

Impact of Elevated Estrogen on the Testis with Potential Implications for Prostate  
Carcinogenesis

by

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## ABSTRACT

Loss of the androgen-regulated gene *Nkx3.1* leads to prostatic intraepithelial neoplasia (PIN), a precursor to prostate cancer (PCa). Elevated testicular estrogen may disrupt hormonal balance in the prostate and downregulate *Nkx3.1* expression by indirectly suppressing androgen production via the hypothalamic-pituitary-gonadal axis. Previous research has shown that increased levels of estrogen within the prostate can induce PIN. However, the effects of elevated estrogen levels in the testis, particularly when *Nkx3.1* is absent and their potential downstream impact on prostate carcinogenesis remain unknown. This study investigates the effects of systemically elevated 17 $\beta$ -estradiol (E2) levels on the testis in the absence of *Nkx3.1*. Testicular morphology and connective tissue organisation were assessed by histological analysis following prolonged exposure to elevated E2 via subcutaneous implantation of 90-day extended-release pellets. Cancer-related gene expression was analysed by RT-qPCR after acute administration of elevated E2 by subcutaneous injection. Alterations in testicular morphology and connective tissue organisation were observed; however, no significant changes in gene expression were detected in *Nkx3.1*-deficient mice in response to E2 treatment. *Nkx3.1*-intact mice exhibited reduced *Esr1*, increased *Esr2*, increased *Myc* and unchanged *Trp53* expression levels following E2 treatment. These findings show that elevated testicular E2 induces tissue abnormalities in the absence of *Nkx3.1* and regulates cancer-related gene expression when *Nkx3.1* is present, suggesting that *Nkx3.1* may modulate testicular response to estrogen, with potential implications for prostate carcinogenesis.

## GENERAL SUMMARY

Prostate cancer often develops from early tissue changes in the prostate called prostatic intraepithelial neoplasia (PIN), which can occur when the *Nkx3.1* gene important for prostate growth and maintenance stops functioning properly. Estrogen produced in the testis may affect hormone balance in the prostate and reduce *Nkx3.1* activity. Previous studies have shown that high estrogen in the prostate can lead to PIN. In this study, we investigated the effects of high levels of estrogen on the testis when *Nkx3.1* is absent. The results showed that elevated estrogen caused changes in testicular tissue but did not significantly change the activity of genes linked to cancer when *Nkx3.1* was absent. However, when *Nkx3.1* was present high estrogen levels led to significant changes in the activity of these genes. Our findings suggest that *Nkx3.1* may control how the testis responds to estrogen, which could influence prostate health.

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## LIST OF ABBREVIATIONS

ADT:	Androgen deprivation therapy
AR:	Androgen receptor
BRCA1:	Breast cancer 1
BRCA 2:	Breast cancer 2
cDna:	Complementary DNA
c-Myc:	Cellular Myc
CO <sub>2</sub> :	Carbondioxide
CRPC:	Castration-resistant prostate cancer
CYP19A1:	Cytochrome P450 family 19 subfamily A member 1
DEPC:	Diethyl Pyrocarbonate
dH <sub>2</sub> O:	Deionized water
DHT:	5 $\alpha$ -dihydrotestosterone
DNA:	Deoxyribonucleic acid
dNTP:	Deoxynucleotide triphosphate
E2:	17 $\beta$ -estradiol
ECM:	Extracellular matrix
EDTA:	Ethylenediaminetetraacetic acid
ER $\alpha$ :	Estrogen receptor alpha
ER $\beta$ :	Estrogen receptor beta
ER:	Estrogen receptor
ER $\alpha$ KO:	Estrogen receptor alpha knockout
ER $\beta$ KO:	Estrogen receptor beta knockout
Esr1:	Estrogen receptor 1 gene
Esr2:	Estrogen receptor 2 gene

FGFs:	Fibroblast Growth Factors
FOXA1:	Forkhead box protein A1
FSH:	Follicle stimulating hormone
GnRH:	Gonadotropin-releasing hormone
H <sub>2</sub> O:	Water
H&E:	Hematoxylin and eosin
HGPIN:	High-grade PIN
HOXB13:	Homeobox B13
HPG axis:	Hypothalamic-pituitary-gonadal axis
LGPIN:	Low-grade PIN
LH:	Luteinizing Hormone
LNCap:	Lymph Node Carcinoma of the Prostate (Androgen-dependent human prostate cancer cell line)
MAPK/ERK:	Mitogen-activated protein kinase/ extracellular signal-regulated kinase
MOPS:	3-(N-morpholino)propanesulfonic acid
mRNA:	Messenger ribonucleic acid
NaOH:	Sodium hydroxide
Nkx3.1:	Nkx3 homeobox 1
PI3K/AKT:	Phosphoinositide 3-kinase/AKT
P53:	Tumour suppressor protein encoded by the TP53 gene
P63:	Tumour protein 63
PBO:	Placebo
PC-3:	Prostate cancer 3 (Androgen-independent human prostate cancer cell line)
PCa:	Prostate cancer
PCR:	Polymerase chain reaction

PIN:	Prostatic intraepithelial neoplasia
RDD:	RNase-Free DNase reaction buffer
RLT:	RNA lysis buffer
RNA:	Ribonucleic acid
RPE/C:	RNA wash buffer II
RPM:	Revolutions per minute
RT-qPCR:	Real time quantitative reverse transcription polymerase chain reaction
RT:	Reverse transcriptase
RWI:	RNA wash buffer I
SEM:	Standard error of the mean
SHH:	Sonic Hedgehog
SRD5A1/2:	Steroid -5 $\alpha$ -reductase 1 and Steroid -5 $\alpha$ -reductase 2
TAE:	Tris-Acetate-EDTA
Trp53:	Tumour protein 53
UGS:	Urogenital sinus
UV:	Ultraviolet

## **LIST OF APPENDICES**

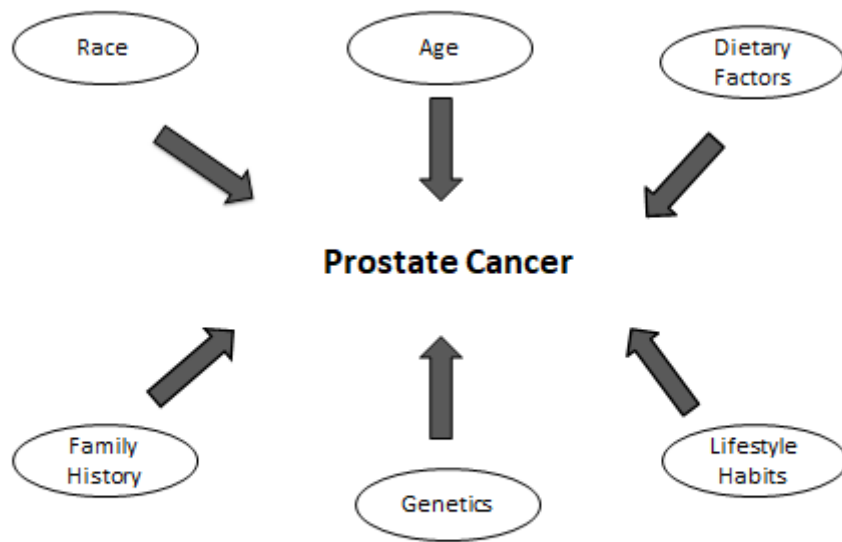
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## **CHAPTER 1: INTRODUCTION**

### **1.1 Epidemiology of Prostate Cancer**

Prostate cancer (PCa) is one of the most prevalent malignancies among men worldwide and a leading cause of cancer-related death [17, 89]. According to global cancer statistics, it is the second most frequently diagnosed cancer in men, following lung cancer and poses a major public health challenge [24].

Age is the most significant risk factor for this disease, with the highest incidence observed in men aged 65 and older [57, 78, 122, 146]. In addition to age, several other risk factors including race, family history, genetics, environmental exposures, lifestyle habits and dietary factors have been implicated in prostate carcinogenesis [13, 146].



**Figure 1: Schematic of prostate cancer risk factors.**

Risk factors for prostate cancer include race, age, dietary factors, family history, genetics and lifestyle factors.

The incidence of PCa varies widely across different populations and ethnic groups [32, 54, 108]. Men of African descent exhibit the highest incidence and have a greater tendency to develop more aggressive pathological features, leading to poorer survival outcomes and higher mortality rates [75, 146]. In contrast, Asian men have the lowest incidence rate, which may be attributed to lifestyle, environmental and genetic factors [61, 73, 82]. Most prostate tumours result from acquired somatic mutations; however, some arise from inherited germline mutations [107, 141]. A positive family history increases the likelihood of developing PCa, particularly in men with first-degree relatives affected by the disease, with the risk being higher when the relative has early-onset PCa diagnosed before age 55, suggesting a genetic predisposition [15]. Mutations in genes such as *HOXB13*, *BRCA1* and *BRCA2* have been associated with an increased risk of developing PCa [19, 62, 141].

PCa is a heterogeneous disease with multiple histological subtypes. Adenocarcinoma is the most common, accounting for approximately 95% of all PCa cases, while rarer subtypes include neuroendocrine PCa, small cell carcinoma and squamous cell carcinoma [106, 131].

PCa progression ranges from localized to advanced, aggressive metastatic disease. It is classified into low-, intermediate- and high-risk groups based on the Gleason score, prostate-specific antigen levels and clinical staging [7, 141].

A number of environmental, dietary and lifestyle factors such as high-fat diets, smoking, physical inactivity have also been linked to PCa incidence [86, 109, 144]. Treatment strategies typically include prostatectomy, chemotherapy, radiation therapy, immunotherapy and hormonal therapy [7, 120, 129, 141].

## 1.2 Prostate Gland Anatomy and Development

The human prostate gland is a walnut-sized organ located beneath the urinary bladder, anterior to the rectum and encircling the proximal urethra [10, 114]. It is the largest male sex accessory gland and a vital component of the male reproductive system, playing a crucial role in supporting sperm nourishment and motility by secreting essential proteins, enzymes, prostate-specific antigen and other nutrients into the seminal fluid [10, 26, 114, 138].

The prostate develops from the budding of the epithelium within the endodermal layer of the urogenital sinus (UGS), a structure derived from the caudal hindgut endoderm [10, 26, 87]. Prostate organogenesis is regulated by androgens synthesized by the fetal testes during embryogenesis, around 8-9 weeks gestation in humans and gestational days 13-15 in mice [26, 37, 53, 114].

During embryonic development, fetal testicular androgens act by binding to the androgen receptor (AR) in the surrounding mesenchyme, which originates from the mesodermal layer of the UGS [26, 53, 114]. AR signalling in the mesenchymal cells stimulates the secretion of paracrine factors, including Fibroblast Growth Factors (FGFs) and Sonic Hedgehog (SHH), which mediate epithelial-mesenchymal interactions and drive growth and differentiation of the gland [26, 114].

Elongation and branching of epithelial buds during embryonic development lead to the formation of glandular heterogeneous structures, including ducts and acini, which are typically lined by pseudostratified columnar epithelium. During pubertal development, these glandular structures undergo functional differentiation, enabling the production, secretion and transportation of prostatic fluid, a key component of seminal fluid [59, 114].

As the prostate continues to develop during puberty, the surrounding mesenchyme differentiates into fibromuscular stroma composed of smooth muscle cells and connective tissue, which provide structural support and regulate epithelial function in the mature prostate [114].

### **1.2.1 Cell Types in Prostate Gland**

Intercellular communication through ligand-receptor interactions is crucial for tissue development, maintenance and function [135]. The interactions between the glandular epithelial cells of the prostate and the surrounding fibromuscular stromal cells are essential for normal prostate development and differentiation [38, 43, 142]. Disruptions in signal transduction pathways can impair normal prostate homeostasis and contribute to disease onset and progression [56, 67].

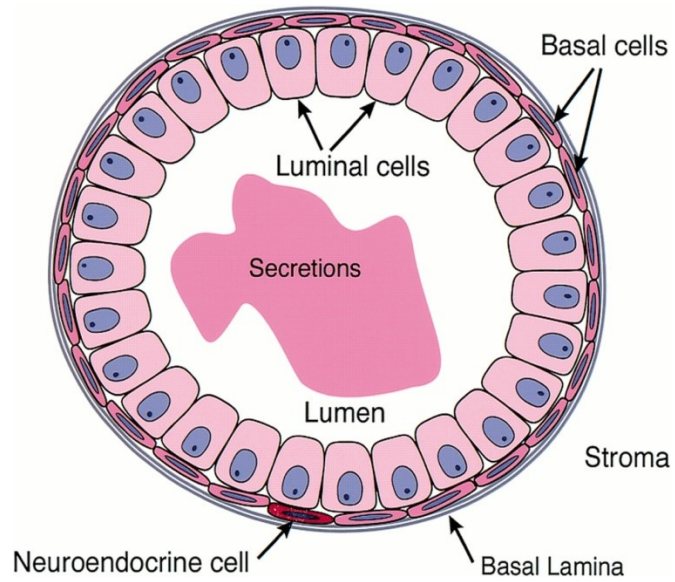
The glandular epithelium, which forms the initial structures of the prostate, consists of three major cell types: differentiated columnar luminal cells, which produce the functional prostatic secretory proteins involved in seminal fluid production; flat basal cells, which act as progenitor cells supporting the maintenance of the epithelial tissue and rare neuroendocrine cells, which are involved in hormone secretion [113, 149].

Luminal cells make up the majority of the epithelial cell population, typically exhibiting a wavy, papillary-like appearance with eosinophilic cytoplasm and round nuclei. Basal cells, located between the basement membrane and the luminal cells have pale cytoplasm and oval-shaped nuclei. Neuroendocrine cells are sparsely distributed throughout the epithelium, constituting only a small fraction of the total prostatic epithelial cells and are mainly found in the basal layer [7].

Secretory luminal cells express both cytokeratin 8 and AR and are dependent on AR signalling for growth and survival. Basal cells express cytokeratin 5 and the transcription factor p63 and are

androgen-independent due to low or absent AR expression, allowing them to survive in the absence of androgen signalling [68, 113].

The fibromuscular stroma surrounding the epithelial layer consists of fibroblasts and smooth muscle cells embedded within an extracellular matrix (ECM) rich in collagen and elastic fibers, with endothelial and immune cells also present within the stroma [111, 142, 149].



**Figure 2: Cell types in the prostate gland.**

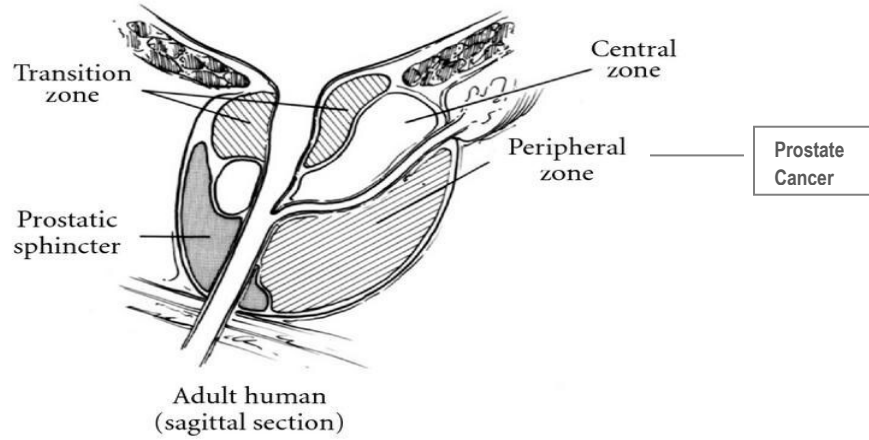
The prostate epithelium comprises three major cell types: luminal, basal and neuroendocrine cells. Figure reproduced from Abate-Shen and Shen [1].

### **1.2.2 Morphology of the Prostate**

The normal adult male prostate is morphologically composed of three distinct anatomical regions: the peripheral, transition and central glandular zones, surrounded by the anterior fibromuscular stroma [7, 10, 26, 142].

The central zone surrounds the ejaculatory ducts and is located posterior to the transition zone and proximal to the urethra. The transition zone encircles the proximal prostatic urethra, while the peripheral zone forms the outermost region of the gland, encompassing both the central and transition zones [142].

PCa primarily arises from the uncontrolled proliferation of abnormal luminal epithelial cells within the peripheral zone, which is the largest region of the prostate, comprising 70–75% of the gland and containing the majority of the prostatic glandular epithelium [7, 26, 53, 142, 149].



**Figure 3: Anatomy of the human prostate.**

The human prostate gland is composed of three morphological zones: the peripheral, transition and central zones. Figure adapted from McNeal [100] and Valkenburg and Williams [140].

### **1.3 Testis Development**

The testis is a paired male gonadal organ located within the scrotum, playing a central role in male reproductive physiology and fertility. Testicular development begins early in embryogenesis from the gonadal ridge, which arises from the intermediate mesoderm. This developmental process is initiated by the expression of the sex-determining region Y (*SRY*) gene on the Y chromosome, which activates a cascade of downstream genes, including *SOX9*, to promote testis differentiation [76, 70].

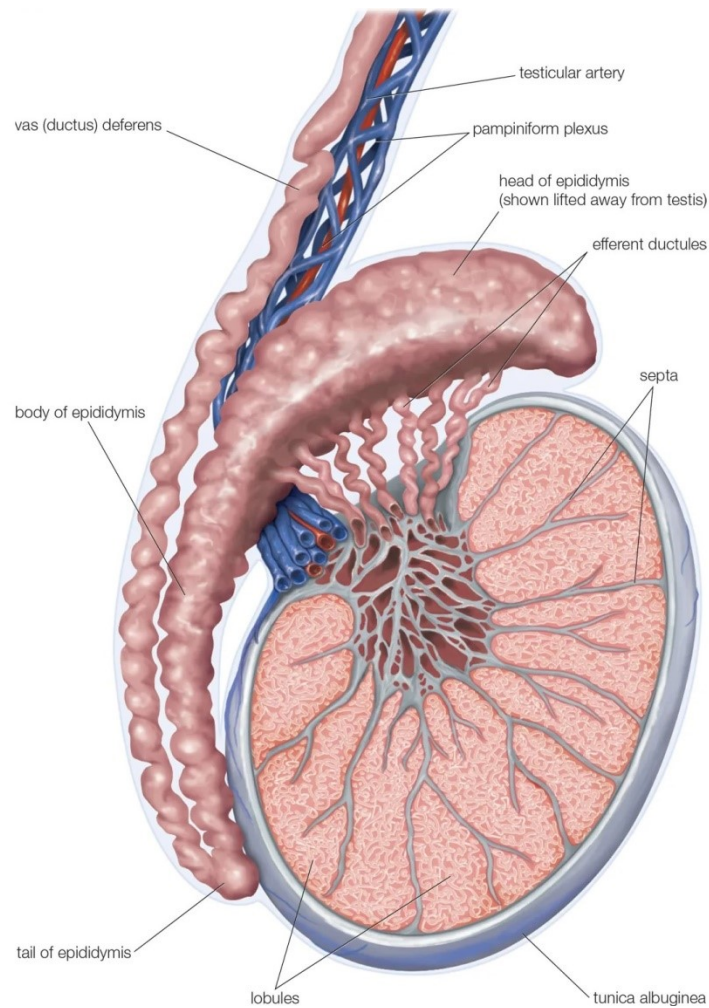
Sertoli cells surround germ cells to form testis cords, which later elongate and differentiate into seminiferous tubules. The spaces between the cords develop into the interstitium where fetal Leydig cells differentiate and begin producing testosterone around week 8 of gestation. By the end of gestation, testes descend into the scrotum. After birth, the seminiferous tubules continue to mature and initiate spermatogenesis at the onset of puberty [76, 70].

#### **1.3.1 Morphology of the Testis**

Testis tissue is divided into two main compartments: the seminiferous tubules and the interstitium. The seminiferous tubules comprise approximately 60-80% of the testis and contain germ cells along with two somatic cell types: Sertoli cells and peritubular cells. The germinal epithelium, also known as seminiferous epithelium surrounds the lumen of the tubules and is the site where germ cells differentiate into spermatozoa through meiosis. Differentiated germ cells are protected by blood–testis barrier, formed by the Sertoli cells, which support and maintain spermatogenesis after the onset of puberty [145].

The interstitium constitutes about 12-15% of the testis and occupies the space between the seminiferous tubules. It contains Leydig cells that synthesize and secrete male steroid hormones,

androgens. The interstitial compartment also includes loose connective tissue, immune cells, blood vessels, lymphatic vessels and nerves [145].



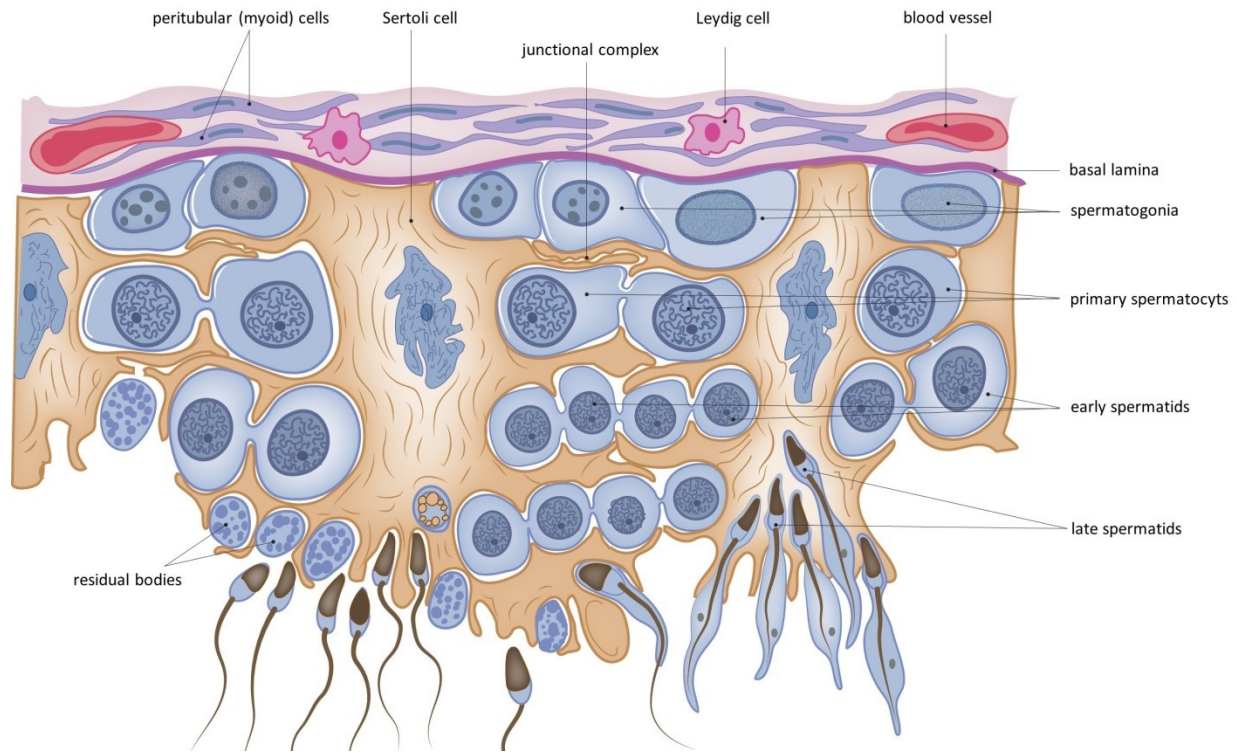
**Figure 4: Anatomy of the testis.**

The human testes are paired male gonadal organs located within the scrotum outside the abdominal cavity, as part of the male reproductive system. Figure adapted from Encyclopædia Britannica [71].

### **1.3.2 Testicular Cell Types**

Cell-cell interactions between testicular cells are critical for normal testis formation and function. Leydig cells account for about 10-20% of the interstitial tissue and are rich in smooth endoplasmic reticulum and mitochondria. The peritubular cells, present between the tubules and interstitial space and produce smooth muscle fibers that enable contractility, facilitating the transport of mature sperm through the testicular ducts to the epididymis. These cells also secrete components of the ECM, including collagen, which is essential for maintaining the structural integrity of the connective tissue [145].

Sertoli cells are situated on the basement membrane of the seminiferous tubules and extend towards the lumen. They comprise about 35-40% of the seminiferous epithelium and function as the supporting framework of the germinal epithelium. Germ cells are located within the seminiferous tubules and are responsible for the process of sperm production [145].



**Figure 5: Seminiferous tubule of the testis.**

The seminiferous tubules of the testis, where sperm is produced, are surrounded by a basement membrane and consist of germ cells supported by Sertoli cells. Leydig cells are located in the interstitial space between the tubules. Figure adapted from Ron et al. [133].

## **1.4 The Role of Hormones in Prostate and Testis**

### **1.4.1 Androgens**

It has been well-established that the prostate is an androgen-dependent organ which fails to develop without androgen-induced AR transcriptional activity [37]. The development of the prostate is mediated by circulating androgenic steroid hormones synthesized primarily by Leydig cells in the testes [81, 104]. In the testis, functional AR is essential for steroidogenic function, spermatogenesis, male fertility and the regulation of male secondary sexual characteristics [99, 145].

Tissue recombination experiments by Cunha and colleagues demonstrated that stromal AR signalling is essential for normal prostate development, regulating epithelial-mesenchymal paracrine interactions during prostate epithelial development [37]. Another study by Lee et al. supported these findings and provided further insights into the molecular mechanisms of mesenchymal AR signalling [87].

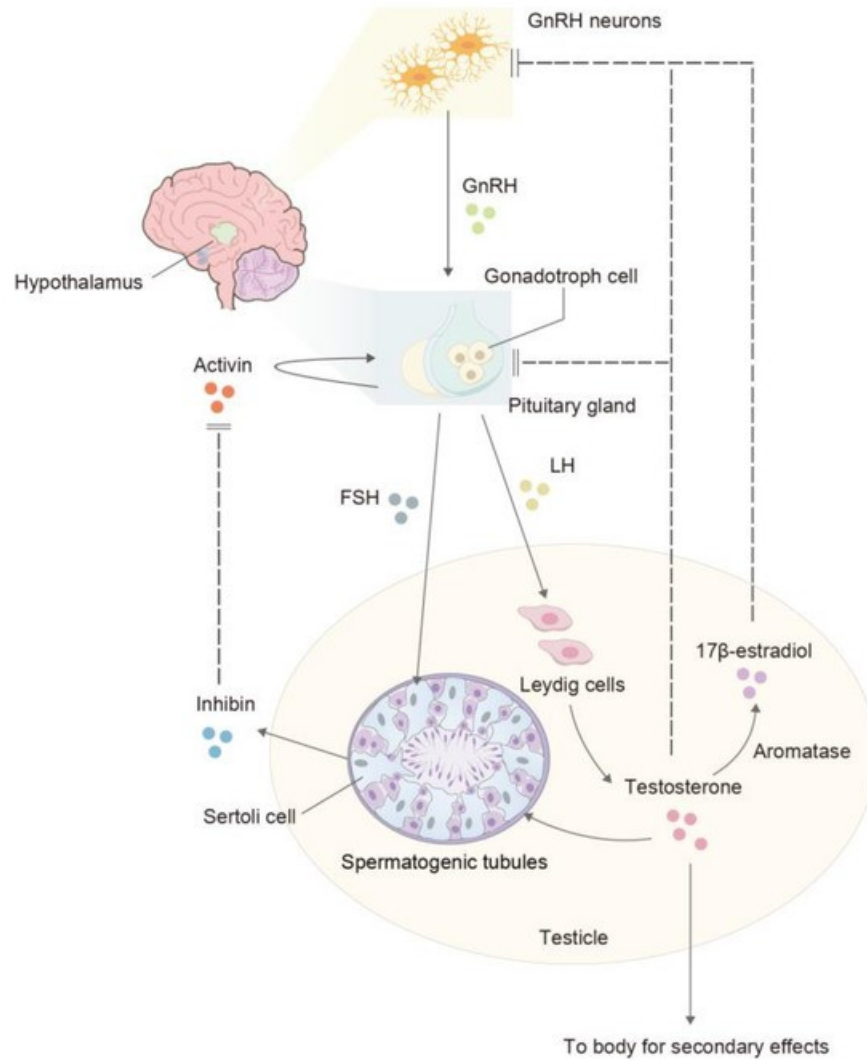
#### **1.4.1.1 Androgen Biosynthesis**

Androgen synthesis in adult males is tightly regulated by the hypothalamic–pituitary–gonadal (HPG) axis [3]. Pulsatile release of hypothalamic gonadotropin-releasing hormone (GnRH) stimulates the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland. The luteinizing hormone then signals to the Leydig cells of the testis to produce and secrete testosterone into circulation, which is the most abundant and predominant androgen in males [65, 102, 104].

5 $\alpha$ -dihydrotestosterone (DHT), a more potent metabolite of testosterone, is the principal androgen bound to the AR in prostate cells [150] and serves as the primary androgen responsible for regulating prostatic development [37, 53, 65].

Testosterone is converted to DHT by steroid-5 $\alpha$ -reductases (SRD5A1/2), first within the stromal cells of the developing UGS during early prostate development and later in both the prostate epithelium and stroma in the mature prostate via the DHT synthesis pathway [37, 53, 142].

DHT binds to the AR with higher affinity [81] compared with testosterone, thereby activating critical AR signalling pathways that regulate prostate growth and function [37, 102].



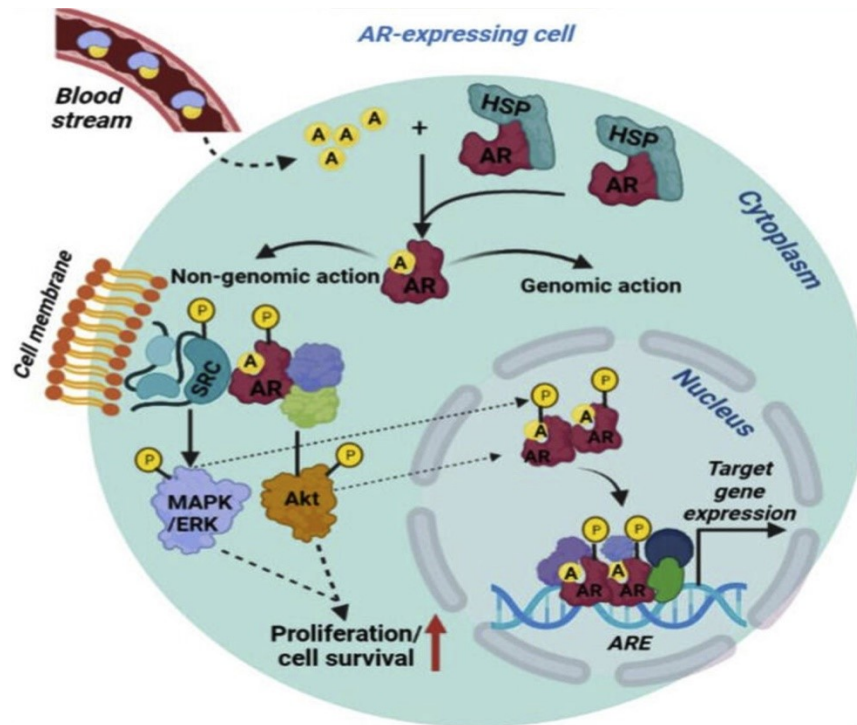
**Figure 6: Regulation of androgen synthesis by the HPG axis.**

The hypothalamus regulates the biosynthesis of pituitary hormones LH and FSH through GnRH. LH then induces Leydig cells of the testis to secrete testosterone. Figure reproduced from Li et al. [90].

#### **1.4.1.2 Mechanism of Action: Androgen Receptor Signalling**

Normal prostate growth, differentiation, function and maintenance of cellular homeostasis are regulated by androgenic signalling through activation of the AR a ligand-dependent nuclear hormone transcription factor and a member of the steroid hormone receptor superfamily present in both the prostate epithelium and stroma [3, 39, 87]. Similarly, normal testis function and maintenance of tissue homeostasis are also regulated by androgenic signalling via AR activity in peritubular, Sertoli and Leydig cells [99, 145].

Once androgens are released into the blood stream, they enter AR-expressing cells in the target organ, where they bind to and activate the AR [65, 104]. AR signalling is initiated when ligands testosterone or dihydrotestosterone (DHT) bind to the AR in the cytoplasm [146]. Upon binding, the AR-ligand complex translocates to the nucleus, where it interacts with pioneer factors, such as FOXA1, to facilitate chromatin remodelling before binding to chromatin. The AR-ligand complex then binds directly to specific DNA sequences, known as androgen response elements, in the promoter or enhancer regions of target genes and recruits co-activators to regulate the transcription of androgen-responsive genes [23, 103].



**Figure 7: Androgen receptor signalling.**

Androgens are released into the bloodstream, where they enter the AR-expressing cells and activate the AR. In the genomic action, the AR-ligand complex binds ARE in the target gene and initiates gene expression. In the non-genomic action, the AR-ligand can bind membrane-bound receptors and initiate signalling cascades that promote cell proliferation and survival. Figure adapted from Naamneh et al. [104].

## **1.4.2 Estrogen**

Androgens are known to have a major role in male reproductive organs; however, normal tissue development and function also depend on the actions of sex steroid hormones, estrogens. Estrogens play essential physiological roles in the prostate, influencing embryonic developmental processes, regulating cellular differentiation, ductal branching morphogenesis and maintaining tissue homeostasis [16]. In the testis, estrogens regulate steroidogenesis and the proliferation, maturation, differentiation and apoptosis of germ cells during spermatogenesis, thereby contributing to the maintenance of testicular homeostasis [34, 28].

### **1.4.2.1 Estrogen Biosynthesis**

Androgens produced by the testes and in circulation in the blood stream are metabolized into estrogen; predominantly estradiol (E2), the most potent form of estrogen in men. This conversion is catalyzed by the aromatase enzyme (cytochrome P450 19A1), which is encoded by the *CYP19A1* gene [16, 77].

In the male body system aromatase is expressed in various tissues, including the testes, adipose tissue, prostate, brain, blood vessels and skin [23]. The prostate expresses aromatase at a relatively low level compared to these other tissues, enabling local estrogen biosynthesis primarily in stromal cells [16, 23]. In the testis, estrogen synthesis occurs in the germ cells, Leydig and Sertoli cells and these cells serve as the major source of estrogens in the male reproductive tract [16].

Estrogen has significant direct and indirect effects on the prostate gland. Elevated testicular estrogen exerts systemic endocrine effects by signalling to the hypothalamus and pituitary gland to decrease the production and release of GnRH and LH, respectively. The suppression of LH reduces stimulation of Leydig cells in the testes, resulting in a decline in testosterone production

[65, 77]. This negative feedback loop in the HPG axis disrupts systemic hormonal balance, indirectly altering the androgen-estrogen ratio of the prostate [51, 124].

Furthermore, estrogen can be synthesized locally within the prostate through the aromatization of testosterone to estrogen and acts in a paracrine manner by binding to intraprostatic estrogen receptors, directly influencing prostate tissue [31, 51, 124]. Notably, the reduction in androgen levels as a result of increased estrogen levels in the testis may also indirectly downregulate *Nkx3.1* expression.

#### **1.4.2.2 Mechanism of Action: Estrogen Receptor Signalling**

Estrogen exerts its biological effects through cell signalling via two major receptor subtypes: estrogen receptor alpha ( $ER\alpha$ ) and estrogen receptor beta ( $ER\beta$ ), which are encoded by the estrogen receptor 1 (*ESR1*) and estrogen receptor 2 (*ESR2*) genes, respectively [16, 31, 34, 77]. Both  $ER\alpha$  and  $ER\beta$  are members of the nuclear receptor superfamily and function as ligand-activated transcription factors in the prostate [16] and testis [28].

In the testis,  $ER\alpha$  is highly expressed in Leydig cells of the interstitial tissue and is also present in Sertoli cells and germ cells within the seminiferous tubules.  $ER\beta$  is predominantly expressed in Sertoli cells and germ cells, with lower levels of expression in Leydig cells compared to  $ER\alpha$  [28].

In prostate tissue,  $ER\alpha$  is primarily localized in stromal cells and expressed at lower levels in the androgen-independent basal cell layer of the epithelium.  $ER\beta$ , the most prevalent estrogen receptor (ER) in the human prostate, is predominantly expressed in the androgen-dependent luminal epithelial cells [16, 21, 120].

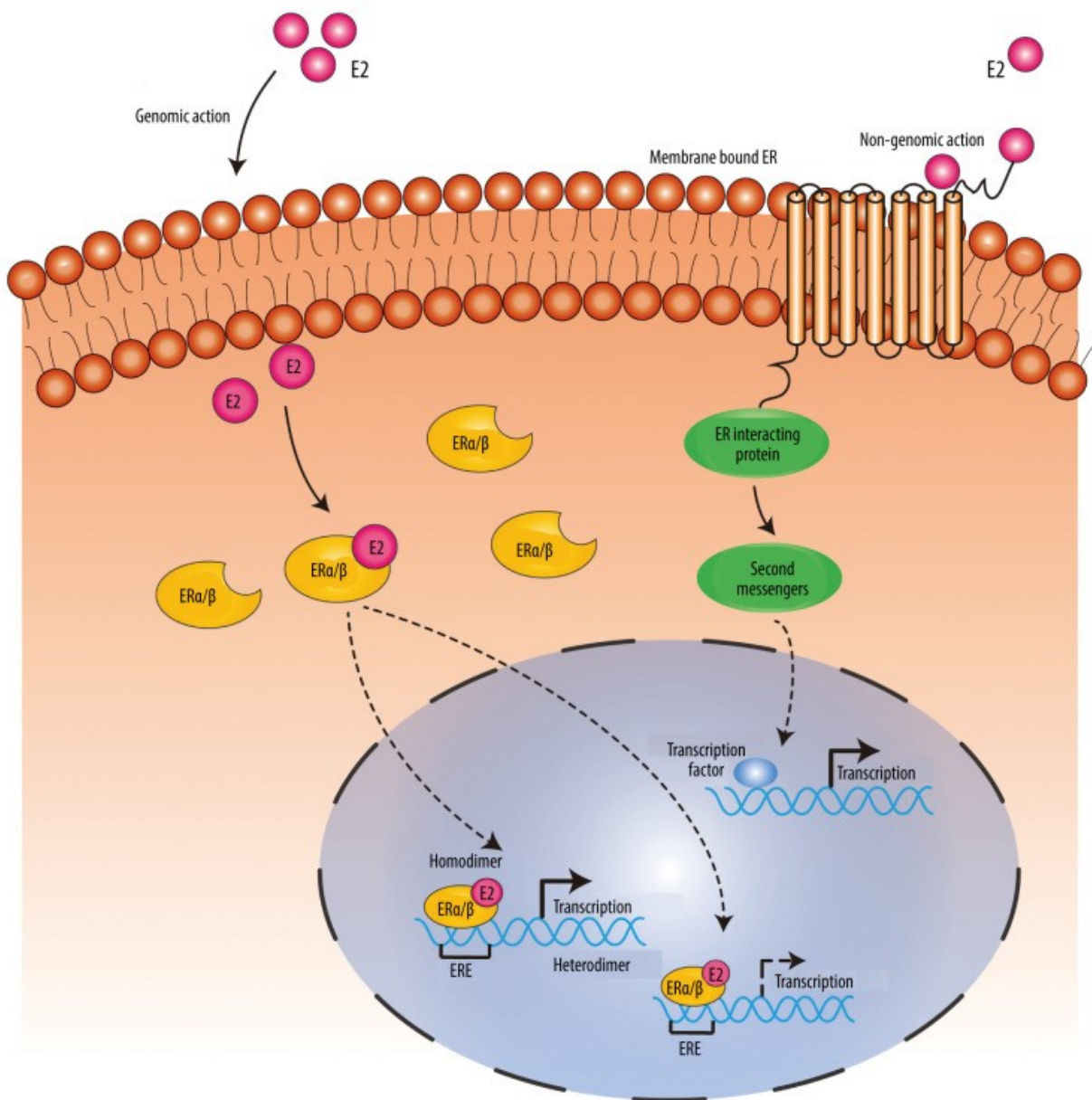
The classical mechanism of ER action involves estrogen binding to and activating either ER $\alpha$  or ER $\beta$  receptors in the cytoplasm [3, 23]. Upon ligand binding, the activated receptor undergoes a conformational change, dimerizes and translocates to the nucleus. In the nucleus, the ligand-receptor complex binds to specific estrogen response elements in the promoter region of target genes, directly regulating their transcription in response to estrogen [3, 16, 23, 77].

In addition to their genomic actions, ERs also initiate non-genomic signalling through cytoplasmic and membrane-associated receptors such as G-protein-coupled estrogen receptors. These receptors rapidly activate key intracellular signalling cascades including the mitogen-activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK) and phosphoinositide 3-kinase/AKT (PI3K/AKT) pathways. These pathways influence gene transcription indirectly by activating a network of downstream effectors, driving immediate responses in prostate [16, 77] and testicular cells [29, 34]. Both genomic and non-genomic signalling pathways play critical roles in regulating cellular processes such as growth, survival, apoptosis and differentiation in prostate [16, 23, 77, 91] and testicular cells [29, 34].

ER $\alpha$ , primarily mediates the proliferative and pro-inflammatory effects of estrogen on prostate tissue by regulating stromal cell differentiation and function. ER $\alpha$ 's effects on stromal cells lead to the activation of growth factors and other paracrine signals that indirectly modulate the proliferation and differentiation of epithelial cells. On the other hand, ER $\beta$  is often associated with anti-proliferative and pro-differentiation effects, modulating epithelial processes, maintaining cellular differentiation and tissue homeostasis [31, 121].

ER $\alpha$  primarily regulates steroidogenesis and modulates testosterone synthesis in Leydig cells; it influences testicular structure and function by acting on Sertoli cells. ER $\alpha$  activity also

contributes to fluid reabsorption in the efferent ducts, which is essential for maintaining sperm transport and testicular morphology. In contrast, ER $\beta$  regulates spermatogenesis and helps maintain testicular homeostasis, with Sertoli cells providing critical support for germ cell maturation within the seminiferous tubules [29].



**Figure 8: Estrogen receptor signalling.**

In genomic action, estrogen regulates the transcription of target genes by binding to and activating ER receptors in the cytoplasm or nucleus. In non-genomic action, estrogen acts by binding to receptors on the cell membrane. Figure reproduced from Li et al. [91].

## **1.5 Hormonal Imbalance and Its Impact on Prostate Health**

The interplay between androgen and estrogen, as well as the balance of their respective receptors and downstream signalling pathways is essential for maintaining prostate health. The male body tightly regulates this hormonal balance and disruptions in their interactions can impair prostate development and cellular homeostasis.

Aromatization of androgens to estrogen within the prostate tissue can lead to elevated estrogen levels potentially due to increased aromatase activity. Elevated estrogen levels negatively influence AR signalling and disrupt hormone balance within the prostate. Local prostatic estrogen directly regulates the expression of AR co-regulators and co-repressors, influencing AR transcriptional activity and modulating the differentiation and function of androgen-responsive genes [77].

Elevated levels of estrogen primarily originating from the testes and in systemic circulation indirectly suppress androgen synthesis, thereby altering androgen signalling through the negative feedback regulation of the HPG axis [65, 77]. This disruption in circulating systemic hormone levels indirectly impacts prostate cells by downregulating AR activity within the prostate and increasing local estrogen levels, creating an imbalance that alters normal cellular signalling in adulthood [96, 127].

Research has shown that hormonal imbalance may impair prostate development and the maintenance of prostate health. Elevated levels of estrogen in the prostate have been associated with the development of PIN in adulthood [49, 123]. Prins and colleagues also found that exposure to high levels of estrogen during early development in rodents is associated with

increased susceptibility to hyperplasia and dysplasia, predisposing the prostate to neoplasia with aging through altered gene expression and cellular differentiation [115].

Their findings demonstrated that elevated estrogen levels alter the expression of steroid receptors (AR, ER $\alpha$  and ER $\beta$ ) and disrupt normal receptor signalling, leading to morphological and cellular differentiation abnormalities. These changes were linked to alterations in the expression of key prostatic developmental genes such as *Nkx3.1*, *Hoxb-13*, *Fgf10* and *Shh*, highlighting how hormonal imbalance can impact prostate health at any stage of development [115 – 117].

Estrogen plays a significant regulatory role in the prostate by modulating the expression and activity of ARs, influencing how the prostate responds to androgen signalling. This cross-talk between estrogen and androgen signalling pathways is critical for maintaining prostate gland homeostasis. Hormonal imbalance - whether due to elevated estrogen, reduced androgen levels or impaired receptor signalling - may drive aberrant cellular differentiation and increase susceptibility to neoplastic transformation.

### **1.5.1 Sex Steroid Hormones and Prostate Cancer Pathogenesis**

PCa is a well-established hormone responsive malignancy, with sex steroid hormone signalling pathways playing a central role in its development, maintenance and progression through the activation of a complex regulatory network.

The AR is a key driver of prostate tumour growth, regulating the expression of genes that influence cell proliferation, differentiation, and survival in prostate tissue. Estrogen also influences prostate carcinogenesis primarily through ER $\alpha$  and ER $\beta$ , with ER $\alpha$  often linked to pro-tumourigenic activity promoting inflammation and proliferation, while ER $\beta$  is associated with anti-proliferative effects mediating differentiation and apoptosis.

Both androgen and estrogen have been reported to stimulate proliferation of prostate cancer cells in in vitro studies, mainly through receptor-mediated mechanisms. Studies using animal models suggest that the combined actions of androgens and estrogens may be required for the malignant transformation of prostate epithelial cells.

The complex interplay between androgens and estrogens in prostate biology underscores their critical roles in PCa development, maintenance and progression.

#### **1.5.1.1 Androgen Receptor Signalling in Prostate Cancer**

Androgen, particularly its biologically active form, DHT, is the major regulator of neoplastic growth in prostate tissue and the survival of PCa, as it is expressed throughout the various stages of the disease [103]. Molecular alterations to AR-mediated transcription can promote abnormal cell proliferation, inhibit apoptosis and enhance the survival of neoplastic cells [40, 103, 150]. Overactivation of the AR signalling pathway is a hallmark of prostate carcinogenesis [40].

Androgen deprivation therapy (ADT) is a well-established standard treatment for PCa. ADT involves deprivation of testicular androgen synthesis and inhibition of AR signalling through castration or drug treatment. It exerts a suppressive effect on tumour growth, leading to rapid luminal epithelial cell apoptosis and subsequent prostate involution [10].

Despite the diminished androgen activity, cancer progression persists, as ADT frequently fails and individuals ultimately develop resistance. This results in tumour recurrence and the development of an androgen-independent form of PCa, known as castration-resistant prostate cancer (CRPC), characterized by the reactivation of AR signalling despite low circulating androgen levels, which may eventually progress to metastatic CRPC [40, 141, 146].

Resistance to ADT often arises from genetic alterations in the AR, such as gene amplification, gain-of-function mutations, alternative splicing variants or altered expression of AR coactivator and corepressor proteins leading to persistent AR activity and continued tumour growth [120]. In particular, AR binding sites exhibit an increased mutation rate compared to other transcription factors, which is specific to PCa [103]. Studies have found that somatic mutations of the AR protein induce uncontrolled cellular proliferation and tumourigenesis, ultimately leading to prostate tumour development [103]. Additionally, AR splice variants, such as AR-V7, enable AR signalling in the absence of androgens, rendering ADT less effective [63, 148].

#### **1.5.1.2 Estrogen's Role in Prostate Carcinogenesis**

Estrogen and its receptors have been implicated in tumor cell proliferation and cancer progression. Although androgen signalling is the primary driver of prostate tumour growth, cell survival and disease progression [77, 120], numerous studies support a physiological role for estrogens in prostate carcinogenesis, particularly in relation to altered receptor cell signalling and hormonal imbalance in males [85].

It has been proposed that PCa does not develop in the absence of estrogen, even when androgen levels are elevated. In studies using aromatase knockout mice, which cannot synthesize estrogen, the absence of estrogen inhibited malignant transformation of the prostatic epithelium despite increased androgen levels [85, 120]. This underscores the essential role of estrogen in prostate carcinogenesis, suggesting that androgen alone is insufficient to drive tumourigenesis.

##### **1.5.1.2.1 Estrogen Receptor Signalling and Prostate Cancer**

The presence of ER $\alpha$  and ER $\beta$  in neoplastic prostate tissues suggests their involvement in the development and progression of PCa [16, 85]. These receptor subtypes mediate distinct and opposing effects on cellular functions, potentially due to their cellular localization [95]. ER $\alpha$

activation is often associated with pro-proliferative, anti-apoptotic and tumour promoting responses in the prostate, whereas ER $\beta$  may act as an onco-suppressor, exhibiting anti-proliferative, proapoptotic and tumour-suppressive effects [77, 120]. Disruptions in estrogen signalling, influencing the ER $\alpha$  to ER $\beta$  ratio, have been linked to PCa progression, with loss of ER $\beta$  and activation of ER $\alpha$  observed in human prostate tumours [77, 120].

In vivo studies have shown that ER $\alpha$  activation in ER $\beta$  knockout (ER $\beta$ KO) mice triggers abnormal cell proliferation and the development of premalignant lesions. Researchers observed epithelial hyperplasia and enhanced expression of AR-regulated genes in the prostates of ER $\beta$ KO mice, promoting the initiation of PCa [16, 85]. In contrast, prostates of ER $\alpha$  knockout (ER $\alpha$ KO) mice do not show neoplastic growth, but exhibit increased apoptotic activity, preventing PCa development. Findings from this study suggest that ER $\beta$  activation promotes tumor-suppressive functions, supporting its protective role in regulating cell differentiation [16, 85, 91]. In vitro studies have further highlighted the oncogenic role of ER $\alpha$  in promoting prostate tumor formation, with increased expression levels of ER $\alpha$  observed in PCa cells, while ER $\beta$  was found to be expressed at low levels [45, 85, 120]. This suggests that the ratio of ER $\alpha$ /ER $\beta$  is an important factor in estrogen-induced proliferation.

#### **1.5.1.2.2 Interactions between ER $\beta$ and Androgen Signalling**

ER $\beta$  activation in the prostate may prevent PCa initiation by downregulating androgen signalling and restricting androgen-induced cell proliferation, either directly or via ER $\alpha$  inhibition [16, 77, 85, 120]. ER $\beta$  and AR are co-regulatory receptor proteins influencing prostate cancer progression. They interact with each other to modulate upstream regulators and downstream target genes of AR at the transcriptional or post-transcriptional levels [91].

#### **1.5.1.2.3 The Proposed Dual Role of ER $\beta$ in Prostate Cancer**

While most studies suggest that ER $\beta$  mediates anti-proliferative effects that counteract the proliferative effects of androgens on the epithelium, some studies propose a dual role for ER $\beta$ , suggesting potential carcinogenic effects [77, 91, 120, 121]. ER $\beta$  has been shown to drive cell cycle progression in the androgen dependent human prostate cancer cell line, LNCap, stimulating proliferation [91, 120]. Moreover, another study demonstrated that activation of ER $\beta$  is associated with increased migration, invasion and proliferation of androgen-independent prostate cancer cells (PC-3) [94]. The proposed dual functionality of ER $\beta$  in PCa may be due to multiple splice variants of ER $\beta$  and the differences in their effects [45, 91].

#### **1.5.1.2.4 Roles of ER $\alpha$ and ER $\beta$ in Castration-Resistant Prostate Cancer**

Estrogen signalling pathways have also been implicated in the development and progression of CRPC. Studies have shown that, in response to ADT, when systemic androgen levels were reduced, prostate tumour cells upregulated intratumour androgen synthesis and sustained activation of AR signalling. This enabled continued tumour cell proliferation and the development of CRPC. Simultaneously, local production of E2 increased, largely due to elevated aromatase enzyme activity, contributing to enhanced metastasis in PCa cells [77].

After ADT, low androgen levels have been shown to reduce the functional ability of ER $\beta$  to suppress tumour growth, while increasing ER $\alpha$  activity and promoting prostatic epithelial proliferation of PCa cells and further progression to CRPC [77, 120].

ER $\alpha$  expression has been found to be upregulated in the prostatic epithelium in advanced human prostate cancers, including CRPC and metastatic PCa, while ER $\beta$  expression is lost in higher-grade PCa or CRPC [77, 120]. Clinical evidence has shown increased ER $\alpha$  expression and decreased or lost ER $\beta$  expression in biopsies from patients with high-grade carcinomas [27, 45].

A study by QU et al. revealed that castration resistance is linked to the presence of ER $\alpha$  mutations and low expression levels of ER $\beta$  [119]. Another study examining primary tumour tissues from patients who had undergone radical prostatectomy found that reduced ER $\beta$  levels are associated with CRPC [77].

The increased expression of ER $\alpha$  observed during the progression of PCa to CRPC suggests that ER signalling can bypass AR for tumour growth following ADT [77]. Interestingly, differentially expressed isoforms of ER $\beta$ , particularly ER $\beta$ 1, which may interact with estrogen signalling pathways that promote tumour growth and metastasis, have been suggested to play a pro-tumourigenic role in the progression to CRPC, potentially driven by interactions within the tumour microenvironment [77].

### **1.5.2 Age-Related Hormonal Imbalance and Carcinogenesis**

PCa often develops at an advanced age in older men when testosterone levels decline and estradiol levels become elevated [27, 44, 51]. Testosterone levels peak during early adulthood and gradually decrease with age, while estrogen levels remain relatively stable or slightly decrease in early adulthood but increase with age [33, 36, 51]. This hormonal shift in aging men may create a microenvironment conducive for the neoplastic transformation of prostate cells [27, 35, 126].

The age-related decline in testosterone production by the testes leads to an increased estrogen-to-androgen ratio in circulation [8, 46, 69]. This hormonal imbalance is associated with increased aromatase activity in the prostate, resulting in elevated local estrogen levels [36, 112]. Enhanced aromatase activity in other peripheral tissues, such as adipose tissue, also contributes to higher systemic estrogen levels [16, 112].

Studies have shown that PCa cell lines exhibit aberrant expression of the aromatase protein [16]. Additionally, increased aromatase expression and local estrogen production have been observed in advanced prostate cancer tissues, further supporting the link between estrogen signalling and prostate tumourigenesis [91, 92, 101].

These hormonal changes disrupt the balance between the ER and AR signalling pathways in the prostate. The interactions between these signalling pathways may amplify the effects of age-related hormonal imbalance. Age-related changes in AR sensitivity, such as AR overactivation, AR gene mutations, or the emergence of splice variants of AR, may alter AR and ER interactions and promote PCa progression [27, 63, 120]. Moreover, elevated estrogen levels can further enhance AR sensitivity and modulate downstream targets and signalling pathways of the AR, contributing to PCA progression [27, 63, 120].

In particular, the age-related decrease in androgens may diminish the tumor-suppressive effects of ER $\beta$ , whereas elevated estrogen levels may enhance ER $\alpha$  activity, promoting prostatic epithelial cell proliferation [16]. Prostate tissues from aging men are often hyperproliferative and show signs of prostatic intraepithelial neoplasia (PIN), a well-established precursor to PCa [85, 120]. Alterations in the ER signalling pathways - upregulation of ER $\alpha$  and loss of ER $\beta$  expression - have been confirmed in high-grade prostate lesions and CRPC [85].

Age-related hormonal imbalance disrupts cellular signalling pathways that maintain prostate homeostasis. Androgen-driven genes, such as *Nkx3.1*, play a crucial role in regulating prostate epithelial differentiation and drive PCa initiation and progression. Molecular studies have also identified several estrogen-responsive genes implicated in PCa progression including *Esr1* (ER $\alpha$ ), *Esr2* (ER $\beta$ ), *Myc* and *Trp53*, which contribute to enhanced epithelial proliferation and

resistance to apoptosis in PCa cells [16]. Furthermore, dysregulation of downstream signalling pathways, such as PI3K/AKT, MAPK/ERK and Wnt/ $\beta$ -catenin, may amplify the oncogenic effects of hormone signalling, driving prostate tumourigenesis [16].

Studies suggest that age-related hormonal imbalance may serve as a significant risk factor for PCa initiation. Elevated serum estrogen levels have been linked to an increased risk of PCa [16]. Epidemiological studies have reported that men, who exhibit higher serum E2 levels, are at a higher risk of developing PCa [27]. These findings suggest that increased estrogen levels may contribute to the initiation of PCa.

## **1.6 *Nkx3.1***

*Nkx3.1* is a member of the NK homeobox gene family, essential for male reproductive health and exhibits tissue-specific expression in both the prostate and testis [10, 12, 22, 55, 110, 128, 132]. It is predominantly expressed in the lateral region of the prostate UGS epithelium during embryogenesis, contributing to prostate bud formation. It continues to be expressed in the luminal epithelial cells of the gland throughout all stages of prostate differentiation [10, 142]. Additionally, *Nkx3.1* is expressed at low levels in Sertoli cells [11] and spermatogenic germ cells within the seminiferous tubules of the testes [132].

### **1.6.1 Functional Role of *Nkx3.1* in Prostate and Testis**

NKX3.1 is an androgen-regulated transcription factor that regulates the expression of genes involved in cellular growth, differentiation and the maintenance of homeostasis in the male reproductive system [12, 134].

It serves as a key regulator of embryonic and adult prostate development and is essential for ductal morphogenesis, epithelial cell differentiation and secretory function [53].

In the testis, *Nkx3.1* is involved in maintaining testicular development and function, supporting spermatogenesis [12, 55, 110, 132].

### **1.6.2 *Nkx3.1* and Androgen Receptor Signalling**

*Nkx3.1* is a key downstream target of AR signalling and the AR positively regulates *Nkx3.1* expression and activity [10, 12]. In turn, NKX3.1 negatively modulates AR gene transcription, influencing AR levels and AR-regulated pathways, which are crucial for maintaining normal prostate cell proliferation and survival [10, 12]. AR overactivation or mutations might disrupt this regulatory balance.

Experimental studies have demonstrated that both NKX3.1 and AR form a feedback signalling loop [12, 88]. The expression levels of *Nkx3.1* mRNA decrease significantly in response to castration [20], while it increases during sexual maturation [128] and upon androgen stimulation in LNCaP [66], establishing that *Nkx3.1* expression is androgen-dependent.

Furthermore, a study by Lei and colleagues demonstrated through luciferase reporter assays that *Nkx3.1* acts as a negative modulator of AR [88]. Overexpression of *Nkx3.1* inhibited mRNA and protein levels of AR in cell cultures, whereas prostates from *Nkx3.1* knockout mice exhibited increased AR levels [88]. These findings suggest that normal levels of *Nkx3.1* prevent excessive AR activation, which is essential for maintaining prostate homeostasis.

Given the interactions between AR and ER pathways, estrogens may indirectly modulate *Nkx3.1* expression and activity by influencing AR signalling, either through alterations in AR co-regulators or via crosstalk between the ER and AR pathways.

### 1.6.3 *Nkx3.1* in Prostate Cancer

In addition to being a prostate epithelium-specific marker, *Nkx3.1* is a well-established tumour suppressor gene downregulated in PCa [12, 25]. It plays a crucial role in preventing abnormal cell growth and differentiation within the prostate. Immunohistological analysis of human prostate tumours has shown that NKX3.1 protein expression was significantly reduced in PCa patients, with NKX3.1 levels decreasing as PCa progressed [10]. It has also been shown that the upregulation of *Nkx3.1* decreased cell proliferation in vitro and cancer growth in vivo [12].

The *Nkx3.1* gene is located on chromosome 8p21, a genomic region that frequently undergoes loss of heterozygosity and is often deleted in both PIN and PCa [10, 141].

The critical role of *Nkx3.1* in prostate development is demonstrated by defects observed in *Nkx3.1*-deficient mouse models. Null mutation of the *Nkx3.1* gene leads to reduced prostatic protein secretion, impaired ductal branching and the development of prostatic lesions, including epithelial hyperplasia and dysplasia [10, 53]. These morphological alterations closely resemble the phenotype of pre-cancerous lesions of the prostate; PIN [2, 79, 110]. Loss of *Nkx3.1* is associated with the downregulation of genes critical for normal prostate differentiation [10, 53].

Heterozygous *Nkx3.1* mutant mice also develop PIN-like lesions, indicating haploinsufficiency for this phenotype [2, 110]. Loss of heterozygosity is a common genetic alteration in PCa [25] and *Nkx3.1* haploinsufficiency is considered an initiating event in prostate carcinogenesis [10, 58]. These findings indicate that loss of NKX3.1 protein function is a key driver of prostate tumourigenesis, contributing to disease initiation and progression [141].

## 1.7 Prostatic Intraepithelial Neoplasia

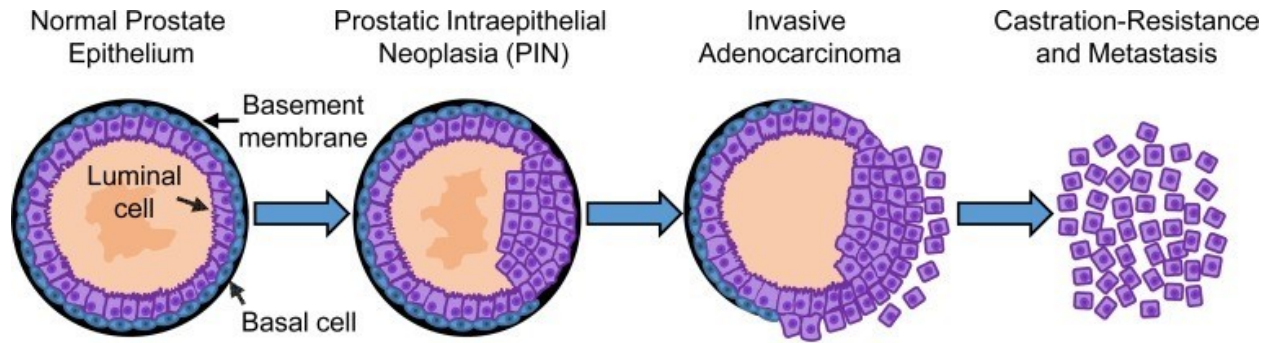
PIN is a well-established precursor to PCa, characterized by morphological alterations in luminal epithelial cells, including abnormal cell proliferation and dysplasia along the ducts [139, 141]. Histologically, PIN is defined by increased cellularity, enlarged hyperchromatic nuclei and prominent nucleoli. It is classified into low-grade PIN (LGPIN) and high-grade PIN (HGPIN) based on cellular morphology [139, 141].

HGPIN is widely considered a precursor to PCa, exhibiting distinct and more pronounced cellular abnormalities that contribute to disease progression compared to LGPIN [139]. It is characterized by nuclear and cytoplasmic features resembling invasive adenocarcinoma, with affected glands displaying cellular crowding and nuclear pseudostratification [139]. The malignant transformation of the prostatic epithelium begins with the development of HGPIN, progresses to localized PCa, then advanced prostate adenocarcinoma and ultimately metastatic PCa [10, 141]. As men advance in age, PIN lesions frequently develop in the peripheral zone and may progress to PCa [142].

Several lines of evidence have established that HGPIN is strongly associated with an increased risk of developing PCa [10, 139]. The loss of *Nkx3.1*, an early event in prostate carcinogenesis is associated with PIN, supporting its role in disease initiation. *Nkx3.1*-deficient mouse models exhibit morphological changes that closely resemble HGPIN in humans. Additionally, reduced *Nkx3.1* expression has been reported in HGPIN, further supporting its role as a tumour suppressor gene [10].

In HGPIN, the expression of ER $\alpha$  is up-regulated suggesting its involvement in the malignant transformation of the prostatic epithelium, while ER $\beta$  expression is reduced in luminal prostatic

epithelial cells of HGPIN, contributing to tumour progression [16, 21]. Experimental studies using testosterone and estradiol-treated mouse models have demonstrated that ER $\alpha$ KO mice fail to develop HGPIN, whereas ER $\beta$ KO mice developed HGPIN, likely due to increased proliferation and decreased apoptosis [16]. These findings indicate that ER signalling contributes to HGPIN development and further implicate hormonal imbalance in the early stages of PCa initiation.



**Figure 9: Prostate cancer progression.**

Prostate cancer progresses from normal prostate tissue to PIN, then to invasive adenocarcinoma and ultimately advances to castration-resistant and metastatic disease. Figure reproduced from Rybak et al. [125].

## 1.8 Molecular Genetics of Prostate Cancer

The development and progression of PCa is complex and not fully understood. Multiple genetic alterations, initiated by several mechanisms, have been implicated in prostate carcinogenesis. Prostate tumour growth is associated with changes in the expression of various developmental and regulatory genes involved in cell proliferation, apoptosis and differentiation, which are crucial for maintaining normal prostate homeostasis.

Although the loss of *Nkx3.1* alone is a key initiating factor in PCa development, it is not sufficient on its own to induce the neoplastic transformation of non-malignant cells. Additional genetic alterations, such as mutations in tumor suppressor genes like *TP53* or oncogenes such as *MYC*, are typically required for malignancy [143].

### 1.8.1 *MYC*

*MYC* is a proto-oncogene that encodes the nuclear phosphoprotein c-Myc (cellular Myc), which functions as a transcription factor regulating cell growth, differentiation, metabolism and cell cycle progression [50, 118].

c-Myc signalling has a tumor-promoting role, driving cell proliferation and inhibiting differentiation [50]. *MYC* is located at chromosome 8q24, which is a locus often amplified in PCa [14, 93].

In prostate carcinogenesis, c-Myc activation can occur through various mechanisms including gene amplification, mutations or translocations [42]. Overexpression and amplification of c-Myc are frequently observed in both early and metastatic disease [118].

Overexpression of c-Myc has been proposed as an initiating event in PCa, driving the onset of HGPIN and its progression to invasive adenocarcinoma [139, 141]. Immunohistochemical (IHC)

analysis of prostates from transgenic mice overexpressing c-Myc has shown the development of morphological and molecular alterations resembling human HGPIN [9, 74, 139]. Overexpression of c-Myc in normal luminal cells of murine prostate is also sufficient to initiate PCa [118].

Furthermore, *MYC* is commonly amplified in human prostate cancer tumours [141], with overexpression observed in metastatic PCa patients and associated with poor survival [84]. This provides evidence that dysregulation of *MYC* expression is a key driving force of PCa initiation [118].

Additionally, an inverse relationship between c-Myc and NKX3.1 levels has been observed during PCa progression [10]. Overexpression of c-Myc has been shown to repress NKX3.1 expression [74]. Normal prostate luminal cells express high levels of NKX3.1, whereas PIN lesions with c-Myc overexpression exhibit significantly reduced NKX3.1 expression [74]. This repression of NKX3.1 by c-Myc may contribute to disease progression, as studies suggest that NKX3.1 loss, combined with c-Myc activation, promotes prostate tumourigenesis [141].

Conversely, NKX3.1 opposes c-Myc's transcriptional activity in PCa. NKX3.1 has been shown to interact with c-Myc and modulate its transcriptional function, thereby antagonizing c-Myc-driven activation of overlapping downstream target genes and pathways [79]. This suggests that NKX3.1 may act as a critical regulator of c-Myc-dependent oncogenic activity, and its loss could further enhance cMyc-driven PCa progression [9, 10].

### **1.8.2 *TP53***

The tumour suppressor gene *TP53*, which encodes the p53 protein, plays a critical role in cell cycle regulation, DNA damage repair and apoptosis [12, 97, 146]. It functions as a transcription factor, regulating the expression of target genes involved in cell differentiation, cell cycle arrest

and cellular senescence in response to cellular stress [64, 97, 146]. *TP53* is located on chromosome 17p 13.1, a region frequently lost in advanced PCa and metastasis [12].

*TP53* is commonly mutated in PCa and associated with disease progression [52, 64, 97, 146]. Inactivation of p53 through mechanisms such as loss of heterozygosity (LOH) or loss of function mutations results in uncontrolled cell proliferation due to impaired cell cycle regulation and evasion of apoptosis [97, 146]. Conditional inactivation of *Trp53* in mouse models has been shown to lead to prostate adenocarcinoma [141].

Mutations in *TP53* can promote PCa progression, particularly in combination with the overexpression of the *MYC* oncogene [141, 143]. While *MYC* is a key driver of uncontrolled proliferation, its overexpression in the absence of growth factors, can also induce apoptosis in normal and pre-malignant cells. *MYC* overexpression induces the expression of *TP53*, activating pro-apoptotic pathways through both p53 dependent and independent mechanisms [5, 42]. c-Myc promotes apoptosis by indirectly increasing p53 levels and in turn, p53 suppresses excessive c-Myc activation, forming a regulatory feedback loop [5]. Studies show that p53 activation leads to the downregulation of *Myc* mRNA levels, reducing its oncogenic activity [5]. However, in PCa, mutations in p53, along with upregulation of survival factors and activation of anti-apoptotic pathways prevent c-Myc-induced apoptosis, enabling c-Myc-driven proliferation [84].

Additionally, the *Nkx3.1* gene has been shown to modulate p53 protein levels and transcriptional activity, while *Nkx3.1* expression may be influenced by p53 signalling [12]. Lei et al. found that *Nkx3.1* expression significantly increased p53 protein levels whereas knockout of *Nkx3.1* led to reduced p53 levels in both in vivo studies and in vitro experiments using LNCaP cells. They

further demonstrated that *Nkx3.1* overexpression stabilized p53 protein [88]. On the other hand, overexpression of p53 inhibited activation of NKX3.1 by androgens [146].

## **1.9 Rationale, Hypothesis and Objectives**

The androgen-regulated gene *Nkx3.1* is essential for maintaining prostate homeostasis. Its loss is considered an initiating event in prostate carcinogenesis and is associated with the development of PIN. Evidence suggests that estrogens play a critical role in prostate carcinogenesis. Estrogens, whether produced through local aromatization or derived from systemic circulation, influence prostate gland development and function [23, 51, 124].

Elevated testicular estrogen may indirectly alter hormonal balance in the prostate and downregulate *Nkx3.1* expression by reducing systemic androgen levels through negative feedback on the HPG axis. Previous studies have shown that elevated estrogen levels in the prostate can lead to the development of PIN. However, the effect of elevated E2 levels in the testis, particularly when *Nkx3.1* is lost remains poorly understood.

*Nkx3.1*-deficient mice serve as a model for early prostate carcinogenesis. The *Nkx3.1* gene is also expressed in the testis, where it maintains tissue homeostasis and its loss is associated with testicular abnormalities [11, 132]. Estrogen is a key regulator of testicular structure and function and elevated testicular E2 may disrupt these processes, potentially leading to systemic hormonal imbalance. This model allows us to investigate whether elevated estrogen in the absence of *Nkx3.1* induces structural and molecular changes in the testis, which may lead to downstream effects that could potentially contribute to prostate carcinogenesis.

Therefore, the goal of this research is to study the effect of elevated estrogen levels on the testis in the absence of *Nkx3.1*. We hypothesized that elevated testicular E2 levels would alter

testicular architecture and induce distinct changes in the expression of cancer-related genes in the absence of the *Nkx3.1* gene.

### **Objectives**

- Assess morphological changes in testicular architecture in response to elevated E2 levels and *Nkx3.1* loss
- Examine the effect of elevated E2 levels on the organisation of testicular connective tissue in the absence of *Nkx3.1*
- Evaluate the influence of elevated testicular E2 on the expression of cancer-related genes following *Nkx3.1* loss

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Animal Husbandry

#### 2.1.1 Mouse Model

*Nkx3.1* knockout mice (B6N;129S6-*Nkx3.1*<sup>tm2Mms/J</sup>) were obtained from The Jackson Laboratory (JAX stock #016541). In this *Nkx3.1*<sup>lacZ</sup> mutant strain, a  $\beta$ -galactosidase (*lacZ*) cassette disrupts the NK-3 transcription factor, locus 1 gene, abolishing gene function [18, 130].

To establish the colony, Heterozygous *Nkx3.1*<sup>+/*tm2Mms*</sup> males and females were bred to generate wild-type *Nkx3.1*<sup>+/+</sup>, heterozygous *Nkx3.1*<sup>+/*tm2Mms*</sup> and homozygous *Nkx3.1*<sup>tm2Mms/*tm2Mms*</sup> offspring.

To maintain the colony and prevent genetic drift, we backcrossed the male heterozygous *Nkx3.1*<sup>+/*tm2Mms*</sup> offspring to female C57BL/6NJ wildtype mice. The resulting offspring from this cross were either wild-type *Nkx3.1*<sup>+/+</sup> or heterozygous *Nkx3.1*<sup>+/*tm2Mms*</sup>.

Heterozygous *Nkx3.1*<sup>+/*tm2Mms*</sup> male and female mice were then bred to produce litters consisting of wild-type *Nkx3.1*<sup>+/+</sup>, heterozygous *Nkx3.1*<sup>+/*tm2Mms*</sup> and homozygous *Nkx3.1*<sup>tm2Mms/*tm2Mms*</sup> offspring.

The following genotype abbreviations will be used throughout this thesis for clarity:

Homozygous mutant *Nkx3.1*<sup>tm2Mms/*tm2Mms*</sup>: *Nkx3.1*<sup>-/-</sup>

Wild-type *Nkx3.1*<sup>+/+</sup>: *Nkx3.1*<sup>+/+</sup>

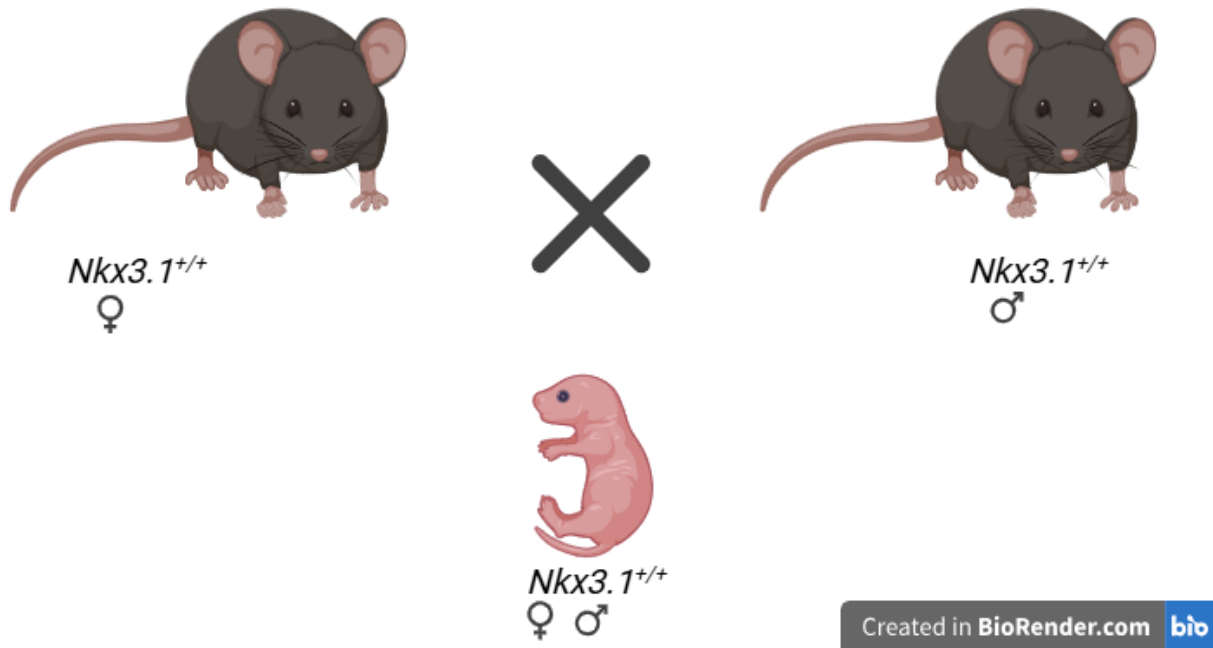
### 2.1.2 Animal Housing and Care

All procedures involving animal care were conducted in accordance with protocols approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (19-01-KK, Appendix 1). Animals were housed in the Animal Resource Centre, operated by Animal Care & Veterinary Resources at Memorial University of Newfoundland. Mice were housed in individual ventilated cages with a floor area of 501 cm<sup>2</sup> (GM500, Tecniplast Canada) and provided with Bed-O'Cobs corn cob bedding (The Andersons, Maumee, OH, USA). The animal room was maintained at a temperature of 22–25°C and a relative humidity of 40–60%, with a 12-hour light: 12-hour dark light cycle. The animals were provided with food, 2018 Teklad Global 18% Protein Rodent Diet, (Inotiv, Appendix 2) and administered water *ad libitum*.

### 2.1.3 Mating

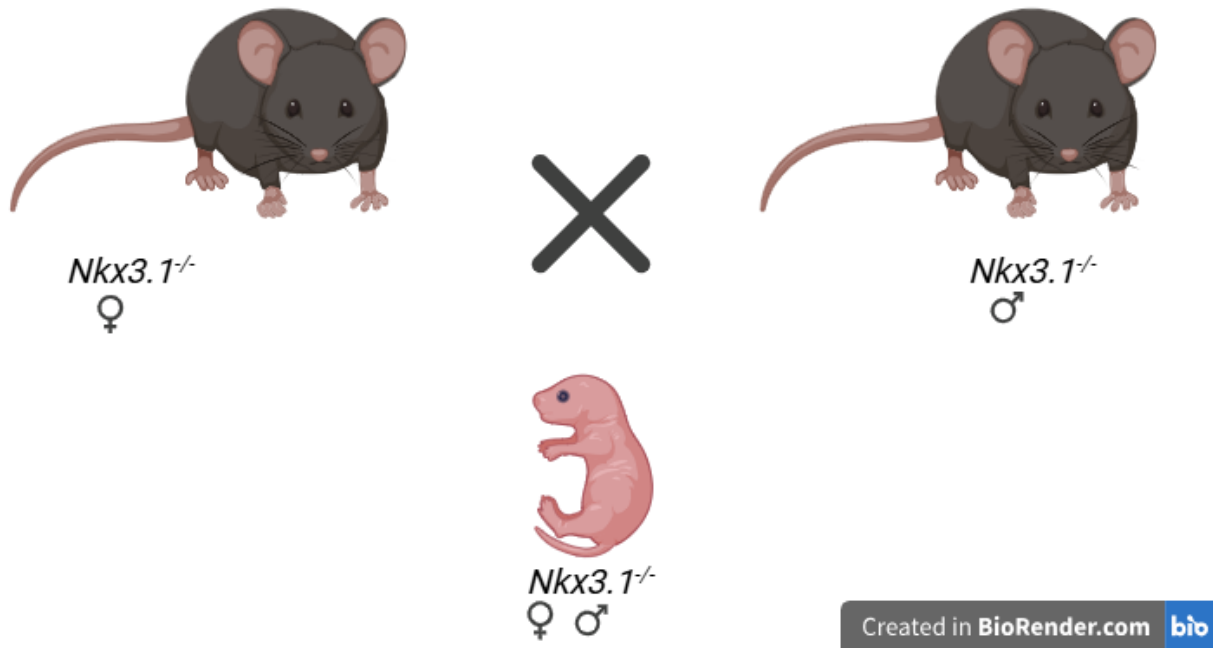
To generate the experimental animals, female *Nkx3.1*<sup>-/-</sup> mice were paired with male *Nkx3.1*<sup>-/-</sup> mice, while female *Nkx3.1*<sup>+/+</sup> mice were paired with male *Nkx3.1*<sup>+/+</sup> mice for controls. Females were monitored for signs of pregnancy starting approximately 14 days after pairing.

After birth, litters were monitored until weaning at postnatal day 21, after which pups were separated from their mothers and grouped by sex. A maximum of four mice were housed per cage. Only male pups were used for this study. Homozygous mutant *Nkx3.1*<sup>-/-</sup> males served as the experimental group, while wild-type *Nkx3.1*<sup>+/+</sup> males served as the control group.



**Figure 10: Mating scheme for generating *Nkx3.1*<sup>+/+</sup> control mice.**

Female and male *Nkx3.1*<sup>+/+</sup> mice were continuously paired. Females were checked for pregnancy two weeks after pairing and pregnant females were left to deliver naturally. All offspring from this cross were *Nkx3.1*<sup>+/+</sup> and only male pups were used as the control group in this study. Figure was created in <https://BioRender.com>.



**Figure 11: Mating scheme for generating *Nkx3.1*<sup>-/-</sup> experimental mice.**

Female and male *Nkx3.1*<sup>-/-</sup> mice were continuously paired. Females were checked for pregnancy two weeks after pairing and pregnant females were left to deliver naturally. All offspring from this cross were *Nkx3.1*<sup>-/-</sup> and only male pups were used as the experimental group in this study.

Figure was created in <https://BioRender.com>.

## **2.2 Genotyping**

### **2.2.1 Ear Tissue Collection**

For genotyping, a 2–3 mm ear tissue sample was collected from each mouse using a sterile ear punch while the animal was briefly anesthetized with isoflurane (Baxter, Deerfield, IL).

Ear tissues were immediately placed into labelled 2 mL Eppendorf tubes containing 100  $\mu$ L of Extraction Solution (Sigma-Aldrich, Oakville, ON) and 25  $\mu$ L of Tissue Preparation Solution (Sigma-Aldrich, Oakville, ON). The samples were vortexed and then centrifuged briefly using a benchtop centrifuge (Fisher Scientific, Ottawa, ON) to spin down the content of the tubes.

The tubes were then placed in an incubator (ThermoFisher Scientific, Ottawa, ON) and the tissues were left to incubate overnight at 55°C to allow digestion, during which proteinase K breaks down cellular components, releasing DNA from the cells for extraction.

### **2.2.2 DNA Extraction**

The digested ear samples were incubated to 95°C for 3 minutes to inactivate proteinase K and prevent further digestion of the DNA. Following incubation, 100  $\mu$ L of neutralization buffer (Sigma-Aldrich, Oakville, ON) was added to the extracted DNA solution to stabilize the pH. The solution was vortexed to ensure thorough mixing and then briefly centrifuged in a bench top centrifuge. The DNA extract was stored at -20°C to prevent degradation and for subsequent polymerase chain reaction (PCR) analysis.

### 2.2.3 PCR

PCR was performed using REDEExtract-N-Amp<sup>TM</sup> Tissue PCR Kit (Sigma-Aldrich, Oakville, ON) to determine the genotype of the mice. The PCR reaction was carried out on DNA samples obtained from both *Nkx3.I<sup>+/+</sup>* and *Nkx3.I<sup>-/-</sup>* mice.

The PCR master mix was prepared by mixing 10  $\mu$ L of REDEExtract-N-Amp PCR reaction mix, 0.5  $\mu$ L of forward primer (10  $\mu$ M), 0.5  $\mu$ L of reverse primer (10  $\mu$ M) and 5  $\mu$ L of autoclaved water. The primer sequences used are listed in Table 1 (JAX, USA).

16  $\mu$ L of the PCR master mix was transferred to a 0.2 mL PCR tube (Fischer Scientific, Toronto, ON). 4  $\mu$ L of DNA extract was added to each PCR tube labelled according to the DNA samples. The reaction mixture in each tube was briefly centrifuged in a benchtop centrifuge to ensure thorough mixing. The PCR tubes were then placed in a thermal cycler [MasterCycler Gradient 5331] (Eppendorf, AG, Hamburg) for amplification.

The PCR thermal cycling conditions were as follows: Step 1: Initial denaturation at 94°C for 3 minutes; Step 2: Denaturation at 94°C for 20 seconds; Step 3: Annealing at 60°C for 1 minute; Step 4: Extension at 72°C for 1 minute; Steps 2-4 were repeated for 30 cycles; Step 5: Final extension at 72°C for 10 minutes and Step 6: Final hold at 4°C indefinitely.

**Table 1: Primer Sequences used in Genotyping**

	<b>Forward Primer (5'-3')</b>	<b>Reverse Primer (5'-3')</b>	<b>Reference</b>
<i>Nkx3.1</i> : Wildtype	CTC CGC TAC CCT AAG CAT CC	GAC ACT GTC ATA TTA CTT GGA CC	(JAX, USA)
<i>Nkx3.1</i> : Mutant	CCG ACG GCA CGC TGA TTG AAG	TGC ACC GGG CGG GAA GGA T	(JAX, USA)

#### 2.2.4 Gel Electrophoresis

After PCR amplification, gel electrophoresis was performed to analyze the PCR products. A 1% agarose gel was prepared by dissolving 1.0 g of agarose powder in 100 mL of 1X TAE buffer (10 mL 10XTAE buffer pH 8.5 (Thermo Fischer Scientific, Lithuania) diluted with 90 mL deionized water [dH<sub>2</sub>O]).

The solution was heated at intervals until agarose was fully dissolved and then cooled to approximately 60°C. 1 µL of RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology, Gyeonggi-do, Korea) was added to the dissolved agarose-TAE solution and gently swirled to mix.

The gel solution was poured into a gel casting mold sealed at both ends and fitted with a gel comb to create loading wells. The gel was allowed to solidify for 45 minutes before being placed in a gel electrophoresis chamber. A 1X TAE gel running buffer was poured into the electrophoresis chamber until the gel was fully submerged and the gel comb was then carefully removed.

For each DNA sample, a 2.5  $\mu\text{L}$  aliquot of the PCR product was mixed with 0.5  $\mu\text{L}$  of loading dye and 2  $\mu\text{L}$  of  $\text{dH}_2\text{O}$ . 5  $\mu\text{L}$  of the PCR product and loading dye mixture was loaded directly into each well of the agarose gel. The gel was run at 60V for 1 hour.

Following electrophoresis, the gel was imaged under UV light using the Gel Doc EZ Imager (Bio-Rad, Hercules, CA). DNA bands were visualized with the Image Lab Software (Bio Rad, Hercules, CA) to differentiate between  $Nkx3.I^{+/+}$  and  $Nkx3.I^{-/-}$  genotypes based on fragment size.

### **2.3 Experimental Design**

After genotyping, twelve 20-week-old mice were randomly assigned to two groups based on their genotype: six homozygous mutant  $Nkx3.I^{-/-}$  mice and six wild-type  $Nkx3.I^{+/+}$  mice. Each genotype group was then further divided into estradiol-treated (experimental) and placebo (control) sub-groups, with three mice per sub-group.

The four mouse groups used in this study were as follows:

$Nkx3.I^{+/+}$  Placebo

$Nkx3.I^{+/+}$  Estradiol-treated

$Nkx3.I^{-/-}$  Placebo

$Nkx3.I^{-/-}$  Estradiol-treated

## **2.4 Histological Analysis**

### **2.4.1 Hormone Treatment**

To evaluate the effects of elevated estrogen levels in the absence of *Nkx3.1*, mice were treated with either 17 $\beta$ -estradiol or placebo. Mice were anesthetized using isoflurane gas, administered via a calibrated vaporizer. The anesthetic concentration was gradually increased from 1% to 3% with an oxygen flow rate of 3–5 L/min. Anaesthesia depth was confirmed by the absence of a reflex response to hind limb stimulation. Moisture gel was applied to the eyes to prevent dryness during the procedure. After anaesthesia administration, the surgical site was prepared with a depilatory agent (Nair® lotion) and cleaned with water-soaked gauze to ensure sterility and clear exposure of the skin.

Mice in the treatment group were implanted subcutaneously with 0.5mg 17 $\beta$ -estradiol 90-day extended-release pellets (Innovative Research of America, Sarasota, FL). Control mice received matched placebo pellets (Innovative Research of America, Sarasota, FL). This method is widely used in murine models to reliably elevate systemic estradiol (E2) levels. A sterilized trocar, preloaded with the E2 hormone pellet was inserted into the surgical site to create a subcutaneous channel, after which the pellet was gently placed under the skin. Once the pellet was in place, the trocar was carefully removed and the incision site was inspected to confirm proper pellet placement. Mice were monitored for recovery post-surgery. Body weight was recorded prior to pellet implantation and measured weekly thereafter.

### **2.4.2 Tissue Collection**

Ninety days after E2 treatment, bilateral testes from two mice per group were dissected for histological analysis. Tissues were fixed in 10% buffered formalin (ThermoFisher, Mississauga,

ON) and sent to the Department of Histology at Memorial University of Newfoundland for paraffin embedding and sectioning.

### **2.4.3 Histological Processing and Staining**

Two paraffin-embedded sections were cut per mouse, one from each testis at 4  $\mu\text{m}$  thickness. Both sections were mounted on two slides: one stained with hematoxylin and eosin (H&E) to assess tissue morphology and the other with one-step trichrome to evaluate connective tissue organisation. Thus, for each mouse, two slides were prepared, each containing sections from both testes. After H&E and one-step trichrome staining, the stained testes sections were examined qualitatively at 100X magnification using an Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan). Samples were compared to assess differences in tissue morphology and connective tissue organisation.

## **2.5 Gene Expression Analysis**

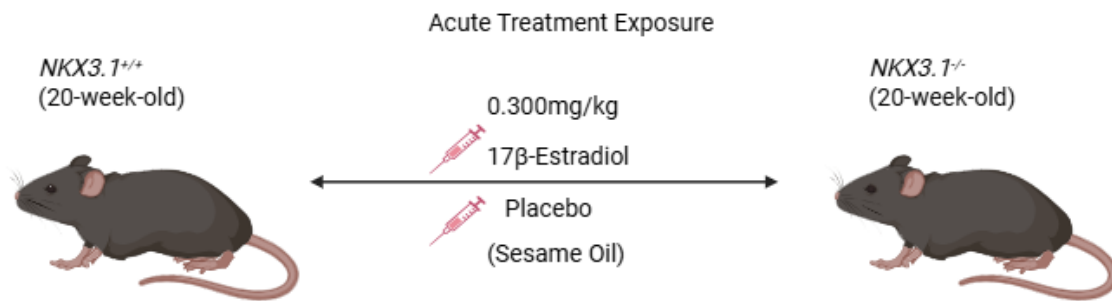
### **2.5.1 Hormone Treatment**

Twenty-week-old male mice were anesthetized with isoflurane (Baxter, Deerfield, IL) administered via a calibrated vaporizer. The anesthetic concentration was gradually increased from 1% to 3% with an oxygen flow rate of 3–5 L/min. Anesthesia depth was confirmed by the absence of a reflex response to hind limb stimulation.

17 $\beta$ -Estradiol (Sigma-Aldrich, Oakville, ON) was dissolved in 100 mL of sesame oil to prepare a 0.125 mg/mL E2 solution, which was administered as the experimental treatment, while sesame oil was used as the placebo treatment.

Mice in the treatment group received a subcutaneous injection of the E2 solution at a dose of 0.300mg/kg body weight, whereas mice in the control group received an equivalent volume of

sesame oil only as a placebo. This method has been validated for elevating circulating estradiol levels in murine models. Mice were monitored for recovery post-surgery.



Created in BioRender.com 

**Figure 12: Schematic of treatment administration.**

E2 and PBO treatment administration in 20-week-old *Nkx3.1<sup>+/+</sup>* and *Nkx3.1<sup>-/-</sup>* mice. Figure was created in <https://BioRender.com>.

### **2.5.2 Tissue Collection**

Mice were euthanized using CO<sub>2</sub> and testis tissue samples were collected two hours post-treatment. Testes were placed individually into labelled 1.5 mL Eppendorf tubes and their weights were measured. Directly after harvesting, tissues were immediately snap-frozen in liquid nitrogen, which rapidly permeated the tissues to stabilize and protect RNA. Snap-freezing prevented RNA degradation and preserved the integrity of the RNA for subsequent analysis. The samples were stored at -80°C for long-term preservation.

### **2.5.3 RNA Isolation**

RNA was isolated from testes ( $\leq 30$  mg of tissue) using the QIAwave RNA Mini Kit (QIAGEN, Toronto, ON). Tissues were disrupted and homogenized in a denaturing buffer, which immediately inactivated RNases ensuring the purification of intact RNA. 600  $\mu$ L of buffer RLT (QIAGEN, Toronto, ON) was added to a 2 mL RNase-free microcentrifuge tube (ThermoFisher Scientific, Burlington, ON) containing the testis tissue and the lysate was centrifuged (Eppendorf, Hamburg, Germany) for three minutes at 10,000 RPM. The supernatant was carefully transferred to a clean microcentrifuge tube to remove tissue remnants.

Then, 600  $\mu$ L of 70% ethanol was added to the supernatant to promote RNA binding to the RNeasy membrane. After brief vortexing, the supernatant was transferred to an RNase mini spin column (QIAGEN, Toronto, ON) and centrifuged for 30 seconds at 10,000 RPM. Contaminants were washed away from the silica membrane by adding 350  $\mu$ L of buffer RW1 (QIAGEN, Toronto, ON) to the spin column and centrifuging for 15 seconds at 10,000 RPM.

To eliminate genomic DNA contamination, on-column DNase digestion was performed using the RNase-Free DNase set (QIAGEN, Toronto, ON). DNase 1 stock solution was prepared by dissolving lyophilized DNase 1 [1500 Kunitz units] (QIAGEN, Toronto, ON) in 550  $\mu$ L RNase-

free water (QIAGEN, Toronto, ON). The reconstituted DNase 1 was gently mixed, aliquoted into single-use portions and stored at -20°C. 70 µL of buffer RDD (QIAGEN, Toronto, ON) was added to 10 µL of DNase 1, mixed and centrifuged. For DNase digestion, 80 µL of the DNase 1 incubation mix was added directly to the RNeasy spin column membrane and incubated at room temperature for 15 minutes.

After incubation, DNase 1 was washed away by adding 350 µL of buffer RW1 and centrifuging at 10,000 RPM for 15 seconds. Contaminants were removed by adding 500 µL buffer RPE/C to the spin column membrane (2×) and centrifuging briefly. The spin column was then carefully placed in a labelled 1.5 mL microcentrifuge tube. RNA was eluted by adding RNase-free water directly onto the membrane of the column and centrifuging briefly. RNA aliquots were stored at -80°C.

#### **2.5.3.1 Quality Control**

Quality control was conducted to ensure that the isolated RNA was of high quality, free of contaminants and suitable for downstream analyses, including real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). All equipment and reagents used in RNA isolation and quantification were RNase-free to prevent RNA degradation.

#### **2.5.3.2 Spectrophotometry**

The concentration and the absorbance ratio were measured using a NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific, Wilmington, USA) to get an estimate of the RNA yield and determine the purity of the sample.

RNA concentration was quantified by measuring absorbance at the wavelength of 260 nm (A<sub>260</sub>) and the ratio of absorbance at 260 nm to 280 nm (A<sub>260</sub>/A<sub>280</sub>) was used to assess RNA purity.

### **2.5.3.3 Gel Electrophoresis**

RNA integrity was assessed by agarose gel electrophoresis using MOPS buffer. To prepare 10X MOPS buffer, 41.8 g of MOPS was dissolved in 700 mL of RNase-free H<sub>2</sub>O (ThermoFisher Scientific, Mississauga) and the pH was adjusted to 7.0 with 2M NaOH. Then, 20 mL of DEPC-treated 1M sodium acetate and 20 mL of DEPC-treated 0.5M EDTA were added to the solution. The volume was adjusted to 1 L with DEPC-treated H<sub>2</sub>O and the solution was sterilized using a 0.45 µm Millipore filter.

A 1.2% agarose gel was prepared by dissolving 1.2 g of agarose powder (ThermoFisher Scientific, Burlington, ON) in 73 mL of dH<sub>2</sub>O. The solution was heated at intervals until fully dissolved and then cooled to 60°C. In a separate tube, 10 mL of 10X MOPS and 17 mL of formaldehyde (ThermoFisher Scientific, Ottawa, ON) were mixed, pre-warmed and then added to the agarose solution. 1 µL of RedSafe nucleic acid staining was added to the solution and gently swirled to mix. The solution was then poured into the gel casting mold fitted with a gel comb to create wells and allowed to polymerize for 45 minutes. Once solidified, the gel was placed in an electrophoresis chamber. 1X MOPS running buffer was added until the gel was fully submerged and then the gel comb was carefully removed.

For each RNA sample, 0.5 µL of RNA loading dye (ThermoFisher Scientific, Ottawa, ON), 4 µL of RNase-free H<sub>2</sub>O and 0.5 µL of RNA were mixed in an RNase-free 2 mL microcentrifuge tube. The mixture was incubated at 60°C for 5 to 10 minutes to denature RNA and then briefly

centrifuged. 5  $\mu$ L of the sample was loaded into the gel wells and electrophoresis was carried out at 70V for 1 hour.

After electrophoresis, the RNA bands were visualized under UV light using the Gel Doc EZ Imager and analyzed with the Image Lab Software.

## **2.6 Synthesis of Complementary DNA**

Complementary DNA (cDNA) was synthesized in triplicate from 2  $\mu$ g of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). In the initial step of the cDNA synthesis protocol, a 2x RT master mix was prepared using reverse transcription reagents. The components of the 2x RT master mix were as follows: 2.0  $\mu$ L of 10x RT Buffer, 0.8  $\mu$ L of 25x dNTP Mix (100 mM), 2  $\mu$ L of 10x RT Random Primers and 1  $\mu$ L of Multiscribe Reverse Transcriptase. 10  $\mu$ L of the 2x RT master mix was transferred into individual 0.2 mL PCR tubes (ThermoFisher Scientific, Ottawa, ON) and 10  $\mu$ L of RNA sample was added to each tube. The mixture was gently mixed by pipetting and then centrifuged to spin down the contents. This procedure was performed on ice to prevent potential RNA degradation and maintain the stability of the reaction components before the reverse transcription step.

The reaction mixture was then transferred to the thermal cycler for cDNA synthesis. The PCR thermal cycling conditions were as follows: Step 1: Incubation at 25°C for 10 minutes; Step 2: Incubation at 37°C for 120 minutes; Step 3: Incubation at 85°C for 5 minutes; Step 4: Hold at 4°C. The reaction volume was set to 20  $\mu$ L. cDNA was stored at -80°C for long-term use.

## **2.7 RT-qPCR**

RT-qPCR was performed using PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, Ottawa, Ontario) according to the manufacturer's protocol. DNA samples and primers

were thawed on ice, vortexed to mix and then briefly centrifuged. Primer sequences are listed in Table 2 (Integrated DNA Technologies, Coralville, Iowa). The qPCR reaction master mix was prepared for each primer in a 2 mL microcentrifuge tube. The components of the PCR reactions included 10  $\mu$ L of PowerUp™ SYBR™ Green Master Mix, 1  $\mu$ L of primer and 8  $\mu$ L of RNase-Free Water (plus 10% overage to be used as no template control to ensure that the reactions were not contaminated and primers were not amplifying non-specific products). The components were mixed thoroughly and then briefly centrifuged to spin down the contents.

19  $\mu$ L of the reaction mix for each primer was transferred to each well of an optical plate [MicroAmp Fast 96-Well Reaction Plate (0.1 mL)] (ThermoFisher Scientific, Ottawa, ON) and 1  $\mu$ L of cDNA was added to each well. The negative control in the bottom of the well included all reagents except the DNA template. The plate was sealed with an optical adhesive cover and centrifuged with a MPS 1000 Mini Plate Spinner (Labnet, Woodbridge, NJ) to eliminate any air bubbles and spin down the contents. The experiment was conducted in triplicate.

The reaction plate was then placed in a ViiA™ 7 Real-Time PCR System (ThermoFisher, Carlsbad, CA). The PCR thermal cycling conditions were as follows: Step 1: UDG activation at 50°C for 2 minutes; Step 2: Polymerase activation at 95°C for 2 minutes; Step 3: Denaturation at 95°C for 15 seconds; Step 4: Annealing/extension at 60°C for 1 minute. Steps 3 and 4 were repeated for 40 cycles. The dissociation curve conditions were: Step 1: 95°C for 15 seconds; Step 2: 60°C for 1 minute; Step 3: 95°C for 15 seconds.

**Table 2: Primer Sequences used in RT-qPCR Analyses**

	<b>Forward Primer (5'-3')</b>	<b>Reverse Primer (5'-3')</b>
<i><b>β-actin</b></i>	GGC TGT ATT CCC CTC CAT CG	CCA GTT GGT AAC AAT GCC ATGT
<i><b>Gapdh</b></i>	AGG TCG GTG TGA ACG GAT TTG	TGT AGA CCA TGT AGT TGA GGT CA
<i><b>Esr1</b></i>	ATG AAA GGC GGC ATA CGG AAA G	CAC CCA TTT CAT TTC GGC CTT C
<i><b>Esr2</b></i>	CCA GAC TGC AAG CCC AAA TGT	AGA AGC GAT GAT TGG CAG TGG
<i><b>Myc</b></i>	ATG CCC CTC AAC GTG AAC TTC	CGC AAC ATA GGA TGG AGA GCA
<i><b>Trp53</b></i>	GTC ACA GCA CAT GAC GGA GG	TCT TCC AGA TGC TCG GGA TAC

## 2.8 Statistical Analysis

Data were analyzed using the  $2^{-\Delta\Delta Ct}$  method and normalized to the reference genes Gapdh and  $\beta$ -Actin. Differences between groups were assessed using unpaired two-tailed t-test. P-value < 0.05 was considered statistically significant. Statistical analyses were performed using R statistical software (version 4.3.2, R foundation for statistical computing, Vienna, Austria).

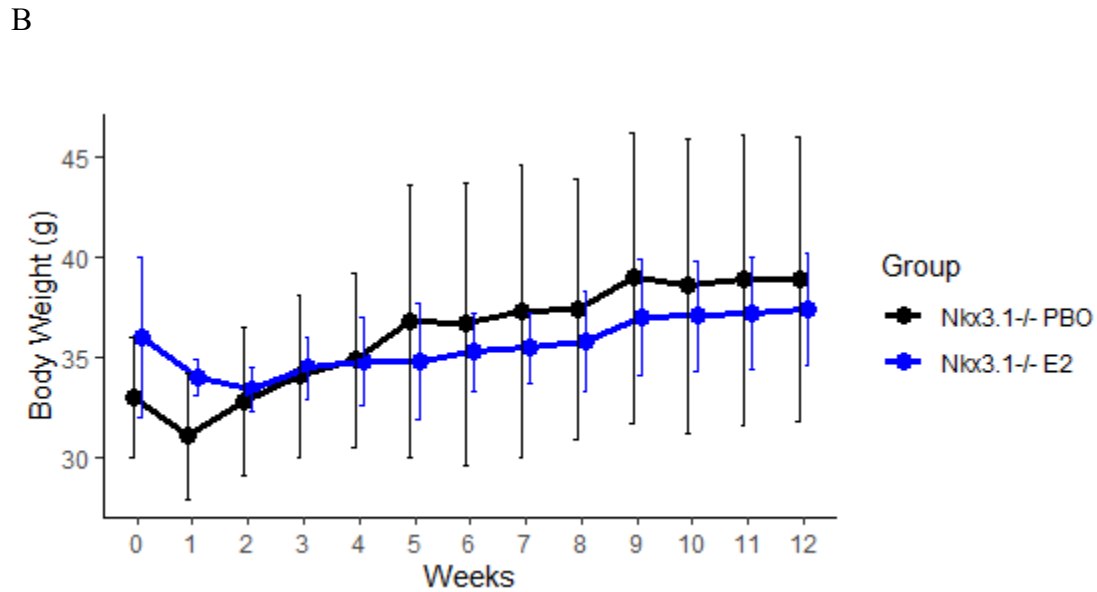
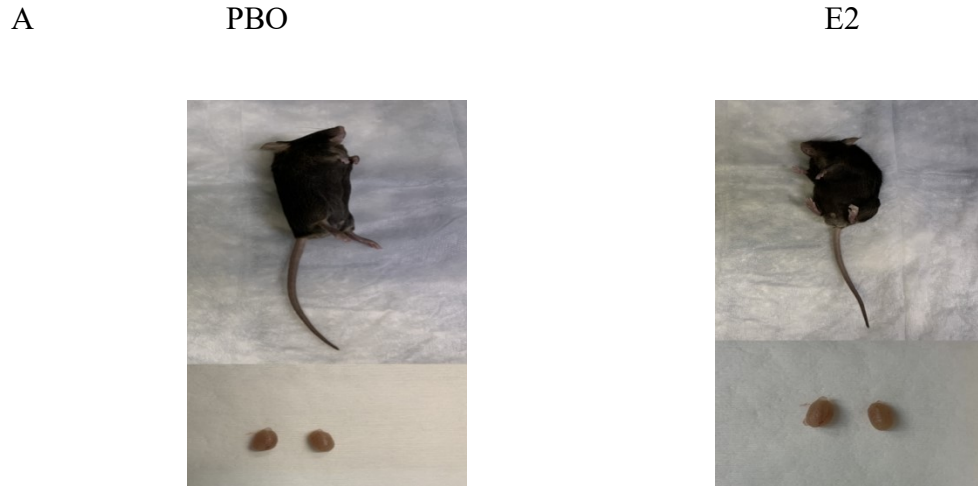
## CHAPTER 3: RESULTS

### 3.1 Effect of Estradiol Treatment on Body and Testis Weight

We aimed to assess whether prolonged E2 exposure affects testis development in *Nkx3.1*<sup>-/-</sup> mice and evaluate whether body weight differs between E2-treated and placebo-treated mice. Differences may reflect developmental abnormalities in response to elevated E2 levels in the testis.

Body weight was recorded prior to pellet implantation and monitored weekly over the 90-day treatment period. Testis tissues were harvested and examined for changes in size in both treatment and control groups.

We found that prolonged E2 exposure had no impact on testicular development in *Nkx3.1*<sup>-/-</sup> mice. Furthermore, there was no statistically significant difference in body weight between the E2-treated and placebo-treated mice groups at any time point, suggesting that elevated testicular E2 did not affect overall growth.



**Figure 13: Effect of estradiol treatment on body weight.**

(A) Representative images of *Nkx3.1<sup>-/-</sup>* mice and testes after 90-days of treatment with PBO or E2. (B) Average body weight ( $\pm$  SEM) of *Nkx3.1<sup>-/-</sup>* mice over 13 weeks of PBO or E2 treatment. Although no statistically significant difference was observed between the groups ( $p > 0.05$ ), mice receiving E2 showed a trend toward lower body weight compared to PBO-treated mice from week 5 through the end of the treatment period. Statistical analysis was done by Student's t-test.

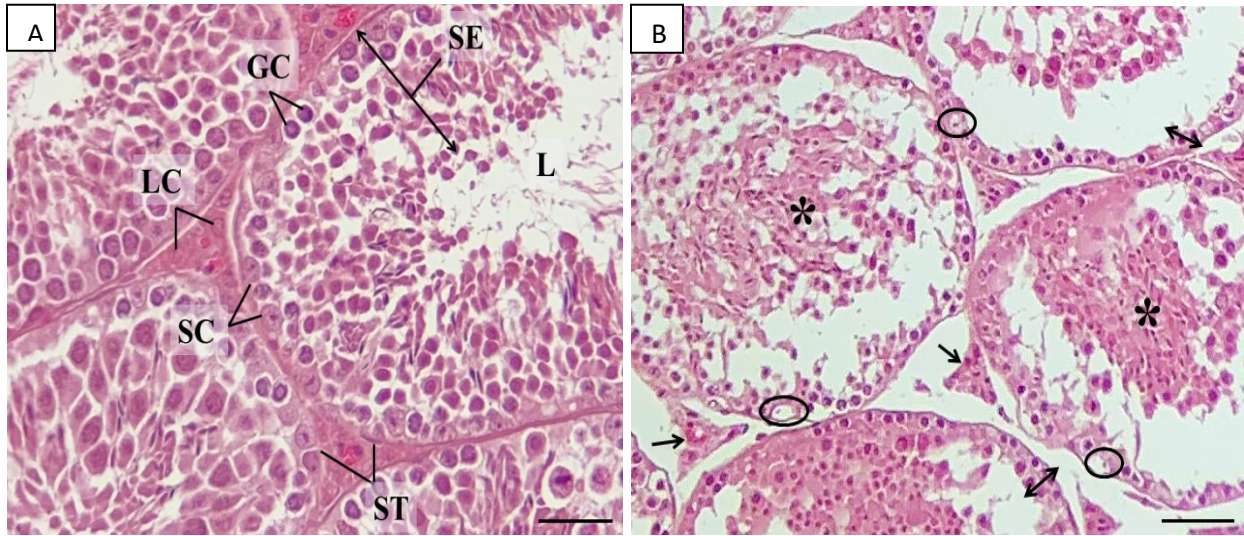
### **3.2 Histological Evaluation of *Nkx3.1*<sup>-/-</sup> Testes Following E2 or Placebo Treatment**

To determine whether elevated E2 levels in the absence of *Nkx3.1* resulted in testicular abnormalities, we compared tissue morphology and connective tissue organisation in testes from *Nkx3.1*<sup>-/-</sup> mice treated with E2 or placebo. H & E staining was used to evaluate tissue morphology, including changes in the structure of the seminiferous tubules and epithelium, as well as the morphology of germ cells, Sertoli cells and Leydig cells. One-step trichrome staining was used to assess connective tissue organisation.

#### **3.2.1 Effect of Estradiol Treatment on Testicular Morphology**

E2-treated *Nkx3.1*<sup>-/-</sup> testes showed disrupted architecture, characterized by irregularly shaped seminiferous tubules and thinner seminiferous epithelium. Germ cells within the seminiferous epithelium appeared diminished. The lumen was enlarged and contained detached germ cells along with vacuolated and fragmented cellular debris. Sertoli cells exhibited signs of degeneration, including vacuolation along the basement membrane and a decreased number of cells. Additionally, Leydig cell clusters within the interstitial tissue were disorganized.

In contrast, placebo-treated *Nkx3.1*<sup>-/-</sup> testes displayed more rounded and densely packed seminiferous tubules with compact lumina. The seminiferous epithelium appeared more structurally intact containing a higher density of germ cells and Sertoli cells. The interstitial tissue was less disrupted, with Leydig cell clusters appearing more organized.

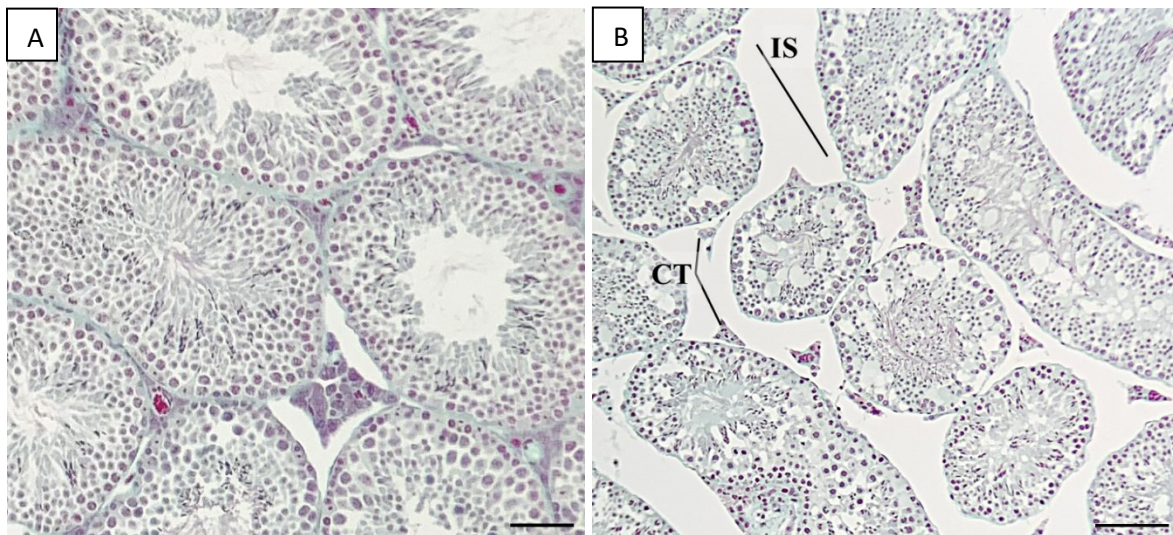


**Figure 14: Histological analysis of the testis using H&E staining.**

(A) Placebo-treated *Nkx3.1*<sup>-/-</sup> testis showing seminiferous tubules (STs), seminiferous epithelium (SE), lumen (L), germ cells (GCs), Leydig cells (LCs) and Sertoli cells (SCs). Magnification: 100x; scale bar = 200μm (B) E2-treated *Nkx3.1*<sup>-/-</sup> testis showing irregularly shaped seminiferous tubule with thinned epithelium and reduced germ cell population (double arrow), enlarged lumen containing fragmented and vacuolated detached germ cells (asterisk), vacuolated Sertoli cells (circle) and disorganized Leydig cell clusters (arrow). Magnification: 100x; scale bar = 100μm. n = 2 mice per group.

### 3.2.2 Effect of Estradiol Treatment on Testicular Connective Tissue

The testes of *Nkx3.1*<sup>-/-</sup> mice treated with elevated E2 exhibited a visibly expanded interstitial space between seminiferous tubules. The interstitial connective tissue showed signs of disruption, including loss of its normal structural organisation. Testes from the placebo-treated control group displayed more intact connective tissue structure, with less disrupted interstitial area compared to those in the E2-treated group.



**Figure 15: Histological analysis of the testis using one-step trichrome staining.**

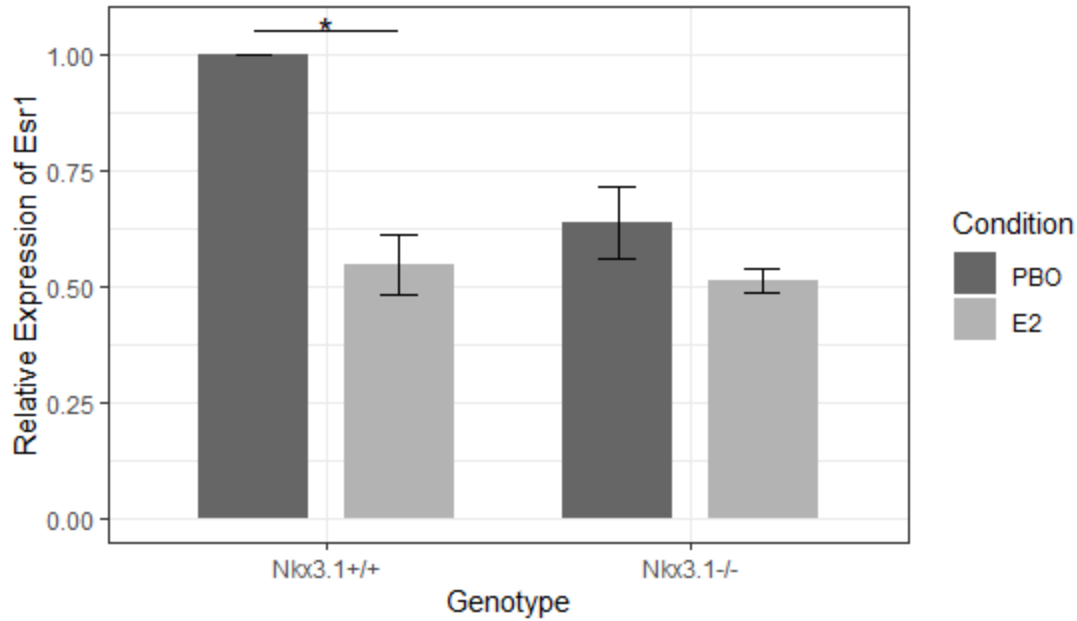
(A) Placebo-treated *Nkx3.1*<sup>-/-</sup> testis showing connective tissue and interstitial areas. Magnification: 100x, scale bar = 100 $\mu$ m (B) E2-treated *Nkx3.1*<sup>-/-</sup> testis displaying disrupted connective tissue (CT) and enlarged interstitial space (IS). Magnification: 100x, scale bar = 100 $\mu$ m. n = 2 mice per group.

### 3.3 Expression of Estrogen Responsive Genes Assessed by RT-qPCR

To investigate whether elevated testicular E2 also induces distinct changes in the expression of cancer-related genes, RT-qPCR analysis was performed. We assessed the expression of *Esr1*, *Esr2*, *Trp53*, and *Myc* in the testes of *Nkx3.1<sup>-/-</sup>* and *Nkx3.1<sup>+/+</sup>* mice following E2 or placebo treatment.

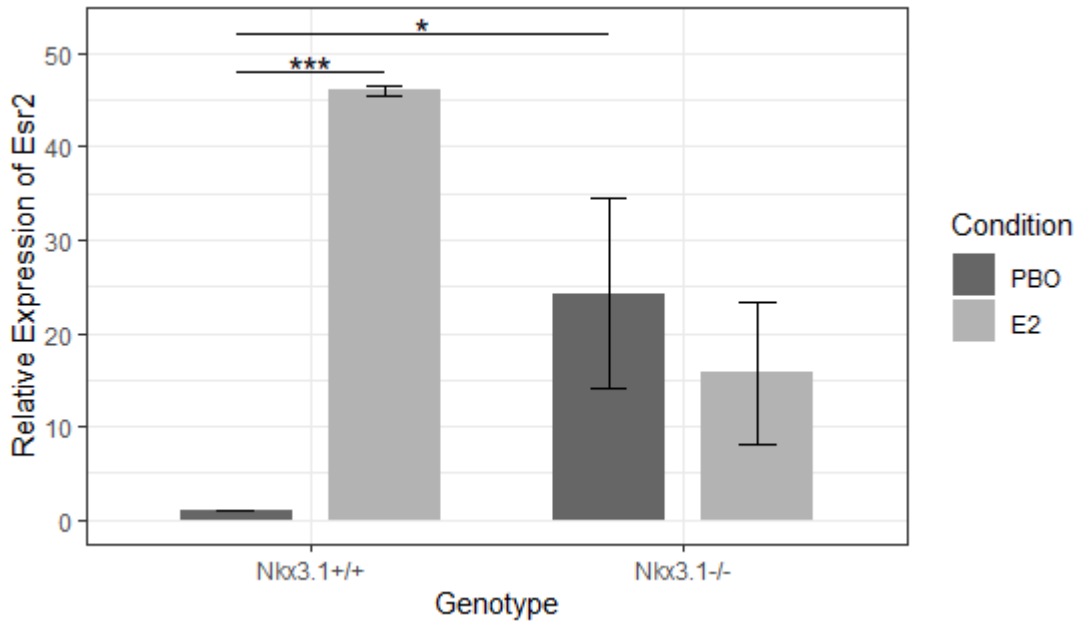
In *Nkx3.1<sup>+/+</sup>* mice, E2 treatment significantly decreased *Esr1* expression and increased *Esr2* and *Myc* expression compared to placebo controls. There were no significant changes observed in *Trp53* expression. In contrast, *Nkx3.1<sup>-/-</sup>* mice showed no significant changes in the expression of *Esr1*, *Esr2*, *Trp53* or *Myc* in response to E2 treatment.

However, when comparing genotypes, placebo-treated *Nkx3.1<sup>-/-</sup>* mice exhibited significantly higher *Esr2* expression than placebo-treated *Nkx3.1<sup>+/+</sup>* mice. Placebo-treated *Nkx3.1<sup>-/-</sup>* mice also showed a significant increase in *Myc* expression compared to placebo-treated *Nkx3.1<sup>+/+</sup>* mice.



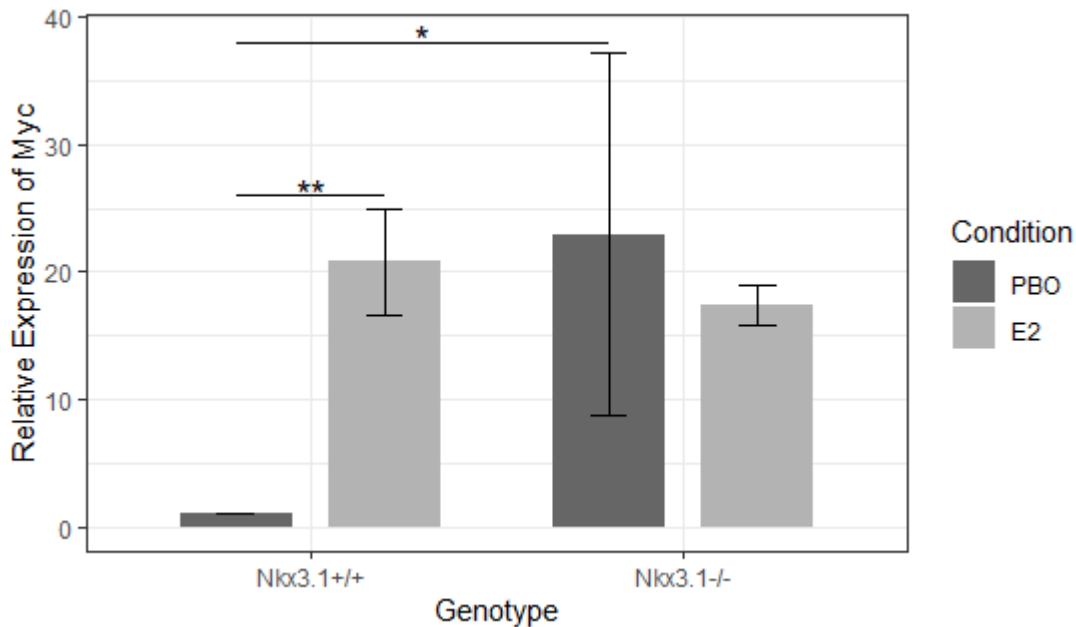
**Figure 16: Relative expression of *Esr1* in *Nkx3.1*<sup>+/+</sup> and *Nkx3.1*<sup>-/-</sup> testes following treatment with E2 or PBO.**

Bar graphs represent *Esr1* gene expression normalized to housekeeping genes and expressed relative to *Nkx3.1*<sup>+/+</sup> placebo. Error bars show mean  $\pm$  SEM from three biological replicates, each measured in technical triplicates. Elevated testicular E2 differentially influenced *Esr1* expression in *Nkx3.1*<sup>+/+</sup> mice, but had no statistically significant effect in *Nkx3.1*<sup>-/-</sup> mice. Statistical analysis was performed using Student's t-test (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001).



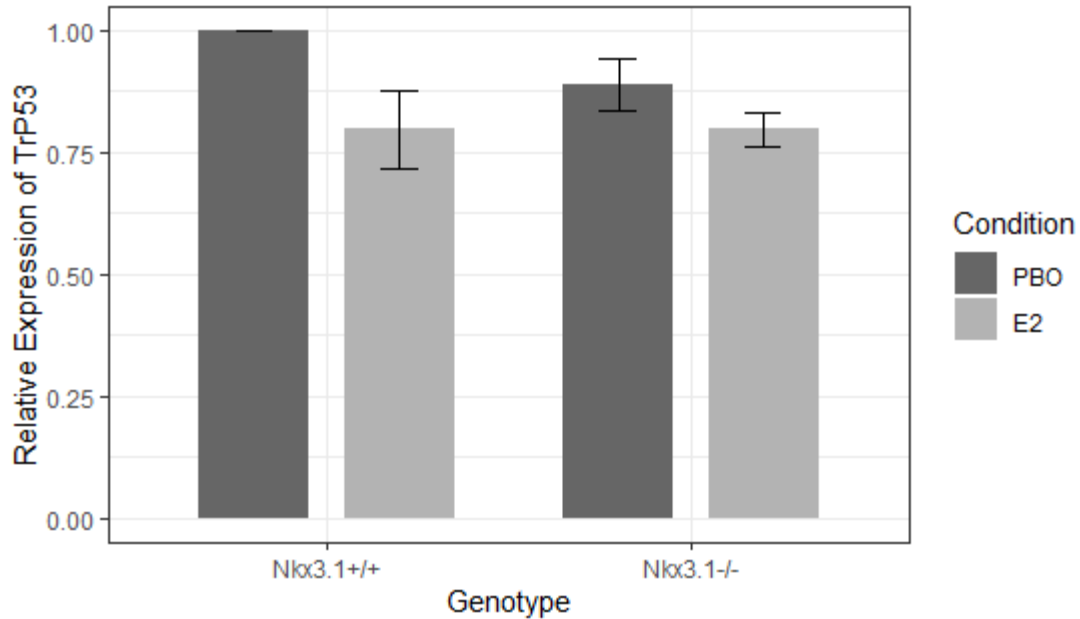
**Figure 17: Relative expression of *Esr2* in *Nkx3.1*<sup>+/+</sup> and *Nkx3.1*<sup>-/-</sup> testes following treatment with E2 or PBO.**

Bar graphs represent *Esr2* gene expression normalized to housekeeping genes and expressed relative to *Nkx3.1*<sup>+/+</sup> placebo. Error bars show mean ± SEM from three biological replicates, each measured in technical triplicates. Elevated testicular E2 differentially influenced *Esr2* expression in *Nkx3.1*<sup>+/+</sup> mice, but had no statistically significant effect in *Nkx3.1*<sup>-/-</sup> mice. *Esr2* expression also differed significantly between genotypes under placebo treatment. Statistical analysis was performed using Student's t-test (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001).



**Figure 18: Relative expression of *Myc* in *Nkx3.1*<sup>+/+</sup> and *Nkx3.1*<sup>-/-</sup> testes following treatment with E2 or PBO.**

Bar graphs represent *Myc* gene expression normalized to housekeeping genes and expressed relative to *Nkx3.1*<sup>+/+</sup> placebo. Error bars show mean ± SEM from three biological replicates, each measured in technical triplicates. Elevated testicular E2 differentially influenced *Myc* expression in *Nkx3.1*<sup>+/+</sup> mice, but had no statistically significant effect in *Nkx3.1*<sup>-/-</sup> mice. *Myc* expression also differed significantly between genotypes under placebo treatment. Statistical analysis was performed using Student's t-test (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001).



**Figure 19: Relative expression of *Trp53* in *Nkx3.1*<sup>+/+</sup> and *Nkx3.1*<sup>-/-</sup> testes following treatment with E2 or PBO.**

Bar graphs represent *Trp53* gene expression normalized to housekeeping genes and expressed relative to *Nkx3.1*<sup>+/+</sup> placebo. Error bars show mean ± SEM from three biological replicates, each measured in technical triplicates. Elevated testicular E2 did not differentially influence *Trp53* expression in *Nkx3.1*<sup>+/+</sup> and *Nkx3.1*<sup>-/-</sup> mice. Statistical analysis was performed using Student's t-test (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001).

## CHAPTER 4: DISCUSSION

This study focused on determining the effects of elevated E2 on testicular structure and the expression of cancer-related genes in the absence of *Nkx3.1*. Our study used *Nkx3.1*-deficient mice and administered estradiol via subcutaneous injection and extended-release pellets, well-established methods commonly used to elevate serum estradiol concentrations [137].

*Nkx3.1* acts as a tumor suppressor and is a key regulator of differentiation and homeostasis in both the prostate and testis. Its loss is an early event in prostate carcinogenesis and has been associated with impaired testicular function. Estrogen interacts with androgen-regulated pathways and plays a critical role in maintaining the structure and function of the testis. Elevated estrogen in the testis can suppress androgen synthesis via negative feedback on the HPG axis. Therefore, elevated estrogen levels may disrupt testicular structure and function, as well as downregulate the expression of the androgen-regulated gene *Nkx3.1*.

We used *Nkx3.1*-deficient mice to examine how the absence of *Nkx3.1* in the testis, under conditions of elevated E2, might alter testicular structure and function. These testicular changes could have implications for prostate carcinogenesis, as elevated estrogen of testicular origin may act systemically to alter the hormonal milieu of the prostate, while loss of *Nkx3.1* may impair testicular function and contribute to systemic hormonal imbalance.

We hypothesized that elevated testicular E2 levels would alter testicular structure and induce distinct changes in the expression of cancer-related genes in the absence of the *Nkx3.1* gene. Findings from this study may provide insights into how elevated testicular E2 levels could contribute to prostate carcinogenesis.

Our key findings from this study revealed that, in response to elevated E2 levels:

- 1) *Nkx3.1*<sup>-/-</sup> testes exhibited irregular seminiferous tubules, degenerated seminiferous epithelium, disorganized cellular morphology, expanded interstitial space and altered connective tissue architecture.
- 2) Gene expression in *Nkx3.1*<sup>-/-</sup> testes remained unchanged for *Esr1*, *Esr2*, *Trp53* and *Myc*.
- 3) In *Nkx3.1*<sup>+/+</sup> testes, elevated E2 reduced *Esr1* and increased *Esr2* and *Myc* expression.

#### **4.1 Elevated Systemic E2 Levels Disrupts Testicular Architecture When *Nkx3.1* Is Absent**

The testes of *Nkx3.1*<sup>-/-</sup> mice exposed to prolonged E2 treatment exhibited more severe tissue abnormalities compared to those in the placebo group. These results align with previous reports demonstrating that elevated E2 levels can damage normal testicular tissue [30, 41, 80]. The observed alterations in E2-treated *Nkx3.1*<sup>-/-</sup> mice are likely a result of elevated testicular E2 levels, as *Nkx3.1*<sup>-/-</sup> mice receiving placebo treatment displayed mild alterations likely due to the *Nkx3.1* gene mutation. Our findings suggest that testicular structure is more severely disrupted when *Nkx3.1* gene is absent and E2 levels are elevated.

In the E2-treated *Nkx3.1*<sup>-/-</sup> mice, the testes displayed irregular and loosely packed seminiferous tubules, indicating reduced cellular density and disrupted organisation, likely due to increased cell atrophy. In contrast, placebo-treated *Nkx3.1*<sup>-/-</sup> mice had more rounded and densely packed seminiferous tubules with higher cellular density, although abnormalities were still present due to the *Nkx3.1* mutation.

Additionally, the testes of *Nkx3.1*<sup>-/-</sup> mice exposed to elevated E2 levels showed increased interstitial space and disorganised Leydig cell clusters. In comparison, the interstitial space between tubules in placebo-treated *Nkx3.1*<sup>-/-</sup> testes exhibited less disrupted distribution of Leydig cells. These findings suggest that elevated E2 levels may disrupt the regulatory mechanisms controlling testosterone production in *Nkx3.1*<sup>-/-</sup> testes, as Leydig cells are responsible for testosterone synthesis [81, 104]. This is consistent with the known inhibitory effect of testicular E2 on LH, which suppresses Leydig cell function and reduces testosterone levels [65, 77].

Similar observations were seen in the *Nkx3.1*<sup>+/+</sup> testes where there was a decrease in testicular cells [30] and a decrease in testosterone [80] in response to estradiol treatment.

The *Nkx3.1* gene negatively modulates AR activity [10, 12]; therefore, its loss would be expected to enhance AR signalling. In our results, elevated testicular E2 may reduce testosterone production through its observed deleterious effects on Leydig cells, implying that elevated E2 levels may directly impair Leydig cell function even in the absence of *Nkx3.1*.

Sertoli cells also appeared disrupted in *Nkx3.1*<sup>-/-</sup> testes exposed to elevated E2, exhibiting signs of degeneration including vacuolation and a lower number of cells lining the basement membrane. These abnormalities may impair germ cell development and disrupt spermatogenesis, as Sertoli cells play a central role in regulating germ cell maturation and supporting normal spermatogenesis [136, 147].

Furthermore, in E2-treated *Nkx3.1*<sup>-/-</sup> testes, degeneration of the seminiferous epithelium was observed, leading to epithelial thinning and germ cell loss, indicative of impaired spermatogenesis. These changes were possibly due to testicular atrophy and increased apoptosis triggered by elevated E2 levels. The lumen was enlarged and contained detached germ cells

showing vacuolation, along with fragmented cellular debris, likely due to epithelial degeneration. In contrast, the lumen of the testes from placebo-treated mice appeared more compact with minimal space, suggesting mildly active spermatogenesis due to the *Nkx3.1* mutation. These observations support the idea that elevated testicular E2, in the absence of *Nkx3.1*, further impairs germ cell survival and differentiation, resulting in germ cell apoptosis, ultimately disrupting spermatogenesis.

This is in line with previous findings of reduced spermatogenic activity, increased germ cell apoptosis and subsequent germ cell loss in the seminiferous epithelium of normal murine testes following chronic E2 treatment [30, 41, 80].

#### **4.2 Elevated Systemic E2 Alters Testicular Connective Tissue in the Absence of *Nkx3.1***

In *Nkx3.1*<sup>-/-</sup> testes treated with elevated E2, widened interstitial space was observed between seminiferous tubules and the interstitial connective tissue appeared disrupted, with loss of its normal architecture. These changes suggest ECM remodelling and possible fibrosis, which may disrupt the testicular microenvironment. This potential fibrotic remodelling could disrupt the smooth muscle layer, impairing its contractile function and potentially compromising the structural integrity of the seminiferous tubules. It may also contribute to stromal remodelling, potentially fostering a pro-tumourigenic microenvironment.

In contrast, in placebo-treated control testes, the interstitial space between seminiferous tubules appeared compact and well-organised, with a relatively intact connective tissue structure. These findings indicate that the testicular microenvironment exhibits minimal disruption despite the

loss of *Nkx3.1*, suggesting that the observed interstitial alterations are induced by the combined effect of elevated E2 and *Nkx3.1* deficiency.

Sertoli cells are involved in the deposition and regulation of ECM components [98], so the widened interstitial space and disrupted connective tissue architecture observed in E2-treated *Nkx3.1*<sup>-/-</sup> testes may reflect E2-induced impairment of Sertoli cell function.

### **4.3 Elevated Systemic E2 in the Absence of *Nkx3.1* Does Not Affect the Expression of Cancer-Related Genes**

While testicular cell morphology was altered by elevated E2 levels in the absence of *Nkx3.1*, the expression of the estrogen-responsive genes *Esr1*, *Esr2*, *Trp53* and *Myc*, which are related to cancer, remained unchanged. However, these findings are the opposite of what was expected. Since elevated E2 levels in the absence of *Nkx3.1* resulted in changes in morphological features and composition of testicular tissue, we expected to see changes in the expression of these estrogen-responsive genes, as altered tissue structure is often associated with molecular changes.

The fact that high testicular E2 levels did not significantly influence the expression of these cancer-related genes is likely because other pathways such as AR signalling pathways might be compensating for the loss of *Nkx3.1* and elevated levels of E2. This could indicate a compensatory response, where increased levels of E2 in the absence of *Nkx3.1* trigger the activation of other signalling pathways to counterbalance their effects on gene expression.

### **4.4 Elevated Systemic E2 Influences the Expression of Cancer-Related Genes in the *Nkx3.1*<sup>+/+</sup> Testis and May Indirectly Affect Prostate Carcinogenesis**

The testes of *Nkx3.1*<sup>+/+</sup> mice showed reduced expression of *Esr1* in response to E2 treatment. The *Esr1* gene encodes the ER $\alpha$  protein, which is primarily expressed in testicular stromal cells

[60, 83]. The observed reduction in *Esr1* expression suggests that elevated testicular E2 could impact signalling by stromal cells, potentially influencing testicular development and function. It is possible that elevated testicular E2 may alter ER $\alpha$ -driven stromal function, creating a tumour-promoting microenvironment.

ER $\alpha$  plays a role in modulating AR activity and E2 regulates testicular steroidogenesis via ER $\alpha$  [6]. Therefore, the reduction in *Esr1* expression suggests that elevated testicular E2 may alter AR signalling and steroidogenesis in *Nkx3.1*<sup>+/+</sup> testes.

Interestingly, while the testes of *Nkx3.1*<sup>+/+</sup> mice treated with E2 showed reduced *Esr1* expression, *Esr2* expression was significantly increased. *Esr2* encodes the ER $\beta$  protein, which regulates cell differentiation and predominantly mediates estrogenic effects to maintain normal testicular function [47]. Therefore, the upregulation in ER $\beta$  in the testis may represent a feedback mechanism to compensate for the downregulation of ER $\alpha$ .

In *Nkx3.1*<sup>+/+</sup> testes, *Myc* expression significantly increased following E2 treatment, suggesting that the presence of *Nkx3.1* facilitates E2-driven upregulation of *Myc*. Although, *Nkx3.1* has been associated with reduced *Myc* expression [79], the increase in *Myc* expression observed in our study in the presence of *Nkx3.1*, suggests that elevated testicular E2 is reducing the suppressive effect of *Nkx3.1* and promoting *Myc* expression. *Myc* is a well-known oncogene that regulates cell proliferation; therefore its upregulation as a result of elevated testicular E2 levels may activate pathways that promote abnormal cell growth.

Interestingly, no significant changes in *Trp53* expression were observed. E2 is known to modulate *Trp53* activity [16] but the unchanged expression despite elevated E2 levels in the

testis may be due to *Nkx3.1* stabilizing *Trp53*. *Nkx3.1* may be interacting with other signalling pathways to prevent E2 levels from altering *Trp53* levels.

These results imply that elevated E2 levels can alter gene expression independently of *Nkx3.1* loss. Since the testis contributes to the systemic hormonal milieu that influences the prostate, these findings suggest that elevated testicular E2 may indirectly promote a pro-tumorigenic environment in the prostate, even when *Nkx3.1* is present.

## CONCLUSION

Although mild tissue changes were observed in the testes of *Nkx3.1*<sup>-/-</sup> mice receiving placebo, the alterations seen in testes exposed to elevated levels of E2 suggest that E2 exposure exacerbates testicular defects in the absence of *Nkx3.1*. These findings show that prolonged E2 exposure severely alters testicular morphological structure and connective tissue organisation when *Nkx3.1* is absent. These changes could have downstream effects on prostate health given the role of the testis in regulating hormonal balance.

While no significant changes in the selected cancer-related genes were observed in *Nkx3.1*-deficient testes exposed to elevated E2, the disruption of testicular architecture seen under these conditions suggests that molecular alterations are occurring. Even though the expression of these genes remained unchanged, elevated testicular E2 may still significantly influence other estrogen-responsive, cancer-related genes when *Nkx3.1* is absent.

The *Nkx3.1*<sup>+/+</sup> testes had significant changes in the expression of the cancer-related genes in response to E2 treatment, suggesting that *Nkx3.1* may play a role in modulating gene expression in response to elevated E2 levels in the testis. Although *Nkx3.1* does not directly influence estrogen, it may indirectly affect ER signalling through its modulation of AR activity; however, this remains to be elucidated.

Taken together, our findings suggest that elevated estrogen levels lead to structural and functional disruptions within the testis, which may have potential implications for prostate carcinogenesis.

## FUTURE DIRECTIONS

We were unable to detect changes in the expression of *Esr1*, *Esr2*, *Trp53* and *Myc* by RT-qPCR in *Nkx3.I<sup>-/-</sup>* testes. Therefore, additional cancer-related genes could be analysed in order to further evaluate the effect of elevated E2 on *Nkx3.I<sup>-/-</sup>* testes. IHC analysis could be performed to determine whether gene expression changes between *Nkx3.I<sup>-/-</sup>* and *Nkx3.I<sup>+/+</sup>* testes are reflected at the protein level. Assessing intra-testicular and serum E2 concentrations would also help confirm elevated E2 levels and clarify their effects on testicular structure and gene expression, including their potential role in prostate carcinogenesis.

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# APPENDICES

## Appendix 1: Animal Research Ethics Approval

Email

<https://memorial.researchservicesoffice.com/Romeo.Researcher/Research...>

**From** ambakwe@mun.ca  
**To** Kao, Kenneth(Principal Investigator) <KKAO@MUN.CA >  
**Subject** Your Annual Report has been approved  
**Attachments**



**MEMORIAL UNIVERSITY**  
 Animal Care Committee (ACC)  
 St. John's, NL, Canada A1C 5S7  
 Tel: 709 777-6620 acs@mun.ca  
<https://www.mun.ca/research/about/acs/acc/>

**Dear: Dr. Kenneth Kao, Faculty of Medicine/Division of BioMedical Sciences**

Researcher Portal File No.: 20230906  
 Animal Care File: 19-01-KK  
 Entitled: (19-01-KK) The role of estrogen on the initiation of prostate cancer  
**Related Awards:**

Awards File No	Title	Status	
20192883	Characterization of a novel Myc-Pygo2 ribonucleoprotein complex that drives prostate cancer cell proliferation	Completed	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20201714	Evidence to assess risk factors for prostate cancer in transgender women	Completed	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20231461	Role of estrogen on the initiation of prostate cancer	Active	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20240829	Unraveling the Link between Ageing, Estrogen, and Susceptibility to Prostate Adenocarcinoma	Denied	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses
20250126	Role of estrogen on the initiation of prostate cancer	Denied	1. Research Initiatives & Services (RIS) – St. John's and Grenfell Campuses

Approval Date: October 01, 2022  
 Next Annual Report Due: October 01, 2025  
 Ethics Clearance Expires: October 01, 2025

**Your Annual Report was reviewed by the ACC and approved.**

**Animal use records will be compiled and reported to the Canadian Council on Animal Care.**

**NOTE: You can access a copy of this email at any time under the “Shared Communications” section of the Logs tab of your file in the Memorial Researcher Portal.**

Please note that approval of the protocol or amendment does not guarantee space for animal housing or procedures. Coordination with Animal Care & Veterinary Resources is required prior to ordering animals.

Sincerely,

**ANULIKA MBAKWE**  
 ACC Coordinator | Department of Animal Care & Veterinary Resources (ACVR)  
 Animal Resource Centre (ARC) | Room H-1A100 |  
 Memorial University of Newfoundland | Research  
 T: 709-864-3763 | ambakwe@mun.ca | www.mun.ca/acs

## Appendix 2: Teklad Global 18% Protein Rodent

2018

Teklad Global 18% Protein Rodent Diet

**Product Description-** 2018 is a fixed formula, non-autoclavable diet manufactured with high quality ingredients designed to support gestation, lactation, and growth of rodents. 2018 excludes alfalfa meal, which lowers phytoestrogen (coumestrol) content, and reduces chlorophyll, improving optical imaging clarity. A moderate inclusion of soybean meal results in an expected isoflavone range of 225-340 mg/kg diet (daidzein + genistein aglycone equivalents). Absence of fish meal minimizes the presence of nitrosamines.

**Related codes 2018C (certified), 2918 (irradiated), 2918C (irradiated, certified), 2018X (extruded), 2918X (irradiated, extruded), 2018S/SC (sterilizable, certified), 2018SX (sterilizable, extruded).**

Macronutrients		
Crude Protein	%	18.4
Fat (ether extract) <sup>a</sup>	%	6.0
Carbohydrate (available) <sup>b</sup>	%	44.2
Crude Fiber	%	3.8
Neutral Detergent Fiber <sup>c</sup>	%	14.7
Ash	%	5.5
Energy Density <sup>d</sup>	kcal/g (kJ/g)	3.1 (13.0)
Calories from Protein	%	24
Calories from Fat	%	18
Calories from Carbohydrate	%	58
Minerals		
Calcium	%	1.0
Phosphorus	%	0.7
Non-Phytate Phosphorus	%	0.4
Sodium	%	0.2
Potassium	%	0.6
Chloride	%	0.4
Magnesium	%	0.2
Zinc	mg/kg	70
Manganese	mg/kg	100
Copper	mg/kg	15
Iodine	mg/kg	6
Iron	mg/kg	200
Selenium	mg/kg	0.23
Amino Acids		
Aspartic Acid	%	1.4
Glutamic Acid	%	3.4
Alanine	%	1.1
Glycine	%	0.8
Threonine	%	0.7
Proline	%	1.6
Serine	%	1.1
Leucine	%	1.8
Isoleucine	%	0.8
Valine	%	0.9
Phenylalanine	%	1.0
Tyrosine	%	0.6
Methionine	%	0.4
Cystine	%	0.3
Lysine	%	0.9
Histidine	%	0.4
Arginine	%	1.0
Tryptophan	%	0.2

Teklad Diets are designed and manufactured for research purposes only.



Teklad Diets, Madison, WI | Teklad@inotivco.com | 800.483.5523 | inotivco.com



**Ingredients** (in descending order of inclusion)- Ground wheat, ground corn, wheat middlings, dehulled soybean meal, corn gluten meal, soybean oil, calcium carbonate, dicalcium phosphate, brewers dried yeast, iodized salt, L-lysine, DL-methionine, choline chloride, magnesium oxide, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B<sub>12</sub> supplement, folic acid, biotin, vitamin D<sub>3</sub> supplement, cobalt carbonate.

Standard Product Form: Pellet

Vitamins		
Vitamin A <sup>e,f</sup>	IU/g	15.0
Vitamin D <sub>3</sub> <sup>e,g</sup>	IU/g	1.5
Vitamin E	IU/kg	110
Vitamin K <sub>3</sub> (menadione)	mg/kg	50
Vitamin B <sub>1</sub> (thiamin)	mg/kg	17
Vitamin B <sub>2</sub> (riboflavin)	mg/kg	15
Niacin (nicotinic acid)	mg/kg	70
Vitamin B <sub>6</sub> (pyridoxine)	mg/kg	18
Pantothenic Acid	mg/kg	33
Vitamin B <sub>12</sub> (cyanocobalamin)	mg/kg	0.08
Biotin	mg/kg	0.40
Folate	mg/kg	4
Choline	mg/kg	1200
Fatty Acids		
C16:0 Palmitic	%	0.7
C18:0 Stearic	%	0.2
C18:1ω9 Oleic	%	1.2
C18:2ω6 Linoleic	%	3.1
C18:3ω3 Linolenic	%	0.3
Total Saturated	%	0.9
Total Monounsaturated	%	1.3
Total Polyunsaturated	%	3.4
Other		
Cholesterol	mg/kg	--

**Shelf life:** With proper storage, diet is suitable for use out to 9 months.

[www.inotivco.com/shelf-life-of-diets-used-in-research](http://www.inotivco.com/shelf-life-of-diets-used-in-research)

<sup>a</sup> Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

<sup>b</sup> Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

<sup>c</sup> Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

<sup>d</sup> Energy density is a calculated estimate of *metabolizable energy* based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

<sup>e</sup> Indicates added amount but does not account for contribution from other ingredients.

<sup>f</sup> 1 IU vitamin A = 0.3 µg retinol

<sup>g</sup> 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.