

Investigation of circular RNA and androgen receptor signalling in cancer: RSK crosstalk and androgen response element variation

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Statement of Originality

I declare that this thesis is my own work and has not been submitted for any other degree or qualification. All sources of information have been properly acknowledged.

Abstract

Cancer incidence rates continue to increase, and hence there is a need to better understand the molecular mechanisms that regulate disease development and progression. This project focussed on three aspects of cancer biology: circRNA-mediated regulatory networks in oral and prostate cancer, the impact of regulatory single nucleotide polymorphisms (rSNPs) within androgen response elements (AREs) on androgen receptor (AR) activity, and the role of ribosomal S6 kinases (RSKs 1-4) in modulating AR signalling.

Computational analysis of publicly available RNA sequencing datasets identified dysregulated circRNAs in oral squamous cell carcinoma (OSCC) and prostate cancer. Differential expression and predicted circRNA-miRNA-mRNA interactions suggested potential miRNA sponging mechanisms involved in cancer-related pathways. In OSCC, hsa_circ_0056440 was strongly upregulated, while hsa_circ_0064977 and hsa_circ_0010794 were identified as candidate regulatory circRNAs in prostate cancer. Functional growth assays in 22Rv1 prostate cancer cells provided evidence that these circRNAs may influence cell proliferation.

Reporter assays investigating rSNP-containing AREs demonstrated androgen-dependent transcriptional activation following mibolerone treatment, consistent with AR-mediated regulation. The MAF SNP-containing construct showed reduced androgen-dependent reporter activity, suggesting that this variant may impair AR-mediated transcription. To further investigate potential effects on AR-DNA interactions, bacterial protein production optimisation was performed for the AR DNA-binding domain (DBD), enabling recovery of soluble GST-AR DBD to support future DNA-binding studies. In parallel, the potential crosstalk between AR signalling and RSKs was explored. RSK2 showed the strongest suppressive effect on reporter activity, whereas RSK1 produced the strongest increase in cell proliferation, highlighting potentially isoform-specific effects of RSKs on AR signalling and prostate cancer cell behaviour.

Overall, this study used computational and experimental approaches to investigate circRNA regulatory networks, androgen receptor signalling, and associated regulatory variants in cancer, identifying candidates for further functional investigation.

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Abbreviations

Abbreviation	Full Form
ADAR	Adenosine Deaminase Acting on RNA
ADT	Androgen Deprivation Therapy
AIM	Autoinduction Medium
ANOVA	Analysis of Variance
AR	Androgen Receptor
ARE	Androgen Response Element
ARE-LUC	Androgen Response Element- Luciferase Reporter Construct
AR-V7	Androgen Receptor Variant 7
ATCC	American Type Culture Collection
BBC3	BCL2 Binding Component 3
BBS	BES-Buffered Saline
BCL2	B-cell Lymphoma 2
BES	N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid
BOS- β -gal	BOS Beta-Galactosidase Reporter Construct
BPH	Benign Prostatic Hyperplasia
BSA	Bovine Serum Albumin
circRNA	Circular RNA
ciRNA	Circular Intronic RNA
CCND1	Cyclin D1
CRPC	Castration-Resistant Prostate Cancer
CT	Computed Tomography
CXCR1	C-X-C Motif Chemokine Receptor 1

Abbreviation	Full Form
DBD	DNA-Binding Domain
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DRE	Digital Rectal Examination
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ElciRNA	Exon-intronic Circular RNA
EMT	Epithelial-Mesenchymal Transition
ENA	European Nucleotide Archive
ERK	Extracellular Signal-Regulated Kinase
ESR1	Oestrogen Receptor 1
EtOH	Ethanol
EXO1	Exonuclease 1
FCS	Foetal Calf Serum
FOXM1	Forkhead Box M1
FUS	Fused in Sarcoma
GPCR	G Protein-Coupled Receptor
GRE	Glucocorticoid Response Element
GST	Glutathione S-Transferase
GST-AR DBD	Glutathione S-Transferase Tagged Androgen Receptor DNA-Binding Domain
h	Hour
hsa	<i>Homo sapiens</i>

Abbreviation	Full Form
huR	Human Antigen R
HPV	Human Papillomavirus
HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IRES	Internal Ribosome Entry Site
kDa	Kilodalton
LBD	Ligand-Binding Domain
LB	Luria-Bertani
LHX2	LIM Homeobox 2
log2FC	Log2 Fold Change
LUC	Luciferase
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog.
MAPK	Mitogen-Activated Protein Kinase
miRNA	MicroRNA
MIB	Mibolerone
min	Minute
mL	Millilitre
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
MRI	Magnetic Resonance Imaging
m6A	N6-Methyladenosine
Mut	Mutant
MYC	Myelocytomatosis Oncogene

Abbreviation	Full Form
cMYC	MYC proto-oncogene
ng	Nanogram
NGS	Next-Generation Sequencing
NR4A3	Nuclear Receptor Subfamily 4 Group A Member 3
ns	Not Significant
NTD	N-Terminal Domain
OD ₆₀₀	Optical Density at 600 nm
OPMDs	Oral Potentially Malignant Disorders
ORF	Open Reading Frame
OSCC	Oral Squamous Cell Carcinoma
p-value	Probability Value
PBS	Phosphate-Buffered Saline
PB-PROM-LUC	Probasin Promoter Luciferase Reporter Construct
PCA	Principal Component Analysis
pcDNA3.1	Plasmid Cytomegalovirus DNA 3.1 Expression Vector
PCa	Prostate Cancer
PIN	Prostatic Intraepithelial Neoplasia
POMC	Proopiomelanocortin
PSA	Prostate-Specific Antigen
pSV-AR	Simian Virus Promoter-Androgen Receptor Expression Vector
PUMA	p53 Upregulated Modulator of Apoptosis
PVDF	Polyvinylidene Fluoride
qPCR	Quantitative Polymerase Chain Reaction

Abbreviation	Full Form
QKI	Quaking Protein
RBP	RNA-Binding Protein
RFP	Red Fluorescent Protein
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute Medium
RSK	Ribosomal S6 Kinase
rSNP	Regulatory Single Nucleotide Polymorphism
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SNP	Single Nucleotide Polymorphism
TAT-GRE-E1B-LUC	Tyrosine Aminotransferase-Glucocorticoid Response Element-E1B Luciferase Reporter Construct
TERT	Telomerase Reverse Transcriptase
UCSC	University of California, Santa Cruz Genome Browser
VIM	Vimentin
w/v	weight per Volume
WT	Wild Type
ZnCl ₂	Zinc Chloride
ZEB1	Zinc Finger E-box Binding Homeobox 1

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Chapter 1

Introduction

1.1 Cancer Overview

Cancer is a major health concern characterised by the uncontrolled growth of abnormal cells that no longer respond to normal regulatory signals. It develops through the accumulation of genetic and molecular alterations that disrupt normal cellular regulation (Roy & Saikia, 2016).

Prostate cancer (PCa) and oral squamous cell carcinoma (OSCC) are clinically important but biologically distinct malignancies. Prostate cancer is the most common cancer in men in the UK, with around 57,900 new cases diagnosed annually (Cancer Research UK, 2026). Oral cancer also represents an increasing health burden in the UK, with reports indicating around 10,825 new cases each year and a marked rise in incidence over the past decade (Oral Health Foundation, 2024). Although these cancers differ in tissue origin and clinical behaviour, they share a common feature of dysregulated gene expression and altered regulatory networks. PCa is strongly influenced by hormone-dependent signalling pathways (Messner et al, 2020), whereas OSCC is more commonly associated with environmental and lifestyle-related risk factors such as tobacco use, alcohol consumption, and human papillomavirus infection (Lv et al, 2025). Together, these characteristics make them useful models for studying molecular mechanisms involved in cancer development and progression.

Recent advances in cancer biology have highlighted the importance of gene regulatory mechanisms beyond protein-coding genes alone. In particular, non-coding RNAs have emerged as important contributors to cancer-associated molecular changes and may provide useful biomarkers and therapeutic targets (Yan & Bu, 2021). This provides an important foundation for examining the regulatory mechanisms explored in this study.

1.2 Circular RNAs

Circular RNAs (circRNAs) are a distinct group of RNA molecules that arise from a non-canonical splicing event known as back-splicing. In this process, a spliceosome facilitates the linkage of a downstream splice donor to an upstream splice acceptor, resulting in a closed-loop RNA structure that lacks 5' and 3' ends (Yu & Kuo, 2019). This circular configuration provides increased stability compared to linear RNAs by protecting circRNAs from exonuclease-mediated degradation (Xu et al, 2024).

1.2.1 Types of circRNA

CircRNAs are generated during the transcript splicing process and depending on how splicing generates them, there are three main types of circRNAs: exonic circRNAs (ecircRNAs), circular intronic RNAs (ciRNAs), and exon-intron circRNAs (EciRNAs) (Patop et al, 2019). ecircRNAs are formed entirely from exonic sequences and are primarily located in the cytoplasm, where they play a crucial role in post-transcriptional gene regulation, often by functioning as microRNA (miRNA) sponges. This activity allows them to modulate the availability of miRNAs and, consequently, the expression of their target mRNAs (Kristensen et al, 2019). ciRNAs, on the other hand, are generated from intronic regions and are typically confined to the nucleus. These circRNAs are known to influence the transcriptional activity of their parent genes, likely by interacting with transcriptional regulators or components of the RNA polymerase II complex. The third type, EciRNAs, contains both exonic and intronic sequences and shares a nuclear localisation with ciRNAs (Yuan et al, 2024). The distinct localisation and composition of these circRNAs types contribute to their diverse functional roles, particularly in the regulation of gene expression in cancer.

1.2.2 Biogenesis of circRNA

The biogenesis of circRNAs begins with transcription by RNA polymerase II, the same enzyme responsible for generating linear mRNAs. The back-splicing event that produces circRNAs is carried out by the spliceosome (Patop et al, 2019). Circular RNA formation occurs through several distinct mechanisms, each involving specific molecular interactions. One well-established pathway is exon skipping, where certain exons are omitted during pre-mRNA splicing. This process can result in a lariat structure, which may later be circularised if not debranched (Yang et al, 2018) (Figure 1.1C). Lariat-driven circularisation can give rise to ciRNAs, which are derived exclusively from intronic regions. These intronic lariats escape debranching due to the presence of conserved GU-rich elements near the 5' splice site and C-rich elements near the branch point site, which together stabilise the lariat and promote its circularisation into ciRNA (Zhang et al, 2013) (Figure 1.1A). Another key mechanism is intron pairing-driven circularisation (cis-acting). In this case, complementary sequences within the flanking introns, such as Alu repeats, align and bring the appropriate splice sites into proximity, thereby facilitating back-splicing (Yang et al, 2018)(Figure 1.1B).

Another pathway involves RNA-binding proteins (RBPs) (trans-acting) that recognise and bind to specific motifs in the intronic regions flanking the exons. These RBPs, such as Quaking (QKI), Muscleblind (MBL), Fused in Sarcoma (FUS), and Epithelial Splicing Regulatory Protein 1 (ESRP1), can dimerise and physically loop the RNA, promoting the joining of downstream 5' splice sites to upstream 3' splice sites to generate circRNAs (Ashwal-Fluss et al, 2014; Conn et al, 2015; Errichelli et al, 2017; Patop et al, 2019; Teplova et al, 2013; Yu & Kuo, 2019) (Figure 1.1D). In addition, the expression of some circRNA may depend on a combination of factors including heterogeneous nuclear ribonucleoproteins (hnRNPs) and SR proteins (Serine/Arginine-rich proteins), suggesting a multifactorial regulatory network

(Kramer et al, 2015). In contrast, some RBPs act as inhibitors of circRNA formation. For example, ADAR (adenosine deaminase acting on RNA) suppresses circRNA biogenesis by converting adenosine residues into inosines within double-stranded RNA regions. This disrupts the base-pairing required to bring splice sites together, thus reducing circRNA formation (Ivanov et al, 2015).

Interestingly, circRNA biogenesis is not only influenced by splicing but also by the efficiency of cleavage and polyadenylation, processes important for mRNA maturation (Liang et al, 2017). CircRNAs often compete with canonical linear splicing, meaning that reduced linear splicing efficiency may favour circRNA production. This balance may be influenced by changes in splicing regulators such as SR protein SF2 (splicing factor 2) or key components of the spliceosome complex like small nuclear ribonucleoprotein particle U1 subunit 70K and C (snRNP-U1-70K, snRNP-U1-C) (Patop et al, 2019). Overall, circRNA formation is governed by both cis-acting elements and trans-acting factors, and disruption of this balance can contribute to the altered circRNA expression observed in cancer.

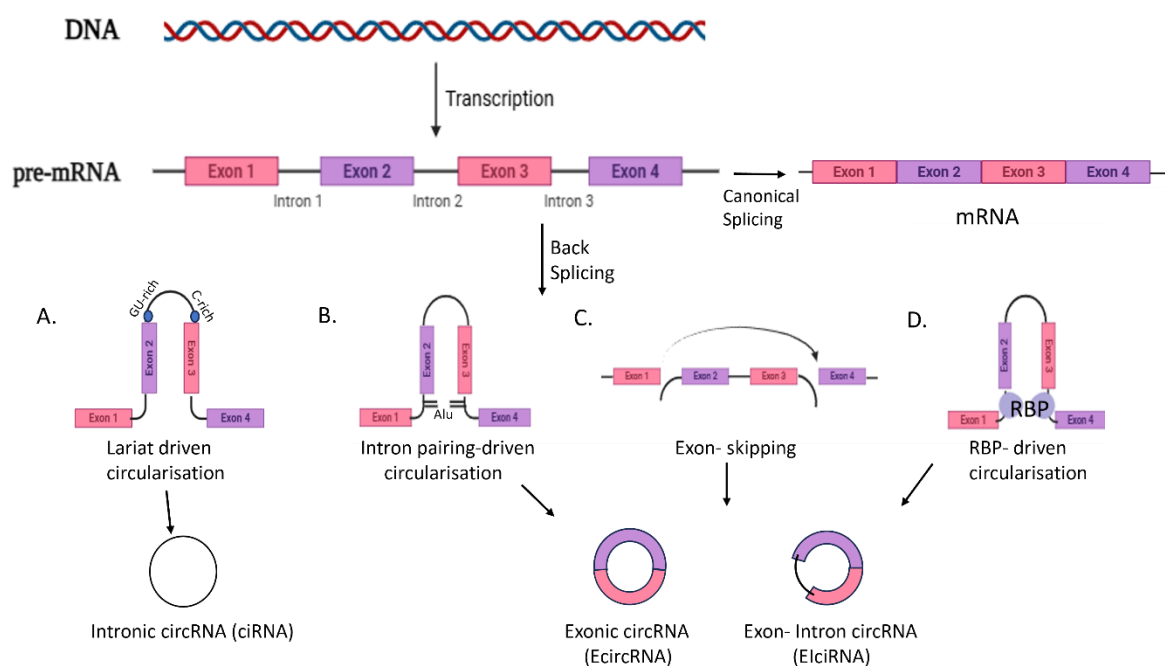


Figure 1.1. Biogenesis of circRNA. Schematic summary of the different forms of circRNA biogenesis: A. lariat-driven circularisation, B. intron pairing-driven circularisation, C. exon-skipping, and D. RBP-driven circularisation. Created using BioRender.com and Microsoft PowerPoint.

1.2.3 Transcriptional Regulation of circRNA Expression

Circular RNAs (circRNAs) do not usually possess independent promoters or enhancers. Instead, their expression is largely determined by the regulatory elements and transcription factors that control their host genes (Patop et al, 2019; Zhang et al, 2014). As a result, changes in host gene transcription can directly influence the abundance of circRNAs produced from the same locus.

Because circRNAs are generated from the same primary transcripts as their linear counterparts, transcription factors that regulate host gene expression can also indirectly affect circRNA levels. For example, GLI Family Zinc Finger 2 (GLI2), Myelocytomatosis Oncogene (MYC), and Oestrogen Receptor 1 (ESR1) have been reported to influence the expression of specific circRNAs through their effects on host gene transcription (Kristensen et al, 2019).

Interestingly, this regulatory relationship can also form feedback loops. Recent evidence suggests that circRNAs themselves can modulate the activity of transcription factors or influence chromatin states, which may further affect the expression of both circRNAs and their host genes (Patop et al, 2019). This highlights a complex relationship between circRNA and gene transcription, where circRNAs are products of gene transcription but can also, in turn, contribute to its regulation.

1.2.4 Functions of circRNA

1.2.4.1 miRNA sponging

MicroRNAs (miRNAs) are short, noncoding RNA molecules, typically about 22 nucleotides long, that play a crucial role in regulating gene expression after transcription. They usually function by binding to specific messenger RNAs (mRNAs) and preventing these mRNAs from being translated into proteins, thereby reducing gene expression at the post-transcriptional level (Li et al, 2021a) (Figure 1.2). In recent years, circRNAs have emerged as important regulators within this pathway. Once thought to be mere byproducts of splicing (Kristensen et al, 2019), circRNAs are now recognised for their diverse roles in cellular processes, with one of their most prominent functions being the ability to act as miRNA sponges (Figure 1.3A). By binding to and sequestering particular miRNAs, circRNAs prevent these miRNAs from associating with their target mRNAs, thereby reducing this repression and allowing gene expression to proceed (Kristensen et al, 2019) (Figure 1.2). A well-known example is ciRS-7 (also known as CDR1as), which contains multiple binding sites for miR-7 and inhibits its activity (Hansen et al, 2013). This interaction has been linked to both cancer biology and neural development, highlighting the broader impact of circRNAs in gene regulation networks (Memczak et al, 2013).

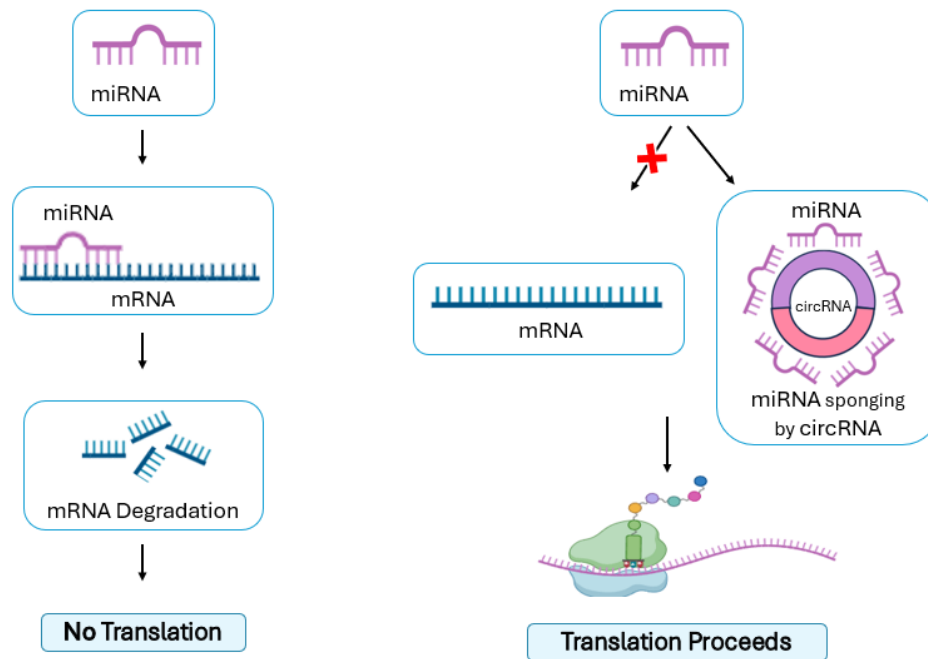


Figure 1.2. Schematic representation of circRNA-mediated miRNA sponging. In the absence of circRNA, miRNAs bind to complementary sequences on target mRNAs, leading to mRNA degradation and inhibition of translation. In the presence of circRNA, miRNAs are sequestered through binding to circRNA molecules, preventing their interaction with target mRNAs and allowing translation to proceed. Created using BioRender.com and Microsoft PowerPoint.

1.2.4.2 Transcriptional regulation

In addition to miRNA sponging, some circRNAs, particularly those localised in the nucleus, such as ciRNAs and ElciRNAs, can regulate the transcription of their host genes (Li et al, 2015). ElciRNAs can associate with U1 small nuclear ribonucleoprotein (snRNP), allowing the resulting complex to interact with RNA polymerase II at the promoter regions of their host genes, leading to increased gene expression (Li et al, 2015). Similarly, ciRNAs, which arise from intron lariats that are not debranched, can enhance the transcription of their host genes by influencing the activity of elongating RNA polymerase II (Zhang et al, 2013) (Figure 1.2E).

1.2.4.3 Protein interactions with RNA-binding proteins

CircRNAs also interact with RNA-binding proteins (RBPs), functioning as decoys, scaffolds, or recruiters in various post-transcriptional processes. Circ-FOXO3 (circular RNA derived from *forkhead box O3 (FOXO3)* gene) inhibits cell cycle progression by forming a complex with cyclin-dependent kinase 2 (CDK2) and cyclin-dependent kinase inhibitor 1A (CDKN1A/p21). This prevents CDK2 from interacting with cyclins, thereby arresting the cell cycle (Du et al, 2016) (Figure 1.2B and C).

1.2.4.4. Translation into proteins

Some exonic circular RNAs (ecircRNAs) can be translated into proteins or peptides despite lacking a traditional 5' cap structure. Translation may occur through cap-independent mechanisms involving internal ribosome entry sites (IRES) or N6-methyladenosine (m6A) modifications (Di Timoteo et al, 2020) (Figure 1.2D).

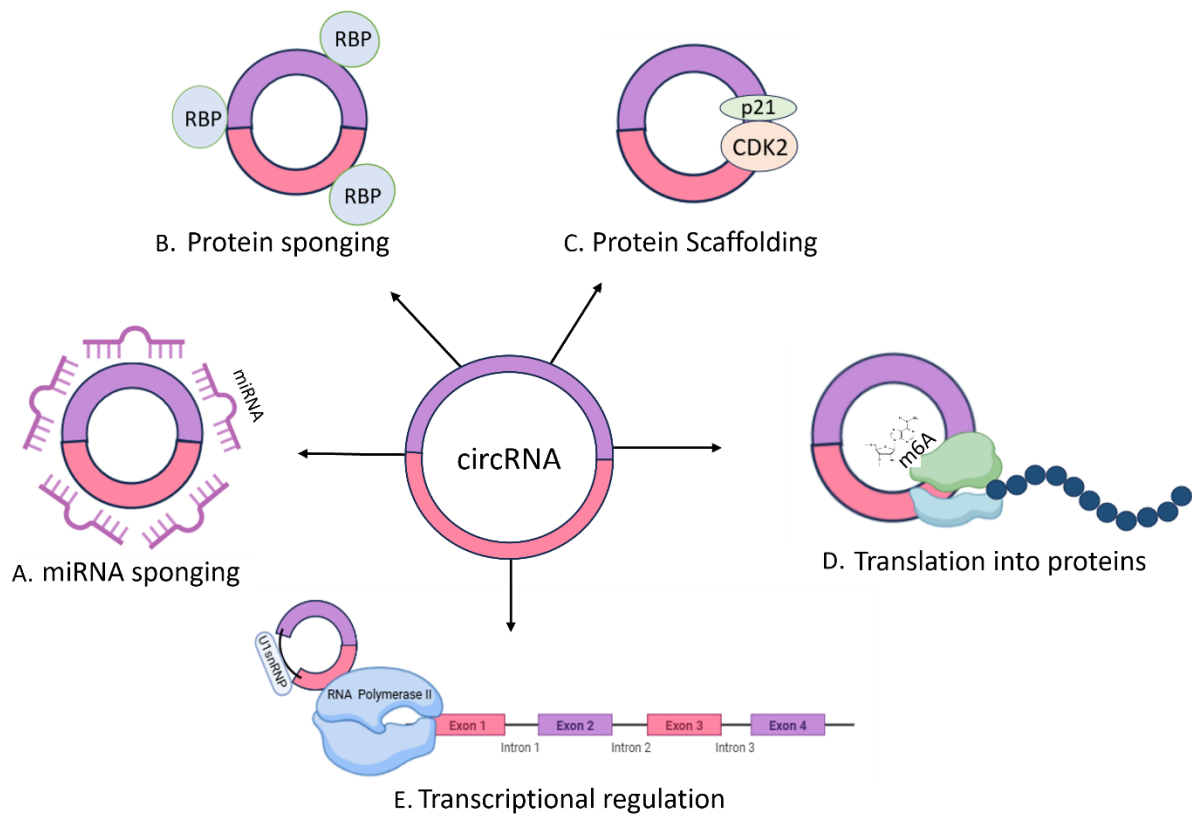


Figure 1.3. Functions of circRNAs. Schematic summarising the role of circRNAs in A. protein scaffolding, B. protein sponging, C. miRNA sponging, D. translation into proteins, and E. transcriptional regulation. Created using BioRender.com and Microsoft PowerPoint.

Finally, circRNAs have been implicated in a variety of diseases, including cancer, neurological disorders, and cardiovascular conditions. Their stability, abundance, and tissue-specific expression patterns make them promising candidates as biomarkers and potential therapeutic targets (Kristensen et al, 2019). Together, these diverse mechanisms demonstrate that circRNAs function as key regulators of gene expression at multiple levels, supporting their emerging role in cancer development and progression. Overall, the different functions of circRNAs suggest that they may contribute to cancer development through several regulatory mechanisms. This makes their role in cancer, including prostate and oral cancer, an important area of study.

1.3 Prostate Cancer

The prostate is a dense fibromuscular gland situated in the pelvis. Its main function is to produce an alkaline fluid that helps to protect sperm in the acidic environment of the vagina. The gland is shaped like an inverted cone and is about the size of a walnut. It lies just below the urinary bladder. Clinically, it is described as having two lateral lobes and one median lobe (Rao, 2023).

1.3.1 Anatomy of Prostate Gland

Anatomically, the prostate is divided into three zones. The peripheral zone forms the largest part of the gland, and it is the main site where prostate cancer develops (Aaron et al, 2016). The central zone surrounds the ejaculatory ducts and has different structural features. The transition zone lies around the proximal urethra and is the site where benign prostatic hyperplasia usually develops. The anterior fibromuscular stroma forms the front portion of the gland and mainly provides support (Aaron et al, 2016). The major blood supply comes from the inferior vesical artery, with additional branches from the middle rectal and internal pudendal arteries. The veins around the gland form the prostatic venous plexus, which drains into the internal iliac vein. Sympathetic nerve supply is through the hypogastric nerves, while parasympathetic nerve supply comes from the pelvic nerves (Rao, 2023).

1.3.2 Common Diseases of Prostate Gland

The three most common clinical problems involving the prostate are benign prostatic hyperplasia (BPH), prostate cancer and prostatitis (Rao, 2023). BPH is a nodular enlargement of the transition zone. It narrows the urethra or pushes upward into the bladder, causing difficulty in passing urine. Prostate cancer usually presents as an adenocarcinoma and commonly arises in the peripheral zone. Patients may have increased urinary frequency, nocturia or a weak stream, and advanced disease can cause lower back pain due to spread to the lower lumbar vertebrae through venous connections. Prostatitis is an inflammation of the gland, commonly due to bacterial infection, and presents with urinary symptoms and pelvic discomfort (Rao, 2023).

1.3.3 Detection and Diagnostic Methods of Prostate Cancer

Prostate cancer is one of the most significant health concerns affecting men worldwide, ranking as the second most common cancer in males (Cronin et al, 2021). It accounts for approximately 28% of all cancer diagnoses in men (Cancer Research UK, 2026). The disease can be highly variable, ranging from slow-growing, non-aggressive tumours to fast-spreading, aggressive forms (Nguyen-Nielsen & Borre, 2016). Early detection is crucial and mainly involves measuring prostate-specific antigen (PSA) levels through a blood test, along with a digital rectal examination (DRE) to physically check the prostate for abnormalities. Newer blood tests like the Prostate Health Index and the 4Kscore use different PSA-related markers to understand whether a raised PSA is more likely due to cancer or a benign problem. If a person is considered at higher risk, they are usually sent for a transrectal

ultrasound-guided biopsy for confirmation. Imaging techniques such as MRI, CT scans, and bone scans are also important for identifying the extent of the disease and planning treatment (Leslie et al, 2024) (Figure 1.4).

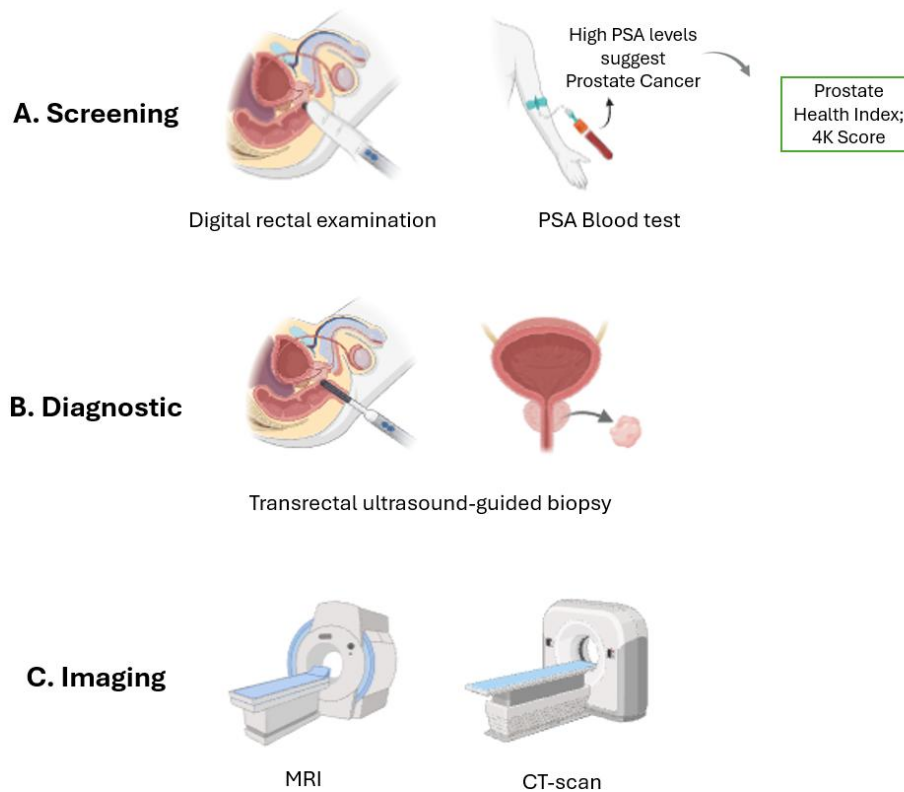


Figure 1.4. Detection methods for prostate cancer. Summary of the major approaches used for prostate cancer detection and evaluation. (A) Screening methods include digital rectal examination (DRE), prostate-specific antigen (PSA) blood test, Prostate Health Index (PHI), and the 4Kscore test, which are used to identify individuals at increased risk. (B) Diagnostic method involves transrectal ultrasound (TRUS)-guided biopsy, performed to confirm the presence of cancer. (C) Imaging techniques such as magnetic resonance imaging (MRI) and computed tomography (CT) scans are used for tumour localisation, staging, and assessment of disease extent. Created using BioRender.com

1.3.4 Prostate Cancer Development and Progression

Prostate cancer develops when prostate epithelial cells acquire molecular alterations that promote uncontrolled growth. Disease progression ranges from low-risk and intermediate-risk localised cancer to high-risk, locally advanced and metastatic cancer (Almeeri et al, 2024). Early detection allows curative treatment, but late-stage PCa is incurable. Prostate cancer develops gradually through several stages, starting with a condition called prostatic intraepithelial neoplasia (PIN), which involves early abnormal changes in prostate cells. If

these early lesions progress, PIN can progress to localised prostate cancer, then to advanced prostate adenocarcinoma that invades nearby tissues, and eventually to metastatic prostate cancer that spreads to other parts of the body (Leslie et al, 2024). Cancer cells can invade surrounding tissues like the seminal vesicles or rectum or spread through blood and lymph to distant organs (Figure 1.5). The connection between the prostatic venous plexus and vertebral veins explains the frequent spread to bones (Almeeri et al, 2024; Jung et al, 2016).

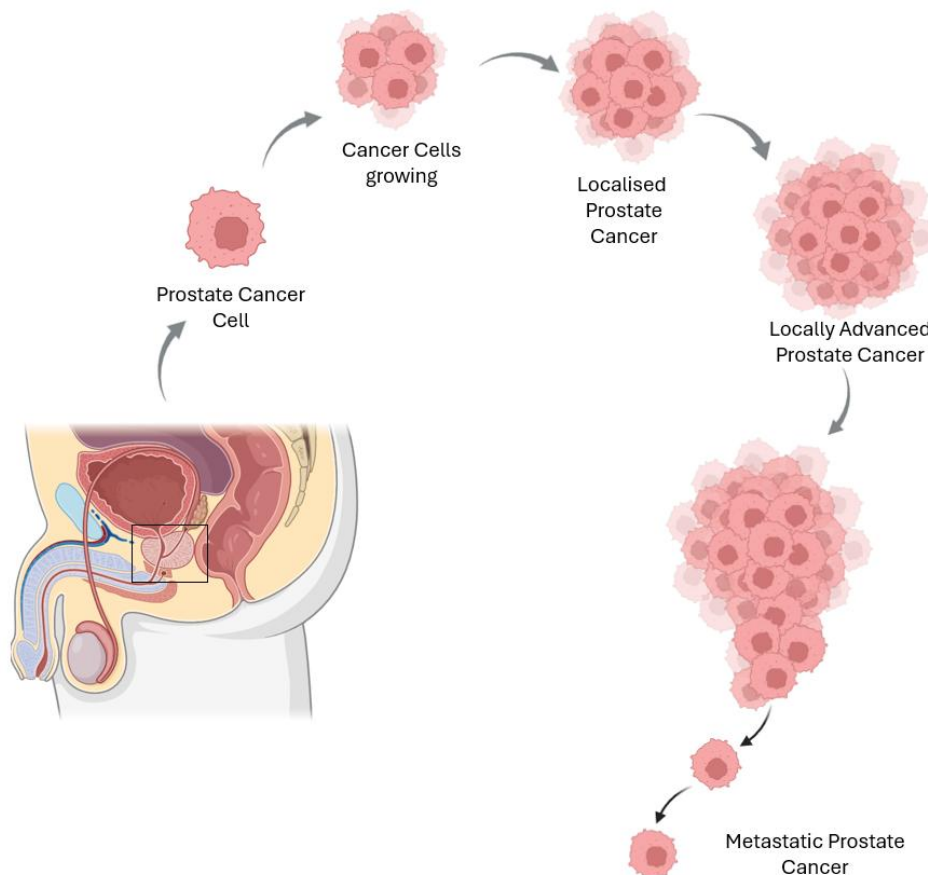


Figure 1.5. Schematic representation of prostate cancer development. The progression of prostate cancer from a single abnormal cell within the prostate gland to the formation of small clusters of cancer cells, followed by localised prostate cancer, locally advanced disease, and ultimately metastatic prostate cancer. Created using BioRender.com.

1.3.5 Risk Factors and Molecular Changes

Several risk factors contribute to the likelihood of developing prostate cancer. The well-established risk factors for prostate cancer are increasing age, Black ethnicity, and a family history of the disease (Rawla, 2019). Other factors such as hormonal influences, diet, obesity, smoking, chronic inflammation, and exposure to radiations and chemicals like cadmium, have been suggested. However, their exact roles in prostate cancer development

are still uncertain and not clearly proven (Cuzick et al, 2014; Rawla, 2019) (Figure 1.6). Inherited susceptibility has also been linked to several loci and genes, including HPC1/RNASEL, BRCA1/2, and regions on the X chromosome associated with hereditary prostate cancer (Rawla, 2019).

At the molecular level, prostate cancer is associated with alterations in transcriptional regulation, metabolism, and signalling pathways. For example, reduced activity of the ZIP1 zinc transporter contributes to decreased zinc accumulation and altered citrate metabolism, changes that may support tumour growth and progression (Almeeri et al, 2024).

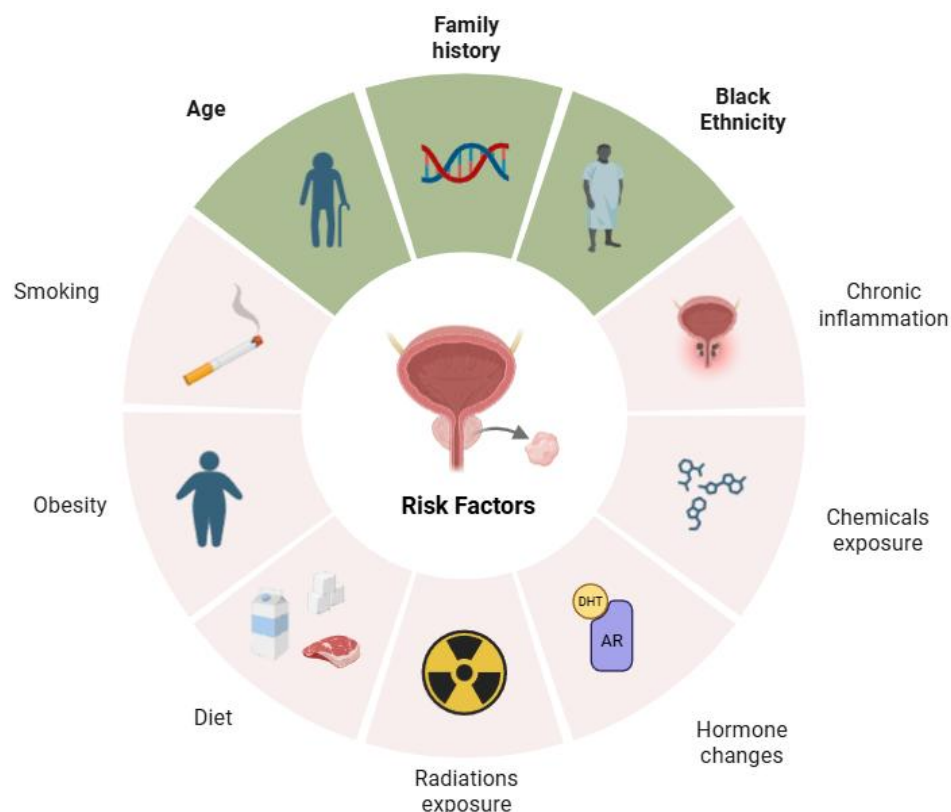


Figure 1.6. Risk factors for prostate cancer. Factors associated with prostate cancer risk. Established risk factors include age, family history, and Black ethnicity. Other potential risk factors include smoking, obesity, diet, radiation exposure, hormonal changes, chemical exposure, and chronic inflammation. Created using BioRender.com

1.3.6 Signs and Symptoms of Prostate Cancer

PCa can be asymptomatic in early stages. When symptoms appear, they commonly involve urinary difficulty, interrupted flow, nocturia, painful urination or blood in urine. In advanced stages, patients may experience bone or back pain, weight loss, swelling of the legs,

testicular discomfort and fatigue. Bone involvement may lead to fractures or spinal cord compression (Almeeri et al, 2024).

1.3.7 Treatment and Clinical Relevance of Androgen Receptor Signalling

Management of prostate cancer depends on disease stage, tumour aggressiveness, and patient factors. Localised disease may be treated with active surveillance, surgery, or radiotherapy, whereas advanced disease often requires systemic therapy (Leslie et al, 2024). A key feature of prostate cancer biology is its strong dependence on androgen receptor (AR) signalling, which drives tumour growth and progression and forms the basis for use of androgen deprivation therapy (ADT). Although ADT is initially effective in many patients, resistance frequently develops, leading to castration-resistant prostate cancer (CRPC) and highlighting the importance of understanding AR regulation in disease progression (Chaturvedi & Dehm, 2019).

1.4 Androgen Signalling and the Androgen Receptor

Testosterone and dihydrotestosterone (DHT) are the principal androgens involved in maintaining normal prostate function through activation of the AR. In prostate cancer, however, AR signalling also promotes tumour growth and survival, and this dependence persists even as tumours progress (Almeeri et al, 2024).

The AR is a ligand-activated transcription factor that plays a central role in prostate cancer development and progression. The AR protein is organised into distinct functional domains, including an N-terminal domain (NTD), a central DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD) (Figure 1.7). In androgen-responsive tissues such as the prostate, testosterone is converted to the more potent androgen dihydrotestosterone (DHT) by the enzyme 5 α -reductase. In the absence of androgens, AR is associated with heat-shock proteins (HSPs) in the cytoplasm. Upon androgen binding, these chaperone proteins dissociate, allowing AR to undergo conformational changes, dimerise, and translocate to the nucleus (Messner et al, 2020) (Figure 1.8).

Once in the nucleus, AR binds to androgen response elements (AREs) located in regulatory regions of target genes. This interaction is mediated by the DNA-binding domain, which contains two zinc finger motifs responsible for DNA recognition and receptor dimerisation. The P-box within the DBD contributes to DNA sequence specificity, while the D-box facilitates dimer formation, enabling stable binding to target DNA. This interaction enables AR to regulate transcription of genes involved in cell growth, metabolism, and survival. The NTD and LBD further support transcriptional activity by enabling recruitment of coactivators and other regulatory proteins (Messner et al, 2020).

Prostate cancer cells rely heavily on AR signalling for survival, which forms the basis of androgen deprivation therapy (ADT) (Chaturvedi & Dehm, 2019). While initially effective,

many tumours eventually progress to castration-resistant prostate cancer (CRPC), where tumour growth continues despite low androgen levels due to mechanisms that maintain or bypass AR activity (Bluemn et al, 2017; Fujita & Nonomura, 2019; Wang et al, 2018). This highlights the importance of understanding AR regulation beyond ligand binding alone.

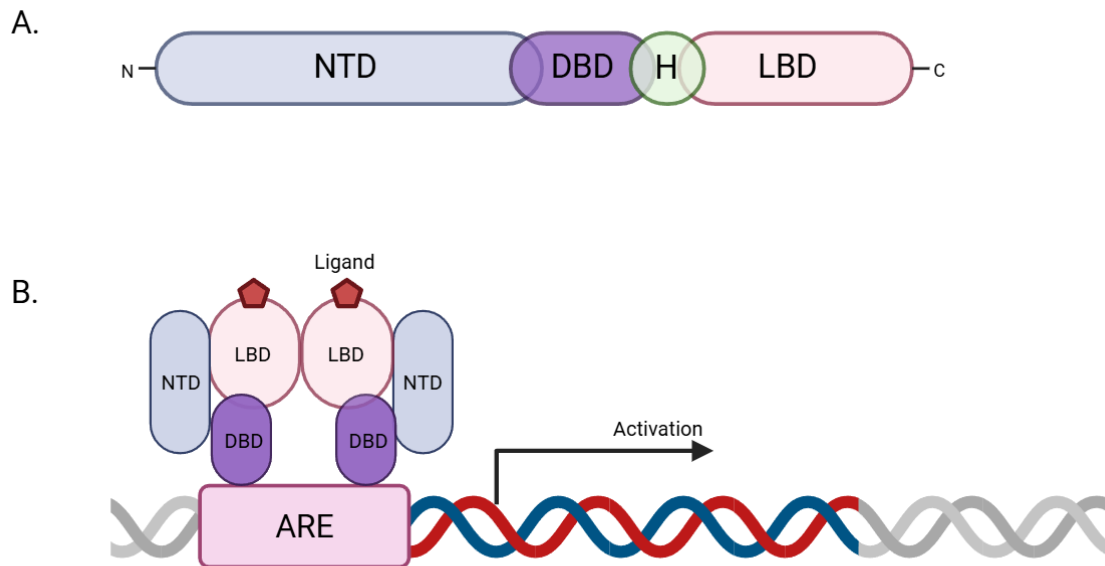


Figure 1.7. Structure and DNA-binding mechanism of the androgen receptor. (A) Schematic representation of the androgen receptor domain organisation, showing the N-terminal domain (NTD), DNA-binding domain (DBD), hinge region (H), and ligand-binding domain (LBD), (B) Upon ligand binding to the LBD, AR undergoes dimerisation and binds to androgen response elements (AREs) within target DNA through the DNA-binding domain. Created using BioRender.com

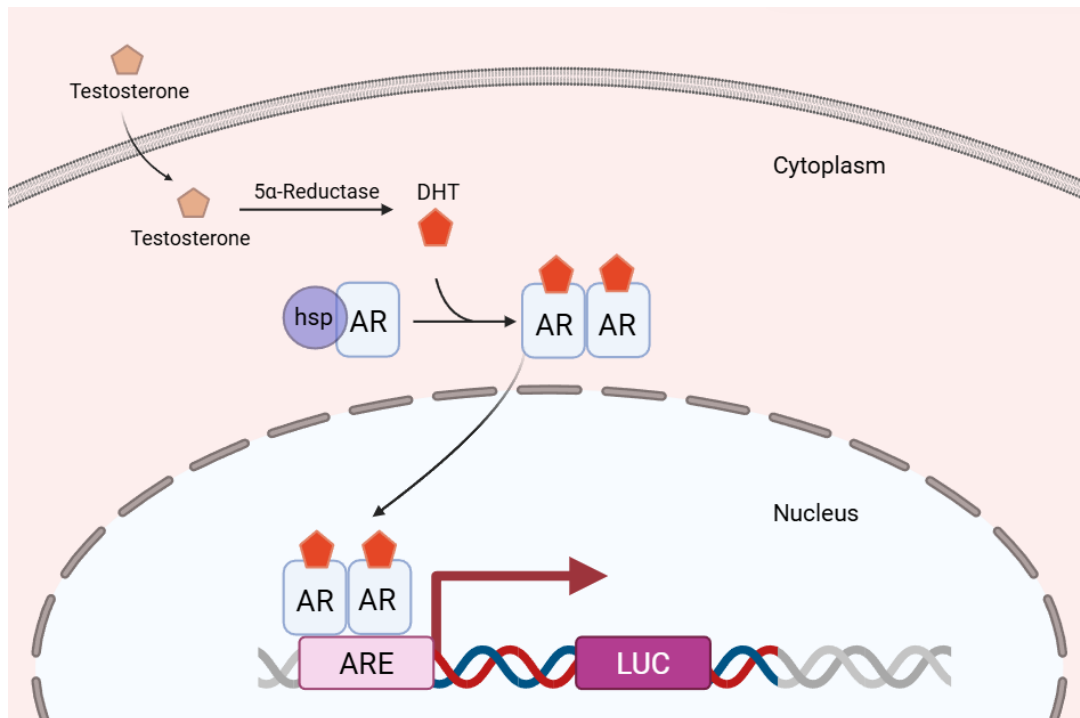


Figure 1.8. Androgen receptor (AR) signalling mechanism. Testosterone is converted to dihydrotestosterone (DHT) by 5α-reductase. In the cytoplasm, AR is associated with heat shock proteins (HSPs). Upon DHT binding, AR dissociates from HSPs, dimerises, and translocates to the nucleus, where it binds androgen response elements (AREs) to activate transcription. Created using BioRender.com

Although androgen binding is a primary driver of AR activation, increasing evidence indicates that AR activity is also influenced by additional regulatory mechanisms, including DNA sequence variation and intracellular signalling pathways. These factors contribute to the complexity of AR-mediated transcriptional regulation in prostate cancer.

1.4.1 Androgen Receptor Signalling and Regulatory Variation in Prostate Cancer

The DNA-binding domain (DBD) plays a central role in mediating AR-DNA interactions, meaning that alterations in DNA sequence may influence AR function (Messner et al, 2020). As the DBD directly interacts with AREs, even small changes in DNA sequence can affect receptor binding and transcriptional activity (Messner et al, 2020; Stelloo et al, 2019). Although ARE-like sequences are widely distributed across the genome, only a subset are functionally bound by AR *in vivo*, suggesting that local DNA sequence context plays an important role in determining effective binding (Stelloo et al, 2019).

Single nucleotide polymorphisms (SNPs) located within or near AREs may influence AR-mediated gene regulation (Lu et al, 2012). Prostate cancer-associated SNPs have been

reported to be enriched in genomic regions containing AR binding sites, suggesting that sequence variation in these regions may contribute to prostate cancer susceptibility and progression (Allemailem et al, 2021; Lu et al, 2012). To investigate these effects at a mechanistic level, recombinant protein systems such as GST-tagged AR DBD constructs provide a useful experimental approach. These systems allow direct assessment of AR binding to specific DNA sequences *in vitro*. Importantly, they enable evaluation of DNA-binding effects independently of other domains of the receptor, allowing comparison between wild-type and variant ARE sequences (Zhou et al, 2010).

The importance of the DBD is further supported by studies showing that it can be targeted therapeutically. Small molecules designed to inhibit the AR DBD suppress transcriptional activity of both full-length AR and constitutively active splice variants, indicating that disruption of DNA binding alone may be sufficient to impair AR-driven transcription (Dalal et al, 2014).

1.5 Kinase-Mediated Regulation of Androgen Receptor Signalling: Focus on RSK

In addition to DNA sequence variation, androgen receptor (AR) activity is also regulated by intracellular signalling pathways. Although AR function is mainly driven by androgen binding and interaction with DNA, post-translational modifications, particularly phosphorylation, are also important in regulating AR transcriptional activity. These modifications provide a way through which extracellular signals can influence nuclear receptor function (Wen et al, 2020).

One of the major pathways involved in this regulation is the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signalling pathway, which transmits signals from the cell surface to the nucleus and regulates processes such as proliferation, survival, and gene expression. Ribosomal S6 kinases (RSKs) are key downstream effectors of this pathway and are activated through ERK-mediated phosphorylation. The RSK family consists of four isoforms (RSK1-4), which are serine/threonine kinases involved in signal transduction and regulation of cellular processes relevant to cancer progression (Cronin et al, 2021).

Emerging evidence suggests that RSK signalling plays a role in prostate cancer by linking growth factor signalling pathways to transcriptional regulation. RSK proteins have been implicated in processes such as proliferation, cell cycle control, and therapeutic resistance, and have also been reported to modulate AR activity in prostate cancer models (Cronin et al, 2021). Together, these findings highlight that AR signalling in prostate cancer is influenced by multiple regulatory mechanisms, including differences in androgen response element sequence, intracellular signalling pathways, and non-coding RNA regulation.

1.6 Role of circRNAs in Prostate Cancer

In addition to protein-coding genes, circRNAs have emerged as important regulators of prostate cancer biology (Li et al, 2021a). Dysregulated circRNA expression has been associated with tumour growth, metastasis, and therapeutic resistance, indicating that circRNAs may contribute functionally to disease progression as well as serve as potential biomarkers (Li et al, 2021a).

1.6.1 miRNA Sponging as a Key Mechanism of circRNA Function

One of the best-characterised functions of circRNAs in prostate cancer is their ability to act as microRNA sponges. By sequestering specific miRNAs, circRNAs relieve repression of target mRNAs and thereby promote expression of genes involved in tumour growth, metastasis, and resistance (Kristensen et al, 2019). For example, circABCC4 has been associated with poor patient survival and promotes prostate cancer progression by sponging miR-1182, leading to increased FOXP4 expression. Similarly, circFMN2 increases LIM Homeobox 2 (LHX2) through miR-1238, while circ0092339 is upregulated in castration-resistant prostate cancer and enhances tumour growth by sponging miR-940, thereby increasing c-MYC expression (Weidle & Birzele, 2025).

Together, these findings suggest that circRNAs contribute to prostate cancer progression by regulating oncogenic pathways through miRNA sponging (Weidle & Birzele, 2025).

1.6.2 Interaction of circRNA with RNA-Binding Proteins (RBPs)

circRNAs can interact with RNA-binding proteins (RBPs) to regulate gene expression and promote cancer progression. They may act as scaffolds, decoys, or recruiters for RBPs, influencing transcription, mRNA stability, splicing, or protein localisation (Feng et al, 2019). A well-characterised example in prostate cancer is circ0005276, which is derived from the *XIAP* gene. Circ0005276 binds the RBP FUS (Fused in Sarcoma) and recruits it to the promoter of its host gene, XIAP. FUS then enhances XIAP transcription. XIAP is known to promote cell survival, proliferation, migration, and epithelial-mesenchymal transition (EMT) in prostate cancer. Silencing either circ0005276 or FUS reduces XIAP expression and inhibits tumour cell growth and migration. This demonstrates that circ0005276 functions as a scaffold for FUS, enabling transcriptional activation of an oncogenic target in prostate cancer (Feng et al, 2019).

1.7 Androgen Receptor Regulation of circRNA Expression in Prostate Cancer

Recent studies indicate that AR signalling also influences circRNA expression in prostate cancer. Although AR does not directly interact with circRNAs, it can regulate their expression indirectly through transcriptional and post-transcriptional mechanisms.

1.7.1 Indirect Transcriptional Control via Host Genes

AR binds to AREs within target gene promoters or enhancers, thereby altering transcriptional activity. Because circRNAs are generated from the same precursor transcripts, androgen stimulation can increase or decrease circRNA expression depending on whether AR activates or represses the relevant target gene. Increased target gene transcription may raise circRNA abundance by providing more precursor RNA, whereas AR-mediated repression may reduce circRNA production (Patop et al, 2019; Zhang et al, 2014).

1.7.2 Post-transcriptional Regulation via Splicing Machinery and RNA-Binding Proteins

Beyond simply altering the quantity of precursor RNA, AR signalling can influence how precursor transcripts are spliced. This includes shifting the balance between linear splicing and back-splicing, which is essential for circRNA formation. This regulation is mediated through RNA-binding proteins (RBPs) and splicing factors that interact with pre-mRNA (Kong et al, 2021). Two such RBPs are Wilms Tumour 1-Associating Protein (WTAP) and Trinucleotide Repeat-Containing Gene 6 (TNRC6). WTAP is mainly involved in the formation of androgen-repressed circRNAs, while TNRC6 is associated with androgen-induced circRNAs. Silencing either protein alters the expression of several androgen-responsive circRNAs. This suggests that AR may influence circRNA biogenesis at the post-transcriptional level by influencing these splicing regulators (Kong et al, 2021).

1.7.3 m6A-Mediated Regulation of circRNA Biogenesis

WTAP is also part of the m6A methyltransferase complex, together with METTL3 and METTL14, linking epitranscriptomic regulation to circRNA expression (Kong et al, 2023). A well-studied example is circDDIT4, a circRNA derived from the *DNA Damage-Inducible Transcript 4* gene (hsa_circ_0018744). It is an androgen-repressed circRNA, meaning that its expression is downregulated by androgen stimulation, and its levels are decreased in prostate cancer samples. Its biogenesis is significantly influenced by m6A modification, with the WTAP-METTL3-METTL14 complex promoting its formation, while the demethylase FTO reduces its levels (Kong et al, 2023).

1.7.4 Feedback Loops Between AR Signalling and circRNAs

The relationship between AR signalling and circRNAs may also involve feedback mechanisms. For example, circ0005276 regulates *XIAP* expression through FUS-mediated transcriptional activation, suggesting that circRNAs may indirectly influence oncogenic pathways linked to AR signalling (Feng et al, 2019; Patop et al, 2019).

1.7.5 AR-Derived circRNAs in CRPC

Recent research has shown that the *AR* gene also produces circRNAs, with at least 13 different types identified in CRPC (castration-resistant prostate cancer) tissues (Cao et al, 2019). One of these, circAR3, is formed from exon 3 of the *AR* gene and is highly expressed

in early-stage or low-grade prostate tumours but reduced in aggressive and advanced cancers, suggesting a potential role in disease progression. However, plasma circAR3 levels increase with disease progression and become undetectable following radical prostatectomy, confirming its tumour origin. These findings suggest that AR-derived circRNAs, particularly circAR3, may serve as non-invasive blood-based biomarkers for prostate cancer diagnosis and monitoring without the need for tissue biopsy (Luo et al, 2019b).

In conclusion, AR regulates circRNA expression in prostate cancer through a combination of mechanisms. It modifies host gene transcription, influences RBP-mediated splicing and back-splicing, interacts with m6A-related factors affecting circRNA biogenesis, and participates in feedback loops involving circRNAs and transcriptional machinery. AR-derived circRNAs may also serve as valuable biomarkers, especially in advanced disease. Together, these findings reveal a complex regulatory landscape connecting AR signalling and circRNA biology in prostate cancer progression.

1.8 Oral Cancer

1.8.1 Introduction to Oral Squamous Cell Carcinoma

CircRNA dysregulation is not only relevant to prostate cancer but has also been shown to play an important role in other cancer types, including oral cancer. Oral squamous cell carcinoma (OSCC) is a common and aggressive malignancy arising from the mucosal epithelium of the oral cavity (Tan et al, 2023). Oral cancer represents a growing health burden in the UK, with around 10,825 new cases reported annually and incidence rising substantially over the past decade (Oral Health Foundation, 2024). OSCC is more common in males and can severely affect speech, swallowing, taste, and overall quality of life (Tan et al, 2023).

1.8.2 Clinical Features and Common Sites

Early OSCC lesions often appear as red (erythroplakia) or mixed red and white patches in the mouth, usually with a slightly irregular surface and clearly defined borders (Tan et al, 2023). These lesions are frequently painless in the early stages, making early detection challenging (Bagan et al, 2010). As the disease progresses, symptoms such as ulceration, induration, and pain or discomfort may become evident. One of the hallmark clinical signs is ulceration with a hard base and irregular margins. Other clinical features include decreased mobility of the tongue, dysphagia and paraesthesia (Tan et al, 2023). The tongue, particularly the posterolateral border, is the commonly affected site, followed by the floor of the mouth, soft palate, gingiva, buccal mucosa, and hard palate. The cancer spreads mainly through lymphatic pathways to neck lymph nodes and can metastasise to distant organs such as lungs and bones (Bagan et al, 2010; Linsen et al, 2018; Tan et al, 2023).

1.8.3 Risk Factors and Etiology

Patients with oral potentially malignant disorders (OPMDs), including oral leukoplakia, oral erythroplakia, oral submucous fibrosis, and oral lichen planus, have a higher risk of developing OSCC (Warnakulasuriya, 2020). These disorders often show no symptoms initially but may present with redness, pain, or ulceration as they progress (Bagan et al, 2010). The development of OSCC is driven by a combination of environmental, lifestyle, viral, and genetic factors. Tobacco use, alcohol consumption, and betel quid chewing are well-established risk factors, as these substances contain carcinogens such as polycyclic aromatic hydrocarbons, nitrosamines, and ethanol. Long-term exposure to these risk factors can induce genetic mutations and chronic irritation of the oral mucosa, which can lead to the development of OPMDs and their progression into OSCC (Tan et al, 2023). Infection with high-risk strains of human papillomavirus (HPV), particularly HPV-16 and HPV-18 has also been linked to OSCC, as the virus can disrupt normal cell cycle control and promote malignant transformation (Parshad et al, 2015)(Figure 1.9).

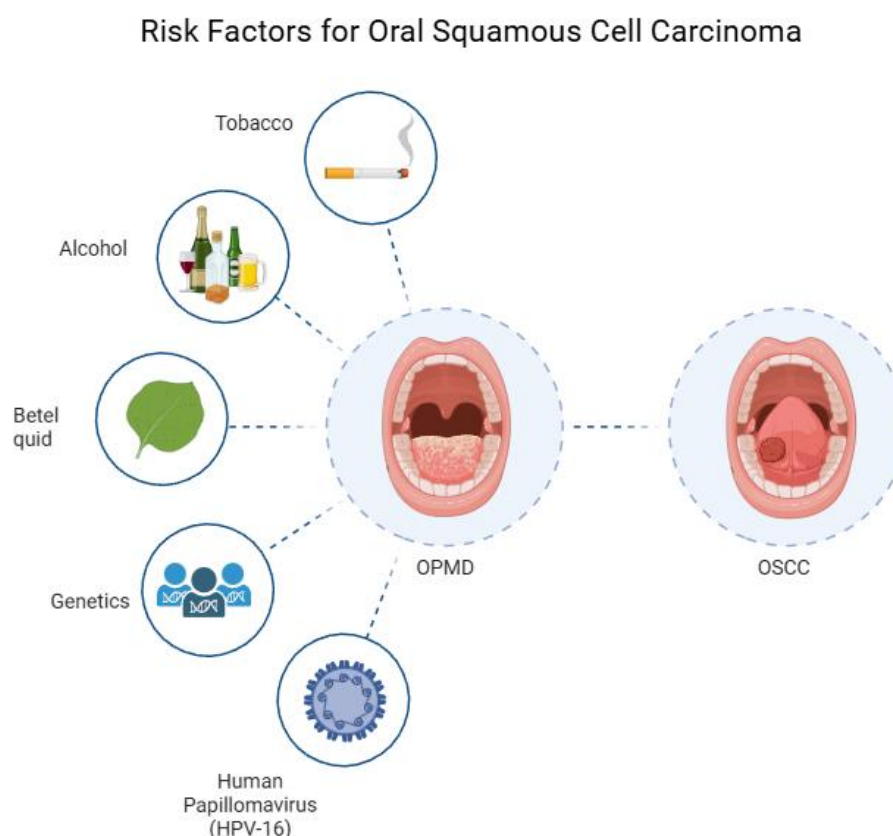


Figure 1.9. Risk Factors for Oral Squamous Cell Carcinoma. Major risk factors including tobacco, alcohol, betel quid chewing, infection with high-risk human papillomavirus, and genetic predisposition contribute to the development of oral potentially malignant disorders (OPMDs), which may progress to oral squamous cell carcinoma (OSCC). Created using BioRender.com

1.8.4 Diagnostic and Therapeutic Challenges

Despite advances in surgery, radiotherapy, and chemotherapy, OSCC continues to show high rates of recurrence and poor long-term survival, largely because many cases are diagnosed at a late stage (Lv et al, 2025; Rivera, 2015). OPMDs provide an opportunity for earlier detection, but they are often asymptomatic and therefore overlooked (Tan et al, 2023). These limitations highlight the need for more sensitive biomarkers and improved therapeutic targets. In this context, circRNAs have attracted increasing interest because of their stability, cancer-specific expression, and potential relevance in diagnosis, prognosis, and targeted intervention (Lv et al, 2025; Rivera, 2015).

1.8.5 Key circRNAs in OSCC

In OSCC, circ_0000745 is upregulated relative to normal tissue and promotes tumour cell growth through both miRNA- and protein-mediated mechanisms. It acts as a sponge for miR-488, thereby relieving repression of *Cyclin D1* (*CCND1*), and also interacts with the RNA-binding protein human antigen R (HuR) to further enhance *CCND1* expression (Li et al, 2021b).

CircPVT1 (hsa_circ_0001821) is upregulated in OSCC and functions as an oncogenic circRNA by promoting tumour cell proliferation, migration, and invasion while reducing apoptosis. Mechanistically, it acts by sponging miR-143-3p, which results in increased *SLC7A11* expression and activation of the MAPK signalling pathway. Suppression of circPVT1 has been shown to inhibit OSCC progression in both *in vitro* and *in vivo* models, supporting its potential relevance as a biomarker and therapeutic target (Wang et al, 2022).

1.9 Study Overview and Rationale

The background presented above highlights the importance of understanding the different molecular mechanisms that contribute to cancer development and progression. In this study, different aspects of molecular regulation in cancer were investigated with a focus on circRNAs and AR signalling. The overall research approach is shown in Figure 1.10 and included three main areas of investigation.

First, computational approaches were used to investigate circRNA dysregulation and predict potential circRNA-associated regulatory networks in OSCC and prostate cancer. Although circRNAs have been increasingly studied in cancer, their dysregulation and potential regulatory interactions are not fully understood. Therefore, circRNA expression was investigated in OSCC and prostate cancer to identify dysregulated circRNAs and explore their potential interactions with miRNAs and target genes.

Second, since prostate cancer is highly dependent on AR signalling, the study investigated whether variation within AREs could affect AR activity at the DNA level. AREs are important regulatory elements through which AR regulates the expression of androgen-responsive genes. Therefore, sequence variation within these regions may affect AR-mediated transcription. Selected ARE variants were investigated to determine whether they could affect AR activity. The AR DNA-binding domain was also expressed and purified as a recombinant GST-AR DBD protein for use in a DNA-binding assay to investigate whether the different ARE sequences affected AR binding.

Finally, AR activity can also be influenced by intracellular signalling pathways. Therefore, the study investigated whether different RSK isoforms could affect AR activity. RSK proteins are involved in intracellular signalling and may also affect AR-mediated transcription. The effect of individual RSK isoforms on AR activity was therefore investigated.

These three areas investigated different aspects of molecular regulation in cancer. The circRNA analysis focused on changes in circRNA expression and their potential regulatory interactions, while the ARE and RSK experiments investigated factors that may affect AR activity.

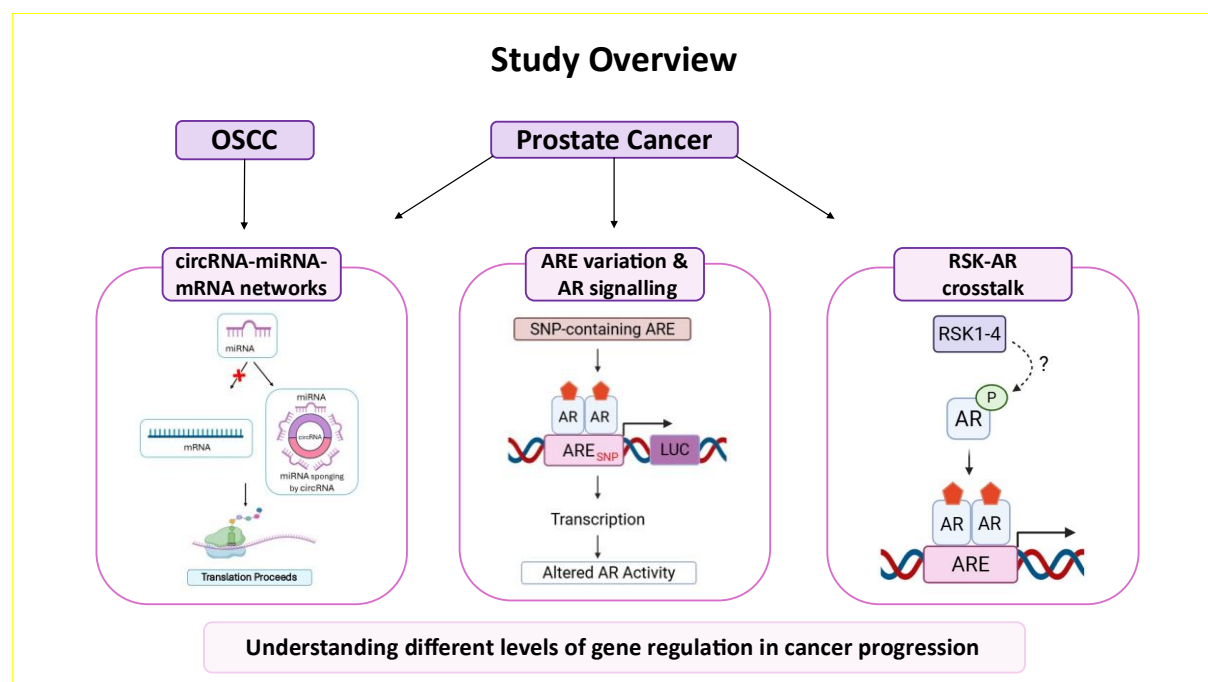


Figure 1.10. Study overview. The study investigated circRNA dysregulation and predicted regulatory networks, followed by investigation of AR activity at the DNA and intracellular signalling levels.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Reagents: Chemicals and kits

All chemicals and kits used in this study, along with their suppliers, are listed below (Table 2.1-2.6).

Table 2.1. Cell Culture and Hormone Treatment Reagents

Reagent	Catalogue No.	Supplier
Charcoal-stripped foetal calf serum (csFCS)	10611235	HyClone®, Fisher Scientific
DMEM (without phenol red and L-glutamine)	17-205-CV	Corning
Dulbecco's Modified Eagle's Medium (DMEM)	10-013-CV	Corning
Foetal calf serum (FCS)	10014813	HyClone®, Fisher Scientific
L-Glutamine-Penicillin-Streptomycin solution	G1146	Sigma-Aldrich
Mibolerone	SML0438	Sigma-Aldrich
PBS tablets	P32080-100T	Melford
RPMI-1640 (without phenol red and L-glutamine)	17-105-CV	Corning
RPMI-1640 medium	10-040-CV	Corning
Trypsin-EDTA (0.05%)	25-052-CI	Corning

Table 2.2. Molecular Biology and Cloning Reagents

Reagent	Catalogue No.	Supplier
Absolute ethanol	E/0600DF/17	Fisher BioReagents
Agarose	BP1356-100	Fisher BioReagents
Ampicillin sodium salt	A9518	Sigma-Aldrich
Calcium chloride (CaCl ₂)	223506	Sigma-Aldrich
DNA gel loading dye	R0611	Thermo Fisher Scientific
EDTA	E9884	Sigma-Aldrich
FastDigest EcoRI	FD0274	Thermo Fisher Scientific
FastDigest NotI	FD0594	Thermo Fisher Scientific
GeneJET Plasmid Miniprep Kit	K0502	Thermo Fisher Scientific
GeneRuler 1 kb DNA ladder	SM0311	Thermo Fisher Scientific
Lennox LB agar	L24030-2000.0	Melford
Miller's LB broth	L24400-2000.0	Melford
Nuclease-free water	AM9937	Invitrogen
QIAGEN Plasmid Plus Midi Kit	12943	QIAGEN
SYBR Safe	S33102	Invitrogen
Tris base	BP152-1	Fisher Scientific
Tryptone	LP0042	Oxoid
Yeast extract	LP0021	Oxoid

Table 2.3. Protein Analysis and Western Blot Reagents

Reagent	Catalogue No.	Supplier
4X Laemmli sample buffer	1610747	Bio-Rad
Acrylamide/Bis-acrylamide solution	J60868.K2	Thermo Scientific
Ammonium persulphate (APS)	248614	Sigma-Aldrich
β-mercaptoethanol	125472500	Thermo Scientific
Bovine serum albumin (BSA)	A2153	Sigma-Aldrich
Brilliant Blue R-250	BP101-50	Fisher BioReagents
DL-Dithiothreitol (DTT)	43816	Sigma-Aldrich
Glycine	G/0800/60	Fisher Scientific
Methanol	20847.307	VWR
PageRuler Prestained Protein Ladder	26616	Thermo Scientific
Protease inhibitor cocktail III	P50750-1.0	Melford
PVDF membrane	IPVH00010	Millipore
Sodium deoxycholate	D6750	Sigma-Aldrich
Sodium dodecyl sulphate (SDS)	S/P530/53	Fisher Scientific
SuperSignal West Pico PLUS substrate	34580	Thermo Fisher Scientific
TEMED (N,N,N',N'-tetramethylethylenediamine)	T9281	Sigma-Aldrich
Triton X-100	T8787	Sigma-Aldrich
Tween-20	P1379	Sigma-Aldrich
Western HRP substrate	WBLUF0500	Millipore

Table 2.4. Reporter Assay and Transfection Reagents

Reagent	Catalogue No.	Supplier
5X Reporter Lysis Buffer	E397A	Promega
FuGENE HD transfection reagent	E2311	Promega
Galacto Reaction Buffer Diluent	T2073	Thermo Fisher Scientific
Galacton-Plus substrate	T2117	Thermo Fisher Scientific
Luciferase assay system	E1500	Promega
Opti-MEM I Reduced Serum Medium	31985-062	Gibco
Tropix Accelerator II	T2222	Thermo Fisher Scientific

Table 2.5. Bacterial Culture and GST Protein Purification Reagents

Reagent	Catalogue No.	Supplier
Coomassie Brilliant Blue R-250	BP101-50	Fisher BioReagents
Glucose	G/0500/53	Fisher Scientific
Glycerol	G5516-1L	Sigma-Aldrich
Isopropyl β -D-1-thiogalactopyranoside (IPTG)	I6758	Sigma-Aldrich
L-Glutathione (reduced)	G4251	Sigma-Aldrich
Magnesium chloride (MgCl ₂)	M/0600/53	Fisher Scientific
Magnesium sulphate (MgSO ₄)	11625871	Fisher Scientific
Zinc chloride (ZnCl ₂)	208086	Sigma-Aldrich

Table 2.6. General Chemicals and Buffer Components

Reagent	Catalogue No.	Supplier
BES (N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid)	14853	Sigma-Aldrich
ddH ₂ O (molecular biology grade)	SH30538.03	HyClone
Glacial acetic acid	A/0400/PB17	Fisher Scientific
Hydrogen Chloride (HCl)	H/1100/PB17	Fisher Scientific
Potassium Chloride (KCl)	P9541	Sigma-Aldrich
Sodium Chloride (NaCl) (5 M)	S/3160/60	Alfa Aesar
Sodium Hydroxide (NaOH)	S/4920/53	Fisher Scientific

2.1.2 Buffers and Solutions

Buffers and solutions used in this study are listed below (Table 2.7-2.12).

Table 2.7. Agarose Gel Electrophoresis solutions

Name	Recipe	Sterilisation	Storage
Agarose gel (1%)	1 g agarose dissolved in 100 mL 1X TAE by heating until fully melted. The solution was cooled (~50 °C), SYBR Safe added, and the gel cast using a comb.	-	Freshly prepared
Tris-acetate-EDTA (TAE, 1X)	40 mM Tris base, 1.114 mL acetic acid and 1 mM EDTA made up to 1 L with ddH ₂ O.	-	RT

Table 2.8. Bacterial Culture Solutions

Name	Recipe	Sterilisation	Storage
Ampicillin stock (100 mg/mL)	1 g ampicillin dissolved in 10 mL ddH ₂ O and used at 100 µg/mL final concentration.	0.22 µm filter	–20 °C
Glucose stock (1 M)	90.08 g glucose dissolved in 500 mL ddH ₂ O.	0.22 µm filter	RT
LB agar plates	40 g LB agar powder dissolved in 1 L ddH ₂ O, autoclaved, cooled before supplementation with antibiotics where required, and poured into Petri dishes while molten.	Autoclave	4 °C
Luria broth (LB)	25 g LB powder dissolved in 1 L ddH ₂ O, pH adjusted to 7.2 where necessary, supplemented with antibiotics when required	Autoclave	4 °C
Magnesium chloride stock (1 M)	101.655 g MgCl ₂ dissolved in 500 mL ddH ₂ O.	Autoclave	RT
Magnesium sulphate stock (1 M)	120.366 g MgSO ₄ dissolved in 500 mL ddH ₂ O.	Autoclave	RT
SOC medium (Super Optimal broth with Catabolite repression)	20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 0.18 g KCl, 10 mL MgCl ₂ , 10 mL MgSO ₄ and 20 mL glucose stock made up to 1 L with ddH ₂ O.	Autoclave (before glucose)	4 °C

Table 2.9. Transfection Buffers

Name	Recipe	Sterilisation	Storage
BES-buffered saline (BBS, 2X)	50 mM BES (10.66 g), 280 mM NaCl (16.36 g), 1.5 mM Na ₂ HPO ₄ (0.21 g) in 1 L ddH ₂ O, adjusted to pH 6.95.	0.22 µm filter	–20 °C
Calcium chloride (2.5 M)	138.73 g CaCl ₂ dissolved in 500 mL ddH ₂ O.	0.22 µm filter	–20 °C

Table 2.10. Reporter Assay Buffer solutions

Name	Recipe	Storage
Reporter lysis buffer (1X)	Prepared by diluting 5X stock solution with ddH ₂ O to 1X concentration.	RT

Table 2.11. Western Blot Buffers and Solutions

Name	Recipe	Sterilisation	Storage
Ammonium persulphate (APS, 10%)	1 g APS dissolved in 10 mL ddH ₂ O.	-	-20 °C
Blocking solution	5% skimmed milk prepared in PBS-T.	-	4 °C (use within 24 h)
BSA standards	10 µg/µL stock prepared and serially diluted to generate standard curve.	-	-20 °C
PBS (1X)	PBS tablets dissolved in ddH ₂ O to required volume.	Autoclave	RT
PBS-T (0.1%)	0.1% Tween-20 prepared in PBS.	-	4 °C
Resolving gel (10%)	Per gel, a 10% resolving gel was prepared consisting of 1.24 mL 40% acrylamide/bis-acrylamide solution, 1.875 mL of 1 M Tris-HCl (pH 8.9), 2.085 mL ddH ₂ O and 50 µL of 10% SDS. Immediately prior to pouring, 50 µL of 10% APS and 5 µL TEMED were added.	-	Fresh
Resolving gel (12%)	Per gel, a 12% resolving gel was prepared consisting of 1.50 mL 40% acrylamide/bis-acrylamide solution, 1.875 mL of 1 M Tris-HCl (pH 8.9), 1.52 mL ddH ₂ O and 50 µL of 10% SDS. Immediately prior to pouring, 50 µL of 10% APS and 5 µL TEMED were added.	-	Fresh
Running buffer (10X)	250 mM Tris, 1.92 M glycine and 0.5% SDS prepared in ddH ₂ O. For 1 L: 30 g Tris, 144.5 g glycine and 5 g SDS, made up to 1 L with ddH ₂ O.	-	RT
Running buffer (1X)	Prepared by diluting 10X running buffer 1:10 in ddH ₂ O.	-	RT

Name	Recipe	Sterilisation	Storage
Semi-dry transfer buffer	20 mM Tris, 150 mM glycine and 20% methanol prepared in ddH ₂ O. For 1 L: 2.44 g Tris, 11.26 g glycine, 200 mL methanol, made up to 1 L with ddH ₂ O.	-	4 °C
Stacking gel	Per gel, a 5% stacking gel was prepared consisting of 0.32 mL 40% acrylamide/bis-acrylamide solution, 0.94 mL of 1 M Tris-HCl (pH 6.8), 1.19 mL ddH ₂ O and 25 µL of 10% SDS. Immediately prior to pouring, 25 µL of 10% APS and 2.5 µL TEMED were added.	-	Fresh
Tris-HCl buffer (1 M)	12.114 g Tris base dissolved in ddH ₂ O to a final volume of 100 mL and adjusted to pH 6.8 or 8.9 using 4 M HCl, as required.	Autoclave	RT

Table 2.12. GST-AR DBD Expression and Purification Reagents

Name	Recipe	Storage
Coomassie staining solution	0.1% (w/v) Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid in ddH ₂ O	RT
Destaining Solution	40% methanol, 10% acetic acid in ddH ₂ O	RT
Elution buffer (reduced glutathione)	10 mM reduced glutathione prepared by dissolving 0.0307 g in 10 mL PBS	4 °C (freshly prepared)
IPTG (1 M stock)	IPTG powder dissolved in ddH ₂ O to a final concentration of 1 M	-20 °C
Zinc chloride (1 M stock)	ZnCl ₂ dissolved in ddH ₂ O to a final concentration of 1 M	RT

2.1.3 Cell culture and treatment

Mammalian cell culture media used in this study are summarised in Table 2.13.

Table 2.13. Mammalian cell culture media

Cell line	Media	Storage
COS-1	Complete DMEM supplemented with 10% FCS and 1% L-glutamine-penicillin-streptomycin (final concentration: 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin)	4 °C (use within 1 month)
COS-1	Phenol red-free DMEM supplemented with 5% charcoal-stripped FCS (csFCS) and 1% L-glutamine-penicillin-streptomycin	4 °C (use within 1 month)
22Rv1	Complete RPMI-1640 supplemented with 10% FCS and 1% L-glutamine-penicillin-streptomycin (final concentration: 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin)	4 °C (use within 1 month)
22Rv1	Phenol red-free RPMI-1640 supplemented with 5% charcoal-stripped FCS (csFCS) and 1% L-glutamine-penicillin-streptomycin	4 °C (use within 1 month)

Reagents used for cell treatment are listed in Table 2.14.

Table 2.14. Reagents for cell treatment

Treatment	Stock preparation	Final concentration	Target Receptor
Ethanol	100% ethanol	Equivalent vehicle volume	Control
Mibolerone	1 μ M stock in 100% ethanol	0.1 nM, 1 nM	AR agonist

Cell lines used in this study are listed in Table 2.15. All cell lines were obtained from the American Type Culture Collection (ATCC).

Table 2.15. Mammalian cell lines

Cell line	Description	Source
22Rv1	Human prostate carcinoma epithelial cell line expressing endogenous androgen receptor	ATCC
COS-1	African green monkey kidney fibroblast-like cell line	ATCC

2.1.4 Plasmids and molecular constructs

Plasmids and constructs used in this study are listed in Table 2.16. All plasmids carry ampicillin resistance unless otherwise stated.

Table 2.16. Plasmids and constructs

Plasmid / Construct	Description
pARE-LUC	Androgen response element luciferase reporter
pBOS- β -galactosidase	β -galactosidase control plasmid for transfection normalisation
pGL4-CXCR1-Mut	Mutant CXCR1 androgen response element reporter construct
pGL4-CXCR1-WT	Wild-type CXCR1 androgen response element reporter construct
pGEX-GST-AR DBD	GST-tagged androgen receptor DNA-binding domain construct
pGL4-MAF-Mut	Mutant MAF expression construct
pGL4-MAF-WT	Wild-type MAF expression construct
PB-PROM-LUC	Promoter luciferase reporter construct
pcDNA3.1-Empty	Mammalian expression empty vector
pcDNA3.1-RSK1	Mammalian expression plasmid for RSK1
pcDNA3.1-RSK2	Mammalian expression plasmid for RSK2
pcDNA3.1-RSK3	Mammalian expression plasmid for RSK3
pcDNA3.1-RSK4	Mammalian expression plasmid for RSK4
pcDNA3.1-circ_0010794	Circular RNA overexpression construct (hsa_circ_0010794)
pcDNA3.1-circ_0064977	Circular RNA overexpression construct (hsa_circ_0064977)
pSG5-Empty	Empty vector control (pSG5 backbone)
pSV-AR	Androgen receptor expression plasmid
pcDNA3.1-RFP-Empty	Red fluorescent protein expression control plasmid
pcDNA3.1-RFP-RSK1	RFP-tagged RSK1 expression construct
pcDNA3.1-RFP-RSK2	RFP-tagged RSK2 expression construct
pcDNA3.1-RFP-RSK3	RFP-tagged RSK3 expression construct
pcDNA3.1-RFP-RSK4	RFP-tagged RSK4 expression construct
TAT-GRE-E1B-LUC	Steroid-responsive luciferase reporter construct

2.1.5 Antibodies for Western blotting

Antibodies used for Western blotting are listed in Table 2.17.

Table 2.17. Antibodies used for Western blotting

Antibody	Catalogue No.	Supplier	Dilution (WB)
Primary antibodies			
Androgen receptor (AR) (rabbit)	AB108341	Abcam	1:1000
α -Tubulin (rabbit)	11224-1-AP	Proteintech	1:2000
RSK1 (mouse)	sc-393147	Santa Cruz Biotechnology	1:1000
RSK2 (mouse)	sc-9986	Santa Cruz Biotechnology	1:1000
RSK3 (mouse)	sc-517283	Santa Cruz Biotechnology	1:1000
RSK4 (mouse)	sc-100424	Santa Cruz Biotechnology	1:1000
Secondary antibodies			
HRP-conjugated anti-mouse IgG (goat)	SA00001-1	Proteintech	1:2000
HRP-conjugated anti-rabbit IgG (goat)	SA00001-2	Proteintech	1:2000

2.1.6 Computational tools and resources

Bioinformatics tools and online resources used for computational analysis are summarised in Table 2.18.

Table 2.18. Bioinformatics tools and online resources

Resource	Purpose	Website
ENA	RNA-seq dataset retrieval	https://www.ebi.ac.uk/ena/
UCSC Genome Browser	Genomic coordinate verification and sequence extraction	https://genome.ucsc.edu/
circBase	circRNA sequence retrieval	https://www.circbase.org/
Galaxy Europe (DESeq2 v2.11.40.8+galaxy2)	Differential expression analysis (DESeq2)	https://usegalaxy.eu/
SeedVicious v1.1	miRNA binding site prediction	https://seedvicious.essex.ac.uk/
miRTarBase 2025	Validated target gene identification	https://mirtarbase.cuhk.edu.cn/
RStudio (v2025.05.1+513))	Statistical analysis and data visualisation	https://posit.co/download/rstudio-desktop/
GraphPad Prism (v10)	Statistical analysis and data visualisation	https://www.graphpad.com/
Microsoft Excel (Microsoft 365)	Data organisation and plotting	https://www.microsoft.com/excel

2.2 Methods

2.2.1 Computational analysis of circRNA-miRNA-mRNA regulatory interactions

Computational analysis was performed to identify dysregulated circular RNAs (circRNAs) and investigate their potential circRNA-miRNA-mRNA regulatory interactions in oral squamous cell carcinoma (OSCC) and prostate cancer.

2.2.1.1 RNA-seq dataset acquisition

Publicly available RNA sequencing datasets were obtained from the European Nucleotide Archive (ENA). For OSCC, dataset PRJNA542863 (European Nucleotide Archive, 2020), comprising six paired tumour and normal oral mucosa tissue samples, was analysed. For prostate cancer, dataset PRJNA552058 (European Nucleotide Archive, 2019), comprising 15 paired tumour and matched non-tumour tissue samples, was used. Initial mapped read count files for circRNAs, coding genes, and microRNAs were previously generated in our lab. These mapped read count files were subsequently used for downstream differential expression analysis.

2.2.1.2 Differential expression analysis

Differential expression analysis of circRNAs and target genes was performed using Galaxy Europe (DESeq2 v2.11.40.8+galaxy2) (The Galaxy Community, 2024). CircRNAs and target genes were considered significantly dysregulated using the thresholds $|\log_2 \text{fold change}| \geq 1$ and adjusted p-value ≤ 0.05 . Principal component analysis (PCA) was performed on normalised circRNA expression data generated from DESeq2 analysis to evaluate overall sample clustering and variation between tumour and control samples. Differential expression results were visualised using volcano plots, and expression profiles of selected circRNAs across tumour and control samples were further assessed by heatmap analysis using RStudio (v2025.05.1+513).

2.2.1.3 circRNA annotation and sequence retrieval

For each cancer dataset, the top ten upregulated and top ten downregulated circRNAs were initially selected based on differential expression analysis for downstream investigation. Genomic coordinates of selected circRNAs were examined using the UCSC Genome Browser (hg38 assembly) (Perez et al, 2025). CircRNA annotations and genomic coordinates were obtained from circBase (Glazar, 2014), which provides coordinates based on the hg19 genome assembly. Where required, hg19 coordinates were converted to hg38 coordinates using the UCSC Genome Browser liftOver tool, and corresponding sequences were extracted from the hg38 human reference genome.

2.2.1.4 miRNA target prediction

Potential microRNA binding sites within selected circRNAs were predicted using SeedVicious (Marco, 2018). Mature high-confidence dominant-arm human microRNA sequences

provided by the supervisor were used as input in FASTA format. Target prediction was performed using canonical target site analysis, with the option to merge microRNAs from the same seed family enabled, allowing related miRNAs sharing identical seed sequences to be analysed as a single functional family.

Candidate circRNAs were prioritised for further downstream analysis based on the presence of multiple predicted binding sites for the same miRNA seed family, as this was considered indicative of stronger miRNA sponging potential. CircRNAs containing a high number of repeated sites for individual miRNA families were therefore selected for target validation and regulatory network analysis.

2.2.1.5 Target gene validation

Experimentally validated target genes of selected miRNAs were identified using miRTarBase (Cui et al, 2025). Priority was given to targets supported by strong experimental evidence, including reporter assay, western blot, and quantitative Polymerase Chain Reaction (qPCR), while interactions supported only by weaker high-throughput evidence, such as microarray or next-generation sequencing (NGS), were considered lower confidence. In the final analysis, emphasis was placed on targets supported by at least three strong evidence records to improve confidence in the predicted regulatory interactions.

2.2.1.6 Regulatory network construction

To assess potential circRNA-mediated miRNA sponging relationships, differential expression patterns of circRNAs, corresponding miRNAs, and validated downstream target genes were compared. For significantly upregulated circRNAs, miRNAs showing opposite fold change trends, including negative fold change values, were preferentially examined together with target genes showing increased expression. Conversely, for downregulated circRNAs, miRNAs with increased expression and target genes with reduced expression were assessed. Where required, biologically consistent fold change direction was prioritised during miRNA selection, even if the magnitude of change did not fully meet the circRNA and target gene threshold criteria.

For target gene validation, mapped gene read count datasets corresponding to each cancer cohort were analysed using DESeq2 in Galaxy to obtain log2 fold change and adjusted p-values. These results were integrated with circRNA and miRNA expression profiles to identify candidate circRNA-miRNA-mRNA regulatory axes consistent with a putative miRNA sponging mechanism.

2.2.2 Plasmid constructs, bacterial transformation and plasmid preparation

Plasmid constructs used in this study were obtained from existing laboratory resources and are summarised in Table 2.16. The *MAF* and *CXCR1* wild-type and mutant ARE reporter plasmids were provided as pre-existing constructs. These plasmids were initially verified by diagnostic restriction digestion, followed by agarose gel electrophoresis to assess whether

the expected digestion pattern was obtained. Constructs showing the expected digestion profile were subsequently sent for Sanger sequencing for further confirmation.

For circRNA overexpression experiments, plasmid constructs expressing hsa_circ_0010794 and hsa_circ_0064977 were commercially synthesised (Twist Bioscience). The constructs included flanking intronic sequences to facilitate circularisation and were provided in a pcDNA3.1 expression vector.

Where plasmid amplification was required, constructs were transformed into competent *Escherichia coli* (*E. coli*) cells, cultured under antibiotic selection, and purified for downstream experimental use.

2.2.2.1 Bacterial strains and culture

For bacterial transformation and plasmid amplification, the high-efficiency XL1-Blue strain of competent *E. coli* (Thermo Scientific) was used. All bacterial procedures were carried out under sterile conditions to minimise contamination. Bacterial cultures were maintained in Luria broth (LB) or on LB agar plates supplemented with the appropriate antibiotic.

2.2.2.2 Bacterial transformation

For transformation, 50 µL of XL1-Blue competent cells were thawed on ice and gently mixed with approximately 10 ng of plasmid DNA. The cell-DNA mixture was incubated on ice for 30 min, followed by heat shock at 42 °C for 45 s in a water bath. The cells were then immediately returned to ice for 2 min.

Following heat shock, 450 µL of pre-warmed SOC medium was added, and the cells were incubated at 37 °C for 1 h with shaking to allow recovery and expression of antibiotic resistance. The appropriate volume of transformed cell suspension was then spread onto LB agar plates supplemented with ampicillin (100 µg/mL) and incubated overnight at 37 °C to allow colony formation.

2.2.2.3 Plasmid DNA preparation (mini-, midi- and maxi-prep)

A single transformed bacterial colony was picked from the LB agar plate using a sterile pipette tip and used to inoculate 5 mL of LB medium supplemented with 5 µL ampicillin. The culture was incubated at 37 °C with shaking for 16 h. For miniprep, plasmid DNA was isolated directly from the 5 mL overnight culture using the GeneJET Plasmid Miniprep Kit (Thermo Scientific), according to the manufacturer's instructions. For large scale plasmid preparation, the overnight culture was transferred into 200 mL of LB medium containing 200 µL ampicillin and incubated overnight at 37 °C with shaking. Plasmid DNA was then isolated using the Qiagen Plasmid Midi/Maxiprep Kit, according to the manufacturer's guidelines. The concentration and purity of the extracted plasmid DNA were measured using a NanoDrop spectrophotometer (LabTech).

2.2.2.4 Plasmid verification

Plasmid verification was carried out by diagnostic restriction digestion using FastDigest restriction enzymes (Thermo Scientific), according to the manufacturer's instructions. The digested plasmids were analysed by 1% agarose gel electrophoresis to confirm the presence and integrity of the plasmid DNA. Selected samples were further confirmed by Sanger sequencing.

2.2.2.5 Glycerol stock preparation

For long-term storage, 200 µL of sterile glycerol was mixed with 800 µL of bacterial culture. The glycerol stocks were stored at -80 °C.

2.2.2.6 Agarose gel electrophoresis

Agarose (1.5 g, Melford) was dissolved in 150 mL of 1X Tris-Acetate-EDTA (TAE) buffer (1% agarose gel). The mixture was heated in a microwave until the agarose was completely dissolved and then allowed to cool to approximately 50 °C. To enable DNA visualisation, 3 µL of SYBR Safe was added to the cooled agarose solution. The solution was poured into a gel tray with a comb in place and allowed to solidify. Once set, the comb was removed to create wells. DNA samples mixed with DNA gel loading dye (Thermo Scientific) and a 1 kb DNA ladder (Thermo Scientific) were loaded onto the gel. Electrophoresis was carried out at 100 V and 100 mA for 45 min and separated DNA bands were visualised using a UV transilluminator.

2.2.3 Mammalian cell culture and passaging

All cell lines were maintained at 37 °C in a humidified incubator with 5% carbon dioxide (CO₂). Cells were regularly observed under a microscope to monitor confluency and overall cell morphology. Once the cells reached approximately 70-80% confluency, they were passaged, typically twice per week. The culture medium was aspirated, and the cells were washed twice with 1X PBS. To detach the cells from the flask surface, 0.5 mL of trypsin was added, and the cells were incubated at 37 °C for 2-5 min until detached. Complete growth medium was then added to neutralise the trypsin, and the cell suspension was gently pipetted up and down to break up any cell clumps. The cells were either transferred into fresh culture flasks for continued maintenance or seeded into appropriate plates for experimental analysis. For hormone treatment experiments, cells were maintained in hormone-depleted medium for 24-72 h prior to ligand treatment. Details of the mammalian cell lines used are provided in Table 2.15.

2.2.4 Transfection and Luciferase reporter assays

2.2.4.1 Reporter enzyme assays: luciferase and β -galactosidase

For reporter enzyme assays, cells were seeded in 24-well plates in hormone-depleted medium and incubated overnight prior to transfection. COS-1 cells were seeded at approximately 50,000 cells per well. 22Rv1 cells were seeded to achieve optimal confluency for transfection.

2.2.4.2 Calcium phosphate transfection in COS-1 cells

Transfections in COS-1 cells were carried out using the calcium phosphate method. Cells were co-transfected with pSV-AR, BOS β -galactosidase plasmid for normalisation, and the appropriate luciferase reporter construct. For ARE SNP reporter assays, cells were transfected with either *MAF* or *CXCR1* wild-type or mutant androgen response element (ARE) reporter constructs. Each well received 100 ng pSV-AR, 100 ng BOS β -galactosidase plasmid, and 1 μ g reporter plasmid.

For RSK reporter assays, COS-1 cells were co-transfected with pSV-AR, BOS β -galactosidase plasmid, and either ARE-LUC, PB-PROM, or TAT-GRE-E1B-LUC reporter constructs together with PSG5-empty vector or RSK1, RSK2, RSK3, or RSK4 expression plasmids. Each well received approximately 50 ng pSV-AR, 100 ng BOS β -galactosidase plasmid, 1 μ g reporter construct, and 200 ng of the appropriate empty vector or RSK expression plasmid.

For all calcium phosphate transfections, DNA mixtures were prepared in sterile water, combined with CaCl_2 and 2X BES-buffered saline (BBS), vortexed briefly, and incubated at room temperature for 15 min to allow precipitate formation before being added dropwise to the cells. Following transfection, cells were treated with ethanol vehicle control or 1 nM mibolerone prior to luciferase and β -galactosidase assays. Luciferase activity was normalised to β -galactosidase activity to control for differences in transfection efficiency.

2.2.4.3 FuGENE transfection in 22Rv1 cells

For reporter assays in 22Rv1 cells, transfections were performed using FuGENE HD in Opti-MEM. Cells were transfected with TAT-GRE-E1B-LUC reporter plasmid, BOS β -galactosidase plasmid, and either pcDNA3.1 empty vector or RSK1, RSK2, RSK3, or RSK4 expression constructs. As 22Rv1 cells express endogenous androgen receptor, pSV-AR was not included.

A master DNA mix was prepared containing 12.5 μ g TAT-GRE-E1B-LUC and 1.25 μ g BOS in Opti-MEM. The mixture was vortexed, briefly centrifuged, and divided equally into five tubes corresponding to each condition. To each tube, 2.25 μ g of the respective expression plasmid (empty vector or RSK1-4) was added. FuGENE HD reagent was then added at an approximate ratio of 3:1 (μ L reagent: μ g DNA), mixed gently, and incubated at room temperature for 15 min. Transfection complexes were added to cells at 50 μ L per well.

2.2.4.4 Hormone treatment

Mibolerone stock solution was prepared in ethanol and diluted in hormone-depleted medium to the required final concentrations. For *MAF* and *CXCR1*-associated reporter

assays, cells were treated with ethanol vehicle control, 0.1 nM mibolerone, or 1 nM mibolerone. For RSK overexpression experiments, cells were treated with ethanol vehicle control or 1 nM mibolerone only. Ethanol was included as a vehicle control at an equivalent final concentration in all experiments

2.2.4.5 Cell lysis and reporter assay

Following treatment, culture medium was removed and cells were washed twice with 1X PBS. Cells were lysed using 60 μ L of 1X Promega Reporter Lysis Buffer per well. Plates were incubated at -80 °C for 15 min, followed by thawing, and lysates were kept on ice prior to analysis. For luciferase assays, 20 μ L of lysate was transferred into a 96-well plate and mixed with 20 μ L luciferase reagent. The plate was covered with foil and incubated on a shaker at room temperature for 15 min before luminescence was measured using a CLARIOstar plate reader. Luciferase activity was normalised to β -galactosidase activity.

For β -galactosidase assays, 5 μ L of lysate was transferred into a 96-well plate and mixed with substrate solution. The plate was incubated on a shaker at room temperature for 1 h, followed by addition of accelerator solution and a further 15 min incubation before measurement.

2.2.5 Western blotting

2.2.5.1 Cell harvesting and lysis

Cells were first washed twice with ice-cold 1X PBS to remove any remaining culture medium. The cells were then scraped, collected into microcentrifuge tubes, and centrifuged at approximately 13,000 g for 1 min at 4 °C. The supernatant was discarded, and the cell pellet was resuspended in the appropriate lysis buffer containing protease inhibitor cocktail.

The lysates were sonicated using a Bioruptor Plus (Diagenode) for four cycles of 30 s ON and 30 s OFF at high power. Following sonication, the samples were centrifuged again at approximately 13,000 g for 10 min at 4 °C, and the supernatant containing the protein lysate was transferred into a fresh pre-cooled microcentrifuge tube.

2.2.5.2 Protein quantification

Protein concentration was determined using bovine serum albumin (BSA) standards. A standard curve was generated using serial dilutions, and protein concentration of samples was calculated based on absorbance measurements obtained using a NanoDrop spectrophotometer. Sample volumes were adjusted to ensure equal protein loading.

2.2.5.3 SDS-PAGE

Protein lysates were mixed with 4X Laemmli sample buffer and heated at 95 °C for 5 min to denature the proteins. The samples were then briefly cooled on ice and mixed before loading. A volume of 25 μ L of each sample was loaded into wells of a 10% polyacrylamide gel. 5 μ L of pre-stained protein ladder was loaded into a separate well for molecular weight

reference. Electrophoresis was carried out in 1X running buffer at 120 V and 100 mA for approximately 2 h 30 min.

2.2.5.4 Protein transfer and immunoblotting

Following electrophoresis, proteins were transferred onto a PVDF membrane which had been pre-activated in 100% methanol. Protein transfer was carried out using a semi-dry transfer system. After transfer, the membrane was blocked for 1 h at room temperature in blocking solution to reduce non-specific binding. The membrane was then incubated with the relevant primary antibody overnight at 4 °C with gentle rocking.

The following day, membranes were washed three times with PBS-T for 10 min each and then incubated with the appropriate secondary antibody for 1 h at room temperature. Following secondary antibody incubation, membranes were washed three times with PBS-T for 5 min each, followed by a final wash with PBS. Protein bands were visualised using the Odyssey LI-COR imaging system. Primary and secondary antibodies used for Western blotting are listed in Table 2.18.

2.2.6 Cell growth assay following RSK overexpression and androgen treatment

To examine the effect of RSK isoform overexpression on 22Rv1 cell growth, a fluorescence-based proliferation assay was performed in 96-well plates. 22Rv1 cells were seeded in hormone-depleted RPMI-1640 medium supplemented with charcoal-stripped foetal bovine serum and transfected with red fluorescent protein (RFP)-tagged constructs, including pcDNA3.1-RFP-Empty (control), RSK1, RSK2, RSK3, or RSK4.

For each well, transfection complexes were prepared using 50 ng plasmid DNA together with FuGENE in Opti-MEM and incubated for 15 minutes before addition to the cells. Following transfection, cells were treated with either ethanol (EtOH) vehicle or 1 nM mibolerone (MIB). Cell growth was monitored over time by quantifying the number of RFP-positive cells at multiple time points. Mean RFP-positive cell counts were calculated for each condition and plotted as growth curves with error bars representing variation between replicate wells.

2.2.7 Fluorescence-based cell proliferation assay for circRNA overexpression

To assess the effect of circRNA overexpression on prostate cancer cell proliferation, a fluorescence-based live-cell imaging assay was performed using 22Rv1 cells. Cells were seeded in 96-well plates and transfected with pcDNA3.1 constructs expressing hsa_circ_0010794 or hsa_circ_0064977. An RFP expression plasmid (pcDNA3.1-RFP-Empty) was co-transfected to enable visualisation of transfected cells. Control wells were transfected with empty vector. Transfection complexes were prepared using FuGENE HD in

Opti-MEM and added to cells according to the manufacturer's instructions. Following transfection, cells were maintained in complete RPMI medium.

Cell proliferation was monitored using the Incucyte live-cell imaging system, with images acquired at regular time intervals. The number of RFP-positive cells was quantified over time to assess cell growth. To account for differences in transfection efficiency, cell counts were normalised to the initial time point (baseline normalisation). Growth curves were generated to compare proliferation across conditions.

2.2.8 GST-AR DBD expression and purification

2.2.8.1 Bacterial expression optimisation

The glutathione S-transferase (GST)-tagged androgen receptor DNA-binding domain (GST-AR DBD) construct was transformed into BL21 competent *E. coli* cells for recombinant protein expression. A single transformed colony was inoculated into LB medium supplemented with ampicillin and incubated overnight at 37 °C with shaking. The overnight culture was then inoculated into fresh LB medium and grown at 37 °C until an optical density at 600 nm (OD_{600}) of approximately 0.6 was reached.

Protein expression was optimised by testing different induction conditions, including IPTG concentrations ranging from 0.05 to 1.0 mM, incubation temperatures of 20 °C and 37 °C, and induction periods between 2 and 18 h. In selected experiments, $ZnCl_2$ (10-100 μ M) was added simultaneously with IPTG to assess its effect on protein expression and solubility. Expression was further assessed using LB broth and autoinduction medium (AIM). Cell lysis conditions were evaluated using PBS alone or PBS supplemented with 0.5 mM DTT. Cell disruption was carried out using EmulsiFlex homogenisation and sonication, followed by centrifugation to separate soluble and insoluble protein fractions for SDS-PAGE analysis.

Based on optimisation experiments, comparison with previously reported methods (Lee et al, 2024), and group discussion, the final conditions selected for large-scale GST-AR DBD expression were induction at OD_{600} approximately 0.6 with 0.2 mM IPTG and 10 μ M $ZnCl_2$, followed by incubation at 20 °C for 18 h with shaking at 180 rpm.

2.2.8.2 Large-scale bacterial expression and cell lysis

For large-scale protein expression, an overnight starter culture of transformed BL21 cells was used to inoculate fresh LB medium supplemented with ampicillin. Cultures were grown until OD_{600} approximately 0.6, at which point a 1 mL uninduced sample was collected. Protein expression was induced by addition of 0.2 mM IPTG together with 10 μ M $ZnCl_2$, and the culture was incubated at 20 °C for 18 h with shaking at 180 rpm.

Following induction, cells were harvested by centrifugation at 4 °C and the supernatant was discarded. The cell pellet was resuspended in PBS containing protease inhibitor cocktail and incubated at room temperature for 30 min. Samples were collected at key stages to monitor

protein solubility and distribution. Cell lysis was then performed using an EmulsiFlex homogeniser for three cycles, with the first cycle carried out at 500 psi and the second and third cycles at 10,000 psi. The lysate was subsequently sonicated for 10 cycles using 40 s ON and 20 s OFF settings. All samples were centrifuged at 4 °C to separate soluble and insoluble fractions, which were retained for SDS-PAGE analysis.

2.2.8.3 Purification of GST-AR DBD using GST affinity chromatography

The soluble lysate fraction containing GST-AR DBD was purified using a GST affinity column. Prior to sample loading, the column was equilibrated with PBS. The clarified lysate was then passed through the column, and the unbound material was collected as the flow-through fraction. The flow-through was reapplied to the column to improve binding efficiency. The column was subsequently washed with PBS to remove non-specifically bound proteins.

The fusion protein was eluted using reduced glutathione prepared in PBS. Elution fractions were collected sequentially in 1 mL volumes, and approximately eight to nine individual fractions were obtained. Flow-through, wash, and elution fractions were retained for SDS-PAGE analysis. Fractions were analysed to assess the presence and enrichment of GST-AR DBD following affinity purification.

2.2.8.4 SDS-PAGE and Coomassie staining of protein fractions

Protein samples and purification fractions were mixed with 4X Laemmli sample buffer and heated at 95 °C for 5 min prior to electrophoresis. Samples were separated using 12% SDS-PAGE gels at 120 V for approximately 1 h 20 min, until the dye front reached the bottom of the gel. Following electrophoresis, gels were stained using Coomassie Blue staining solution for 1 h and destained for approximately 30 min, followed by additional washes with water until protein bands were clearly visible. Protein bands were visualised using the Odyssey LI-COR imaging system. Fractions containing GST-AR DBD were identified based on the presence of a band at the expected molecular weight of approximately 38 kDa, allowing assessment of protein expression and elution efficiency.

2.2.9 Statistical analysis

Data were analysed using Microsoft Excel or GraphPad Prism (version 10). Luciferase activity was normalised to β -galactosidase activity, and IncuCyte fluorescence data were normalised to baseline (time 0). Mean values $\pm$ SEM (standard error of the mean) were calculated from independent replicates. Statistical significance was determined using an unpaired two-tailed Student's t-test or two-way ANOVA, with $p < 0.05$ considered significant.

Chapter 3

Results

3.1 Identification of Dysregulated circRNAs in Prostate and Oral Cancer

Circular RNAs (circRNAs) are increasingly recognised as regulators of gene expression in cancer, particularly at the post-transcriptional level (Patop et al, 2019). Dysregulated circRNA expression has been reported in both oral squamous cell carcinoma (OSCC) and prostate cancer, suggesting their involvement in tumour-associated molecular processes.

Based on this, a bioinformatics approach was undertaken to identify significantly dysregulated circRNAs in publicly available RNA sequencing datasets from OSCC and prostate cancer and to investigate their potential circRNA-miRNA-mRNA regulatory interactions, with the aim of prioritising biologically relevant candidates for further study.

3.1.1 Identification of Dysregulated circRNAs from RNA-seq Datasets

To identify circular RNAs associated with oral squamous cell carcinoma (OSCC) and prostate cancer, differential expression analysis was performed using publicly available RNA-seq datasets obtained from the European Nucleotide Archive (ENA). Dataset PRJNA542863 was analysed for OSCC, while dataset PRJNA552058 was used for prostate cancer.

Principal component analysis (PCA) was performed to assess overall sample clustering based on circRNA expression profiles. In the OSCC dataset, tumour and normal samples formed distinct clusters, indicating clear differences between conditions (Figure 3.1). Similarly, prostate cancer samples showed overall separation between tumour and normal tissues, although partial overlap between a small number of samples was observed, suggesting biological heterogeneity or sample variability (Figure 3.2).

Differential expression analysis using Galaxy identified a large number of significantly dysregulated circRNAs in both datasets. In the OSCC dataset, approximately 2,800 circRNAs were identified as upregulated and around 1,000 as downregulated under the selected thresholds of $|\log_2 \text{fold change}| \geq 1$ and adjusted $p \leq 0.05$ (Figure 3.3). In the prostate cancer dataset, 713 circRNAs were upregulated and 397 were downregulated under the same criteria (Figure 3.4). The expression profiles of the most strongly dysregulated circRNAs across tumour and control samples were further visualised by heatmap analysis, which showed clear clustering between disease and control groups (Figure 3.5 and 3.6). This separation suggests that circRNA expression profiles may distinguish tumour from control samples.

These findings demonstrate widespread circRNA dysregulation in both OSCC and prostate cancer and provide candidate circRNAs for further regulatory analysis. Based on the

magnitude of dysregulation and separation between tumour and control samples, selected candidate circRNAs were prioritised for downstream investigation of their potential post-transcriptional regulatory roles.

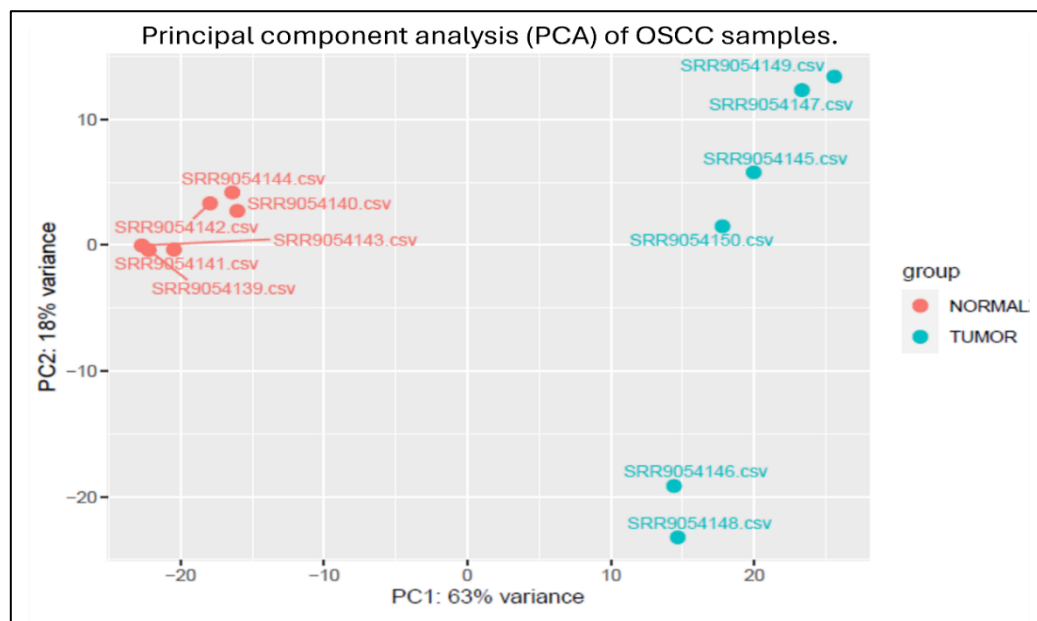


Figure 3.1. Principal Component Analysis (PCA) of OSCC Samples. Tumour and normal samples formed distinct clusters based on circRNA expression profiles. PC1 and PC2 explained 63% and 18% of the total variance, respectively.

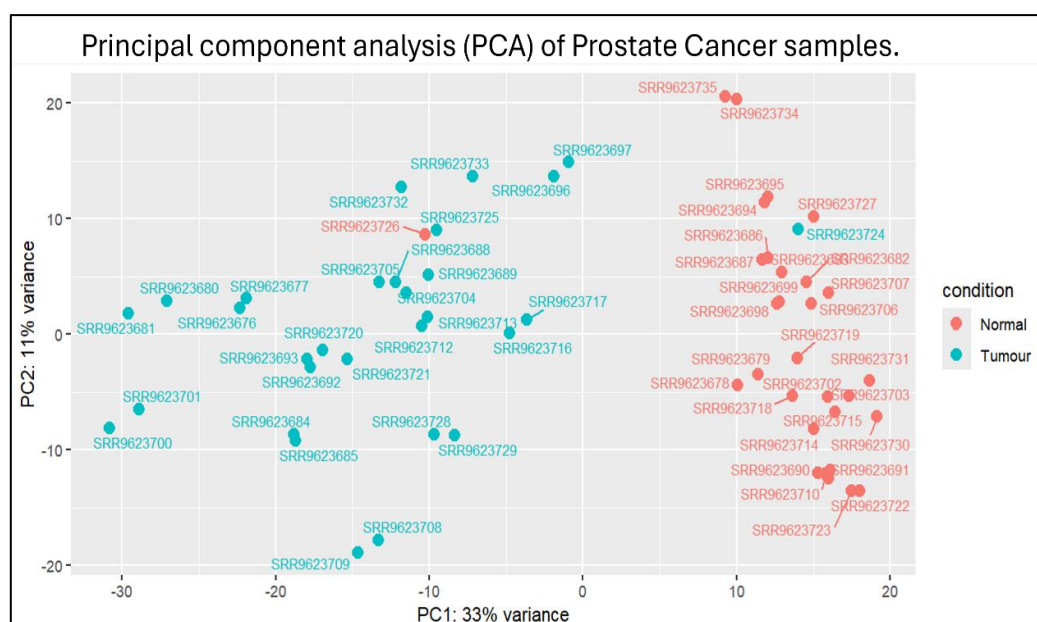


Figure 3.2. Principal component analysis (PCA) of prostate cancer samples. Tumour and normal samples showed overall separation based on circRNA expression profiles, although partial overlap between a small number of samples was observed. PC1 and PC2 explained 33% and 11% of the total variance, respectively.

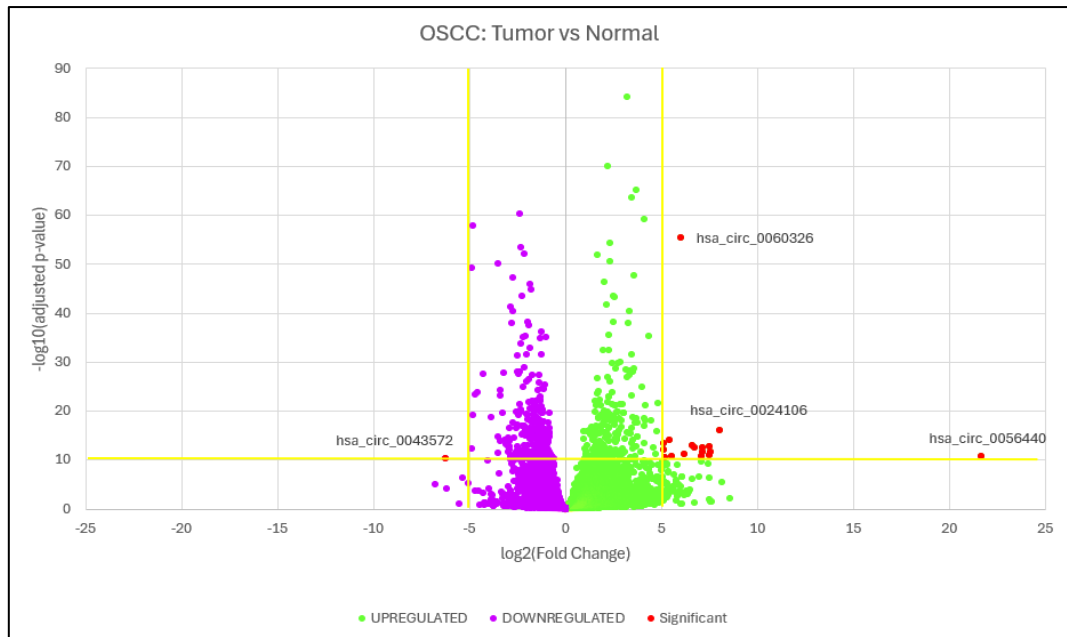


Figure 3.3. Differential expression analysis identifies dysregulated circRNAs in OSCC. Volcano plot showing significantly upregulated and downregulated circRNAs identified from OSCC RNA-seq dataset PRJNA542863 using $|\log_2 \text{fold change}| \geq 1$ and adjusted $p \leq 0.05$.

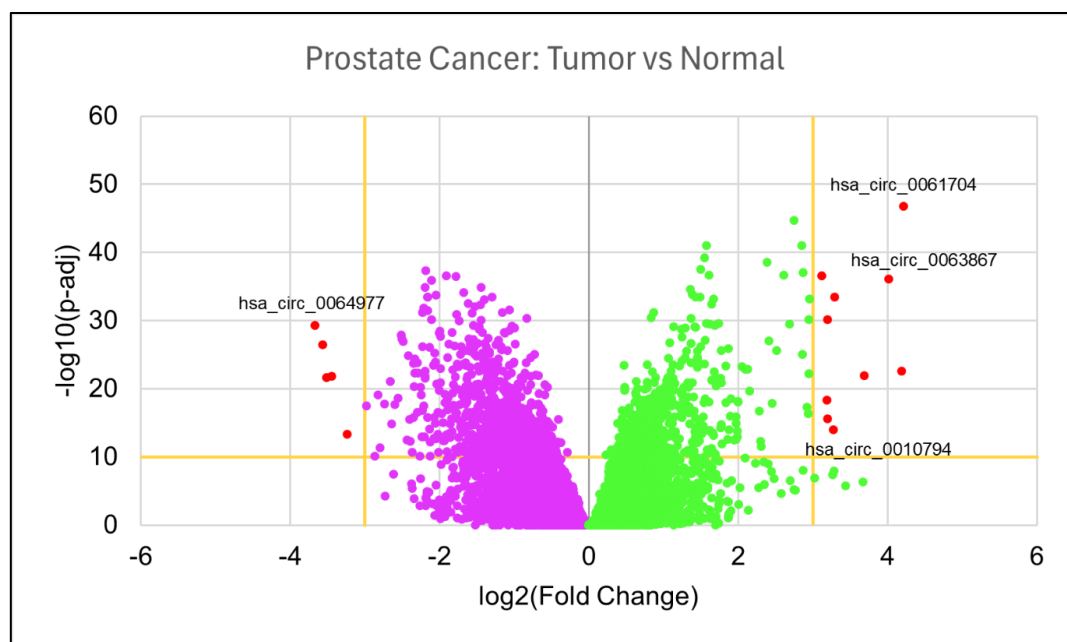


Figure 3.4. Differential expression analysis identifies dysregulated circRNAs in prostate cancer. Volcano plot showing significantly upregulated and downregulated circRNAs identified from prostate cancer RNA-seq dataset PRJNA552058. Differentially expressed circRNAs were selected using thresholds of $|\log_2 \text{fold change}| \geq 1$ and adjusted $p \leq 0.05$.

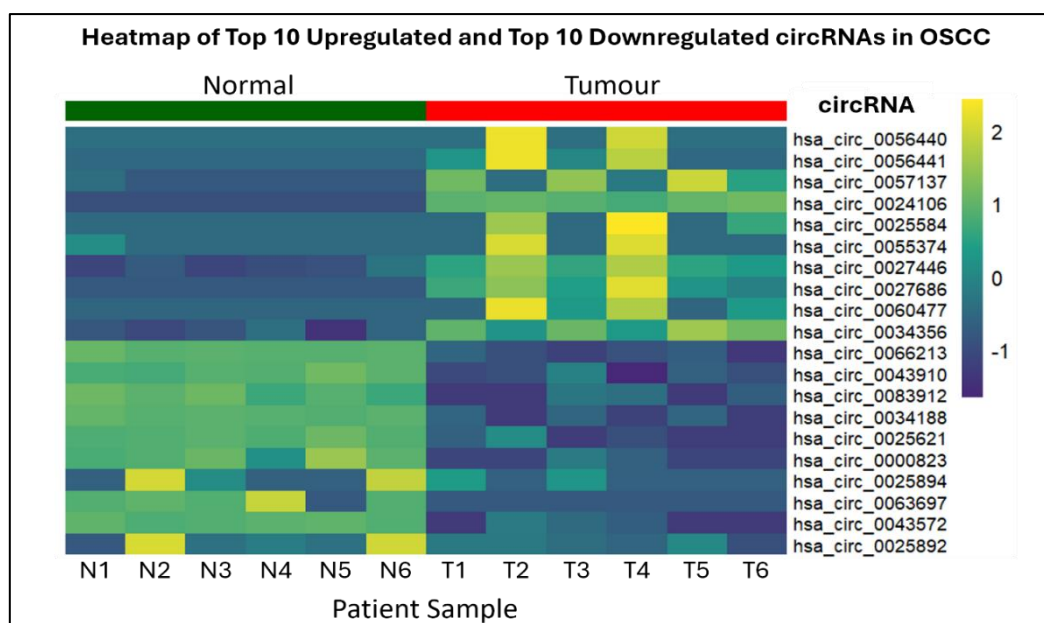


Figure 3.5. Top 10 upregulated and top 10 downregulated circRNAs distinguish tumour and control samples in OSCC. Heatmap showing the expression profiles of the top 10 upregulated and top 10 downregulated circRNAs across tumour and control samples in the OSCC dataset. Rows represent individual circRNAs and columns represent individual samples. Expression values are displayed as row-wise z-scores.

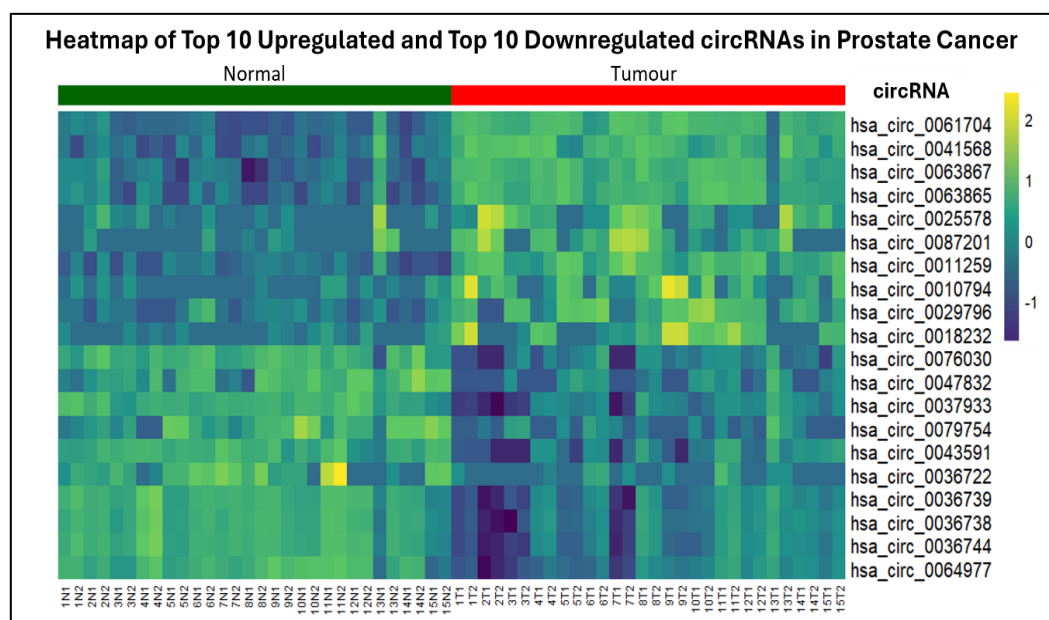


Figure 3.6. Top 10 upregulated and top 10 downregulated circRNAs distinguish tumour and control samples in prostate cancer Heatmap showing the expression profiles of the top 10 upregulated and top 10 downregulated circRNAs across tumour and control samples in the prostate cancer dataset. Rows represent individual circRNAs and columns represent individual samples. Expression values are displayed as row-wise z-scores.

3.1.2 circRNA-miRNA-mRNA Regulatory Interaction Analysis in Oral Cancer

To investigate the potential regulatory role of dysregulated circRNAs in OSCC, highly differentially expressed candidates were selected for downstream circRNA-miRNA-mRNA interaction analysis. One of the most strongly upregulated circRNAs identified was hsa_circ_0056440. Regulatory network analysis using a microRNA target prediction software (SeedVicious) indicated that this circRNA may interact with several miRNAs, including: hsa-miR-150-5p, hsa-miR-138-5p, hsa-miR-370-3p and hsa-miR-222-3p. Experimentally validated downstream targets identified through miRTarBase included genes associated with epithelial-mesenchymal transition, apoptosis and tumour progression (Ma et al, 2022; Ma et al, 2021). These included *ZEB1* (Zinc Finger E-box Binding Homeobox 1), *VIM* (Vimentin), *FOXM1* (Forkhead Box M1), *BBC3* (BCL2 Binding Component 3) and *ESR1*.

The observed expression pattern was consistent with a potential miRNA sponging mechanism, whereby upregulation of hsa_circ_0056440 may reduce the availability of interacting miRNAs and consequently contribute to increased expression of downstream target genes associated with OSCC progression (Figure 3.7). Taken together, these findings suggest that hsa_circ_0056440 may contribute to OSCC progression through post-transcriptional regulatory mechanisms.

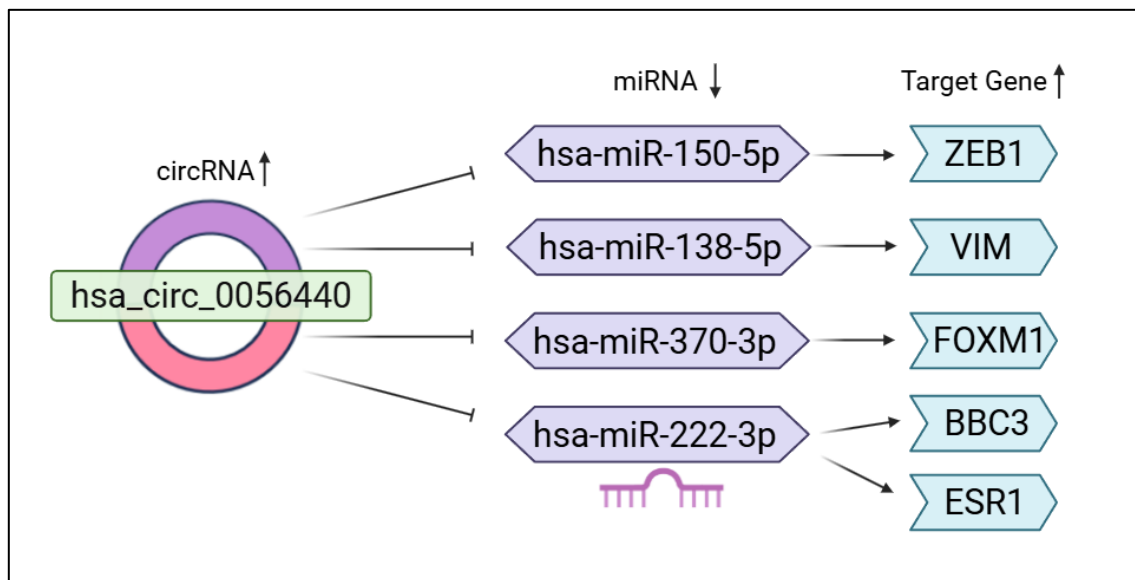


Figure 3.7. Predicted regulatory network of hsa_circ_0056440 in OSCC. Predicted circRNA-miRNA-mRNA interaction network showing potential regulatory interactions between hsa_circ_0056440, associated miRNAs, and downstream target genes implicated in OSCC progression.

3.1.3 circRNA-miRNA-mRNA Regulatory Interactions in Prostate Cancer

To explore potential regulatory mechanisms in prostate cancer, significantly dysregulated circRNAs were analysed for predicted miRNA interactions and downstream target genes. One of the most significantly downregulated circRNAs identified was hsa_circ_0064977. Predicted miRNA interactions identified using SeedVicious included hsa-miR-508-3p and hsa-miR-488-3p, with experimentally validated targets from miRTarBase including *NR4A3* (*Nuclear Receptor Subfamily 4 Group A Member 3*) and *POMC* (*Proopiomelanocortin*), respectively.

A second significantly dysregulated circRNA, hsa_circ_0010794, was found to be upregulated and showed predicted interactions with hsa-miR-138-5p and hsa-miR-1908-5p. Downstream targets included *TERT* (*Telomerase Reverse Transcriptase*) and *EXO1* (*Exonuclease 1*), which are involved in telomerase activation, tumour progression and poor prognosis in prostate cancer (Graham & Meeker, 2017; Luo et al, 2019a). These findings suggest that dysregulated circRNAs may contribute to altered post-transcriptional regulatory networks in prostate cancer (Figure 3.8).

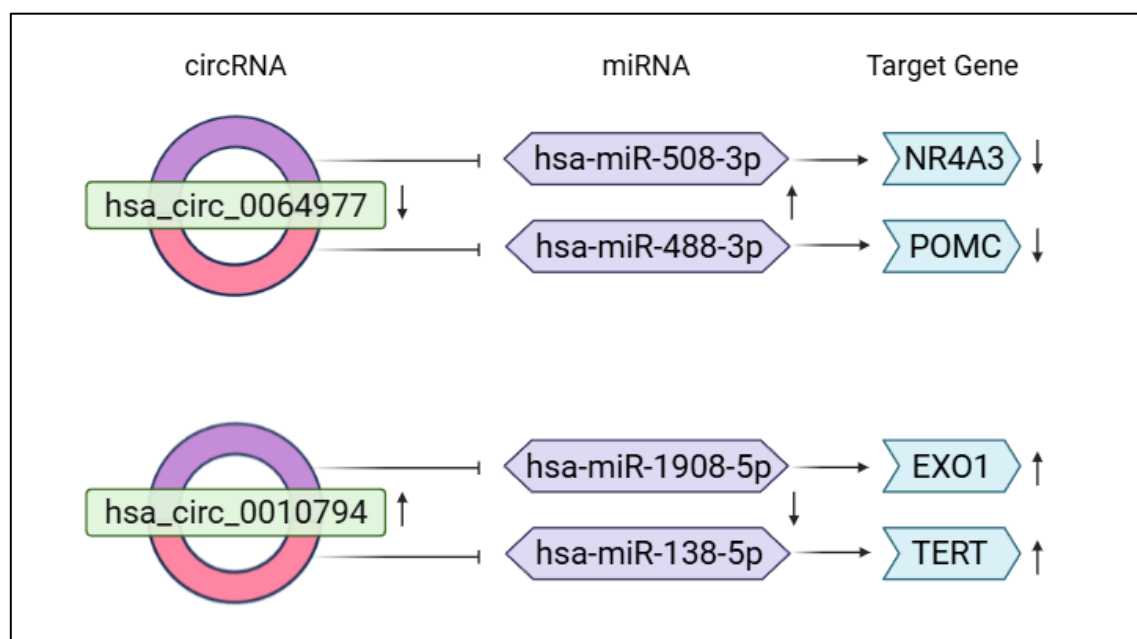


Figure 3.8. Predicted circRNA-mediated regulatory network in prostate cancer. Predicted circRNA-miRNA-mRNA regulatory network involving hsa_circ_0064977 and hsa_circ_0010794, associated miRNAs, and downstream target genes implicated in prostate cancer progression.

3.1.4 Functional Validation of Candidate circRNAs in 22Rv1 Cells

To functionally validate the computational findings, candidate circRNAs identified in the prostate cancer dataset were selected for overexpression analysis in 22Rv1 cells.

hsa_circ_0010794, which was found to be upregulated in the computational analysis, and hsa_circ_0064977, which was downregulated, were transiently overexpressed and cell proliferation was monitored over a 144-hour time course.

Overexpression of hsa_circ_0010794 resulted in a significant increase in cell proliferation compared to the pcDNA3.1 empty vector control, with differences becoming significant from 32 hours onwards and maintained across later time points. In contrast, hsa_circ_0064977 showed a trend towards reduced proliferation; however, this effect was not statistically significant at any time point (Figure 3.9).

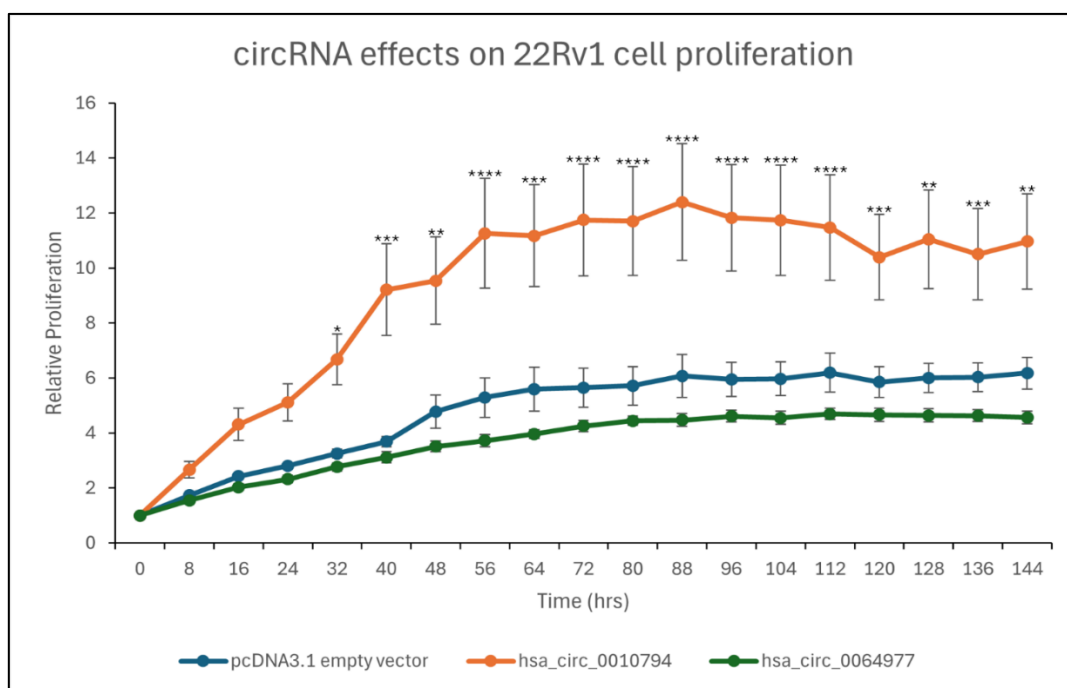


Figure 3.9. hsa_circ_0010794 enhances cell proliferation, while hsa_circ_0064977 shows a reduced proliferation trend in 22Rv1 cells. 22Rv1 cells were transfected with pcDNA3.1 empty vector control, hsa_circ_0010794 or hsa_circ_0064977 expression constructs together with an RFP reporter. Cell proliferation was monitored over time by quantifying RFP-positive cells using live-cell fluorescence IncuCyte imaging. Fluorescence signal was normalised to baseline (time 0) and expressed relative to control. Data represent mean $\pm$ SEM from four independent replicates. Statistical significance was determined using two-way ANOVA with Dunnett's multiple comparisons test, comparing each circRNA to pcDNA3.1 empty vector at individual time points. hsa_circ_0010794 showed a significant increase in proliferation from 32 hours onwards, whereas hsa_circ_0064977 showed no significant effect. Two-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Overall, this analysis identified widespread circRNA dysregulation in OSCC and prostate cancer, with distinct expression patterns separating tumour and control samples. Predicted circRNA-miRNA-mRNA networks highlighted potential regulatory interactions involving key cancer-associated genes. Functional validation in 22Rv1 cells further supported the relevance of selected prostate cancer-associated circRNAs, linking computational predictions to observable cellular effects. Following the identification of potential post-transcriptional regulatory mechanisms in cancer, the study next examined a different level of gene regulation involving AR signalling. More specifically, the effect of single nucleotide polymorphisms within androgen response element (ARE) sequences on receptor activity was investigated.

3.2 Functional Characterisation of Androgen Response Elements and Androgen Receptor DNA Binding

Androgen receptor (AR) signalling regulates gene expression through binding to androgen response elements (AREs), and variation within these regulatory sequences can influence transcriptional activity (Messner et al, 2020; Stelloo et al, 2019).

Recent analysis from the Brooke-Marco group identified several single nucleotide polymorphisms (SNPs) located within predicted AREs (unpublished data) that may alter an individual's risk of developing the disease. However, the functional consequences of these variants on AR-mediated transcription remain unclear.

To address this, luciferase reporter assays were used to compare the transcriptional activity of wild-type and SNP-containing ARE sequences following androgen stimulation. In parallel, the AR DNA-binding domain (DBD) was expressed and purified as a GST-tagged protein to support future mechanistic studies investigating AR-DNA interactions.

3.2.1 Functional Analysis of Regulatory SNPs within Androgen Response Elements

Two SNPs predicted to be present within AREs were selected for functional studies: one present in the regulatory region of the oncogene *MAF* and the other in *CXCR1* (a GPCR linked to prostate cancer progression). The *MAF* variant shows a population-specific distribution, with an allele frequency of 15% in European populations, 2% in African populations, and an absence in Asian populations.

To assess their functional significance, the corresponding ARE sequences were cloned into the pGL4.1 luciferase reporter plasmid, with and without the SNP variant. Reporter constructs were co-transfected with the androgen receptor expression plasmid pSV-AR into

COS-1 cells. Cells were treated with the synthetic androgen mibolerone at concentrations of 0.1 nM and 1 nM to activate AR signalling, and reporter assays were performed.

As a positive control, the TAT-GRE-E1B-LUC androgen-responsive reporter was used. As expected, this reporter was activated in an androgen-dependent manner (Figure 3.10A). For the wild-type MAF construct, luciferase activity also significantly increased following androgen stimulation (Figure 3.10B), demonstrating that this ARE is functional.

In contrast, the mutant MAF construct containing the SNP variant showed reduced reporter activity following androgen treatment (Figure 3.10C). The SNP therefore appears to disrupt the ARE, resulting in reduced androgen regulation. Reporter assays using the CXCR1-associated ARE constructs showed no consistent androgen-dependent response and displayed variability between replicates (Figure 3.11).

Western blot analysis was performed to confirm AR protein expression in the transfected COS-1 cells used for the reporter assays (Figure 3.10 and 3.11). Distinct bands corresponding to the expected molecular weight of AR were detected across treatment conditions (0, 0.1 and 1 nM mibolerone).

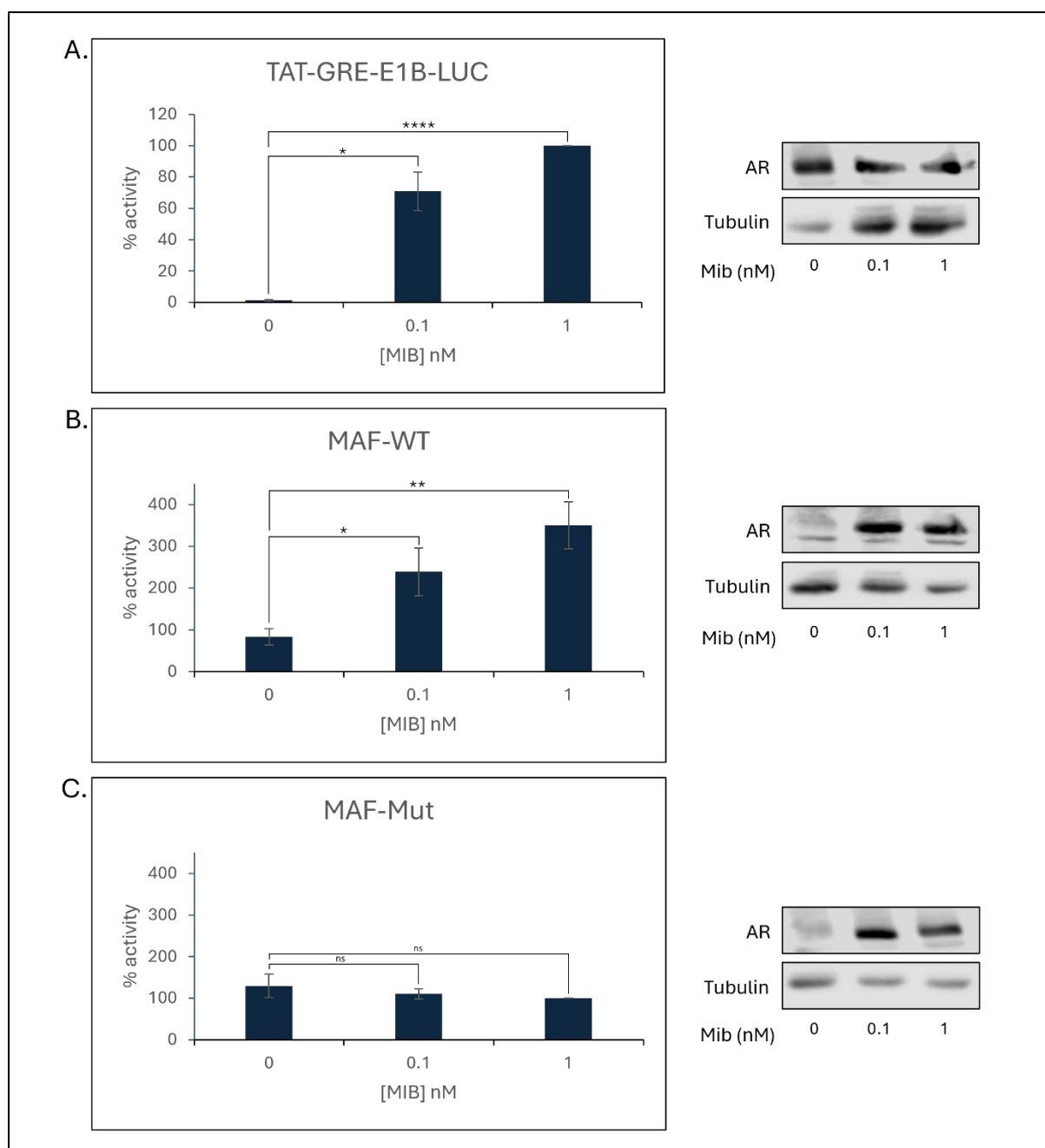


Figure 3.10. A SNP in *MAF* abolishes AR-mediated transcription. COS-1 cells were transfected with (A) TAT-GRE-E1B-LUC, (B) wild-type MAF ARE reporter, or (C) SNP-containing MAF ARE reporter, together with AR and β -galactosidase expression plasmids, followed by treatment with increasing concentrations of mibolerone. Luciferase activity was normalised to β -galactosidase and expressed relative to untreated control. Western blot analysis was performed to confirm androgen receptor (AR) expression under each treatment condition. Data represent the mean of four independent experiments $\pm$ SEM. Statistical significance was determined using a two-tailed Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, not significant).

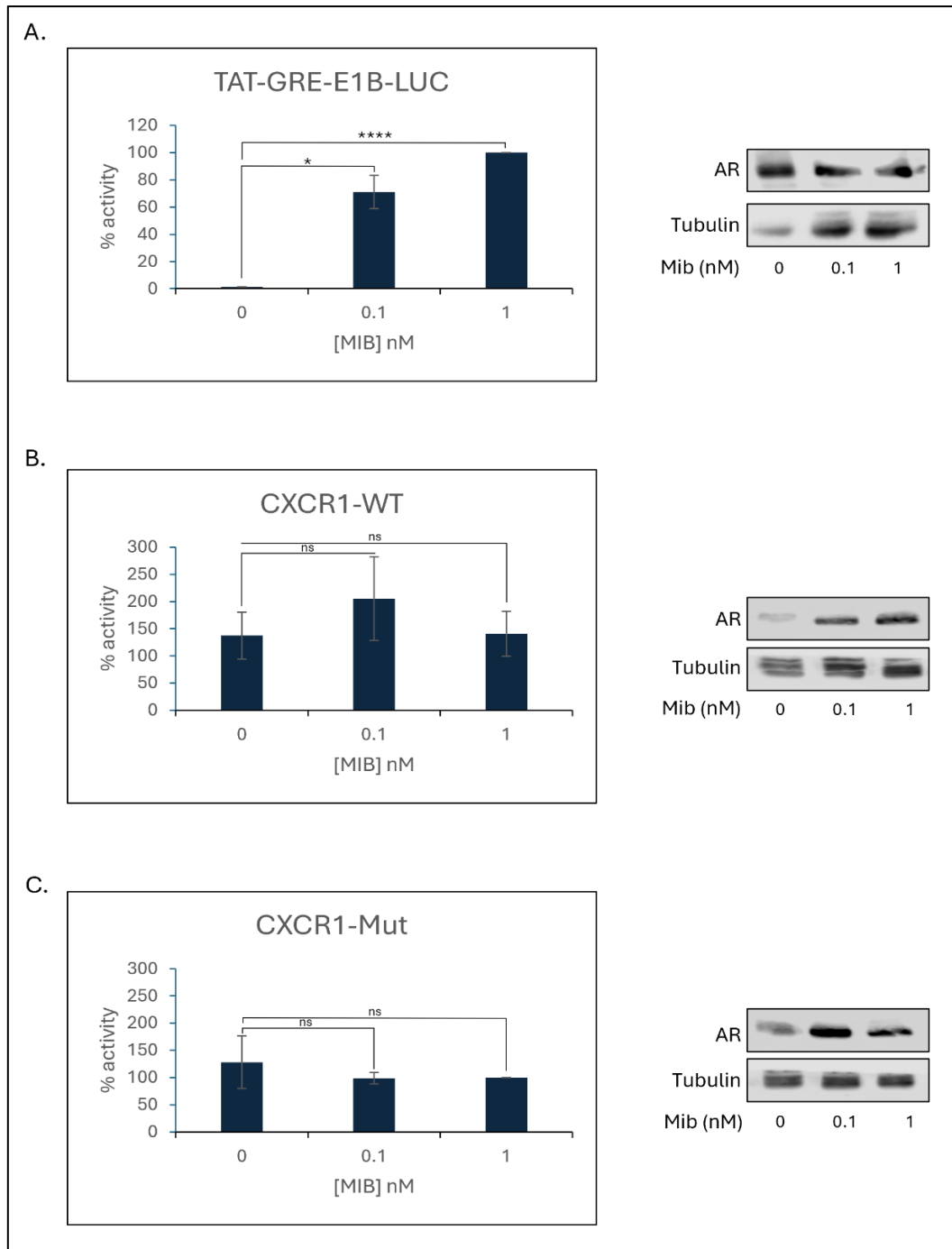


Figure 3.11. CXCR1-associated ARE does not show consistent AR regulation. COS-1 cells were transfected with (A) TAT-GRE-E1B-LUC (positive-control data shown in Figure 3.10A), (B) CXCR1 wild-type ARE reporter, or (C) CXCR1 SNP-containing ARE reporter, together with AR and β -galactosidase expression plasmids, followed by treatment with increasing concentrations of mibolerone. Luciferase activity was normalised to β -galactosidase and expressed relative to untreated control. Western blot analysis was performed to confirm androgen receptor (AR) expression under each treatment condition. Data represent the mean of four independent experiments $\pm$ SEM. Statistical significance was determined using a two-tailed Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, not significant).

3.2.2 Expression and purification of GST-tagged AR DNA-Binding Domain

To further investigate the potential effects of the regulatory SNPs on AR-DNA interactions, the AR DBD was expressed in *E. coli* BL21 cells as a GST-tagged fusion protein, and multiple conditions were assessed to optimise protein expression and solubility. Initial optimisation experiments examined the effect of IPTG concentration and ZnCl₂ supplementation on GST-AR DBD expression and solubility (Figure 3.12A and B). A protein band corresponding to the expected molecular weight of GST-AR DBD (~38 kDa) was detected under induced conditions. However, most of the protein was found in the insoluble pellet fraction, while only a smaller amount was detected in the soluble supernatant. Changing IPTG and ZnCl₂ concentrations did not significantly improve soluble protein recovery.

Expression was further assessed using LB broth and autoinduction medium (AIM) at 20 °C and 37 °C (Figure 3.12C). GST-AR DBD expression was detected under all conditions, with slightly improved recovery in the soluble fraction observed in autoinduction medium at 20 °C. However, the majority of the protein remained in the insoluble fraction.

To assess whether reducing conditions affect protein recovery, PBS alone and PBS supplemented with DTT were compared (Figure 3.12D). No clear difference in soluble protein recovery was observed between the conditions, although more protein appeared to be present in the pellet fraction following lysis in PBS alone. A shorter induction period of 2 hours was also tested (Figure 3.12E), but this resulted in weak GST-AR DBD expression and low soluble protein recovery.

Based on optimisation experiments, previously reported methods (Lee et al, 2024), and group discussion, final conditions were selected for larger-scale protein production. An overnight GST-AR DBD culture was added into 500 mL LB medium and grown at 37 °C until an OD₆₀₀ of approximately 0.6 was reached. Cultures were then supplemented with 10 µM ZnCl₂ and induced with 0.2 mM IPTG before incubation at 20 °C for 18 hours. Under these conditions, SDS-PAGE analysis showed a clear enrichment of a ~38 kDa band in induced samples compared with uninduced controls, confirming GST-AR DBD expression (Figure 3.13A).

Comparison of protein extraction before cell disruption, after Emulsiflex treatment, and following sonication showed no clear difference in GST-AR DBD recovery, suggesting that sonication did not noticeably improve protein extraction compared with Emulsiflex treatment alone (Figure 3.13B). Analysis of soluble and insoluble fractions confirmed that most GST-AR DBD remained in the pellet fraction, although some protein was also detected in the soluble fraction (Figure 3.13C).

During glutathione affinity purification, the lysate flow-through was passed through the column twice before PBS washing. SDS-PAGE analysis showed a strong ~38 kDa band in the flow-through fraction, indicating that some GST-AR DBD did not bind to the glutathione resin, while no detectable protein was observed in the PBS wash fractions (Figure 3.13D).

Elution with 10 mM reduced glutathione resulted in recovery of GST-AR DBD, with faint bands observed in elution 1 (E1) at approximately 38 kDa and 26 kDa, consistent with GST-AR DBD and free GST, respectively (Figure 3.13D). No clear bands were detected in the remaining elution fractions.

Following larger-scale purification, the strongest ~38 kDa band was observed in elution 1 (E1), with gradually weaker signal intensity across elutions 2-6 (Figure 3.13E). A lower molecular weight band of approximately 26 kDa, consistent with free GST, was also observed, particularly in the early elution fractions.

These findings confirmed successful expression and initial purification of GST-tagged AR DBD, although a large proportion of the protein remained insoluble.

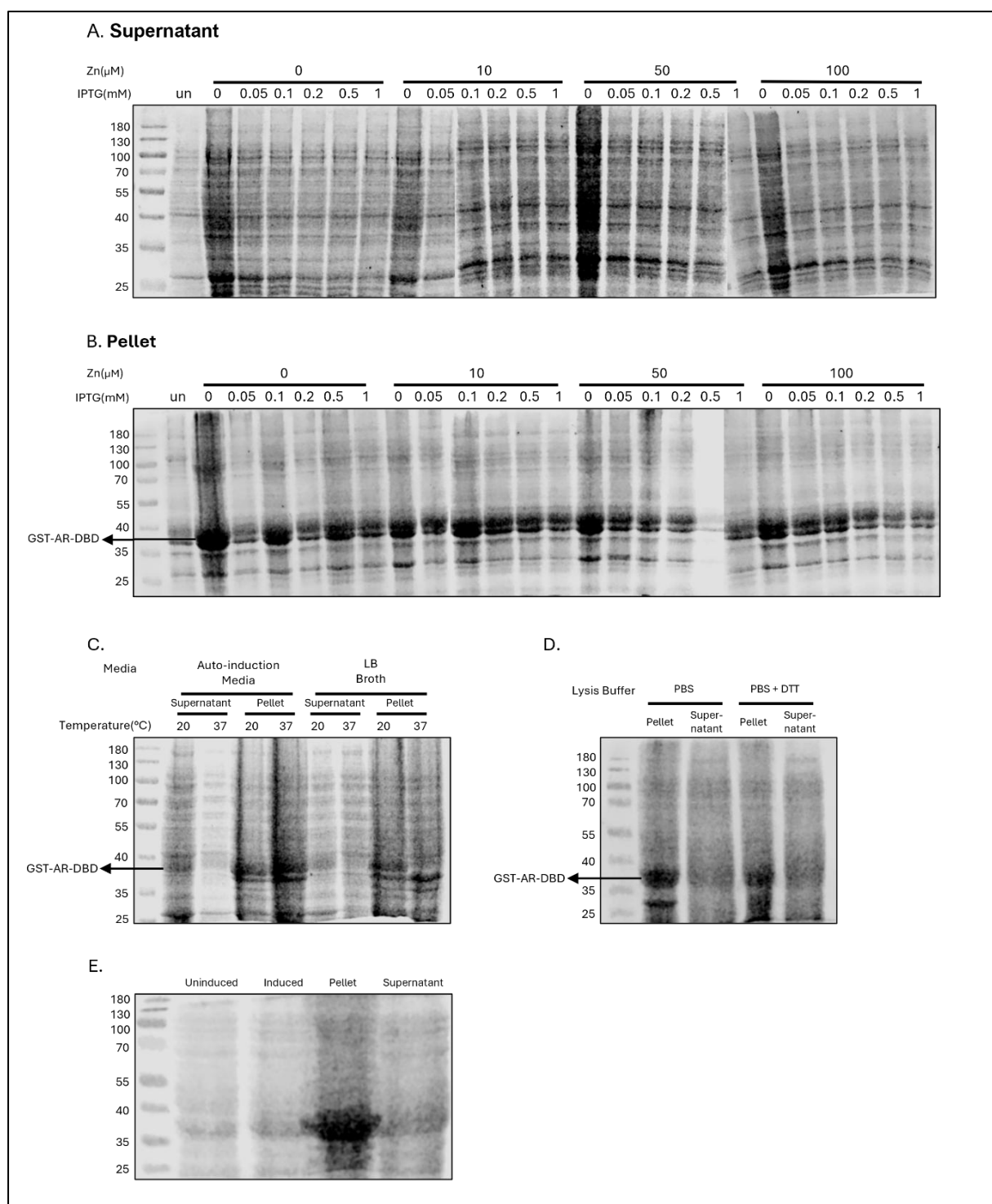


Figure 3.12. Optimisation of GST-AR DBD expression and solubility. SDS-PAGE analysis was performed to optimise GST-AR DBD expression and solubility under different induction and lysis conditions. (A) Soluble (supernatant) fractions from LB-grown cultures induced with varying concentrations of IPTG and ZnCl₂, (B) Corresponding insoluble (pellet) fractions showing accumulation of GST-AR DBD (~38 kDa), (C) Comparison of GST-AR DBD expression in LB and AIM under different temperature conditions, (D) Effect of different lysis buffers on GST-AR DBD solubility, and (E) GST-AR DBD expression after 2 hours of induction.

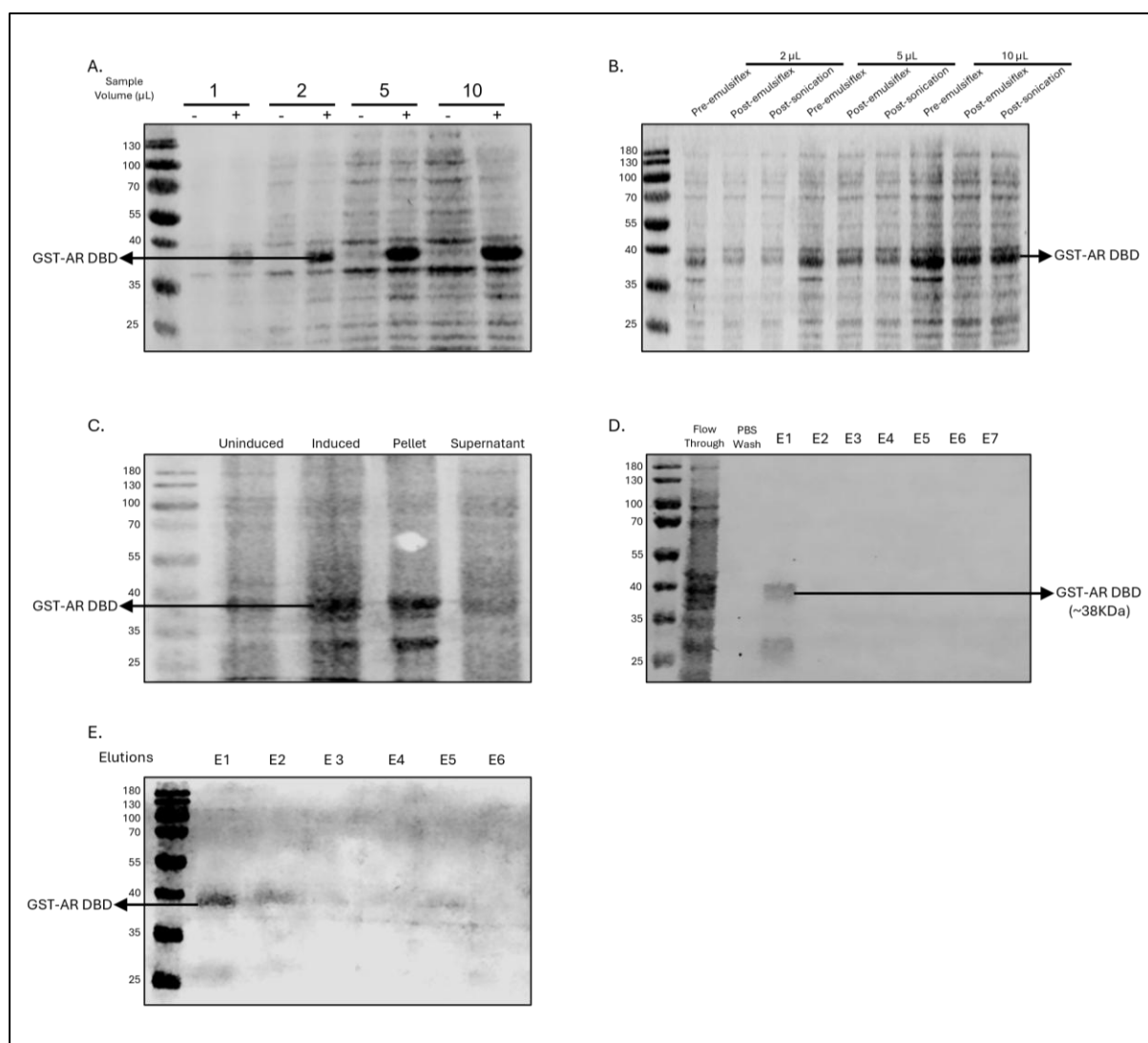


Figure 3.13. Expression, solubility and purification of GST-AR DBD. SDS-PAGE analysis of GST-AR DBD expression, solubility and purification in *E. coli* BL21 cells. (A) IPTG-induced expression of GST-AR DBD, showing a distinct band at ~38 kDa following induction, (B) Protein extraction efficiency following cell lysis under different conditions, (C) Distribution of GST-AR DBD between soluble and insoluble fractions, indicating predominant localisation in the insoluble fraction, (D) Protein fractions collected during glutathione affinity purification, including flow-through, wash and elution fractions, and (E) Elution profile showing enrichment of GST-AR DBD (~38 kDa), with stronger recovery in the first elution fraction and a lower molecular weight band consistent with free GST.

Overall, these findings demonstrate that sequence variation within AREs can significantly alter AR-mediated transcription, as shown by the disruption of the *MAF* ARE by a single nucleotide change. In contrast, *CXCR1*-associated AREs did not show consistent androgen responsiveness under the conditions tested, suggesting context-dependent regulatory activity. In parallel, successful expression and initial purification of the AR DNA-binding

domain provide a foundation for future studies investigating the molecular basis of AR-DNA interactions and the functional impact of regulatory variation.

3.3 Functional Characterisation of RSK-Androgen Receptor Crosstalk in Prostate Cancer

While the previous section examined AR regulation at the level of ARE sequence variation and DNA binding, AR activity can also be influenced by intracellular signalling pathways, including kinase-mediated regulation (Wen et al, 2020). One such family of potential regulators is the Ribosomal S6 Kinases (RSKs), which are downstream of MAPK/ERK signalling and have been implicated in transcriptional regulation of various transcription factors, including the androgen receptor, and cancer progression (Cronin et al, 2021).

This chapter aimed to investigate the functional relationship between RSK isoforms and AR signalling by assessing their effects on AR-mediated transcription and androgen-dependent cell proliferation. Luciferase reporter assays were used to evaluate transcriptional activity across different androgen-responsive promoters, while fluorescence-based growth assays were performed to examine the impact of RSK isoforms on prostate cancer cell proliferation under androgen-stimulated conditions.

3.3.1 Functional Analysis of RSK-Mediated Regulation of AR Transcriptional Activity

To investigate whether RSK isoforms influence AR-mediated transcriptional regulation, luciferase reporter assays were performed in COS-1 and 22Rv1 cells using androgen-responsive reporter constructs.

Initial validation experiments were carried out to confirm that reporter activation was AR dependent. Mibolerone induced strong reporter activity only in the presence of the pSV-AR expression plasmid, confirming androgen-dependent AR activation (Figure 3.14A). In the absence of pSV-AR, only minimal reporter activation was observed.

For the ARE-LUC reporter, strong androgen-induced transcriptional activation was observed in the empty vector control. Co-expression of all four RSK isoforms significantly reduced reporter activity to varying degrees, with RSK2 showing the greatest suppression followed by RSK1, RSK3 and RSK4 (Figure 3.14B).

Similarly, for the PB-PROM reporter, androgen-induced transcription was highest in the empty vector control. Among the RSK isoforms, RSK2 and RSK4 significantly reduced androgen-induced transcription compared to the empty vector control, while RSK1 and RSK3 did not reach significance (Figure 3.14C).

In contrast, for the TAT-GRE-E1B-LUC reporter, RSK isoforms enhanced androgen-responsive transcription compared to empty vector control, with only RSK4 showing a statistically significant increase in activity (Figure 3.14D).

Western blot analysis confirmed AR expression in pSV-AR-transfected cells, with higher expression observed following mibolerone treatment. No AR signal was detected in cells transfected without pSV-AR. RSK1 expression was confirmed in RSK1-transfected cells. The RSK2 antibody produced bands of similar intensity across all lanes, including empty vector controls, suggesting detection of endogenous RSK2 in COS-1 cells. As a result, overexpression of RSK2 could not be clearly distinguished from endogenous protein under these conditions. The RSK3 antibody appeared to mainly detect RSK1, likely due to sequence similarity between isoforms. No signal was detected using the RSK4 antibody. Equal protein loading was confirmed by α -tubulin immunoblotting.

Overall, these results indicate that the effect of RSK isoforms on AR transcriptional activity is promoter-dependent.

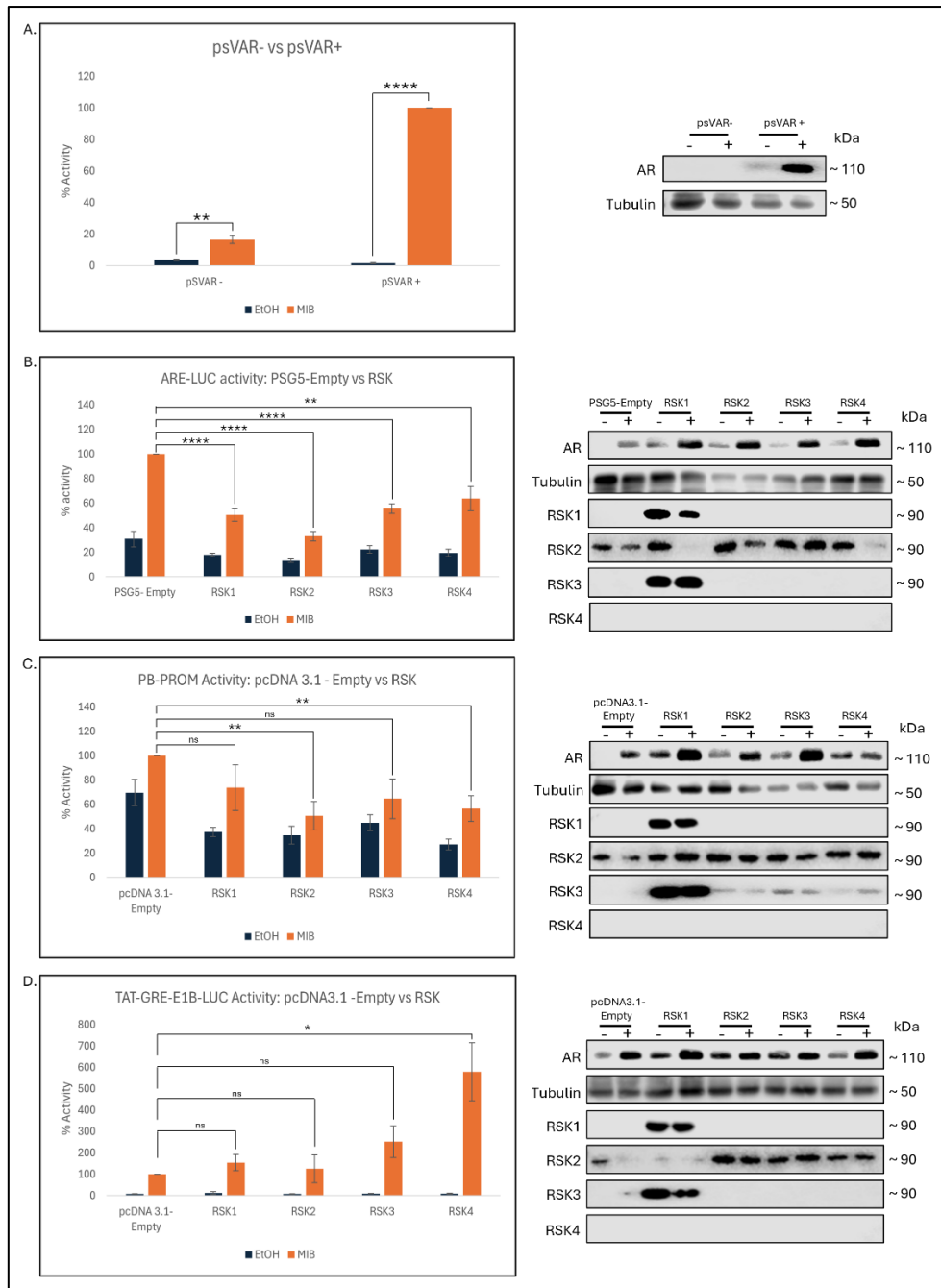


Figure 3.14. RSK isoforms differentially regulate AR-mediated transcription in a promoter-dependent manner. COS-1 cells were transfected with (A) TAT-GRE-E1B-LUC reporter under pSV-AR- and pSV-AR+ conditions, (B) ARE-LUC, (C) PB-PROM or (D) TAT-GRE-E1B-LUC constructs, together with empty vector or RSK1, RSK2, RSK3 or RSK4 expression plasmids, followed by treatment with ethanol (EtOH) or mibolerone (MIB). Androgen-responsive transcription varied across reporters, with RSK isoforms showing differential effects depending on promoter context. Western blot analysis was performed to confirm expression of RSK isoforms, androgen receptor (AR) and α -tubulin. Data represent the mean of four independent experiments $\pm$ SEM. Statistical significance was determined using a two-tailed Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, not significant).

3.3.2 RSK Isoform Effects in 22Rv1 Prostate Cancer Cells

To assess whether the RSK-mediated effects observed in COS-1 cells were also present in a prostate cancer-relevant model, reporter assays were performed in 22Rv1 cells using the TAT-GRE-E1B-LUC construct. Unlike COS-1 cells, 22Rv1 cells express endogenous AR and were therefore not transfected with the pSV-AR expression plasmid.

Mibolerone treatment produced a clear increase in reporter activity across all transfection conditions, confirming that 22Rv1 cells retain androgen-responsive AR signalling under these experimental conditions (Figure 3.15). However, overexpression of RSK1, RSK2, RSK3 or RSK4 did not produce a significant change in mibolerone-induced reporter activity compared to the empty vector control in any condition tested. The differences observed between isoforms were small and variable, and none reached statistical significance.

These findings indicate that RSK isoforms do not significantly modulate AR-mediated transcription from the TAT-GRE-E1B-LUC reporter in 22Rv1 cells under these conditions, in contrast to the clear effects observed in COS-1 cells. This difference likely reflects the more complex endogenous AR signalling environment in 22Rv1 cells compared to the exogenous AR system used in COS-1 cells.

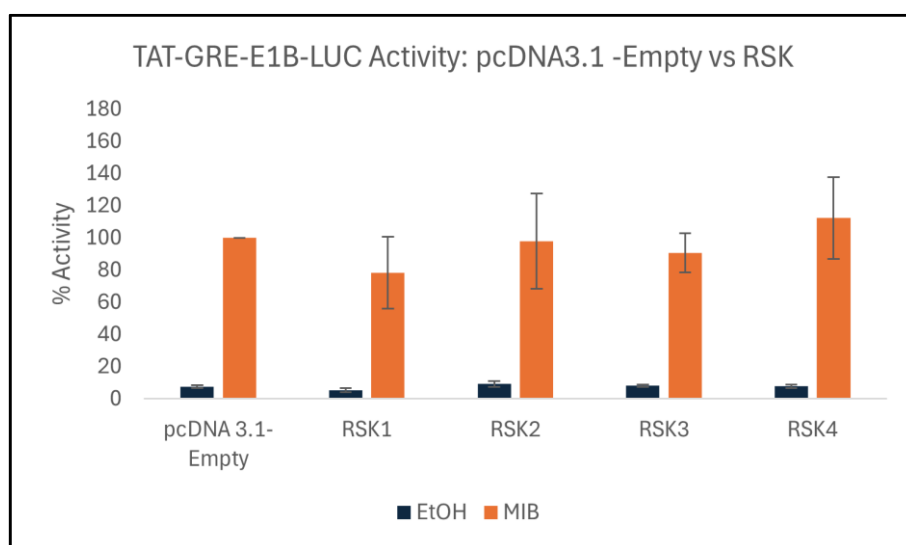


Figure 3.15. RSK isoforms show limited modulation of AR-mediated transcription from the TAT-GRE-E1B-LUC reporter in 22Rv1 cells. 22Rv1 cells were transfected with TAT-GRE-E1B-LUC reporter together with pcDNA3.1 empty vector or RSK1, RSK2, RSK3 or RSK4 expression constructs and treated with ethanol (EtOH) or mibolerone (MIB). Androgen stimulation increased reporter activity across all conditions, with limited and variable modulation observed upon expression of RSK isoforms. Data represent the mean of four independent experiments $\pm$ SEM. Statistical significance was determined using a two-tailed Student's t-test.

3.3.3 Differential effects of RSK isoforms on 22Rv1 cell proliferation

As RSK isoforms showed limited effects on AR-mediated transcription in 22Rv1 cells, their effects on a cellular outcome of androgen signalling were next examined using a fluorescence-based growth assay. The effect of RSK isoform overexpression on 22Rv1 cell proliferation was assessed in the presence and absence of androgen stimulation. Overall, mibolerone treatment increased cell growth compared with the corresponding ethanol-treated controls across all groups, indicating that androgen stimulation promotes proliferation in 22Rv1 cells (Figure 3.16).

RSK1 overexpression showed the strongest increase in cell growth compared with the empty vector control under both treatment conditions (Figure 3.16A). RSK1-transfected cells showed a steeper increase in RFP-positive cell number over time, with a greater effect following mibolerone treatment. Statistical analysis showed significant increases in proliferation at multiple time points, particularly under mibolerone-treated conditions.

RSK2 overexpression showed a moderate increase in cell growth, especially following mibolerone treatment, although these changes were not statistically significant compared with the empty vector control (Figure 3.16B).

RSK3 overexpression showed a decrease in cell growth compared with the empty vector control under both treatment conditions (Figure 3.16C). RSK3-transfected cells showed lower RFP-positive cell numbers over time compared with the corresponding empty vector controls under both ethanol- and mibolerone-treated conditions. Statistical significance was observed at selected time points.

RSK4 overexpression showed only a small increase in cell growth compared with the empty vector control (Figure 3.16D). Statistical significance was observed only at a single later time point under ethanol-treated conditions.

Overall, these findings suggest that RSK isoforms have different effects on 22Rv1 cell proliferation. RSK1 showed the strongest growth-promoting effect, while RSK2 and RSK4 showed weaker effects. In contrast, RSK3 showed a reduction in cell proliferation.

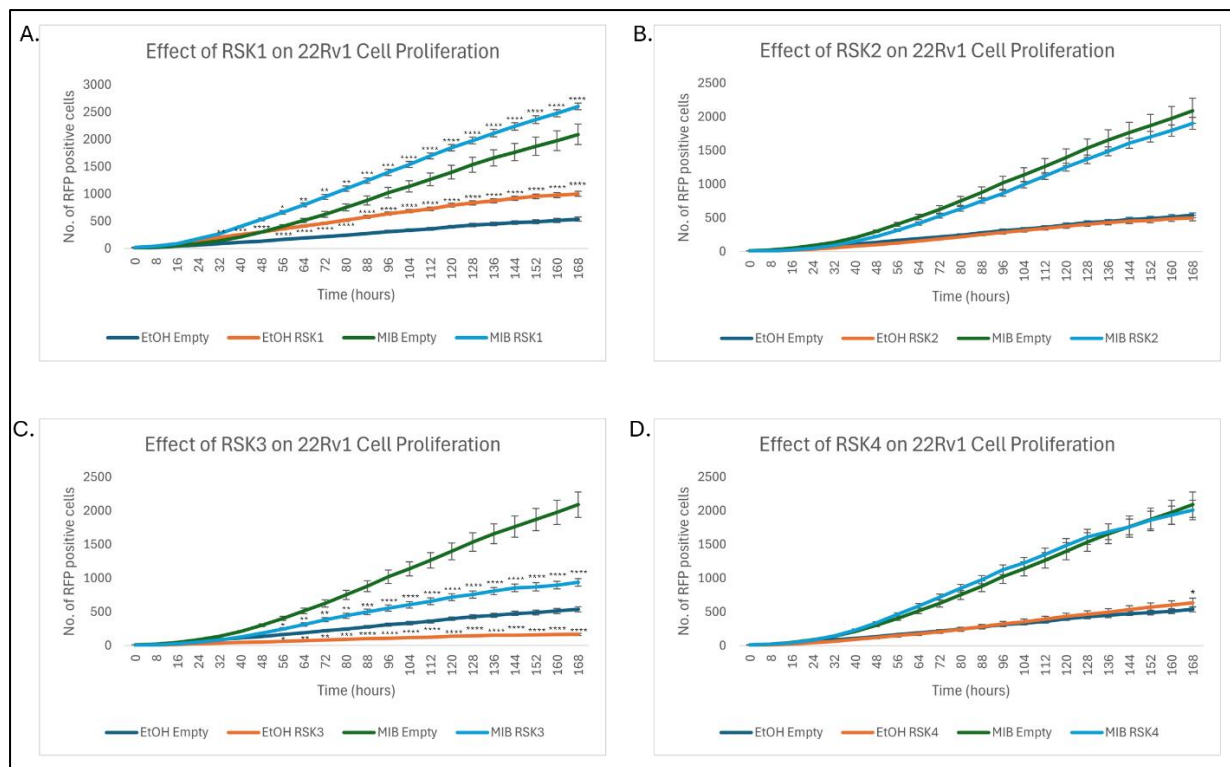


Figure 3.16. Effects of RSK isoform overexpression on 22Rv1 cell proliferation under androgen-stimulated and control conditions. 22Rv1 cells were transfected with RFP-tagged empty vector, RSK1, RSK2, RSK3, or RSK4 expression constructs and treated with either ethanol (EtOH) or 1 nM mibolerone (MIB). Cell proliferation was monitored over time by quantifying RFP-positive cells using live-cell fluorescence IncuCyte imaging. (A) RSK1, (B) RSK2, (C) RSK3, and (D) RSK4 proliferation profiles compared with empty vector control. Data represent mean RFP-positive cell count $\pm$ SEM from replicate wells across the assay time course. Statistical significance was determined using two-way ANOVA followed by Dunnett's multiple comparisons test against the empty vector control at individual time points. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To summarise, these results suggest that RSK isoforms differentially regulate androgen receptor-mediated transcription in a promoter-dependent manner, with distinct effects observed across different androgen-responsive elements. While these regulatory effects were more evident in COS-1 cells, they were less pronounced in the prostate cancer-derived 22Rv1 model, suggesting that RSK-mediated modulation of AR activity may depend on cellular context. In contrast, RSK isoforms showed different effects on cell proliferation, with RSK1 showing the strongest growth-promoting effect, while RSK2 and RSK4 showed weaker effects and RSK3 showed reduced proliferation. Together, these findings indicate that RSK isoforms may influence AR signalling and prostate cancer cell behaviour through distinct and potentially isoform-specific mechanisms.

Chapter 4

Discussion

This study investigated multiple layers of gene regulation in cancer, including circRNA-mediated regulatory networks, androgen receptor (AR) signalling, and the role of RSK isoforms in prostate cancer. By combining computational analysis with experimental validation, this work provides insight into how these mechanisms may contribute to cancer progression.

4.1 Computational Identification of Dysregulated circRNAs and Predicted Regulatory Networks

The observed circRNA dysregulation suggests widespread changes in gene regulation in both OSCC and prostate cancer. The larger number of differentially expressed circRNAs in OSCC may indicate a more pronounced alteration of circRNA expression compared with prostate cancer. In OSCC, hsa_circ_0056440 was selected for further analysis because of its strong upregulation and predicted interactions with multiple cancer-associated miRNAs. Based on the miRNA sponging model, increased circRNA expression may reduce the availability of target miRNAs, which can lead to increased expression of downstream genes (Kristensen et al, 2019). Predicted circRNA-miRNA-mRNA interactions were assessed to determine whether they followed this expected pattern.

Two predicted axes were most consistent with this model. First, hsa_circ_0056440 may interact with miR-370-3p, which showed lower expression in the dataset, with increased expression of *FOXM1*. *FOXM1* is a transcription factor known to promote proliferation, invasion and epithelial-mesenchymal transition (EMT) in head and neck cancers (Ma et al, 2021). Previous studies have also reported reduced miR-370-3p expression in early-stage OSCC tissues compared with healthy controls, suggesting a possible tumour-suppressive role for this miRNA in oral cancer (Taherkhani et al, 2022). Although miR-370-3p expression was not found to be significantly altered, its lower expression together with increased *FOXM1* expression may suggest a possible hsa_circ_0056440/miR-370-3p/*FOXM1* regulatory relationship.

Similarly, hsa_circ_0056440 may interact with miR-150-5p, which showed lower expression, with increased expression of *ZEB1*. *ZEB1* is a key regulator of EMT and is linked to tumour invasion and poor prognosis in OSCC (Ma et al, 2022). Previous studies have also reported altered miR-150-5p expression in oral cancer and suggest a possible tumour-suppressive role for this miRNA (Chang et al, 2018; Koshizuka et al, 2018). Although miR-150-5p was not found to be significantly altered in OSCC, its lower expression together with increased *ZEB1*

expression may suggest a possible hsa_circ_0056440/miR-150-5p/*ZEB1* regulatory relationship.

Vimentin (*VIM*), another EMT-related marker, was also upregulated (Mogre et al, 2022). hsa_circ_0056440 may interact with miR-138-5p, a predicted regulator of *VIM*. Previous studies have reported reduced miR-138-5p expression in OSCC, suggesting a possible tumour-suppressive role for this miRNA (Zhang et al, 2017). As miR-138-5p expression data were not available in the present dataset, this predicted regulatory relationship could not be fully assessed.

However, not all predicted interactions followed the expected circRNA-miRNA-mRNA relationship. hsa_circ_0056440 may also interact with miR-222-3p and *BBC3* (*PUMA*), but this pattern did not match previous findings. miR-222-3p has been reported to be upregulated in OSCC and is generally considered to have oncogenic functions (Yang et al, 2020), whereas *BBC3*, a pro-apoptotic gene, is usually reduced in head and neck cancers (Tajnik et al, 2012). However, in this analysis, miR-222-3p did not show increased expression and *BBC3* was instead upregulated. This difference may be due to variation between datasets, differences between tumour samples, or other regulatory mechanisms affecting gene expression. Similarly, *ESR1* did not show a clear relationship with its predicted miRNA regulators, and its role in OSCC is still not fully understood (Almouhanna & Hess, 2024). Overall, these findings suggest that hsa_circ_0056440 may contribute to tumour progression through proliferation- and EMT-related pathways, while also highlighting the limitations of computational prediction and the need for further experimental validation.

In prostate cancer, hsa_circ_0064977 was selected for further analysis because of its strong downregulation. Predicted circRNA-miRNA-mRNA interactions were assessed to determine whether they followed this expected pattern. One predicted axis that was partly consistent with this model involved miR-508-3p and *NR4A3*. hsa_circ_0064977 may interact with miR-508-3p, alongside reduced expression of the *NR4A3* gene, which has previously been reported to function as a tumour suppressor in prostate cancer and may suppress tumour growth, invasion and metastasis (Lin et al, 2022). However, miR-508-3p has previously been reported to be reduced in prostate cancer tissues and cell lines (Che et al, 2022), which differs from the trend observed in the present dataset. Although miR-508-3p was not significantly altered, its increased expression together with reduced *NR4A3* expression may suggest a potential hsa_circ_0064977/miR-508-3p/*NR4A3* regulatory relationship. Similarly, hsa_circ_0064977 may interact with miR-488-3p, with *POMC* identified as a predicted target gene. However, the role of *POMC* in prostate cancer remains poorly understood, and this predicted relationship was therefore not explored further.

To further investigate the potential role of hsa_circ_0064977, a growth assay was performed in 22Rv1 cells. Overexpression of hsa_circ_0064977 showed a consistent trend towards reduced proliferation compared with the empty vector control. Although this effect did not reach statistical significance, it may suggest a possible tumour-suppressive role for

hsa_circ_0064977 in prostate cancer. However, further experimental validation would be required to confirm its biological significance and underlying regulatory mechanisms.

In contrast to hsa_circ_0064977, hsa_circ_0010794 was upregulated and showed a more consistent pattern. One predicted axis that was partly consistent with this model involved miR-138-5p and *TERT*. hsa_circ_0010794 may interact with miR-138-5p, alongside increased expression of the *TERT* gene, which contributes to telomerase activation, a process commonly observed in prostate cancer and associated with tumour progression (Graham & Meeker, 2017). Previous studies have also reported a tumour-suppressive role for miR-138-5p in prostate cancer through inhibition of cell proliferation and chemoresistance (Liu et al, 2023). Although miR-138-5p was not significantly altered in the present dataset, increased *TERT* expression together with strong upregulation of hsa_circ_0010794 may suggest a potential hsa_circ_0010794/miR-138-5p/*TERT* regulatory relationship.

Similarly, hsa_circ_0010794 may interact with miR-1908-5p, alongside increased expression of the *EXO1* gene, which has previously been linked to prostate cancer progression and poor prognosis (Luo et al, 2019a). Although miR-1908-5p expression data were not available in the present dataset, previous studies have reported reduced miR-1908-5p expression in prostate cancer (Wang et al, 2017), which may support a potential hsa_circ_0010794/miR-1908-5p/*EXO1* regulatory relationship.

To further investigate the role of hsa_circ_0010794, a growth assay was performed in 22Rv1 cells. Overexpression of hsa_circ_0010794 significantly increased cell proliferation, supporting a possible oncogenic role for this circRNA in prostate cancer. Together, these findings suggest that hsa_circ_0010794 may contribute to prostate cancer progression through proliferation-related pathways. Overall, these findings suggest that hsa_circ_0056440 and hsa_circ_0010794 may have oncogenic roles, whereas hsa_circ_0064977 may have a more complex and potentially tumour-suppressive role. However, these regulatory interactions are based mainly on computational prediction, and further experimental validation will be required to confirm these mechanisms.

4.2 Functional Impact of ARE Sequence Variation on AR-Mediated Transcription

Reporter assays were used to assess whether the identified ARE sequences were functionally responsive to androgen signalling and whether the associated SNPs affected this response.

The wild-type MAF construct showed a clear dose-dependent increase in luciferase activity following mibolerone treatment, consistent with AR-mediated transcriptional activation. The SNP-containing construct showed no such response, suggesting that the rs250165 variant may disrupt androgen-dependent transcription at this element. This is consistent with

evidence that single nucleotide changes within or adjacent to the ARE core can reduce AR binding affinity and impair transcriptional activation (Stelloo et al, 2019).

Interestingly, this SNP shows population-specific variation, being present in approximately 15% of European populations, compared with 2% in African populations, and was absent in Asian populations in the dataset examined. As the SNP-containing construct showed reduced androgen responsiveness, it is possible that individuals carrying this variant may be at reduced risk of prostate cancer and/or disease progression. Therefore, SNPs such as this may have potential prognostic value, although further studies are required to investigate this relationship.

Neither the wild-type nor SNP-containing CXCR1 constructs showed a consistent or dose-dependent androgen-dependent response. COS-1 cells were used for these reporter assays because of their high transfection efficiency, which makes them suitable for transient reporter experiments. However, the absence of the chromatin context or cofactors required for AR binding at this locus may have contributed to the lack of response (Stelloo et al, 2019). It may also reflect that COS-1 cells are not prostate-derived and may lack some prostate-specific cofactors required for AR activity of specific promoters. Therefore, AR-mediated transcription in this system may not fully represent regulation in prostate cancer cells, as AR activity is highly dependent on cellular context and cofactor availability (Leach et al, 2022).

These findings suggest that sequence variation within regulatory elements can influence AR-dependent gene regulation, while also showing that not all predicted AREs are functionally active. Further studies in prostate-derived models would be needed to better understand the biological and clinical relevance of these variants. As the effects of the MAF SNP may result from altered AR-DNA interactions, the AR DNA-binding domain was expressed and purified to provide a basis for future studies investigating this interaction.

4.3 Expression and Purification of AR DNA-Binding Domain

Expression of the GST-tagged AR DNA-binding domain (DBD) was successfully achieved following IPTG induction, with a clear band observed at the expected size of approximately 38 kDa on SDS-PAGE. This confirms that the protein was expressed under the conditions used.

However, analysis of the soluble and insoluble fractions showed that most of the protein was present in the insoluble fraction, indicating limited solubility. A smaller amount was detected in the soluble fraction, which allowed purification to be carried out using glutathione affinity chromatography. This limited solubility is not unusual in *E. coli* expression, as recombinant proteins can sometimes misfold and accumulate in insoluble inclusion bodies (Rosano & Ceccarelli, 2014). Future optimisation, such as changing induction temperature or using solubility-enhancing tags, may improve protein recovery.

The elution fractions showed a band at the expected size, with the strongest signal observed in the early fractions, suggesting successful binding and recovery of the GST-tagged protein. An additional band at around 26 kDa was also observed in some fractions, likely corresponding to free GST.

Overall, these results show that the AR DBD can be expressed and partially purified, although solubility remains a limitation. As AR-mediated transcription depends on DNA binding through its DNA-binding domain, successful production of this protein provides a basis for future studies examining AR-DNA interactions. Such experiments may help confirm the effects of SNPs on AR-DNA interactions. In addition to regulation at the level of DNA binding, AR activity can also be influenced by intracellular signalling pathways. This was further investigated by examining the role of RSK isoforms in AR-mediated transcription and prostate cancer cell proliferation.

4.4 RSK-Mediated Regulation of AR Transcriptional Activity and Cell Proliferation

RSK isoforms are downstream effectors of the MAPK/ERK pathway and have been linked to regulation of AR signalling in prostate cancer (Cronin et al, 2021). This part of the study aimed to assess whether RSK isoforms influence AR-mediated transcription and androgen-dependent cell proliferation.

In COS-1 cells, reporter assays showed that RSK isoforms reduced AR-mediated transcription from the ARE-LUC and PB-PROM reporters, although the extent of suppression varied between isoforms. RSK2 showed the strongest effect across both reporters, while RSK1 and RSK3 did not significantly affect PB-PROM activity. In contrast, the TAT-GRE-E1B-LUC reporter showed an opposite pattern, where RSK isoforms increased androgen-induced transcription, with only RSK4 reaching significance. This suggests that RSK effects on AR activity are not uniform and depend on the promoter being analysed.

The opposite trends observed across the reporter constructs may reflect differences in promoter context and cofactor recruitment. Although all reporters are androgen responsive, differences in regulatory elements (e.g. direct versus palindromic) may influence how RSK isoforms affect AR-mediated transcription. AR activity is known to depend on regulatory context and interacting proteins, which may explain the promoter-dependent effects observed in the present study (Leach et al, 2022). Therefore, the effects of RSK isoforms on AR activity may be promoter dependent, although the underlying mechanism was not directly investigated in the present study. The RSK protein expression results in COS-1 cells were less clear, as the Western blot did not show clear isoform-specific detection. As COS-1 cells are derived from African green monkey rather than human cells, species differences in the RSK proteins may have contributed to differences in their detection by the antibodies used. However, this cannot be confirmed from the present data.

In 22Rv1 cells, RSK overexpression did not produce a clear or significant change in AR-mediated transcription from the TAT-GRE-E1B-LUC reporter. This differs from the COS-1 results and likely reflects the more complex signalling environment in 22Rv1 cells. Unlike COS-1 cells, which rely on exogenous AR expression, 22Rv1 cells express endogenous AR and additional signalling components, which may reduce or mask the effect of individual RSK isoforms (Wen et al, 2020). 22Rv1 cells also express AR splice variants, including AR-V7, which may further complicate AR-dependent transcriptional responses (Wadosky & Koochekpour, 2017). Modulation of endogenous RSK expression (e.g. siRNA depletion) was not assessed, which limits interpretation. Therefore, it is unclear whether the observed effects reflect physiologically relevant changes or non-physiological overexpression.

As RSK isoforms showed limited effects on AR-mediated transcription in 22Rv1 cells, their effects on cell proliferation were next examined to determine whether changes in RSK expression were associated with a cellular response. Mibolerone treatment increased cell growth compared with ethanol-treated controls across all groups, consistent with androgen-driven proliferation. Among the isoforms, RSK1 showed the strongest increase in cell proliferation, with significant effects observed at multiple time points, particularly in the presence of mibolerone. RSK2 overexpression showed a moderate increase in cell growth, although this did not reach statistical significance. In contrast, RSK3 overexpression was associated with reduced cell proliferation compared with the empty vector control under both treatment conditions. RSK4 showed only limited effects on cell proliferation.

Interestingly, RSK2 showed the strongest suppressive effect in reporter assays, whereas RSK1 had the strongest effect on cell proliferation. This suggests that RSK1 may influence prostate cancer cell growth through mechanisms that are not solely dependent on AR-mediated transcription.

Overall, these findings suggest that RSK isoforms can influence AR transcriptional activity and prostate cancer cell behaviour in an isoform-specific and context-dependent manner (Cronin et al, 2021). Although RSK-mediated effects on transcription differed between COS-1 and 22Rv1 cells, this may reflect differences in cellular context and AR-cofactor interactions, which are known to influence androgen signalling in prostate cancer (Brooke et al, 2026; Leach et al, 2022). Importantly, the transcriptional effects did not directly match the proliferation patterns, suggesting that RSK isoforms may influence prostate cancer cell growth through mechanisms beyond AR-mediated transcription alone. Further studies investigating downstream signalling pathways and modulation of endogenous RSK expression would help clarify the mechanisms through which RSK isoforms influence AR signalling and prostate cancer cell growth.

Chapter 5

Conclusion

This study investigated multiple levels of gene regulation in cancer, including circRNA-mediated regulatory networks, androgen receptor (AR) signalling, and the role of RSK isoforms in prostate cancer. A combination of computational and experimental approaches was used to better understand how these mechanisms may contribute to cancer progression.

Computational analysis identified widespread circRNA dysregulation in both oral squamous cell carcinoma (OSCC) and prostate cancer. Among the circRNAs analysed, hsa_circ_0056440 and hsa_circ_0010794 showed patterns consistent with potential oncogenic roles, while hsa_circ_0064977 showed a more complex pattern and may have a tumour suppressor-like function. However, the effect of hsa_circ_0064977 on cell proliferation did not reach statistical significance. These findings support the idea that circRNAs can influence cancer-related pathways but also highlight the limitations of computational predictions and the need for further experimental validation.

Functional analysis of androgen response elements showed that sequence variation can directly affect AR-mediated transcription. The disruption of androgen responsiveness in the *MAF* SNP construct demonstrates how small genetic changes can influence gene regulation. In contrast, the lack of response observed for *CXCR1* suggests that not all predicted regulatory elements are functionally active.

Expression and partial purification of the AR DNA-binding domain confirmed that the protein can be successfully produced, although solubility remains a limitation. This provides a foundation for future studies aimed at directly examining AR-DNA interactions.

Investigation of RSK isoforms showed that they differentially affect prostate cancer cell proliferation and AR signalling in an isoform-specific and context-dependent manner. RSK1 showed the strongest association with increased cell proliferation, particularly under androgen-stimulated conditions, while other isoforms showed more moderate or limited effects. However, as this study relied on overexpression, the physiological relevance of these findings remains to be fully established.

Overall, this study highlights the complexity of gene regulation in cancer and demonstrates that circRNAs, regulatory SNPs, and kinase signalling pathways can all contribute to tumour progression. Importantly, it underscores the need to integrate multiple layers of regulation rather than focussing on individual pathways in isolation, to fully understand tumour biology. Further experimental work will be required to validate these interactions and to better understand their biological significance.

References

- Aaron, L., Franco, O. E. & Hayward, S. W. (2016) Review of Prostate Anatomy and Embryology and the Etiology of Benign Prostatic Hyperplasia. *Urol Clin North Am*, 43(3), 279-88.
- Allemailem, K. S., Almatroudi, A., Alrumaihi, F., Makki Almansour, N., Aldakheel, F. M., Rather, R. A., Afroze, D. & Rah, B. (2021) Single nucleotide polymorphisms (SNPs) in prostate cancer: its implications in diagnostics and therapeutics. *Am J Transl Res*, 13(4), 3868-3889.
- Almeeri, M. N. E., Awies, M. & Constantinou, C. (2024) Prostate Cancer, Pathophysiology and Recent Developments in Management: A Narrative Review. *Curr Oncol Rep*, 26(11), 1511-1519.
- Almouhanna, F. & Hess, J. (2024) An ESR1-Related Gene Signature Identifies Head and Neck Squamous Cell Carcinoma with Imputed Susceptibility to Endocrine Therapy. *Int J Mol Sci*, 25(2).
- Ashwal-Fluss, R., Meyer, M., Pamudurti, N. R., Ivanov, A., Bartok, O., Hanan, M., Evantal, N., Memczak, S., Rajewsky, N. & Kadener, S. (2014) circRNA biogenesis competes with pre-mRNA splicing. *Mol Cell*, 56(1), 55-66.
- Bagan, J., Sarrion, G. & Jimenez, Y. (2010) Oral cancer: clinical features. *Oral Oncol*, 46(6), 414-7.
- Bluemn, E. G., Coleman, I. M., Lucas, J. M., Coleman, R. T., Hernandez-Lopez, S., Tharakan, R., Bianchi-Frias, D., Dumpit, R. F., Kaipainen, A., Corella, A. N., Yang, Y. C., Nyquist, M. D., Mostaghel, E., Hsieh, A. C., Zhang, X., Corey, E., Brown, L. G., Nguyen, H. M., Pienta, K., Ittmann, M., Schweizer, M., True, L. D., Wise, D., Rennie, P. S., Vessella, R. L., Morrissey, C. & Nelson, P. S. (2017) Androgen Receptor Pathway-Independent Prostate Cancer Is Sustained through FGF Signaling. *Cancer Cell*, 32(4), 474-489.e6.
- Brooke, G. N., Leach, D. A., Culley, R. L., Azadova, A., Latonen, L., Rees, E., Alkheilewi, M. A., Pine, A. C., Fioretti, F. M., Reader, C. S., Powell, S. M., Reebye, V., Waxman, J., Visakorpi, T. & Bevan, C. L. (2026) Disruption of androgen receptor-cofactor interactions by the RNA-binding protein FUS/TLS alters androgen signalling in prostate cancer. *Oncogene*, 45(8), 757-773.
- Cao, S., Ma, T., Ungerleider, N., Roberts, C., Kobelski, M., Jin, L., Concha, M., Wang, X., Baddoo, M., Nguyen, H. M., Corey, E., Fazli, L., Ledet, E., Zhang, R., Silberstein, J. L., Zhang, W., Zhang, K., Sartor, O., Dong, X., Flemington, E. K. & Dong, Y. (2019) Circular RNAs add diversity to androgen receptor isoform repertoire in castration-resistant prostate cancer. *Oncogene*, 38(45), 7060-7072.
- Chang, Y. A., Weng, S. L., Yang, S. F., Chou, C. H., Huang, W. C., Tu, S. J., Chang, T. H., Huang, C. N., Jong, Y. J. & Huang, H. D. (2018) A Three-MicroRNA Signature as a Potential Biomarker for the Early Detection of Oral Cancer. *Int J Mol Sci*, 19(3).
- Chaturvedi, A. P. & Dehm, S. M. (2019) Androgen Receptor Dependence. *Adv Exp Med Biol*, 1210, 333-350.
- Che, P., Jiang, S., Zhang, W., Zhu, H., Hu, D. & Wang, D. (2022) A novel prognostic model based on three clinic-related miRNAs for prostate cancer. *Front Surg*, 9, 872953.
- Conn, S. J., Pillman, K. A., Toubia, J., Conn, V. M., Salmanidis, M., Phillips, C. A., Roslan, S., Schreiber, A. W., Gregory, P. A. & Goodall, G. J. (2015) The RNA binding protein quaking regulates formation of circRNAs. *Cell*, 160(6), 1125-34.

Cronin, R., Brooke, G. N. & Prischi, F. (2021) The role of the p90 ribosomal S6 kinase family in prostate cancer progression and therapy resistance. *Oncogene*, 40(22), 3775-3785.

Cui, S., Yu, S., Huang, H.-Y., Lin, Y.-C.-D., Huang, Y., Zhang, B., Xiao, J., Zuo, H., Wang, J., Li, Z., Li, G., Ma, J., Chen, B., Zhang, H., Fu, J., Wang, L. & Huang, H.-D. (2025) miRTarBase 2025: updates to the collection of experimentally validated microRNA–target interactions. *Nucleic Acids Research*, 53(D1), D147-D156.

Cuzick, J., Thorat, M. A., Andriole, G., Brawley, O. W., Brown, P. H., Culig, Z., Eeles, R. A., Ford, L. G., Hamdy, F. C., Holmberg, L., Illic, D., Key, T. J., La Vecchia, C., Lilja, H., Marberger, M., Meyskens, F. L., Minasian, L. M., Parker, C., Parnes, H. L., Perner, S., Rittenhouse, H., Schalken, J., Schmid, H. P., Schmitz-Dräger, B. J., Schröder, F. H., Stenzl, A., Tombal, B., Wilt, T. J. & Wolk, A. (2014) Prevention and early detection of prostate cancer. *Lancet Oncol*, 15(11), e484-92.

Dalal, K., Roshan-Moniri, M., Sharma, A., Li, H., Ban, F., Hessein, M., Hsing, M., Singh, K., LeBlanc, E., Dehm, S., Tomlinson Guns, E. S., Cherkasov, A. & Rennie, P. S. (2014) Selectively targeting the DNA-binding domain of the androgen receptor as a prospective therapy for prostate cancer. *J Biol Chem*, 289(38), 26417-26429.

Di Timoteo, G., Dattilo, D., Centrón-Broco, A., Colantoni, A., Guarnacci, M., Rossi, F., Incarnato, D., Oliviero, S., Fatica, A., Morlando, M. & Bozzoni, I. (2020) Modulation of circRNA Metabolism by m(6)A Modification. *Cell Rep*, 31(6), 107641.

Du, W. W., Yang, W., Liu, E., Yang, Z., Dhaliwal, P. & Yang, B. B. (2016) Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2. *Nucleic Acids Res*, 44(6), 2846-58.

Errichelli, L., Dini Modigliani, S., Laneve, P., Colantoni, A., Legnini, I., Capauto, D., Rosa, A., De Santis, R., Scarfò, R., Peruzzi, G., Lu, L., Caffarelli, E., Shneider, N. A., Morlando, M. & Bozzoni, I. (2017) FUS affects circular RNA expression in murine embryonic stem cell-derived motor neurons. *Nat Commun*, 8, 14741.

European Nucleotide Archive (2019) PRJNA552058, 2019. Available online: <https://www.ebi.ac.uk/ena/browser/view/PRJNA552058> [Accessed].

European Nucleotide Archive (2020) PRJNA542863, 2020. Available online: <https://www.ebi.ac.uk/ena/browser/view/PRJNA542863> [Accessed].

Feng, Y., Yang, Y., Zhao, X., Fan, Y., Zhou, L., Rong, J. & Yu, Y. (2019) Circular RNA circ0005276 promotes the proliferation and migration of prostate cancer cells by interacting with FUS to transcriptionally activate XIAP. *Cell Death Dis*, 10(11), 792.

Fujita, K. & Nonomura, N. (2019) Role of Androgen Receptor in Prostate Cancer: A Review. *World J Mens Health*, 37(3), 288-295.

Glazar, P. P., P.; Rajewsky, N. (2014) circBase: a database for circular RNAs. *RNA*, 20(11), 1666-1670.

Graham, M. K. & Meeker, A. (2017) Telomeres and telomerase in prostate cancer development and therapy. *Nat Rev Urol*, 14(10), 607-619.

Hansen, T. B., Jensen, T. I., Clausen, B. H., Bramsen, J. B., Finsen, B., Damgaard, C. K. & Kjems, J. (2013) Natural RNA circles function as efficient microRNA sponges. *Nature*, 495(7441), 384-8.

Ivanov, A., Memczak, S., Wyler, E., Torti, F., Porath, H. T., Orejuela, M. R., Piechotta, M., Levanon, E. Y., Landthaler, M., Dieterich, C. & Rajewsky, N. (2015) Analysis of intron sequences reveals hallmarks of circular RNA biogenesis in animals. *Cell Rep*, 10(2), 170-7.

Jung, S. H., Shin, S., Kim, M. S., Baek, I. P., Lee, J. Y., Lee, S. H., Kim, T. M., Lee, S. H. & Chung, Y. J. (2016) Genetic Progression of High Grade Prostatic Intraepithelial Neoplasia to Prostate Cancer. *Eur Urol*, 69(5), 823-30.

Kong, Z., Lu, Y., Wan, X., Luo, J., Li, D., Huang, Y., Wang, C., Li, Y. & Xu, Y. (2021) Comprehensive Characterization of Androgen-Responsive circRNAs in Prostate Cancer. *Life (Basel)*, 11(10).

Kong, Z., Lu, Y., Yang, Y., Chang, K., Lin, Y., Huang, Y., Wang, C., Zhang, L., Xu, W., Zhao, S. & Li, Y. (2023) m6A-Mediated Biogenesis of circDDIT4 Inhibits Prostate Cancer Progression by Sequestering ELAVL1/HuR. *Mol Cancer Res*, 21(12), 1342-1355.

Koshizuka, K., Hanazawa, T., Kikkawa, N., Katada, K., Okato, A., Arai, T., Idichi, T., Osako, Y., Okamoto, Y. & Seki, N. (2018) Antitumor miR-150-5p and miR-150-3p inhibit cancer cell aggressiveness by targeting SPOCK1 in head and neck squamous cell carcinoma. *Auris Nasus Larynx*, 45(4), 854-865.

Kramer, M. C., Liang, D., Tatomer, D. C., Gold, B., March, Z. M., Cherry, S. & Wilusz, J. E. (2015) Combinatorial control of Drosophila circular RNA expression by intronic repeats, hnRNPs, and SR proteins. *Genes Dev*, 29(20), 2168-82.

Kristensen, L. S., Andersen, M. S., Stagsted, L. V. W., Ebbesen, K. K., Hansen, T. B. & Kjems, J. (2019) The biogenesis, biology and characterization of circular RNAs. *Nat Rev Genet*, 20(11), 675-691.

Leach, D. A., Fernandes, R. C. & Bevan, C. L. (2022) Cellular specificity of androgen receptor, coregulators, and pioneer factors in prostate cancer. *Endocr Oncol*, 2(1), R112-r131.

Lee, X. Y., Van Eynde, W., Helsen, C., Willems, H., Peperstraete, K., De Block, S., Voet, A. & Claessens, F. (2024) Structural mechanism underlying variations in DNA binding by the androgen receptor. *J Steroid Biochem Mol Biol*, 241, 106499.

Leslie, S., Soon-Sutton, T. & Skelton, W. (2024) *Prostate Cancer*. Treasure Island (FL): StatPearls Publishing.

Li, F., Yang, Q., He, A. T. & Yang, B. B. (2021a) Circular RNAs in cancer: Limitations in functional studies and diagnostic potential. *Semin Cancer Biol*, 75, 49-61.

Li, K., Fan, X., Yan, Z., Zhan, J., Cao, F. & Jiang, Y. (2021b) Circ_0000745 strengthens the expression of CCND1 by functioning as miR-488 sponge and interacting with HuR binding protein to facilitate the development of oral squamous cell carcinoma. *Cancer Cell Int*, 21(1), 271.

Li, Z., Huang, C., Bao, C., Chen, L., Lin, M., Wang, X., Zhong, G., Yu, B., Hu, W., Dai, L., Zhu, P., Chang, Z., Wu, Q., Zhao, Y., Jia, Y., Xu, P., Liu, H. & Shan, G. (2015) Exon-intron circular RNAs regulate transcription in the nucleus. *Nat Struct Mol Biol*, 22(3), 256-64.

Liang, D., Tatomer, D. C., Luo, Z., Wu, H., Yang, L., Chen, L. L., Cherry, S. & Wilusz, J. E. (2017) The Output of Protein-Coding Genes Shifts to Circular RNAs When the Pre-mRNA Processing Machinery Is Limiting. *Mol Cell*, 68(5), 940-954.e3.

Lin, H. Y., Ko, C. J., Lo, T. Y., Wu, S. R., Lan, S. W., Huang, C. A., Lin, Y. C., Lin, H. H., Tu, H. F., Lee, C. F., Hsiao, P. W., Huang, H. P., Chen, M. J., Chang, K. H. & Lee, M. S. (2022) Matriptase-2/NR4A3 axis switches TGF- β action toward suppression of prostate cancer cell invasion, tumor growth, and metastasis. *Oncogene*, 41(20), 2833-2845.

Linsen, S. S., Gellrich, N.-C. & Kruskemper, G. (2018) Age- and localization-dependent functional and psychosocial impairments and health related quality of life six months after OSCC therapy. *Oral Oncology*, 81, 61-68.

Liu, L., Zhang, Y., Hu, X., Zhang, H., Jiang, C., Guo, Y. & Cang, S. (2023) MiR-138-5p inhibits prostate cancer cell proliferation and chemoresistance by targeting APOBEC3B. *Transl Oncol*, 35, 101723.

Lu, Y., Sun, J., Kader, A. K., Kim, S. T., Kim, J. W., Liu, W., Sun, J., Lu, D., Feng, J., Zhu, Y., Jin, T., Zhang, Z., Dimitrov, L., Lowey, J., Campbell, K., Suh, E., Duggan, D., Carpten, J., Trent, J. M., Gronberg, H., Zheng, S. L., Isaacs, W. B. & Xu, J. (2012) Association of prostate cancer risk with SNPs in regions containing androgen receptor binding sites captured by ChIP-On-chip analyses. *Prostate*, 72(4), 376-85.

Luo, F., Wang, Y. Z., Lin, D., Li, J. & Yang, K. (2019a) Exonuclease 1 expression is associated with clinical progression, metastasis, and survival prognosis of prostate cancer. *J Cell Biochem*, 120(7), 11383-11389.

Luo, J., Li, Y., Zheng, W., Xie, N., Shi, Y., Long, Z., Xie, L., Fazli, L., Zhang, D., Gleave, M. & Dong, X. (2019b) Characterization of a Prostate- and Prostate Cancer-Specific Circular RNA Encoded by the Androgen Receptor Gene. *Mol Ther Nucleic Acids*, 18, 916-926.

Lv, J. L., Ma, R., Ren, Y. S., Liang, Q. Y., Zhang, H. M., Dong, G. C. & Li, J. (2025) CircRNA: the potential biomarkers and therapeutic targets in oral squamous cell carcinoma (OSCC). *Front Oncol*, 15, 1555002.

Ma, J., Yao, Y., Tao, D. T., Yin, J. Y., Chen, L., Cui, Y., Wang, Y., Hu, C. L., Tian, M. X. & Wang, Q. (2022) Comprehensive and Integrated Analysis Identifies ZEB1 as a Key Novel Gene in Oral Squamous Cell Carcinoma. *Contrast Media Mol Imaging*, 2022, 4486104.

Ma, X., Zhang, H., Li, Q., Schiferle, E., Qin, Y., Xiao, S. & Li, T. (2021) FOXM1 Promotes Head and Neck Squamous Cell Carcinoma via Activation of the Linc-ROR/LMO4/AKT/PI3K Axis. *Front Oncol*, 11, 658712.

Marco, A. (2018) SeedVicious: analysis of microRNA target and near-target sites. *PLOS ONE*, 13(4), e0195532.

Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., Rybak, A., Maier, L., Mackowiak, S. D., Gregersen, L. H., Munschauer, M., Loewer, A., Ziebold, U., Landthaler, M., Kocks, C., le Noble, F. & Rajewsky, N. (2013) Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature*, 495(7441), 333-8.

Messner, E. A., Steele, T. M., Tsamouri, M. M., Hejazi, N., Gao, A. C., Mudryj, M. & Ghosh, P. M. (2020) The Androgen Receptor in Prostate Cancer: Effect of Structure, Ligands and Spliced Variants on Therapy. *Biomedicines*, 8(10).

Mogre, S., Makani, V., Pradhan, S., Devre, P., More, S., Vaidya, M. & Dmello, C. (2022) Biomarker Potential of Vimentin in Oral Cancers. *Life (Basel)*, 12(2).

Nguyen-Nielsen, M. & Borre, M. (2016) Diagnostic and Therapeutic Strategies for Prostate Cancer. *Seminars in Nuclear Medicine*, 46(6), 484-490.

Parshad, S., Nandi, S., Marwah, N., Mehta, P., Tripathi, M., Netrapal, Gogna, S. & Karwasra, R. K. (2015) Human papillomavirus 16 and 18 in squamous cell carcinoma of oral cavity and sexual practices: A pilot study at a Tertiary Care Hospital of North India. *Natl J Maxillofac Surg*, 6(2), 185-9.

Patop, I. L., Wüst, S. & Kadener, S. (2019) Past, present, and future of circRNAs. *Embo j*, 38(16), e100836.

Perez, G., Barber, G. P., Benet-Pages, A., Casper, J., Clawson, H., Diekhans, M., Fischer, C., Gonzalez, J. N., Hinrichs, A. S., Lee, C. M., Nassar, L. R., Raney, B. J., Speir, M. L., van Baren, M. J., Vaske, C. J., Haussler, D., Kent, W. J. & Haeussler, M. (2025) The UCSC Genome Browser database: 2025 update. *Nucleic Acids Res*, 53(D1), D1243-d1249.

Rao, S. O. B. S. (2023) *Anatomy, Abdomen and Pelvis, Prostate*. Treasure Island (FL), USA: StatPearls Publishing.

Rawla, P. (2019) Epidemiology of Prostate Cancer. *World J Oncol*, 10(2), 63-89.

Rivera, C. (2015) Essentials of oral cancer. *Int J Clin Exp Pathol*, 8(9), 11884-94.

Rosano, G. L. & Ceccarelli, E. A. (2014) Recombinant protein expression in *Escherichia coli*: advances and challenges. *Front Microbiol*, 5, 172.

Roy, P. & Saikia, B. (2016) Cancer and cure: A critical analysis. *Indian Journal of Cancer*, 53(3), 441-442.

Stelloo, S., Bergman, A. M. & Zwart, W. (2019) Androgen receptor enhancer usage and the chromatin regulatory landscape in human prostate cancers. *Endocr Relat Cancer*, 26(5), R267-r285.

Taherkhani, A., Dehto, S. S., Jamshidi, S. & Shojaei, S. (2022) Pathogenesis and prognosis of primary oral squamous cell carcinoma based on microRNAs target genes: a systems biology approach. *Genomics Inform*, 20(3), e27.

Tajnik, M., Stražišar, M., Volavšek, M., Boštjančič, E. & Glavač, D. (2012) BBC3 is down-regulated with increased tumor size independently of p53 expression in head and neck cancer. *Cancer Biomark*, 11(5), 197-208.

Tan, Y., Wang, Z., Xu, M., Li, B., Huang, Z., Qin, S., Nice, E. C., Tang, J. & Huang, C. (2023) Oral squamous cell carcinomas: state of the field and emerging directions. *International Journal of Oral Science*, 15(1), 44.

Teplova, M., Hafner, M., Teplov, D., Essig, K., Tuschl, T. & Patel, D. J. (2013) Structure-function studies of STAR family Quaking proteins bound to their in vivo RNA target sites. *Genes Dev*, 27(8), 928-40.

The Galaxy Community (2024) The Galaxy platform for accessible, reproducible, and collaborative data analyses: 2024 update. *Nucleic Acids Research*, 52(W1), W83-W94.

Wadosky, K. M. & Koochekpour, S. (2017) Androgen receptor splice variants and prostate cancer: From bench to bedside. *Oncotarget*, 8(11), 18550-18576.

Wang, G., Zhao, D., Spring, D. J. & DePinho, R. A. (2018) Genetics and biology of prostate cancer. *Genes Dev*, 32(17-18), 1105-1140.

Wang, L. Y., Cui, J. J., Zhu, T., Shao, W. H., Zhao, Y., Wang, S., Zhang, Y. P., Wu, J. C. & Zhang, L. (2017) Biomarkers identified for prostate cancer patients through genome-scale screening. *Oncotarget*, 8(54), 92055-92063.

Wang, S., Li, W., Yang, L., Yuan, J., Wang, L., Li, N. & Zhao, H. (2022) CircPVT1 facilitates the progression of oral squamous cell carcinoma by regulating miR-143-3p/SLC7A11 axis through MAPK signaling pathway. *Funct Integr Genomics*, 22(5), 891-903.

Warnakulasuriya, S. (2020) Oral potentially malignant disorders: A comprehensive review on clinical aspects and management. *Oral Oncology*, 102, 104550.

Weidle, U. H. & Birzele, F. (2025) Prostate Cancer: De-regulated Circular RNAs With Efficacy in Preclinical In Vivo Models. *Cancer Genomics Proteomics*, 22(2), 136-165.

Wen, S., Niu, Y. & Huang, H. (2020) Posttranslational regulation of androgen dependent and independent androgen receptor activities in prostate cancer. *Asian J Urol*, 7(3), 203-218.

Xu, F., Xiao, Q., Du, W. W., Wang, S. & Yang, B. B. (2024) CircRNA: Functions, Applications and Prospects. *Biomolecules*, 14(12).

Yan, H. & Bu, P. (2021) Non-coding RNA in cancer. *Essays Biochem*, 65(4), 625-639.

Yang, K., Chen, Y., Cui, Z., Chen, H., Yang, L., Zhao, J., Luo, F., Man, S., Zhao, J. & Jin, L. (2020) MicroRNA-222-3p participates in the development of oral squamous cell carcinoma by targeting CDKN1B. *J Oral Pathol Med*, 49(7), 621-629.

Yang, L., Fu, J. & Zhou, Y. (2018) Circular RNAs and Their Emerging Roles in Immune Regulation. *Frontiers in Immunology*, Volume 9 - 2018.

Yu, C.-Y. & Kuo, H.-C. (2019) The emerging roles and functions of circular RNAs and their generation. *Journal of Biomedical Science*, 26(1), 29.

Yuan, H., Liao, X., Hu, D., Guan, D. & Tian, M. (2024) Back to the Origin: Mechanisms of circRNA-Directed Regulation of Host Genes in Human Disease. *Noncoding RNA*, 10(5).

Zhang, Q., He, Y., Nie, M. & Cai, W. (2017) Roles of miR-138 and ISG15 in oral squamous cell carcinoma. *Exp Ther Med*, 14(3), 2329-2334.

Zhang, X. O., Wang, H. B., Zhang, Y., Lu, X., Chen, L. L. & Yang, L. (2014) Complementary sequence-mediated exon circularization. *Cell*, 159(1), 134-147.

Zhang, Y., Zhang, X. O., Chen, T., Xiang, J. F., Yin, Q. F., Xing, Y. H., Zhu, S., Yang, L. & Chen, L. L. (2013) Circular intronic long noncoding RNAs. *Mol Cell*, 51(6), 792-806.

Zhou, X. E., Suino-Powell, K., Ludidi, P. L., McDonnell, D. P. & Xu, H. E. (2010) Expression, purification and primary crystallographic study of human androgen receptor in complex with DNA and coactivator motifs. *Protein Expr Purif*, 71(1), 21-7.

Cancer Research UK. Prostate cancer statistics. Available at:
<https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/prostate-cancer> (Accessed: 27 April 2026).

Oral Health Foundation. Oral cancer statistics and facts. Available at:
<https://www.dentalhealth.org/thestateofmouthcancer> (Accessed: 27 April 2026).