes involved in growth, differentiation, homeostasis and apoptosis.

Once dissociated from androgen, the AR is transported back to the cytoplasm. It can then be recycled for multiple rounds between cytoplasm and nucleus for the activation of target genes or undergoes proteosomal degradation.

1.3.1.3 Non-genomic signalling pathway

In the genomic signalling pathway, the AR initiates the transcription of its regulated genes and the transcription activity normally peaks within hours (Cato et al. 1988;Foradori et al. 2008), rarely, in minutes (Groner et al. 1983). Recent evidence showed androgens can exert their effects in a time frame of minutes or even seconds in a plasma membrane mediated fashion (Falkenstein et al. 2000;Foradori et al. 2008). Therefore, it has been proposed that some of these effects involve a plasma membrane-located AR acting through a non-transcriptional/translational machinery activation pathway (Fig 1.5) (Falkenstein et al. 2000). However, it should be noted that some of these non-genomic effects may ultimately alter transcriptional/ translational activity through second messenger activation (Foradori et al. 2008). In this pathway, activated plasma membrane AR (mAR) can interact with different

pathways to exert its rapid regulatory functions: 1) interaction with L-type calcium channels leads to the inflow of calcium. This increases intracellular Ca^{2+} concentration and in turn activates protein kinase A, protein kinase C and mitogen-activated protein kinase pathways; 2) interaction with IP3 receptors leads to the release of calcium from the endoplasmic reticulum. This activates the RAS/MEK/ERK pathway; 3) interaction with the Src kinase pathway leads to the activation of mitogen-activated protein kinase (Foradori et al. 2008;Li and Al-Azzawi 2009;Rahman and Christian 2007).

Some of the non-genomic AR actions may affect its genomic action. An example is the activation of mitogen-activated protein kinase which may also increase the AR transactivation activity through the phosphorylation of the AR and its coregulators (Chmelar et al. 2007).

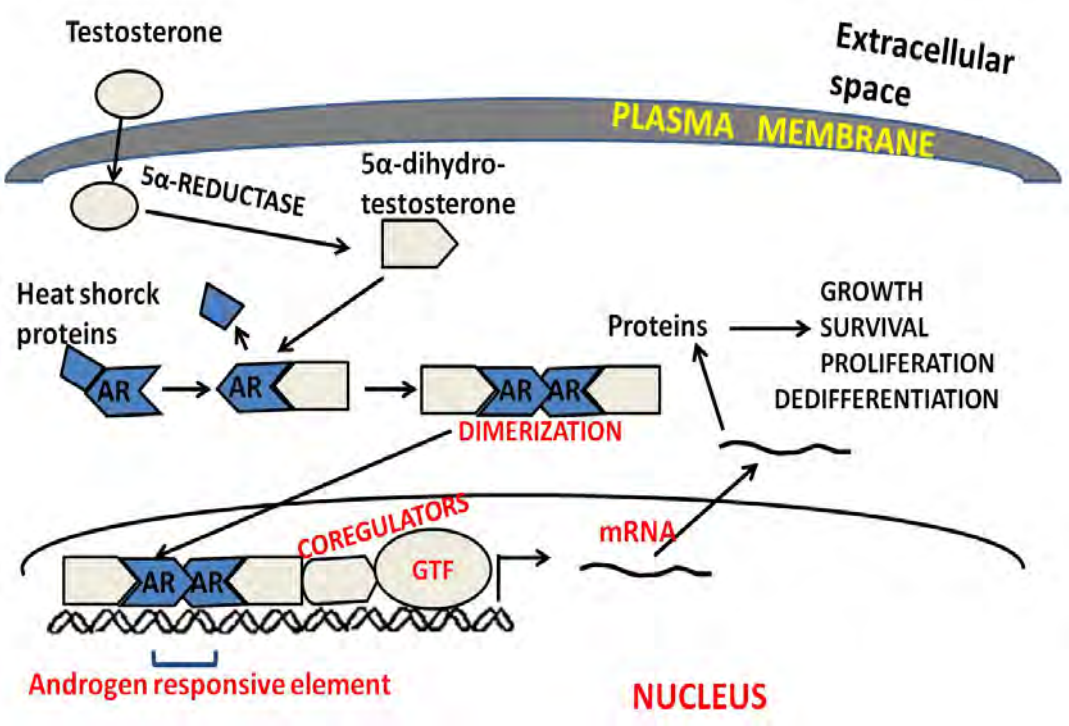


Figure 1. 4 Genomic AR signalling pathway

The AR genomic pathway exerts regulatory function through initiation or inhibition of transcription of genes. This pathway includes steps, such as mature AR (in the cytoplasm) activated by androgen, forming AR dimers, translocating to the nucleus, binding AREs, initiating or inhibiting transcription of genes (involved in growth,survival, proliferation or differentiation).

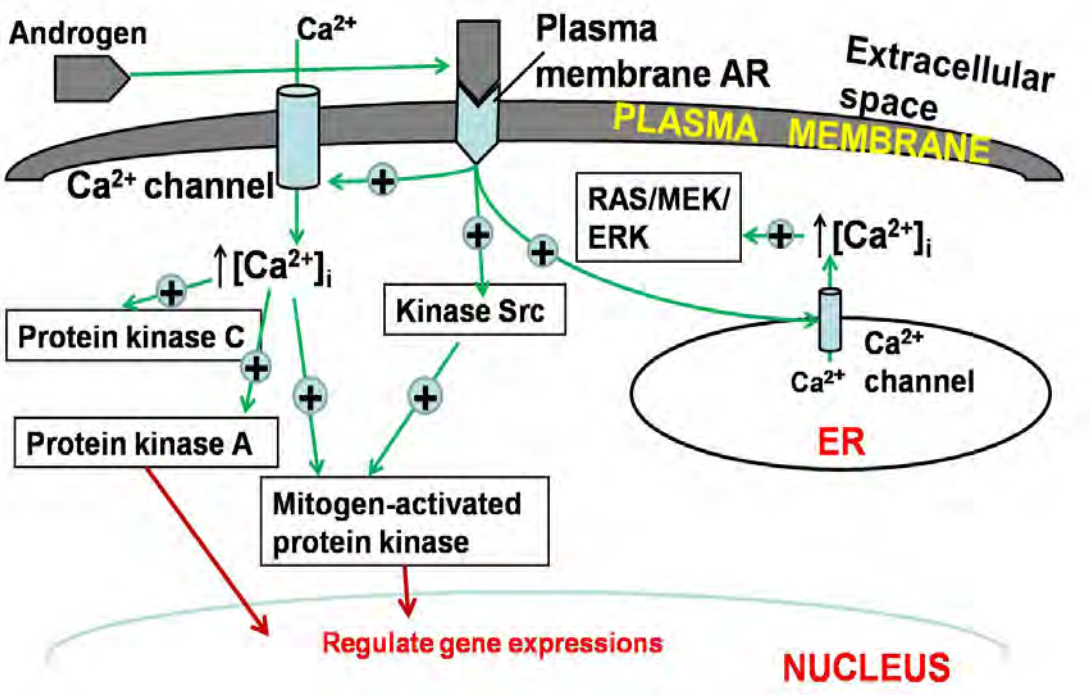


Figure 1. 5 Non-genomic AR signalling pathway

The non-genomic AR pathway exerts regulatory functions by regulating various non-transcriptional/translational pathways. However, some of these pathways may ultimately lead to genomic actions.

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

There is a significant amount of evidence showing that AR protein and mRNA are expressed in nearly every stage of human prostate cancer, especially in a majority of the hormone-refractory prostate cancers (Chmelar et al. 2007;Chodak et al. 1992;Dehm and Tindall 2006;Ruizeveld de Winter et al. 1994;Yuan et al. 2006). This indicates the majority of hormone-refractory cancers are not due to the selection of AR-negative colonies, instead their survival, growth and proliferation are still regulated by the AR. Various alterations of AR signalling pathways have been evident in hormone-refractory prostate tumours (Linja et al. 2001;Small et al. 2004;Titus et al. 2005). These indicate the possible underlying mechanisms for prostate cancer's progress to the hormone-refractory stage. The following topic is going to be discussed in the coming sections: alterations in AR signalling in the genomic pathway that have been observed in prostate cancers, and the possible mechanisms and the advantages these modifications can provide for the tumours in hormone therapy.

1.3.2.1 *In situ* synthesis of androgen in prostate tumours

There is evidence that in hormone-refractory prostate cancer or prostate cancer that is undergoing hormone therapy, the prostatic DHT levels remain at levels that are capable of activating AR (Mohler et al. 2004). In addition, it was reported that in hormone-refractory prostate cancer, there were changes in the expression of genes that regulate the formation of androgen in situ within the tumour, especially, the genes for both androgen synthesis and catabolism (McPhaul 2008). All these suggested a mechanism of *in situ* synthesis of testosterone or DHT from other androgen sources, such as adrenal androgens (McPhaul 2008). This provides a sufficient and stable intracellular level of androgen for tumour cells to activate the AR despite the low circulating androgen level during the hormone therapy.

1.3.2.2 Modifications of the AR gene in prostate tumours

The AR gene modifications that accompany tumorigenesis include gene mutations and gene amplification (Fig 1.6 A).

Observations of the AR mutation frequency at different prostate cancer stages showed a relationship between mutation frequency, cancer stages and the selection pressure imposed by treatment (McPhaul 2008;Tilley et al. 1996). In localized

primary tumours, the mutation frequency was reported to be approximately 5%, whereas it was approximately 20% in locally-metastatic tumour specimens (Marcelli et al. 2000;McPhaul 2008). The distally-metastatic tumour samples have been shown to have a mutation frequency of 50% (Taplin et al. 1999;Taplin et al. 2003). A higher mutation frequency and the clustering of specific mutations were observed in patients treated with anti-androgen treatment (Taplin et al. 1995). The majority of AR mutations that have been found in prostate cancer samples are point mutations leading to single amino acid changes. These mutations occurred mainly on the ligand-binding domain of the AR protein (Heinlein and Chang 2004). This results in a broadening of the range of agonists that can activate AR. A good example is the AR T876A mutation that is thought to be the most frequent mutation of this type (Gaddipati et al. 1994;Heinlein and Chang 2004;Taplin et al. 1999). This mutation was observed in the LNCaP prostate cancer cell line and it allowed the activation of the AR by antiandrogens HF, cyproterone acetate and other steroid hormones, such as DHEA, androstenediol, estradiol and progesterone (Veldscholte et al. 1990). These suggest a mechanism of hormone-refractory tumour growth through utilizing other steroid hormones or even androgen antagonists to escape the hormone therapy.

AR gene amplification is rarely found in untreated primary prostate cancer. However, in 23-28% of primary prostate cancer after hormone treatment, AR gene

amplification was observed (Koivisto et al. 1997;Koivisto et al. 1996). AR gene amplification was also observed in metastases and hormone-refractory prostate cancer, 22% and 20-30% of cancers, respectively. It has been suggested that AR gene amplification could elevate AR gene expression in hormone-refractory prostate cancer so that the prostate cancer cells could proliferate in a low androgen environment. However, it is still not known if this results in an increase in AR protein levels. Another possibility is this event is a reflection of the increasing level of genomic instability during prostate cancer progression.

1.3.2.3 Androgen independent activation of the AR

Apart from activation by agonists, such as DHT, the AR can also be activated by growth factors in an agonist-independent manner possibly through a combination of the post-translational modification of the AR and alterations of the assembly of transcription machinery (Fig 1.6 B) (Bunone et al. 1996;Craft et al. 1999;Glass et al. 1997;Janknecht and Hunter 1996). There is evidence showing growth factors such as keratinocyte growth factor, insulin-like growth factor 1, interleukin-6 (a cytokine), epidermal growth factor, receptor-tyrosine kinases (eg HER-2/neu) and protein kinase-A can activate the AR in the absence of androgen in prostate cancer cell line and model studies (Craft et al. 1999;Culig et al. 1994;Jenster 2000;Nazareth and Weigel 1996). The androgen-independent activation of AR by the growth factors is

through phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase pathways (Bennett et al. 2010;Ramsay and Leung 2009). In addition, there is evidence demonstrating the activation of the phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase pathways and increase of receptor-tyrosine kinases in more aggressive primary and hormone-refractory prostate cancers (Craft et al. 1999;Gioeli et al. 1999;Signoretti et al. 2000). These studies indicate that agonist-independent activation of the AR by growth factor pathways is one of the underlying mechanisms for the development of hormone-refractory tumours.

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

It has been observed that the expression of multiple AR coregulators was altered in prostate cancer specimens and cell lines (Chmelar et al. 2007). A combination of SRC-1 and TIF-2 was found to be over-expressed in hormone-refractory prostate cancer (Gregory et al. 2001). AR coregulators ARA70 and Tat interactive protein, 60kDa (Tip60), were also shown to be over-expressed in CWR22 prostate xenograft after androgen withdrawal (Gregory et al. 1998;Halkidou et al. 2003). There is evidence showing that AR coregulators can even promote wild-type AR activation with antiandrogens, adrenal androgens and estradiol (Miyamoto et al. 1998).

Therefore it was suggested that the alteration of coregulator levels may play a role in the progression of hormone-refractory prostate cancer (Chen et al. 2004).

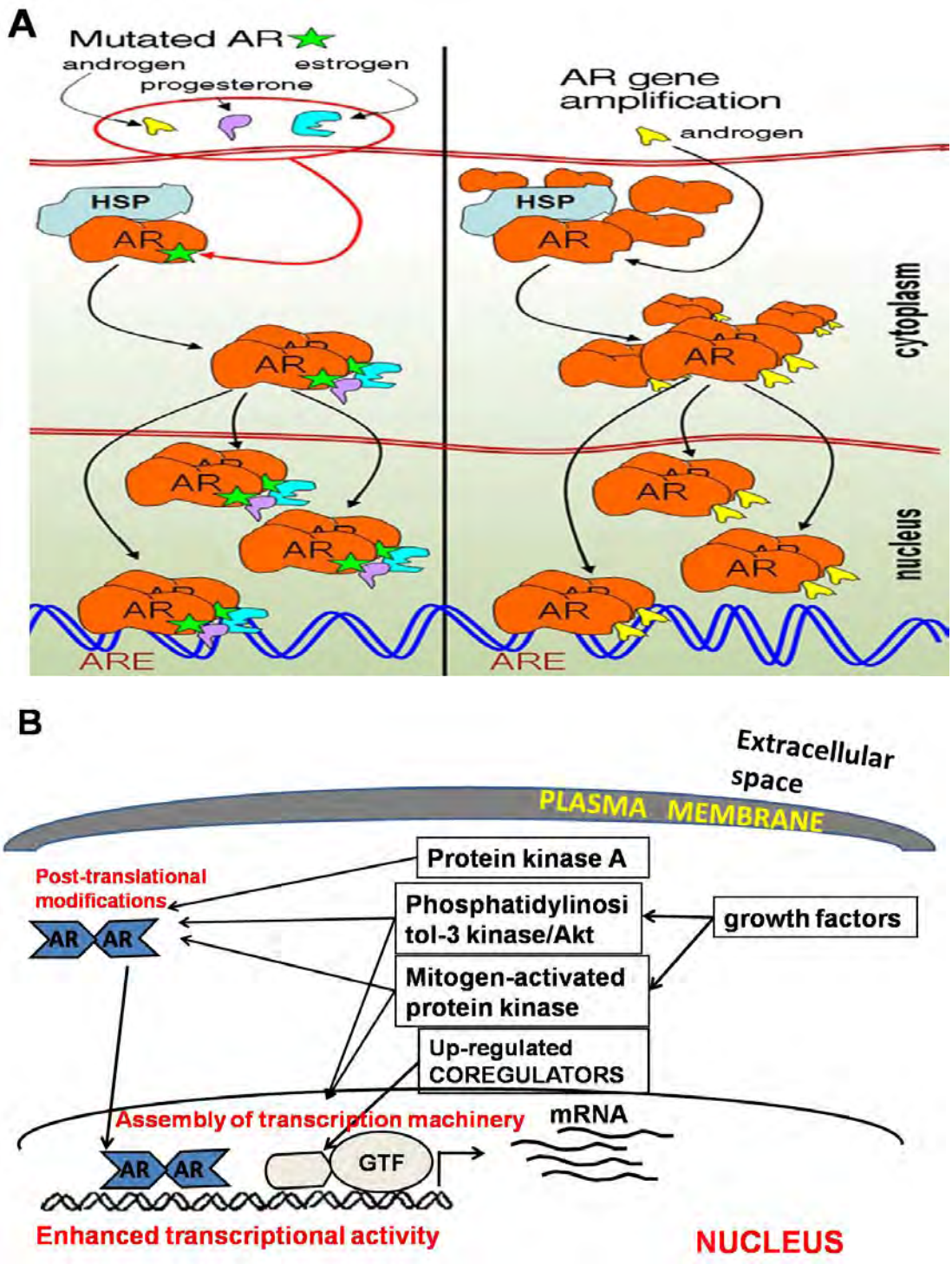


Figure 1. 6 Abnormal AR signalling in prostate cancer

A. Modifications of the AR gene (adapted from (Bennett et al. 2010)). **B.** Androgen independent activation of the AR.

1.3.3 The Androgen-responsive element

The specific DNA sequence that the AR binds, and through which it exerts its transcriptional regulatory function, is called the androgen response element (ARE). AREs defined by traditional *in vitro* binding studies consist of two copies of consensus hexamers, 5'-TGTTCT-3', with a three nucleotide separation in the middle. This is due to the two DNA-binding domains in an AR dimer binding to each hexamer and the space between the two DNA-binding domains matches the three-nucleotide span (Claessens et al. 2008). Based on the arrangement of the two hexamers, these AREs are divided into two groups, classical AREs and selective AREs. For the classical ARE, the arrangement of the hexamers is in an inverted repeat, eg. 5'-AGAACAnnnTGTTCT-3'. Classical AREs are not only recognized by the AR, but also by glucocorticoid, progestagen and mineralocorticoid receptors (Claessens et al. 2008). For selective ARE, the arrangement is the partial direct repeat of 5'-TGTTCT-3', eg. 5'-AGAACTnnnAGAACA-3'. The selective AREs can only be recognized by the AR. Many consensus ARE motifs have been proposed from various *in vitro* AR binding studies for bioinformatics predictions of the putative AREs. Among them, several widely used ones are AGAACAnnnTGTTCT, GGTACANNRTGTTCT, AGWACATNWTGTTCT and WGAGCANRN (Claessens et al. 2008;Lin et al. 2009;Zhang and Barritt 2004).

The employment of an AR *in vivo* binding detection technique, the chromatin immunoprecipitation (ChIP) assay, has demonstrated that the above ARE consensus motifs are enriched in AR binding sites. However, they represent only a small proportion of the *in vivo* AR binding sites (Lin et al. 2009;Massie et al. 2007). Therefore, new consensus ARE motifs, such as AGAACC, CGAGCTCTTC, have been proposed based on an analysis of these *in vivo* AR binding sites (Lin et al. 2009;Massie et al. 2007). In addition, it has been shown that the promoter is not the preferred region of AREs, since they can be located further upstream from the transcription start site, within the gene body or 3' downstream from the transcription start site of the gene (Jariwala et al. 2007;Lin et al. 2009;Massie et al. 2007).

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

The search for AREs in a genomic DNA segment can be carried out in two ways: 1) functional assays to detect the androgen responsiveness and thus the presence of an ARE; 2) directly detect or bioinformatically predict the AR binding site on the DNA segment (Maston et al. 2006). For the functional assay method, a reporter-gene assay is the versatile and commonly used approach. For the direct identification of the AR binding site, chromatin immunoprecipitation (ChIP) assay is now a widely used and powerful method. DNase I hypersensitive site mapping has also been used to search for AREs. These methods will be described in the following sections, the focus will be on reporter-gene assays and ChIP assays as they were used in the studies described in this thesis.

1.4.1 Reporter-gene assay

Reporter-gene assays have been widely used for transcriptional regulatory element activity studies (Alam and Cook 1990;Maston et al. 2006;Naylor 1999;Nordeen 1988). Several types of commonly used reporter genes are: chloramphenicol acetyltransferase, β-galactosidase, green fluorescent protein and luciferase gene (Naylor 1999).

Firefly (Photinus pyralis) and Renilla (Renilla reniformis) luciferase are two of the most commonly used forms of luciferase for reporter-gene assays because of their properties: 1) they do not require post-translational processing for luciferase activity; 2) they have high sensitivity and broad linear range (7-8 orders of magnitude and detection sensitivity of≤1 femtogram for firefly luciferase, 6 orders of magnitude and detection sensitivity of around 30 femtograms for Renilla luciferase) (Naylor 1999)(Technical Manual Revised 08/2006, Part# TM040, Promega). When performing luciferase reporter-gene assays, it is common to construct a firefly luciferase reporter-gene expression construct containing the genomic DNA of interest and co-transfect it with a standard amount of Renilla luciferase reporter vector (used as transfection efficiency control) into mammalian cells (Zhang et al. 2003). The structure of the reporter-gene construct depends on the region of the genomic segment to be studied (Fig 1.6). When a promoter region is to be studied, the region is cloned into a promoterless vector immediately upstream of a reporter gene (Schuur et al. 1996). When studying a regulatory element, such as an enhancer, it is normally cloned into a plasmid upstream site (sometimes it can also be the downstream) of a reporter gene, the expression of which is driven by a minimal promoter, such as SV40 (Maston et al. 2006).

Using the luciferase reporter-gene assay as an example, the normal strategy includes construction of a reporter-gene construct containing the genomic DNA segment of

interest, transfection of mammalian cells, appropriate androgen treatments, cell harvest and luciferase analysis. It is common to dissect the DNA segment into smaller restriction endonuclease-digested fragments to further localize the ARE. Each of these fragments needs to be tested in a reporter-gene construct using the strategy described above. This is followed by introducing point mutations into the putative ARE and confirming their function in a reporter-gene construct, tested as above.

The identified ARE would normally be tested for its interaction with the AR in an *in vitro* DNA-protein binding test, such as the electrophoretic mobility-shift assay (EMSA) or DNase I foot-printing (Riegman et al. 1991;Schuur et al. 1996).

1.4.2 Chromatin immunoprecipitation (ChIP) assay

The ChIP assay is a powerful technique that detects the *in vivo* binding site of transcription factors within a chromatin context. The ChIP assay includes the following steps: 1) appropriate treatments of cells to promote the AR binding to chromatin or no treatment for AR binding as a control; 2) chemical crosslinking of chromatin and AR in living cells; 3) cell lysis to release the chromatin; 3) fragmentation of the chromatin; 4) immunoprecipitation of anti-AR antibody and AR together with its bound chromatin fragment; 5) reversing the crosslinking and

purifying the enriched DNA; 6) downstream analysis of the DNA to identify regions that correspond to the enriched DNA fragments.

Based on the downstream analysis method, ChIP assays can be divided into standard ChIP assays (including ChIP-qPCR, ChIP-PCR, ChIP-southern blot) ChIP-tag library and sequencing (including ChIP-SACO, ChIP-STAGE and ChIP-PET), ChIP-microarray, ChIP-seq, ChIP-display and ChIP-DSL (Collas and Dahl 2008;Massie and Mills 2008).

ChIP-qPCR uses quantitative PCR as a downstream analysis method for purified DNA from ChIP. In this method, locus specific primers are designed and verified followed by the qPCR analysis of the particular immunoprecipitated region to determine whether the DNA for that region is enriched. For ChIP-qPCR, a typical upper limit for average chromatin size after fragmentation is between 700 bp and 1kb. ChIP-qPCR may be combined with reporter gene assay to identify AREs. This is for the following two reasons: 1) the resolution of ChIP-qPCR is between 700 bp and 1 kb so that further analysis to locate AREs is required; 2) not all the identified AR binding sites are functional therefore a functional test is needed (Maston et al. 2006).

1.4.3 DNase I hypersensitive site mapping

DNase I hypersensitive site mapping is based on the fact that transcription factor binding sites on chromatin tend to be more sensitive to DNase I digestion. This is due to the relaxed of chromatin formation at these sites (Maston et al. 2006). This method is typically combined with reporter-gene assays to test the functionality of the potential AR binding sites identified and to locate the ARE (Cleutjens et al. 1997).

Since the study carried out in this thesis was to search for AREs in *TRPM8,* the current knowledge of TRPM8 is to be described in the following sections.

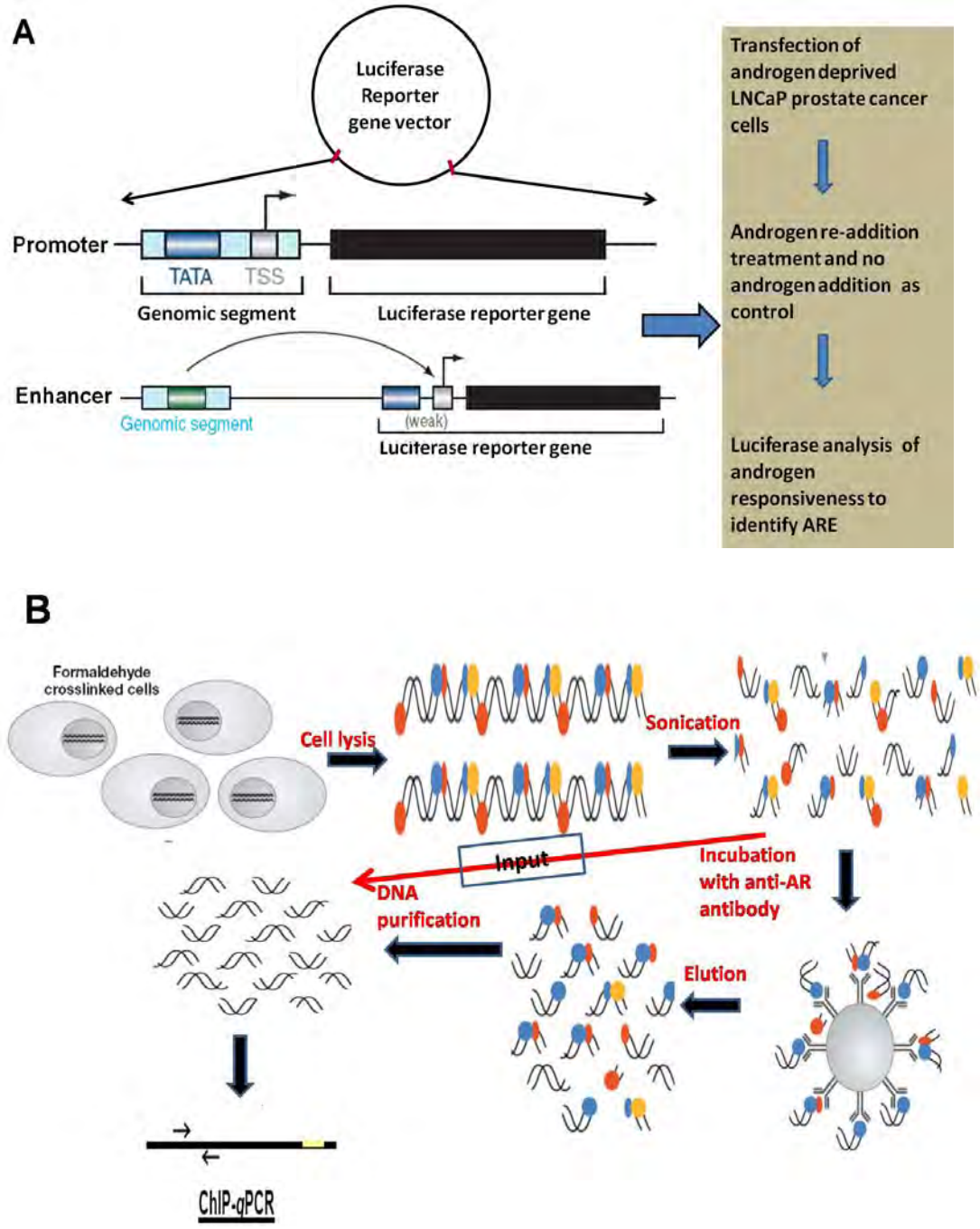


Figure 1. 7 Approaches for searching for ARE

A. Reporter-gene assay approach using a luciferase reporter gene as an example. Steps include construction of a luciferase reporter vector, transfection of mammalian cells, androgen treatment and luciferase analysis. *TSS*, transcription start site. Partly adapted from (Maston et al. 2006). **B.** ChIP assay approach using ChIP-qPCR as an example. Steps include crosslinking, cell lysis, sonication, immunoprecipitation, DNA purification and qPCR analysis. *Input*, a fraction of chromatin reserved without immunoprecipiation and used as control for normalization of qPCR values. Adapted from (Massie and Mills 2008)

1.5 The TRPM8 non-selective cation channel

Transient receptor potential cation channel, subfamily M, member 8 (TRPM8) was first discovered as Trp-p8 in prostate and prostate cancerous tissues followed by its identification in sensory neurones as a cold- and menthol-sensitive receptor, CMR1 (McKemy et al. 2002;Tsavaler et al. 2001). Later it was designated TRPM8.

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

The transient receptor potential (TRP) cation channel superfamily consists of seven subfamilies (Table 1.1 left panel). The members of the TRP superfamily have diverse cation selectivity ranging from cation non-selective to selective of a particular cation. They are expressed in a wide range of tissues or organs responsible for various physiological functions, including the sensing of major classes of external stimuli, such as light, sound, chemical, temperature and touch; sensing the changes in the local cellular environment; smooth muscle contraction; secretion of fluid and hormones and functions in kidney, bladder and bone (Venkatachalam and Montell 2007). The TRP channels have also been shown to be involved in many diseases, especially in cancers (Prevarskaya et al. 2007c;Venkatachalam and Montell 2007).

The transient receptor potential melastatin (TRPM) plasma membrane cation channel subfamily was named after the identification of its first member, melastatin now named TRPM1 (Harteneck 2005). TRPM has eight members with diverse functions (Fig 1.7 right panel). These members were divided into four groups based on phylogenetic similarity: 1) TRPM2 and TRPM8, TRPM1 and TRPM3, TRPM4 and TRPM5, TRPM6 and TRPM7.

	Worms	Flies	Mice	Humans
TRPC	3	3	7	6
TRPV	5	2	6	6
TRPM	4	1	8	8
TRPA	2	4	1	1
TRPN	1	1	0	0
TRPP	1	1	3	3
TRPML	1	1	3	3
Total	17	13	28	27

Channel	Functions
TRPM1	?
TRPM2	Sensor of oxidative stress
TRPM3	Renal osmo-homeostasis
TRPM4	Calcium-activated nonselective cation channel
TRPM5	Calcium-activated nonselective cation channel
TRPM6	Mg^{2+} uptake in kidney and intestine
TRPM7	Cellular Mg^{2+} homeostasis
TRPM8	Cold receptor (<28°C)

Table 1.1 Subfamilies of TRP cation channels and their functions

The left panel shows the subfamilies and number of members across four species. The right panel is the expanded version of the TRPM subfamily from the left panel showing the members and their known functions. Adapted from (Harteneck 2005;Venkatachalam and Montell 2007)

1.5.2 The *TRPM8* gene

The *TRPM8* gene is located on chromosome 2 at region 2q37.1. It spans 102.92 kb and has 26 exons (GenBank ID NM_024080) (Fig 1.9). In Fig 1.9, the 5' regulatory region of the *TRPM8* gene was the region studied in this thesis.

1.5.3 Expression of TRPM8 in normal tissues

TRPM8 has been shown to be expressed mainly in the prostate, tissues of the male genital tract and sensory neurons (Table 1.2). TRPM8 mRNA was first discovered almost exclusively in normal human prostate tissues, with some TRPM8 mRNA in testis and weak TRPM8 mRNA signals in breast, lung and thymus (Tsavaler et al. 2001). In another study, TRPM8 mRNA expression in testis was confirmed and its mRNA expression in bladder urothelium and some tissues in the male genital tract demonstrated (Stein et al. 2004). TRPM8 mRNA or protein or both have been found in sensory neurons including small diameter dorsal root ganglion neurons, trigeminal ganglia neurons, nodose ganglion neurons (Babes et al. 2004;McKemy et al. 2002;Nealen et al. 2003;Peier et al. 2002;Thut et al. 2003;Tominaga and Caterina 2004;Zhang and Barritt 2004) and taste papillae (Abe et al. 2005). In addition, TRPM8 expression has been shown in other tissues including small intestinal mucosa, gastric fundus, vascular smooth muscle, liver and lung epithelium (Henshall et al.

2003;Mustafa and Oriowo 2005;Sabnis et al. 2008;Yang et al. 2006;Zhang et al. 2004).

1.5.4 General properties of TRPM8

TRPM8 is a non-selective cation channel permeable to both divalent and monovalent cations with a relatively higher preference for Ca^{2+} and with a relative permeability for Ca^{2+} against Na^{+} ranging from 0.97 to 3.3 (McKemy et al. 2002;Peier et al. 2002;Voets et al. 2007). There is little selectivity among monovalent cations (McKemy et al. 2002;Voets et al. 2007).

TRPM8 can be activated by natural cooling agents, menthol, eucalyptol, linalool, geraniol, synthetic menthol-derivatives and icilin (for agonists and their potency refer to Table 1.3). Icilin is very potent with an EC50 in the nanomolar range (McKemy et al. 2002;Peier et al. 2002;Voets et al. 2007). It is reported that the most potent agonist is WS-12 (Beck et al. 2007). The least potent agonists are eucalyptol, linalool and geraniol with EC50s in milimolar range. The other agonists have an EC50 in the micromolar range. There is a range of TRPM8 antagonists with the highest potency (IC50) being between 800 nM and 3.5 μM (Table 1.2). However, none of these antagonists is a TRPM8 specific antagonist (Voets et al. 2007).

1.5.5 Structure and mechanisms of activation of TRPM8

Based on the homology to TRP, TRPM8 structure was predicted to have 6 transmembrane domains. A loop between transmembrane domain 5 and 6 is believed to form the outer pole region of the channel (Fig 1.8). TRPM8 functions as a tetramer (Stewart et al. 2010).

It is now known that TRPM8 can be activated by membrane depolarization, a cool temperature (23-28 ℃), cooling chemicals, such as menthol and icilin, phosphatidylinositol 4,5-bisphosphate (PIP_2), lysophosphatidylcholine (LPC) and lysophosphatidylinositol (LPI) (Fig 1.7).

Several mechanisms for TRPM8 activation have been proposed. Voets et al have proposed a voltage dependent mechanism for TRPM8 activation (Voets et al. 2004;Voets et al. 2007). In this mechanism, activation of TRPM8 is caused by the triggering of membrane depolarization. A co-activation mechanism has been proposed for TRPM8 activation by icilin (Chuang et al. 2004). In this mechanism, full scale activation by icilin has to be paired with elevated intracellular Ca^{2+}. Ca^{2+} store-dependent activation of TRPM8 through Ca^{2+}-independent phospholipase A2 (iPLA2) was also proposed for organs not exposed to ambient temperature, such as the prostate (Vanden Abeele et al. 2006).

Tissues	Detection
Small-diameter neurons in trigeminal ganglia, dose root ganglia, nodose ganglia	mRNA Protein
Prostate epithelium	mRNA Protein
Taste papillae	Protein
Testis	mRNA
Seminiferous tubules	mRNA
Scrotal skin	mRNA
Bladder urothelium	mRNA Protein
Urothelial cells in culture	Protein
Thymus	mRNA
Breast	mRNA
Small intestine mucosa	mRNA

Table 1.2 TRPM8 expression patterns in normal tissues

Adapted from (Zhang and Barritt 2006).

1.5.6 Normal physiological functions of TRPM8

A known function of TRPM8 is its cold sensing in sensory neurons. It was reported in a study to identify the menthol receptor in trigeminal neurons (McKemy et al. 2002). In this study, heterologously-expressed TRPM8 exhibited an identical activation current as compared to the current of trigeminal neurons with respect to the selectivity of ions, sensitivity to cold and menthol and current-voltage relationship. A cold sensing function in prostate has also been proposed (Stein et al. 2004).

It has also been proposed that in tissues/organs not exposed to ambient temperature, such as the prostate, TRPM8 may regulate ion and protein secretion, and be involved in prostate epithelial cell proliferation and/or apoptosis (Prevarskaya et al. 2007c;Zhang and Barritt 2006)

Compounds	Indicative EC_{50} or IC_{50} range
Agonists	
Icilin	Nanomolar
Frescolat ML	Micromolar
WS-3	Micromolar
Menthol	Micromolar
Frescolat MGA	Micromolar
Cooling agent 10	Micromolar
PMD-38	Micromolar
WS-23	Micromolar
Coolact P	Micromolar
Geraniol	Millimolar
Linalool	Millimolar
Eucalyptol	Millimolar
Hydroxy-citronellal	Millimolar
Antagonists	
BCTC	Micromolar
Thio-BCTC	Micromolar
2-APB	Micromolar
Capsazepine	Micromolar

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

Due to the variations of the EC50 and IC50 values obtained from different methods and experimental conditions, only an indicative concentration range is listed. Adapted from (Voets et al. 2007)

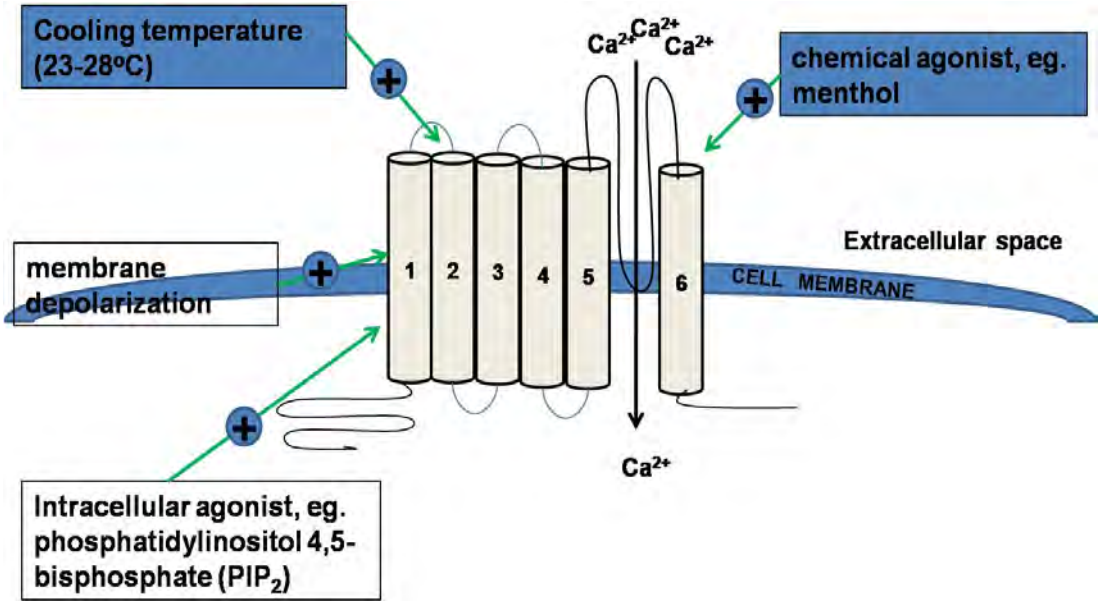


Figure 1. 8 Predicted structure of the TRPM8 channel and activation pathways

Numbers 1-6 represent the predicted 6 domains of TRPM8 proteins; a loop between domains 5 and 6 represents the predicted outer pore region of the channel; '+' activation of TRPM8 channel

1.6 TRPM8 in normal prostate epithelial cells

1.6.1 TRPM8 expression

Tsavaler and colleagues have found *TRPM8* mRNA expression is strong in the basal layer and luminal surface of prostate epithelium (Tsavaler et al. 2001). In a later study, TRPM8 protein was strongly localized in luminal epithelial cells and weak TRPM8 expression was also detected in smooth muscle cells (Bidaux et al. 2005). In addition, TRPM8 protein production was restricted to the luminal epithelial cell surface membrane and indicated the intracellular localization of TRPM8 is on the plasma membrane of prostate luminal epithelial cells.

The intracellular distribution of TRPM8 protein has been shown on both plasma membrane and endoplasmic reticulum (ER) of LNCaP cells (Zhang and Barritt 2004). However, another study has found TRPM8 protein is expressed only on the ER membrane of LNCaP cells (Thebault et al. 2005). The reason for this discrepancy is still unclear. Interestingly, in this study the authors also showed that GFP-tagged TRPM8 heterologously-expressed in LNCaP cells was located only in intracellular areas such as perinuclear membranes. A further study of TRPM8 localization has found functional TRPM8 protein distributed on both plasma membrane and ER in prostate primary epithelial cells from normal prostate and benign prostatic hyperplasia, and prostate primary cancer cells from in situ prostate cancer biopsies

(Bidaux et al. 2007). In addition, the current density of both plasma membrane and ER TRPM8 responding to icilin was higher in prostate primary cancer cells then in prostate primary epithelial cells (Bidaux et al. 2007). Furthermore, the authors have found the disappearance of TRPM8 mediated plasma membrane current in the dedifferentiated prostate primary epithelial cells accompanied a loss of plasma membrane TRPM8 expression as shown by confocal microscopy. The authors thus hypothesized the involvement of a splice variant of TRPM8 for ER expression (Bidaux et al. 2007).

Several hypothetical physiological functions of TRPM8 in the prostate have been proposed: acting as a cold sensor, involvement in ion and protein secretion, as well as regulation of proliferation and / or apoptosis in prostate epithelial cells (Prevarskaya et al. 2007c;Stein et al. 2004;Zhang and Barritt 2006).

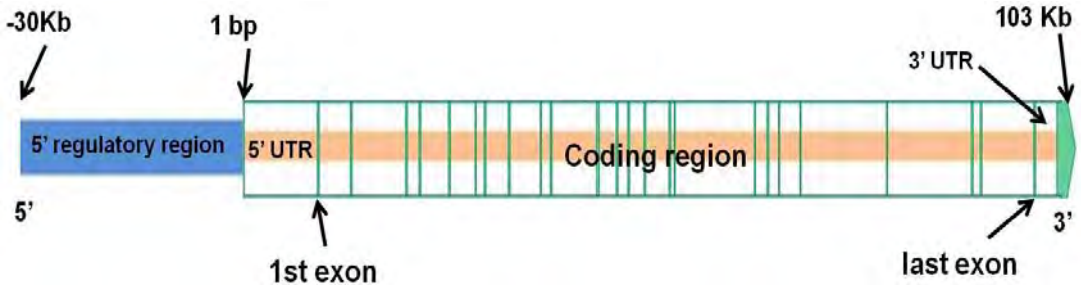


Figure 1. 9 Schematic illustration of the *TRPM8* gene

In this layout, the *TRPM8* gene has been divided into four regions: 30 kb 5' regulatory region (including the *TRPM8* promoter), 5' untranslated region (5' UTR, between the 1st base pair and the 1st exon), coding region, 3' untranslated region (3'UTR, between the last exon and the last base pair of the gene). *Horizontal blue bar*, 30 kb 5' regulatory region. *Vertical Green lines*, exons.

1.6.2 Androgen regulation of *TRPM8* expression

Current evidence shows that *TRPM8* gene expression is androgen-dependent and directly regulated by the AR. *TRPM8* mRNA expression is androgen-dependent in androgen-responsive LNCaP prostate cancer cells (Zhang and Barritt 2004). In this study, culturing LNCaP cells in androgen-deprived medium resulted in a significant decrease in *TRPM8* mRNA. This decrease was reversed after androgen re-addition. In a later study, Bidaux *et al* have shown that *TRPM8* expression required functional AR (Bidaux et al. 2005). In that study, they first confirmed that *TRPM8* expression is androgen-dependent in LNCaP cells then established that anti-androgen treatment of LNCaP cells led to a significant decrease in *TRPM8* expression. The following ectopic AR-expression study using AR deficient PNT1A human prostate cell line showed *TRPM8* expression was restored only in PNT1A cells transfected with functional AR. Bidaux *et al* also carried out single cell RT-PCR and immunohistochemical experiments on primary human prostate cancer cells and found TRPM8 was mainly expressed in androgen-dependent secretory epithelial cells. The expression was down-regulated when the cells lost AR activity.

Based on the above observation, it was proposed that *TRPM8* is directly regulated by the AR through its genomic signalling pathway. As mentioned in the previous section, activated AR is expected to bind to the ARE of its regulated genes to initiate

and/or enhance or inhibit gene transcription. Therefore bioinformatic analysis of the *TRPM8* gene to identify putative AREs has been carried out and several putative AREs have been identified (see Fig 1.10) (Bidaux et al. 2005;Zhang and Barritt 2004;Zhang and Barritt 2006). However, none of these putative AREs has been tested and it is still largely unknown how the AR regulates *TRPM8* gene expression.

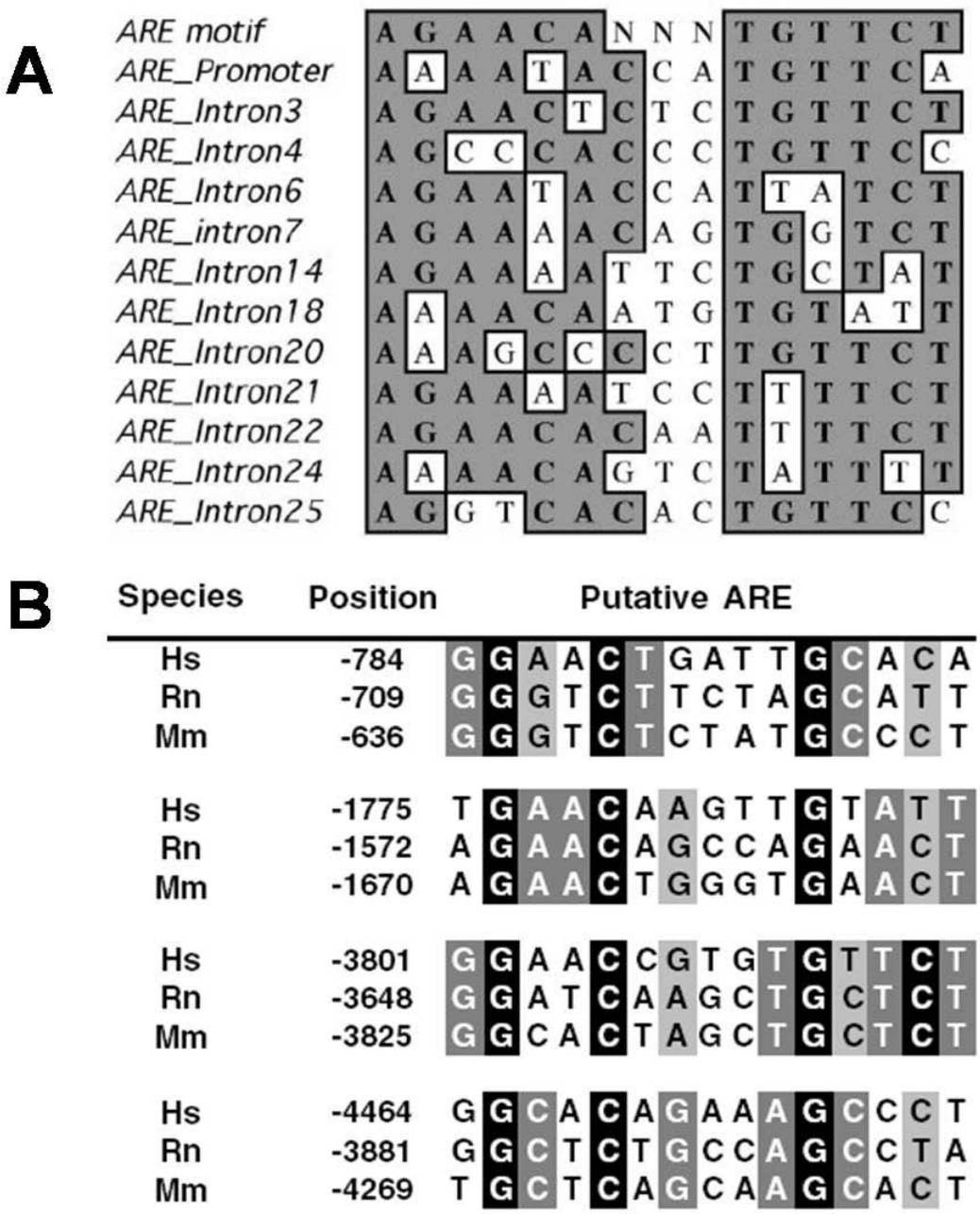


Figure 1. 10 Putative AREs identified in the *TRPM8* gene

A. Study from (Zhang and Barritt 2004). **B.** Search using the ARE motif RKNWCNNNWGHNHW adapted from (Bidaux et al. 2005). The numbers under Position are relative to the transcription start site (which is the 1st base pair)

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

We hypothesized that the AR-regulation of *TRPM8* gene expression is mediated by the AR binding to two sites at the *TRPM8* promoter (Fig 1.11). By binding a proximal promoter region the AR initiates *TRPM8* gene transcription and the transcription will be enhanced by AR binding to an enhancer site in the distal promoter. On each site, there is at least one ARE.

This hypothesis was based on the evidence from previous *TRPM8* studies and the model of AR regulation of the prostate specific antigen (PSA) gene. The loss of *TRPM8* mRNA expression in a prostate cancer xenograft model during the transition to androgen independence and in tissue from patients who underwent anti-androgen treatment has suggested *TRPM8* expression is androgen-dependent (Henshall et al. 2003). In a study carried out by Zhang and Barritt, *TRPM8* expression in an androgen-responsive LNCaP prostate cancer cell line was shown to be androgen-dependent (Zhang and Barritt 2004). A later study by Bidaux *et al* further demonstrated that *TRPM8* expression required functional AR and that its expression is stimulated by androgen (Bidaux et al. 2007;Bidaux et al. 2005). The studies also indicated that the *TRPM8* gene was directly regulated by the AR gene (Bidaux et al. 2005). Bioinformatics analysis of the *TRPM8* gene has also been conducted and

several putative AREs were identified on the *TRPM8* promoter (refer to Fig 1.8) (Bidaux et al. 2005;Zhang and Barritt 2004;Zhang and Barritt 2006).

The expression of PSA protein is known to be directly regulated by the AR and this knowledge has been used for prostate cancer diagnosis. The AR-mediated regulation of the PSA gene serves as a good model since its transcriptional regulation by the AR has been well studied. In this model, the AR regulates PSA gene transcription through binding to two sites on the PSA promoter. The first site is in the proximal promoter, between -156~-396 bp and containing two AREs. The second site, which is in the distal promoter between -3874~-4366 bp and containing 6 AREs, serves as an enhancer (Kim and Coetzee 2004). Among the AREs, there are two strong consensus AREs present in each of the two AR binding sites (Cleutjens et al. 1996;Riegman et al. 1991). The other AREs are weak nonconsensus AREs but needed for optimal androgen inducibility. So based on the above evidence we hypothesized the mechanism of AR regulation of *TRPM8* expression and performed our study on the *TRPM8* promoter.

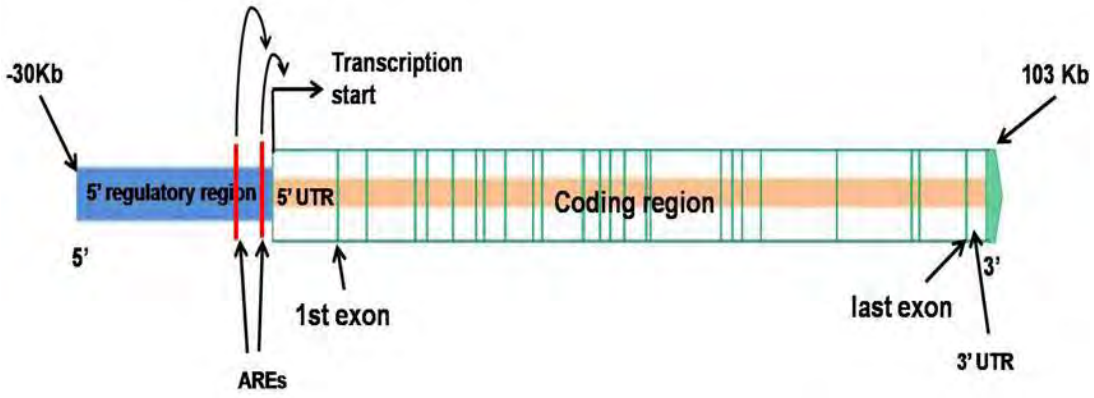


Figure 1. 11 Hypothesized mechanism by which the AR regulates *TRPM8* transcription

Schematically illustrated is the *TRPM8* gene (5' regulatory region, 5' untranslated region, coding region and 3' untranslated region), the proposed locations of the AREs, and how the AREs interact with each other to initiate *TRPM8* gene transcription.

1.7 *TRPM8* expression in prostate cancer

1.7.1 Change in *TRPM8* expression during prostate cancer progression

TRPM8 is expressed at moderate levels in normal prostate but in a range from moderate to high in benign prostate hyperplasia (Tsavaler et al. 2001). Its expression is elevated in early stage tumours (T1 and T2) and decreases to a level comparable to that in normal prostate tissue in advanced stage tumours (T3, T4, N+ or M+) (Fuessel et al. 2003). *TRPM8* expression is lost during the transition to androgen-independent tumours (Bidaux et al. 2005;Henshall et al. 2003).

The expression of *TRPM8* in prostate cancer cell lines has also been investigated. *TRPM8* mRNA and protein are both expressed in androgen-dependent LNCaP cells (Bidaux et al. 2005;Fuessel et al. 2003;Henshall et al. 2003;Kiessling et al. 2003;Tsavaler et al. 2001;Zhang and Barritt 2004). However, its expression in two androgen-insensitive prostate cancer cell lines, PC-3 and DU-145, is controversial. Fuessel *et al* have detected *TRPM8* mRNA in both prostate cancer cell lines (Fuessel et al. 2003). One study has detected functional TRPM8 protein expression in the PC-3 cell line whereas two other studies have not detected *TRPM8* expression in either cell line (Henshall et al. 2003;Tsavaler et al. 2001;Zhang and Barritt 2004).

1.7.2 The relationships between AR activity and *TRPM8* expression

In previous sections we discussed the modifications of the AR gene that increase during the progression of prostate cancer and are possibly related to the stage of prostate cancer and selection pressure from the treatments. Therefore the more advanced a prostate cancer, the higher the deregulation of AR signalling pathways and potentially the higher the AR activities. It is interesting to find that *TRPM8* expression is higher in the early stages of prostate cancer and decreases in more advanced prostate cancer until the loss of expression in the transition to hormone-refractory prostate cancer. So it seems that the descrease of *TRPM8* expression or loss of *TRPM8* expression is an indication of a more deregulated AR signalling (or an indication of highly aberrant AR activities?). How the AR regulates *TRPM8* expression under these conditions is not clear and the physiological mechanisms underlying cancer progression and hormone-refractory cancer transition are not known. More data, such as the correlation between AR activity and *TRPM8* expression in hormone-refractory prostate cancer, is required.

1.7.3 Proposed roles of TRPM8 in prostate cancer progression

It has been proposed that TRPM8 may play a role in the regulation of proliferation, apoptosis and / or inhibiting apoptosis (Prevarskaya et al. 2007c). The increase of

TRPM8 expression in prostate tumour tissues had led to the hypothesis that *TRPM8* was an oncogene (Tsavaler et al. 2001). However, now it does not seem to be likely since TRPM8 may play an important role in normal prostate functions. TRPM8 has also been shown to have a potential pro-proliferative role (Prevarskaya et al. 2007c). Zhang and Barritt have demonstrated TRPM8 is also involved in androgen-dependent LNCaP cell viability although it is still not clear that this role is through a pro-proliferative or anti-apoptotic mechanism (Prevarskaya et al. 2007c;Zhang and Barritt 2004). Recent studies have shown TRPM8 protein expression on both plasma membrane and ER membrane. However, during the dedifferentiation of prostate epithelial cells and the transition of prostate cancer cells from androgen-dependent to androgen-independent, plasma membrane TRPM8 expression is lost (Bidaux et al. 2007;Prevarskaya et al. 2007a;Prevarskaya et al. 2007b;Prevarskaya et al. 2007c;Thebault et al. 2005;Zhang and Barritt 2004). These findings have suggested that TRPM8 may regulate both cell proliferation and apoptosis (Prevarskaya et al. 2007c). The change in the intracellular distribution of TRPM8 may alter the cellular calcium signature that in turn may increase cell proliferation or apoptosis.

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

It has been demonstrated TRPM8 is comparable to PSA as a diagnostic marker for prostate cancer due to its distinct expression patterns among normal prostate cancer, early stage prostate cancer, advanced stage prostate cancer and the transition to the hormone-refractory stage (Fuessel et al. 2003;Henshall et al. 2003;Zhang and Barritt 2006). Since TRPM8 may play an important role in prostate cancer carcinogenesis and progression, it is also a potential pharmaceutical target for treatment. It has been demonstrated that sustained activation of TRPM8 can lead to LNCaP cell death and can inhibit prostate tumour growth in a mouse model (Zhang and Barritt 2004;Zhang and Barritt 2006). TRPM8 was also used as the vaccination target in a Phase I clinical trial that showed promising outcomes (Fuessel et al. 2006).

1.8 Aims

Evidence has shown that TRPM8, an AR-regulated protein, may play an important role in prostate cancer onset and early progression. It is a potential pharmaceutical target to treat prostate cancer as well as a potential diagnostic marker. Since the AR plays a central role in prostate cancer carcinogenesis and progression, it is important to understand the mechanism of AR-regulation of *TRPM8* expression. Therefore the

general aim of this study was to explore how the AR regulates *TRPM8* gene transcription. The specific aims were:

I. Search for androgen-responsive elements in the 6 kb promoter region of *TRPM8*

II. Search for androgen-responsive elements in the 30 kb 5' regulatory region of the *TRPM8* gene by ChIP assay

III. Evaluation of the regulatory function of Androgen Receptor Binding Site 1 using a luciferase reporter-gene assay

Chapter 2

Materials and Methods

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Plasmid vectors

pGL3 Basic vector, pGL3 Control vector, pGL3 Promoter vector, phRL-null vector and pGEM-T EASY vector system I were from Promega (NSW, Australia); pGL-PROBPr-luc was previously constructed by Dr. Lei Zhang.

2.1.2 Reagents, chemicals and other materials

Roswell Park Memorial Institute medium 1640 (RPMI 1640), RPMI 1640 (phenol red free), Lipofectamine 2000, Fetal Bovine Serum (FBS), Opti-Mem I medium, Glycogen, M-MLV Reverse Transcriptase, dNTP set, Rnase Out Ribonuclease inhibitor, Express SYBR Green ER qPCR Supermix Universal, AccuPrime Taq DNA Polymerase High Fidelity, Salmon Sperm DNA, TRIzol reagent and UltraPure distilled water were from Invitrogen (Mt Waverley, Victoria Australia); AR(N-20) antibody and Normal Rabbit IgG were from Santa Cruz Biotech, Inc.(CA, USA); Protein G Sepharose 4 Fast Flow were from GE Healthcare Life Sciences (NSW, Australia); Protease inhibitor cocktail tablets were from Roche Applied Science (NSW, Australia); JM109 Competent cells, Dual-Luciferase Reporter Assay kit and

T4 DNA ligase were from Promega (NSW, Australia); QIAfilter Plasmid Maxi kit, QIAGEN plasmid Midi kit, QIAprep Spin Miniprep Kit, QIAquick Gel Extraction Kit, MinElute PCR Purification Kit, DNeasy Tissue Kit, Proteinase K, RNAse A were from QIAGEN (VIC, Australia); Charcoal/Dextran Treated FBS were from Hyclone (Utah, USA); Agarose, DNAse I, EDTA, Glucose, Glycine, HEPES, Phenol, Penicillin, Transfer Ribonucleic Acid (tRNA, from baker's yeast), Sodium Bicarbonate, Sodium Pyruvate, Streptomycin, TBE buffer, TAE buffer, Triton X-100, Trizma Base were from Sigma-Aldrich (NSW, Australia); all restriction enzymes were from New England Biolabs (Herts, UK)

The source of some other materials and instruments used will be indicated where is appropriate in this thesis.

2.2 Mammalian cell culture

In the following subsections, LNCaP human prostate cancer cells (CRL-1740, obtained from ATCC) were always kept in a 37 °C incubator supplemented with 5% (v/v) CO_2 in appropriate medium as indicated below.

2.2.1 Maintenance of cells

LNCaP human prostate cancer cells were maintaining in 75 mm^2 tissue culture flask (Greiner Bio-One, Germany) in Complete RPMI [RPMI 1640 supplemented with 10% (v/v) FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), sodium pyruvate (110mg/ml), glucose (2.5g/L) and HEPES (10 mM)] (www.atcc.com). Cells were subcultured every six days with ratio of 1: 6 into a new flask.

2.2.2 Androgen deprivation of LNCaP cells for luciferase reporter gene assay

Androgen depleted Complete RPMI used had same component as mentioned above except for the following difference: RPMI 1640 (phenol red free), 10% (v/v) Dextran / Charcoal stripped FBS. For luciferase reporter gene assays, LNCaP cells cultured in androgen depleted Completed RPMI for four days (around 96 hr) in 75 cm^2 tissue culture flask before seeded into 24-well plastic plate (Greiner Bio-One, Germany) and cultured for another two days (around 48 hr) in androgen depleted Complete RPMI (antibiotics free). Androgen treatment (refer to Section 2.2.4) of LNCaP cells was then started. One hour after androgen treatment started, LNCaP cells underwent transfection (refer to Section 2.3).

2.2.3 Androgen deprivation of LNCaP cells for ChIP assay

LNCaP cells were cultured in androgen depleted Completed RPMI for 72 hr in 75 cm^2 tissue culture flask before seeded into a 145 mm (diameter) tissue culture dish (Greiner Bio-One, Germany). They were further cultured for another 72 hr in the same medium. Androgen treatment was then performed (refer to Section 2.2.5).

2.2.4 Androgen treatment of LNCaP cells for luciferase reporter gene assay

For the +DHT treatment, the androgen depleted Complete RPMI was replaced with androgen depleted Complete RPMI (containing 10nM DHT) 1 hr before transfection and continued in culture in the same medium for another 48 hr after transfection. For the –DHT treatment (used as control), the androgen depleted Complete RPMI was replaced with androgen depleted Complete RPMI (containing ethanol) 1 hr before transfection and continued in culture in the same medium for another 48 hr after transfection.

2.2.5 Androgen treatment of LNCaP cells for ChIP assay

For the +DHT treatment, the androgen depleted Complete RPMI was replaced with androgen depleted Complete RPMI (containing 10nM DHT) and continued in culture

for another 4 hr. For the –DHT treatment (used as control), the androgen depleted Complete RPMI was replaced with androgen depleted Complete RPMI (containing ethanol) and cultured for another 4 hr.

2.3 Transient transfection of LNCaP Cells with plasmids

LNCaP cells ($2x10^5$) were seeded into each well of a poly-L-lysine coated 24-well plastic plate and cultured for 48 hr. One hour before transfection, the androgen treatment started (refer to Section 2.2.4) and at the beginning of transfection the volume of medium in each well was adjusted to 500 µl. In the transfection, every well of cells was transfected with same amount of molecule for each plasmid using 1µg of pGL-TRPM8pr-luc as standard. Other plasmids' amounts were adjusted accordingly based on the ratio between their molecule sizes (measured in number of base pairs) and the molecule size of pGL-TRPM8pr-luc (measured in number of base pairs). For every well of cells each of the luciferase plasmids was co-transfected with 0.01 µg of hphRL-null (a Renilla luciferase vector used for transfection efficiency control) were used.

Lipofectamine 2000 was used as Transfection reagent. The ratio of DNA: Lipofectamine 2000 used was 1:2. The preparation of transfection mixture, DNA-Lipofectamine 2000, was following the manufacturer's protocol. 20 min after the

preparation of DNA- Lipofectamine 2000, 100 µl of the DNA- Lipofectamine 2000 were then added into each single well. The cells were then incubated in 37 ^{o}C tissue culture incubator supplemented with 5% (v/v) CO_2 for 6 hr. The medium from each well was then replaced with corresponding fresh medium. 48 hr after transfection the cells were ready for luciferase analysis.

2.4 Competent *E.coli* transformation

Competent *E. coli* (100µl) were gently mixed with up to 10 µl of DNA (1-50 µg, or 0.1 ng for competent cell control plasmid) and incubated on ice for 20 min before a 30-second heat-shock at 42^{o}C. Competent *E. coli* were then further incubated on ice for 5 min before spread onto antibiotics (100 µg/ µl ampicillin) containing agar plates. The agar plates were then incubated at 37^{o}C for overnight (16 hr). For transformation of ligation products of AR Binding Site 1 and pGEM-T EASY, LB agar plates containing IPTG/X-Gal and 100µg/ml ampicillin were used.

2.5 DNA and RNA purification

2.5.1 Genomic DNA purification

LNCaP cells ($5x10^6$) cultured in Complete RPMI in a 37^{o}C incubator supplemented with 5% (v/v) CO_2 were harvested and genomic DNA was purified from them using

DNeasy Tissue Kit following the manufacturer's instructions. Purified LNCaP genomic DNA was kept at 4^{o}C. For long term storage, they were aliquoted into small volume and stored at -20^{o}C.

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

DNA products from PCR reactions or from restriction enzyme reactions which only produced single band analyzed by agarose gel electrophoresis were directly purified by QIAquick Gel Extranction kit followed the manufacturer's instructions with the following modifications: when purifying same type of DNA from multiple tubes, in the elution step the eluate of the first tube can be repeatedly used to elute DNA from the other tubes in order to increase the DNA concentration. However, the volume of elution buffer, buffer EB, needed to be calculated accordingly so that when eluting the last tube the volume of the eluate would be at least 30 µl. DNA products (≤ 4 kb) from the above reactions were purified by MinElute PCR Purification Kit following manufacturer's protocol to increase the concentration of the eluate.

2.5.3 DNA purification from agarose gel

Restriction enzyme digested DNA samples were firstly separated on TAE agarose gel [1% (v/v)] by gel electrophoresis (refer to Section 2.7). After 20-30 min of GelRed (Biotium INC., CA USA) staining, the gel was analysed on Ultraviolet Transilluminator (Ultra-Lum Inc, Carson CA 90746, USA). The required bands were then quickly cut off from the gel under low strength UV light. The gel slices containing needed DNA fragments were then purified by using QIAquick Gel Extranction kit following manufacturer's instruction. The final elution volume was between 30-50 µl.

2.5.4 Plasmid DNA Mini-prep

A single colony of *E.coli* from a selective agar plate was inoculated into 5 ml of selective LB medium and shaking overnight (12-16 hr) at 37 ^{o}C. Volume of 3 to 4 ml of the bacteria was harvested by centrifugation at 6,800 Xg for 3 min at room temperature. The cell pellets were proceeded with QIAprep Spin Miniprep Kit following manufacturer's protocol.

2.5.5 Plasmid DNA Midi-prep or Maxi-prep

Aliquote, 150 µl (Midi-prep) or 250 µl (Maxi-prep), of stater *E.coli* culture from

Section 2.5.4 was diluted to 100ml (Midi-prep) or 250 ml (Maxi-prep) in selective LB medium and was shaked for 12- 16 hr at 37^{o}C. The bacteria were harvested and plasmid DNA was purified by QIAGEN plasmid Midi kit or QIAfilter Plasmid Maxi kit according to manufacturer's protocols.

2.5.6 Total RNA extraction and purification

LNCaP cells from each well of a 6-well plate were lysed in 1 ml of TRYzol reagent and following manufacturer's protocol to purify total RNA which is briefly described below. After lysis in 1 ml TRYzol reagent in a 1.5 ml eppendrof tube, 200 µl of chloroform was added followed by vortexing and centrifugation at 12,000 Xg. The top aqueous layer was transferred into a new 1.5 ml eppendrof tube followed by DNA precipitation by adding 500ul of isopropyl alcohol. The DNA pellets were then wash twice by 1 ml of 75% ethanol (pre-chill at -20℃ for 1hr) followed by air dry at room temperature for 10 min. The final RNA pellets were dissolved in 30 µl of UltraPure distilled water.

2.6 DNA and RNA quantification and purity assessment

The DNA or RNA quantification was carried out on NanoDrop 8000 (Thermo Scientific) by loading 2µl of each sample for each detection following

manufacturer's instruction. The purity of samples was assessed by ratio of UV absorbance of A260/A280 (considered as pure if ≥ 1.8 for DNA or ≥ 2.0 for RNA) and A260/A230 (considered as pure if between 1.8 and 2.2).

2.7 Agarose gel electrophoresis

A particular percentage (w/v) TBE (or TAE) agarose gel was prepared by dissolving appropriate amount of agarose into 35-40 ml of 1X TBE (or 1X TAE) buffer. DNA sample was prepared by mixing 100- 500 ng of DNA, 2 µl 6 X gel loading dye and appropriate volume of 1X TBE (or 1X TAE) buffer to make up total volume of 12 µl. DNA samples were then loaded into the gel and run for 1 hr at 110 V in 1X TBE buffer for TBE agarose gels, or for 1.5 hr at 80 V in 1X TAE buffer for TAE agarose gels. The gel was then staining in 3X GelRed stain (Biotium INC., CA USA) for 25-30 min before analysis by ImageMaster VDS-CL (Amersham Pharmacia Biotech, now GE Healthcare Life Sciences) under the UV light following manufacturer's instructions.

2.8 PCR

2.8.1 Primers and oligos

All the oligo and primers were synthesized by GeneWork (SA, Australia)

2.8.1.1 Oligos for reverse transcription

Oligo $(dT)_{21}$: 5'-TTTTTTTTTTTTTTTTTTTTT-3'

Random HexPrimer primer

2.8.1.2 qPCR primers for Human *TRPM8* mRNA amplification

htrpm8qS1(1547-1556 bp, exon 12): 5'-ACGCTTGTGTACCGGAATCT-3'

htrpm8qa1(1693-1713 exon12 &13): 5'-CGAGTAATAGGAGACACGTCG-3'

The amplicon size: 167 bp. The positions are relative to the first nucleotide of the reference human *TRPM8* mRNA sequence, NM_024080.4 (from NCBI)

2.8.1.3 qPCR primers for Human *HPRT* mRNA amplification

hHPRTqS (465-493 bp, exon 3 & 4):

5'-ATCAGACTGAAGAGCTATTGTAATGACCA-3'

hHPRTqA (673-695 bp, exon 7):

5'-TGGCTTATATCCAACACTTCGTG-3'

The amplicon size: 231 bp. The positions are relative to the first nucleotide of the reference human *HPRT*1 mRNA sequence, NM_000194.2

2.8.1.4 qPCR primers for ChIP assay-qPCR

The design, validation procedure of the qPCR primers is described in Chapter 4. The primer details refer to Appendix 1

2.8.1.5 PCR primers for amplifying 6 kb *TRPM8* promoter

hTRPM8pro-1Sense:

5'-TATATCCCGGGAATCTGTATAAGGGCCTACAAAGTCCT-3'

hTRPM8pro-1Antisense:

5'-ATAATCCCGGGTTGTCAAGCAGGATTTTCTTTCACCCAA-3'

The amplicon size was 6084 bp amplifying *TRPM8* promoter from -6042 to +20 relative to transcription start site.

2.8.1.6 PCR primers for amplifying AR Binding Site 1

ARBS_1_S: 5'-TTGTGGCATTTGTCAATACGATGA-3'

ARBS_1_An: 5'-CAGATTCCTCTGCTACATGTCACAA-3'

The amplicon size: 1298 bp.

2.8.2 Reverse transcription

Reverse transcription (RT) was carried out by using 1 µg of DNAse I treated total RNA from each sample. RT was performed either immediately after the step of deactivation of DNAse I at 70^{o}C for 10 minutes or after the RNA being thawed from -80^{o}C followed by denaturation at 70^{o}C for 10 min. RT was carried out using M-MLV Reverse Transcriptase following manufacturer's protocol which is briefly described below. 1 µg of DNAse I treated total RNA was mixed with 0.5 µl of Oligo $(dT)_{21}$ (100µM), 0.5 µl of Random HexPrimer (100uM), 1 µl of dNTPs (20mM), 0.5 µl of RNAse Out (RNAse inhibitor), 2 µl DTT (0.1 M) and 4 µl of 5X first-strand buffer in a final reaction volume of 20 µl. The mixture was incubated at 37^{o}C for 50 min followed by the deactivation of Reverse Transcriptase at 70^{o}C for 15 min.

2.8.3 Conventional PCR

2.8.3.1 PCR amplifying 6 kb *TRPM8* promoter

The PCR was prepared with 1 unit of AccuPrime Taq DNA Polymerase High Fidelity, 5µl of 10X buffer II, 5µl of LNCaP genomic DNA (from Section 2.5.1), 200µM of primers (hTRPM8pro-1Sense and hTRPM8pro-1Antisense) and appropriate volume of UltraPure distilled water to a final volume of 50µl. This mixture was then overlaid with mineral oil. The PCR condition was: 1 cycle at 94^{o}C

for 2 min followed by 40 cycles of 94^{o}C for 20 sec, 55^{o}C for 30 sec, 68^{o}C for 8 min; with a final enlongation at 68^{o}C for 7 min.

2.8.3.2 PCR amplifying Androgen Receptor Binding Site 1

The PCR was prepared with 1 unit of AccuPrime Taq DNA Polymerase High Fidelity, 5µl of 10X buffer II, 5µl of LNCaP genomic DNA (from Section 2.5.1), 200µM of primers (ARBS_1_S and ARBS_1_An) and appropriate volume of UltraPure distilled water to a final volume of 50µl. This mixture was then overlaid with mineral oil. The PCR condition was: 1 cycle at 94^{o}C for i min followed by 40 cycles of 94^{o}C for 20 sec, 58^{o}C for 30 sec, 68^{o}C for 2 min.

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

The qPCR was performed by mixing 2µl of cDNA, 10µl Express SYBR Green ER qPCR Supermix Universal, 200nM primers and appropriate volume of UltraPure distilled water to a final volume of 20 µl and running on Rotor Gene 6000 (QIAGEN) realtime PCR machine. The qPCR condition: 1 cycle at 50^{o}C for 2 min, 1 cycle at 95^{o}C for 2 min followed by 40 cycles of 95^{o}C for 15 sec and 60^{o}C for 1 min. The condition for melting curve: ramping from 60^{o}C to 95^{o}C, 1^{o}C a step and wait for 5 sec in each step. Raw data was processed in Rotor Gene 6000 software by

'comparative quantitation' method under the 'Analysis' function. The 'Take off ' cycle number from each sample was exported as an Excel file and then imported into Q-Gene (Muller et al. 2002;Simon 2003) software for further calculation. In Q-Gene, house keeping gene *HPRT* mRNA amount from each sample was used to normalize the *TRPM8* mRNA amount from the corresponding sample using the equation, $NE = \frac{(E_{hprt})^{Ct_{hprt}}}{(E_{trpm8})^{Ct_{trpm8}}}$ (NE, normalized gene expression; E_{hprt}= qPCR amplification efficiency of *HPRT* gene; E_{trpm8}= qPCR amplification efficiency of *TRPM8* gene; Ct_{hprt}= threshold cycle number of the qPCR amplification of *HPRT* gene, here we used 'take off' cycle number of *HPRT* gene; Ct_{trpm8}= 'take off' cycle number of *TRPM8* gene). The mean value of the triplicated 'NE' from each sample was used as final value shown in Chapter 3.

2.8.4.1 Determination of qPCR amplification efficiency of *TRPM8* gene and *HPRT* gene

In order to determine the qPCR amplification efficiency of each gene, a standard curve (log amount of cDNA VS mean Ct value) of each gene was generated (Fig 2.1 B). Detail of each step is described below.

A pool of series dilutions (6.25, 12.5, 25, 50, 100, 200, 400X based on the total RNA concentration) of control cDNA (generated from LNCaP cells cultured in Complete

RPMI) was created. qPCRs for *TRPM8* gene and *HPRT* gene were performed using this cDNA pool. An Excel file containing 'take off' cycle number of each sample was exported from Rotor Gene 6000 software as mentioned above. The 'take off' values were then imported into Q-Gene (Fig 2.1 A) to generate standard curves of *TRPM8* gene and *HPRT* gene (Fig 2.1 B). The amplification efficiencies of the two genes were then calculated from the slopes (k) of the standard curves using the equation, $E = 10^{\frac{-1}{k}}$.

2.8.4.2 qPCR analysis of *TRPM8* and *HPRT* mRNA quantity

cDNA concentration (25X dilution) had been chosen from each sample as template to perform qPCR using both *TRPM8* primers and *HPRT* primers. The qPCR condition was mentioned above. After the qPCR run was completed, the melting curve was also generated for quality control purpose. The 'take off' cycle number of each sample was obtained and imported into Q-Gene and the final normalized *TRPM8* mRNA amounts were calculated (Fig 2.2). For each run of qPCR, a plate control was used to monitor the amplification efficiency of each qPCR run so that only the data from the qPCR runs having same amplification efficiency were chosen for normalized *TRPM8* mRNA calculation. The plate control used 6.25X and 12.5X dilutions from the dilution pool of control cDNA. When comparing the plate controls of two qPCR runs, only when the difference between two mean 'take off' cycle

numbers for 6.25X dilution or for 12.5X dilution was within 1, respectively, we deemed the two qPCR runs have same amplification efficiency.

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

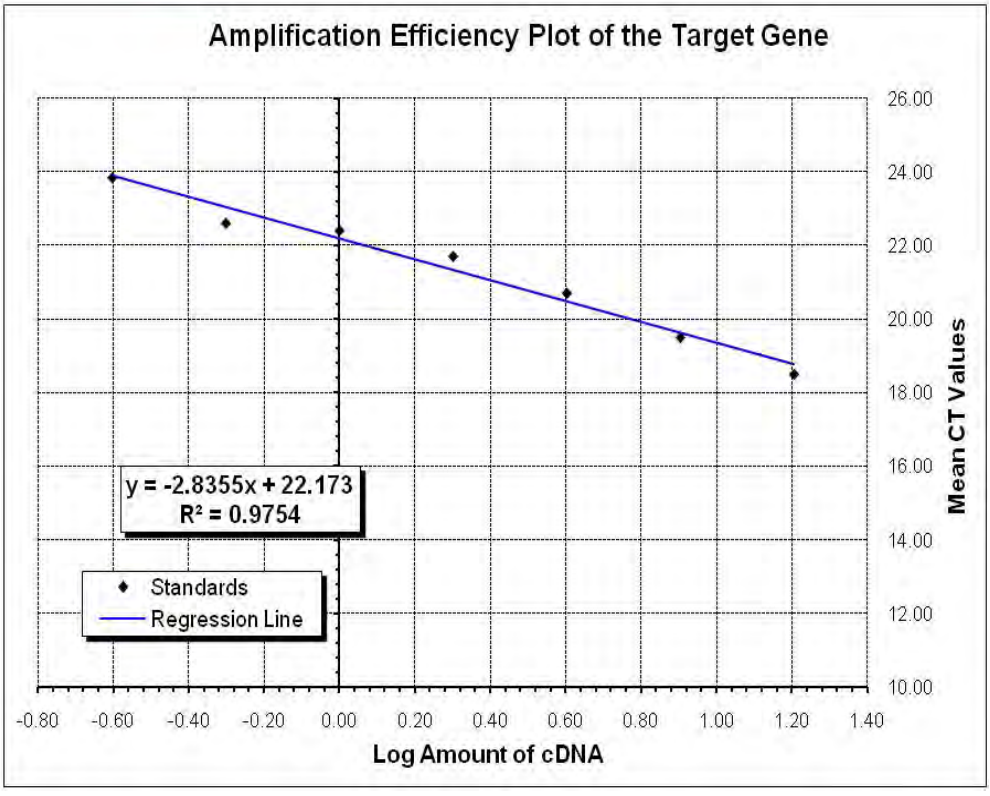
A. Data table for standard curve. *CT Values* column used 'take off' cycle number. *Amount of cDNA* column, used the amount RNA, unit was ng. *Mean CT Values* column, used average value of the triplicates in the *CT Values* column. **B.** Amplification efficiency plot of the target gene. The amplification efficiency was calculated using slope value, -2.8335.

A

Description	CT Values	Amount of cDNA	Mean CT Values	Log Amount of cDNA
trpm8 6.25X	18.5 18.6 18.4	16.00	18.50	1.20
12.5X	19.4 19.5 19.6	8.00	19.50	0.90
25X	20.6 20.7 20.8	4.00	20.70	0.60
50X	21.6 21.8 21.7	2.00	21.70	0.30
100X	22.3 22.4 22.5	1.00	22.40	0.00
200X	22.4 22.6 22.8	0.50	22.60	-0.30
400X	23.7 23.8 24	0.25	23.83	-0.60

B

Slope: -2.8355 E_{target} = 2.2525



A

Well	Description	CT of Target Gene	CT of Reference Gene	Normalized Expression
A1	P1_1	25.2	24.6	1.45E+02
A2		25.5	24.5	1.02E+02
A3		25.5	24.5	1.02E+02
A4	P1_2	24.5	25.1	4.29E+02
A5		24.5	25.2	4.76E+02
A6		24.6	25.1	3.96E+02
A7	P1_3	22.9	23.5	3.01E+02
A8		22.9	23.5	3.01E+02
A9		22.8	23.5	3.26E+02

B

Mean of Triplicates			
Description	Mean Normalized Expression	SE of Mean Normalized Expression	SE of Mean Normalized Expression in %
P1_1	1.17E+02	1.42E+01	12.15
P1_2	4.34E+02	2.33E+01	5.37
P1_3	3.09E+02	8.49E+00	2.74

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

A. The Calculation of normalized *TRPM8* mRNA quantities. *CT of Target Gene* column, 'take off' cycle number of *TRPM8* gene. *CT of Reference Gene* column, 'take off' cycle number of *HPRT* gene. *Normalized Expression* column, normalized *TRPM8* mRNA quantities. **B.** The mean value of the normalized *TRPM8* mRNA quantity. *Mean Normalized Expression* column, mean value of the normalized *TRPM8* mRNA quantity. The mean was calculated using the equation, MNE= (NE1+NE2+NE3)/3.

2.8.5 qPCR analysis of DNA from ChIP Assay

2.8.5.1 Performing qPCR

The qPCR was performed using Express SYBR Green ER qPCR Supermix Universal kit on a Rotor Gene 6000 Machine. The qPCR condition was same as the one mentioned in Section 2.8.4.2. The melting curve was also generated after each run of qPCR for each PCR sample. For each sample, 2µl of DNA from each of the fractions: anti-AR immunoprecipitation fraction, normal rabbit IgG immunoprecipitation fraction and Input fraction, was used as template to perform qPCR with each primer pair, respectively. For each primer pair, two qPCR runs were needed to amplify all the samples. The plate control used DNA from the Input fraction from biological replicate 1 undergone +DHT treatment.

2.8.5.2 Raw data processing

Raw data was processed by Rotor Gene 6000 software, melting curve of each PCR sample from each primer pair was checked to make sure only same single product was amplified in each reaction. This was judged by peak number (=1), peak temperature (less then 1^{o}C difference). The threshould for each PCR run was manually set and Ct value was obtained by 'Quantitation Analysis' method in 'Analysis' function of the software. The mean Ct values of plate control samples

between two qPCR runs of each set were compared. Only the samples from each set of two qPCR runs with the Ct difference of plate control within 1 cycle were chosen.

2.8.5.3 Further data processing with an Excel SuperArray ChIP-qPCR Data Analysis Template

The Ct value of each sample was exported into to the Excel SuperArray ChIP-qPCR Data Analysis Template (SA Biosciences, USA) to calculate the relative DNA quantity of each amplicon from each immunoprecipitation fraction. This was expressed as a percentage of that from Input fraction (% Input). This was calculated using the following two equations: 1) % Input =$(2^{-\Delta Ct})$X100%; 2) ΔCt= Ct[IP] - Ct[Input X DF] (Ct[IP], Ct values from immunoprecipitation fractions; Ct[Input X DF], Ct values from Input fractions minus 6.644 since Input fraction has been diluted 100X more than the other fractions as mentioned above, which equals to 6.644 cycles ($\log_2$ 100=6.644).)

2.8.5.4 Final data presentation

The mean % Input value of each amplicon of a primer pair from anti-AR immunoprecipitation fractions of samples undergone either +DHT or –DHT treatment, respectively, was used for final data presentation. The mean % Input

values of the same amplicon were compared between +DHT and –DHT treatment. The ratio of them, +DHT /–DHT, was shown as fold enrichment to demonstrated the enrichment of each amplicon in +DHT treatment compared to –DHT treatment. This represented the enrichment of genomic DNA tile that the amplicon was on.

2.9 Enzymatic reactions on DNA or RNA

2.9.1 DNAse I treatment of total RNA

Aliquoted 5µg of total RNA from each sample and treated with DNAse I according to manufacturer's protocol which is briefly described below. 5 µg of total RNA were mix with 2 µl of DNAse I and 2 µl of 10X buffer in a total volume of 20 µl. The mixture was then incubated at room temperature for 15 min. After incubation, the DNAse I was deactivated by addition of 2 µl of Stop Solution and a further incubation at 70^{o}C for 10 min. The sample was then chilled on ice and stored at -80^{o}C.

2.9.2 Restriction enzyme digestion

Restriction enzyme digestions were carried out by mixing 0.5-1 µg of DNA with 10 to 20 units of restriction enzyme in appropriate volume of 10X restriction enzyme buffer and UltraPure distilled water to final volume of either 10µl or 50 µl followed

by 37^{o}C incubation for 2-4 hr. The restriction enzymes were deactivated at appropriate temperature for 15 min according to the manufacturer's instructions (some restriction enzymes do not have deactivation temperature).

2.9.3 DNA ligation

The recipient vectors undergone restriction enzyme digestion were firstly purified. The purified vectors were firstly dephosphorylated (except for pGEM-T EASY vector) by shrimp alkaline phosphatase (United States Biochemical, Ohio, USA) following manufacturer's instruction followed by DNA purification before ligation. During ligation 50-100ng of purified linearized plasmid vector or 50 ng of pGEM-T EASY vector and appropriate amount of insert DNA were mixed at 1:1 molar ratio (amount of insert DNA was calculated using the equation: $\frac{ng\ of\ vector\ X\ Kb\ size\ of\ insert}{Kb\ size\ of\ vector} X\ molar\ ratio\ of\ \frac{insert}{vector} = ng\ of\ insert$) together with 1 µl of 10X ligase buffer and appropriate amount of UltraPure distilled water to a final volume of 10 µl. Ligation was performed at room temperature for 3 hr or 4^{o}C overnight followed by inactivation at 70^{o}C for 10 min.

2.9.4 DNA recirculization

The recirculization was carried out by mixing around 140 ng of *pfu* blunted plasmid DNA (Section 2.9.5), 1 unit of T4 DNA ligase, 5µl of 10X ligase buffer, and appropriate amount of UltraPure distilled water to a final volume of 50 µl and incubated at 18 to 20 ^{o}C for 16 hr followed by inactivation at 70^{o}C for 10 min.

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

DNA blunting was carried out by adding 1.3 Unit of *pfu* (Fermentas Life Sciences, Ontario, Canada) into 300 ng of purified restriction enzyme digested DNA together with 5 µl of 10 X *pfu* buffer, 200µM of dNTP mix and appropriate volume of UltraPure distilled water to a final volume of 50 µl. Overlay with 30 µl of mineral oil and incubated in Thermal Sequencer (FTS-1, Corbett Research) for 30 min at 37 ^{o}C.

2.10 Construction of luciferase reporter vectors

The maps of the luciferase reporter vectors mentioned below refer to Appendix 2.

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

pGL-TRPM8pr-luc and pGL-R-TRPM8pr-luc were constructed by the following

steps. 1) PCR amplifying 6 kb *TRPM8* promoter (-6042 to +20 relative to transcription start site) from LNCaP cell genomic DNA. The PCR primer introduced a restriction enzyme *XmaI* site on each end of the PCR product; 2) restriction enzyme *XmaI* digestion of both the PCR product and recipient vector pGL3 Basic; 3) ligation of the PCR product and pGL3 Basic. 4) Competent E.coli transformation of ligation products. 5) E.coli colony screening using restriction enzyme mapping to select colonies containing correct plasmids, respectively.

Two deletion constructs, pGL-TRPM8pr-luc-*KpnI*/*NheI*-del and pGL-TRPM8pr-luc-*KpnI*/*AflII*-del, were generated from pGL-TRPM8pr-luc through the following steps: 1) restriction enzyme *KpnI* and *NheI* digestion or *KpnI* and *AflII* digestion to remove a 16 bp *KpnI* / *NheI* fragment or a 5954 bp *KpnI*/*AflII* fragment from pGL-TRPM8pr-luc, respectively; 2) DNA purification to remove the 16 bp *KpnI* / *NheI* fragment or the 5954 bp *KpnI*/*AflII* fragment from the reaction mixture; 3) recirculization of the plasmids; 4) transformation of competent E.coli; 5) colony screening by DNA sequencing (performed by SouthPath and Flinders Sequencing Facility). The methods for the techniques for each step are described below.

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

pGEM-T EASY-ARBS1 was generated through the following steps. 1) PCR amplification of AR Binding Site 1 from LNCaP cell genomic DNA. 2) Ligation of pGEM-T EASY (a linear vector with 3'-T overhang) and PCR amplified AR Binding Site 1 fragment (a step of addition of 5'-A overhang was perform prior to ligation).3) *E.coli* transformation with the ligation products. 4) Colony screening for colonies containing the correct plasmid by DNA sequencing.

pGL-ARBS1EN/SV40-luc, pGL-R-ARBS1EN/SV40-luc and pGL-R-ARBS1EN/TRPM8pr-luc were constructed in the following steps: 1) restriction enzyme *NotI* digestion of pGEM-T EASY-ARBS1 to release a 1334 bp *NotI*/*NotI* fragment containing the AR Binding Site 1, *NotI*-ARBS1-*NotI* ; 2) restriction enzyme *NotI* digestion of the recipient vectors, pGL3 Promoter and pGL-TRPM8pr-luc; 3) ligation of *NotI*-ARBS1-*NotI* and pGL Promoter or *NotI*-ARBS1-*NotI* and pGL-TRPM8pr-luc; 4) competent *E.coli* transformation of the ligation products; 5) colony screening to select the colonies containing the correct plasmids by restriction enzyme mapping (details refer to Chapter 5).

2.11 Luciferase assay

Dual-Luciferase Reporter Assay kit was used to perform luciferase assay. The firefly luciferase was firstly measured by the addition of Luciferase Assay Reagent II (LARII). This generated a stabilized luminescent signal. Following the addition of Stop & Glo reagent to the same sample, the firefly luciferase reaction was quenched, simultaneously initiated Renilla luciferase reaction and generated a stabilized signal. The chemistry of firefly luciferase reaction and Renilla luciferase reaction is shown in Fig 2.3. The procedure for luciferase assay included the following steps: reagent preparation, cell lysis, luminescence measurement and data process. These will be described in details below.

2.11.1 Reagent preparation

The reagents used included 1X Passive Lysis Buffer (PLB), LARII and Stop & Glo Reagent. 1X PLB was used for cell lysis. It was prepared by diluting 1 volume of 5X PLB in 4 volumes of distilled water and stored at 4 ^{o}C until use. LARII was used to generate luminescence from firefly luciferase. It was prepared by resuspending the lyophilized Luciferase Assay Substrate in Luciferase Assay Buffer II. Stop & Glo Reagent was used to quench the firefly luciferase reaction and generated luminescence from Renilla luciferase reaction. It was prepared by diluting one volume of 50X Stop & Glo Substrate in 50 volumes of Stop & Glo Buffer.

2.11.2 Cell lysis

At 48 hr after transfection, LNCaP cells in each well of a 24-well plate were lyzed using 150-200 µl of 1X Passive lysis buffer for 30 min on a shaking platform at room temperature. 20 µl of the lysate were transferred into each well of an OptiPlate-96 (96-well plate from Perkinelmer) for following luminescence measurement.

2.11.3 Luminescence measurement for firefly luciferase and Renilla luciferase activity

The luminescence was measured by Topcount NXTv2.12 (Perkinelmer) with the following key parameters: Assay Type using luminescence, Count Time was 10 sec and counting once for each well and each plate. 50 µl of the LAR II were firstly added into each well where the lysate was.The luminescent from firefly luciferase was then measured. 50 µl of the Stop & Glo reagent was then added into each well where the LAR II was added. The luminescent from Renilla luciferase was measured.

2.11.4 Data processing

The unit of luminescence value was count per second (CPS). The luminescence values of firefly luciferase were normalized by the luminescence values of renilla luciferase. The final data presentation was shown as normalize firefly luciferse.

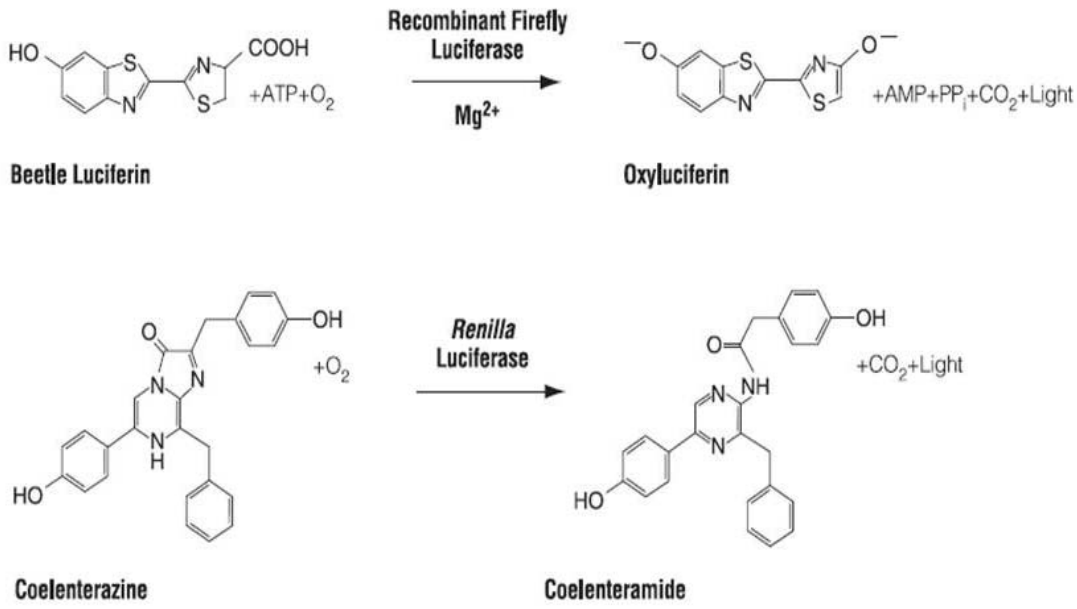


Figure 2. 3 Chemistry of firefly luciferase and Renilla luciferase reactions

Adapted from Dual-Luciferase Reporter Assay System Technical Manual (Promega Part# TM040)

2.12 ChIP assay

The original protocol was kindly provided by Buchanan *et al* (Molecular Ageing Laboratory, University of Adelaide) and had been modified. The main modifications were listed below: 1) optimizing sonication condition to suite the sonicator we used; 2) scaling up the amount of LNCaP cells to use and adjusting the amount of other reagents used accordingly. ChIP assay included several steps: cell treatment; crosslinking; cell lysis and sonication; immunoprecipitation; elution and reverse of crosslinking; DNA purification (Fig1.7 B, a schematic diagram for ChIP assay).

2.12.1 Cell treatment

LNCaP cell line was chosen. In total, 20 dishes of LNCaP cells were used. Two androgen treatments, +DHT and -DHT treatment, were carried out (The androgen treatment refers to Section 2.2.5) with 10 dishes of cells for each treatment. In each treatment, 5 biological replicates were included with 2 dishes of cells pooled as a biological replicate.

2.12.2 Crosslinking

After androgen treatment, the medium was removed followed by the addition of 20 ml of 1% formaldehyde and 10 min room temperature incubation. The crosslinking

was stoped by the addition of 1 ml of glycine (2.5 M). The crosslinking by formaldehyde fixed ARs on chromatin intracellularly at the sites they bound to exert their transactivation functions. The fixation formed chromatin-AR complex.

2.12.3 Cell lysis and sonication

After the crosslinking, each two dishes of cells with same androgen treatment were 2X washed with ice cold 1X PBS, scraped with 16 ml of ice cold 1X PBS, and pooled together in a 50 ml centrifuge tube. Cells were pelleted by 500Xg centrifugation at 4^{o}C followed by cell lysis. Cell lysis was performed by resuspending cell pellets in 700 µl of 1% SDS Lysis buffer, transferring into a 1.5 ml eppendorf tube and incubating on ice for 10 min. Cell lysis released the chromatin from cells and the chromatin was subsequently sheared into smaller pieces by sonication.

Sonication was performed on Misonix Sonicator S-4000 (Qsonica, LLC., CT USA). The samples were sonicated in ice-water bath in the same eppendorf tubes where they were lysed. The sonication used microtip with following condition: energy level of 39 % amplitude, 3 seconds on and 1 min off time with total on time of 1 min. The sonication resulted in smaller chromatin-AR complex.

2.12.4 Immunoprecipiation

After sonication, centrifugation was performed to remove the cell debris. Cell lysate containing chromatin from each tube (each sample) was collected and split into two fractions: Input fraction (25 µl) and immunoprecipitation fraction (500µl, was later further split into two 250 µl fractions as listed below). Input fraction was reserved and stored at -20^{o}C without undergoing immunoprecipitation. It was later used in qPCR analysis as control to normalize the values from immunoprecipitation fraction of the same sample. The immunoprecipitation fraction was diluted 10 times in dilution buffer followed by preclearing for 1 hr using 360µl of Protein G Sepharose 4 Fast Flow blocked by 160 µl of tRNA.

The precleared immunoprecipitation fraction from each sample was then further split into two equal halves (each half containing 250 µl of original cell lysate): 1) anti-AR immunoprecipitation fraction; 2) normal rabbit IgG immunoprecipitation fraction (used for background control for unspecific immunoprecipitation and subsequent unspecific binding of Sepharose beads). By this step, each of the cell lysate was split into three fractions (anti-AR immunoprecipitation fraction, normal rabbit IgG immunoprecipitation fraction and Input fraction). The anti-AR immunoprecipitation fraction from each sample was then incubated with 26µg of anti-AR antibody (AR (N-20), raised in rabbit) overnight (16 hr) on a shaking platform at 4^{o}C. The normal

rabbit IgG immunoprecipitation fraction from each sample was incubated with 26µg normal rabbit IgG under the same condition. During the overnight incubation, the majority of chromatin-AR complex bound to AR (N-20) and formed chromatin-AR-AR (N-20) complex.

The next day, 180 µl of blocked (by 80 µl of tRNA) Protein G Sepharose 4 Fast Flow was added to each of the immunoprecipitation fraction and rotated for 1 hr at 4^{o}C. During this step, the chromatin-AR-AR (N-20) complex bound to Sepharose beads.

2.12.5 Elution and reversal of crosslinking

Protein G Sepharose 4 Fast Flow from each tube was gently pelleted by centrifugation at 5000Xg followed by one wash with 1 ml of low salt, high salt and LiCl immune complex wash buffer, respectively. Two washes with 1X TE buffer were then performed. Each of the above washes was performed at 4^{o}C for 5 min on a rotator followed by a gentle centrifugation at 5000Xg to pellet the Sepharose beads.

The elution step was performed by resuspending the Protein G Sepharose 4 Fast Flow in each tube with 350µl of elution buffer and incubating on a room temperature rocking platform for 15 min. This was repeated once and two batches of eluate from

one tube were combined. This eluted chromatin-AR complex from Sepharose beads. At this step, the reserved Input fraction was thawed and 700 µl of elution buffer was added.

The crosslinking of the chromatin in each tube was subsequently reversed by the addition of 28 µl of NaCl (5M) followed by 65^{o}C incubation for overnight (16 hr). This separated the chromatin from chromatin-AR complex.

2.12.6 DNA purification

After the reverse of crosslinking, the sample in each tube was treated by RNAse If (New England Biolabs, UK) at 37^{o}C for 1 hr followed by 1 hr Proteinase K (Qiagen) treatment at 45^{o}C. DNA was subsequently extracted by phenol/chormoform and precipitated by pure ethanol together with Glycogen (used as DNA carrier). The DNA pellets were washed once with 70% ethanol followed by airdry.

The dried DNA pellets were then resuspended and dissolved in 120 µl of UltraPure distilled water. DNA from immunoprecipitation fractions were further diluted 2.5X in UltraPure distilled water. DNA from Input fractions were further diluted 25X in UltraPure distilled water. This made the final concentration of DNA from Input fraction 100 times more diluted than that from the immunoprecipitation fractions.

2.13 Statistical analysis

The data have been expressed as mean ± standard deviation (SD) of the number of biological replicates indicated. Degrees of significance between two groups were determined using paired Student's T-test.

Chapter 3

Search for Androgen-Responsive Elements in the 6 kb Promoter Region of *TRPM8*

CHAPTER 3: SEARCH FOR ANDROGEN-RESPONSIVE ELEMENTS IN THE 6 KB PROMOTER REGION OF *TRPM8*

3.1 Introduction

TRPM8 has been suggested to be involved in prostate cancer carcinogenesis and early progression (Bidaux et al. 2007;Prevarskaya et al. 2007a;Prevarskaya et al. 2007c;Tsavaler et al. 2001;Zhang and Barritt 2004). The *TRPM8* mRNA level is elevated in early stage prostate cancer tissue (Henshall et al. 2003). Since AR plays a central role in prostate cancer onset and progression (Li and Al-Azzawi 2009) and TRPM8 is directly regulated by AR (Bidaux et al. 2007;Bidaux et al. 2005;Zhang and Barritt 2004), the elevation of *TRPM8* mRNA levels in the early stage of prostate cancer indicates some earlier events that have modified the regulation of *TRPM8* gene transcription. However, the mechanism underlying the regulation of *TRPM8* gene transcription by AR is still largely unknown. As an AR regulated gene, sequences surrounding the *TRPM8* gene should contain AREs to which AR can bind and exert its regulatory function. The promoter region of the *TRPM8* gene has been analysed for putative AREs using computer software to detect consensus ARE motifs. Five putative AREs were identified (Bidaux et al. 2005;Zhang and Barritt 2004). However, none of these putative AREs had been tested.

In this chapter, the aim was to look for AREs in the promoter region of the *TRPM8* gene. This was done by inserting the 6 kb *TRPM8* promoter DNA into a luciferase reporter vector. The androgen activation of the ARE on *TRPM8* promoter can be assessed by induced luciferase activity. In order to do this, it was first necessary to identify likely AREs in the promoter region and thereby decide the region of the promoter sequence to study. It was also very important to know the endogenous *TRPM8* gene expression pattern and hence how the *TRPM8* promoter responds to androgen deprivation and to androgen addition over time. In this chapter, putative AREs have been identified in a consensus cross-species bioinformatics search, the region of the promoter for further study was defined, conditions for androgen activation and deactivation of *TRPM8* gene expression studied, the *TRPM8* promoter inserted into a luciferase expression system and the response to androgen investigated.

3.2 Results

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

In order to identify putative AREs, up to 6 kb of the *TRPM8* promoter sequence from human (NT_005120.15), chimpanzee (NC_006470.2) and olive baboon (DP000516.1) were analysed using Vector NTI Advance (Invitrogen) and the

commonly used TRANSFAC ARE motif (AGAACAnnnTGTTCT) (Zhang and Barritt 2006). Five putative AREs were identified in human *TRPM8* promoter with high homology to TRANSFAC motif (Fig 3.1 A & B). Cross species analysis of the 6 kb 5' flanking region of the *TRPM8* gene from human, chimpanzee and baboon showed four out the five putative AREs were conserved (Fig 3.1 C) across the three species and the genomic location of these four conserved putative AREs were similar across the three species (Fig 3.1 D). Therefore, it was considered probable that at least one functional ARE was likely to be among these four putative AREs. However, validation using the luciferase reporter gene assay was required.

3.2.2 Androgen induced endogenous *TRPM8* gene expression

Based on the predictions above, the 6 kb *TRPM8* promoter contains at least one ARE. We had predicted that under the control of a 6 kb *TRPM8* promoter, luciferase gene expression should show similar expression patterns as that of endogenous *TRPM8* mRNA. Therefore it was important to first obtain information on the pattern of *TRPM8* gene expression, then use this information to optimize cell culture and androgen treatment conditions to be applied in luciferase reporter gene experiments.

The first study addressed the decrease in endogenous *TRPM8* expression during androgen deprivation. This was done by measuring *TRPM8* mRNA levels at time

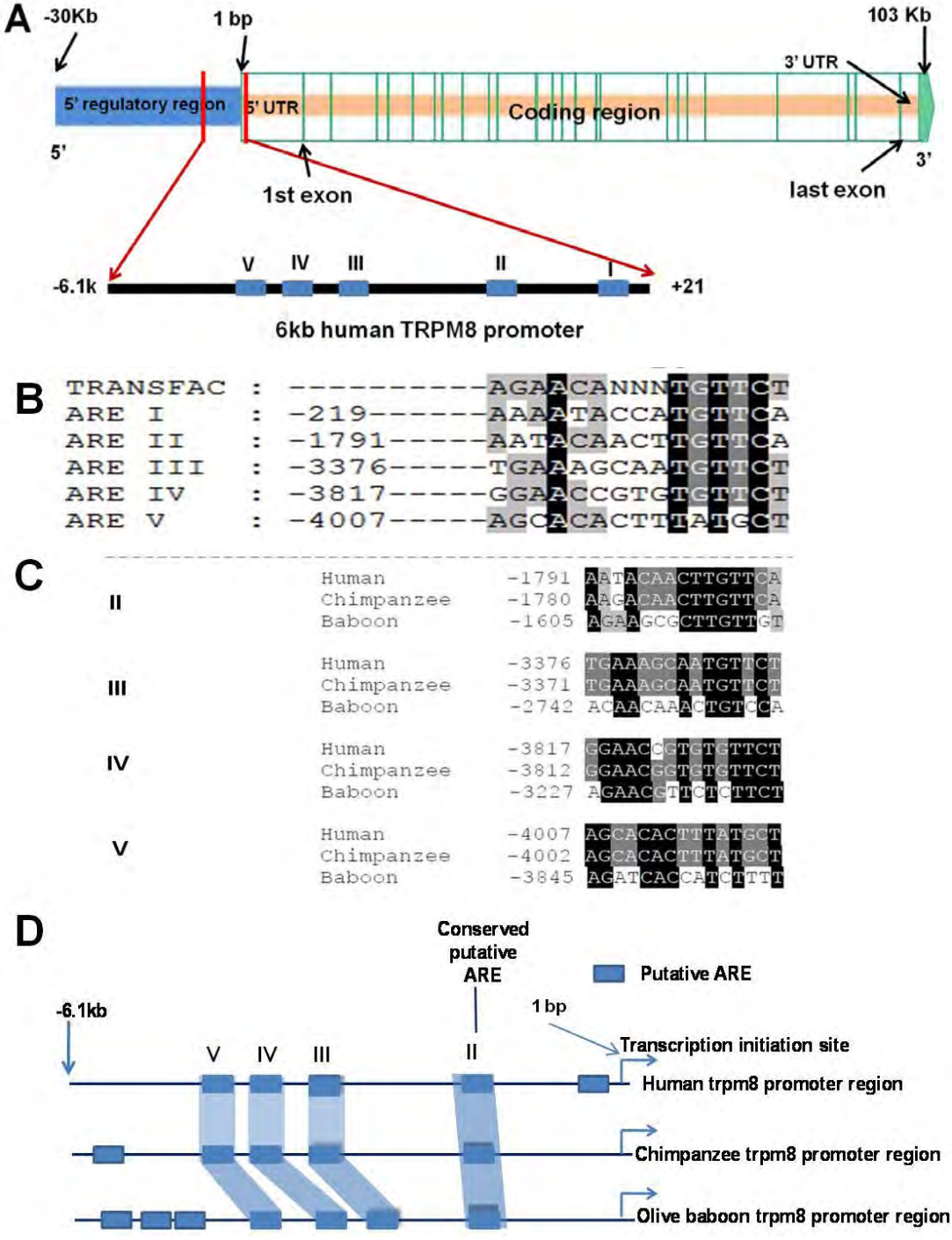
points: 0 hr, 48 hr, 96 hr, and 144 hr after the removal of androgen from the media. *TRPM8* mRNA levels were analysed by RT-qPCR. The human hypoxanthine guanine phosphoribosyl transferase (*HPRT*) mRNA levels from the same samples of each time point were used as internal normalization controls. The *TRPM8* mRNA from LNCaP cells cultured in Complete RPMI medium was used as an external control. The normalized *TRPM8* mRNA from each sample was finally expressed as the percentage of the external control. The results (Fig. 3.2) showed that under androgen depletion, the *TRPM8* mRNA level continued to drop to a basal level (2.5±0.5%) at 144 hr. A sharp decrease (14.9±3.4%) of the *TRPM8* mRNA level occurred within the first 48 hr period.

The second investigation was a time course of the restoration of endogenous *TRPM8* expression levels by re-addition of androgen to LNCaP cells that were cultured in androgen depleted Complete RPMI for 6 days. This was done by measuring the *TRPM8* mRNA amount at time points, 0 hr, 12 hr, 24 hr, 48 hr, 72 hr, and 96 hr after re-addition of androgen (10 nM DHT). *TRPM8* mRNA levels were analysed by RT-qPCR. *TRPM8* mRNA from LNCaP cells cultured in complete RPMI medium was used as an external control. The *TRPM8* mRNA quantity from each sample was expressed as the percentage of the control. The data (Fig 3.3) showed that the *TRPM8* mRNA level rose continuously with two fluctuations: one between 12 hr and 48 hr, and the other between 72 hr and 96 hr. A sharp rise (from 2.0±1% to 47.7±4%)

occurred at 12 hr. At 72 hr the *TRPM8* mRNA level was restored to 79.9±10.5% of the control. The fluctuations detected caused a relative lower quantity of *TRPM8* mRNA at 48 hr compared to 12hr and 24 hr, and a lower quantity at 96 hr compared to 72 hr.

Figure 3. 1 Informatics analysis for putative AREs

The TRANSFAC consensus ARE motif was used for the putative ARE search. **A.** Schematic view of human *TRPM8* gene and its 30kb 5' regulatory region. The 6 kb promoter is indicated between the two vertical red lines and its expanded view is in the lower panel. The 5 blue horizontal bars indicate identified putative AREs. **B.** Five putative AREs identified in the 6kb 5' region of the human *TRPM8* gene using the TRANSFAC ARE motif. **C.** Cross species analysis of the five putative AREs identified four conserve putative AREs across three species. **D.** A schematic diagram showing the location of the conserved AREs in the *TRPM8* gene across three species.


A
-30Kb
1 bp
103 Kb
3' UTR
5' regulatory region
5' UTR
Coding region
5'
3'
1st exon
last exon
V
IV
III
II
I
-6.1k
+21
6kb human TRPM8 promoter
B
TRANSFAC : ----------AGAACANNNTGTTCT
ARE I : -219------AAAATACCATGTTCA
ARE II : -1791-----AATACAACTTGTTCA
ARE III : -3376-----TGAAAGCAATGTTCT
ARE IV : -3817-----GGAACCGTGTGTTCT
ARE V : -4007-----AGCACACTTTATGCT
C
II
Human -1791 AATACAACTTGTTCA
Chimpanzee -1780 AAGACAACTTGTTCA
Baboon -1605 AGAAGCGCTTGTTGT
III
Human -3376 TGAAAGCAATGTTCT
Chimpanzee -3371 TGAAAGCAATGTTCT
Baboon -2742 ACAACAAACTGTCCA
IV
Human -3817 GGAACCGTGTGTTCT
Chimpanzee -3812 GGAACGGTGTGTTCT
Baboon -3227 AGAACGTTCTCTTCT
V
Human -4007 AGCACACTTTATGCT
Chimpanzee -4002 AGCACACTTTATGCT
Baboon -3845 AGATCACCATCTTTT
D
Conserved putative ARE
Putative ARE
-6.1kb
1 bp
V
IV
III
II
Transcription initiation site
Human trpm8 promoter region
Chimpanzee trpm8 promoter region
Olive baboon trpm8 promoter region

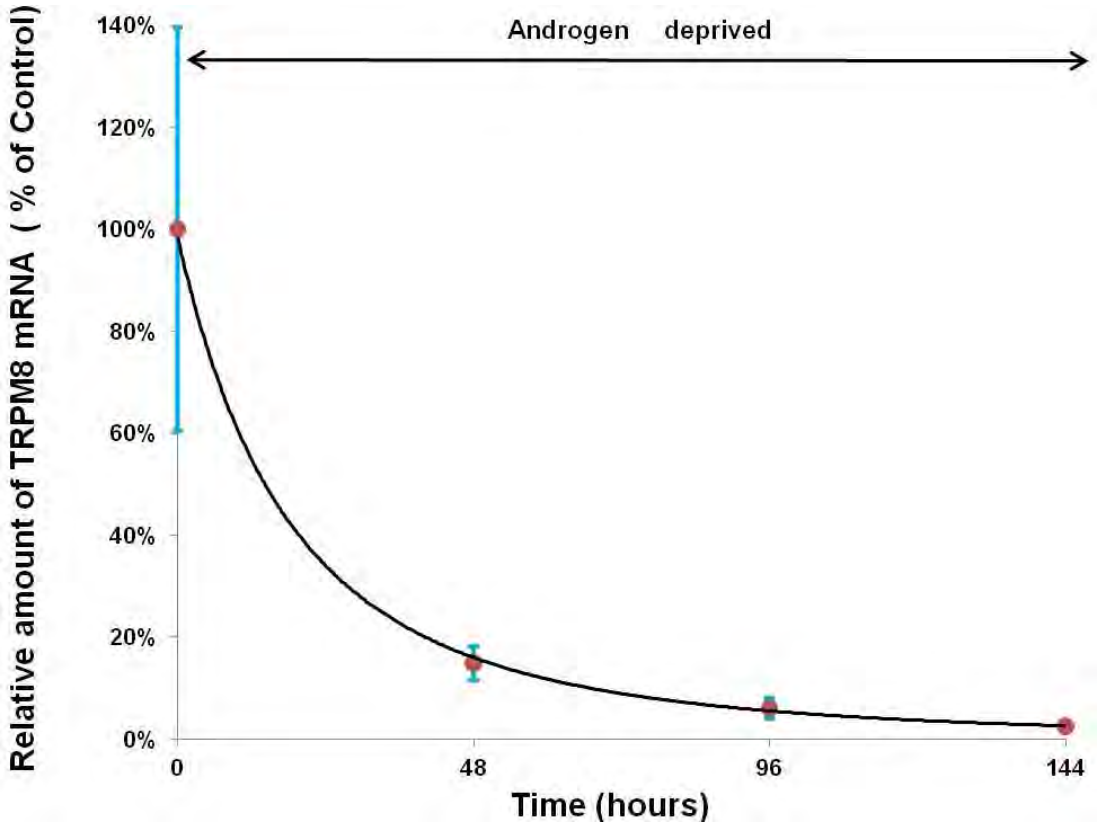


Figure 3. 2 Time course showing a decrease in *TRPM8* mRNA level in LNCaP cells following removal of androgen

LNCaP cells were cultured in androgen depleted Complete RPMI for up to 144 hr. *TRPM8* mRNA levels in LNCaP cells was analysed at 0, 48, 96 and 144 hr. For the 0 hr time point 5×10^6 LNCaP cells cultured in Complete RPMI were harvested for analysis. For the 48 hr time point 2×10^6 LNCaP cells were seeded into each well of a 6-well plate together with androgen depleted Complete RPMI and incubated for 48 hr before harvested. For the96 and 144 hr time points LNCaP cells were initially cultured in androgen depleted Complete RPMI in 75 cm^2 tissue culture flasks for 48 or 96 hr, respectively. 2×10^6 LNCaP cells from each flask were then seeded in each well of a 6-well plate with the same medium and cultured for another 48 hours followed by cell harvest and analysis. The data shown are expressed as Mean±SD, n=3. This graph is representative of two experiments.

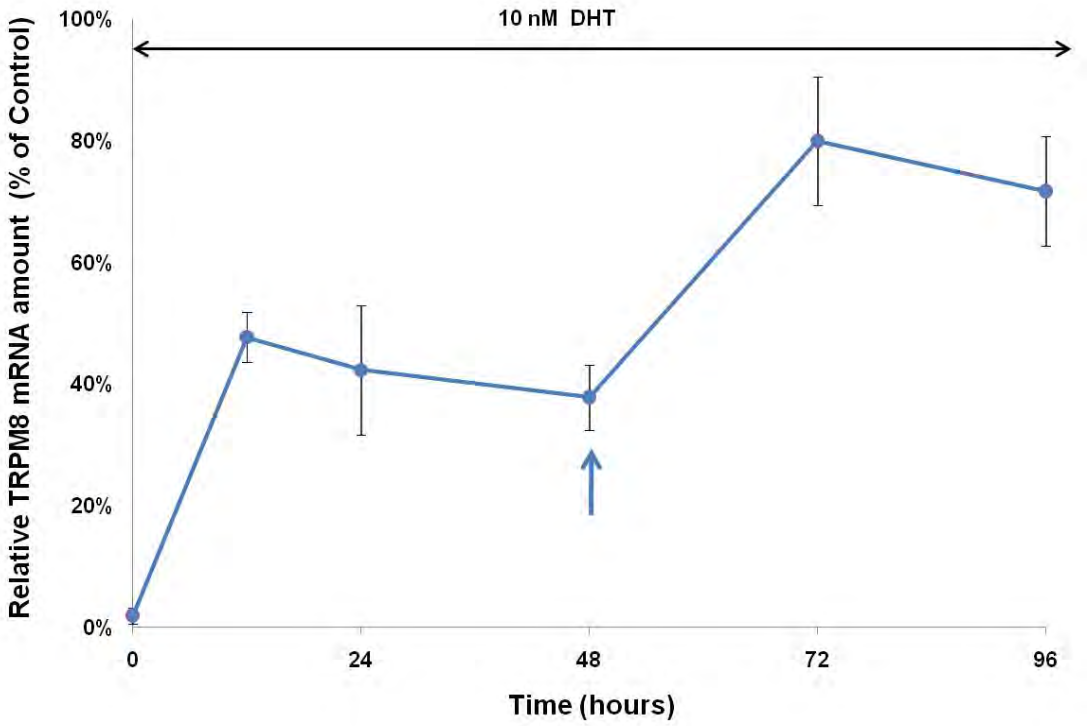


Figure 3. 3 Time course showing the induction of *TRPM8* mRNA by 10 nM DHT in androgen deprived LNCaP cells

The 0 hr time point represents the *TRPM8* mRNA level in LNCaP cells cultured in androgen depleted Complete RPMI for 144 hr. Medium was replaced with androgen depleted Complete RPMI (containing 10 nM DHT) and LNCaP cells were cultured for up to 96 hr. *TRPM8* mRNA levels in LNCaP cells were analysed at 0, 12, 24, 48, 72 and 96 hr. LNCaP cells were cultured in androgen depleted Complete RPMI for 96 hr before 1.5×10^5 of these cells were seeded in each well of a 6-well plate. At the 0 hr time point, LNCaP cells was harvested for analysis before medium was replaced with RPMI containing 10 nM DHT. The remaining LNCaP cells were then harvested following the addition of 10 nM DHT at the chosen time points for analysis. The blue arrow indicates the time point when cell culture medium was replaced. Data shown are expressed as Mean±SD, n=3. This graph is representative of two experiments.

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

We further compared how the quantity of *TRPM8* mRNA changes among cells cultured in steroid-free growth medium for 48, 96 or 144 hr followed by re-exposure to 10 nM DHT for 48 hr. The results (Fig. 3.4) showed that 48 hr of androgen deprivation prior to 48 hr androgen treatment gives only a 1.8±0.4 fold change, while androgen deprivation for 96 hr gives a bigger change of 6.9±2.6 fold ($p< 0.05$). The largest change, 10.5±2.3 fold ($p<0.01$) occurred following 144 hr of androgen deprivation prior to the 48 hr androgen treatment. This demonstrated that given the same androgen treatment, different androgen deprivation time causes different *TRPM8* mRNA induction. A 48 hr androgen depletion gave no statistically significant induction of *TRPM8*, whereas 144 hr of androgen depletion gave the highest induction with $p< 0.01$. Based on our prediction, under the control of the *TRPM8* promoter, luciferase gene expression pattern was expected to be similar. Thus 144 hr prior to 48 hr 10 nM DHT treatment would be expected to give a good induction of luciferase reporter gene expression.

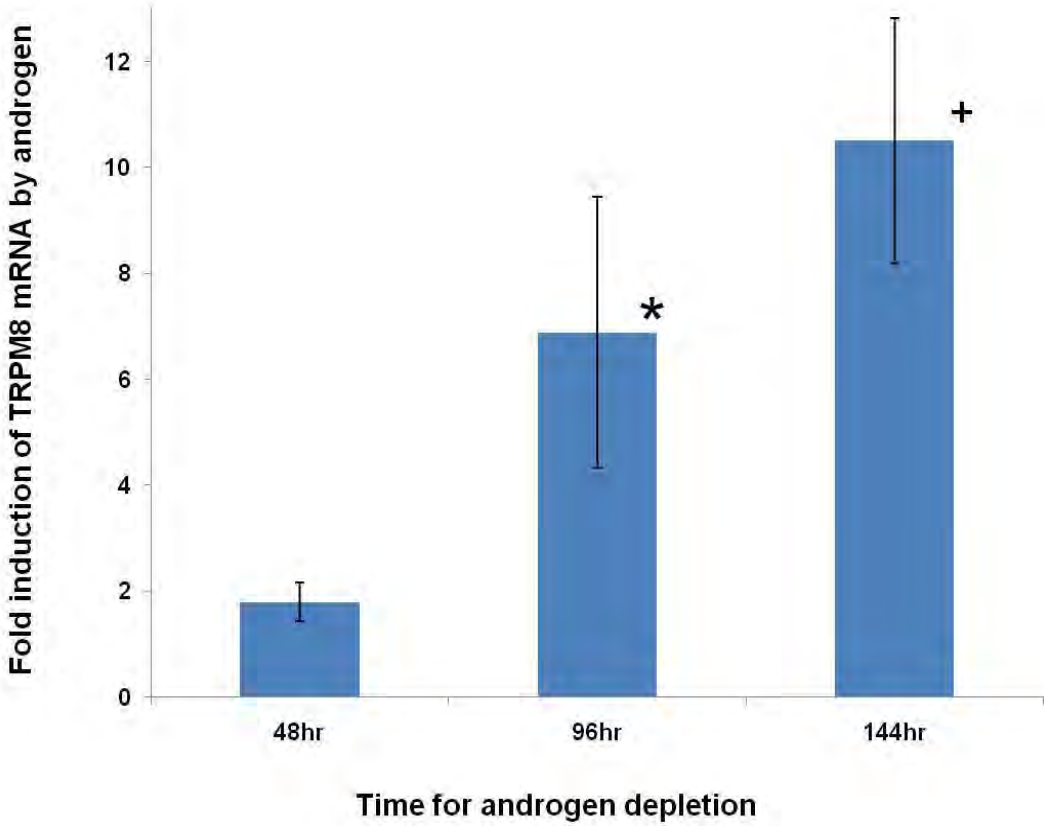


Figure 3. 4 Effect of different times for androgen depletion on the induction of *TRPM8* expression in LNCaP cells

LNCaP cells cultured in androgen depleted Complete RPMI for 48 hr, 96 hr or 144 hr followed by incubation for 48 hr with10 nM DHT. Fold change was 1.8, 6.9, and 10.5, respectively. Data shown are expressed as Mean±SD, n=3. Data are representative of two experiments. *, $p<0.05$. +, $p< 0.01$

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

3.2.4.1 Overall strategy

There were two stages employed for the luciferase reporter gene assays using the 6 kb *TRPM8* promoter. 1) Luciferase analysis of the androgen responsiveness of the whole 6 kb *TRPM8* promoter. 2) If androgen responsiveness was present, serial deletions within the promoter insert were created to locate the androgen responsive region. For confirmation of putative AREs, point mutations were to create within each ARE to nullify their potentiating effects. The first stage can be divided into three steps: 1) construction of the *TRPM8* promoter containing luciferase reporter gene plasmids; 2) transfection of LNCaP cells with the plasmids followed by androgen treatment; 3) cell harvest and luciferase expression analysis. Since we did not observe androgen responsiveness using the 6 kb TRPM8 promoter, we did not enter the second stage. We will further discuss the findings from the first stage of experiments.

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

The purpose of constructing pGL-TRPM8pr-luc, containing 6.1 kb of *TRPM8* promoter inset in the clockwise orientation, was to investigate the androgen responsiveness of the 6.1 kb promoter. The deletion construct, pGL-TRPM8pr-luc-*KpnI*/*AflII*-del, was generated by deleting a putative E-Box and all five putative AREs, and leaving a 128 bp core *TRPM8* promoter (containing a putative TATA box and transcription start site) for further testing of the *TRPM8* promoter's response to androgen. It has been reported that a putative E-box, at the *KnpI*/*NheI* site in the recipient vector backbone may cause false positives in the luciferase assays upon androgen stimulation (Annicotte et al. 2001). Therefore pGL-TRPM8pr-luc-*KpnI*/*NheI*-del was constructed by removing the E-box to control for the putative E-box false positive effect. Along with the negative control plasmid, pGL3 Basic, another negative control plasmid using pGL-R-TRPM8pr-luc with the *TRPM8* promoter insert in the opposite orientation was also constructed. Since promoter activity is orientation-dependent, pGL-R-TRPM8pr-luc should exhibit no *TRPM8* promoter activity.

pGL-TRPM8pr-luc was constructed by inserting the 6.1kb promoter region of the *TRPM8* gene (-6043/+21 amplified by PCR) into the *XmaI* site of the pGL3 Basic vector (Promega) upstream of the luciferase gene in correct orientation (schematic demonstration is shown in Fig 3.5 A). Orientation and correct insertion were confirmed by restriction enzyme mapping (gel images see Fig 3.7 A & B). *XmaI* digestion was used to indentify plasmids with the correct 6.1 kb *TRPM8* promoter insert; *XbaI* and *HindIII* digestions were used to identify the orientation of the *TRPM8* promoter. Their expected restriction fragments were as follows:

1) *XmaI* digestion: two bands at 6.1 kb and 4.8 kb.
2) *XbaI* digestion: two bands at 6.2kb and 4.7 kb.
3) *HindIII* digestion: three bands at 6.1 kb, 4.7 kb and 94 bp

The deletion plasmid, pGL- TRPM8pr-luc-*KpnI*/*NheI*-del (designated as pGL-TRPM8-minEbox), was obtained by deleting a 16 bp *KpnI*/*NheI* fragment (see schematic illustration in Fig 3.6 A) containing a putative E-box. The construction of pGL-TRPM8pr-luc-*KpnI*/*AflII*-del, designated as pGL-TRPM8-minARE, was acheived by deleting a 5954 bp region containing the putative E-box and all five putative AREs. This left 128 bp (-108/+21) of the *TRPM8* promoter in the construct (see schematic demonstration in Fig 3.6 B). The two deletion plasmids were

confirmed by sequencing using sequencing primers RVprimer 3 and GLprimer 2 (Promega), respectively.

The method of construction of pGL-R-TRPM8pr-luc was essentially the same as for pGL-TRPM8pr-luc. The only difference was the reverse orientation of the *TRPM8* promoter within it (schematic map see Fig 3.5 B). The correct insert and orientation was confirmed using restriction enzyme mapping (gel image see Fig 3.7 C & D). After confirming by *XmaI* digestion (refer to *XmaI* digestion for pGL-TRPM8pr-luc above) that the *TRPM8* promoter insert was correct, the orientation was confirmed using *HindIII* and *XbaI* digestion. The expected restriction fragment lengths were as follows:

1) *XbaI* digestion: two bands at 7.6 kb and 3.3 kb.
2) *HindIII* digestion: three bands at 4.9 kb, 4.7 kb and 1.4 k b.

The recipient vector pGL3 Basic was also used as a negative control. The positive control construct used was pGL-PROBpr-luc ,which contains three copies of partial rat probasin promoter region (-244 to -96 relative to the transcription start site) in a head to tail configuration (Zhang et al 2003).

3.2.4.3 Transfection of LNCaP cells, androgen treatment and luciferase analysis

Each of the plasmids mentioned above was co-transfected into LNCaP cells together with a Renilla luciferase plasmid, phRL-null, which was used as a transfection efficiency control. After transfection, LNCaP cells, deprived of androgen for 6 days, were further incubated for 48 hr with either 10 nM DHT-containing medium (+DHT treatment) or medium containing the same volume of ethanol (-DHT treatment). Cells were then harvested and luciferase analysed. Measured firefly luciferase values were normalized by Renilla luciferase values. LNCaP cells transfected with pGL-PROBpr-luc showed 1457 fold luciferase induction in the +DHT group compared to the –DHT group. This showed the luciferase reporter assay system was working and had reasonable sensitivity. In Fig 3.8 A, pGL-TRPM8pr-luc transfected LNCaP cells only showed 1.8-fold ($p > 0.5$) induction of luciferase with +DHT treatment compared to –DHT treatment which means insignificant androgen responsiveness. The results of the deletion construct, pGL-TRPM8-minEbox, did not demonstrate any response to androgen. The other deletion construct, pGL-TRPM8-minARE, did not show any androgen response either. However, we have interestingly found that pGL-TRPM8-minARE transfected cells showed an increase in luciferase activity in an androgen-independent manner, i.e. this appeared in both +DHT and –DHT groups (see Fig 3.8 B)

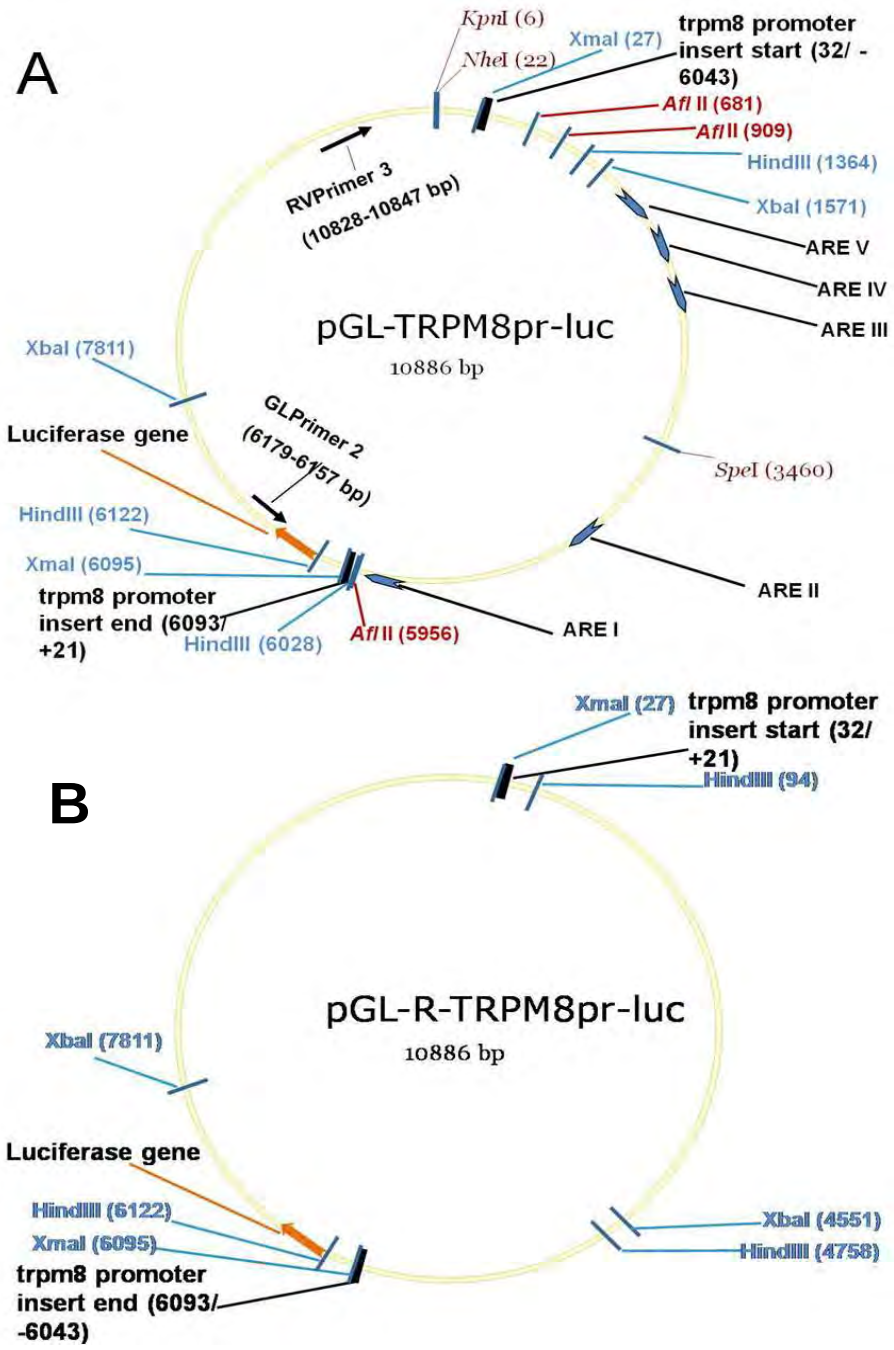


Figure 3. 5 Schematic illustration of the plasmid maps of pGL-TRPM8pr- luc and pGL-R-TRPM8pr-luc

The locations of the *TRPM8* promoter, luciferase reporter gene, putative AREs, restriction enzyme sites for mapping the correct insert and orientation, and restriction enzyme sites for the deletion constructs are shown. **A.** Map of pGL-TRPM8pr-luc. **B.** Map of pGL-R-TRPM8pr-luc. *Number in brackets*, location on the plasmid relative to the start of the sequence. For *TRPM8* promoter start and end sites, the 2nd number refers to the genomic location relative to the transcription start site.

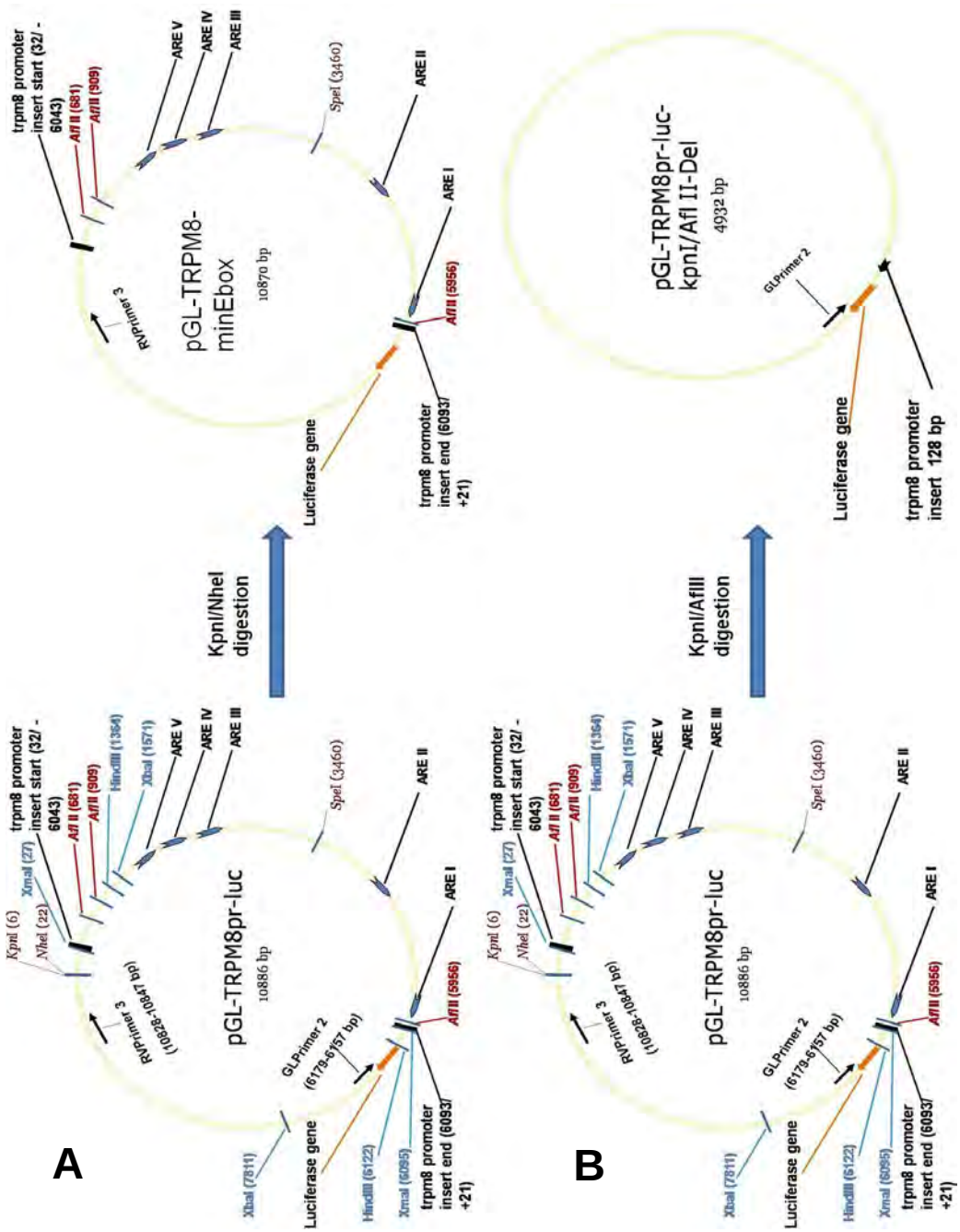


Figure 3. 6 Schematic illustration of the construction of pGL-TRPM8pr-luc-*KpnI*/*NheI*-del and pGL-TRPM8pr-luc-*KpnI*/*AflII*-del

A. Construction of pGL-TRPM8pr-luc-*KpnI*/*NheI*-del. B. Construction of pGL-TRPM8pr-luc-*KpnI*/*AflII*-del.

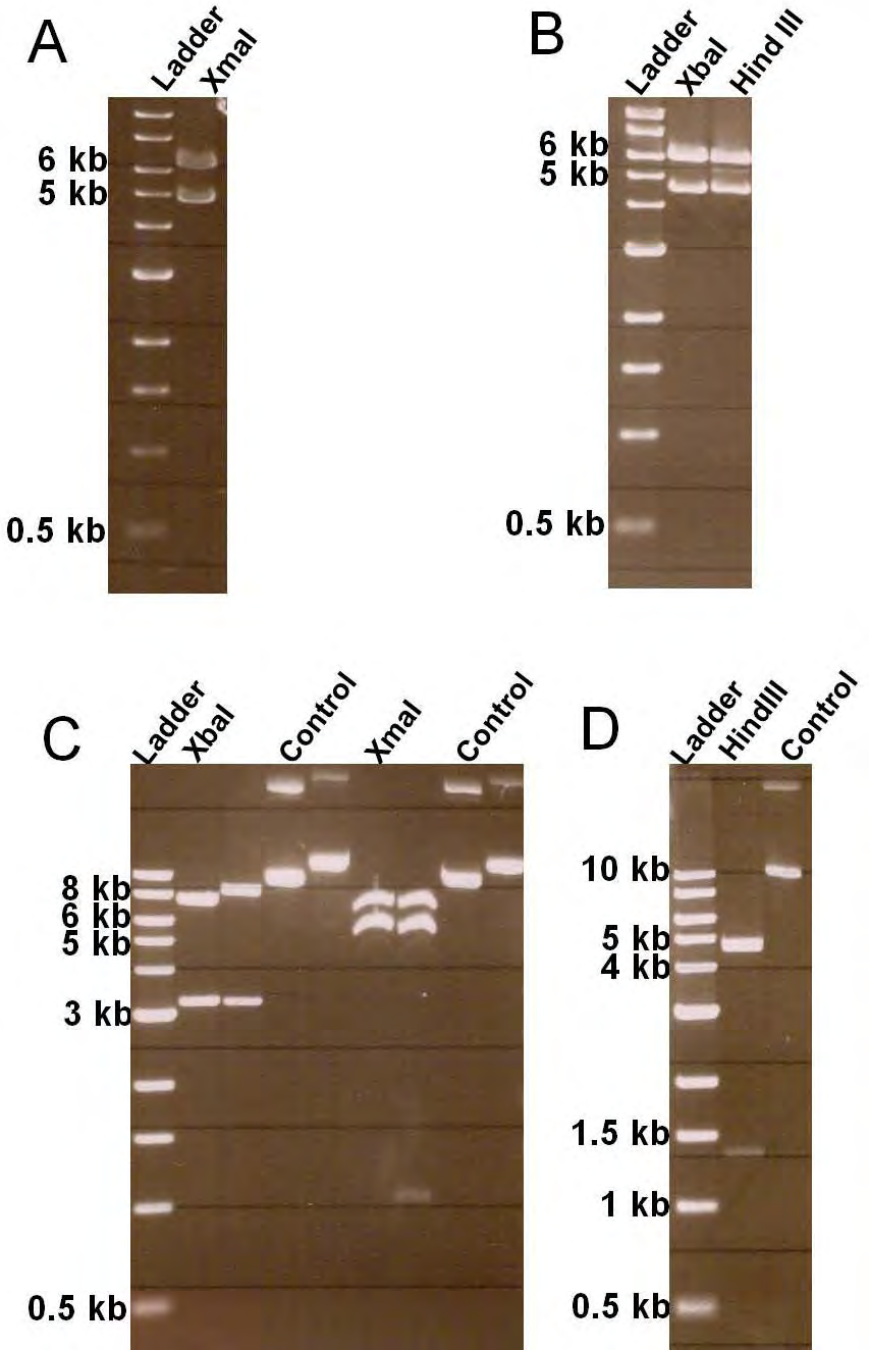


Figure 3. 7 Agarose gel image of restriction enzyme mapping for pGL-TRPM8pr-luc and pGL-R- TRPM8pr-luc

A. *XmaI* digestion for pGL-TRPM8pr-luc. **B.** *XbaI* and *HindIII* digestions for pGL-TRPM8pr-luc. **C.** *XmaI* and *XbaI* digestion for pGL-R-TRPM8pr-luc. **D.** *HindIII* digerstion for pGL-R-TRPM8pr-luc. *Ladder*, 1 kb DNA size ladder. Enzyme names refer to the corresponding restriction endonuclease. *Control*, non-digested circular plasmid sample. Only relevant lanes are marked.

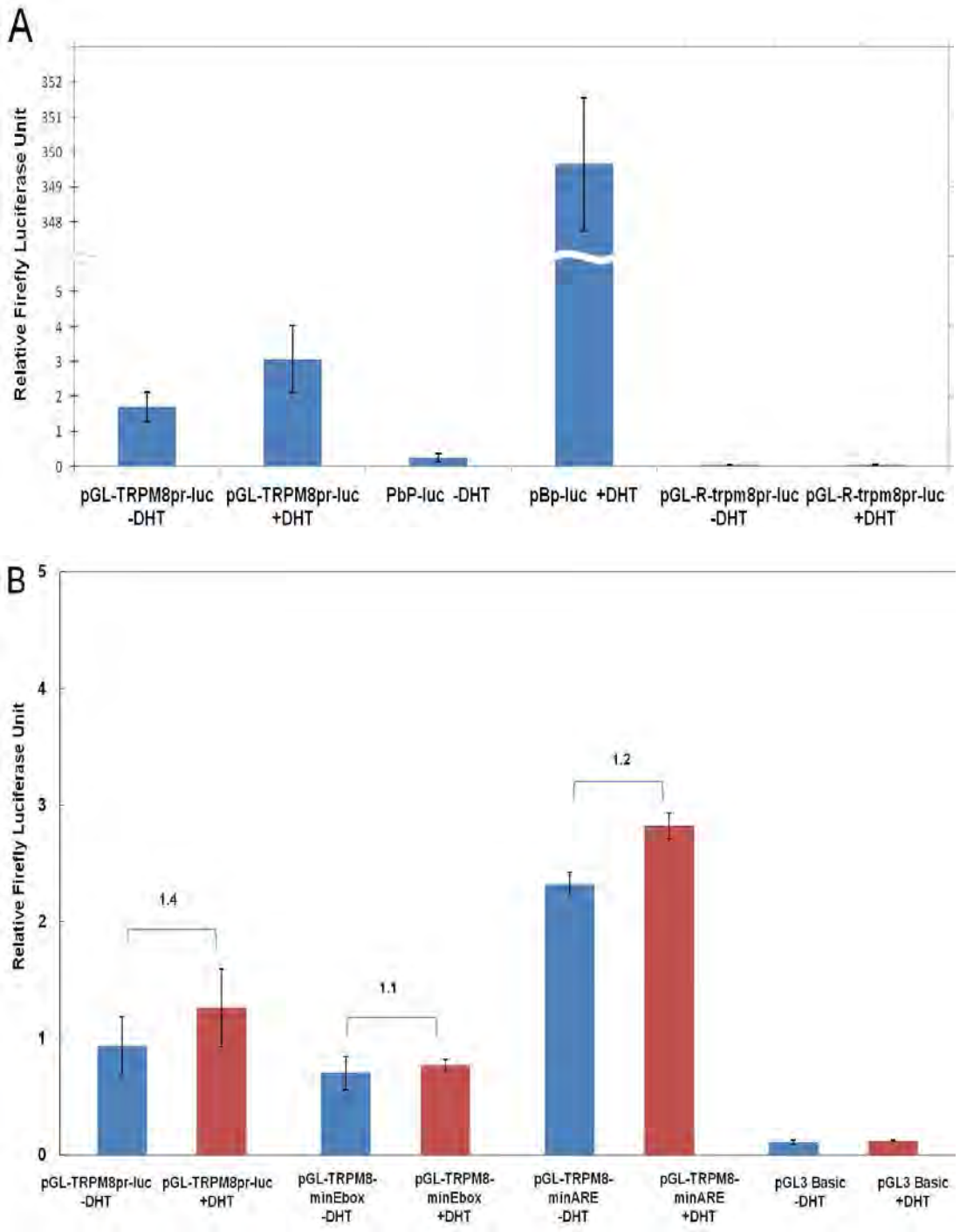


Figure 3. 8 Luciferase analysis of constructs that contain various sizes of the 5' flanking region of the *TRPM8* gene

A. Luciferase analysis for pGL-TRPM8pr-luc. **B.** Luciferase analysis of deletion constructs. Data shown are Mean ± SD, n=3. *Relative firefly luciferase unit*, a ratio which is calculated by normalization of a value of firefly luciferase to the respective value of Renilla luciferase.

3.3 Discussion

In this chapter, we first undertook bioinformatics analysis of the *TRPM8* promoter region and identified five putative AREs. Among them, four were highly conserved across species. We then studied the endogenous *TRPM8* gene expression pattern and used the information obtained to optimize the conditions for later luciferase reporter gene experiments. We constructed four *TRPM8* promoter-containing luciferase reporter plasmids, in particular, pGL-TRPM8pr-luc and pGL-TRPM8-minARE. The former contains the 6.1 kb *TRPM8* promoter, the later contains a 128 bp *TRPM8* minimal promoter. Luciferase analysis of the reporter plasmids demonstrated both 6.1 kb and 128 bp *TRPM8* promoter did not show androgen responsiveness. In addition, the *TRPM8* minimal promoter, or the deletion of the 6 kb *TRPM8* promoter, showed higher luciferase in both +DHT and –DHT groups when compared to the entire 6.1kb containing construct.

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

It is reported that androgen stimulation of *TRPM8* mRNA is concentration dependent: 0.1nM > 1nM > 10nM > 100nM (Bidaux et al. 2005). We therefore tested the androgen dose response of the 6 kb *TRPM8* promoter fragment (pGL-TRPM8pr-luc)

with the luciferase assay, and found a different pattern: 0.1nM < 1nM < 10nM (data not shown here). So 10 nM DHT was selected for the luciferase reporter gene expression study.

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

The luciferase reporter gene assay results showed the 6.1 kb *TRPM8* promoter is not androgen responsive although androgen induced some slight increase of luciferase (Fig 3.8).

It is possible that due to low system detection sensitivity, there are functional AREs (maybe weak AREs) present in the 6.1 kb promoter region but not detectable. The fact that a slight increase of luciferase was always observed in the 6.1 kb promoter +DHT group (Fig 3.8 and some other data not shown) suggests a possibility that weak AREs may be present. In Fig 3.8 A, the high basal level of luciferase from pGL-TRPM8pr-luc –DHT (when compared to PbP-luc –DHT and pGL-R-TRPM8pr-luc -DHT) may reduce the system's detection sensitivity of an androgen response with the 6.1 kb promoter region. Since pGL-TRPM8pr-luc contains the complete 6.1 kb of the *TRPM8* promoter (compared to Pbp-luc which only contains partial rat probasin promoter), and the high basal level of luciferase from pGL-TRPM8pr-luc –DHT appeared to be orientation-dependent (compared to pGL-R-

TRPM8pr-luc –DHT whose 6.1 kb promoter region is in reverse orientation), it indicates some orientation-dependent mechanism specifically associated with the 6.1 kb promoter region that causes the high basal transcription activities. This could be the result of AR binding (either dependent or independent of androgen activation), or some other transcription regulator. AR silencing and/or study of the 6.1 kb promoter region in an AR-deprived cell line may help us to determine whether AR is involved (refer to Section 6.5.3). In addition, the response to androgen of the 6.1 kb promoter fragment is different from that of *TRPM8* mRNA in both the induction scale and the response to DHT concentration. Bidaux et al reported that DHT stimulation of *TRPM8* mRNA production is dose-dependent (0.1nM > 1nM > 10nM > 100 nM) whereas for the 6.1 kb promoter fragment the effect of DHT in my experiments is 0.1nM < 1nM < 10nM (data not shown here) (Bidaux et al. 2005). This suggests that cooperation of the 6.1 kb promoter and transcription regulators located in other regions is needed to exert full transcription regulation. The model of regulation of transcription of the *PSA* gene can be used to explain this. Schuur et al found in the *PSA* gene, several weak AREs together with a strong ARE (as an enhancer) that exerted the full stimulation by androgen (Schuur et al. 1996).

Another possibility for the lack of androgen responsiveness is that no functional ARE is present in the 6.1 kb *TRPM8* promoter region.

Whether a portion of the AREs or no ARE is present in the 6.1 kb promoter region, expanding the search for AREs to a broader promoter region and combining other approaches with the current luciferase reporter gene system would be important for the determination of AR regulation of *TRPM8* expression.

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

It was interesting to observe that when compared to pGL-TRPM8pr-luc, pGL-TRPM8-minARE showed much higher luciferase expression in an androgen-independent manner. This indicates that a possible silencer element is present in the deleted region of the *TRPM8* promoter.

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

Before carrying out the induction time course experiments, we predicted that the time course curve would show *TRPM8* mRNA levels change as a function of time, increase smoothly and reach a plateau after a certain time. In the actual experiments, we had observed that the overall trend of *TRPM8* mRNA level was rising throughout the time, however, with two fluctuations occurring between 12 and 48 hr, 72 and 96

hr (Fig 3.3). This may be due to the fluctuation of DHT concentration in the medium. After 48 hr most of the androgen in the medium would be degraded (personal communication, Dr G Buchanan). In the curve, the lowest *TRPM8* mRNA level (except for 0 hr) was at 48 hr. It was the time point when medium was replaced, however, the cells were harvested before the medium replacement. This made the DHT concentration at the time point the lowest one over the whole 96 hr period. In particular, enrichment with fresh medium at 48 hr led to a sharp increase of *TRPM8* mRNA level at the following time point, 72 hr.

3.3.5 Interference by phenol red

Phenol red is commonly used as a pH indicator in mammalian cell culture medium. However, in the endogenous *TRPM8* mRNA expression experiments and following luciferase reporter vector experiments, phenol red was removed from the medium and phenol red free RPMI medium was used. This was because, at the beginning when phenol red-containing medium was used, high background androgen-like activity was observed even under androgen-depleted conditions. This was observed at normal LNCaP cell proliferation and high background luciferase values etc (data not shown), and meant that the hormone was not effectively depleted from medium. Upon further investigation it was noted that in other androgen regulated gene studies, using the LNCaP cell line, phenol red was omitted from media. However, no further

information on phenol red was mentioned in those studies. The literature revealed two separate studies that cast light on this. One study (Berthois et al. 1986) showed phenol red was an estrogen-like compound. The second study showed that AR in LNCaP cells had a mutation which allowed it to be activated by estrogen. Putting them together, it was obvious that in my experiments phenol red in the medium activated AR in LNCaP cells which subsequently led to a high AR activity in the androgen depleted LNCaP cells. The subsequent removal of phenol red confirmed that it was the factor that posed a high background artifact.

Chapter 4

Search for Androgen-Responsive Elements in the 5’ Regulatory Region of the *TRPM8* Gene by ChIP Assay

CHAPTER 4: SEARCH FOR ANDROGEN-RESPONSIVE ELEMENTS IN THE 5' REGULATORY REGION OF THE *TRPM8* GENE BY CHIP ASSAY

4.1 Introduction

The experiments described in Chapter 3 did not detect any ARE in the 6.1 kb of the *TRPM8* promoter. This raised the possibility that ARE could be located elsewhere in the gene. Recent findings from others confirmed that AREs do not only locate at the promoter region, but could also be in the 5' region further upstream within the gene body or 3' downstream of an AR regulated gene (Lin et al. 2009;Massie et al. 2007). For *TRPM8*, one possibility was the AREs located at the 5' region further upstream from the *TRPM8* gene. The decision to further explore the 5' regulatory region of the *TRPM8* gene for ARE was based on several lines of evidence which suggest that it is likely that an ARE can be found in the 5' regulatory region of the *TRPM8* gene. Firstly, although luciferase reporter gene assay did not detect significant androgen responsiveness of the 6.1 kb *TRPM8* promoter, the possibility of having ARE cannot be completely excluded. Secondly, the model of AR regulation of the Prostate Specific Antigen gene (Fig 4.1) suggests the possible of the presence of a 5' upstream ARE in the *TRPM8* gene. Thirdly, two ChIP assay studies, one ChIP display and a ChIP-seq, have shown that 21% or 16% of the identified AR binding sites were within 10 kb or 30 kb of the 5' regulatory region of AR regulated genes,

respectively (Jariwala et al. 2007;Lin et al. 2009). This means if there was an ARE located somewhere of the *TRPM8* gene, we would have about 20% chance of finding it within the 30 kb 5' regulatory region. Lastly the data from a ChIP-seq study on a human breast cancer cell line, ZR-75-1, from Buchanan et al (personal communication, Dr. Grant Buchanan) showed a low affinity AR binding site between 28 and 29 kb of the 5' regulatory region of the *TRPM8* gene (sequence in Fig 4.2).

The aim of this chapter was to employ a series of ChIP assays to search for AREs in the 30 kb 5' flanking region and the first kilo basepair of 5' untranslated region (5' UTR) of the *TRPM8* gene (Fig 4.3). As a transcription factor, AR exerts its regulation on the transcription of genes by binding to AREs in the genes. Chromatin Immunoprecipitation (ChIP) assay has been used to search for novel in vivo AR or other TF binding sites under the context of chromatin (Jariwala et al. 2007;Massie et al. 2007;Massie and Mills 2008;Orlando and Paro 1993). For the detailed description of the ChIP assay refer to Chapter 1.

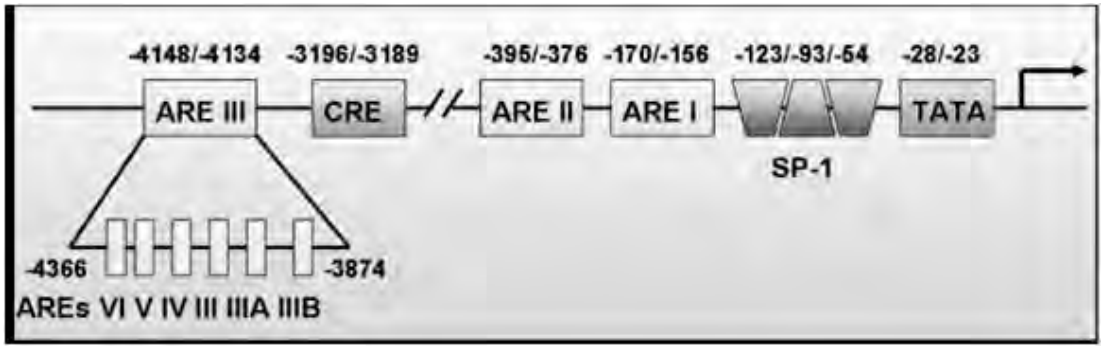


Figure 4. 1 Model of ARE layout in the PSA gene

Numbers shown are locations relative to the transcription start site of the PSA gene. Adapted from (Kim and Coetzee 2004)

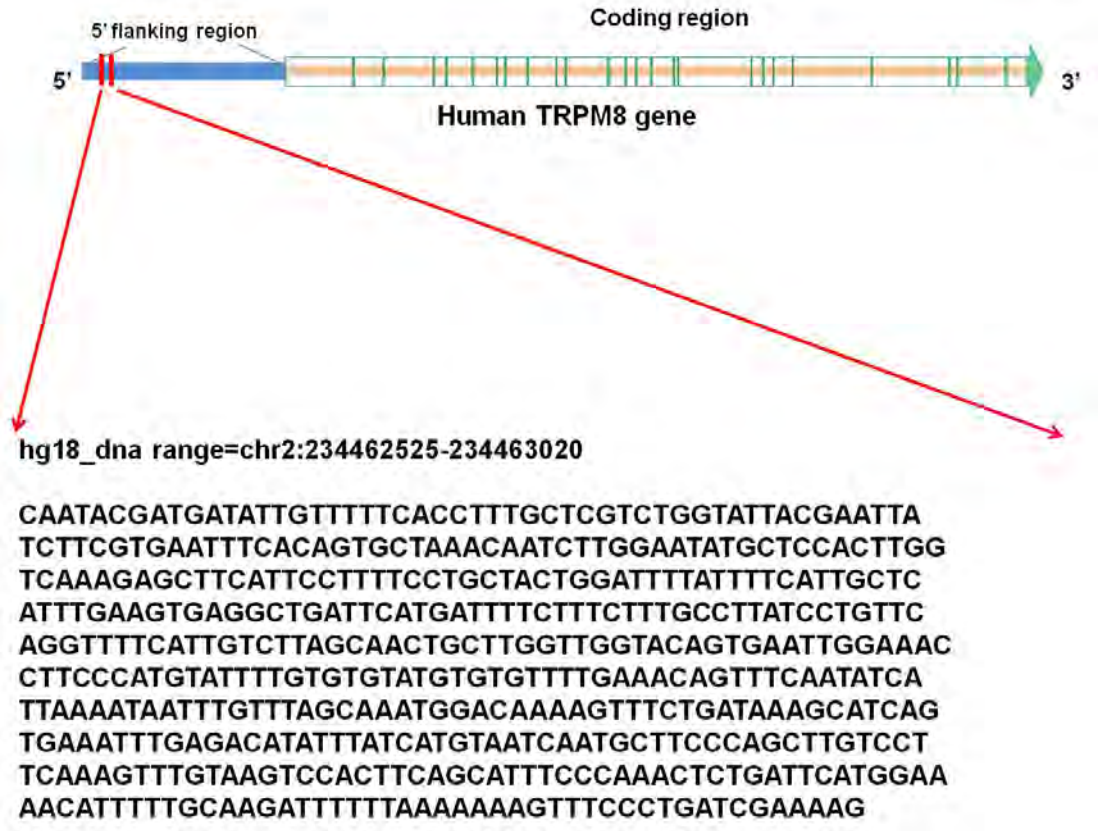


Figure 4. 2 ChIP-seq data of the low affinity AR binding site from Buchanan et al

This figure shows the approximate location of the low affinity AR binding site on the *TRPM8* gene. The upper part of the figure shows the schematic demonstration of the *TRPM8* gene. the lower part shows the DNA sequence of the low affinity AR binding site.

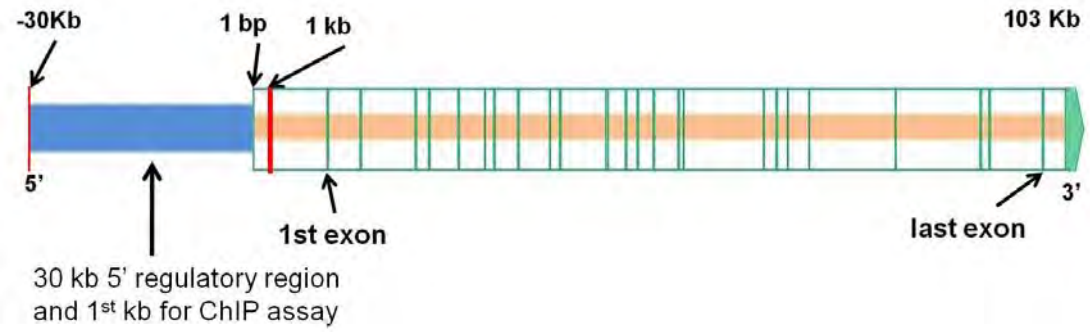


Figure 4. 3 Schematic illustration of the human *TRPM8* gene and the 5' regulatory region to be studied using a ChIP assay

The region between the two red vertical lines is the 30kb 5' regulatory region and 1st kb of the *TRPM8* gene that was investigated in this chapter.

4.2 Results

4.2.1 Overall strategy

The search for *in vivo* AR binding sites that containing AREs had three stages: 1) Primer design, synthesis and validation. 2) ChIP assay. Immunoprecipitation of AR-bound genomic DNA and purification. 3) qPCR analysis of the purified genomic DNA using the designed primers to look for genomic regions with enriched DNA quantity. The significantly enriched region identified was considered to be an AR binding site which may contain ARE.

4.2.2 Primer design, synthesis and validation

We split the 30 kb 5' regulatory region and 1st kb into 31 regions, designated as tiles, with each 1 kb long followed by designing primers that specifically amplify a portion of each tile. In total 32 primer pairs whose amplicons spanned 31 kb of human *TRPM8* gene (the genomic location of the amplicon for each primer pair is shown in Fig 4.6, the sequence of each primer pair and the size of each amplicon are listed in Appendix I) were synthesized and validated.

4.2.2.1 Primer design

The genomic sequence of the *TRPM8* gene was obtained from Genbank (Genbank ID: NC 000002). The sequence of the 30 kb of 5' regulatory region and the first kilobase of *TRPM8* gene was then imported into software Primer Express 3.0 (Applied Biosystems) to design qPCR primers. The amplicon size of each pair of primers was limited to between 75 and 150 bp.

4.2.2.2 Primer synthesis and validation

The validation of primers had two phases: 1) validation of the amplicon size by PCR-gel electrophoresis, 2) confirmation of the amplicon by sequencing.

After primer synthesis, the first phase of the validation was to employ actual PCR using the synthesized primers and LNCaP genomic DNA as template followed by gel electrophoretic analysis of the size and number of the PCR products. All synthesized primer pairs, except for -21 kb, passed the validation (for gel images see Fig 4.4 and 4.5 A and B). The -21 kb primer pair was then re-designed and subsequently passed the first phase of validation (gel image see Fig 4.5 C). The last phase of validation was the sequencing of the purified PCR products that passed the second phase validation. The sequencing confirmed the sequence of the PCR products.

Two control primer pairs were also synthesized: positive control primer pair that amplifies -170~ -400 bp of the promoter region (containing two known AREs) from prostate specific antigen (PSA, Chr 19) (Jia et al. 2003) and a negative control primer pair targeting the region known without AR binding sites Chr 20: 44141632-44141782 (Peters et al. 2009).

4.2.3 Optimization of chromatin fragmentation for ChIP assay

In a ChIP assay, chromatin fragmentation is a very important step as it determines the sensitivity and resolution of the ChIP assay. The optimal average size for fragmented chromatin for ChIP-qPCR is between 200 bp and 1 kb. In the current study, the approach for chromatin fragmentation was sonication. The sonication conditions require optimization for every sonicator due to their output energy difference.

4.2.3.1 Sonication optimization for Misonix Sonicator S-4000

S-4000 sonicator with microtip was used for chromatin fragmentation. For the optimization, we first tested different output energy levels to determine the suitability for eppendorf tubes with 700-1000µl SDS lysis buffer. Output energy level 39% amplitude was chosen. We then fixed the pulse length to 3 seconds and tested the

chromatin sharing effects with various numbers of pulses. Results showed that sonication between 15 and 25 pulses gave suitable size (Gel image of DNA size see Fig 4.7 A). This was repeated once with two biological replicates (see Fig 4.7 B). From the results, 20 pulses of sonication with 3 seconds each pulse was chosen as the sonication condition for the following ChIP assay.

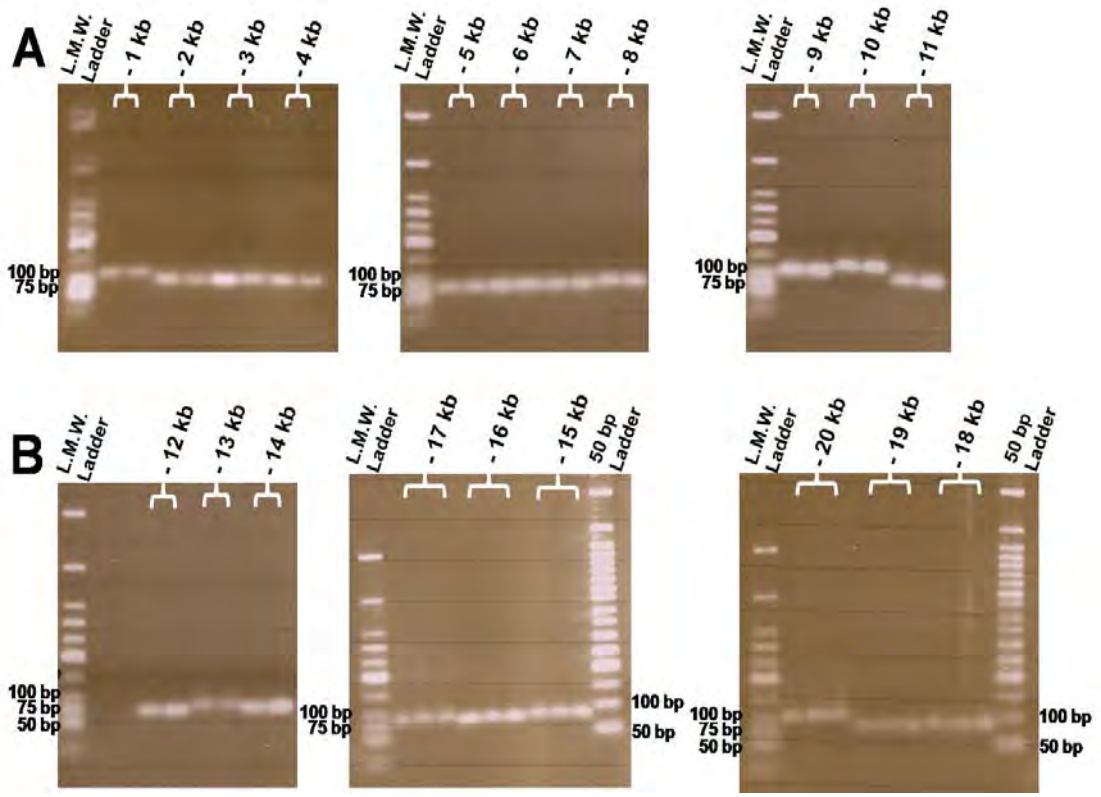


Figure 4. 4 PCR- gel electrophoresis validation of the size of amplicons from -1 kb to -20 kb primer pairs

A. Validation of amplicons from -1 kb to -11 kb primer pairs. The size of band from each amplicon matched the expected size: 99 bp (-1kb), 75 bp (-2kb), 77 bp(-3kb), 75 bp (-4kb), 75 bp (-5kb), 75 bp(-6kb), 75 bp (-7kb), 81 bp (-8kb), 102 bp (-9kb), 116 bp (-10kb), 83 bp (-11kb). *L. M. W. Ladder*, low molecular weight DNA ladder. **B.** Validation of amplicons from -12 kb to -20 kb primer pairs. The size of band from each amplicon matched the expected size: 75 bp (-12kb), 85 bp (-13kb), 75 bp (-14kb), 80 bp (-15kb), 75 bp (-16kb), 80 bp (-17kb), 84 bp (-18kb), 79 bp (-19kb), 101 bp (-20kb).

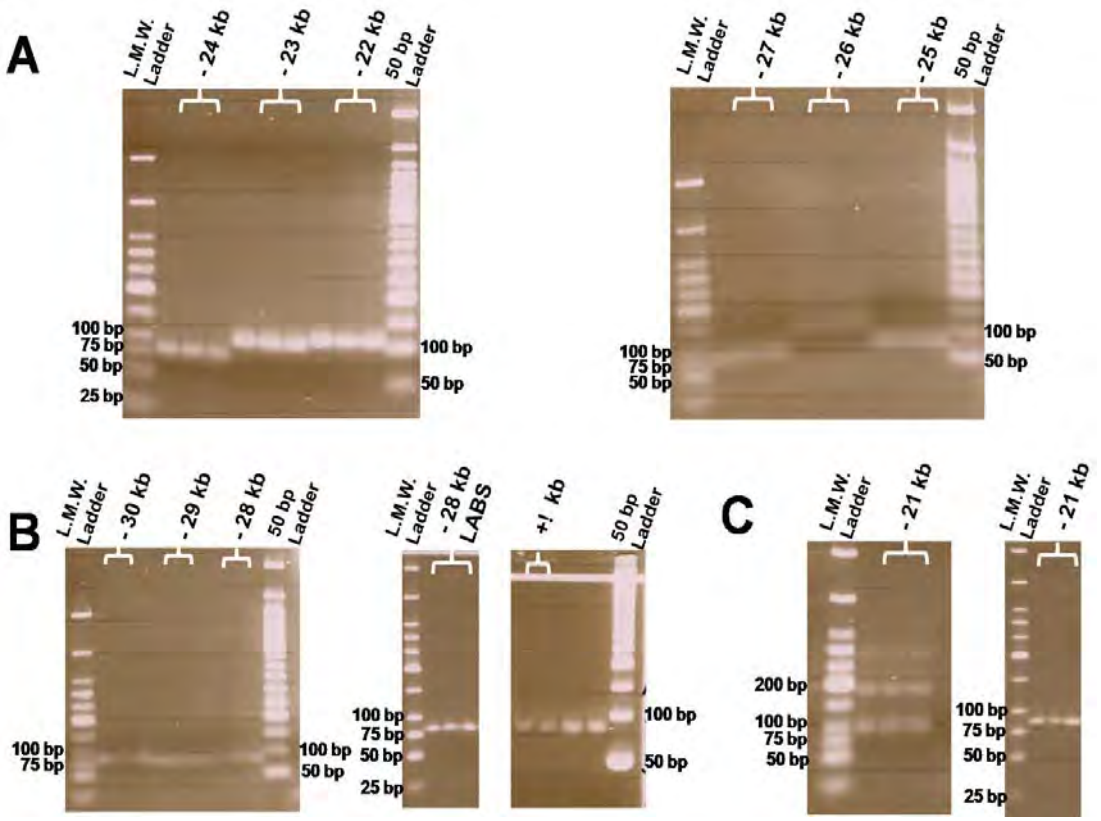
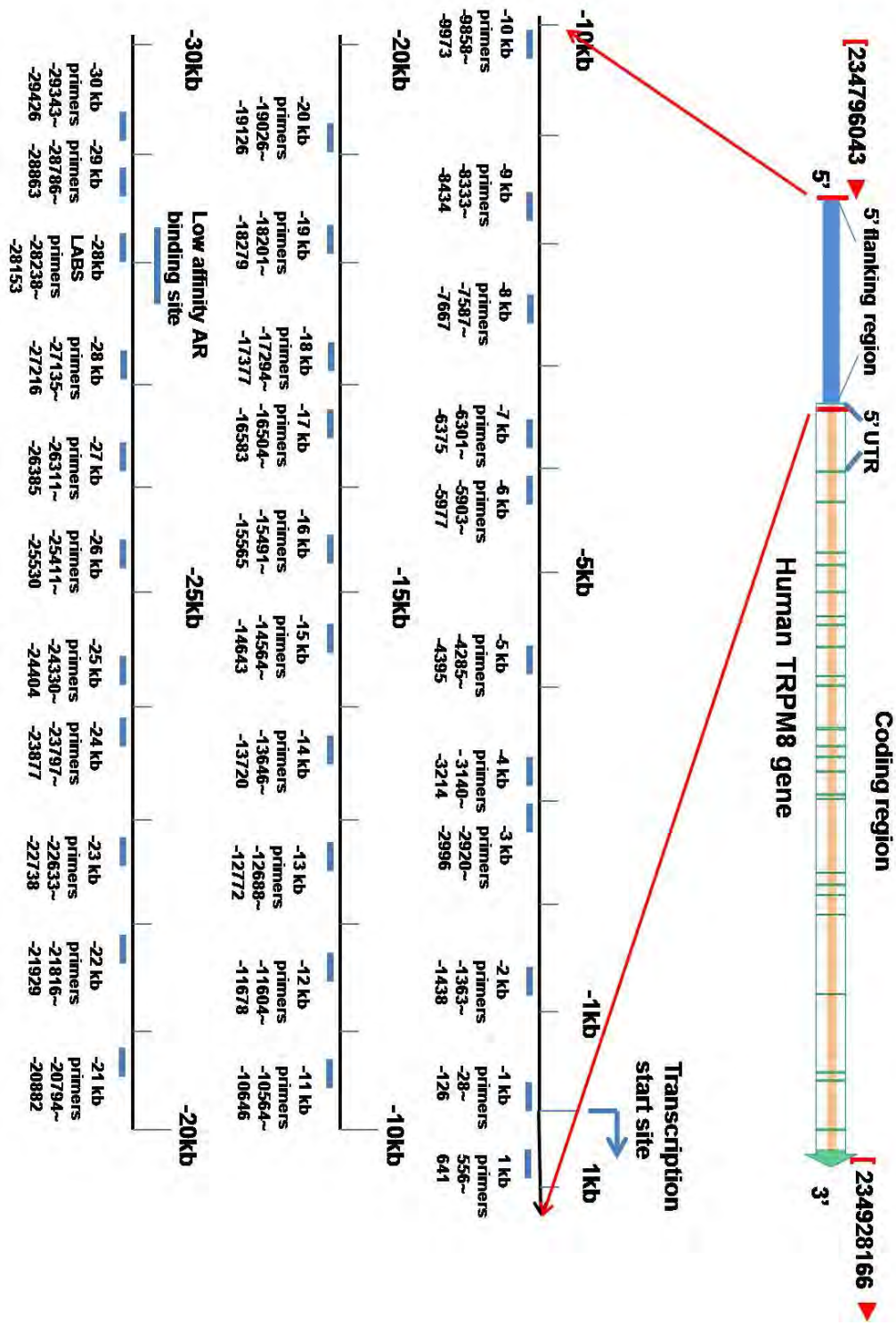


Figure 4. 5 PCR- gel electrophoresis validation of the size of amplicons from -21 kb to -30 kb primer pairs

A. Validation of amplicons from -22 kb to -27 kb primer pairs. The size of band from each amplicon matched the expected size: 114 bp (-22kb), 106 bp (-23kb), 81 bp(-24kb), 75 bp (-25kb), 120 bp (-26kb), 75 bp (-27kb). *L. M. W. Ladder*, low molecular weight DNA ladder. **B.** Validation of amplicons from -28 kb to -30 kb and +1 kb primer pairs. The size of band from each amplicon matched the expected size: 82 bp (-28kb), 85 bp (-28kb LABS), 78 bp (-29kb), 84 bp (-30kb), 85 bp (+1kb). **C.** Validation of amplicon from -21 kb primer pair. Left panel, the first designed -21 kb primers did not pass the validation because multiple bands shown for this amplicon. Right panel, single band at correct size (89 bp) of the amplicon from re-designed -21 kb primers.

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

Top panel was a schematic demonstration of the *TRPM8* gene. The numbers at both 5'and 3'end of the gene represented the genomic location of this region. The lower panel was the enlarged 5' flanking region and the first kilo base of the 5' untranslated region (5' UTR). This enlarged region was divided into 31 tiles with 1 kb length each tile and the location of the 32 qPCR amplicons were shown underneath each tile. *Horizontal blue bars underneath each tile*, qPCR amplicons. *Horizontal blue bar above the -28 and -29 tiles*, low affinity AR binding site from Buchanan et al. *Numbers underneath each horizontal blue bar*, location of the amplicon relative to the transcription start site.


[34796043
5'
5' flanking region
5' UTR
Coding region
3'
[34928166
Human TRPM8 gene
-10kb
-5kb
-1kb
1kb
Transcription start site
-10 kb primers -9858~ -9973
-9 kb primers -8333~ -8434
-8 kb primers -7587~ -7667
-7 kb primers -6301~ -6375
-6 kb primers -5903~ -5977
-5 kb primers -4285~ -4395
-4 kb primers -3140~ -3214
-3 kb primers -2920~ -2996
-2 kb primers -1363~ -1438
-1 kb primers -28~ -126
1 kb primers 556~ 641
-20kb
-15kb
-10kb
-20 kb primers -19026~ -19126
-19 kb primers -18201~ -18279
-18 kb primers -17294~ -17377
-17 kb primers -16504~ -16583
-16 kb primers -15491~ -15565
-15 kb primers -14564~ -14643
-14 kb primers -13646~ -13720
-13 kb primers -12688~ -12772
-12 kb primers -11604~ -11678
-11 kb primers -10564~ -10646
-30kb
Low affinity AR binding site
-25kb
-20kb
-30 kb primers -29343~ -29426
-29 kb primers -28786~ -28863
-28kb LABS primers -28238~ -28153
-28 kb primers -27135~ -27216
-27 kb primers -26311~ -26385
-26 kb primers -25411~ -25530
-25 kb primers -24330~ -24404
-24 kb primers -23797~ -23877
-23 kb primers -22633~ -22738
-22 kb primers -21816~ -21929
-21 kb primers -20794~ -20882

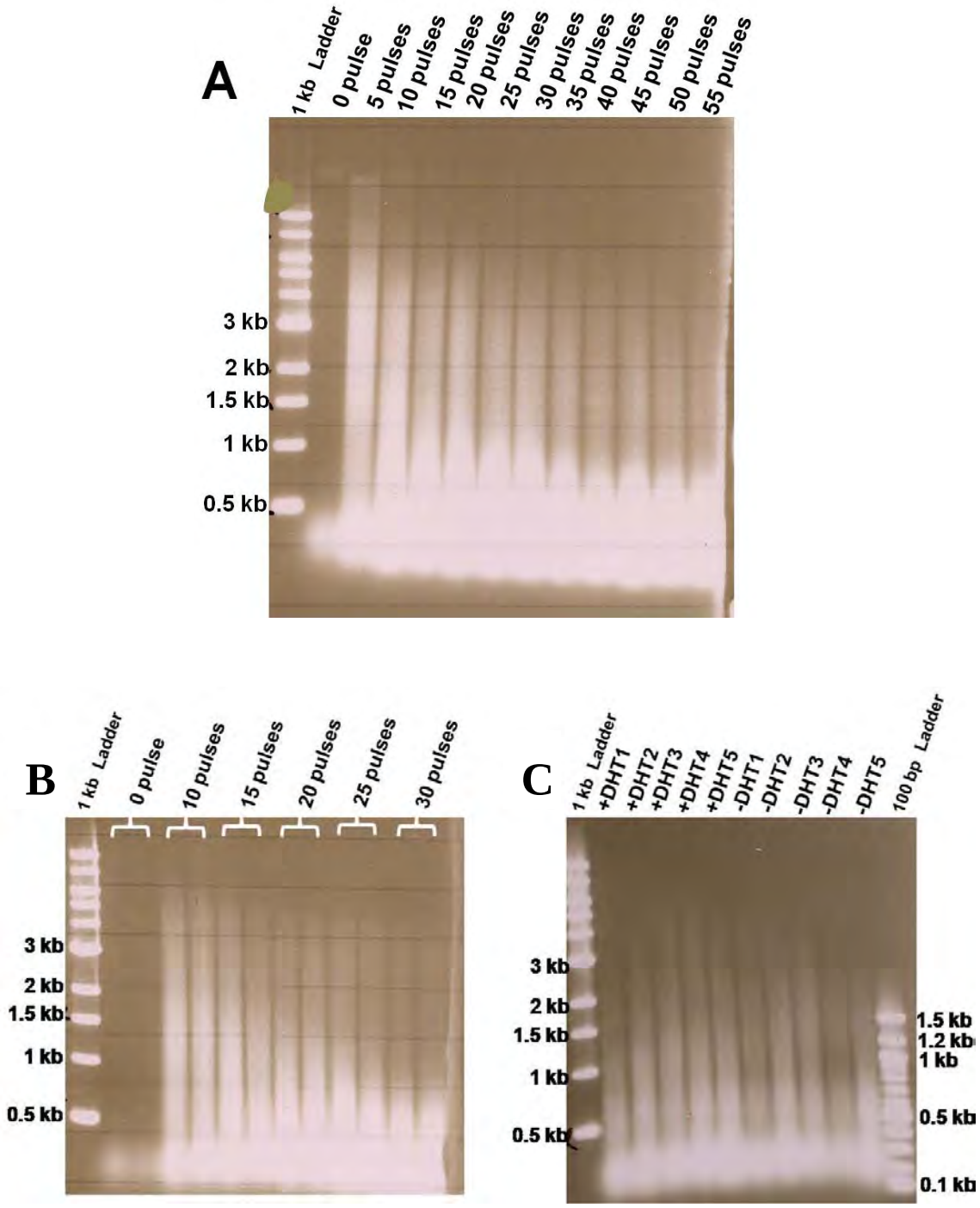


Figure 4. 7 Optimization of sonication conditions using a Misonix Sonicator S-4000 with microtip

A. First optimization using microtip. **B.** A repeat optimization with duplicated samples using microtip. **C.** Sonication results of the ChIP assay samples with average chromatin size between 200 and 700 bp.

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

4.2.4.1 ChIP assay

In ChIP-qPCR analysis, each sample was split into three fractions: Input fraction (non-immunoprecipitation control fraction, used for data normalization), anti-AR antibody immunoprecipitation fraction and anti-rabbit IgG immunoprecipitation fraction (negative control fraction for non-specific immunoprecipitation). For each sample, every kilobase genomic region was analyzed by qPCR for the relative quantity of its DNA from anti-AR antibody immunoprecipitation fraction and the quantity was expressed as a percentage of that from the Input fraction. The change in the quantity of immunoprecipitated DNA of each kilobase region between +DHT treated sample and –DHT treated sample was calculated. The AR binding site would be shown as a kilobase region whose immunoprecipitated DNA quantity was enriched in the +DHT treated sample when compared to the –DHT treated sample.

In order to reduce the endogenous AR binding activity to the basal level, LNCaP cells were cultured in androgen depleted Complete RPMI for 6 days prior to androgen treatment. The androgen treatment employed was culturing LNCaP cells in androgen depleted Complete RPMI (containing 10nM DHT) (+DHT) or in androgen

depleted Complete RPMI (containing ethanol) (-DHT) for 4 hours. The ChIP procedure followed and is described in Chapter 2. Sonication used the optimized condition mentioned above. At the end of the ChIP procedure, the fragmented chromatin size was checked by gel electrophoresis using the purified DNA. The result showed that the average size of fragmented chromatin was between 200 and 700 bp (see Fig 4.7 C).

4.2.4.2 qPCR analysis

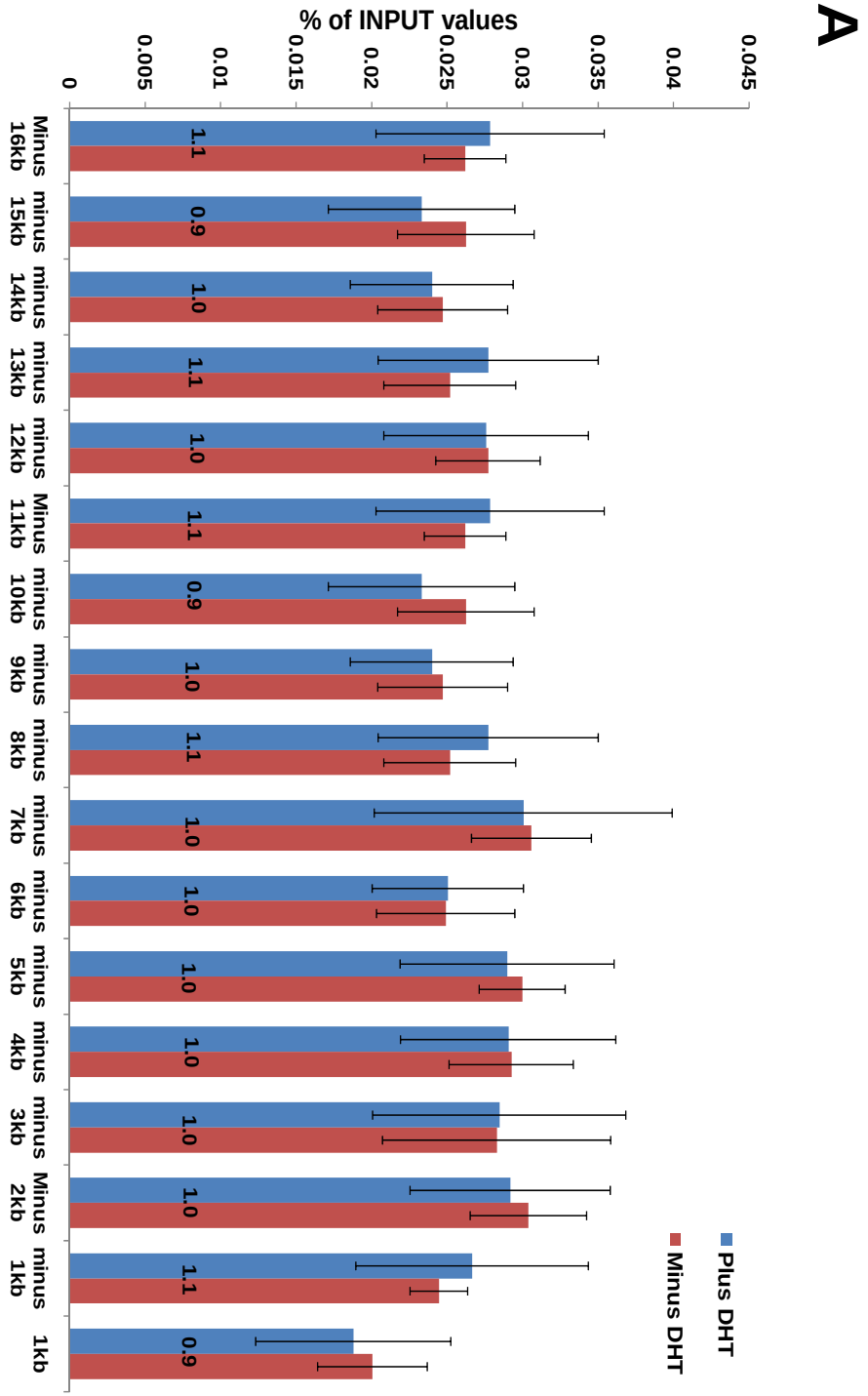
The purified DNA from each sample was analysed using the primer pairs mentioned in Section 4.2.2. The results showed that the positive control region on the *PSA* promoter harbouring ARE had a 1.9 fold enrichment ($p < 0.01$) in +DHT treated samples compared with –DHT treated samples. This was similar to the value reported in the paper from which this primer pair was obtained (Jia et al. 2003). At the same time, the negative control amplicon showed no enrichment (1 fold, $p > 0.05$) in +DHT treated region compared with –DHT treated region. All these showed that the ChIP – qPCR analysis system has reasonable sensitivity with low background.

In each of the 32 regions, only the region (designated as Androgen Receptor Binding Site 1, ARBS1) covered by the amplicon of primer pair -28 kb LABS showed a 1.5

fold enrichment (p =0.03) in +DHT treated samples compared with -DHT treated samples (see Fig 4.8 B). The other regions showed no significant enrichment (between 0.9 and 1.2 fold, p > 0.05) in +DHT treated samples compared with -DHT treated samples (see Fig 4.8).

4.2.5 Determination of the boundaries of the ARBS1 region

Since the sonication-sheared chromatin fragment size was on average from 200 to 700 bp, the ARBS1 should be within the 1.4 kb region of DNA centred at the middle of the -28kb_LABS primers' amplicon (from around -28900 bp to -27500 bp, Fig 4.9). However, -29 kb primers and -28kb primers did not detect any enrichment which meant an AR binding site was unlikely within 700 bp from the centres of their amplification regions. If considering the low detection sensitivity at the boundary of the 700 bp region covered by both -28kb and -29 kb primers, it would be safer to offset the 700 bp to 500 bp. This expanded the ARBS1 region from around -27676 to -28325 (blue colour shaded area in Fig 4.9).


A
% of INPUT values
0
0.005
0.01
0.015
0.02
0.025
0.03
0.035
0.04
0.045
Minus 16kb
minus 15kb
minus 14kb
minus 13kb
minus 12kb
Minus 11kb
minus 10kb
minus 9kb
minus 8kb
minus 7kb
minus 6kb
minus 5kb
minus 4kb
minus 3kb
Minus 2kb
minus 1kb
1kb
1.1
0.9
1.0
1.1
1.0
1.1
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1.0
1.0
1.0
1.0
1.1
0.9
Plus DHT
Minus DHT

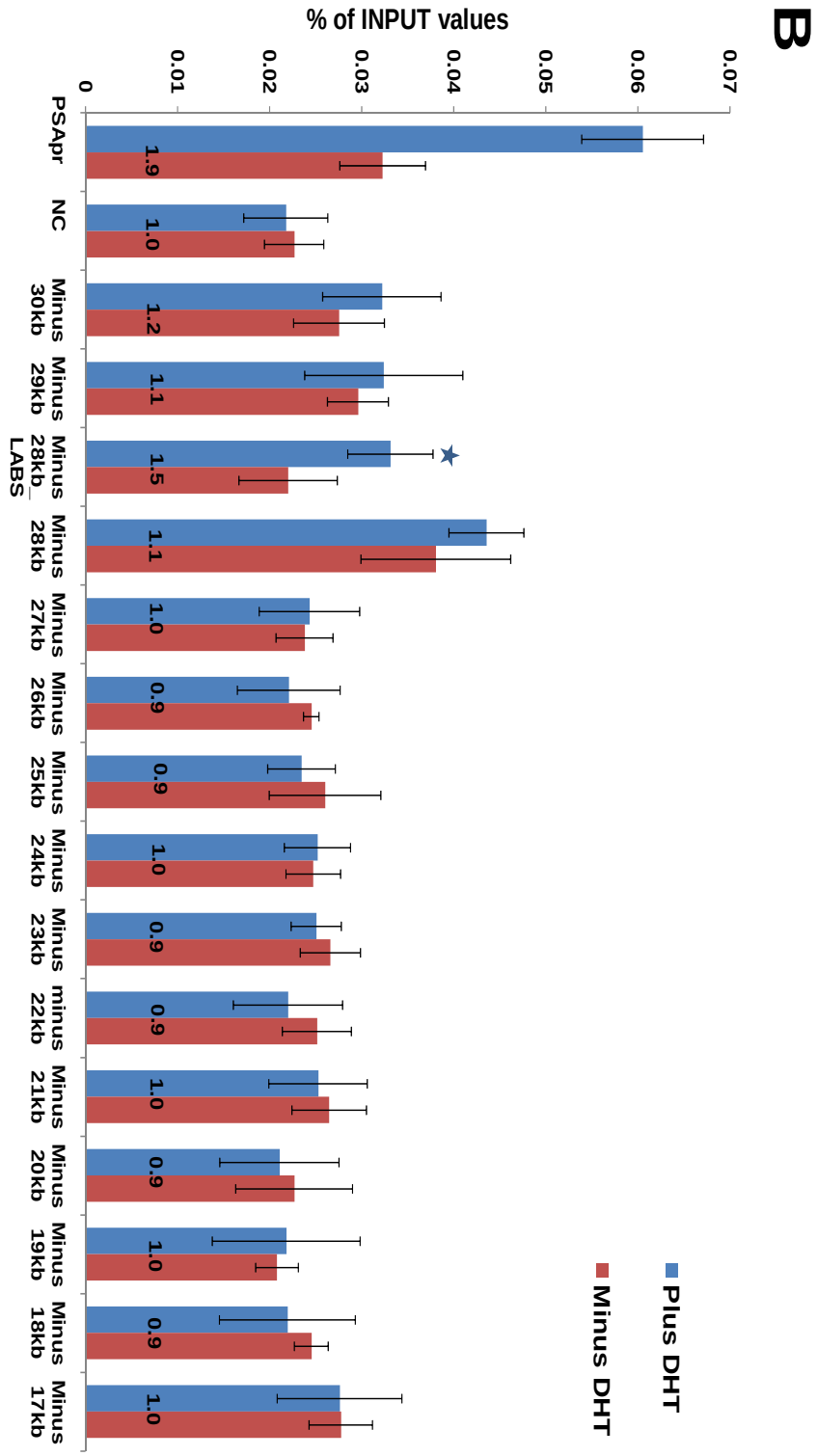

B
% of INPUT values
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
PSApr
NC
Minus 30kb
Minus 29kb
Minus 28kb_LABS
Minus 28kb
Minus 27kb
Minus 26kb
Minus 25kb
Minus 24kb
Minus 23kb
minus 22kb
Minus 21kb
Minus 20kb
Minus 19kb
Minus 18kb
Minus 17kb
1.9
1.0
1.2
1.1
1.5
1.1
1.0
0.9
0.9
1.0
0.9
0.9
1.0
0.9
0.9
1.0
1.0
Plus DHT
Minus DHT

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

A. Enrichment of tiles from 1kb to -16 kb. **B**. Enrichment of tiles from -17kb to -30 kb. 20 dishes of LNCaP cells (1.5X 10^{7} cells/145mm dish) were cultured in androgen depleted Complete RPMI for 6 days prior to 4 hr +DHT or –DHT treatment (The androgen treatment refers to Section 2.2.5) with 10 dishes of cells for each treatment. In each treatment, 5 biological replicates were included with 2 dishes of cells pooled as one biological replicate. ChIP assay was then carried out as described in Chapter 2. Values are presented as the percentage of Input. Data shown here are Means ± SD (n=5). This is representative of two similar experiments. *Name underneath each pair of columns*, name of the tile the column pair represents. *Blue column*, 4hr 10 nM DHT treatment. *Red column*, 4 hr ethanol treatment. *Numbers in the middle of the column pairs*, enrichment of DHT treatment against ethanol treatment. *, p=0.03.

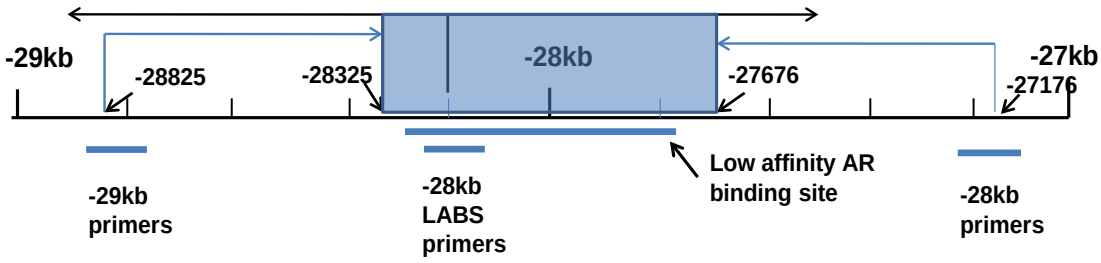


Figure 4. 9 Schematic representation of the location of the ARBS1 at approximately -28 kb of the *TRPM8* gene.

Here we demonstrated how the boundaries of ARBS1 were determined. One tick represents 200 bp distance. *Numbers*, locations relative to TSS. *Short horizontal blue bars*, regions amplified by three primer sets. *Long horizontal blue bar*, low affinity AR binding site from Buchanan et al. *Blue colour shaded area*, identified ARBS1 region.

4.3 Discussion

In summary, we have designed and validated 32 pairs of genomic DNA qPCR primers, then optimized sonication conditions to give us the required average chromatin size and found one AR binding site, designated as AR Binding Site 1, at around -28kb upstream of the *TRPM8* gene in the following ChIP assay. We then determined the boundaries of ARBS1 so that we could clone it and characterize its function in later experiments

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

The selection of 4 hr for DHT stimulation for ChIP assay was based on the following evidence. i) As discussed in Chapter 1, in the genomic AR signalling pathway, activated AR will translocate to the nucleus to bind to AREs before initiating the transcription of the genes. This suggests that sufficient AR binding to AREs is earlier than the major increase of mRNA level. ii) Dr Buchanan suggested that strong increases of expressions of AR-directly regulated genes often appeared from 4 to 8 hr after androgen stimulations (personal communication, Dr. Grant Buchanan). iii) Our experiment showed that *TRPM8* mRNA level started to increase as early as 3 hr after DHT stimulation (data not shown here), and one study from others showed a similar pattern (Bidaux et al. 2005). All these suggest there should be sufficient AR binding

at 4 hr after androgen stimulation. In addition, 4 hr or less of androgen treatment has been commonly used by various ChIP studies for AR-directly regulated genes (Jariwala et al. 2007;Jia et al. 2008;Jia et al. 2006;Massie et al. 2007).

However, there is evidence suggesting longer androgen stimulation may lead to more AR binding (Jia et al. 2004;Louie et al. 2003;Wang et al. 2005;Wang et al. 2007). This could be a potential way to increase ChIP assay sensitivity.

4.3.2 ARBS1 is an AR binding site

Here we determined ARBS1 as an AR binding site based on the following three reasons: 1) The ChIP assay system employed has reasonable detection sensitivity and resolution. The positive control, that detecting AR binding at the proximal promoter of the *PSA* gene (from -395 to -156 bp relative to the transcription start site), provided 1.9 fold enrichment (Fig 4.8B) which was similar to the results obtained using the same primers in a study previously undertaken (Jia et al. 2003). It is reported that there are several AREs on the promoter region of the *PSA* gene: ARE I and II on the proximal promoter region (from -395 to -156 bp), ARE III and other 5 weak AREs on the enhancer element located between -4366 and -3874 bp relative to the transcription start site(Kim and Coetzee 2004). Although the *PSA* proximal

promoter has two AREs, it requires the enhancer element to fully exert its function upon androgen stimulation (Kim and Coetzee 2004;Schuur et al. 1996). In addition, the two AREs at the proximal promoter show only marginal AR enrichment upon androgen stimulation, whereas the enhancer element shows around 5 times higher AR enrichment (Jia et al. 2003;Jia et al. 2006). The negative control region showed only 1 fold enrichment, demonstrating a reasonably low background in the system. 2) The results for the region showed statistical significance ($p=0.03$) and they represented average values of five biological replicates ($n=5$) with triplicate determinations for qPCR for each biological replicate. Other regions showed enrichment in a tight range (0.9-1.1 fold). In addition, this was a repeat of a similar experiment which showed similar results at the same location (data not shown); 3) the result was in agreement with the data from Buchanan et al which indicated a low affinity AR binding site within this region.

4.3.3 Sonication conditions

In the optimization of sonication we chose relatively higher output energy with shorter pulse length and with more pulse times instead of lower output energy with longer pulse length with less pulse times. At the beginning lower output energy was expected to give a better fine tune fragment size so I included in Brandson sonicator two different lower output energy levels. However, it turned out that the lower output

energy tended to produce a higher proportion of shorter fragments (between 100 and 400 bp) while the majority chromatin size was still longer than 3 kb (data not shown). Shorter average chromatin size would increase the resolution of analysis, however, it may also reduce the detection sensitivity. A good balance between them is ensuring an average chromatin size of around 1kb. In addition, the primers were designed to cover 1 kb genomic tiles. Shorter chromatin size will result in shorter coverage length by each amplicon, therefore more primers would be needed.

Another consideration for higher output energy with shorter pulse length was to reduce the heat build up and at the same time retain sonication efficiency. This is because shorter pulse time with 1 min interval would effectively reduce the heat build up as during the sonication the chromatin bound AR is prone to heat degradation. The sonication energy is normally higher in the first few seconds of each pulse.

Chapter 5

Evaluation of the Regulatory Function of the Androgen Receptor Binding Site 1 using Luciferase Reporter Vectors

CHAPTER 5: EVALUATION OF THE REGULATORY FUNCTION OF THE ANDROGEN RECEPTOR BINDING SITE 1 USING LUCIFERASE REPORTER VECTORS

5.1 Introduction

As described in the previous chapter, results obtained using ChIP assays identified AR Binding site 1 (ARBS1) located at around 28 kb upstream of the *TRPM8* gene. As pointed out previously, the AR binding sites identified by ChIP assays may turn out to be either functional or non-functional (Birney et al. 2007;Jia et al. 2008;Li et al. 2008). Moreover, among the functional sites identified, the regulation could be either enhancement or inhibition upon androgen treatment (Jia et al. 2008). Therefore it was important to test the function of ARBS1 as well as the orientation and distal promoter dependence of its function. This was carried out using the luciferase reporter gene assay which is described below.

The overall strategy to characterize the regulatory function of ARBS1 was separated in three stages: 1) Construction of luciferase reporter gene vectors; 2) Transient transfection of LNCaP cells with the constructed plasmids and androgen treatment (refer to Chapter 2); 3) LNCaP cell harvest and luciferase reporter gene expression assays. A schematic demonstration of the strategy is shown in Fig 5.1 and is described in detail in the following.

In stage one, in order to test the orientation and promoter dependence of the function of the presumed enhancer within ARBS1, it was planned to construct four luciferase reporter gene plasmids. Two constructs used the pGL3 Promoter as a recipient vector (for convenience we designated it is as SV40-empty), containing a heterologous promoter SV40, with ARBS1 insert in both clockwise (pGL-ARBS1EN/SV40-luc, for convenience designated as SV40-ARBS1) and counterclockwise (pGL-R-ARBS1EN/SV40-luc designated as SV40-R-ARBS1) orientations, respectively. The ARBS1 insert was at a site upstream of the SV40 promoter and served as a potential enhancer or silencer. The other two constructs used pGL-TRPM8pr-luc (designated as TRPM8-empty), containing endogenous 6 kb *TRPM8* promoter, as a recipient vector with ARBS1 insert in both orientations (pGL-ARBS1EN/TRPM8pr-luc, designated as TRPM8-ARBS1, and pGL-R-ARBS1EN/TRPM8pr-luc, designated as TRPM8-R-ARBS1, respectively). In these two constructs, The ARBS1 insert was at a site upstream of the *TRPM8* promoter and served as a potential enhancer or silencer. The construction was performed by the following steps: 1) Cloning ARBS1 (amplified by PCR) into a linear vector, pGEM-T Easy (containing 5'-T overhangs on both ends so that PCR products, normally containing 3'-A overhangs added by Taq DNA polymerase, can be directly ligated), to form the plasmid pGEM-T Easy-ARBS1. 2) Subcloning an ARBS1 containing *NotI*/*NotI* fragment from pGEM-T Easy-ARBS1 into the *NotI* restriction site on SV40-empty and TRPM8-empty, respectively. Since the *NotI*/*NotI* fragment can be inserting into either plasmid with

two orientations, this will theoretically form all four designed constructs. 3) Transformation of *E.coli* and positive colony (containing one of the four constructs) screening by restriction enzyme mapping. In the study carried out here, however, only three of the four designed constructs (excluding TRPM8pr-ARBS1) were obtained. In stage 2, these three constructs were transiently transfected into LNCaP cells together with Renilla luciferase reporter vector phRN-null (as transfection effiency control). In stage 3, the normalized firefly luciferase activities of +DHT and ethanol -DHT treated LNCaP cells transfected with the same plasmid, were then compared.

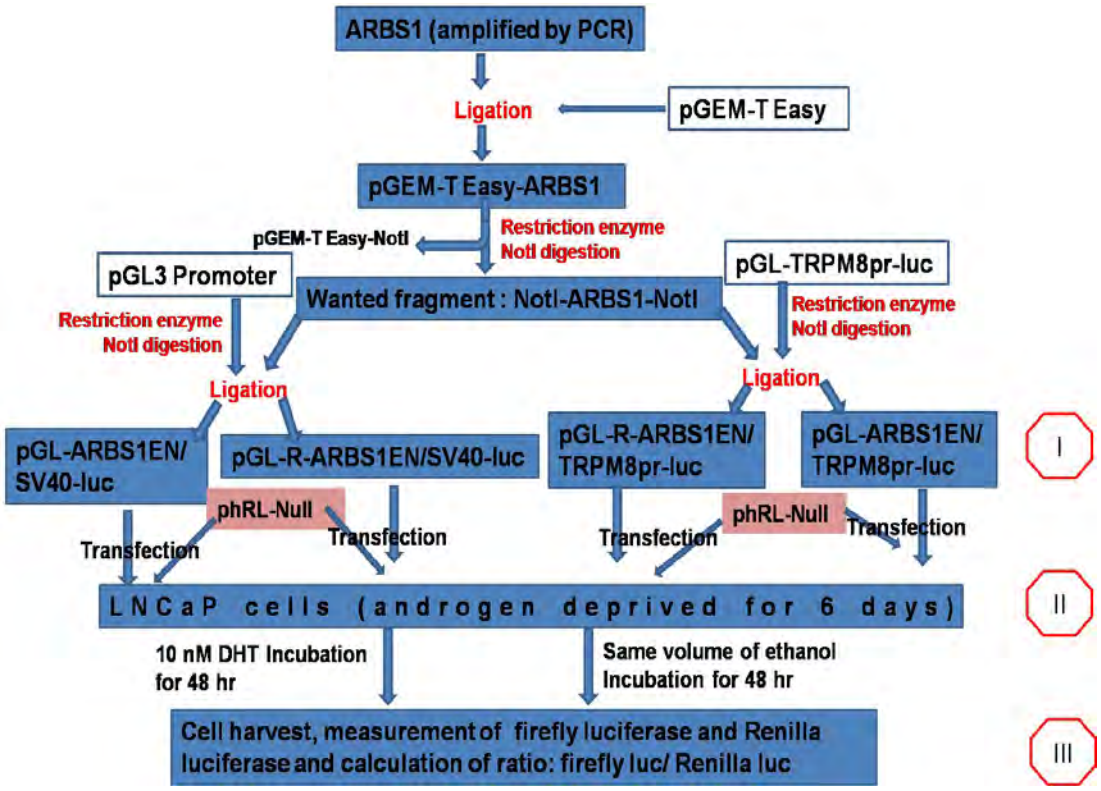


Figure 5. 1 Schematic diagram of the strategy for ARBS1 regulatory function analysis using a luciferase reporter gene assay.

The whole strategy is divided into three stages**.** *I, II or III in octagon***,** indicates the endpoint of each stage. *Words in red color*, ligation or restriction enzyme digestion reactions. *Blue-line rectangles*, recipient plasmid for ligation reactions. *Blue color shaded rectangles*, end point of each step. *Light red color shaded rectangles*, Renilla luciferase plasmid co-transfected with each construct as transfection efficiency control.

5.2 Results

5.2.1 Construction of pGEM-T Easy-ARBS1 plasmid

5.2.1.1 PCR amplification of AR binding site 1 (ARBS1)

A 1298 bp long region (primers and sequence refer to Fig 5.2 A) was amplified. The gel electrophoresis image was shown in Fig 5.2 B. The gel electrophoresis confirmed in principle that this PCR product is ARBS1.

5.2.1.2 Ligation of ARBS1 and linear vector pGEM-T Easy

The PCR product from the last step was ligated with pGEM-T Easy vector to form the designated construct, pGEM-T Easy-ARBS1. Ligation products were transformed into competent *E.coli.* JM109. Seven colonies were randomly chosen and plasmid DNA Miniprep carried out using QIAprep Spin Miniprep kit (details refer to Chapter 2). The purified plasmid DNA samples were sequenced. Sequencing results showed plasmid DNA from one colony had the insert with the identical sequence to ARBS1 as shown in Fig 5.2A (sequencing result shown in Appendix III).

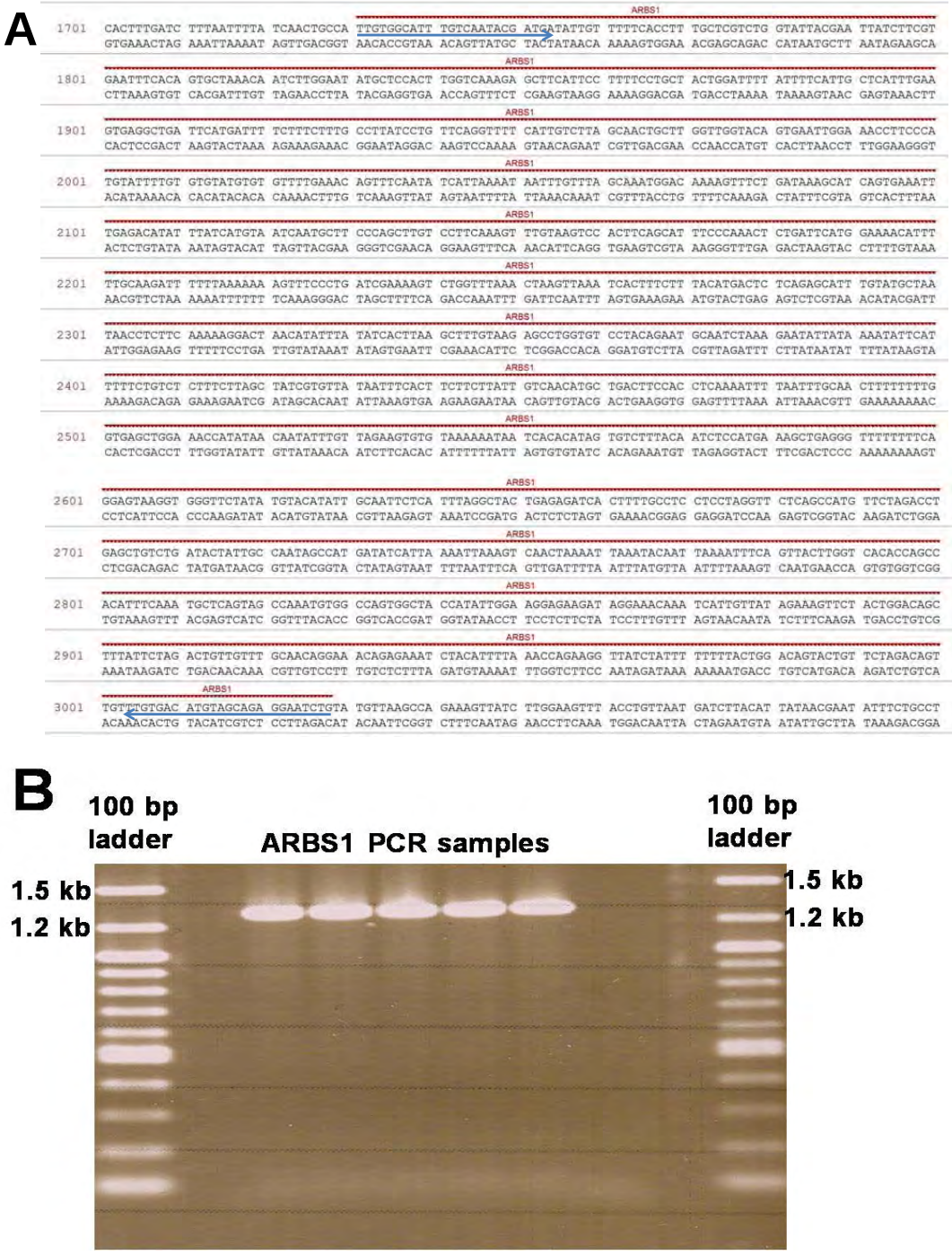


Figure 5. 2 PCR amplification of ARBS1

A. The sequence of ARBS1 and its PCR primers. The double-strand DNA sequences are shown here, the sequence mentioned here is based on the sense strand sequence. *Blue right arrow*, forward primer sequence. *Blue left arrow*, reverse primers sequence. *Top strand*, the sense strand. *Bottom strand*, the antisense strand. **B.** Gel image of ARBS1 PCR product. The bands of ARBS1 PCR products were all around 1.3 kb which matched the 1298 bp size of ARBS1.

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

5.2.2.1 Releasing ARBS1, linearizing SV40-empty and TRPM8-empty by *NotI* digestion

In this and subsequent steps, the designed luciferase reporter gene constructs were to be generated. This was done by releasing the ARBS1 from pGEM-T Easy-ARBS1 and subsequently subcloning into the recipient plasmids, SV40-empty and TRPM8-empty, respectively (schematic demonstration shown in Fig 5.3 and Fig 5.4). First, restriction enzyme *NotI* digestions were carried out on SV40-empty, TRPM8-empty and pGEM-T Easy-ARBS1. This was to linearize the recipient vectors and to release from pGEM-T Easy-ARBS1 the *NotI*/*NotI* fragment that contained ARBS1, designated as *NotI*-ARBS1-*NotI* (sequence see Fig 5.5A). The DNA fragments with correct size were first gel purified, followed by gel electrophoresis confirmation of their size. Based on the pattern and size of the DNA fragments shown on the gel, it was confirmed that the linearized recipient vectors and *NotI*-ARBS1-*NotI* were obtained (gel image shown in Fig 5.5B).

5.2.2.2 Ligation of *NotI*-ARBS1-*NotI* fragment with the linearized SV40-empty or TRPM8-empty.

In this step, ligation of the *NotI*-ARBS1-*NotI* and linearized vectors, SV40-empty or TRPM8-empty was performed. The ligation products were transformed into *E.coli*. The next day, five *E.coli*. colonies were randomly chosen from the agar plates for SV40-based and TRPM8-based constructs, respectively. Each of these 10 colonies underwent plasmid DNA Miniprep. Purified plasmids were then subjected to two rounds of restriction enzyme mapping tests to identify the designed constructs.

The first round of restriction enzyme mapping was to identify the correct plasmids (with correct ARBS1 insert without considering the orientations at this stage) and their correlated colonies. This was performed by *NheI* (linearized the plasmids) and NotI (released the recipient vectors and the inserts) digestions, respectively. For TRPM8-based constructs, the expected patterns were: 1) single band at size of 12220 bp for *NheI* digestion; 2) two bands at sizes of 10886 bp and 1334 bp for *NotI* digestion (Fig 5.6A). For SV40-based constructs, the expected patterns were: 1) single band at size of 6344 bp; 2) two bands at sizes of 5010 bp and 1334 bp (Fig 5.6B). For SV40-based constructs, plasmids from colonies 1, 3, 4 and 5 were identified to contain the correct ARBS1 insert. For TRPM8-based constructs,

plasmids from colonies 3 and 4 were identified to contain the correct ARBS1 insert. They were chosen for the second round of restriction enzyme mapping.

The second round of restriction enzyme mapping was to identify the orientations of the ARBS1 inserts in the plasmids from those colonies. This was performed by *NheI/SpeI* double digestion for pGL3 promoter-based constructs and *SpeI* digestion for pGL-TRPM8pr-luc-based constructs (Fig 5.6C). The orientations and related patterns were

1) SV40- ARBS1 : two bands at 6143 bp and 201 bp;
2) SV40- R-ARBS1: two bands at 4835 bp and 1509 bp;
3) TRPM8-ARBS1 : two bands at 8581 bp and 3639 bp;
4) TRPM8-R-ARBS1: two bands at 7273 bp and 4947 bp.

The mapping result showed that 1) for SV40-based constructs, the plasmid from colony # 3 showed two bands at around 4.8 kb and 1.5 kb, so it was SV40- R-ARBS1. The plasmid from colony #4 showed two bands at around 6.1 kb and 200 bp, so it was SV40- ARBS1; 2) for TRPM8-based constructs, plasmids from colonies #3 and 4 both showed two bands at 7.3 kb and 4.9 kb, so they were both TRPM8-R-ARBS1. Therefore, three out of the four designed constructs (excluding TRPM8-ARBS1) were obtained for the following luciferase analysis.

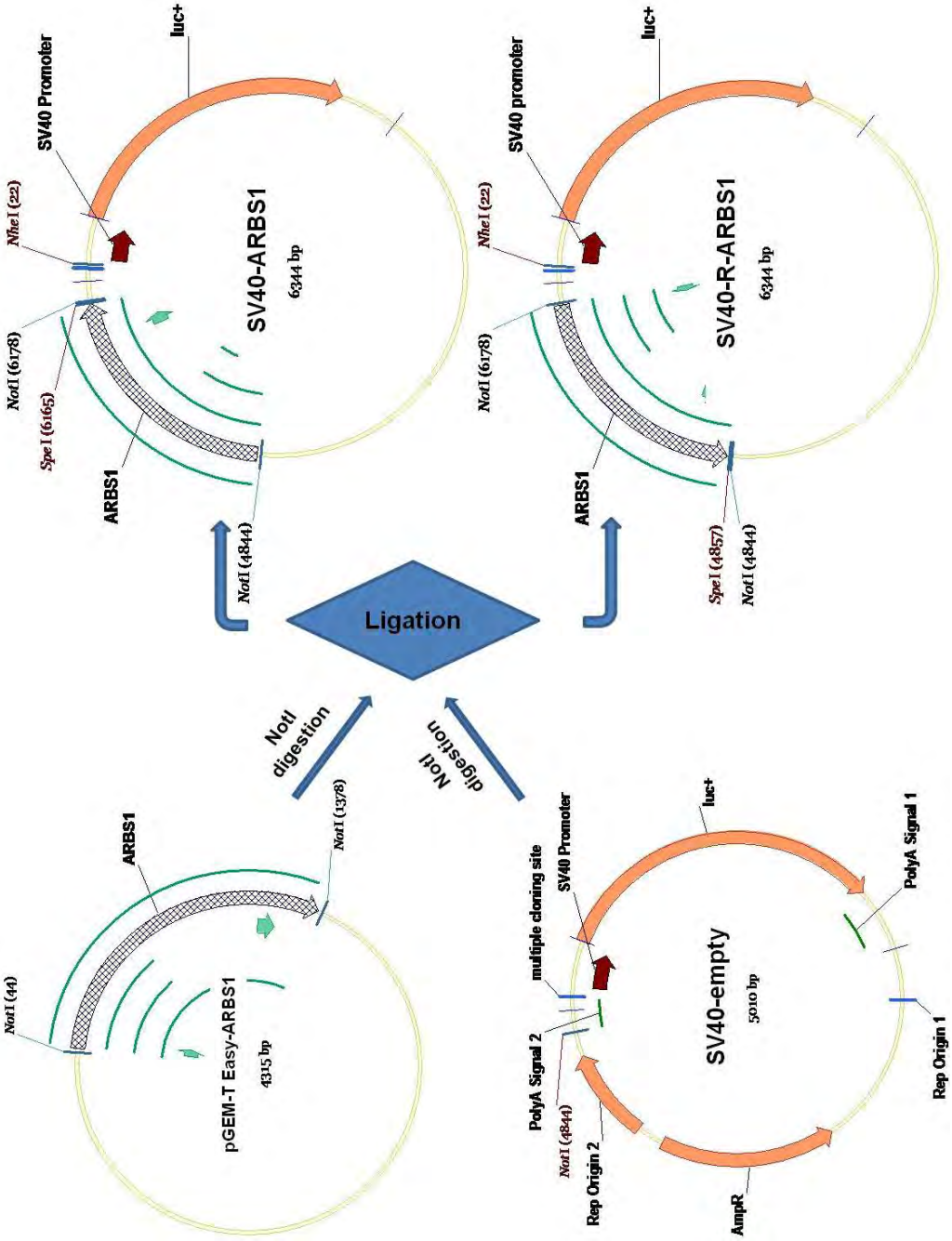


Figure 5. 3 Schematic representation of the construction SV40-ARBS1 and SV40-R-ARBS1

ARBS1 was inserted into plasmid SV40-empty upstream of the SV40 promoter with two possible orientations to form the two constructs, respectively.

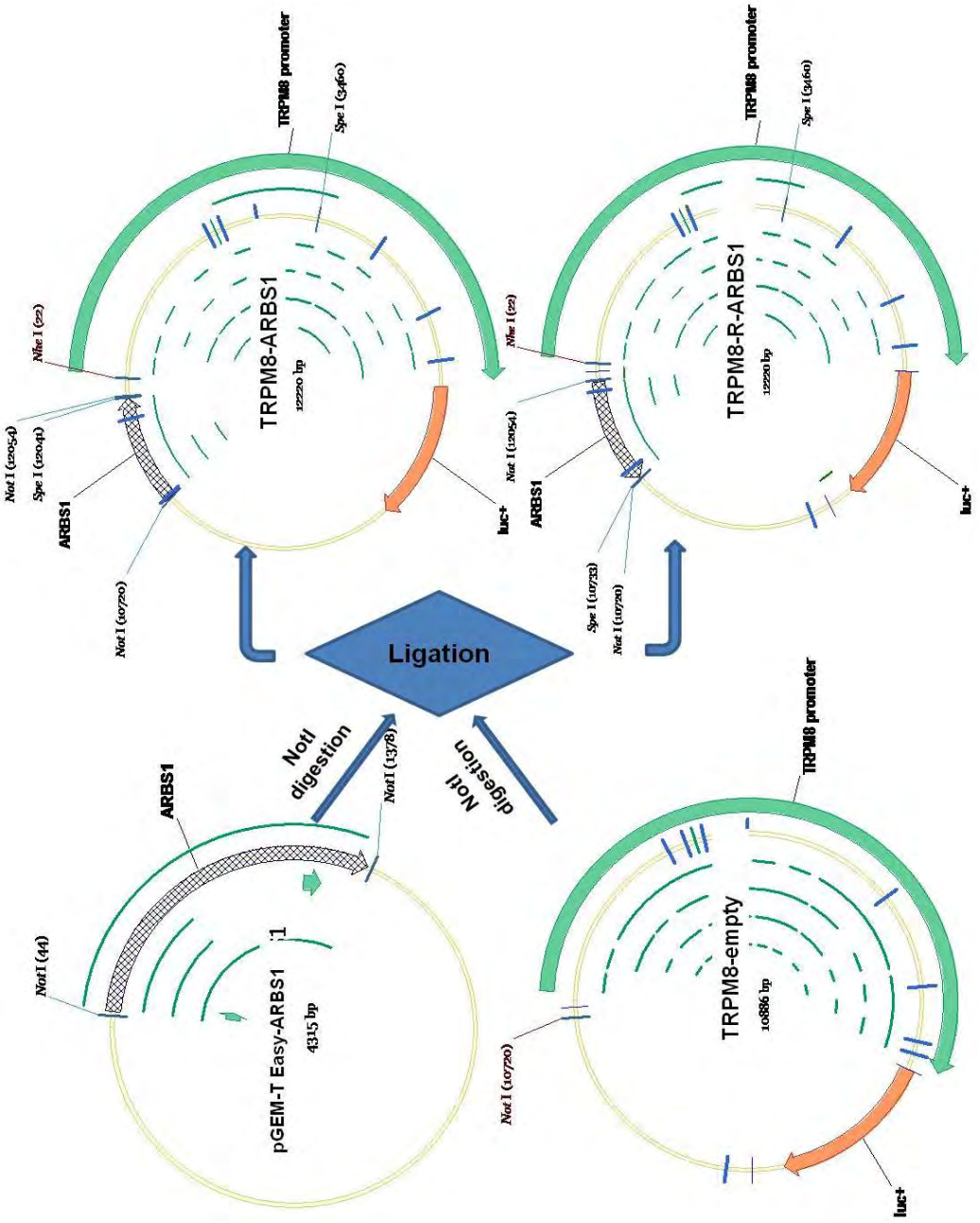


Figure 5. 4 Schematic representation of the construction of TRPM8-ARBS1 and TRPM8--R-ARBS1

ARBS1 was inserted into plasmid TRPM8-empty immediately upstream of the

TRPM8 promoter with two possible orientations to form the two constructs, respectively.

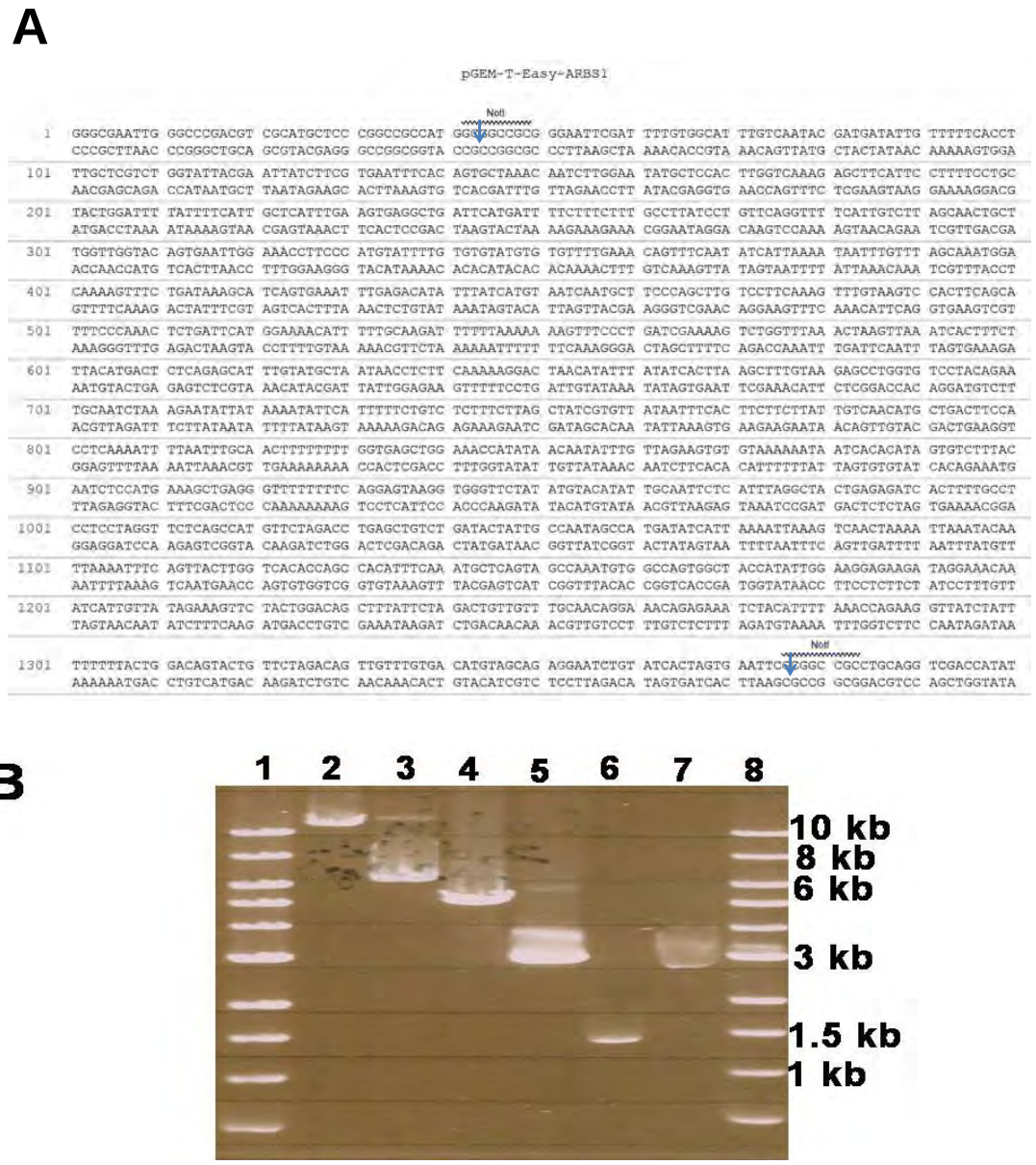


Figure 5. 5 *NotI* restriction enzyme digestion of plasmids used to create constructs

A. Sequence of *NotI*-ARBS1-*NotI*. Double strand DNA sequence is shown here. The sequence refers to the sequence of the sense strand. *Top strand*, sense strand. *Bottom strand*, antisense strand. *Blue arrows*, *NotI* digestion sites. **B.** Gel verification of successful *NotI* digestion of the plasmids and purified 1.3 kb *NotI*-ARBS1-*NotI*. *Lane 8*, 1kb DNA size ladders. *Lane 2* and *3*, TRPM8-empty, *NotI* digestion and non-digestion, respectively. *4* and *5*, SV40-empty, *NotI* digestion and non-digestion, respectively. *6*, purified 1.3 kb *NotI*-ARBS1-*NotI*. *7*, pGEM-T EASY-ARBS1 non-digestion.

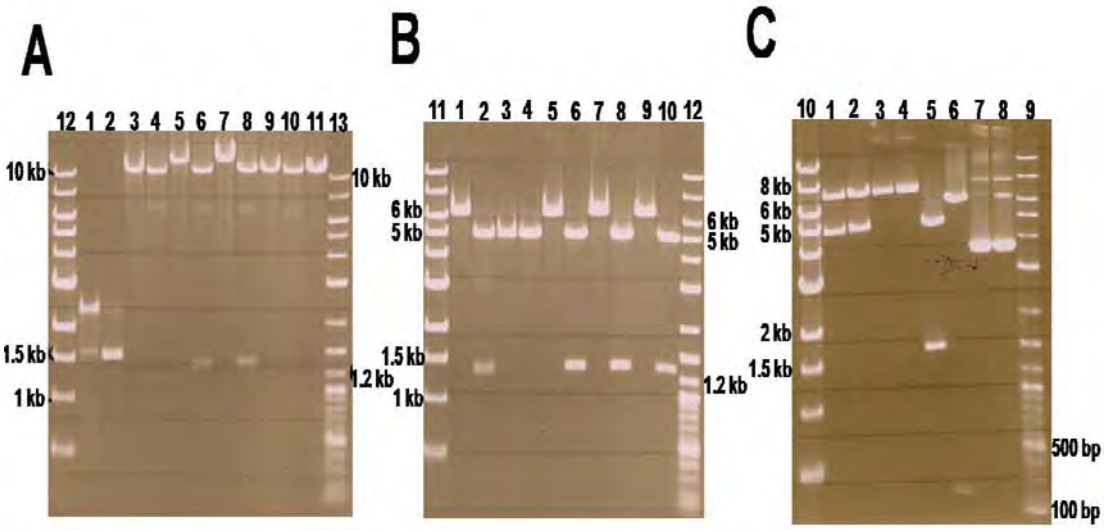


Figure 5. 6 Agarose gel Images of restriction enzyme mapping for construct selection

A. Restriction enzyme mapping of TRPM8-based constructs with the correct insert. In this gel, a positive construct was shown in lanes 5 and 6, the other shown in 7 and 8. *Lanes 1, 3, 5, 7 and 9, NheI* digestons. *Lanes 2, 4, 6, 8 and 10, NotI* digestions. *Lane 11, NheI* digestion of TRPM8-empty (used here as a size marker for 10.8 kb). *Lanes 12,* 1kb DNA size ladder. *Lane 13,* 2-log DNA size ladder. **B.** Restriction enzyme mapping of SV40-based constructs with the correct insert. In this gel, lanes 1 and 2 represented a positive construct; 5 and 6 represented the second one; 7 and 8 the third one; 9 and 10 the fourth one. *Lanes 1, 3, 5, 7 and 9, NheI* digestons. *Lanes 2, 4, 6, 8 and 10, NotI* digestions. *Lanes 11,* 1kb DNA size ladder. *Lane 12,* 2-log DNA size ladder. **C.** Determination of the orientation of the inserts. Lanes 1 and 2 were both TRPM8-R-ARBS1. Lane 5 was SV40-R-ARBS1, and lane 6 was SV40-ARBS1. *Lane 9,* 2-log DNA size ladder. *Lane 10,* 1 kb DNA size ladder.

5.2.3 Effects of androgen treatment on luciferase induction

In order to test the ARBS1's effects on either SV40 promoter- or *TRPM8* promoter-driven firefly luciferase gene expression upon androgen treatment, at stage two, three constructed plasmids (SV40-ARBS1, SV40-R-ARBS1 and TRPM8-R-ARBS1) were transfected into LNCaP cells and followed by androgen treatment (refer to Chapter 2 for details). Meanwhile, the recipient plasmids, SV40-empty and TRPM8-empty, were also transfected as controls, as well as plasmid pGL-PROBPr-luc (designated as positive control vector) which served as a positive control for the whole luciferase reporter gene assay system. A transfection efficiency control, a Renilla luciferase reporter gene plasmid (phRL-Null) was cotransfected with each of the plasmids mentioned above, respectively.

At stage three, after transfection and androgen treatment, the cells were harvested and lysed. The firefly and Rnilla luciferase values from each cell sample were measured and the relative firefly luciferase was calculated (ratio of firefly luciferase/ Renilla luciferase). The difference in the relative firefly luciferase values between –DHT and +DHT treatment of the cells transfected with each plasmid was compared (shown in Fig 5.7). For the positive control vector, the relative firefly luciferase in +DHT treatment was significantly higher than –DHT treatment (954 fold increase). This demonstrated that the whole lucifersase reporter gene assay system was working

and had reasonable sensitivity. A slight increase of relative firefly luciferase was observed in +DHT treatment compared to the corresponding –DHT treatment for SV40-ARBS1 and SV40-R-ARBS1 transfected LNCaP cells (both 1.1 fold, $p > 0.05$). SV40-empty transfected cells had a mild decrease of relative firefly luciferase in +DHT treatment (0.9 fold) (Fig 5.7A). The most interesting finding was the observation of a significant decrease of relative firefly luciferase in +DHT treatment in TRPM8-R-ARBS1 transfected cells (63.4 % of the luciferase value of –DHT treatment, $p=0.009$) whereas TRPM8-empty transfected cells showed a 1.8 fold increase of relative firefly luciferase compared to –DHT treatment (Fig 5.7B).

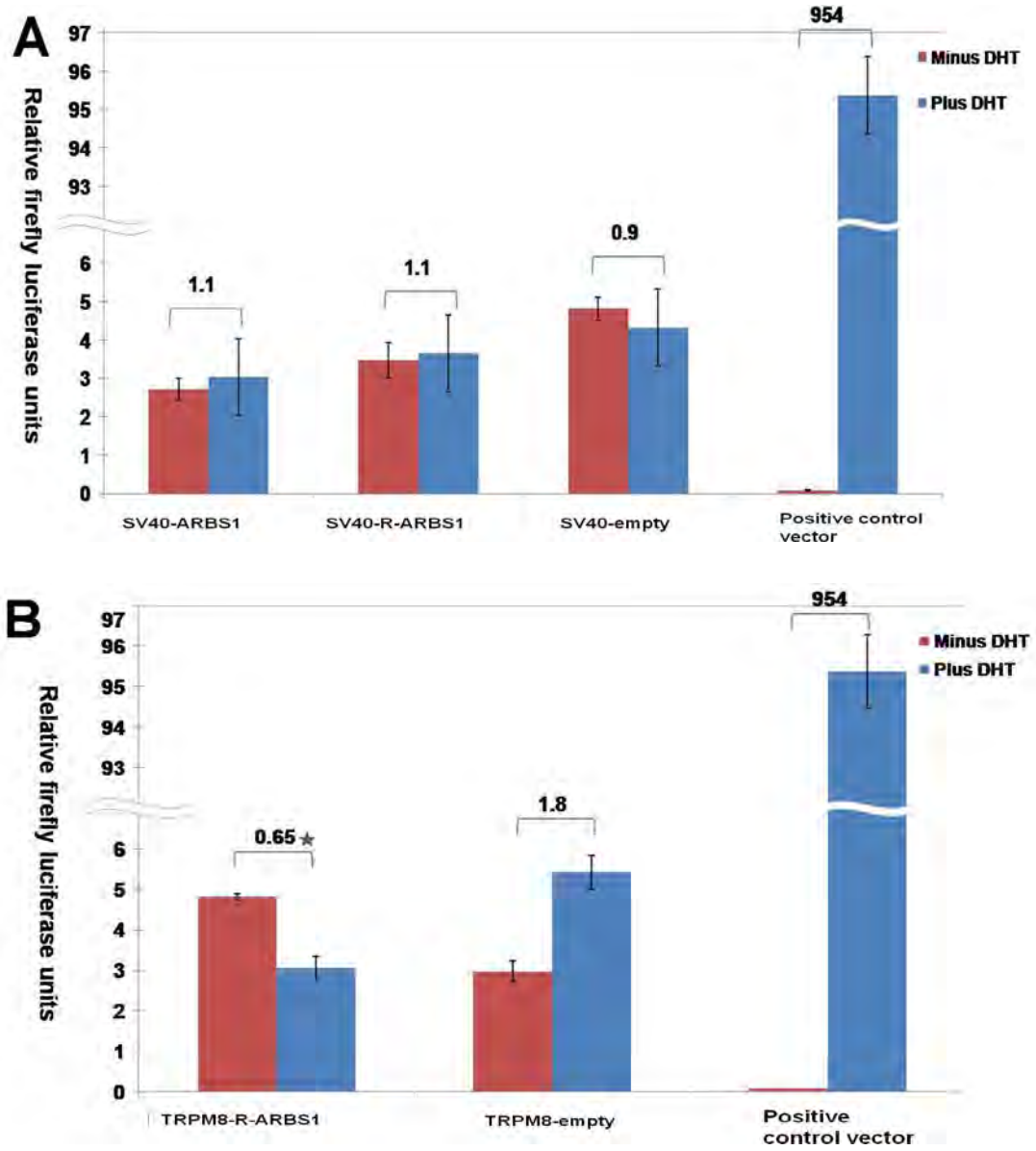


Figure 5. 7 Analysis of ARBS1 regulatory functions on heterologous or endogenous promoters using luciferase reporter gene assays.

Androgen-deprived LNCaP cells transfected with various luciferase (luc.) reporter gene vectors underwent either -DHT or +DHT treatment followed by cell harvest and luc. detection. For each plasmid, relative firefly luc. values were then compared between -DHT and +DHT treatments. While positive controls were effective, no significant changes were observed in SV40-based constructs, however, a significant decrease of firefly luc. was observed in TRPM8-R-ARBS1 transfected cells with +DHT treatment. Relative firefly luc. unit = firefly luc./ Renilla luc. **A.** Luc. results for SV40 based constructs. **B.** Luc. results for TRPM8 based constructs. *Numbers above bars*, fold-change between two treatments. *, $p<0.01$. N=3.

5.3 Discussion

5.3.1 The inhibitory effects of ARBS1 on luciferase gene expression

Luciferase assay showed that ARBS1 only inhibited the luciferase expression when the luciferase gene was driven by the 6 kb *TRPM8* promoter. At the same time 10 nM DHT was presented. A likely explanation for this observation is that ARBS1 has inhibitory function which is androgen- and promoter- dependent (this will be discussed further in Chapter 6). Another possible explanation is that in TRPM8-R-ARBS1, ARBS1 is in a counterclockwise orientation which caused the inhibition through an orientation-related mechanism. It would therefore be important to test this possibility by testing the plasmid, TRPM8-ARBS1, by luciferase assay in the future experiments (refer to Section 6.5.1). The third possibility could be the effects of the long length of the total genomic sequence insert, the 6kb *TRPM8* promoter and 1.3 kb of ARBS1 in a circular vector, on the DNA folding after AR binding leading to inhibition of the luciferase gene expression. However, there is no evidence supporting this possibility.

5.3.2 Colony screening

There are three commonly used colony screening methods: DNA sequencing, PCR-based screening and traditional restriction enzyme mapping. In the stage of

constructing luciferase reporter gene plasmids, two sets of colony screenings were performed. The first one was screening for colony containing pGEM-T-EASY-ARBS1. The second one was screening for the colonies containing the four designed luciferase reporter gene plasmids, respectively.

For the first colony screening, DNA sequencing was used. The main reason for using DNA sequencing here was to choose the correct construct containing ARBS1 sequence free of point mutations since the ARBS1 had been amplified by PCR reaction. It is known that Taq DNA polymerase has a relatively low fidelity with an average of one nucleotide substitution error in every 9000 nucleotides polymerised during PCR (Tindall and Kunkel 1988). AccuPrime Taq DNA Polymerase High Fidelity used here for amplifying ARBS1 claimed to have 9 fold higher fidelity than the normal Taq polymerase (product leaflet, Invitrogen). Assuming that the normal Taq polymerase error rate is 1 in 10,000 nucleotides per PCR cycle (Eckert and Kunkel 1991) therefore when using AccuPrime Taq DNA Polymerase High Fidelity the error rate would be roughly 1 in 90,000 (p) nucleotides per PCR cycle. Thus, for 35 cycles (n) of amplification, the average error frequency (f) at the end of the PCR can be calculated by equation: $f = np/2$ (Eckert and Kunkel 1991). The frequency is around 2 nucleotides per 10,000 nucleotides. Considering that the single copy of PCR product is around 3000 bp, there will be 2 nucleotides mutated per 3-4 copies of

the PCR product. The sequencing result showed one colony out of three chosen ones contained the correct sequence.

The second colony screening used restriction mapping. Although the sequencing method can directly tell if a construct has the correct insert and its orientation, the amount of work would be considerable. Since the *NotI*-ARBS1-*NotI* is around 1.3 kb long, two sequencings are required to cover its whole length: one sequencing uses a forward primer to sequence 700-800 bp of the sense strand of the DNA, the other sequencing uses a reverse primer to sequence another 700-800 bp on the antisense strand of the DNA from reverse direction. If a total of 10 colonies are to be screened, 20 sets of sequencing data are needed to be analysed comparing to three gel images from the restriction mapping method. In addition, JM109 is a widely using *E.coli* strand for cloning and it is known for its low mutation rate. So there is no need to use sequencing to screen out possible mutations. The PCR-based colony screen method is an alternative option to use. It uses PCR and gel electrophoresis to detect the length of insert DNA.

Chapter 6

General Discussions

CHAPTER 6: GENERAL DISCUSSIONS

The main findings from this thesis are:

1) Identification of four highly conserved putative AREs across species;
2) The pattern of endogenous *TRPM8* mRNA changes during androgen deprivation and re-addition;
3) A 6.1 kb *TRPM8* promoter containing luciferase reporter constructs detected insignificant androgen responsiveness;
4) A ChIP-qPCR study identified an androgen receptor binding site, AR Binding Site 1, at around 28 kb upstream of *TRPM8* gene.
5) Promoter regulatory function analysis using a luciferase reporter gene assay showed that ARBS 1 exibited inhibitory function upon androgen stimulation. This inhibitory function seemed to be *TRPM8* promoter dependent.

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

At the beginning of this study, our hypothesis for AR regulation of *TRPM8* gene expression was that the AR initiated *TRPM8* gene transcription by binding to two sites at the *TRPM8* promoter (Fig 6.1 A, also refer to Chapter 1). However, the findings from the current study did not show the presence of an ARE at the 6kb *TRPM8* promoter to initiate the gene transcription although the possibility of AREs

present in the *TRPM8* promoter cannot be excluded, and ARBS 1 inhibits *TRPM8* gene transcription (Fig 6.1B).

Based on the evidence suggesting that the AR initiates *TRPM8* gene transcription, together with the concentration-dependence of the initiation and induction by androgen (Bidaux et al. 2007;Bidaux et al. 2005;Henshall et al. 2003;Zhang and Barritt 2004;Zhang and Barritt 2006), and the finding from the current study, we hypothesize a new model for AR regulation of *TRPM8* gene transcription (Fig 6.1C). The new model consists of three sections: 1) *TRPM8* promoter putative AREs, 2) putative silencer within the AR Binding Site 1, 3) AREs located somewhere else but likely within the gene body. When in the presence of low physiological androgen concentrations (i.e. 0.1 or 1 nM) the AR starts binding to AREs in section 1) and 3), and initiates gene transcription. When androgen concentration increases to 10 nM, the AR starts to bind ARE in ARBS 1(weak binding) to inhibit transcription. When androgen concentration further increases, such as to 100 nM, AR binding to ARBS 1 becomes stronger and exerts stronger inhibitory effects. This may results in a tight regulation of *TRPM8* gene transcription by the AR in an androgen concentration-dependent manner. Since we did not exclude the possibility of having AREs at the *TRPM8* promoter, the AREs in the promoter may exert an initiation and /or enhancer function on *TRPM8* gene transcription. However, it needs to be validated. Other

AREs, except for the one in ARBS 1, are likely to be enhancing the *TRPM8* gene transcription under low androgen concentration, and ARE in ARBS 1 may show AR binding at higher androgen concentration.

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

Firstly, it is possible that functional AREs are among the putative AREs, however, both our luciferase reporter gene system and ChIP assay system did not detect them. There are two possible reasons for this. One reason is high basal level of AR binding to the putative AREs independent of androgen activation. As described in Chapter 1, this could be the result of either one or both of two mechanisms. The first mechanism is AR activation by other ligands, such as DHEA, androstenediol, estradiol and progesterone due to mutation of the AR in LNCaP cells (Veldscholte et al. 1990). The other mechanism is activation through the interactions with other signalling pathways without androgen or other ligands, such as the tyrosine kinase pathway (Jia et al. 2003;Lee et al. 2004). The other possible reason for no detecting AREs is the activation of AR binding in the presence of a basal level of androgen (in the steroid hormone deprived medium) due to its hypersensitivity (Lin et al. 2009). In addition to the two reasons, the observation of a putative androgen independent silencer

within the 6.1 kb *TRPM8* promoter indicates that overall inhibition by the silencer might also reduce the overall detection sensitivity of the luciferase reporter gene assay system.

Secondly, the AGAACAnnnTGTTCT consensus motif of a classical ARE, as discussed in Chapter 1, can also be recognized by other steroid hormone receptors, such as glucocorticoid receptor, progesterone receptor or mineralocorticoid receptor (Claessens et al. 2008; Cleutjens et al. 1996). Therefore, among these conserved putative AREs there may actually be some responsive elements for other steroid hormone receptors.

Thirdly, they are functional responsive elements in other tissues or cell lines but not in the LNCaP cell line due to the lack of the particular steroid hormone signalling co-regulators in LNCaP cells. For example, it was reported that LNCaP cells did not express detectable progesterone receptor. Therefore, it is possible some of these putative AREs are actually progesterone responsive elements, but not functional in LNCaP cells due to the absence of progesterone signalling in LNCaP cells (Webber et al. 1996).

Fourthly, these putative AREs may be functional AREs but have been suppressed in the LNCaP cell line by some epigenetic mechanism, such as DNA methylation, histone modification or the lack of some AR co-regulators (Lin et al. 2009). It has been shown that *TRPM8* expression increased at early stages (stages T1 and T2) of prostate cancer, but decreased to close to normal at advanced stages (T3, T4, N+ or M+) of prostate cancer (Fuessel et al. 2003). In addition, as the LNCaP cell line is a metastasis model of prostate cancer, its *TRPM8* expression has been shown to be much lower than that of early stage prostate cancer (Kiessling et al. 2003). Furthermore, there is evidence of the down-regulation of some AR regulated genes in advanced stages of prostate cancer, and promoter methylation is one of the mechanisms of suppressing gene expression (Hendriksen et al. 2006;Simi et al. 2010). Therefore it is possible that some AREs in LNCaP cells have been deactivated in some way, such as by DNA methylation, to down regulate *TRPM8* expression in LNCaP cells (compared to the *TRPM8* expression level in early stage of prostate cancer tissue) and it would be interesting to test it.

Lastly, bioinformatics analysis for AREs only implies, but does not demonstrate the presence of real AREs (Heemers et al. 2004). This may be due to the probability of random sequence repeating.

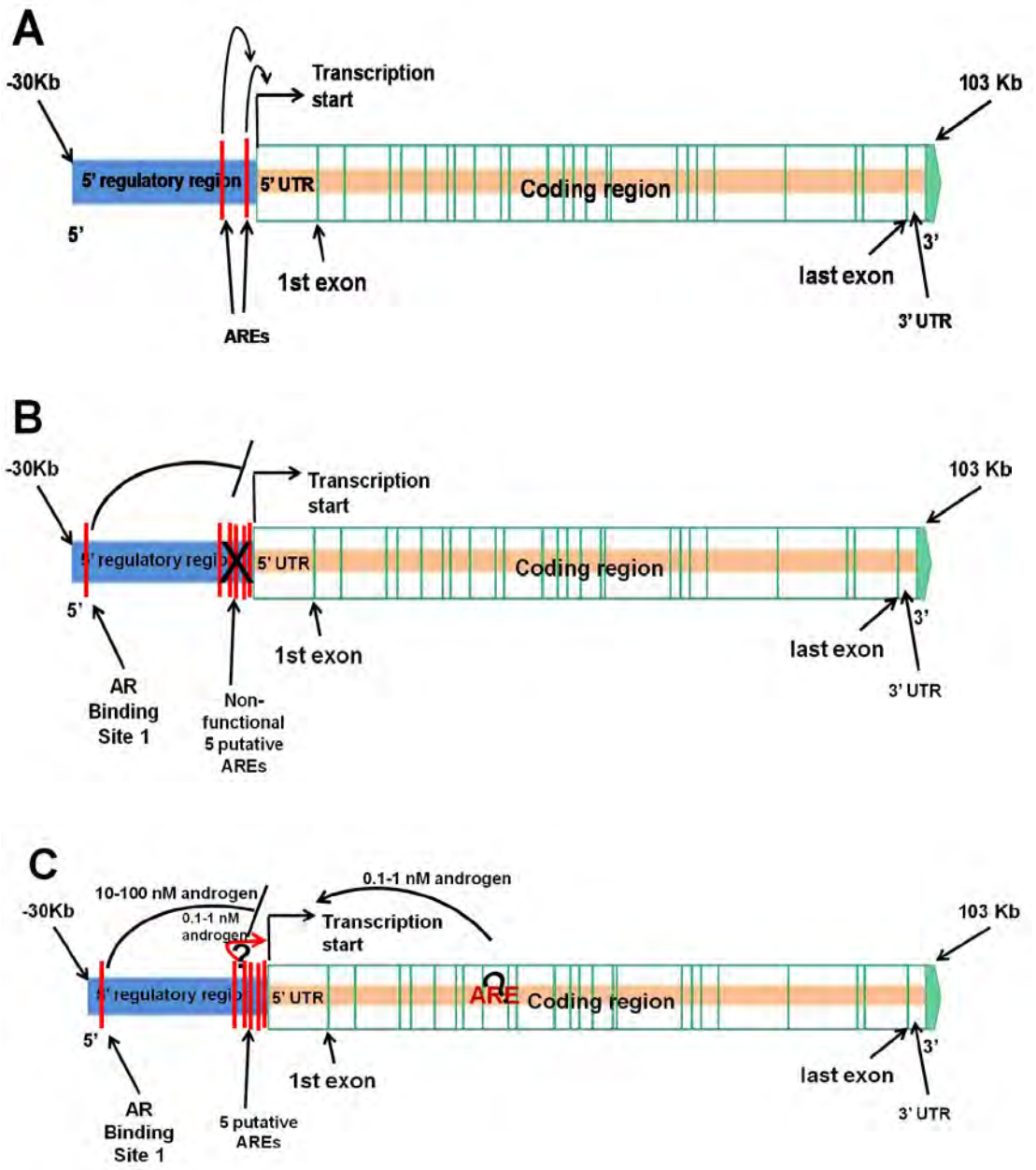


Figure 6. 1 Models for AR regulation of *TRPM8* gene transcription

A. Original hypothesis for the model of AR regulation of *TRPM8* gene expression. **B.** model derived from the current results. **C.** Proposed new model for AR regulation of *TRPM8* gene expression based on the findings from the current study and data from previous studies by others. *0.1-1 nM androgen* or *10-100 nM androgen*, the range of androgen concentrations at which the AREs exert their effects on transcription.

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

The location of AR Binding Site 1, between -27 kb and -29 kb of the 5' flanking region of the *TRPM8* gene, raises a question whether the AR Binding Site 1 belongs to the *TRPM8* gene or to some other gene nearby. We think the AR Binding Site belongs to the *TRPM8* gene although there are three other genes located nearby (Fig 6.2).

If we assume AR Binding Site 1 belongs to one of the other genes, e.g. the MSL3L2 gene and is promoter independent, we then would observe SV40 based constructs showing significant luciferase changes instead of no significant changes. Therefore, at least it is not promoter independent. On the other hand, If assuming that AR Binding Site 1 still belongs to the MSL3L2 gene and is promoter dependent, we then would not observe a significant luciferase decrease in the *TRPM8* base construct (since it is promoter-dependent from assumption we should only see significant luciferase changes in MSL3L2 promoter-based constructs). Therefore, the conclusion is AR Binding Site 1 does not belong to other genes nearby. In addition, the three nearby genes are Male specific lethal 3-like 2 (MSL3L2, Gene ID 151507), Holiday junction recognition P (HJURP, Gene ID 55355) and hypothetical LOC100130859 (Gene ID 100130859). There is no evidence showing that they are AR regulated

genes. Furthermore, there is an evidence of AR binding site located more than 100 kb away from its regulated gene (Jariwala et al. 2007).

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

Numbers above horizontal line, genomic location on chromosome 2. *Black vertical bar*, location of AR Binding Site. *Red arrow*, *TRPM8* gene and its orientation. *Grey arrows*, location and orientation of different nearby genes. Adapted from (*TRPM8* gene (homo sapiens), Gene ID 79054, full report, Entrez Gene, NCBI website).

6.4 AR regulation of *TRPM8* gene expression

The absence of ARE in the *TRPM8* promoter as well as the region further upstream which can initiate *TRPM8* gene transcription, showed that the AR regulation of *TRPM8* gene expression is not what we originally predicted in our hypothesis. In addition, the identification of a putative ARE silencer containing AR Binding Site 1 indicates a more complicated AR regulation mechanism of the *TRPM8* gene. Based on the evidence from previous studies by others and data from the current study, we have proposed a model of AR regulation of *TRPM8* gene expression (refer to section 6.1)

It has been demonstrated that the AR initiates *TRPM8* gene transcription. But in the current study such an ARE (or AREs) has not been identified in the -30 kb of the 5' regulatory region and first kilobase of the 5' untranslated region of the *TRPM8* gene. In addition further upstream from -30 kb there are other genes (see Fig 6.2). Furthermore, no AR binding sites were identified in the 5' flanking region of the *TRPM8* gene according to the data published from one ChIP-chip study that covered the promoter region of 24,275 genes using a promoter array (Massie et al. 2007). Therefore it is likely that this ARE is located in the introns or 3' end of the *TRPM8* gene. The evidence that the AR is able to bind estrogen responsive elements and that *TRPM8* is regulated by the estrogen receptor in breast cancer cells suggests the

possibility of a functional estrogen responsive element in the *TRPM8* gene. This could potentially be the AR binding site for initiating *TRPM8* gene transcription.

The AR Binding Site 1 harbours a low affinity AR binding site, identified by another group (Buchanan *et al*, personal communication). So it is possible that when high AR activity is present, AR may repress *TRPM8* gene transcription through binding with this ARE silencer. This mechanism may explain the observation of a DHT dose response of *TRPM8* mRNA expression (0.1nM >1nM>10nM) in LNCaP cells, with lowest induction at 100nM DHT (Bidaux et al. 2005). In contrast, the DHT dose response of *PSA* is different, mRNA expression in LNCaP cells (0.1nM <1nM <10nM < 100nM) (Zhu et al. 2003).

From luciferase assays on the *TRPM8* promoter, we observed the existence of a potential AR independent silencer. It may be partly due to this silencing activity that the *TRPM8* gene is silenced when AR or androgen is absent. In addition, bioinformatic analysis of the *TRPM8* promoter identified several putative transcription factor binding sites (Zhang and Barritt 2006), among them a putative NKX3.1 binding site being of particular interest since it is an AR regulated repressor. There is evidence for two *TRPM8* isoforms, one full length, the other an N-terminal truncated isoform (Bidaux et al. 2007). The gene expression of the truncated one has

been shown to be less sensitive to androgen regulation and it was suggested the two isoforms play distinct roles in prostate cancer progression (Bidaux et al. 2007). The lower AR sensitivity of the truncated TRPM8 isoform may also be explained by our new proposed model of AR regulation. If there is an alternate promoter for this isoform, which consists of the first several introns and exons, it may potentially bypass the inhibition of a silencer and repressor in the conventional *TRPM8* promoter as well as the inhibition from the putative ARE silencer within AR Binding Site 1. Similarly, this would also nearly abolish the other AREs' regulation. Therefore the transcription of the truncated isoform may drive from the basal transcription activity. However, this assumption needs to be tested.

Taken together, these data showed the strict control of *TRPM8* expression by the AR. This is significant with respect to the putative physiological and pathological functions of TRPM8. It has been suggested that in normal prostate tissue, TRPM8 may play an important role in regulating protein, ion secretion and proliferation and / or apoptosis under the control of the AR. In prostate cancer cells, it may play a role in regulating prostate cancer cell proliferation, with anti-apoptotic functions also under the control of the AR. So the current study provides additional information to help us better understand how the AR regulates *TRPM8* gene expression in the context of prostate cancer.

6.5 Future directions

6.5.1 Testing TRPM8-ARBS1 by luciferase assay

As mentioned in Section 5.3.1, it would be important to test ARBS1's function in TRPM8-ARBS1 by luciferase assay to rule out and /or confirm any orientation related mechanism. This could be achieved by constructing plasmid TRPM8-ARBS1 and testing by luciferase assay using the methods and strategy described in Chapter 5.

6.5.2 Identification of the ARE sequence within AR Binding Site 1

To identify the ARE sequence, we can apply the luciferase reporter assay approach combined with electrophoretic mobility shift assay to test its *in vitro* interaction with the AR. Since AR Binding Site harbours a known low affinity AR binding site, it would be highest priority to search AREs within this low affinity AR binding site. The strategy may be 1) direct point mutations on the identified putative AREs within this region in the current construct, TRPM8-R-ARBS1, to see if a particular point mutation could abolish AR Binding Site 1's inhibitory function; 2) applying EMSA to test the in vitro AR binding ability on the positive putative AREs.

Another alternative strategy may be directly constructing luciferase reporter vectors (*TRPM8* promoter driven) containing each putative ARE and test their functionalities. Similarly, EMSA would be carried out on the positive putative AREs.

Based on the model of AR regulation of the *PSA* gene, it is possible to identify more than one ARE in AR Binding Site 1 (Kim and Coetzee 2004).

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

As described earlier (Sections 3.3.2 and 6.2), it would be important to prevent or rule out the possible effects of androgen-independent activation of the AR in our luciferase reporter gene system. Therefore, reporter gene assays would be conducted in an androgen-deprived cell line, such as PC-3, with transient co-expression of wild-type AR in combination with EMSA and ChIP assay to test *in vitro* AR binding. The strategy is 1) testing the constructs described in Chapter 3 in PC-3 cell line with transient co-expression of wild-type AR; 2) directly cloning each of the putative AREs into pGL3 Promoter vector as an enhancer, respectively, to test their androgen responsiveness in the PC-3 cell line as described in 1); 3) performing a ChIP assay on the 6 kb *trpm8* promoter in PC-3 cells with transient expression of the AR and/or

in LNCaP cells after AR silencing; 4) EMSA can be carried out on the positive putative AREs.

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

We could not experimentally find AREs in the 6.1 kb promoter region and we identified a potential ARE silencer in the 30 kb 5' regulatory region. As described previously, a further search in the 6 kb promoter for ARE is needed. However, it is possible, for some reason, that AREs are in other regions, likely in the 5' untranslated regions and introns (Jariwala et al. 2007;Lin et al. 2009). This could be tested by some high throughput ChIP assay methods, such as ChIP-chip or ChIP-seq.

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

Since the AR Binding Site 1 is promoter-dependent, to identify the specific region on the *TRPM8* promoter that it interacts with would be very useful for us to understand the mechanism of its promoter–dependence, and thus provides more information on AR regulation of the *TRPM8* gene. This can be achieved by a reporter gene assay approach. The strategy would be to clone the silencer ARE into pGL-TRPM8pr-luc,

followed by serial deletions of the *TRPM8* promoter, to see what region is required for the silencer ARE to exert its function.

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

As mentioned above, during the progression of prostate cancer to more advanced stages, some AR-regulated genes have been shown to be down-regulated (Hendriksen et al. 2006). The *TRPM8* gene appears to be one of those genes. Therefore studying the functionalities and roles of the identified AREs in the *TRPM8* gene may help us better understand in a dynamic fashion how AR regulates *TRPM8* gene expression with respect to prostate cancer progression. One way to look at this is to study the AR binding pattern of these AREs in different stages of prostate cancer using cell lines as models and applying a ChIP assay approach. This can also be combined with a study of the pattern of corresponding chromatin modifications, such as histione methylation or acetylation (Jia et al. 2008).

Furthermore, there is a model suggesting that when the AR exerts its repressor function, it binds to ARE then recruits corepressors which in turn recruit histone deacetylases (HDAC) to the transcription complex resulting in chromatin condensation (Jia et al. 2003; Rosenfeld and Glass 2001). It would be interesting to

test if the binding of HDAC is present at the region, as well as on the promoter region when repression occurs, by applying the ChIP assay approach (Jia et al. 2008).

6.5.7 Future directions

This study has shown that AR regulation of *TRPM8* gene transcription is more complicated than we originally thought. AR's regulation of the *TRPM8* gene may involve initiation, enhancement and repression. This strict control of *TRPM8* gene transcription further supports the potential important physiological function of *TRPM8* in normal prostate tissue and pathological roles in prostate cancer carcinogenesis and progression. Therefore the more knowledge we can obtain, potentially the better understanding of prostate cancer we will have. For further exploring the regulation of *TRPM8* expression by the AR, we can look at the mechanism how the AR up-regulates *TRPM8* expression at the early stages of prostate cancer, how it selectively down regulates *TRPM8* gene expression and how this fits into the bigger picture of AR involvement in prostate cancer initiation and progression. It has been shown that TRPM8 has several isoforms and some of them may be important for prostate cancer progression (Bidaux et al. 2007). We need to know how AR regulates the isoform expression, and its physiological significance in terms of the AR regulation of prostate cancer progression as well as normal prostate

functions. Is AR involved in *TRPM8* post-transcription modifications and / or post-translation modifications? All these questions need to be answered.

Appendix

APPENDIX 1: qPCR PRIMERS FOR THE CHIP ASSAY

1.1 Positive control primers (Chr 19, Amplicon size:88 bp)

PSApr fw: 5’-CCTAGATGAAGTCTCCATGAGCTACA-3’

PSApr rv: 5’-GGGAGGGAGAGCTAGCACTTG-3’

1.2 Negative control primers (Chr 20: 44141632-44141782, SPINT3 gene, Amplicon size: 88bp)

NC fw:5’-ATTTTCACTTCTGAGAGTAGCCTGATG-3’

NC rv: 5’- AGTGGCTGCGCGAGGAT-3’

1.3 -1kb region (Amplicon size: 99 bp)

5F126FR: 5’-TCAGCGGCTTAAGTTTTTTGTTC-3’

5F49RV: 5’-CCACAGGAGGAGGAAAGCTTTT-3’

1.4 -2kb region (Amplicon size: 75bp)

5F1438FR: 5’-TGTATTTTTAGTAGAGACAGGGTTTTGC-3’

5F1381RV: 5’- GGCCGATTGCTTGATGTCA-3’

1.5 -3kb region (Amplicon size: 77bp)

5F2996FR: 5’-GGCCAAGAGACAGGGTTATGG-3’

5F2944RV: 5’-GCACATCAAGTCATACATCCTTCCT-3’

1.6 -4kb region (Amplicon size: 75 bp)

5F3214FR: 5’-GACCAGGGTCTTGGCAACAG-3’

5F3140RV: 5’-TCATCAGGAAGAAGAAAGGTTACCA-3’

1.7 -5kb region (Amplicon size: 75 bp)

5F4359FR: 5’-TGCGCCCTTGCTATCATTG-3’

5F4285RV: 5’ TCCATGTCTGACAAGCTCTTACCA-3’

1.8 -6kb region (Amplicon size: 75 bp)

5F5977FR: 5’-TCCTCTCTCCACCTCCTTCACT-3’

5F5903RV: 5’-TGCAGCCCAGGCAGACA-3’

1.9 -7kb region (Amplicon size: 75 bp)

5F6375FR: 5’-AGGCCTCCTGAGCTGAATGA-3’

5F6301RV: 5’-CAATTTGCTGATGAGTTGGAGAAG-3’

1.10 -8kb region (Amplicon size: 81 bp)

5F7667FR: 5’-GCTTTTCATGTTCCCTTTTCCA-3’

5F7587RV: 5’-GACAGGGAAGATGCCGAATG-3’

1.11 -9kb region (Amplicon size 102 bp)

5F8434FR: 5’-AAAAAGACCCTGGAACCAGATG-3’

5F8333RV: 5’-AACTGAGGCACAAACAGATTAAGTAATT-3’

1.12 -10 kb region (Amplicon size: 116 bp)

5F9973FR: 5’-AAAAGTTGCTCAAGATTACACAGGTAAA-3’

5F9858RV: 5’-ACCTGGCTGCCTAAAAATGTATAAG-3’

1.13 -11 kb region (Amplicon size: 83 bp)

5F10646FR: 5’-TGAGGTCAGGATTTCAAGACCAA-3’

5F10564RV: 5’-CCACACCTGGTTACTTTATTGTATTTTT-3’

1.14 -12 kb region (Amplicon size: 75 bp)

5F11678FR: 5’-GTCCCCCTGCCCACCTACT-3’

5F11604RV: 5’-GGGTGTGGCTAGAGCTTGGA-3’

1.15 -13 kb region (Amplicon size: 85 bp)

5F12772FR: 5’- TTTCCTTGCTTTTTCCAACATCTA-3’

5F12688RV: 5’- CAACACGGCTGGCTTTGAG-3’

1.16 -14 kb region (Amplicon size: 75 bp)

5F13720FR: 5’- ACCCAAGGCAACAAAAGTAAAAAC-3’

5F13646RV: 5’- CTGGACACACATCAGCATGGA-3’

1.17 -15 kb region (Amplicon size: 80 bp)

5F14643FR: 5’- TGAGAGTAAAGTCCAGAGGGAGAAG-3’

5F14564RV: 5’- GCTCAGGTGACACCACCAAGA-3’

1.18 -16 kb region (Amplicon size: 75 bp)

5F15565FR: 5’- CAGCCTGCCCTCTTACAGATACA-3’

5F15491RV: 5’- AGGACCCAGTGAAAATAAAAAGGA-3’

1.19 -17 kb region (Amplicon size: 80 bp)

5F16583FR: 5’- GCGTGCCGTGGAAAGATCT-3’

5F16504RV: 5’- ACTTGGGAGGTTGAGGCAAGA-3’

1.20 -18 kb region (Amplicon size: 84 bp)

5F17377FR: 5’-CAGATTGGCAGGAGAGTGAGTTT-3’

5F17294RV: 5’-TTTCCTTGTGGCATCATATGTCTAC-3’

1.21 -19 kb region (Amplicon size: 79 bp)

5F18279FR: 5’- ACCAGCTGGATCTGAGTGTTGA-3’

5F18201RV: 5’- CATGACATCTGGTCACAAAAGCA-3’

1.22 -20 kb region (Amplicon size: 101 bp)

5F19126FR: 5’- CACAAATGTGGTGGCTTAAACAA-3’

5F19026RV: 5’- CCCACAAAAGGAACCGACTCT -3’

1.23 -21 kb region (Amplicon size: 89 bp)

5F20882 FR: 5’-TGTGAGGACCAGCAGCTAAGC-3’

5F20794 RV: 5’-CCCCACTCCCACACTTTGC-3’

1.24 -22 kb region (Amplicon size: 114 bp)

5F21929FR: 5’- TGGGCATTTTCCAGGTCAAA-3’

5F21816RV: 5’- CCAGGGTTGGGACATGTTATG-3’

1.25 -23 kb region (Amplicon size: 106 bp)

5F22738FR: 5’- GCACACCCACGACACCAA-3’

5F22633RV: 5’- CATCCTTGTCTCGTTCCCTAAATAG-3’

1.26 -24 kb region (Amplicon size: 81 bp)

5F23877FR: 5’- GTGGTGTTCCCTTGCCTGTAG-3’

5F23797RV: 5’- GGGAGACGACACAGAGGAGAAG-3’

1.27 -25 kb region (Amplicon size: 75 bp)

5F24404FR: 5’- AAGCTGCCTCACCCAAGGT-3

5F24330RV: 5’- GACCAAGATGGACACAGTGATGA-3’

1.28 -26 kb region (Amplicon size: 120 bp)

5F25530FR: 5’- CAGACCAATATGAATCAATCGATAAGAAT-3’

5F25411RV: 5’- TTGTCACATGTTTCTTGTTCACTTAGA-3’

1.29 -27 kb region (Amplicon size: 75 bp)

5F26385FR: 5’-TGCAACGCTCTCTTCTCTTTCTAG-3’

5F26311RV: 5’- GCCATTCTGGACTGCATGTG-3’

1.30 -28 kb region (Amplicon size: 82 bp)

5F27216FR: 5’- CTTGGTCACACCAGCCACAT-3’

5F27135RV: 5’- TTTCCTATCTTCTCCTTCCAATATGG-3’

1.31 -29 kb region (Amplicon size: 78 bp)

5F28863FR: 5’- TCAGAGCTCCTTGCATTGGA-3’

5F28786RV: 5’- GAGTGTCCCACAGCCAGTAACTG-3’

1.32 -30 kb region (Amplicon size: 84 bp)

5F29426FR: 5’- GGATTACCTCATTTCATCATTAATGC-3’

5F29343RV: 5’- CAGGTGGTCAAGCCAAGTCA-3’

1.33 -28 kbARBS (Amplicon size: 85 bp)

FR: 5’-TTTCACCTTTGCTCGTCTGGTA-3’

RV: 5’-TGACCAAGTGGAGCATATTCCA-3’

1.34 1 kb region (Amplicon size: 85 bp)

5UTRFR: 5’-TTCCGTTCTTCCAGCCTGTT-3’

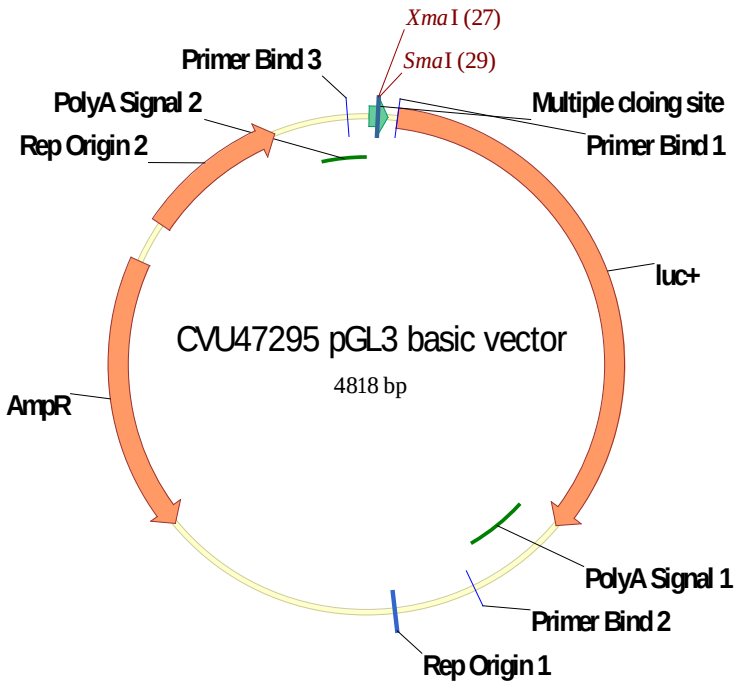
5UTRRV: 5’-TTATTCATCCAGCCCTTCAGTGT-3’

APPENDIX 2: PLASMID CONSTRUCTS USED FOR TRANSIENT TRANSFECTION AND LUCIFERASE REPORTER GENE ASSAYS

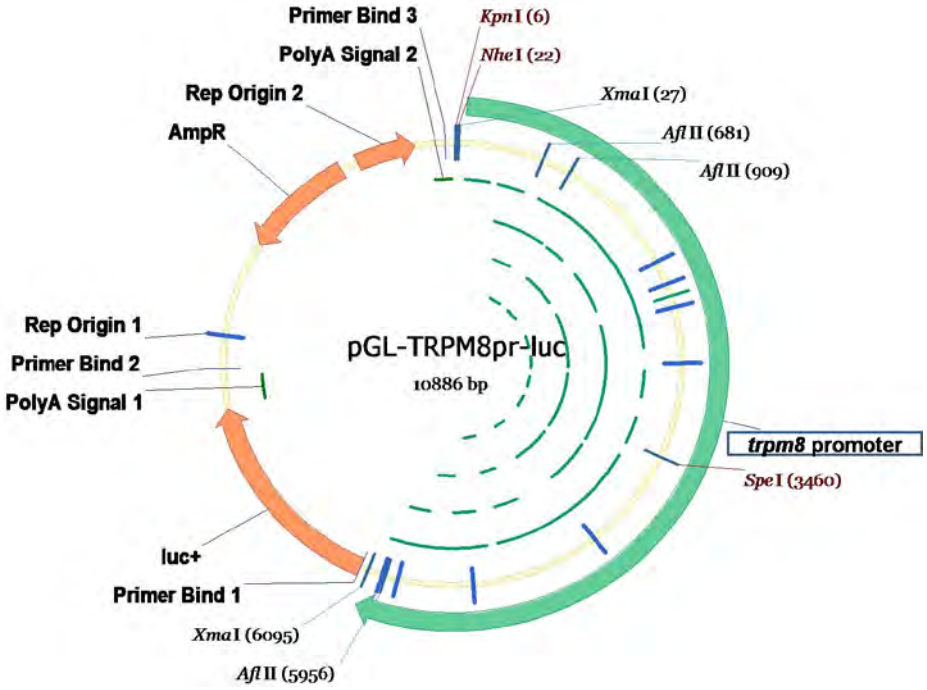
2.1 pGL-TRPM8pr-luc and pGL-R-TRPM8pr-luc

TRPM8 promoter (6 kb, -6042/ +20) was inserted into pGL3 Basic at *XmaI* site in two orientations and formed pGL-TRPM8pr-luc (clockwise orientation of the insert) and pGL-R-TRPM8pr-luc (counterclockwise orientation of the insert)

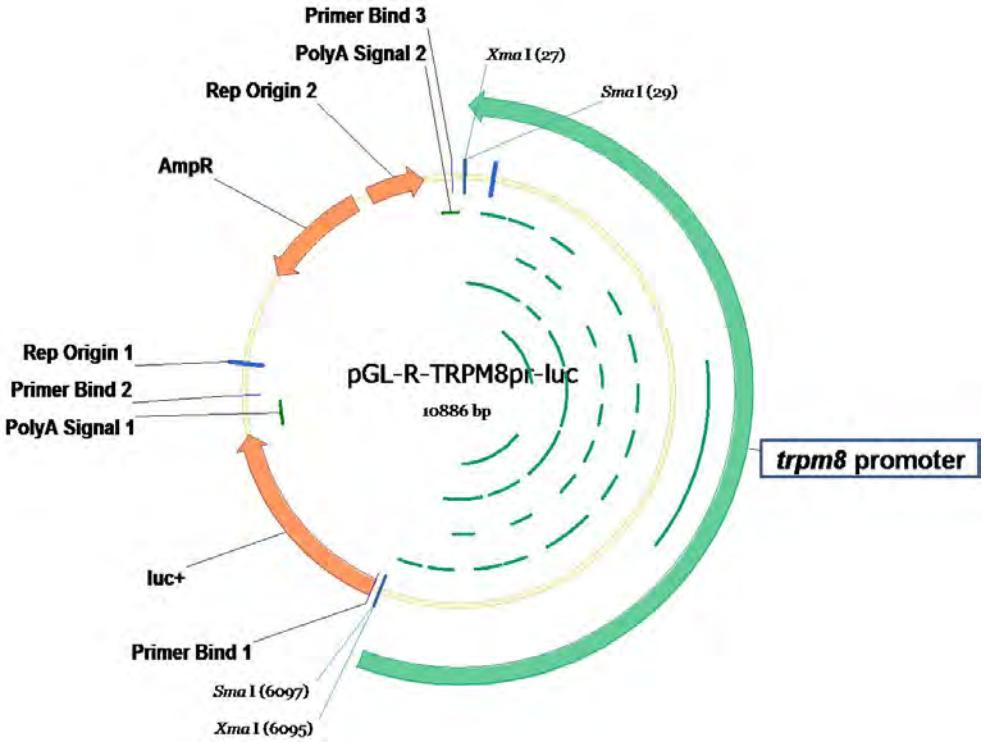
2.1.1 Map of the recipient vector, pGL3 Basic



2.1.2 Map of pGL-TRPM8pr-luc



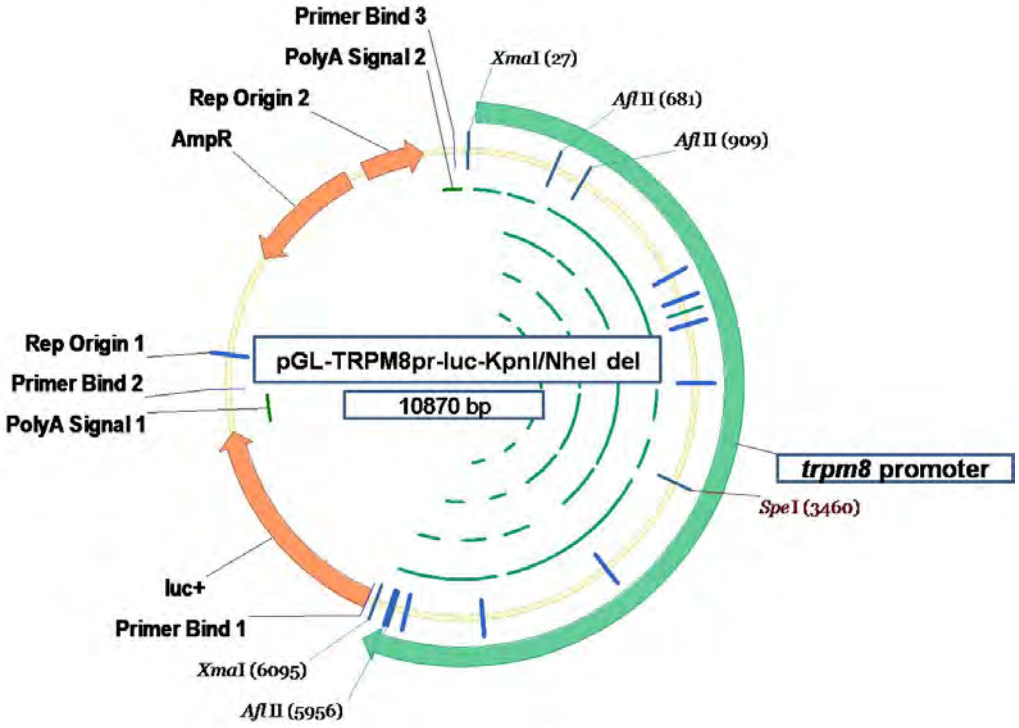
2.1.3 Map of pGL-R-TRPM8pr-luc



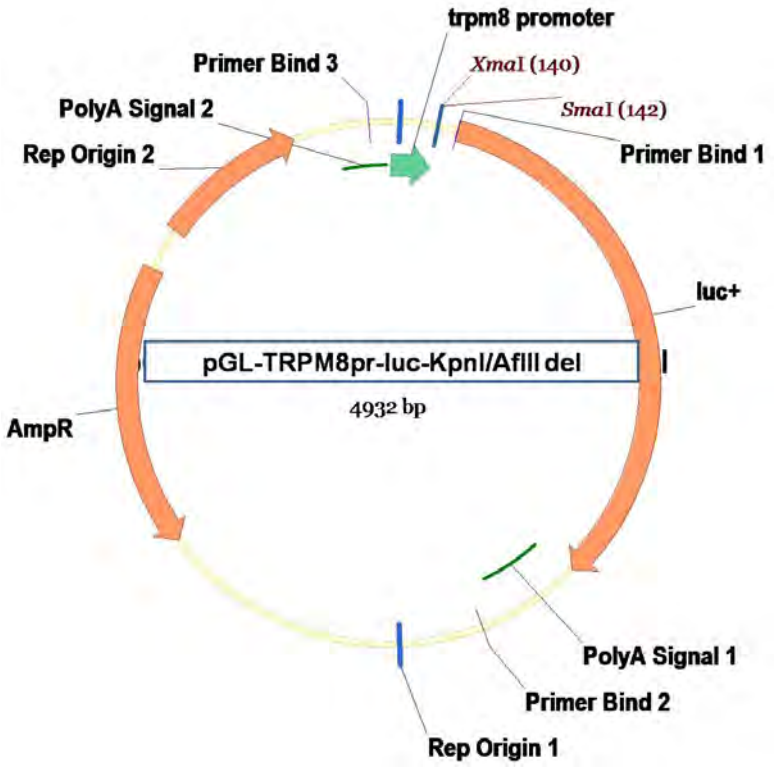
2.2 pGL-TRPM8pr-luc based deletion constructs, pGL-TRPM8pr-luc-*KpnI*/*NheI* del and pGL-TRPM8pr-luc-*KpnI* /*AflII* del

pGL-TRPM8pr-luc-*KpnI*/*NheI* del was constructed by deleting a 16 bp *KpnI*/*NheI* fragment from pGL-TRPM8pr-luc followed by plasmid recirculization. A triple digestion, *Kpn I*/*SpeI*/*AflII*, was performed on pGL-TRPM8pr-luc to remove a 5954 bp *KpnI*/*AflII* fragment followed by plasmid recirculization to construct pGL-TRPM8pr-luc-*KpnI*/*AflII* del.

2.2.1 Map of pGL-TRPM8pr-luc-*KpnI*/*NheI* del



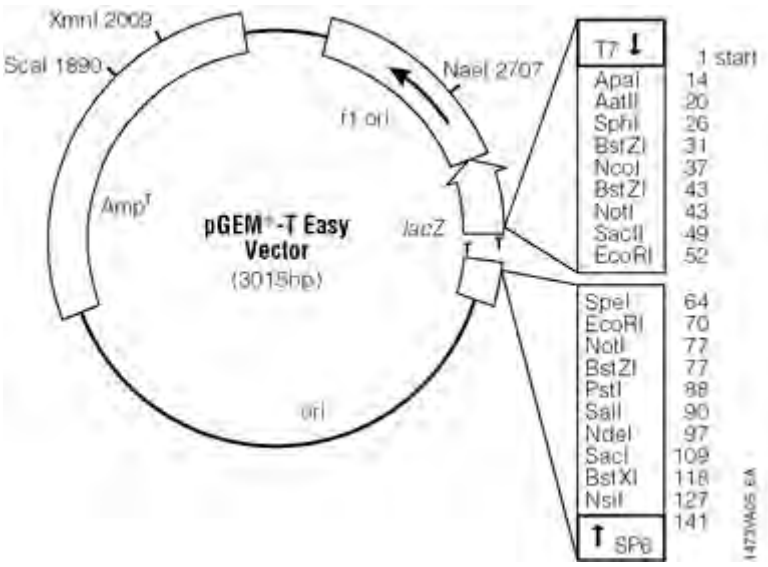
2.2.2 Map of pGL-TRPM8pr-luc-*KpnI/AflII* del



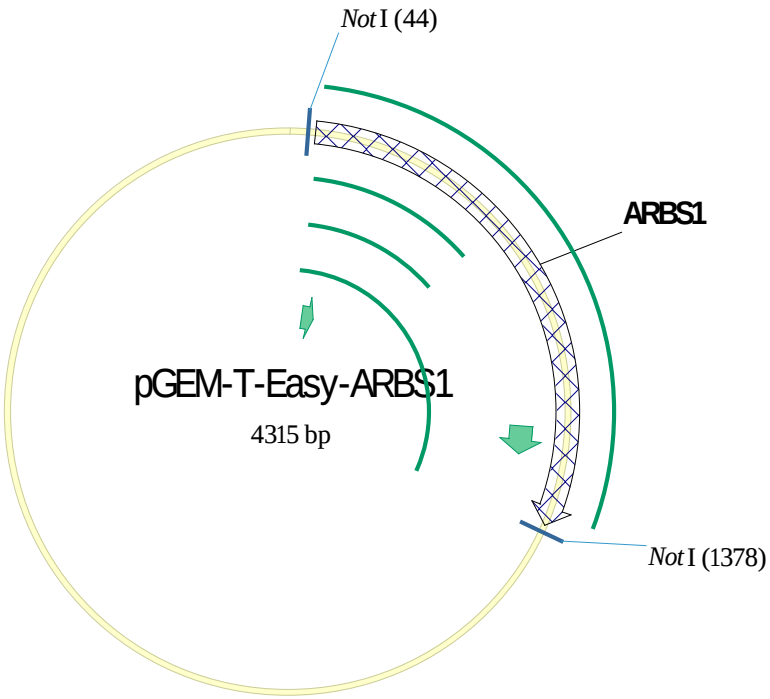
2.3 pGEM-T-EASY-ARBS1

pGEM-T-EASY-ARBS1 was constructed by direct ligating PCR amplified 1298 bp ARBS1 with the linear recipient vector pGEM-T-EASY .

2.3.1 Map of recipient vector, pGEM-T-EASY



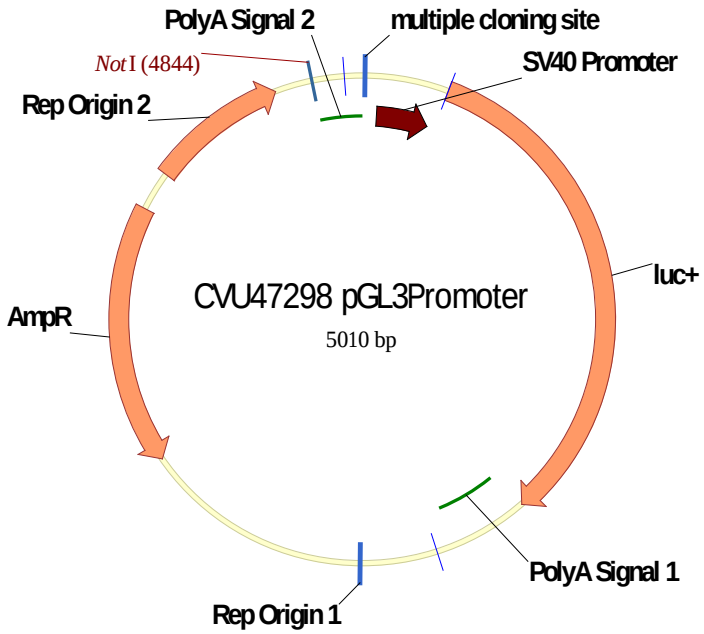
2.3.2 Map of pGEM-T Easu-ARBS1



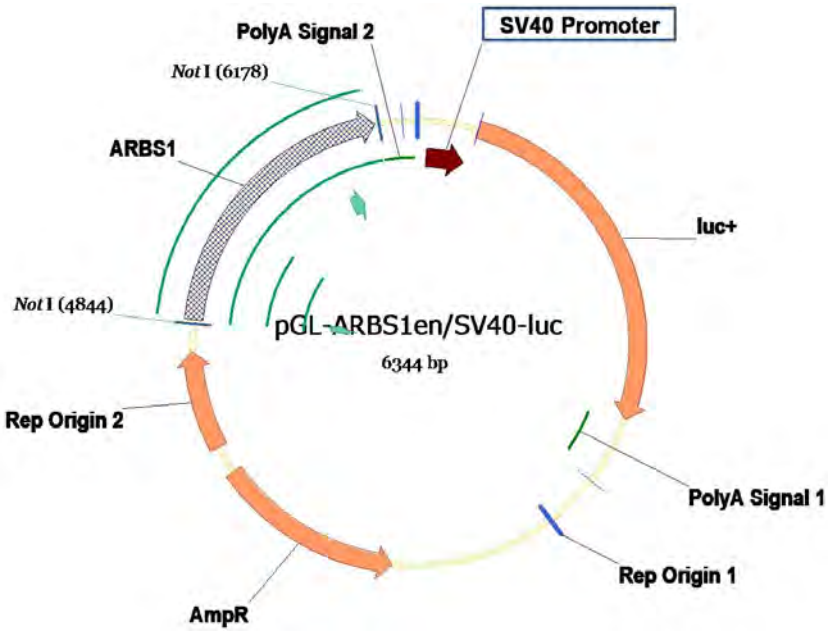
2.4 pGL-ARBS1EN/SV40-luc and pGL-R-ARBS1EN/SV40-luc

pGL-ARBS1EN/SV40-luc and pGL-R-ARBS1EN/SV40-luc were constructed by subcloning the *NotI*/*NotI* fragment from pGEM-T-EASY-ABRS1 into the *NotI* site of pGL3 Promoter vector in both clockwise and counterclockwise orientations.

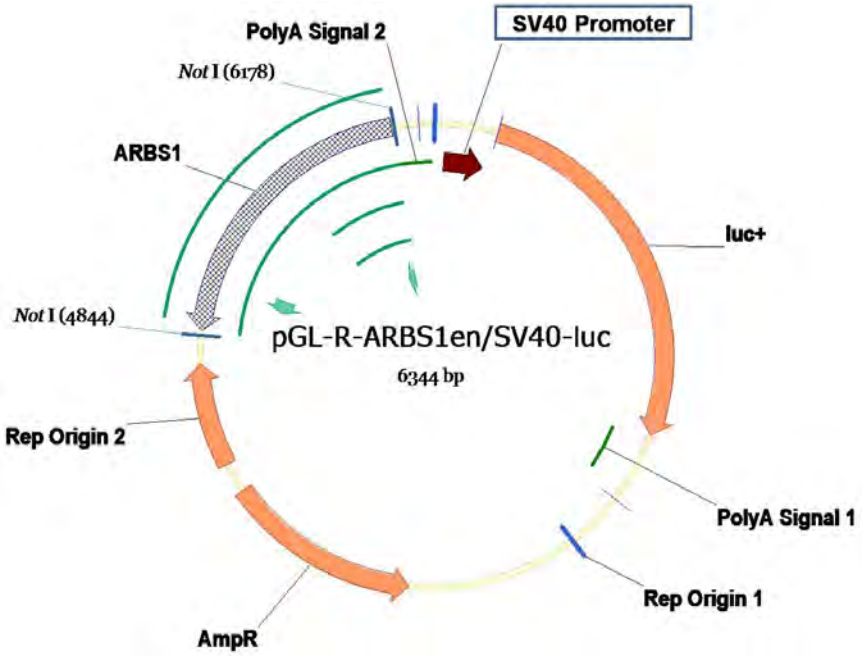
2.4.1 Map of recipient vector, pGL3 Promter



2.4.2 Map of pGL-ARBS1EN/SV40-luc



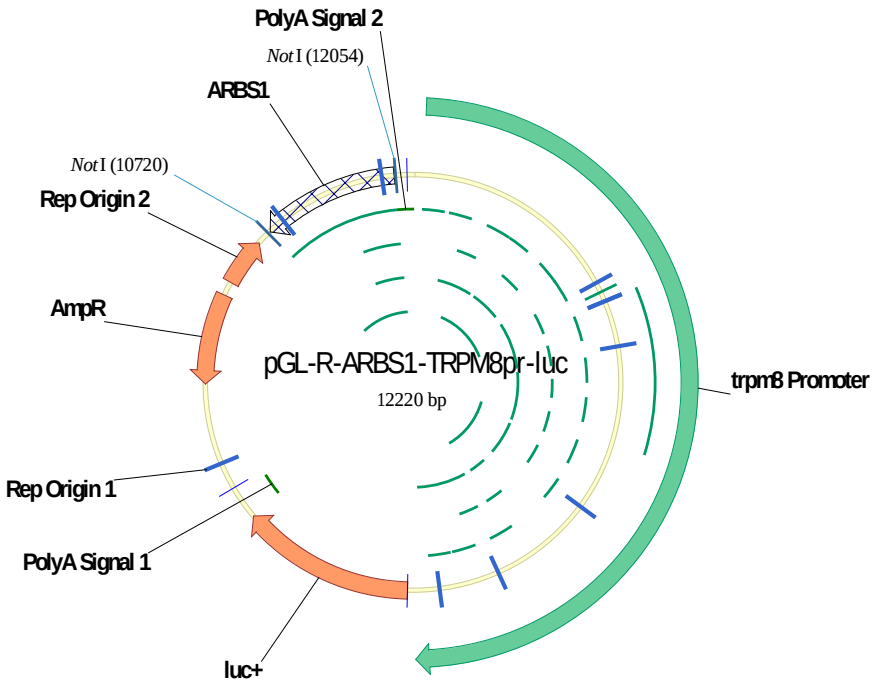
2.4.3 Map of pGL-R-ARBS1EN/SV40-luc



2.5 pGL-R-ARBS1EN/TRPM8pr-luc

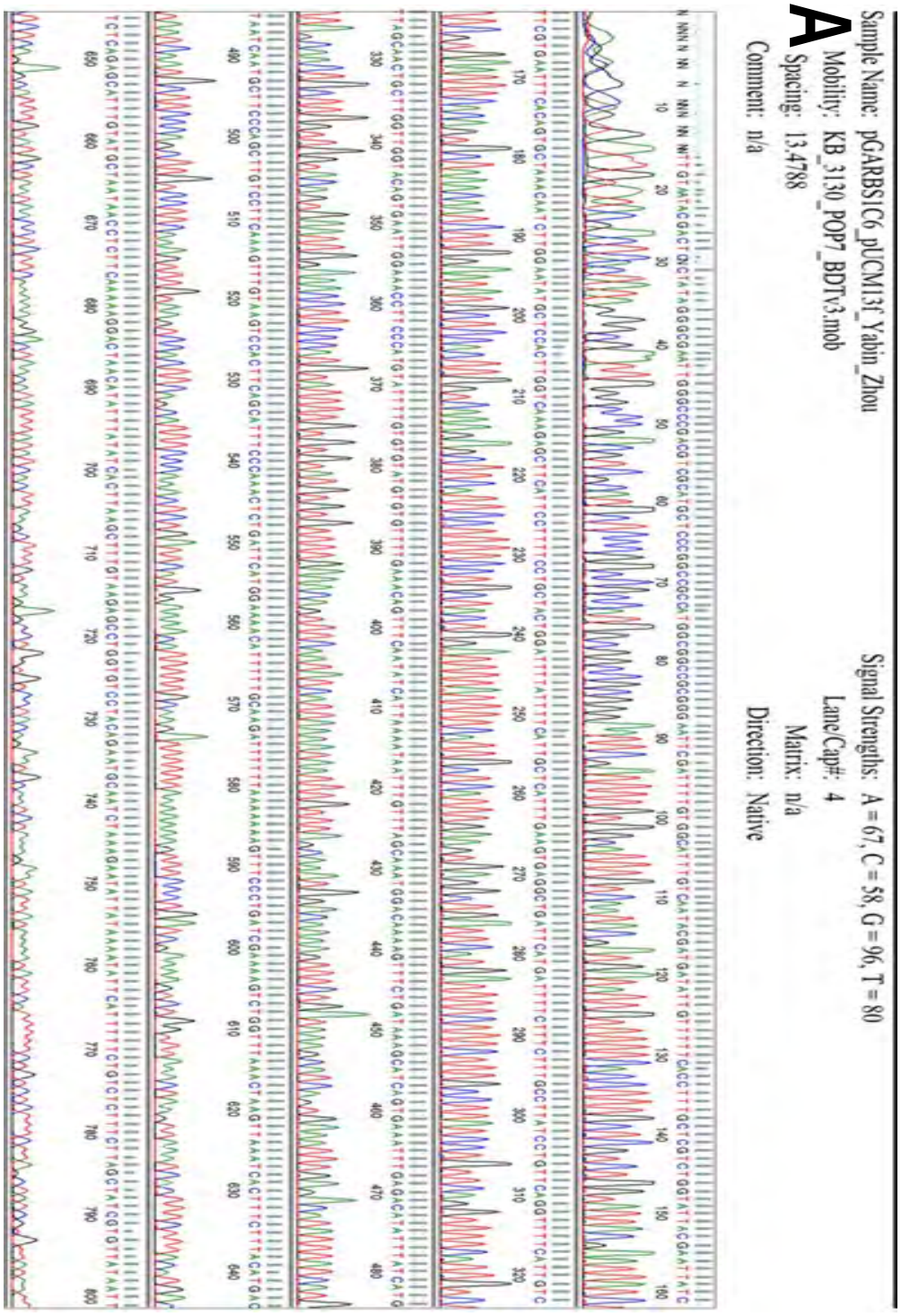
pGL-R-ARBS1EN/TRPM8pr-luc was constructed by subcloning the *NotI*/*NotI* fragment from pGEM-T-EASY-ABRS1 into the *NotI* site of pGL-TRPM8pr-luc plasmid in counterclockwise orientation.

2.5.1 pGL-R-ARBS1EN/TRPM8pr-luc

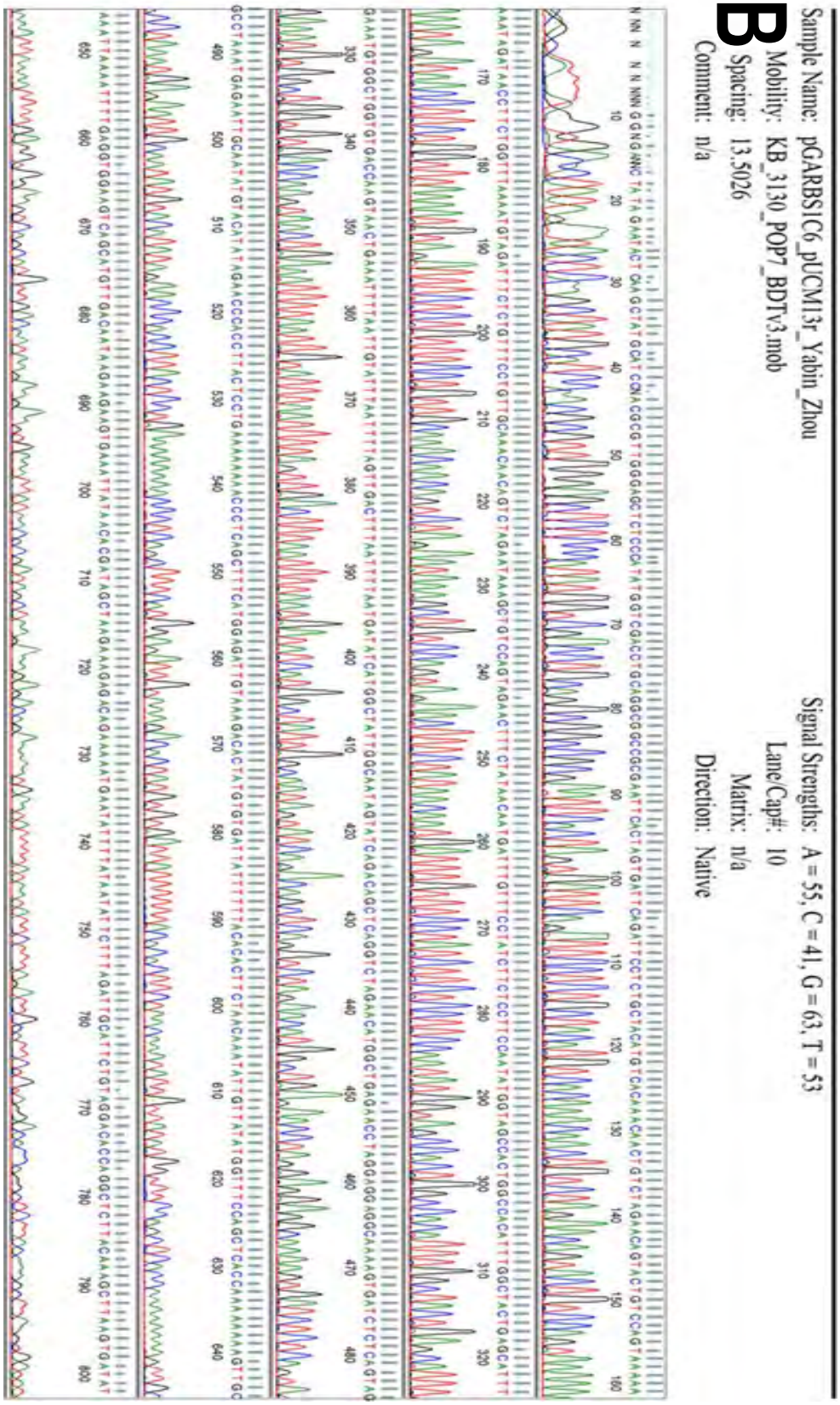


APPENDIX 3: SEQUENCING DATA FOR THE AR BINDING SITE 1 INSERT IN PGEM-T EASY-ARBS1

3.1 Sequencing results of sense strand



3.2 Sequencing results of anti-sense strand



APPENDIX 4: COMPOSITION OF SOLUTIONS

4.1 Solutions for Tissue Culture

4.1.1 Complete RPMI

Stock	Final Concentration
RPMI 1640 solution (containing sodium bicarbonate)	90% (v/v)
FBS	10% (v/v)
Glucose	2.5 g/L
Sodium pyruvate	110mg/L
Hepes (0.5 M, pH7.4)	10mM
Penicillin	100Units/ml
Streptomycin	100µg/ml

4.1.2 Trypsin/EDTA solution (in 1X PBS, pH 7.4)

Stock	Final Concentration
2.5% Trypsin	0.25% (v/v)
0.5M EDTA	0.53mM
Glucose	0.1% (v/v)

4.1.3 10X PBS (pH 7.4)

Stock	Final Concentration
NaCl	80g/L
KCl	2g/L
KH_2PO_4	2.4 g/L
Na2HPO4	11.4g/L

4.2 Solutions for ChIP Assay

4.2.1 SDS Lysis Buffer

Stock	Final Concentration
10% SDS	1% (v/v)
500mM EDTA (pH8.1)	10mM
500mM TrisHCl(pH8.1)	50mM
25x Protease Inhibitor	2X

4.2.2 Dilution buffer

Stock	Final Concentration
10% SDS	0.01% (v/v)
10% Triton X 100	1.1% (v/v)
500mM EDTA(pH8.1)	1.2mM
500mM TrisHCl(pH8.1)	16.7mM
5M NaCl	167mM
25x protease inhibitor	1X

4.2.3 Low salt Immune Complex wash buffer

Stock	Final Concentration
10% SDS	0.1% (v/v)
10% Triton X-100	1%(v/v)
500mM EDTA pH8.1	2mM
500mM TrisHCl pH8.1	20mM
5M NaCl	150mM

4.2.4 High Salt Immune Complex Wash Buffer

Stock	Final Concentration
10% SDS	0.1% (v/v)
10% Triton X-100	1% (v/v)
500mM EDTA pH8.1	2mM
500mM Tris-HCl pH8.1	20mM
5M NaCl	500mM

4.2.5 LiCl Immune Complex Wash Buffer

Stock	Final Concentration
1M LiCl	0.25M
10% Igepal CA-630	1% (v/v)
10% Deoxycholate	1% (v/v)
500mM EDTA pH8.1	1mM
500mM TrisHCl pH8.1	10mM

4.2.6 1 X TE pH8.1

Stock	Final Concentration
500mM TrisHCl pH8.1	10mM
500mM EDTA pH8.1	1mM

4.2.7 Elution Buffer: 500ul per IP and input sample

Stock	Final Concentration
10% SDS	1% (v/v)
1M $NaHCO_3$	0.1M

4.3 Solutions for Bacterial Culture

4.3.1 LB Medium (pH 7.5)

Stock	Final Concentration
Tryptone	10g/L
Yeast extract	5g/L
NaCl	5 g/L

4.3.2 LB Agar

Stock	Final Concentration
Agar	15g/L
LB medium	1L

Bibliography

BIBLIOGRAPHY

ABATE-SHEN, C. & SHEN, M. M. (2000) Molecular genetics of prostate cancer. *Genes & Development,* 14**,** 2410-34.

ABE, J., HOSOKAWA, H., OKAZAWA, M., KANDACHI, M., SAWADA, Y., YAMANAKA, K., MATSUMURA, K. & KOBAYASHI, S. (2005) TRPM8 protein localization in trigeminal ganglion and taste papillae. *Brain Res Mol Brain Res,* 136**,** 91-8.

ALAM, J. & COOK, J. L. (1990) Reporter genes: application to the study of mammalian gene transcription. *Anal Biochem,* 188**,** 245-54.

ANNICOTTE, J. S., SCHOONJANS, K., HABY, C. & AUWERX, J. (2001) An E-box in pGL3 reporter vectors precludes their use for the study of sterol regulatory element-binding proteins. *Biotechniques,* 31**,** 993-4, 996.

BABES, A., ZORZON, D. & REID, G. (2004) Two populations of cold-sensitive neurons in rat dorsal root ganglia and their modulation by nerve growth factor. *Eur J Neurosci,* 20**,** 2276-82.

BECK, B., BIDAUX, G., BAVENCOFFE, A., LEMONNIER, L., THEBAULT, S., SHUBA, Y., BARRIT, G., SKRYMA, R. & PREVARSKAYA, N. (2007) Prospects for prostate cancer imaging and therapy using high-affinity TRPM8 activators. *Cell Calcium,* 41**,** 285-94.

BENNETT, N. C., GARDINER, R. A., HOOPER, J. D., JOHNSON, D. W. & GOBE, G. C. (2010) Molecular cell biology of androgen receptor signalling. *Int J Biochem Cell Biol,* 42**,** 813-27.

BERTHOIS, Y., KATZENELLENBOGEN, J. A. & KATZENELLENBOGEN, B. S. (1986) Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci U S A,* 83**,** 2496-500.

BIANCHINI, F., KAAKS, R. & VAINIO, H. (2002) Overweight, obesity, and cancer risk. *Lancet Oncol,* 3**,** 565-74.

BIDAUX, G., FLOURAKIS, M., THEBAULT, S., ZHOLOS, A., BECK, B., GKIKA, D., ROUDBARAKI, M., BONNAL, J. L., MAUROY, B., SHUBA, Y., SKRYMA, R. & PREVARSKAYA, N. (2007) Prostate cell differentiation status determines transient receptor potential melastatin member 8 channel subcellular localization and function. *J Clin Invest,* 117**,** 1647-1657.

BIDAUX, G., ROUDBARAKI, M., MERLE, C., CREPIN, A., DELCOURT, P., SLOMIANNY, C., THEBAULT, S., BONNAL, J. L., BENAHMED, M., CABON, F., MAUROY, B. & PREVARSKAYA, N. (2005) Evidence for specific TRPM8 expression in human prostate secretory epithelial cells: functional androgen receptor requirement. *Endocr Relat Cancer,* 12**,** 367-82.

BIRNEY, E., STAMATOYANNOPOULOS, J. A., DUTTA, A., GUIGO, R., GINGERAS, T. R., MARGULIES, E. H., WENG, Z., SNYDER, M., DERMITZAKIS, E. T., THURMAN, R. E., KUEHN, M. S., TAYLOR, C. M., NEPH, S., KOCH, C. M., ASTHANA, S., MALHOTRA, A., ADZHUBEI, I., GREENBAUM, J. A., ANDREWS, R. M., FLICEK, P., BOYLE, P. J., CAO, H., CARTER, N. P., CLELLAND, G. K., DAVIS, S., DAY, N., DHAMI, P., DILLON, S. C., DORSCHNER, M. O., FIEGLER, H., GIRESI, P. G., GOLDY, J., HAWRYLYCZ, M., HAYDOCK, A., HUMBERT, R., JAMES, K. D., JOHNSON, B. E., JOHNSON, E. M., FRUM, T. T., ROSENZWEIG, E. R., KARNANI, N., LEE, K., LEFEBVRE, G. C., NAVAS, P. A., NERI, F., PARKER, S. C., SABO, P. J., SANDSTROM, R., SHAFER, A., VETRIE, D., WEAVER, M., WILCOX, S., YU, M., COLLINS, F. S., DEKKER, J., LIEB, J. D., TULLIUS, T. D., CRAWFORD, G. E., SUNYAEV, S., NOBLE, W. S., DUNHAM, I., DENOEUD, F., REYMOND, A., KAPRANOV, P., ROZOWSKY, J., ZHENG, D., CASTELO, R., FRANKISH, A., HARROW, J., GHOSH, S., SANDELIN, A., HOFACKER, I. L., BAERTSCH, R., KEEFE, D., DIKE, S., CHENG, J., HIRSCH, H. A., SEKINGER, E. A., LAGARDE, J., ABRIL, J. F., SHAHAB, A., FLAMM, C., FRIED, C., HACKERMULLER, J., HERTEL, J., LINDEMEYER, M., MISSAL, K., TANZER, A., WASHIETL, S., KORBEL, J., EMANUELSSON, O., PEDERSEN, J. S., HOLROYD, N., TAYLOR, R., SWARBRECK, D., MATTHEWS, N., DICKSON, M. C., THOMAS, D. J., WEIRAUCH, M. T., GILBERT, J., et al. (2007) Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature,* 447**,** 799-816.

BUNONE, G., BRIAND, P. A., MIKSICEK, R. J. & PICARD, D. (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J,* 15**,** 2174-83.

CAMPBELL, M. F., WEIN, A. J. & KAVOUSSI, L. R. (2007) Campbell-Walsh urology editor-in-chief, Alan J. Wein ; editors, Louis R. Kavoussi ... [et al.]. 9th ed. Philadelphia, W.B. Saunders.

CARTER, B. S., BEATY, T. H., STEINBERG, G. D., CHILDS, B. & WALSH, P. C. (1992) Mendelian inheritance of familial prostate cancer. *Proc Natl Acad Sci U S A,* 89**,** 3367-71.

CATO, A. C., SKROCH, P., WEINMANN, J., BUTKERAITIS, P. & PONTA, H. (1988) DNA sequences outside the receptor-binding sites differently modulate the responsiveness of the mouse mammary tumour virus promoter to various steroid hormones. *EMBO J,* 7**,** 1403-10.

CHEN, C. D., WELSBIE, D. S., TRAN, C., BAEK, S. H., CHEN, R., VESSELLA, R., ROSENFELD, M. G. & SAWYERS, C. L. (2004) Molecular determinants of resistance to antiandrogen therapy. *Nat Med,* 10**,** 33-9.

CHMELAR, R., BUCHANAN, G., NEED, E. F., TILLEY, W. & GREENBERG, N. M. (2007) Androgen receptor coregulators and their involvement in the development and progression of prostate cancer. *Int J Cancer,* 120**,** 719-33.

CHODAK, G. W., KRANC, D. M., PUY, L. A., TAKEDA, H., JOHNSON, K. & CHANG, C. (1992) Nuclear localization of androgen receptor in heterogeneous samples of normal, hyperplastic and neoplastic human prostate. *J Urol,* 147**,** 798-803.

CHONG, P. & RASHID, P. (2005) Can we prevent prostate cancer? *Aust Fam Physician,* 34**,** 265-7.

CHUANG, H. H., NEUHAUSSER, W. M. & JULIUS, D. (2004) The super-cooling agent icilin reveals a mechanism of coincidence detection by a temperature-sensitive TRP channel. *Neuron,* 43**,** 859-69.

CLAESSENS, F., DENAYER, S., VAN TILBORGH, N., KERKHOFS, S., HELSEN, C. & HAELENS, A. (2008) Diverse roles of androgen receptor (AR) domains in AR-mediated signaling. *Nucl Recept Signal,* 6**,** e008.

CLEUTJENS, K. B., VAN DER KORPUT, H. A., VAN EEKELEN, C. C., VAN ROOIJ, H. C., FABER, P. W. & TRAPMAN, J. (1997) An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. *Mol Endocrinol,* 11**,** 148-61.

CLEUTJENS, K. B., VAN EEKELEN, C. C., VAN DER KORPUT, H. A., BRINKMANN, A. O. & TRAPMAN, J. (1996) Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. *J Biol Chem,* 271**,** 6379-88.

COLLAS, P. & DAHL, J. A. (2008) Chop it, ChIP it, check it: the current status of chromatin immunoprecipitation. *Front Biosci,* 13**,** 929-43.

CRAFT, N., SHOSTAK, Y., CAREY, M. & SAWYERS, C. L. (1999) A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. *Nat Med,* 5**,** 280-5.

CULIG, Z., HOBISCH, A., CRONAUER, M. V., RADMAYR, C., TRAPMAN, J., HITTMAIR, A., BARTSCH, G. & KLOCKER, H. (1994) Androgen receptor activation in prostatic tumour cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res,* 54**,** 5474-8.

CUTRESS, M. L., WHITAKER, H. C., MILLS, I. G., STEWART, M. & NEAL, D. E. (2008) Structural basis for the nuclear import of the human androgen receptor. *J Cell Sci,* 121**,** 957-68.

DAMBER, J. E. & AUS, G. (2008) Prostate cancer. *Lancet,* 371**,** 1710-21.

DE MARZO, A. M., MEEKER, A. K., ZHA, S., LUO, J., NAKAYAMA, M., PLATZ, E. A., ISAACS, W. B. & NELSON, W. G. (2003) Human prostate cancer precursors and pathobiology. *Urology,* 62**,** 55-62.

DEHM, S. M. & TINDALL, D. J. (2006) Ligand-independent androgen receptor activity is activation function-2-independent and resistant to antiandrogens in androgen refractory prostate cancer cells. *J Biol Chem,* 281**,** 27882-93.

ECKERT, K. A. & KUNKEL, T. A. (1991) DNA polymerase fidelity and the polymerase chain reaction. *PCR Methods Appl,* 1**,** 17-24.

EPSTEIN, J. I. & HERAWI, M. (2006) Prostate needle biopsies containing prostatic intraepithelial neoplasia or atypical foci suspicious for carcinoma: implications for patient care. *J Urol,* 175**,** 820-34.

EPSTEIN, J. I., WALSH, P. C., CARMICHAEL, M. & BRENDLER, C. B. (1994) Pathologic and clinical findings to predict tumor extent of nonpalpable (stage T1c) prostate cancer. *JAMA,* 271**,** 368-74.

FALKENSTEIN, E., TILLMANN, H. C., CHRIST, M., FEURING, M. & WEHLING, M. (2000) Multiple actions of steroid hormones--a focus on rapid, nongenomic effects. *Pharmacol Rev,* 52**,** 513-56.

FORADORI, C. D., WEISER, M. J. & HANDA, R. J. (2008) Non-genomic actions of androgens. *Front Neuroendocrinol,* 29**,** 169-81.

FUESSEL, S., MEYE, A., SCHMITZ, M., ZASTROW, S., LINNE, C., RICHTER, K., LOBEL, B., HAKENBERG, O. W., HOELIG, K., RIEBER, E. P. & WIRTH, M. P. (2006) Vaccination of hormone-refractory prostate cancer patients with peptide cocktail-loaded dendritic cells: Results of a phase I clinical trial. *Prostate.*

FUESSEL, S., SICKERT, D., MEYE, A., KLENK, U., SCHMIDT, U., SCHMITZ, M., ROST, A. K., WEIGLE, B., KIESSLING, A. & WIRTH, M. P. (2003) Multiple tumor marker analyses (PSA, hK2, PSCA, trp-p8) in primary prostate cancers using quantitative RT-PCR. *Int J Oncol,* 23**,** 221-8.

GADDIPATI, J. P., MCLEOD, D. G., HEIDENBERG, H. B., SESTERHENN, I. A., FINGER, M. J., MOUL, J. W. & SRIVASTAVA, S. (1994) Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. *Cancer Res,* 54**,** 2861-4.

GIOELI, D., MANDELL, J. W., PETRONI, G. R., FRIERSON, H. F., JR. & WEBER, M. J. (1999) Activation of mitogen-activated protein kinase associated with prostate cancer progression. *Cancer Res,* 59**,** 279-84.

GLASS, C. K., ROSE, D. W. & ROSENFELD, M. G. (1997) Nuclear receptor coactivators. *Curr Opin Cell Biol,* 9**,** 222-32.

GONZALGO, M. L. & ISAACS, W. B. (2003) Molecular pathways to prostate cancer. *J Urol,* 170**,** 2444-52.

GREGORY, C. W., HAMIL, K. G., KIM, D., HALL, S. H., PRETLOW, T. G., MOHLER, J. L. & FRENCH, F. S. (1998) Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. *Cancer Res,* 58**,** 5718-24.

GREGORY, C. W., HE, B., JOHNSON, R. T., FORD, O. H., MOHLER, J. L., FRENCH, F. S. & WILSON, E. M. (2001) A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. *Cancer Res,* 61**,** 4315-9.

GRONER, B., PONTA, H., BEATO, M. & HYNES, N. E. (1983) The proviral DNA of mouse mammary tumor virus: its use in the study of the molecular details of steroid hormone action. *Mol Cell Endocrinol,* 32**,** 101-16.

HALKIDOU, K., GNANAPRAGASAM, V. J., MEHTA, P. B., LOGAN, I. R., BRADY, M. E., COOK, S., LEUNG, H. Y., NEAL, D. E. & ROBSON, C. N. (2003) Expression of Tip60, an androgen receptor coactivator, and its role in prostate cancer development. *Oncogene,* 22**,** 2466-77.

HARTENECK, C. (2005) Function and pharmacology of TRPM cation channels. *Naunyn Schmiedebergs Arch Pharmacol,* 371**,** 307-14.

HE, B., LEE, L. W., MINGES, J. T. & WILSON, E. M. (2002) Dependence of selective gene activation on the androgen receptor NH2- and COOH-terminal interaction. *J Biol Chem,* 277**,** 25631-9.

HEEMERS, H., VERRIJDT, G., ORGANE, S., CLAESSENS, F., HEYNS, W., VERHOEVEN, G. & SWINNEN, J. V. (2004) Identification of an androgen response element in intron 8 of the sterol regulatory element-binding protein cleavage-activating protein gene allowing direct regulation by the androgen receptor. *J Biol Chem,* 279**,** 30880-7.

HEEMERS, H. V., VERHOEVEN, G. & SWINNEN, J. V. (2006) Androgen activation of the sterol regulatory element-binding protein pathway: Current insights. *Mol Endocrinol,* 20**,** 2265-77.

HEINLEIN, C. A. & CHANG, C. (2004) Androgen receptor in prostate cancer. *Endocr Rev,* 25**,** 276-308.

HENDRIKSEN, P. J., DITS, N. F., KOKAME, K., VELDHOVEN, A., VAN WEERDEN, W. M., BANGMA, C. H., TRAPMAN, J. & JENSTER, G. (2006) Evolution of the androgen receptor pathway during progression of prostate cancer. *Cancer Res,* 66**,** 5012-20.

HENSHALL, S. M., AFAR, D. E., HILLER, J., HORVATH, L. G., QUINN, D. I., RASIAH, K. K., GISH, K., WILLHITE, D., KENCH, J. G., GARDINER-GARDEN, M., STRICKER, P. D., SCHER, H. I., GRYGIEL, J. J., AGUS, D. B., MACK, D. H. & SUTHERLAND, R. L. (2003) Survival analysis of genome-wide gene expression profiles of prostate cancers identifies new prognostic targets of disease relapse. *Cancer Res,* 63**,** 4196-203.

JANKNECHT, R. & HUNTER, T. (1996) Transcription. A growing coactivator network. *Nature,* 383**,** 22-3.

JARIWALA, U., PRESCOTT, J., JIA, L., BARSKI, A., PREGIZER, S., COGAN, J. P., ARASHEBEN, A., TILLEY, W. D., SCHER, H. I., GERALD, W. L.,

BUCHANAN, G., COETZEE, G. A. & FRENKEL, B. (2007) Identification of novel androgen receptor target genes in prostate cancer. *Mol Cancer,* 6**,** 39.

JENSTER, G. (2000) Ligand-independent activation of the androgen receptor in prostate cancer by growth factors and cytokines. *J Pathol,* 191**,** 227-8.

JIA, L., BERMAN, B. P., JARIWALA, U., YAN, X., COGAN, J. P., WALTERS, A., CHEN, T., BUCHANAN, G., FRENKEL, B. & COETZEE, G. A. (2008) Genomic androgen receptor-occupied regions with different functions, defined by histone acetylation, coregulators and transcriptional capacity. *PLoS One,* 3**,** e3645.

JIA, L., CHOONG, C. S., RICCIARDELLI, C., KIM, J., TILLEY, W. D. & COETZEE, G. A. (2004) Androgen receptor signaling: mechanism of interleukin-6 inhibition. *Cancer Res,* 64**,** 2619-26.

JIA, L., KIM, J., SHEN, H., CLARK, P. E., TILLEY, W. D. & COETZEE, G. A. (2003) Androgen receptor activity at the prostate specific antigen locus: steroidal and non-steroidal mechanisms. *Mol Cancer Res,* 1**,** 385-92.

JIA, L., SHEN, H. C., WANTROBA, M., KHALID, O., LIANG, G., WANG, Q., GENTZSCHEIN, E., PINSKI, J. K., STANCZYK, F. Z., JONES, P. A. & COETZEE, G. A. (2006) Locus-wide chromatin remodeling and enhanced androgen receptor-mediated transcription in recurrent prostate tumor cells. *Mol Cell Biol,* 26**,** 7331-41.

KELLER, E. T., ERSHLER, W. B. & CHANG, C. (1996) The androgen receptor: a mediator of diverse responses. *Front Biosci,* 1**,** d59-71.

KIESSLING, A., FUSSEL, S., SCHMITZ, M., STEVANOVIC, S., MEYE, A., WEIGLE, B., KLENK, U., WIRTH, M. P. & RIEBER, E. P. (2003) Identification of an HLA-A*0201-restricted T-cell epitope derived from the prostate cancer-associated protein trp-p8. *Prostate,* 56**,** 270-9.

KIM, J. & COETZEE, G. A. (2004) Prostate specific antigen gene regulation by androgen receptor. *J Cell Biochem,* 93**,** 233-41.

KOIVISTO, P., KONONEN, J., PALMBERG, C., TAMMELA, T., HYYTINEN, E., ISOLA, J., TRAPMAN, J., CLEUTJENS, K., NOORDZIJ, A., VISAKORPI, T. & KALLIONIEMI, O. P. (1997) Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. *Cancer Res,* 57**,** 314-9.

KOIVISTO, P., VISAKORPI, T. & KALLIONIEMI, O. P. (1996) Androgen receptor gene amplification: a novel molecular mechanism for endocrine therapy resistance in human prostate cancer. *Scand J Clin Lab Invest Suppl,* 226**,** 57-63.

LEE, L. F., LOUIE, M. C., DESAI, S. J., YANG, J., CHEN, H. W., EVANS, C. P. & KUNG, H. J. (2004) Interleukin-8 confers androgen-independent growth and migration of LNCaP: differential effects of tyrosine kinases Src and FAK. *Oncogene,* 23**,** 2197-205.

LI, J. & AL-AZZAWI, F. (2009) Mechanism of androgen receptor action. *Maturitas,* 63**,** 142-8.

LI, X. Y., MACARTHUR, S., BOURGON, R., NIX, D., POLLARD, D. A., IYER, V. N., HECHMER, A., SIMIRENKO, L., STAPLETON, M., LUENGO HENDRIKS, C. L., CHU, H. C., OGAWA, N., INWOOD, W., SEMENTCHENKO, V., BEATON, A., WEISZMANN, R., CELNIKER, S. E., KNOWLES, D. W., GINGERAS, T., SPEED, T. P., EISEN, M. B. & BIGGIN, M. D. (2008) Transcription factors bind thousands of active and inactive regions in the Drosophila blastoderm. *PLoS Biol,* 6**,** e27.

LIN, B., WANG, J., HONG, X., YAN, X., HWANG, D., CHO, J. H., YI, D., UTLEG, A. G., FANG, X., SCHONES, D. E., ZHAO, K., OMENN, G. S. & HOOD, L. (2009) Integrated expression profiling and ChIP-seq analyses of the growth inhibition response program of the androgen receptor. *PLoS One,* 4**,** e6589.

LINJA, M. J., SAVINAINEN, K. J., SARAMAKI, O. R., TAMMELA, T. L., VESSELLA, R. L. & VISAKORPI, T. (2001) Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res,* 61**,** 3550-5.

LOUIE, M. C., YANG, H. Q., MA, A. H., XU, W., ZOU, J. X., KUNG, H. J. & CHEN, H. W. (2003) Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. *Proc Natl Acad Sci U S A,* 100**,** 2226-30.

LOY, C. J., SIM, K. S. & YONG, E. L. (2003) Filamin-A fragment localizes to the nucleus to regulate androgen receptor and coactivator functions. *Proc Natl Acad Sci U S A,* 100**,** 4562-7.

MARCELLI, M., ITTMANN, M., MARIANI, S., SUTHERLAND, R., NIGAM, R., MURTHY, L., ZHAO, Y., DICONCINI, D., PUXEDDU, E., ESEN, A., EASTHAM, J., WEIGEL, N. L. & LAMB, D. J. (2000) Androgen receptor mutations in prostate cancer. *Cancer Res,* 60**,** 944-9.

MASSIE, C. E., ADRYAN, B., BARBOSA-MORAIS, N. L., LYNCH, A. G., TRAN, M. G., NEAL, D. E. & MILLS, I. G. (2007) New androgen receptor genomic targets show an interaction with the ETS1 transcription factor. *EMBO Rep,* 8**,** 871-8.

MASSIE, C. E. & MILLS, I. G. (2008) ChIPping away at gene regulation. *EMBO Rep,* 9**,** 337-43.

MASTON, G. A., EVANS, S. K. & GREEN, M. R. (2006) Transcriptional regulatory elements in the human genome. *Annu Rev Genomics Hum Genet,* 7**,** 29-59.

MCKEMY, D. D., NEUHAUSSER, W. M. & JULIUS, D. (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature,* 416**,** 52-8.

MCPHAUL, M. J. (2008) Mechanisms of prostate cancer progression to androgen independence. *Best Pract Res Clin Endocrinol Metab,* 22**,** 373-88.

MINELLI, A., BELLEZZA, I., CONTE, C. & CULIG, Z. (2009) Oxidative stress-related aging: A role for prostate cancer? *Biochim Biophys Acta,* 1795**,** 83-91.

MIYAMOTO, H., YEH, S., WILDING, G. & CHANG, C. (1998) Promotion of agonist activity of antiandrogens by the androgen receptor coactivator, ARA70, in human prostate cancer DU145 cells. *Proc Natl Acad Sci U S A,* 95**,** 7379-84.

MOEHREN, U., ECKEY, M. & BANIAHMAD, A. (2004) Gene repression by nuclear hormone receptors. *Essays Biochem,* 40**,** 89-104.

MOHLER, J. L., GREGORY, C. W., FORD, O. H., 3RD, KIM, D., WEAVER, C. M., PETRUSZ, P., WILSON, E. M. & FRENCH, F. S. (2004) The androgen axis in recurrent prostate cancer. *Clin Cancer Res,* 10**,** 440-8.

MULLER, P. Y., JANOVJAK, H., MISEREZ, A. R. & DOBBIE, Z. (2002) Processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques,* 32**,** 1372-4, 1376, 1378-9.

MUSTAFA, S. & ORIOWO, M. (2005) Cooling-induced contraction of the rat gastric fundus: mediation via transient receptor potential (TRP) cation channel TRPM8 receptor and Rho-kinase activation. *Clin Exp Pharmacol Physiol,* 32**,** 832-8.

NANTERMET, P. V., XU, J., YU, Y., HODOR, P., HOLDER, D., ADAMSKI, S., GENTILE, M. A., KIMMEL, D. B., HARADA, S., GERHOLD, D., FREEDMAN, L. P. & RAY, W. J. (2004) Identification of genetic pathways activated by the androgen receptor during the induction of proliferation in the ventral prostate gland. *J Biol Chem,* 279**,** 1310-22.

NAYLOR, L. H. (1999) Reporter gene technology: the future looks bright. *Biochem Pharmacol,* 58**,** 749-57.

NAZARETH, L. V. & WEIGEL, N. L. (1996) Activation of the human androgen receptor through a protein kinase A signaling pathway. *J Biol Chem,* 271**,** 19900-7.

NEALEN, M. L., GOLD, M. S., THUT, P. D. & CATERINA, M. J. (2003) TRPM8 mRNA is expressed in a subset of cold-responsive trigeminal neurons from rat. *J Neurophysiol,* 90**,** 515-20.

NORDEEN, S. K. (1988) Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotechniques,* 6**,** 454-8.

ORLANDO, V. & PARO, R. (1993) Mapping Polycomb-repressed domains in the bithorax complex using in vivo formaldehyde cross-linked chromatin. *Cell,* 75**,** 1187-98.

PEIER, A. M., MOQRICH, A., HERGARDEN, A. C., REEVE, A. J., ANDERSSON, D. A., STORY, G. M., EARLEY, T. J., DRAGONI, I., MCINTYRE, P.,

BEVAN, S. & PATAPOUTIAN, A. (2002) A TRP channel that senses cold stimuli and menthol. *Cell,* 108**,** 705-15.

PETERS, A. A., BUCHANAN, G., RICCIARDELLI, C., BIANCO-MIOTTO, T., CENTENERA, M. M., HARRIS, J. M., JINDAL, S., SEGARA, D., JIA, L., MOORE, N. L., HENSHALL, S. M., BIRRELL, S. N., COETZEE, G. A., SUTHERLAND, R. L., BUTLER, L. M. & TILLEY, W. D. (2009) Androgen receptor inhibits estrogen receptor-alpha activity and is prognostic in breast cancer. *Cancer Res,* 69**,** 6131-40.

POWELL, S. M., CHRISTIAENS, V., VOULGARAKI, D., WAXMAN, J., CLAESSENS, F. & BEVAN, C. L. (2004) Mechanisms of androgen receptor signalling via steroid receptor coactivator-1 in prostate. *Endocr Relat Cancer,* 11**,** 117-30.

PRANGE, W., ERBERSDOBLER, A., HAMMERER, P., GRAEFEN, M., HAUTMANN, S. H., HAUTMANN, R. E., HULAND, H. & HENKE, R. P. (2001) Significance of high-grade prostatic intraepithelial neoplasia in needle biopsy specimens. *Urology,* 57**,** 486-90.

PREVARSKAYA, N., FLOURAKIS, M., BIDAUX, G., THEBAULT, S. & SKRYMA, R. (2007a) Differential role of TRP channels in prostate cancer. *Biochem Soc Trans,* 35**,** 133-5.

PREVARSKAYA, N., SKRYMA, R., BIDAUX, G., FLOURAKIS, M. & SHUBA, Y. (2007b) Ion channels in death and differentiation of prostate cancer cells. *Cell Death Differ,* 14**,** 1295-304.

PREVARSKAYA, N., ZHANG, L. & BARRITT, G. (2007c) TRP channels in cancer. *Biochim Biophys Acta,* 1772**,** 937-46.

RAHMAN, F. & CHRISTIAN, H. C. (2007) Non-classical actions of testosterone: an update. *Trends Endocrinol Metab,* 18**,** 371-8.

RAHMAN, M., MIYAMOTO, H. & CHANG, C. (2004) Androgen receptor coregulators in prostate cancer: mechanisms and clinical implications. *Clin Cancer Res,* 10**,** 2208-19.

RAMSAY, A. K. & LEUNG, H. Y. (2009) Signalling pathways in prostate carcinogenesis: potentials for molecular-targeted therapy. *Clin Sci (Lond),* 117**,** 209-28.

REGISTRIES, A. I. O. H. A. W. A. A. A. O. C. (2007) *Cancer in Australia: an overview, 2006,* Canberra, Australia, Australian Institute of Health and Welfare.

RIEGMAN, P. H., VLIETSTRA, R. J., VAN DER KORPUT, J. A., BRINKMANN, A. O. & TRAPMAN, J. (1991) The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. *Mol Endocrinol,* 5**,** 1921-30.

ROBINSON-RECHAVI, M., CARPENTIER, A. S., DUFFRAISSE, M. & LAUDET, V. (2001) How many nuclear hormone receptors are there in the human genome? *Trends Genet,* 17**,** 554-6.

ROSENFELD, M. G. & GLASS, C. K. (2001) Coregulator codes of transcriptional regulation by nuclear receptors. *J Biol Chem,* 276**,** 36865-8.

RUIZEVELD DE WINTER, J. A., JANSSEN, P. J., SLEDDENS, H. M., VERLEUN-MOOIJMAN, M. C., TRAPMAN, J., BRINKMANN, A. O., SANTERSE, A. B., SCHRODER, F. H. & VAN DER KWAST, T. H. (1994) Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer. *Am J Pathol,* 144**,** 735-46.

SABNIS, A. S., SHADID, M., YOST, G. S. & REILLY, C. A. (2008) Human Lung Epithelial Cells Express a Functional Cold-Sensing TRPM8 Variant. *Am J Respir Cell Mol Biol.*

SAITOH, H., HIDA, M., SHIMBO, T., NAKAMURA, K., YAMAGATA, J. & SATOH, T. (1984) Metastatic patterns of prostatic cancer. Correlation between sites and number of organs involved. *Cancer,* 54**,** 3078-84.

SCHAUFELE, F., CARBONELL, X., GUERBADOT, M., BORNGRAEBER, S., CHAPMAN, M. S., MA, A. A., MINER, J. N. & DIAMOND, M. I. (2005) The structural basis of androgen receptor activation: intramolecular and intermolecular amino-carboxy interactions. *Proc Natl Acad Sci U S A,* 102**,** 9802-7.

SCHRODER, F. H., HERMANEK, P., DENIS, L., FAIR, W. R., GOSPODAROWICZ, M. K. & PAVONE-MACALUSO, M. (1992) The TNM classification of prostate cancer. *Prostate - Supplement,* 4**,** 129-38.

SCHUUR, E. R., HENDERSON, G. A., KMETEC, L. A., MILLER, J. D., LAMPARSKI, H. G. & HENDERSON, D. R. (1996) Prostate-specific antigen expression is regulated by an upstream enhancer. *J Biol Chem,* 271**,** 7043-51.

SIGNORETTI, S., MONTIRONI, R., MANOLA, J., ALTIMARI, A., TAM, C., BUBLEY, G., BALK, S., THOMAS, G., KAPLAN, I., HLATKY, L., HAHNFELDT, P., KANTOFF, P. & LODA, M. (2000) Her-2-neu expression and progression toward androgen independence in human prostate cancer. *J Natl Cancer Inst,* 92**,** 1918-25.

SIMI, L., MALENTACCHI, F., LUCIANI, P., GELMINI, S., DELEDDA, C., ARVIA, R., MANNELLI, M., PERI, A. & ORLANDO, C. (2010) Seladin-1 expression is regulated by promoter methylation in adrenal cancer. *BMC Cancer,* 10**,** 201.

SIMON, P. (2003) Q-Gene: processing quantitative real-time RT-PCR data. *Bioinformatics,* 19**,** 1439-40.

SMALL, E. J., HALABI, S., DAWSON, N. A., STADLER, W. M., RINI, B. I., PICUS, J., GABLE, P., TORTI, F. M., KAPLAN, E. & VOGELZANG, N. J.

(2004) Antiandrogen withdrawal alone or in combination with ketoconazole in androgen-independent prostate cancer patients: a phase III trial (CALGB 9583). *J Clin Oncol,* 22**,** 1025-33.

STAMEY, T. A., MCNEAL, JOHN E. (1992) Adenocarcinoma of The Prostate. IN AL.], P. C. W. E. (Ed.) *Campbell's Urology.* 6th ed. Philadelphia, Saunders.

STEIN, R. J., SANTOS, S., NAGATOMI, J., HAYASHI, Y., MINNERY, B. S., XAVIER, M., PATEL, A. S., NELSON, J. B., FUTRELL, W. J., YOSHIMURA, N., CHANCELLOR, M. B. & DE MIGUEL, F. (2004) Cool (TRPM8) and hot (TRPV1) receptors in the bladder and male genital tract. *J Urol,* 172**,** 1175-8.

STEWART, A. P., EGRESSY, K., LIM, A. & EDWARDSON, J. M. (2010) AFM imaging reveals the tetrameric structure of the TRPM8 channel. *Biochem Biophys Res Commun.*

TAPLIN, M. E., BUBLEY, G. J., KO, Y. J., SMALL, E. J., UPTON, M., RAJESHKUMAR, B. & BALK, S. P. (1999) Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. *Cancer Res,* 59**,** 2511-5.

TAPLIN, M. E., BUBLEY, G. J., SHUSTER, T. D., FRANTZ, M. E., SPOONER, A. E., OGATA, G. K., KEER, H. N. & BALK, S. P. (1995) Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. *N Engl J Med,* 332**,** 1393-8.

TAPLIN, M. E., RAJESHKUMAR, B., HALABI, S., WERNER, C. P., WODA, B. A., PICUS, J., STADLER, W., HAYES, D. F., KANTOFF, P. W., VOGELZANG, N. J. & SMALL, E. J. (2003) Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. *J Clin Oncol,* 21**,** 2673-8.

THEBAULT, S., LEMONNIER, L., BIDAUX, G., FLOURAKIS, M., BAVENCOFFE, A., GORDIENKO, D., ROUDBARAKI, M., DELCOURT, P., PANCHIN, Y., SHUBA, Y., SKRYMA, R. & PREVARSKAYA, N. (2005) Novel role of cold/menthol-sensitive transient receptor potential melastatine family member 8 (TRPM8) in the activation of store-operated channels in LNCaP human prostate cancer epithelial cells. *J Biol Chem,* 280**,** 39423-35.

THUT, P. D., WRIGLEY, D. & GOLD, M. S. (2003) Cold transduction in rat trigeminal ganglia neurons in vitro. *Neuroscience,* 119**,** 1071-83.

TILLEY, W. D., BUCHANAN, G., HICKEY, T. E. & BENTEL, J. M. (1996) Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. *Clin Cancer Res,* 2**,** 277-85.

TINDALL, K. R. & KUNKEL, T. A. (1988) Fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase. *Biochemistry,* 27**,** 6008-13.

TITUS, M. A., SCHELL, M. J., LIH, F. B., TOMER, K. B. & MOHLER, J. L. (2005) Testosterone and dihydrotestosterone tissue levels in recurrent prostate cancer. *Clin Cancer Res,* 11**,** 4653-7.

TOMINAGA, M. & CATERINA, M. J. (2004) Thermosensation and pain. *J Neurobiol,* 61**,** 3-12.

TSAVALER, L., SHAPERO, M. H., MORKOWSKI, S. & LAUS, R. (2001) Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins. *Cancer Res,* 61**,** 3760-9.

TUOHIMAA, P. (2009) Vitamin D and aging. *J Steroid Biochem Mol Biol,* 114**,** 78-84.

VANDEN ABEELE, F., ZHOLOS, A., BIDAUX, G., SHUBA, Y., THEBAULT, S., BECK, B., FLOURAKIS, M., PANCHIN, Y., SKRYMA, R. & PREVARSKAYA, N. (2006) Ca2+-independent Phospholipase A2-dependent Gating of TRPM8 by Lysophospholipids. *J Biol Chem,* 281**,** 40174-82.

VELDSCHOLTE, J., RIS-STALPERS, C., KUIPER, G. G., JENSTER, G., BERREVOETS, C., CLAASSEN, E., VAN ROOIJ, H. C., TRAPMAN, J., BRINKMANN, A. O. & MULDER, E. (1990) A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem Biophys Res Commun,* 173**,** 534-40.

VENKATACHALAM, K. & MONTELL, C. (2007) TRP channels. *Annu Rev Biochem,* 76**,** 387-417.

VOETS, T., DROOGMANS, G., WISSENBACH, U., JANSSENS, A., FLOCKERZI, V. & NILIUS, B. (2004) The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. *Nature,* 430**,** 748-54.

VOETS, T., OWSIANIK, G. & NILIUS, B. (2007) Trpm8. *Handb Exp Pharmacol***,** 329-44.

WANG, Q., CARROLL, J. S. & BROWN, M. (2005) Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. *Mol Cell,* 19**,** 631-42.

WANG, Q., LI, W., LIU, X. S., CARROLL, J. S., JANNE, O. A., KEETON, E. K., CHINNAIYAN, A. M., PIENTA, K. J. & BROWN, M. (2007) A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. *Mol Cell,* 27**,** 380-92.

WEBBER, M. M., BELLO, D. & QUADER, S. (1996) Immortalized and tumorigenic adult human prostatic epithelial cell lines: characteristics and applications. Part I. Cell markers and immortalized nontumorigenic cell lines. *Prostate,* 29**,** 386-94.

WILLIAM K. OH, M. H., ANTHONY V. D'AMICO, JEROME P. RICHIE, AND PHILIP W. KANTOFF. (2003) Neoplasms of the Prostate. IN KUFE, D. W. P., RAPHAEL E.; WEICHSELBAUM, RALPH R.; BAST, ROBERT C., JR.; GANSLER, TED S.; HOLLAND, JAMES F.; FREI III, EMIL, (Ed.) *Cancer medicine 6.* 6th ed. Hamilton, Ont. ; Lewiston, NY, BC Decker.

YANG, X. R., LIN, M. J., MCINTOSH, L. S. & SHAM, J. S. (2006) Functional expression of transient receptor potential melastatin- and vanilloid-related channels in pulmonary arterial and aortic smooth muscle. *Am J Physiol Lung Cell Mol Physiol,* 290**,** L1267-76.

YUAN, X., LI, T., WANG, H., ZHANG, T., BARUA, M., BORGESI, R. A., BUBLEY, G. J., LU, M. L. & BALK, S. P. (2006) Androgen receptor remains critical for cell-cycle progression in androgen-independent CWR22 prostate cancer cells. *Am J Pathol,* 169**,** 682-96.

ZHANG, L. & BARRITT, G. J. (2004) Evidence that TRPM8 is an androgen-dependent Ca2+ channel required for the survival of prostate cancer cells. *Cancer Res,* 64**,** 8365-73.

ZHANG, L. & BARRITT, G. J. (2006) TRPM8 in prostate cancer cells: a potential diagnostic and prognostic marker with a secretory function? *Endocr Relat Cancer,* 13**,** 27-38.

ZHANG, L., BRERETON, H. M., HAHN, M., FROSCIO, M., TILLEY, W. D., BROWN, M. P. & BARRITT, G. J. (2003) Expression of Drosophila Ca2+ permeable transient receptor potential-like channel protein in a prostate cancer cell line decreases cell survival. *Cancer Gene Ther,* 10**,** 611-25.

ZHANG, L., JONES, S., BRODY, K., COSTA, M. & BROOKES, S. J. (2004) Thermosensitive transient receptor potential channels in vagal afferent neurons of the mouse. *Am J Physiol Gastrointest Liver Physiol,* 286**,** G983-91.

ZHU, Y. S., CAI, L. Q., YOU, X., CORDERO, J. J., HUANG, Y. & IMPERATO-MCGINLEY, J. (2003) Androgen-induced prostate-specific antigen gene expression is mediated via dihydrotestosterone in LNCaP cells. *J Androl,* 24**,** 681-7.

ZLOTTA, A. R. & SCHULMAN, C. C. (1999) Clinical evolution of prostatic intraepithelial neoplasia. *Eur Urol,* 35**,** 498-503.