THE UNIVERSITY OF CHICAGO

THE ROLE OF SOX2 IN REGULATING METABOLISM AND ENZALUTAMIDE RESISTANCE IN PROSTATE CANCER

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To my family

Ek sou dit nie kon doen sonder julle nie

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ABSTRACT

While the survival rate is close to 100% for men who are diagnosed with local prostate cancer disease, the survival rate for advanced stage patients is only about 28%. Approximately 10-20% of men present with metastatic disease, and many others progress to metastasis after hormone treatment, surgery or radiotherapy. However, few biomarkers have been effective at predicting which patients will metastasise, especially for patients who have biochemically recurred. As such, understanding why patients metastasise or become resistant to treatments, as well as identifying biomarkers that will predict response to therapy or propensity for more aggressive disease, is crucial to increasing survival of patients.

We show that SOX2 is a marker for patients who metastasise after biochemical recurrence, and that patients with a SOX2-positive tumour will have a shorter time to metastasis and prostate cancer specific survival. Additionally, we show that SOX2 is not acting in its canonical stem cell-like manner in prostate cancer, and in this context does not confer a neuroendocrine phenotype. In castration-resistant prostate cancer, we see that SOX2 regulates both glycolysis and mitochondrial respiration, perhaps through regulation of PFKP, a key glycolytic enzyme, or increasing mitochondrial mass. We also observed that cells without SOX2 were more sensitive to enzalutamide, with decreased growth and a G1 arrest. This may be due to increased DNA damage, and a deficiency in DNA repair pathways.

In this dissertation, we show for the first time a role for SOX2 in prostate cancer metabolism, as well as a novel role for SOX2 in DNA replication and repair. SOX2 can be used to identify patients at a higher risk for metastasis, and can aid in treatment decisions such as a radical prostatectomy for lower risk patients with SOX2-positive tumour, or increased screening for metastases. Alternatively, due to its role in DNA replication and repair, it could be used to predict patient response to radiation therapy, and patients with SOX2-positive tumours may not respond to radiation therapy. Overall, the implications of this work reveal great potential for the use of SOX2 as a biomarker clinically to inform therapeutic decisions and improve patient outcome.

CHAPTER 1

INTRODUCTION

1.1 PROSTATE CANCER INCIDENCE AND RISK

In the United States, prostate cancer is the most common cancer among men, with an estimated 174,650 cases diagnosed in 2019, and the second leading cause of cancer-related death, with an estimated 31,620 deaths in 2019 [1]. Prostate cancer rarely occurs before the age of 45, and mostly affects older men, with 60% of cases diagnosed in men over the age of 65 and the average age of diagnosis being 66 [1]. The world-wide prostate cancer burden is expected to increase to 1.7 million cases diagnosed and 500,000 deaths by 2030, mostly due to growth and ageing of the global population [2]. Variations in prostate cancer incidence rates globally result in part from differences in diagnosis using prostate specific antigen (PSA) testing or during surgery [2]. 72% of prostate cancer cases and 53% of cancer deaths occur in developed countries (all regions of Europe plus North America, Australia/New Zealand, and Japan), which represent less than 20% of the global population. Prostate cancer rates varied 24-fold globally in 2008, with the highest rates of incidence occurring in Australia/New Zealand, and the rate of mortality varied 10-fold, with most deaths occurring in the Caribbean, followed by countries in southern and western Africa. The lowest rates of incidence and mortality were found in Asia and northern Africa, with North America also having a low rate of mortality [2]. Prostate cancer rates have increased globally in many countries, ranging from 2-3% in Sweden to 12-16% in China, but have stabilised in other countries, such as the United States and Australia. This increase is likely due to increased use of PSA testing, and it is estimated that 23-42% of diagnosed cases in Europe and the United States is due to overuse of PSA testing [2]. However, the use of PSA testing cannot fully explain the variations globally in prostate cancer incidence.

Prostate cancer risk factors include increasing age, African ancestry, family history of prostate cancer and certain inherited genetic mutations, as well as certain lifestyle factors

[1]. Men of African origin are more likely to develop prostate cancer and are more than twice as likely to die from prostate cancer as white men. Furthermore, these men tend to develop cancer at a younger age and have more advanced disease at the time of diagnosis [3]. However, these differences in prostate cancer risk by race may be due to additional factors, such as differences in access to care and decision-making process of seeking medical treatment, or differences genetically with different allelic frequencies of microsatellites at the androgen-receptor (AR) locus or polymorphic variations [4]. Prostate cancer is one of the most heritable cancers, and men with a brother or father who had been diagnosed with prostate cancer having a 2- to 4-fold higher risk of developing prostate cancer, with the risk increasing if a brother is diagnosed [5]. Furthermore, the risk of prostate cancer risk also increases with the number of affected relatives [6]. Studies in twins estimate that 57% of the variation in risk can be attributed to genetic factors [7]. Alterations in genes such as BRCA1, BRCA2, and HOXB13, have been implicated in cases of hereditary prostate cancer, as well as other types of cancers. Breast and prostate cancer co-occur in families, and men with familial breast cancer had a 21% greater risk of prostate cancer overall [8]. Further, men with mutations in BRCA2 or HOXB13 genes have a higher risk of developing life-threatening and more aggressive forms of prostate cancer [9]. A broad variety of dietary factors have also been suggested to play a role in the development of prostate cancer according to many epidemiological studies. Fat consumption, in particular polyunsaturated fat, shows a strong correlation with prostate cancer incidence and mortality, potentially as a result of fat-induced alterations in hormonal profiles, the effect of fat metabolites as protein or DNA-reactive intermediates, or fat-induced elevation of oxidative stress [4]. Despite extensive research into identifying additional risk factors, few have definitively proven to indicate increased risk of prostate cancer.

1.2 PROSTATE CANCER DEVELOPMENT

The prostate is a walnut-sized male organ, located in front of the rectum and behind the bladder, with the urethra running through the middle of the prostate. The main function of the prostate is to produce fluid for semen. The prostate is divided into three zones based on their function: the peripheral zone (PZ), the central zone (CZ), and the transition zone (TZ) [10]. The peripheral zone is closest to the rectum, and can felt during a digital rectal exam, while the transition zone is closest to the urethra. Approximately 70-75% of prostate cancers arise within the PZ whereas fewer than 30% of prostate cancer cases arise in the TZ, and almost no cases are found in the CZ [11, 12].

A large study of 1354 radical prostatectomy samples indicate that there is no difference between TZ and PZ with respect to clinical markers such as age at diagnosis, extracapsular extension, lymph node involvement and lymphovascular invasion [13]. However, there appears to be distinct differences in the underlying biology of cancers in each zone, as patients with cancers in the PZ have a worse prognosis in terms of biochemical recurrence, local, and distant metastasis [14]; this is of great interest, since cases in the TZ are often diagnosed at larger tumour volumes and higher PSA levels [14], likely due to the difficulty of detecting cases via traditional methods, such as a digital rectal exam. There also appear to be differences between TZ and PZ cancers in the volume of high-grade disease compared to the total tumour volume, where cancers arising in the PZ have a larger percentage of high-grade disease compared to cancers from the TZ [14]. Other studies have indicated that TZ cancers have lower proliferation rates and maintain diploid DNA status compared to PZ cancers [15, 16], the oncogenic TMPRSS-ERG fusion is rarely found in TZ cancers [17]. Currently the zonal location of the prostate cancer is not utilised for treatment decisions, but the apparent differences in underlying biology between zones may be utilised in the future for diagnostic purposes.

1.3 PROSTATE CANCER DIAGNOSIS AND SURVIVAL

Prostate cancer is most commonly diagnosed through two tests: the prostate-specific antigen (PSA) blood test and a digital rectal exam (DRE). If the results of either of these tests appears abnormal, a prostate biopsy is performed to confirm the presence of prostate cancer. PSA is a protein produced by the cells of the prostate gland, and both normal or cancer cells secrete PSA. PSA is mostly found in semen, but lower levels can also be detected in the blood [18]. The higher the PSA, the greater the chance of prostate cancer, however there is no set cut-off level at which a man has prostate cancer. Doctors tend to use a level of greater than 4.0 ng/mL to determine whether a man requires further testing, but a level below 4.0 ng/mL does not guarantee the absence of cancer. 15% of men with a PSA less than 4.0 ng/mL will have prostate cancer if a biopsy is done, and the chance of prostate cancer diagnosis increases to greater than 50% when the PSA level is greater than 10 ng/mL [18]. There are several factors that can raise PSA levels, and may confound results of the PSA test including presence of benign prostatic hyperplasia (a non-cancerous enlargement of the prostate), older age, prostatitis (inflammation of the prostate gland), or ejaculation. Due to the multitude of factors potentially affecting PSA levels, many doctors do not recommend their use to diagnose prostate cancer at this time [18]. During a digital rectal exam, a doctor probes the prostate through the rectum to feel for bumps or hard areas which may be tumours. A DRE is often utilised for prostate cancer diagnosis, as tumours are most commonly found in the peripheral zone of the prostate, which is closest to the rectum. The DRE is less effective at finding prostate cancer than the PSA test, but may detect cancers in men with normal PSA levels [18].

Despite the difficulties in detecting prostate cancer, the survival rates are high for prostate cancer, with the 5 year survival rate for all stages of the disease being 98% [1]. Most men are diagnosed with localised disease, where 77% of all prostate cancer cases are confined to the prostate and has not spread to other sites [1]. The survival rate is close to 100% for men who are diagnosed with local disease, but the survival rate for advanced stage patients is only

about 28% [19]. Approximately 10-20% of men with prostate cancer present with metastatic disease, and many others progress to metastasis after hormone treatment, surgery or radiotherapy [20]. The treatment options can vary based on the spread of the disease. Men with localised disease have three primary treatment options: expectant management, surgery, and radiation. Expectant management monitors prostate cancer progression without undergoing definitive treatment, and can involves series of PSA testing, physical examinations, biopsies, or a combination of these, with the goal of monitoring patients until other treatment becomes necessary [21]. Surgery and radiation are effective treatments for men with more significant cancer, such as those with a PSA level greater than 10 ng/mL or those with palpable nodes when a DRE was performed [21].

For patients with metastatic disease, androgen deprivation therapy (ADT) continues to be the first-line treatment. ADT prevents the production of testosterone or blocks testosterone from acting on prostate cancer cells via androgen-receptor signalling [21]. The proliferation and invasion of prostate cancer occurs in the androgen receptor (AR)-sensitive and PSA-secreting luminal cells, which rely on androgen signalling for their growth. This reliance on AR-signalling explains the success of androgen-deprivation therapy (ADT) for metastatic prostate cancer, as this leads to inhibition of proliferation and apoptotic death of the cancer cells [22]. Despite initial success of ADT, several mechanisms of resistance emerge, including AR gene mutations leading to promiscuous ligand binding, splice variations lacking the ligand binding domain, amplification, and AR protein over-expression [23]. This continued dependence on the AR signalling axis led to the development of second-line therapies such as enzalutamide and abiraterone, which block AR signalling and production of androgens, respectively [23]. More recently, interest in the use of docetaxel has increased, especially since clinical trials have shown that docetaxel treatment in combination with ADT increased overall survival by 10 months, and extended time to biochemical recurrence and progression [21]. Other treatment options for patients also include Sipuleucel-T, an autologous cellular immunotherapy which increased median survival by 4.1 months compared to

placebo, and cabazitaxel, a novel tubulin-binding taxane which increased median survival by 2.4 months compared to mitoxantrone. However, resistance to these second-line ADT therapies inevitably arises, and coupled with the low increase in survival for other therapies, highlight the need to identify these mechanisms of resistance in order to develop novel therapies for these patients.

1.4 SOX2 IN EMBRYONIC DEVELOPMENT, PROSTATE DEVELOPMENT, AND STEM CELLS

SOX2, a member of the SOXB1 subfamily of SOX transcription factors, is an essential transcriptional regulator in stem cell pluripotency [24]. The 20 members of the SOX gene family all share a highly conserved high-mobility-group (HMG) DNA binding domain [25], and are named after their similarity to the mammalian testis-determining factor Sry, found in the sex-determining region on the Y-chromosome [26]. The SOX proteins are divided into eight subfamilies based on their degree of homology within the HMG domain, other structural regions [27], and function [28]. Members of the same group typically have overlapping expression patterns and share similar functions, whereas members from other subfamilies perform different functions even when co-expressed [29]. Although the other members of the SOXB1 family, S1 and SOX3, have more than 80% homology with SOX2 and are functionally redundant, SOX2 exerts distinct functions in certain biological contexts [24]. The human SOX2 gene is located on chromosome 3 at position q26.3–27 and encodes for a 317 amino acids long protein [30]. The HMG domain of SOX2 is highly conserved among species, and allows for binding of SOX2 to potential protein partners, as well as containing a nuclear localisation and nuclear export signal. The C-terminal transactivation domain functions as the region where SOX2 binds to specific DNA consensus sequences on gene promoter regions, which leads to activation or repression of target genes [28].

SOX2 is expressed early in embryonic development, with SOX2 expression detected at the

2-cell stage of murine embryos, and increasing levels up to the blastocyst stage [31]. SOX2 plays a critical role in the emergence of the pluripotent inner cell mass, since its absence results in embryonic lethality [32]. While SOX2 is homogenously expressed in the inner cell mass, it becomes restricted to certain cell populations when the embryo develops from a single-layered to multi-layered structure during gastrulation [33], and after this process, SOX2 expression is primarily found in primordial germ cells, gut endoderm, presumptive neuroectoderm, sensory placodes and pharyngeal arches [34, 35]. SOX2's activity in early embryonic development is also essential for specification of the neural lineage, and this is done by SOX2's antagonistic effect on transcription factors that would favour development of non-neural cell lineages [33]. SOX2's effects are not only found in ectodermal lineages, but also in endoderm-derived structures, where SOX2 plays a role in the differentiation of tracheal cartilage, the emergence of the lung from the primary lung bud, and development of taste bud sensory cells [36, 37, 38, 39]. Further, different levels of SOX2 expression in the stomach leads to the formation of the forestomach and the posterior stomach and trachea. SOX2's role in the mesoderm is less characterised, some studies suggest SOX2 may also play a role in the development of cells in the mesodermal lineage [33].

SOX2 is highly expressed in embryonic stem cells, and its depletion leads to changes in mouse or human embryonic cell morphology, loss of pluripotent markers and differentiation into trophectoderm [40, 41]. In addition to SOX2, Nanog and octamer-binding transcription factor 4 (OCT4) are also core intrinsic transcription factors that regulate pluripotency [42, 43], and cooperate functionally to promote expression of pluripotency genes and to suppress expression of genes that allow for differentiation [42, 44]. SOX2 and OCT4 are proposed to be lineage-specific regulators of mesendoderm and ectoderm differentiation, respectively [45, 46]. Forced expression of Oct4 in Sox2-null mouse embryonic stem cells rescues the pluripotency of these cells, suggesting one role of Sox2 in maintaining pluripotency is to maintain adequate levels of Oct4 [41]. Sox2 expression is tightly regulated, with lower or higher levels leading to reduced pluripotency in embryonic stem cells, possibly by

reduced promoter/enhancer activity of Sox2/Oct4 target genes [47]. In 2006, Takahashi and Yamanaka reprogrammed somatic cells to induced pluripotent stem cells (iPSCs) by overexpressing four transcription factors, termed the Yamanaka factors. Considering their role in maintaining stemness in ESCs, it is not surprising SOX2 and OCT4 are Yamanaka factors, along with KLF4 and c-MYC [48]. Ectopic SOX2 expression during reprogramming induces expression of endogenous SOX2 and other pluripotency genes, and subsequently the endogenous core transcriptional network is formed, leading to a stable pluripotent state [49]. Beyond its well-known role in ESCs, SOX2 is also important in the adult. SOX2 expression persists in the adult neural stem cells and in neural progenitor cells in the neurogenic regions of several brain regions [33]. SOX2-positive stem cell populations have also been detected in other tissues, such as the progenitors of the retina, esophagus, glandular stomach, trachea, testes, and cervix [33]. Interestingly, when SOX2-positive adult stem cells are depleted in vivo, mice develop signs of premature ageing, such as kyphosis, hair greying, and reduced fat mass, suggesting SOX2 plays an essential role in maintaining adult stem cells [50].

Expression of SOX2 has been examined in both murine and human prostatic tissue. SOX2 is expressed during embryonic prostate budding morphogenesis [51], and in the adult prostate, SOX2 is detected primarily in basal epithelial and neuroendocrine cells [52], and is largely turned off in the luminal compartment following birth [51]. Our group has shown that Sox2 marks a progenitor cell population, and epithelial cells of the proximal prostate are enriched for Sox2 expression compared to distal regions. Through lineage tracing experiments in a mouse model, we demonstrated that the Sox2-positive lineage self-renews and leads to androgen-mediated regeneration of the prostate. Interestingly, following castration, there are Sox2-expressing luminal cells that persist, suggesting Sox2 is capable of castration-resistance and cell survival, in addition to prostate regeneration [52]. This is particularly interesting, since there is considerable evidence of SOX2 in cancer cell survival and proliferation, as well as castration-resistance.

1.5 SOX2 IN CANCER

Aside from its role in stem cells, SOX2 expression has also been detected in many different types of cancers, where its levels are higher in cancer than normal tissue [53]. For example, 85% of glioblastoma multiforme samples show elevated SOX2 compares to normal patient controls [54]. SOX2 amplification is also seen in various cancers, such as glioblastoma, small-cell lung cancer, ovarian, esophageal, and many forms of squamous cell carcinoma [28, 53]. One study found 26% of serous ovarian cancers have amplified levels of SOX2 [55]. An amplification of the SOX2 locus (3q26.33) has also been reported, suggesting copy number alterations may partly explain this increase in SOX2 expression [53]. SOX2 levels are also varied at different stages of cancer progression. In pancreatic ductal adenocarcinomas, SOX2 is rarely expressed in pre-malignant pancreatic intraepithelial neoplasia, but its expression increases 60% when the cancer becomes poorly differentiated and invasive [56]. In ovarian cancers, approximately 55% of normal ovarian epithelial samples express SOX2 (although a low overall percentage of the cells), but this increases to over 90% of serous and mucinous cystadenocarcinomas samples, where the percentage of cells expressing SOX2 is much higher [57]. For gastric cancers, some studies have demonstrated lower SOX2 expression in cancer and metastases than the normal gastric mucosa [58, 59], and varied levels of SOX2 between different mucosal subgroups of gastric cancer [60].

SOX2's role in prognosis and outcome has also been examined in many cancers, where elevated levels of SOX2 are associated with a poor prognosis for patients with breast, colorectal, prostate, esophageal, ovarian, some lung cancers, nasopharyngeal and sinonasal carcinomas [53]. Furthermore, patients with amplified SOX2 expression in sinonasal carcinomas had a higher incidence of recurrence [61], and rectal cancer patients with higher SOX2 levels had shorter disease-free survival after chemoradiation therapy [62]. High expression of SOX2 and OCT4 in esophageal squamous cancer patients was associated with a higher histological grade and TNM stage, suggesting a more dedifferentiated tumour, and decreased survival [63]. Aside from prognosis, the correlation between elevated levels of SOX2 and metastatic

capacity has also been suggested in the majority of cancers [53]. For example, in prostate and colorectal cancer, SOX2-positive tumours are correlated with increased distant and lymphatic metastases [64, 65, 66], and in esophageal squamous cell carcinomas, tumours with more than 50% of the cells expressing SOX2 were correlated with increased vascular and lymphatic invasion and poor differentiation [67]. However, in some cancers, such as gastric and squamous cell lung cancer, low SOX2 expression is associated with poor prognosis, and increased lymph node and distant metastases [58, 68, 69]. As of yet, it is unclear why SOX2 is playing opposing roles in different types of cancers, but clearly the tissue of interest needs to be taken into consideration when thinking about SOX2's role in clinical outcome of patients.

Due to its role in maintaining ESC pluripotency, many studies have examined whether SOX-expressed cells are tumour-initiating populations in cancer, the so-called cancer stem cell (CSC) population. SOX2 has been shown to increase markers of CSCs in pancreatic, lung, and ovarian cancers [70, 71, 72], as well as mediating self-renewal of CSCs in many cancers including breast, gastric, glioma, and osteosarcoma [54, 73, 74, 75]. Putative stem cell markers such as CD133, CD44, and ABCG2 have been used to isolate and enrich for cells capable of forming tumours. In an ovarian cancer cell line, this enriched population had elevated levels of SOX2 mRNA and a higher percentage of tumour initiating cells (TICs) when performing a limiting dilution assay, the gold-standard for assessing the frequency of TICs in a tumour [76]. Experiments in gastric cancer showed a decrease in the number of spheroid colonies formed upon siRNA-mediated SOX2 knock-down [75], and similarly in non-small cell lung cancer SOX2 knock-down led to a 2.5-fold reduction in sphere formation [77]. Experiments isolating SOX2-positive cells indicate that these cells exhibit a higher frequency of TICs compared to SOX2-negative cells by limiting cell dilution tumour assays [78, 79]. Furthermore, these rare SOX2-positive cells are part of a quiescent, slow-cycling cancer stem cell population that can repopulate the tumour when cytotoxic drugs are withdrawn [80, 79].

SOX2 has also been implicated in drug resistance to therapeutics in other cancers. Ovar-

ian cancer cell lines without endogenous SOX2 expression were sensitive to carboplatin, paclitaxel and cisplatin, but became resistant upon stable expression of SOX2 [70]. Similarly, ectopic expression of SOX2 in MCF-7 cells led to resistance to tamoxifen, and this was abrogated upon SOX2 knockdown [81]. Although SOX2 may be leading to drug resistance through quiescent or anti-apoptotic signalling, SOX2 has been shown to upregulate various ATP-binding cassette (ABC) transporters, such as ABCG2, ABCC3, and ABCC6. The impact of SOX2 on ABCG2 has been particularly well examined, where its expression has been upregulated in many TICs [82, 76], and can be considered an additional CSC marker in some cancers [53]. In lung cancer and head and neck squamous cell carcinoma, knockdown of SOX2 leads to decreased expression of ABCG2 and increased chemosensitivity, suggesting ABCG2 has a role in SOX2-mediated drug resistance [83, 77].

1.6 SOX2 IN PROSTATE CANCER

We have previously shown that SOX2 is one of the most differentially and highly expressed genes between the prostate and seminal vesicle, an organ with a similar function and dependence on androgen receptor signalling as the prostate, but which is rarely is the site of tumourigenesis [84]. SOX2's co-factors in ESCs, OCT4 and NANOG, are not expressed in prostate cancer, suggesting SOX2 interacts with novel binding partners in prostate cancer and may regulate unique, non-stem cell gene targets. In human tissues, SOX2 expression is restricted to 75% of normal basal epithelial cells, while prostate tumours are either binary (either SOX2-positive or SOX2-negative, not heterogenous) and SOX2's expression is found in luminal cells. The percentage of SOX2-positive tumours increases with Gleason Score, and nearly all lymph node metastases are SOX2-positive [65]. Further, ectopic expression of SOX2 in a hormone-sensitive prostate cancer cell line is sufficient to allow tumour formation in a castrated host, leading to castration-resistant prostate cancer tumour growth [65]. These data also imply that SOX2 positive tumours could arise from SOX2-positive basal epithelial cells, and thus SOX2 has clinical utility has a biomarker to discern indolent from aggressive

prostate tumours.

Due to its role in maintaining pluripotency and an undifferentiated state, there has been much interest in the role of SOX2 in neuroendocrine prostate tumours. Neuroendocrine (NE) cells are the third type of epithelial cell in the prostate, in addition to basal and luminal cells, and are rare at about 1% of the total epithelial cells [85]. In the normal prostate, NE cells are involved in regulating epithelial cell growth and differentiation in an androgen-independent manner, and in prostate cancer, it has been suggested that the NE cell population expands, and this leads to tumour progression, poor prognosis, and hormone-refractory cancers [86]. NE cells are not easily distinguished under a microscope, and are typically identified through immunohistochemistry using antibodies for markers such as chromogranin A, synaptophysin, and neuron-specific enolase [87]. Two recent studies examined the role of SOX2 in prostate cancer, specifically in the context of the combined loss of TP53 and RB1, which occur in more aggressive forms of prostate cancer [88, 89]. Combined knock down of RB1 and TP53 in a hormone sensitive cell line, LNCaP, led to increased SOX2 levels, enzalutamide resistance, and increased NE markers [89]. Subsequent knock-down of SOX2 in these cells restored enzalutamide sensitivity and reversed the expression of NE markers [89].

While some reports have indicated a role for SOX2 in the androgen-independent NE cells, others have examined SOX2 in the context of Androgen Receptor (AR) -signalling. Previously, we have demonstrated that SOX2 is an AR-repressed gene, where its expression is decreased upon AR signalling in a castration-resistant prostate cancer cell line, CWR-R1, and is restored upon treatment with enzalutamide, a common second-line therapy in prostate cancer that prevents AR signalling [65]. Similarly, expression of AR in PC3 cells, which do not endogenously express AR, led to a reduction in SOX2, further confirming SOX2 is an AR-repressed gene [90]. Collectively, these data suggest that the role of SOX2 in different types of prostate cancers may depend on many factors. As such, there has been great interest in understanding the role of SOX2 in prostate cancer and its targets as key mediators of cancer metastasis and progression to castration-resistance.

1.7 PROSTATE METABOLISM

Benign prostate epithelial cells have a unique metabolism and utilisation of citrate. Unlike other cells that rely on citrate oxidation as a key step in the Krebs cycle for the progression of aerobic respiration, prostate cells are programmed to produce but not oxidise citrate [91]. This is particular to the prostate epithelial cells in the peripheral zone of the prostate, and this specialisation is of particular interest, considering many cancers initiate in the peripheral zone [92]. High levels of zinc transporter proteins allow for the accumulation of zinc in prostate epithelial cells [93], which in turn inhibits the activity of m-aconitase and prevents the oxidation of citrate within the Krebs cycle [94]. Through the accumulation of zinc, and as a result citrate, normal prostate epithelial cells appear to halt the Krebs cycle and act differently to other cells in the body in respect to ATP production. Citrate is then secreted as part of the seminal fluid, and is essential for the normal function of the prostate.

One of the hallmarks of cancer is the so-called Warburg effect, where cancer cells shift their dominant ATP producing pathway away from oxidative phosphorylation to glycolysis. It has been suggested that this occurs to either increase ATP production rapidly, albeit less energetically favourable overall, or to avoid pathways and breakdown products produced by oxidative phosphorylation that can lead to a halt of cell division due to mitochondrial stress [95]. Furthermore, incomplete breakdown of products such as glucose provides the building blocks of other components needed for proliferation [96]. While many solid tumours rely on the Warburg effect, prostate cancer has a unique phenotype, where it is not heavily reliant on glucose, but instead has an increased reliance on oxidative phosphorylation. It has been suggested that this is due in part to decreased activity of the ZIP1 transporters, where the decrease in zinc levels allows for the increased activity of m-aconitase and the TCA cycle [97]. Alterations in other pathways, such as lipid and fatty acid metabolism, have been suggested to allow for the early phases of malignancy and tumour progression, and to allow for energy production, membrane synthesis, and post-translational modification of signalling molecules [98]. It is only when prostate cancer cells become metastatic that glycolysis is upregulated

and the Warburg effect is seen [99]. As such, metabolic pathways and regulation of these pathways vary dramatically in prostate cancer compared to other cancers, and regulatory mechanisms cannot be equated.

SOX2 and its role in metabolism of cancer has not been well examined, and has mostly been examined in the context of glycolysis or oxidative phosphorylation. SOX2 was shown to act with p63 in squamous cell carcinoma to increase expression of SLC2A1, a key glucose transporter gene, leading to elevated glucose influx [100]. Knock-down of SOX2 in gastric cancer cells reduced expression of key glycolytic proteins, HK2, GLUT1 and LDHA, and decreased glucose uptake and lactate production [101]. In melanoma, knock-down of SOX2 in melanoma cells led to an increase in lactate production and glycolytic genes, suggesting SOX2 has a more oxidative phenotype in melanoma cells [102]. While these studies indicate a role for SOX2 in cancer metabolism, there is a clear gap in our knowledge of SOX2 in metabolism, and especially in terms of prostate cancer metabolism.

1.8 DNA DAMAGE REPAIR IN PROSTATE CANCER

Radiosensitivity can be influenced by many factors such as hypoxia, the cell cycle, DNA damage and repair, apoptosis, growth factors and oncogenes, cancer stem cells (CSCs) and the epigenetic modification of genes [103, 104, 105, 106, 107, 108, 109, 110]. Errors in DNA repair pathways are frequently found in cancer, and allow for cell proliferation, metastasis and invasion, angiogenesis and radio-/chemo-resistance [111]. When DNA damage is not severe or irreparable, damaged cells undergo DNA repair either through homologous recombination (HR) or non-homologous end-joining (NHEJ). These pathways are activated based on the stage of the cell cycle that cells are currently within, where HR is performed post-DNA replication in the S and G2/M stage with the aid of a sister chromatid, and NHEJ is performed primarily during the G1 stage. When DNA damage is irreparable, cells undergo cell cycle arrest, apoptosis or senescence [112].

Upregulation of proteins involved in DNA repair is frequently found in many types of

cancers, as cancer cells are able to rapidly repair any DNA breaks or defects and continue proliferating, leading to mutations and chromosomal translocations, in particular through NHEJ [113]. In glioblastoma, increased expression of SOX2 was found with markers of activated DNA damage response (DDR) in response to replication stress: Chk1 and ATR. Further, increased expression of RPA2 was found in GBM patients with SOX2 expression, indicating increased replication fork stalling [114]. In cervical cancer, patients who are radio-resistant have a higher amount of SOX2 and OCT4 in their tumours, and a shorter progression free survival [115]. Higher expression of activated ATM, Rad17, Chk1 and Chk2 were also found in SOX2-expressing cells from glioma xenografts [116]. In prostate cancer, one group found that SOX2 silencing led to DNA damage, although the mechanism is unknown [117].

Clinical observations have indicated that steroid hormones can modulate response to DNA damage in hormone sensitive cancers. In breast cancer, treatment with tamoxifen to inhibit estrogen receptor (ER) signalling leads to increased response to radiation [118], and while the mechanism of this combinatorial effect is unknown, the ER can interact with known DDR genes and modulate expression of DDR genes [119, 120]. For almost twenty years, androgen deprivation therapy (ADT) and radiotherapy have been combined for high-risk prostate cancer [121], and recent studies have indicated AR signalling increases expression of several DDR proteins [113, 121]. One suggested explanation is that activation of AR causes DNA double strand breaks at transcriptional regulation sites as a mechanism of releasing torsional strain to facilitate gene expression [122]. DDR proteins DNAPKcs and Ku70/80 have also been suggested to be AR coactivators [123]. One group discovered a role for AR in prostate cancer cell telomere stability, where its inhibition led to activation of ATM at telomeres [124]. Altogether, there is ample evidence for a role for SOX2 in cancer, and especially in prostate cancer where AR-signalling is being modulated.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Lines and Materials

R1881 was purchased from Sigma-Aldrich (St. Louis, MO) and was stored at -20°C in ethanol. Human prostate cancer cell lines were grown as previously described [125, 126]. Cell authentication of all cells was confirmed via DDC Medical services (Fairfield, OH). All cultures were routinely screened for the absence of mycoplasma contamination using the ATCC Universal Mycoplasma Detection Kit (Manassas, VA). LAPC-4 cells were grown in IMDM (Hyclone) supplemented with 1% Penicillin/Streptomycin (Corning), 10% fetal bovine serum (Atlanta Biologicals), and 1nM R1881 (Sigma-Aldrich). CWR-R1 cells were grown in RPMI-1640 (Gibco) supplemented with 1% Penicillin/Streptomycin (Corning), and 10% fetal bovine serum (Atlanta Biologicals). For enzalutamide treated conditions, 10μM enzalutamide (Selleck Chemicals or Medivation) was added in media with 10% charcoal stripped FBS (Atlanta Biologicals); for all experiments, equimolar vehicle (DMSO) was added. The human embryonic stem cell (hESC) line WA01(H1) was acquired from WiCell (Madison, WI) and cultured using the feeder-independent protocol using mTeSR1 media (Stem Cell Technologies; Vancouver, B.C.). The human ES cells were used within ten passages of thawing, and were dissociated using mTeSR1 media with an Accutase (Millipore; Billerica, MA) digestion.

2.2 CRISPR-Cas9 Targeting

In order to generate CRISPR/Cas9-mediated SOX2 knockout cell lines, parental CWR-R1 cells were co-transfected with pT2-EF1a-Cas9-P2A-puro and pCMV(CAT)T7-SB100 (#34879, Addgene) using Lipofectamine 2000 (Invitrogen, USA) to introduce Cas9 expression. Stable constitutive Cas9 expression was accomplished by SB100 transposase integra-

tion of EF1a-Cas9-P2A-puro. Cas9-expressing cells were selected for and maintained with puromycin (1 mg/mL, Invitrogen) after 48 hrs from transfection. Constitutive Cas9 expression was confirmed by western blot (# 14697, Cell Signaling Technologies) after 1 week of puromycin selection. Two custom crRNAs (IDT) targeting the N-terminus of SOX2 were selected using CHOPCHOP software (SOX2 crRNA #1: 5'- CGGGCCCGCAGCAAACTTCG-3', SOX2 crRNA #2: 5'-CGCCCGCATGTACAACATGA-3') and were individually complexed with the tracrRNA-ATTO 550 (#1075927, IDT) at a 1:1 ratio immediately prior to transfection. A final concentration of 10 nM of the crRNA:tracrRNA duplexes was then transfected into CWR-R1-Cas9 cells using siLentFect Lipid Reagent for RNAi (#1703360, Bio-Rad) following manufacturer guidelines. Limited dilution was then performed to isolate three clonal knockout cell lines, and successful knockout of SOX2 was validated by western blot (anti-SOX2(D6D9), #3579, Cell Signaling Technologies).

2.3 Human subjects and Tissue Microarrays (TMAs)

All human tissues were acquired under an expedited protocol approved and monitored by the University of Chicago Institutional Review Board (IRB). We acquired the 726 Case PSA progression TMA from the Prostate Cancer Biorepository Network (PCBN; http://prostatebiorepository.org). This nested case-control study was designed specifically to evaluate biomarkers for prostate cancer recurrence, beyond the known prognostic factors (age at surgery, race, pathologic stage, and Gleason sum in the prostatectomy specimen). The full nested case-control study includes 524 recurrence cases and a sample of 524 controls identified among 4,860 prostate cancer patients who underwent radical retropubic prostatectomy for clinically localized prostate cancer at Johns Hopkins in Baltimore, MD between 1993 and 2001. The dataset of 1,048 records (524 matched pairs), includes 742 unique men, where some men serve as controls for multiple cases, and some men who are controls were later sampled as cases. The sample does not include men who received hormonal or radiation therapy before their prostatectomy or hormonal adjuvant therapy before their recurrence.

These men were followed for outcome through 2004. Recurrence was defined as biochemical recurrence (serum PSA >0.2 ng/mL), local recurrence, systemic metastases, or death from prostate cancer. For each case, incidence density sampling [127] was used to select a control who had not experienced recurrence by the date of the case's recurrence, and who was similar to the case on age at surgery, race, pathologic stage, and Gleason sum in the prostatectomy specimen. The TMA contains four cores each of normal and cancerous tissue per patient. Tissues were analyzed by a trained genitourinary pathologist and scored on percentage of cells with positive nuclear staining (0 = no staining; 1 = 1–5% positive cells; 2 = 5–50% positive cells; and 3 = 50-100% positive cells). Patients were then divided into two groups for analysis: Patients with a composite score of 0-1 were considered SOX2 negative, whereas patients with a score $\geq$2 were considered SOX2 positive. For images, slides were digitized using a Pannoramic Scan Whole Slide Scanner (Cambridge Research and Instrumentation, Hopkinton, MA) and images captured using the Pannoramic Viewer software version 1.14.50 (3DHistech, Budapest, Hungary).

2.4 Histology and Immunostaining

H&E staining was performed as previously described using a SAKURA Tissue-Tek Prima Autostainer [67]. For immunohistochemistry staining, formalin-fixed, paraffin-embedded (FFPE) slides were deparaffinized in xylene, and hydrated using graded ethanol washes. Tissues were treated with antigen retrieval buffer (S1699 from DAKO; Glostrup, Denmark) in a steamer for 20 minutes. Anti-SOX2 (D6D9 rabbit monoclonal, Cell Signaling Technology; Danvers, MA) was applied for 1 hour at room temperature in a humidity chamber. Following TBS wash, the antigen-antibody binding was detected with Envision+system (K4001, DAKO; Carpinteria, CA) and DAB+Chromogen (DAKO, K3468). Tissue sections were briefly immersed in hematoxylin for counterstaining and were cover-slipped. Staining quantitation was conducted by a genitourinary pathologist.

2.5 Quantitative Real-Time PCR Analyses

RNA was purified using the Qiagen RNeasy Mini Kit with the optional DNAse digestion kit (Qiagen; Valencia, CA) and quality tested using an Agilent Bioanalyzer 2100 (Agilent Technologies; Santa Clara, CA). For standard Q-RT-PCR, extracted RNA was converted to cDNA by reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen; Carlsbad, CA). Expression levels of SOX2 and RPL13A transcript were quantified using Power SYBR Green Master Mix (Invitrogen) using the custom primers: SOX2 (Forward 5'-GCGGAAAACCAAGACGCTC-3'; Reverse 5'-TCAGCGCGTAACTGTCC-3'), RPL13A (Forward 5'-GGAGCAAGGAAAGGGTCTTAG-3'; Reverse 5'-GGTTGCTCTTCCTATTG GTCATA-3'). Standard curves were used to assess primer efficiency and average change in threshold cycle (ΔCT) values was determined for each of the samples relative to endogenous RPL13A levels and compared to vehicle control ($\Delta\Delta$CT). Experiments were performed in triplicate to determine mean standard error, and student's t-tests performed with normalization to control to obtain p-values.

2.6 Western Blotting

Whole-cell lysates of 100,000 or more cells were used per lane. Western blotting was performed as previously reported [67]. Briefly, cells were rinsed with cold PBS and scraped into RIPA buffer supplemented with protease inhibitors, sonicated twice and re-suspended in 4X sample buffer (BioRad; Hercules, CA) supplemented with 10% Beta-mercaptoethanol (Sigma-Aldrich; St. Louis MO). The Pierce BCA protein assay kit (Thermo Fisher Scientific; Grand Island, NY) was used to determine protein concentration. 60μg of protein was loaded on a 10% SDS-polyacrylamide gel and electrophoresed, and transferred to nitrocellulose (LI COR, Odyssey; Lincoln, NE) overnight at 4°C. The membrane was blocked overnight in 5% non-fat milk in TBS at 4°C. Primary antibodies used were: SOX2 (D6D9, Cell Signaling Technology), phosho-BRCA1 (Ser1524, 9009, Cell Signaling Technology), BRCA1 (A8X9F,

Cell Signaling Technology), phospho-Histone H2A.X (Ser139, clone JBW301, EMD Millipore), Histone H2A.X (D17A3, Cell Signaling Technology), CDK2 (78B2, Cell Signaling Technology), CDK4 (D9G3E, Cell Signaling Technology), CDK6 (DCS-83, Santa Cruz), p27 (D69C12, Cell Signaling Technology), p21 (12D1, Cell Signaling Technology), p-p53 (Ser15, 16G8, Cell Signaling Technology), p53 (7F5, Cell Signaling Technology), E2F1 (Cell Signaling Technology), Phospho-Rb (Ser807/811, D20B12, Cell Signaling Technology), Rb (4H1, Cell Signaling Technology), Androgen Receptor (EP670Y, Abcam), AR-V7 (RM7, Revmab Biosciences), Beta Actin (Clone AC-15, Sigma-Aldrich). The secondary antibodies goat anti-mouse IRDye 800 CW or donkey anti-rabbit IRDye 680 from LI-COR Biosciences were used, and images captured using an infrared Odyssey scanner (LI-COR).

2.7 Chromatin Immunoprecipitation and Sequencing (ChIP-Seq) and RNA-Sequencing (RNA-Seq)

2.7.1 SOX2 ChIP-Sequencing

CWR-R1 and WA01 cells were cultured as described as described above, and methods for ChIP were adapted from previously reported methods [128]. DNA and associated proteins were crosslinked with 1% formaldehyde, and lysates were sonicated and immunoprecipitated as described previously [67]. ChIP experiments were conducted using the ChIP Assay Kit and the manufacturer's protocol (EMD Millipore). A polyclonal goat anti-SOX2 mAb (P48431, R&D Systems), or a goat IgG control, were used for immunoprecipitation. Eluted ChIP DNA was purified using the PCR Purification Kit (Qiagen). ChIP Sequencing libraries were generated using the KAPA LTP Library Preparation Kit (#KK8230; Kapa Biosystems, Wilmington, MA). Libraries were sequenced on a HiSeq 2000 sequencing system (Illumina) in a 50bp, single-end run.

2.7.2 RNA-Sequencing

RNA was purified using the 'RNeasy Mini Kit' (Qiagen). RNA quality was evaluated using a 2200 TapeStation system (Agilent), and samples with a RIN score $\geq$7 were selected for library preparation. RNA-sequencing libraries were prepared and amplified according to 'KAPA Stranded mRNA-Seq Kit- Illumina® Platforms' (KAPA Biosystems), using Oligo-dT magnetic beads to enrich for mRNA. Sample libraries fragment sizes were confirmed using a 2200 TapeStation system (Agilent), and then quantified by qPCR using the Library Quantification kit (KAPA). Library adaptors 1 through 12 (KAPA Biosystems) were used to barcode each sample, and libraries were sequenced on a HiSeq 2000 sequencing system (Illumina) in a 100bp, paired-end run.

2.8 Analyses of Sequencing Data

2.8.1 ChIP-seq Peak and Region Analysis

All ChIP-seq reads were analyzed using the FastQC tool suite (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Low quality and adapter sequences were trimmed using Trimmomatic [129]. The trimmed reads were then aligned to the reference genome GRCh38 using BWA [130] and the resulting alignments were sorted and converted to BAM format using Samtools (http://www.htslib.org/doc/samtools.html). Duplicate reads were subsequently removed with Picard tools (http://broadinstitute.github.io/picard/). MACS2 [131] was used for peak detection for SOX2 IP data. Peak images were captured using Integrative Genomics Viewer (IGV; http://software.broadinstitute.org/software/igv/).

2.8.2 Transcription Factor Motif Analysis

SOX2 peak regions that passed earlier selection criteria were then expanded to a uniform range of 250bp around the summit in order to submit the targeted sequences for motif analysis using MEME Suite [132]. In addition, FIMO [133] was employed to scan for all

JASPAR (vertebrates 2016) transcription factor motifs [134]. In the set of SOX2 peaks where a canonical SOX2 motif was discovered, SpaMo (spamo) was used to infer potential binding partners.

2.8.3 RNAseq Differential Expression

All RNA-seq reads were analyzed using the FastQC tool suite. Low quality and adapter sequences were trimmed using trimmomatic [129]. The trimmed reads were then aligned to the reference genome GRCh38 using STAR (star) and the resulting alignments were sorted and converted to BAM format using Samtools. Duplicate reads were subsequently removed with Picard tools. Differentially expressed genes (DEGs) were detected using featureCounts-DESeq2 or EdgeR (read count-based method). Transcripts were further filtered by fold change $\geq$1.5. Biological insights from candidate gene lists were gained by performing gene set enrichment analysis (GSEA) and Ingenuity Pathways Analysis (IPA) (Ingenuity® Systems) to identify functional categories or pathways that are significantly altered under condition. Heatmaps were generated using the Morpheus platform (https://software.broadinstitute.org/morpheus/).

2.9 Analyses of Publicly-Available RNA-Seq Datasets

We obtained raw RNA-Seq FASTQ files of 25 prostate tumours, 3 benign glands, and 51 annotated metastases via dbGAP (phs000310.v1.
p1 for tumours and benign glands; phs000915.v1.p1 for metastases) [135, 136]. These datasets were chosen based upon the quality of mRNA, sequencing depth, and annotation. The tumour datasets are from patients who had undergone prostate surgery without prior therapy, and the metastases are from patients with castration-resistant prostate cancer, some of which had received taxane, enzalutamide, or abiraterone treatment [135, 137]. The quality of raw reads was accessed by FastQC (v0.11.4). All reads were mapped to the human genome

assembly (NCBI build 19) using STAR (v2.5.1b). Alignment metrics were collected by Picard tools (v2.8.1) and RSeQC (v2.6.4) [138]. Transcripts were assembled from the aligned reads using Cufflinks and combined with known gene annotation. The expression level of transcripts was quantified using FPKM (Fragments Per Kilobase of transcript per Million mapped reads)-based and read count-based methods. Transcript expression was normalized across samples. Multiple gene expression correlations were determined using the cBioPortal for Cancer Genomics (www.cbioportal.org) using the Prostate Adenocarcinoma (TCGA, Cell 2015) dataset consisting of 333 samples with annotated mRNA expression z-Scores.

2.10 Cell Proliferation Assays

2.10.1 Cell growth assay

Cells with nuclear GFP were plated at a density of 10,000 cells per well in a 96-well plate, followed by the indicated treatment the following day. Wells were placed in the IncuCyte S3 Live Cell Analysis System (Essen Bioscience, Ann Arbor MI.), and imaged every 4 hours for 120 hours. Cell number was counted for each image using built in analysis software. Cell numbers per subsequent time point were normalised to the initial number of cells (Time 0). All assays were repeated at least 3 times.

2.10.2 Cell cycle assay

Cells were treated for 72 hours with 10μM enzalutamide and were approximately 70% confluent. Cells were fixed with 70% ethanol for 30 minutes. DNA was stained with a Propidium Iodide solution (Biotum #40017, 1:20 dilution), supplemented with RNase A (FisherScientific #EN0531, 1:100 dilution), overnight at 4°C. Cells were analysed using the Cellometer Spectrum (Nexcelom Bioscience, Lawrence, MA).

2.10.3 BrdU assay

Cells were treated for 72 hours with enzalutamide and were approximately 70% confluent, and pulsed with 30μM BrdU for 2 hours. Cells were fixed with 70% ethanol for 30 minutes, and DNA was denatured using 2N hydrochloric acid/0.5% Triton X-100 for 30 minutes. Anti-BrdU-FITC antibody solution (ThermoFisher #11-5071-42, 1:20 dilution) was added for 1 hour. DNA was stained with a Propidium Iodide solution (Biotum #40017, 1:20 dilution), supplemented with RNase A (FisherScientific #EN0531, 1:100 dilution), overnight at 4°C. Cells were analysed by flow cytometry (Gallios Bench-top Analyzer).

2.11 Apoptosis Assays

2.11.1 PI exclusion assay

150,000 cells were plated in 6-well plates and allowed to attach overnight. Cells were treated with vehicle and 10μM enzalutamide for 72 hours. Propidium Iodide (Biotum #40017) was added to each well at a concentration of 1μg/mL, and incubated for 15 minutes at 37°C. Supernatant was collected, as were trypsinised cells. Number of PI positive cells (non-viable cells), were counted using the Cellometer Spectrum (Nexcelom Bioscience, Lawrence, MA).

2.11.2 RealTime-Glo^{TM} Annexin V Apoptosis and Necrosis Assay

10,000 cells were plated in a solid white bottom 96-well plate (Corning Inc. #353296, Corning, NY), and allowed to attach overnight. Cells were treated with vehicle, 10μM enzalutamide or 2mM staurospaurine (positive control) in triplicate, and RealTime-Glo$^{\text{TM}}$ Annexin V Apoptosis and Necrosis kit (Promega #JA1011, Madison, WI) was added as per manufacturer instructions. Briefly, 50μL containing the 10,000 cells were added to each well of a 96-well plate. The next day, 50μL of 4X concentrated drugs (vehicle, enzalutamide and staurospaurine), and 100μL of 2X concentration of the Detection Reagents were added.

Luminescence and fluorescence (485nm/525nm) were measured daily using the Synergy LX Multimode Reader (Biotek, Winooski, VT), and normalised to the blank wells.

2.11.3 TUNEL assay

5,000 cells were plated in a black, clear bottom plate (Corning #3603, Corning, NY) and allowed to attach overnight. Cells were treated for 72 hours with vehicle or 10μM enzalutamide. Cells were processed as per the manual for the Click-iT TUNEL Alexa Fluor 647 Imaging Assay (ThermoFisher Scientific #C10247, Waltham, MA). Briefly, cells were fixed with 4% paraformaldehyde, and permeabilised with 0.25% Triton X-100 in PBS. DNAse I (included in the kit) was used to generate strand breaks in the DNA as a positive control. The TdT reaction cocktail was added to each well, and was followed by the Click-iT reaction cocktail. Each well was imaged using the Keyence BZ-X800 microscope (Keyence, Osaka, Japan).

2.12 Cell Energetics Assays

To determine cell energetics, and glycolysis and oxidative phosphorylation rates, cells were seeded at a density of 50,000 cells per well. Cells were counted and plated in Xfe24 plates 24 hours before assay, and allowed to attach overnight. The assay cartridge was hydrated with Agilent's equilibration buffer in a 0% CO2 chamber at 37°C overnight. One hour prior to assay, culture media was removed and replaced with Seahorse assay media (Seahorse basal RPMI media supplemented with 2mM L-glutamine, and 11mM glucose, for the Cell Energy Phenotype Test and Cell Mito Stress Test). Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using the Agilent Seahorse Xfe24 machine (Santa Clara, CA), according to manufacturer instructions for the Cell Energy Phenotype Test (Catalogue #103325-100), Glycolysis Stress Test, and Cell Mito Stress Test. FCCP and Oligomycin from Cell Energy Phenotype Test kit were used with rotenone (Tocris Bioscience

#3616, Bristol, UK), 2-DG (Biovision Inc. #B1048-500, Milpitas,CA) and Antimycin A (Santa Cruz Biotechnology #SC-202467A, Dallas, TX). Assays were analysed using Wave Software (Seahorse/Agilent, Santa Clara, CA). Cell density and concentration of FCCP (1μM) were optimised according to manufacturer's instructions.

2.13 Cell Migration Assays

Cells were treated for the indicated number of days with vehicle or enzalutamide and 24 hours before plating of migration assay were serum-starved. Cells were plated at 200,000 cells per well into the Transparent PET Membrane inserts (24 well 8.0μm pore size, Corning) in serum-free conditions with the indicated treatments. Wells outside of the inserts contained media with serum and the indicated treatments. Aphidocolin (Cayman Chemical) was added as a proliferation inhibitor. Membranes were fixed and stained 48 hours post-plating with a crystal-violet solution, mounted and imaged using the BZ-X800 All-in-one Fluorescence Microscope (Keyence). Number of cells migrated were counted using ImageJ.

2.14 Mitochondrial DNA quantification

The amount of mitochondria in each sample was quantified using quantitative PCR, as per this protocol [140]. Briefly, genomic DNA was isolated using the Quick DNA Plus Microprep Kit (Zymo Research #D4074). Mitochondrial genes $\text{tRNA}^{\text{Leu(UUR)}}$ and 16S rRNA subunit were amplified and normalised to the nuclear gene nuclear β-2-microglobulin (β2M). Fold change values of SOX2^{KO} or SOX2 overexpression were compared to the control cells.

2.15 Metabolite Analysis

Samples were prepared according to the protocol from Northwestern University's Metabolomics Core Facility. Briefly, cells were grown in 10cm dishes until reaching 60-80% confluency. Media was changed 2 hours before starting cell preparation, where cells were washed

twice with ice-cold saline (not PBS), and 1mL of ice-cold 80% methanol was added. Cells were incubated at -80°C for 20 minutes, and cells were scraped into Eppendorf tubes and subjected to three freeze-thaw cycles between -80°C and 37°C, with vortexing 30 seconds after each thaw. Samples were centrifuged for 15 minutes at 4°C at >15,000 rcf and supernatant was transferred to new tubes. Samples were dried using Speedvac at the Metabolomics facility. Total protein quantification was performed as described above, for normalisation of metabolites for injection.

2.16 Statistical Analyses

2.16.1 Tissue Analyses

Descriptive statistics of all the variables are summarized for the biochemical recurrence (BCR) patients and the control patients (Supplemental Table 1). Two-sided t tests are used to compare the continuous variables and chi-square tests are used to compare the categorical variables between the BCR group and control group of patients. All the statistical analyses used SAS software with significance levels of 0.05. Post-operative outcomes between patients bearing SOX2 positive and negative tumours utilized Chi-square tests.

2.16.2 Phenotypic Analyses

Tukey's multiple comparisons test was used for the Incucyte growth curves, cell cycle, BrdU, γ-H2AX immunofluorescence, PI exclusion, Promega Apoptosis, TUNEL assay to compare all conditions with each other. For the Seahorse assays, Mann-Whitney test was used to compare control cells to the $SOX2^{KO}$ cells.

CHAPTER 3

SOX2 EXPRESSION IN PRIMARY PROSTATE TUMOURS AND METASTASES, AND ITS ASSOCIATION WITH CLINICAL OUTCOME

3.1 Introduction

Prostate cancer is a significant health problem, with greater than 174,650 cases diagnosed in 2019 and an estimated 31,620 deaths in 2019 [125]. While survival is high for patients with localised disease, patients who metastasise have few treatment options available. Metastasis is considered the primary endpoint for prostate cancer specific mortality (PCSM), but biochemical recurrence (BCR) is often used as an early endpoint when deciding if the radical prostatectomy (RP) was successful. BCR is defined as when a patient has a rising prostate-specific antigen (PSA), most commonly >0.2ng/mL [126]. However, not all patients who experience BCR will end up metastasising, and thus, there is a great need for identifying additional biomarkers that will predict patients who will metastasise.

SOX2's role clinically has been examined extensively in multiple types of cancers. Patients with increased levels of SOX2 in breast, colorectal, prostate, esophageal, ovarian, some lung cancers, nasopharyngeal and sinonasal carcinomas have a poor prognosis [53]. Furthermore, there is a strong correlation between elevated levels of SOX2 and metastatic capacity in the many cancers [53]. Esophageal squamous cell carcinomas tumours with more than 50% of the cells expressing SOX2 were correlated with increased vascular and lymphatic invasion and poor differentiation [67], and in prostate and colorectal cancer, SOX2-positive tumours are correlated with increased distant and lymphatic metastases [64, 65, 66].

Given the extensive work indicating SOX2's potential to act as an oncogene in cancer, we had previously examined SOX2 expression in prostate tumours and metastases. We had shown that the percentage of SOX2-positive tumours increases with Gleason Score,

and nearly all lymph node metastases are SOX2-positive [65]. We also investigated SOX2's function in tumour formation, and showed that ectopic expression of SOX2 in a hormone-sensitive prostate cancer cell line is sufficient to allow tumour formation in a castrated host, leading to castration-resistant prostate cancer tumour growth [65]. Given these data, we wanted to explore more closely the role of SOX2 in prostate cancer patient outcome, with the goal of identifying additional biomarkers that can be used to predict patient response to treatment and metastatic outcome.

3.2 Results

3.2.1 SOX2 expression in primary prostate tumours is associated with more rapid time to metastasis but not associated with biochemical recurrence

The previous observation of binary SOX2 expression led us to hypothesize that SOX2 expression in tumours confers an increased risk of cancer progression and confers a worse overall patient prognosis at the time of surgery. To test this hypothesis, SOX2 IHC was performed in a set of annotated tissue microarrays (TMAs) from the Brady Recurrence Nested Case-Control Study provided by the Prostate Cancer Biorepository Network, composed of 1028 tumours tissues recovered from 742 prostate cancer patients who underwent radical retropubic prostatectomy for clinically localized prostate cancer at Johns Hopkins University Medical Center between 1993 and 2001. Clinical information about these patients included time to biochemical recurrence (BCR) and metastases, PSA at time of biochemical recurrence, prostatectomy Gleason score, and adjuvant treatments.

SOX2 expression was found in both benign and malignant tissues (Figure 3.1), consistent with previous findings reported by our group and others [65, 127]. After SOX2 IHC staining, pathologic analyses, and elimination of samples with incomplete data, there remained 477 cases of biochemical recurrence and 248 control cases for statistical analyses (Table 1). As

expected, there were no significant associations between the BCR and control group based upon age, PSA at surgery, race, or pathological extension (Table 1). However, significant differences between BCR and control groups were observed for Gleason Score post-RP, years to BCR, surgical margin status, local recurrence, salvage treatment, metastasis, overall death, and prostate cancer-specific death (Table 2). Surprisingly, analyses of SOX2 expression demonstrated no significant correlation between tumour SOX2 expression and BCR, as well as years to BCR (Figure 3.2, Table 2). Further, there was no correlation between SOX2 expression in primary tumours and surgical margin, pathologic extension, local recurrence, salvage treatment, or metastases after BCR (Table 2). This was surprising, as we had hypothesised that SOX2 expression in the primary tumour would lead to decreased time to BCR.

Given previous research of SOX2 in castration-resistant prostate cancer, we next examined the role of SOX2 in patients who had undergone biochemical recurrence, and the implications clinically of SOX2 in this patient population. This analysis indicated patients bearing SOX2-positive tumours have a more rapid time to metastasis formation after BCR, as well as a higher prevalence of prostate cancer-specific death and overall death (Table 2, Figure 3.3). A Cox multiple logistic regression model combining age, PSA, Gleason Grade, extraprostatic extension, and seminal vesicle invasion demonstrates a significant association with tumour SOX2 expression and metastasis risk (HR=1.496, p=0.0369), in addition to the expected metastatic risk factors Gleason grade, extraprostatic extension, and seminal vesicle invasion (Figure 3.4). These data demonstrate an increased propensity of SOX2-positive tumours for metastasis and prostate cancer-specific death and suggest that SOX2 expression may be associated with therapy resistance after metastatic colonisation.

Table 3.1: Baseline characteristics of cases and controls

	CONTROLS *(N=248)*	**CASES** *(N=477)*	**P-value**
Age at Surgery (Mean ± SD)	59.01 ± 6.33	58.83 ± 6.22	0.7483
PSA at Surgery (Mean ± SD)	11.16 ± 8.96	12.30 ± 10.14	0.0923
Race (%)			0.7398
African-American	9.75%	9.55%	
Caucasian	87.42%	87.33%	
Asian	0%	0.39%	
Other	2.83%	2.73%	
Gleason Score Post-RP (%)			0.0136
GG6	16.98%	12.28%	
GG3+4	43.08%	38.21%	
GG4+3	14.78%	23.59%	
GG8	14.78%	13.65%	
GG9-10	10.38%	12.28%	
Years to BCR (Mean ± SD)	6.59 ± 3.22	2.45 ± 1.85	< 0.001
Years Follow-Up (Mean ± SD)	14.00 ± 6.83	11.53 ± 6.43	< 0.001
Surgical Margin (%)			< 0.001
Negative	80.2%	62.1%	
Positive	19.8%	37.9%	
Pathological Extension (%)			
Organ Confined	21.4%	14.0%	0.1060
Extraprostatic	71.8%	83.6%	0.0252
Lymph Node Positive	10.1%	19.3%	0.0227
Local Recurrence (%)	8.1%	19.1%	< 0.001
Salvage Treatment (%)[a]	19.0%	93.5%	< 0.001
Years to Salvage Treatment (Mean ± SD)	9.78 ± 7.96	5.22 ± 6.26	< 0.001
Distant Metastases (%)	8.1%	41.1%	< 0.001
Time to Metastases (Mean ± SD)	13.39 ± 6.82	9.46 ± 6.48	< 0.001
Overall Death (%)	24.2%	42.1%	< 0.001
PCa-Specific Death (%)	4.0%	29.1%	< 0.001
SOX2-Positive (%)	14.9%	18.0%	0.5013

a: Includes salvage ADT, radiation, or both.

Table 3.2: Post-operative outcomes of SOX2-positive versus SOX2-negative tumours

	SOX2^{Pos} *(N=123)*	**SOX2^{Neg}** *(N=602)*	**P-value**
BCR (%)	71.5%	70.4%	0.6708
Years to BCR (Mean ± SD)	2.39 ± 1.90	2.64 ± 1.94	0.104
PSA at Surgery (Mean ± SD)	10.97 ± 9.71	11.67 ± 9.31	0.085
Gleason Grade Post-RP (%)			0.0870
GG6	13.8%	15.9%	
GG3+4	44.7%	39.4%	
GG4+3	20.3%	16.9%	
GG8	13.0%	13.3%	
GG9-10	8.1%	11.8%	
Surgical Margin (%)			0.9373
Negative	68.3%	68.3%	
Positive	31.7%	31.7%	
Pathological Extension (%)			
Organ Confined	17.1%	16.4%	0.1060
Extraprostatic	81.3%	79.2%	0.4802
Lymph Node Positive	19.5%	15.4%	0.293
Local Recurrence (%)	15.4%	14.0%	0.408
Salvage Treatment (%)[a]	57.7%	64.6%	0.1486
Years to Salvage Treatment (Mean ± SD)	5.06 ± 5.69	6.01 ± 6.87	0.608
Metastases After BCR (%)	51.3%	43.0%	0.127
Time to Metastases after BCR (Mean ± SD)	7.59 ± 5.39	9.85 ± 6.58	**<0.001**
Cancer Specific Survival (%)			**0.0138**
Alive	72.4%	80.9%	
Dead from PCa	27.6%	19.1%	
Overall Death (%)	45.5%	34.1%	**0.018**

a: Includes salvage ADT, radiation, or both.

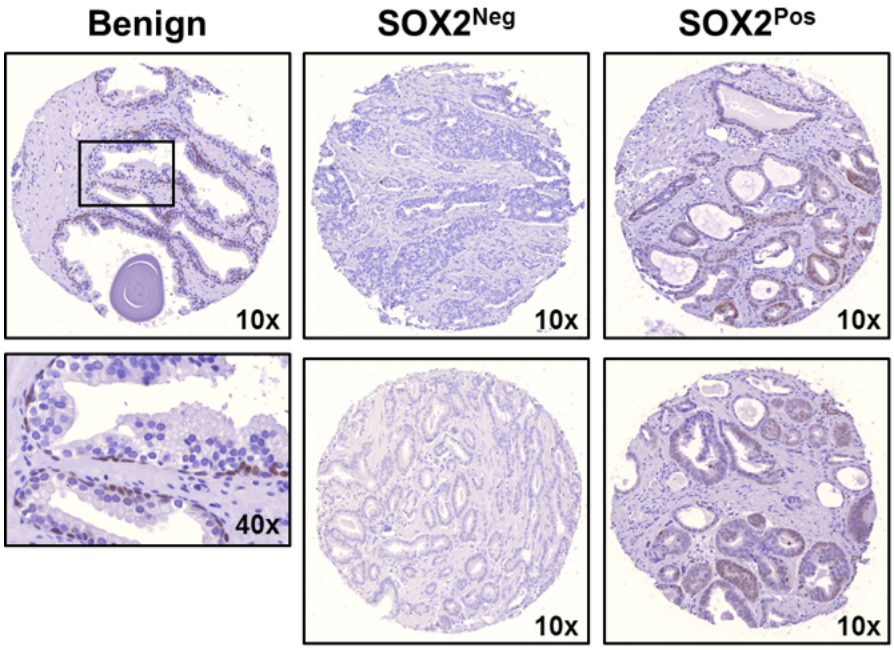

Figure 3.1: SOX2 staining in tissue microarrays. SOX2 is expressed in both benign prostatic tissue and prostate cancer. SOX2 staining is binary, where SOX2 is unexpressed or expressed in almost all cells.

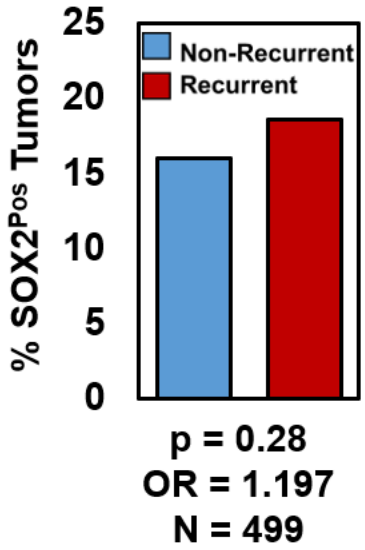

Figure 3.2: SOX2 expression is not associated with biochemical recurrence. Patients SOX2 positive tumours were categorised by whether they biochemically recurred. There was no statistical difference in the number of patients who biochemically recurred compared to those who did not recur.

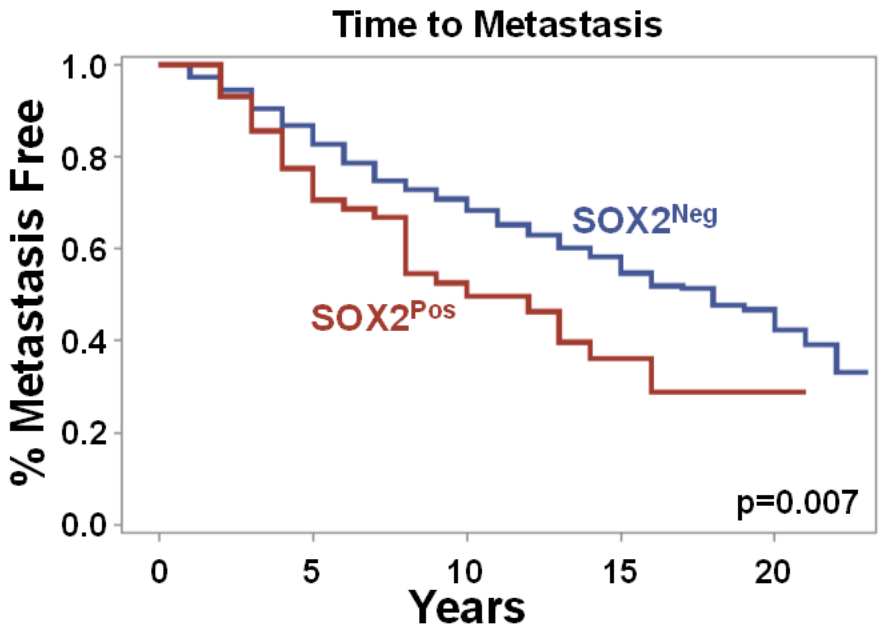

Figure 3.3: Patients who have biochemically recurred and have SOX2 positive tumours metastasise more rapidly than patients who have SOX2 negative tumours. Kaplan Meier curve for time to metastasis was generated for patients who have biochemically recurred, and split by SOX2 expression of primary tumours.

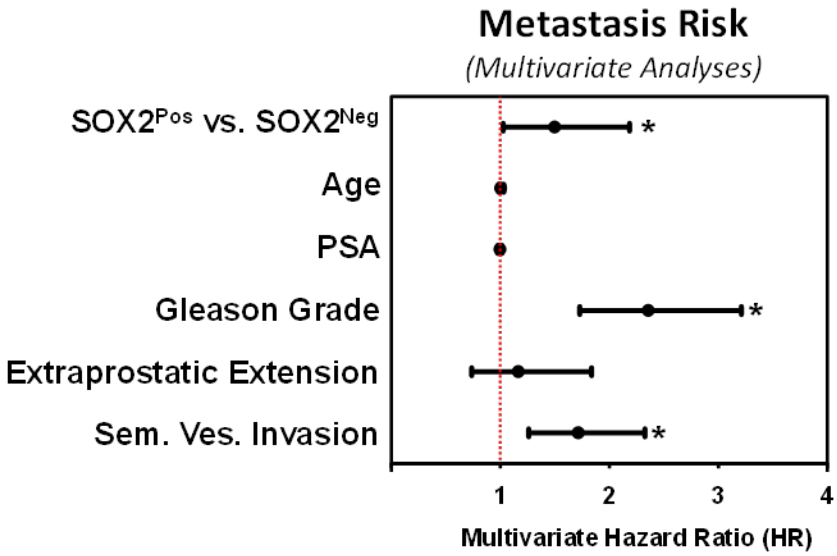

Figure 3.4: SOX2 expression is associated with increased risk of metastasis. Multivariate analysis indicates SOX2 positivity, Gleason Grade and seminal vesicle invasion are factors associated with increased risk of metastasis.

3.2.2 Discussion

SOX2 has been implicated in prostate cancer progression, and has been associated with higher Gleason Score tumours, as well as lymph node metastasis [65, 128]. Here we show that, surprisingly, increased expression of SOX2 in primary tumours does not confer biochemical recurrence, where there was no difference in the number of SOX2-positive tumours that had undergone biochemical recurrence compared to those that do not biochemically recur. However, patients who had biochemically recurred and had SOX2 expression developed metastases much sooner. Furthermore, patients with SOX2 positive tumours had an increased percentage of prostate cancer-specific mortality, as well as overall death. These data suggest an updated view on SOX2's prognostic role as a predictor of metastasis and PCSM in the context of BCR.

Metastatic disease is the primary determinant of survival for prostate cancer, is associated with serious morbidity, and predates PCSM by an average of 3 to 6 years. As such, identifying predictors of metastasis is crucial for predicting patient outcome. BCR is often used as an early endpoint to assess the success of a RP, and is used to determine whether secondary treatments are initiated. Patients are termed to have undergone BCR when there is an increase in the levels of prostate specific antigen (PSA) following RP, although there is much variance in the criteria used to precisely define BCR [129]. A rising PSA level is often the first indication of progression to distant metastasis and PCSM, but not all patients who experience a rising PSA level and subsequent BCR will develop metastases. Furthermore, uniform criteria to define BCR is lacking, and numerous definitions are used, so the overall progression-free probability and risk of subsequent metastatic disease progression can vary dramatically based on the PSA criteria used to define BCR [129]. More than 50 BCR definitions are found in the literature [130], with >0.2ng/mL being the most widespread BCR definition [126], but some studies have indicated that a PSA level of >0.4ng/mL has the greatest association with metastatic disease progression [129, 131]. In short, while PSA levels and BCR are commonly used as predictors of metastatic disease, the lack of consensus

for the definition of BCR and complete predictive capability of BCR for metastasis suggests a great need for alternative markers for prediction of metastasis. Our data here suggests that SOX2 expression in a primary tumour can be one such marker, since expression of SOX2 was associated with an increased risk of metastasis and shorter time to development of metastases. As such, patients with SOX2-positive tumours should be monitored more closely to detect metastases sooner, and may be more amenable to salvage therapies upon detection of biochemical recurrence. Currently, the mechanisms by which SOX2 causes metastasis are not well known, although potentially it could be due to an increase in expression of genes involved with invasion or an upregulation of other processes that allow for increased survival in distant areas of the body. A greater understanding of the mechanisms by which SOX2 leads to metastasis will be crucial to identify additional biomarkers that can be further utilised to identify patients who are more likely to metastasise.

CHAPTER 4

STEM AND NON-STEM CELL FUNCTIONS OF SOX2 IN CASTRATION-RESISTANT PROSTATE CANCER

4.1 Introduction

SOX2 has been most well characterised in stem cells, where its depletion leads to changes in mouse or human embryonic cell morphology, loss of pluripotency markers and differentiation [40, 41]. SOX2 acts with its canonical co-factors, NANOG and OCT4, to maintain pluripotency in stem cells and suppresses expression of genes that lead to differentiation [42, 44, 43]. SOX2 expression is tightly regulated, with levels outside the normal range leading to decreased pluripotency in embryonic stem cells, possibly as a result of reduced promoter/enhancer activity of SOX2/OCT4 target genes [47]. SOX2 gained notoriety in 2006 when Takahashi and Yamanaka reprogrammed somatic cells to induced pluripotent stem cells (iPSCs) by overexpressing SOX2 along with OCT4, KLF4, and c-MYC [48].

Due to its expression in many tissues as an adult stem cell, and its role in maintaining ESC pluripotency, SOX2 has been well examined as a marker of tumour-initiating populations in cancer, the so-called cancer stem cell (CSC) population. Well-known markers of CSCs are increased by SOX2 in pancreatic, lung, and ovarian cancers [70, 71, 72], and it has been suggested that SOX2 mediates the self-renewal of CSCs in breast cancer, gastric cancer, glioma, and osteosarcoma [54, 73, 74, 75]. SOX2 is expressed during embryonic prostate budding morphogenesis [51], and in the adult prostate SOX2 is detected primarily in basal epithelial and neuroendocrine cells [52]. Our group has shown that Sox2 marks a progenitor cell population in the mouse prostate, and this Sox2-positive lineage self-renews and leads to androgen-mediated regeneration of the prostate. Interestingly, following castration, there are Sox2-expressing luminal cells that persist, suggesting Sox2 is capable of castration-resistance and cell survival, in addition to prostate regeneration [52].

Due to its role in maintaining pluripotency and an undifferentiated state, there has been

much interest in the role of SOX2 in neuroendocrine prostate tumours. Neuroendocrine (NE) cells are the third type of epithelial cell in the prostate, in addition to basal and luminal cells, and are rare at about 1% of the total epithelial cells [85]. In prostate cancer, it has been suggested that the NE cell population expands, leading to tumour progression, poor prognosis, and hormone-refractory cancers [86]. Combined knock down of RB1 and TP53 in a hormone sensitive cell line, LNCaP, led to increased SOX2 levels, enzalutamide resistance, and increased NE markers [89]. Subsequent knock-down of SOX2 in these cells restored enzalutamide sensitivity and reversed the expression of NE markers [89]. As such, there is great interest in determining whether SOX2 acts in a stem-like manner in prostate cancer, and whether its expression can lead to a more NE phenotype.

4.2 Results

4.2.1 SOX2 binding characteristics in castration-resistant prostate cancer

The correlation of SOX2 expression with time to metastasis and decreased survival in patients with biochemical recurrence supports a hypothesis whereby SOX2 expression promotes resistance to anti-androgen therapies and progression to a castration-resistant disease [65, 89]. To begin mechanistically defining the function of SOX2 in prostate cancer cells and how it contributes to therapeutic resistance, we performed paired SOX2 chromatin immunoprecipitation sequencing (ChIP-seq) and RNA sequencing (RNA-seq) on a CRPC cell line, CWR-R1, to determine which genes SOX2 binds and potentially regulates in castration-resistant prostate cancer. Motif analyses of the SOX2 bound genes demonstrates strong homology between the SOX2 binding motif in CWR-R1 and the canonical SOX2 binding sequence (Figure 4.1). Analysis of SOX2 ChIP-sequencing revealed that greater than 53% of the SOX2 peaks are within protein coding genes (n=18,284) and SOX2 binds predominantly within 1000 base pairs upstream of the transcription start site of these protein coding genes (Figure 4.2) [132]. RNA expression of SOX2-bound genes was predominantly high,

with 87.7% of genes having an mRNA expression of TPM >10 (Figure 4.3), indicating SOX2 binding is associated with active transcription rather than gene repression. Notable SOX2-bound genes that have robust expression in CRPC cells include BCL2, EZH2, FGFR3, FOXA1, KLK3/PSA, KRAS, MET, TGFRB1, IKZF1, CTNNB1, PARP1, and ONECUT2 (Figure 4.4). It should be noted, however, that these data demonstrate SOX2 binding but are only suggestive of any potential direct transcriptional regulation by SOX2.

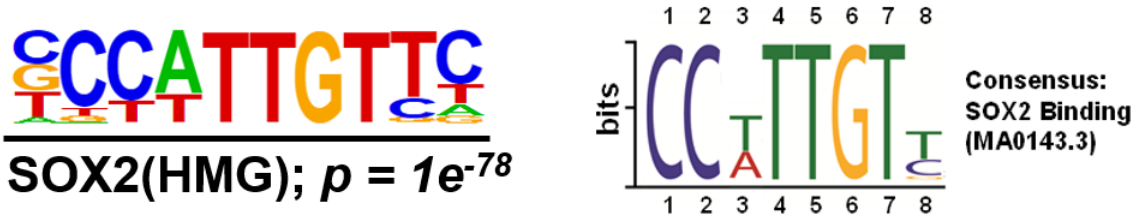

Figure 4.1: SOX2 binds predominantly in the promoter regions of protein coding genes within 100 base pairs of the transcription start site (TSS). SOX2 binding peaks were annotated to the closest gene, and allocated to either protein-coding, non-coding RNA, Pseudo RNA or other RNA. Peaks within the promoter regions of protein-coding genes were split by the distance from the TSS.

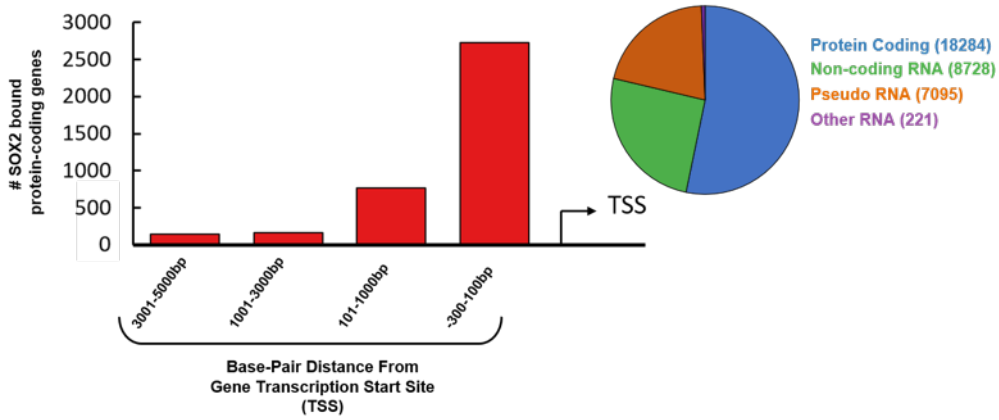

Figure 4.2: SOX2 binds predominantly in the promoter regions of protein coding genes within 100 base pairs of the transcription start site (TSS). SOX2 binding peaks were annotated to the closest gene, and allocated to either protein-coding, non-coding RNA, pseudo RNA or other RNA. Peaks within the promoter regions of protein-coding genes were split by the distance from the TSS.

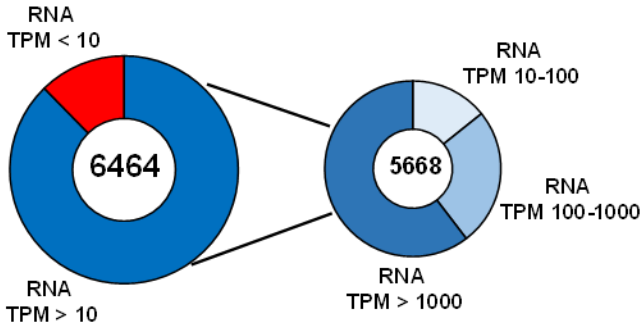

Figure 4.3: RNA expression of SOX2 bound genes indicates SOX2 bound genes are predominantly expressed. Most genes have a TPM >10, indicating SOX2 bound genes are actively expressed and not repressed.

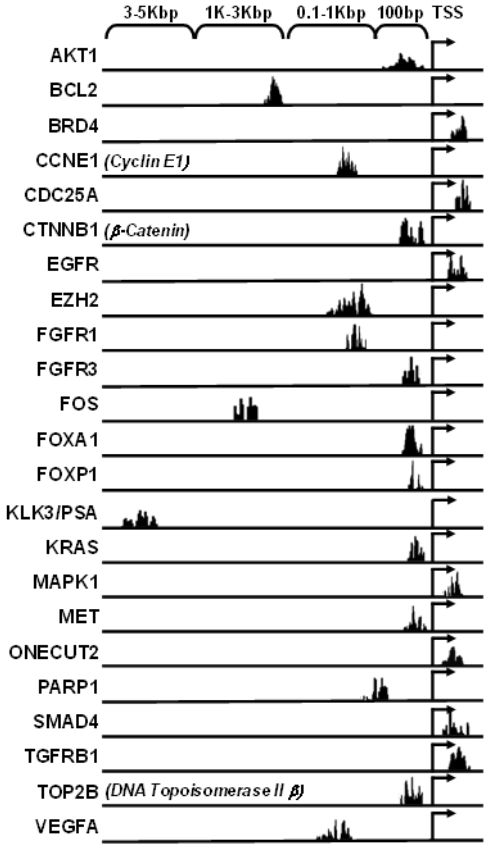

Figure 4.4: SOX2 binds many genes commonly implicated in cancer progression and initiation. IGV tracks indicate SOX2 binding peaks in the promoter regions of many genes implicated in cancer.

4.2.2 SOX2 has both canonical and non-canonical stem cell functions

The canonical function of SOX2 in regulation of embryonic stem cell transcriptional programming and maintenance has been well characterized [133, 134]. To identify novel, prostate cancer-specific SOX2 gene targets in CRPC cells that may also be canonical SOX2 stem cell genes, we conducted parallel SOX2 ChIP-seq in the WA01 embryonic stem cell line, hereafter hESCs, and compared SOX2 target genes to those identified as SOX2 targets in CWR-R1 cells. Of the 3561 genes bound by SOX2 in CRPC, 77.6% (N=2763) are unique to prostate cancer cells, and potentially represent novel SOX2 gene targets in CRPC (Figure 4.5). Of the 1540 genes bound by SOX2 in hESCs, 51.8% (N=798) were commonly bound within CRPC cells (Figure 4.5). To elucidate potential signalling pathways regulated by SOX2, we conducted pathway analysis using Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA). These analyses demonstrated that SOX2 target genes in CRPC were involved in pathways related to common cancer signalling pathways, such as PTEN, TGF-β, and JAK/STAT, whereas genes in the hESCs were not enriched in these cancer pathways (Figure 4.6).

Additional analyses of the overlapping SOX2 target genes between CRPC and hESCs demonstrated that only 6.6% (N=53) of the 798 overlapping gene targets were bound by SOX2 in the prostate cancer cells within 10 base pairs of the SOX2 binding site in the human embryonic stem cells (Figure 4.5), suggesting differential regulation of common genes between CRPC and stem cells. Collectively, the non-canonical and unique gene targeting of SOX2 in CRPC cells suggests alternate, non-stem cell functions for SOX2 in prostate cancer cells, as well potentially novel transcriptional binding partners for SOX2. The association with non-stem cell binding partners in CRPC is further supported by a lack of OCT4 and NANOG expression in CRPC cells [65] compared to embryonic stem cells (Figure 4.7). This large number of unique SOX2-bound genes in prostate cancer led us to further examine the role of a non-canonical stem cell SOX2 function in CRPC cells.

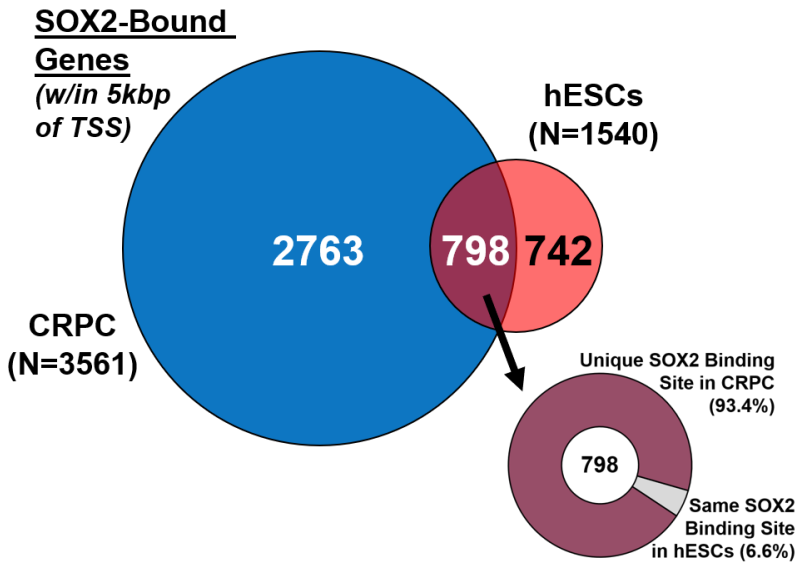

Figure 4.5: The majority of SOX2 bound genes in CRPC do not overlap with genes bound in hESCs. SOX2 binds distinct sets of genes in CWR-R1 cells compared to WA01 cells, and of those commonly bound, SOX2 binds predominantly at unique binding sites (>10 base pairs apart).

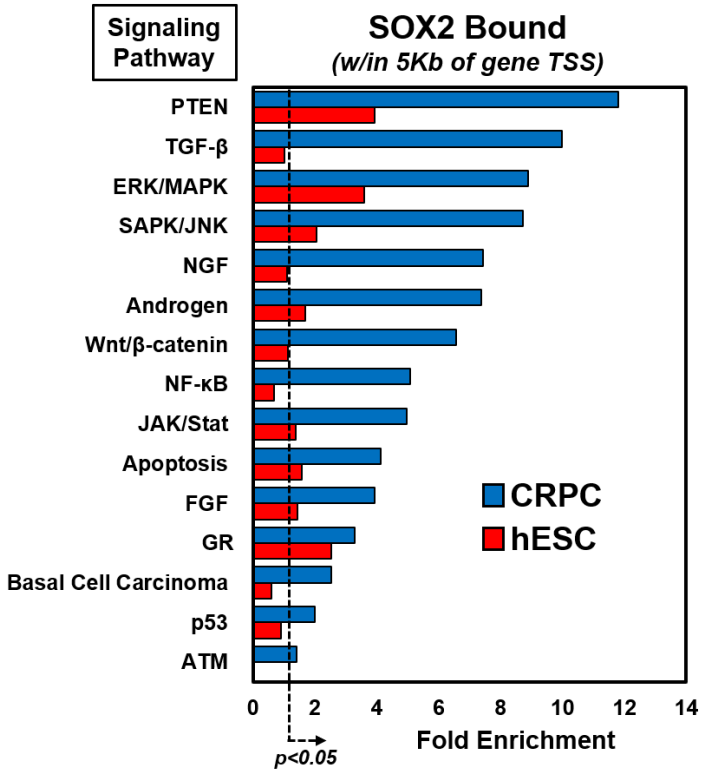

Figure 4.6: Genes bound in CRPC are enriched for common cancer signalling pathways, whereas genes bound in the hESCs are not enriched for these pathways. Genes were input into GO and Ingenuity Pathway Analysis to identify pathways enriched by genes in each cell line.

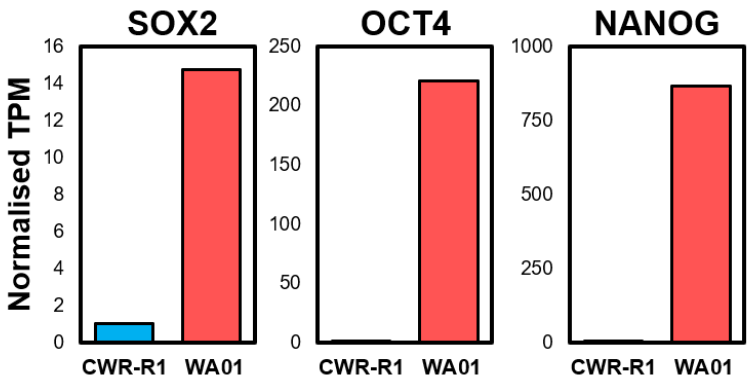

Figure 4.7: Expression of SOX2's canonical co-factors, OCT4 and NANOG, is much higher in hESCs than in CRPC. TPM values of SOX2, OCT4 and NANOG were taken from RNA-sequencing of CWR-R1 and WA01 cells.

4.3 Discussion

To examine whether SOX2 is acting in a similar manner in castration-resistant prostate cancer (CRPC) as in human embryonic stem cells (hESCs), we looked at genes SOX2 was binding in both cell lines. The comparison of the genes that SOX2 binds in both CRPC and hESC cell lines reveals the majority of genes bound by SOX2 in CRPC are not bound in hESCs, and of those that are commonly bound, few genes had SOX2 binding in the same location in the promoter of the gene in both cell lines. Furthermore, the genes bound by SOX2 in CRPC are enriched for common cancer signalling pathways, while those bound in hESCs are not. Together, these results indicate that SOX2 is acting in a non-canonical stem cell manner in CRPC.

SOX2 has been well examined as a marker and driver of cancer stem cells (CSCs). These cells have a unique replicative ability and have been associated with metastasis, drug resistance, and increased colony formation capability in many cancers [54, 70, 71, 72, 73, 74, 75].We showed in the previous chapter that SOX2 is associated with increased metastasis, however the lack of commonly bound genes in hESCs indicates this may not be due to a stem cell phenotype in prostate cancer, but rather may be due to the abundance of cancer signalling pathways. This also suggests that some of the common problems with CSCs, such as

resistance to therapy due to slower division, may not apply in the context of prostate cancer. Clinically, it is possible that patients with SOX2-expression in prostate cancer may respond differently to therapies than patients with SOX2-expression in other cancers. Our group has previously shown that SOX2 is an AR-repressed gene, and that resistance to AR-signalling therapies may be due to the subsequent increase in SOX2 expression [65]. SOX2 has also been implicated in neuroendocrine prostate cancer, a form of prostate cancer that is characterised by loss of androgen-receptor (AR) signalling, resulting in resistance to AR-targeted therapies [135]. While uncommon in a primary tumour, this subtype may emerge from prostate adenocarcinoma due to lineage plasticity following AR-targeted therapies [136, 137]. Some groups have demonstrated that SOX2 can promote lineage plasticity in cells deficient of TP53 and RB1, leading to antiandrogen resistance, and that knock-down of SOX2 in this setting leads to a decrease in the expression of neuroendocrine markers synaptophysin, neuron specific enolase, and chromogranin A [89]. However, within our sequencing dataset, we see mixed expression of these markers, suggesting SOX2 may be a driver of lineage plasticity in some contexts, such as TP53 and RB1 deficient backgrounds, but other genetic factors also need to be considered.

Another aspect that may explain why SOX2 is acting in a different manner to hESCs in prostate cancer could be that SOX2's canonical transcription co-factors, NANOG and OCT4, are not present in CRPC. The expression of NANOG and OCT4 is not detectable in CRPC, and previous work has also shown that prostate cancer cell lines with SOX2 expression do not express OCT4 and NANOG at a protein level [65]. Together with the different binding sites of SOX2 on promoters of commonly bound genes between CRPC and hESCs, these data suggest that SOX2 may be interacting with novel co-factors in CRPC to regulate cancer signalling programs. Identifying the co-factors of SOX2 in CRPC will allow for a better understanding of the regulation of genes and pathways by SOX2 in CRPC. This insight can help to find novel therapies for patients with CRPC.

CHAPTER 5

CHARACTERISATION OF SOX2 IN CASTRATION-RESISTANT PROSTATE CANCER

5.1 Introduction

Castration-resistance is an important clinical problem in prostate cancer, where patients have few options available once they progress following androgen deprivation therapy (ADT). ADT has been the mainstay therapy since the 1940s, when Charles Higgins discovered castration significantly improved the outcomes of patients with advanced prostate cancers [138]. ADT inhibits the production of testosterone and prevents testosterone from activating androgen receptor (AR) signalling in prostate cells [21]. Prostate cancer typically develops from the AR-sensitive and PSA-secreting luminal cells, which rely on androgen signalling for their growth. Since prostate cancer relies heavily on AR-signalling, ADT therapy is hugely successful, leading to inhibition of proliferation and apoptotic death of the cancer cells [22]. However, despite initial success of ADT in most patients, there are several mechanisms of resistance that emerge, such AR gene mutations leading to promiscuous ligand binding, splice variations lacking the ligand binding domain, amplification, and AR protein over-expression [23].

We have previously shown that SOX2 is one of the most differentially and highly expressed genes between the prostate and seminal vesicle, an organ with a similar function and reliance on AR signalling as the prostate, but which is rarely is the site of tumourigenesis [84]. In normal prostate tissues, SOX2 expression is restricted to basal epithelial cells, while in prostate tumours, SOX2's expression is luminal, where tumours are binary for SOX2 expression (either SOX2-positive or SOX2-negative, not heterogenous). We have also seen that the percentage of SOX2-positive tumours increases with Gleason Score, and nearly all lymph node metastases are SOX2-positive [65]. Further, ectopic expression of SOX2 in a hormone-sensitive prostate cancer cell line is sufficient to allow tumour formation in a

castrated host, leading to castration-resistant prostate cancer tumour growth [65], and that AR signalling decreases expression of SOX2, suggesting SOX2 is an AR-repressed gene [65] that may be promoting castration-resistance upon upregulation following AR-inhibition.

Given the work in the previous chapters indicating a role for SOX2 in metastasis in patients with biochemical recurrence, as well as a non-canonical stem cell role in prostate cancer, we sought to determine more fully the phenotype of SOX2 in castration-resistant prostate cancer, as well as the pathways and mechanisms by which it can lead to metastasis.

5.2 Results

5.2.1 Phenotype characterisation of SOX2 in castration-resistant prostate cancer

We have previously demonstrated that constitutive expression of SOX2 in the hormone-sensitive LAPC4 cell line is sufficient to enable castration-resistant tumour formation, and Mu et al. demonstrated that SOX2 depletion in LNCaP-RB/p53 deficient cells abrogates enzalutamide resistance [65, 89]. To more precisely elucidate SOX2-mediated genes that confer therapy resistance, we deleted SOX2 expression in CWR-R1 prostate cancer cells using CRISPR-Cas9 gene targeting. Targeted SOX2 deletion (hereafter referred to as $SOX2^{KO}$) resulted in non-detectable SOX2 mRNA and protein expression among multiple clonal lines (Figures 5.1 and 5.2). Importantly, SOX2 deletion does not impact the expression of AR and AR-V7, a common splice variant that has been implicated in enzalutamide resistance [139], between the different clones and AR-signalling conditions (Figure 5.2).

Deletion of SOX2 led to decreased growth in $SOX2^{KO}$ clones 1 and 2, but not in clone 3, and may be due to the higher residual level of SOX2 mRNA present in clone 3 compared to the other clones (Figure 5.3, left). As anticipated, growth analysis indicates that while $SOX2^{KO}$ leads to decreased growth when treated with enzalutamide in vitro, control cells with SOX2 expression did not show any reduced growth (Figure 5.4); interestingly this

reduction in growth was seen for all clones (Figure 5.3, right). Further analyses were carried out using $SOX2^{KO}$ Clone 1, which had almost undetectable levels of both SOX2 mRNA and protein, and demonstrated a decreased growth in normal conditions as well as substantial sensitivity to enzalutamide compared to the control SOX2-positive cells. Cell cycle analysis indicated that the control and $SOX2^{KO}$ cells proliferate in a similar manner in the vehicle treated condition, but the $SOX2^{KO}$ cells have fewer cells in the S-phase when treated with enzalutamide, and appear to be arrested in the G1 cell-cycle phase (Figure 5.5). This was further supported by a bromodeoxyuridine (BrdU) incorporation assay, where BrdU was incorporated at the same rate in control and $SOX2^{KO}$ cells in the vehicle condition, but $SOX2^{KO}$ cells had decreased BrdU incorporation with enzalutamide treatment, suggesting cells are unable to cycle from the G1 phase to the S phase and undergo DNA replication (Figure 5.6). The decrease in growth of the $SOX2^{KO}$ clone (in the absence of enzalutamide) compared to the control cells was not due to increased apoptosis, as measured by a propidium iodide (PI) exclusion assay, a TUNEL microscopy assay and an apoptosis/necrosis assay (Figure 5.7). To further examine the $SOX2^{KO}$ phenotype, we also performed a transwell migration assay to determine the migratory capacity of the $SOX2^{KO}$ cells when treated with enzalutamide. The $SOX2^{KO}$ cells had decreased migration compared to the control cells (Figure 5.8), further supporting the clinical data phenotype in Figure 1, where patients with tumour SOX2 expression had a decreased time to metastasis.

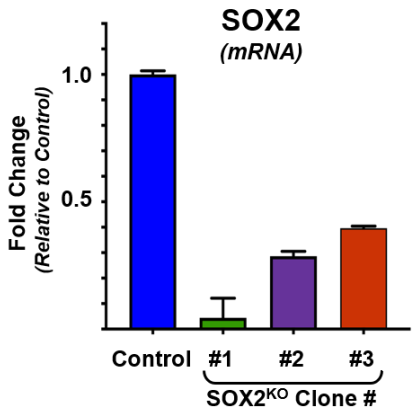

Figure 5.1: SOX2 mRNA expression is reduced in $SOX2^{KO}$ clones. SOX2 was deleted in CWR-R1 cells using CRISPR-Cas9.

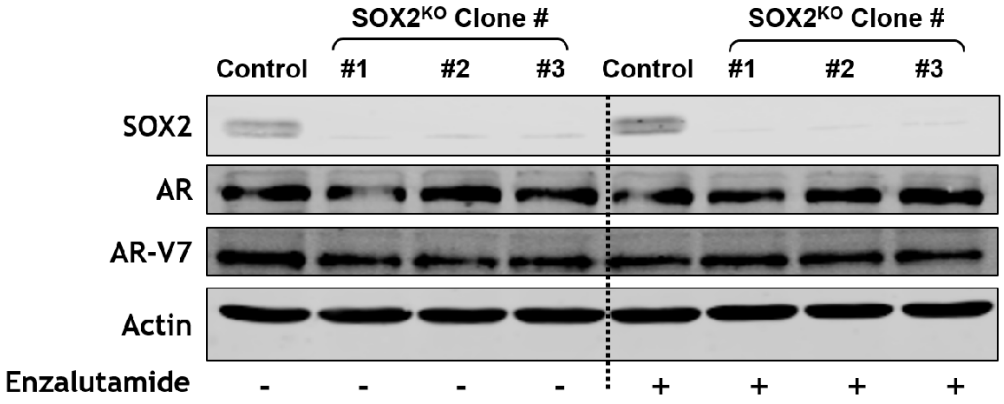

Figure 5.2: SOX2 protein expression is reduced in $SOX2^{KO}$ clones. SOX2 was deleted in CWR-R1 cells using CRISPR-Cas9. Protein levels of the androgen receptor (AR) and AR splice variant 7 (AR-V7), remained at similar levels.

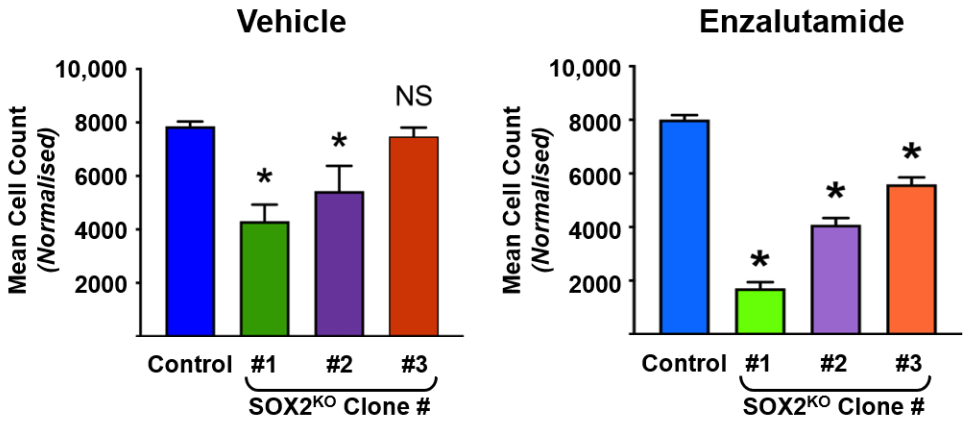

Figure 5.3: Knock out of SOX2 leads to reduced growth in vehicle (left) and enzalutamide treated conditions (right). Cell growth was measured using Incucyte with cells counted every 4 hours for 120 hours. Subsequent cell counts were normalised to number of cells at 0hrs.

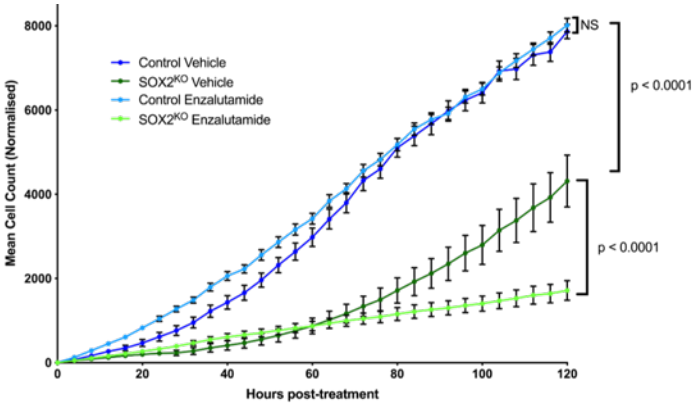

Figure 5.4: Knock out of SOX2 in Clone 1 leads to reduced growth and enzalutamide sensitivity. Cell growth was measured using Incucyte with cells counted every 4 hours for 120 hours. Subsequent cell counts were normalised to number of cells at 0hrs.

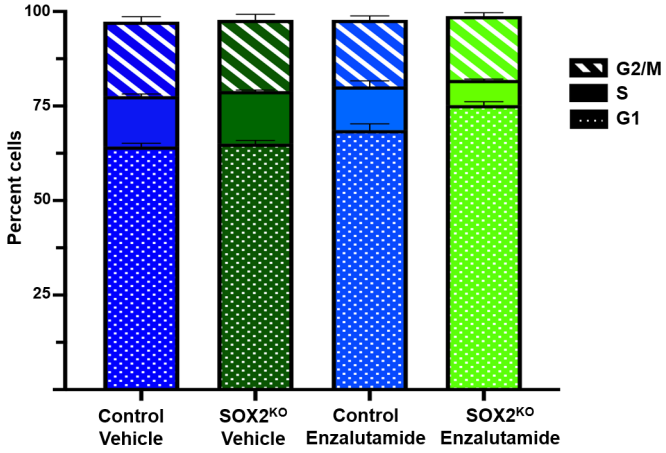

Figure 5.5: Cell cycle analysis indicates SOX2^{KO} cells have a G1 arrest when treated with enzalutamide. Propidium iodide was used to stain DNA to determine at which stage of the cell cycle cells were in.

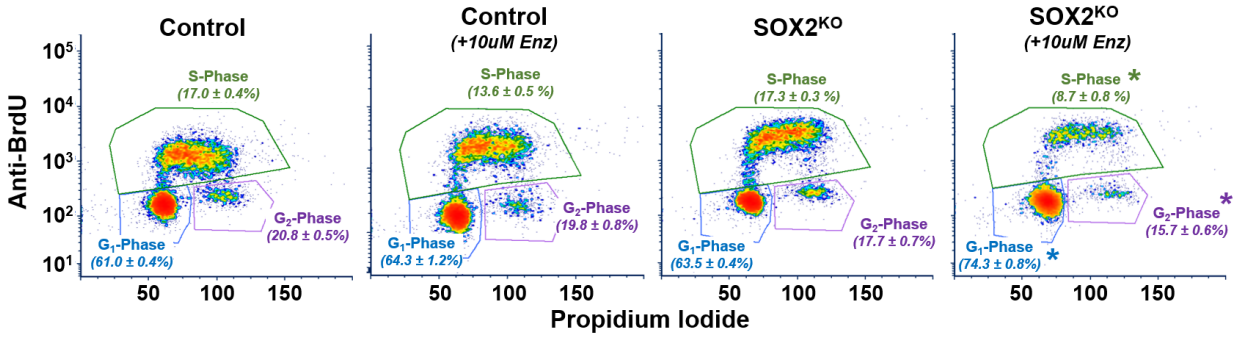

Figure 5.6: BrdU incorporation assay indicates SOX2^{KO} cells have reduced DNA replication when treated with enzalutamide. Cells were pulsed with BrdU (bromodeoxyuridine) and co-stained with propidium iodide to determine cell cycle phase and amount of DNA replication.

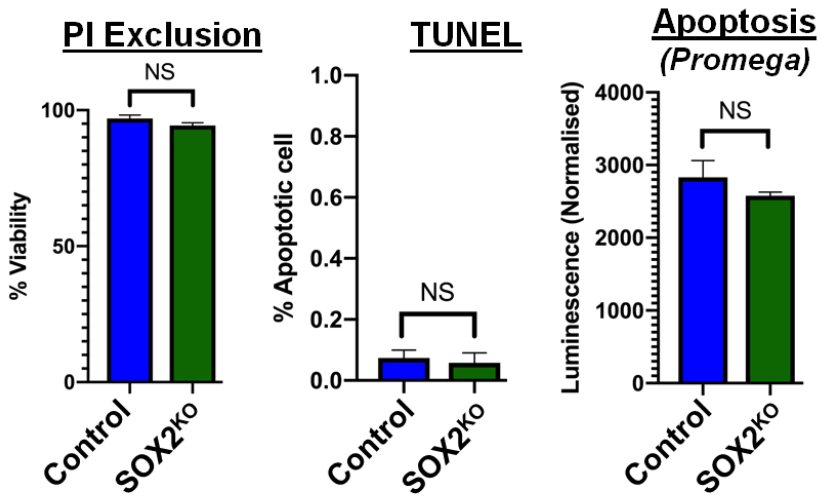

Figure 5.7: Decreased cell growth of $SOX2^{KO}$ cells is not due to increased apoptosis. Three different assays for apoptosis were utilised to examine differences in apoptosis between control and $SOX2^{KO}$ cells. PI exclusion assay indicates cell viability by examining the number of cells that are stained with propidium iodide, indicating cells that have a damaged cell membrane and are unviable. TUNEL assay measures number of apoptotic cells by identifying sites of broken DNA. Promega Apoptosis assay measures apoptosis by measuring binding to proteins on cell membrane that are only on outer membrane when cells have undergone apoptosis.

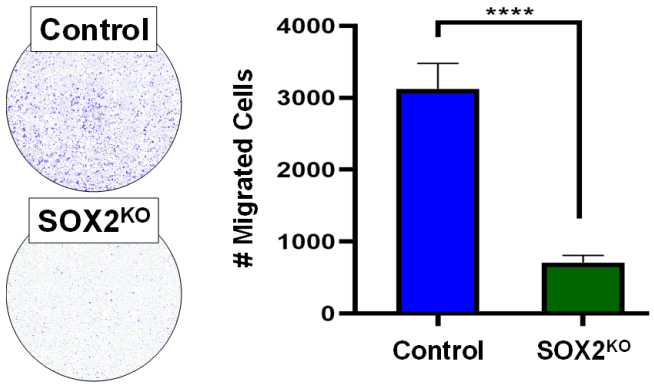

Figure 5.8: Loss of SOX2 leads to decreased migration. Cells were plated in a transwell migration assay, and migrated to serum-rich media over 48 hours. Migrated cells were fixed and stained with crystal violet.

5.2.2 Analyses of castration-resistant prostate cancer cells lacking SOX2 prioritises changes in cellular metabolism

The observation that SOX2^{KO} reduces prostate cancer cell growth without increasing cell death or changes in cell-cycle distribution prompted us to identify the SOX2-mediated mechanisms that led to fewer cells over time. In order to determine the genes that SOX2 regulates in varying AR-signalling conditions, we performed RNA-sequencing on the CWR-R1 control cells and the SOX2^{KO} cells within intact AR signalling (vehicle) and inhibited AR signalling (enzalutamide treated) conditions and identified differentially expressed genes. Principal component analysis (PCA) of the differentially expressed genes (DEGs) indicates samples differ primarily by SOX2 expression, and then by AR signalling condition. This is also seen in the number of DEGs, where over 3000 DEGs are found between control and SOX2^{KO} samples in either treatment condition (3230 in vehicle, 3496 in enzalutamide treated), but only 1042 and 2245 DEGs are found between the vehicle and enzalutamide treated control and SOX2^{KO} cells, respectively (Figure 5.9). Overlaying the DEGs in the vehicle condition with the SOX2 bound gene list from the ChIP-seq in Chapter 4, 781 of the DEGs are also SOX2-bound target genes (Figure 5.10). Further examination of the DEGs reveal that loss of SOX2 leads to a general repressive phenotype, where 59.3% of the genes (N=1915) are downregulated upon SOX2 loss (Figure 5.11, left). This downregulated phenotype is even more clearly seen when the DEGs are combined with the SOX2 ChIP-seq genes, where 70.3% of the SOX2 bound DEGs are downregulated upon SOX2 deletion (Figure 5.11, right).

Gene set enrichment analysis (GSEA) demonstrated many pathways involved with various types of metabolism are altered when SOX2 is deleted, such as oxidative phosphorylation, fatty acid metabolism, amino acid metabolism, and xenobiotic metabolism (Figure 5.12), prioritizing a potential role for SOX2 in regulating cellular metabolism in prostate cancer cells. We thus further refined our candidates to genes that were SOX2-bound (ChIP-Seq), differentially expressed between control and SOX2^{KO} cells (RNA-Seq) and had es-

tablished roles in regulating cellular metabolism (Figure 5.13). Further refinement and clinical validation of SOX2-associated regulation of genes involved in cellular metabolism was conducted using publicly-available RNA-Seq datasets. First, analyses of mRNA data of primary prostate tumours obtained via the TCGA (N=290 patient tumours) demonstrated statistically-significant correlations between SOX2 and ECHS1, SLC46A1, TST, CERK, LPCAT4, and SLC16A3 [140] (Figure 5.14). Second, analyses of RNA-Seq data of metastatic tumours (SU2C/PCF Dream Team) [141] demonstrated statistically-significant differences between primary prostate cancer tumours and metastases for CERK, ECHS1, HS6ST1, LPCAT4, PFKP, TST, SLC16A3, and SLC46A1, genes involved in biosynthesis of several lipids, metabolism of fatty acids, glycolysis and transport of metabolites across membranes (Figure 5.15). Differential correlation between tumours and metastasis could be accounted for by their distinct clinical stage, AR-activation status, and anatomical environment. However, these data support a model whereby SOX2 expression, either within a primary tumour or acquired as a result of therapy-resistance, promotes a distinct cellular metabolism phenotype that is directly regulated by SOX2.

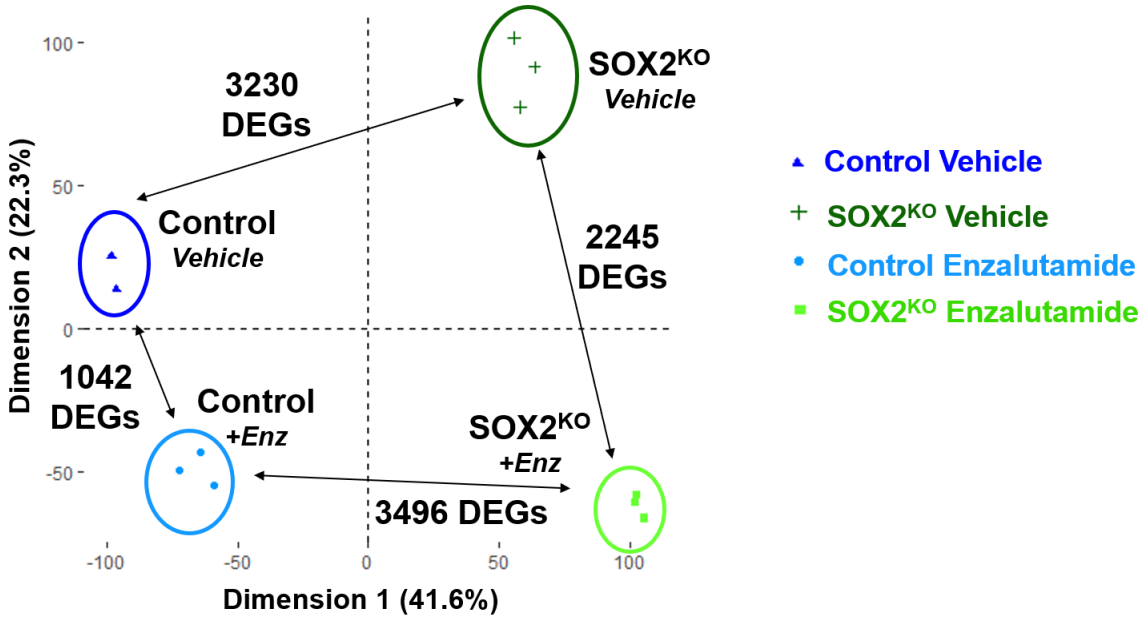

Figure 5.9: Samples differ primarily by SOX2 expression, and then by AR signalling context. Principal component analysis (PCA) groups samples based on differential expression of genes. The first-dimension divides samples into control cells and $SOX2^{KO}$ cells, and the second dimension then splits each of these groups by AR signalling status into vehicle and enzalutamide treated. The number of differentially expressed genes between each group is noted.

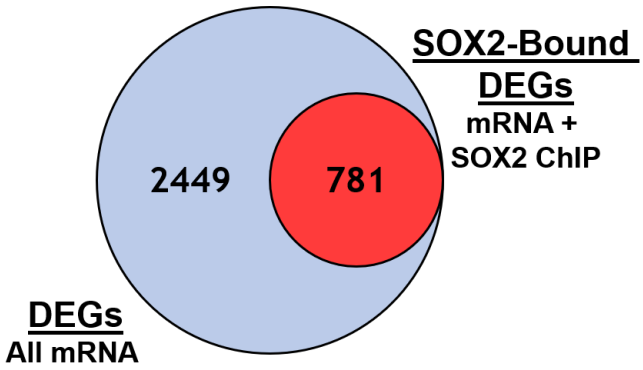

Figure 5.10: 24% of the differentially expressed genes in the vehicle condition are SOX2 target genes and may be regulated by SOX2. The SOX2 ChIP-seq gene list from Chapter 4 was overlaid with the DEGs list between control and $SOX2^{KO}$ cells in the vehicle treated condition.

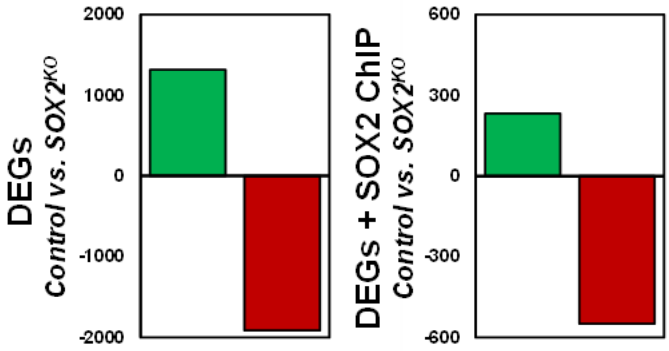

Figure 5.11: Loss of SOX2 leads to a repressed state overall. More differentially expressed genes are downregulated when SOX2 is deleted from CWR-R1 cells (left), and this is seen more clearly when combining the DEGs with SOX2 ChIP-seq (right), where more than half of the genes are downregulated.

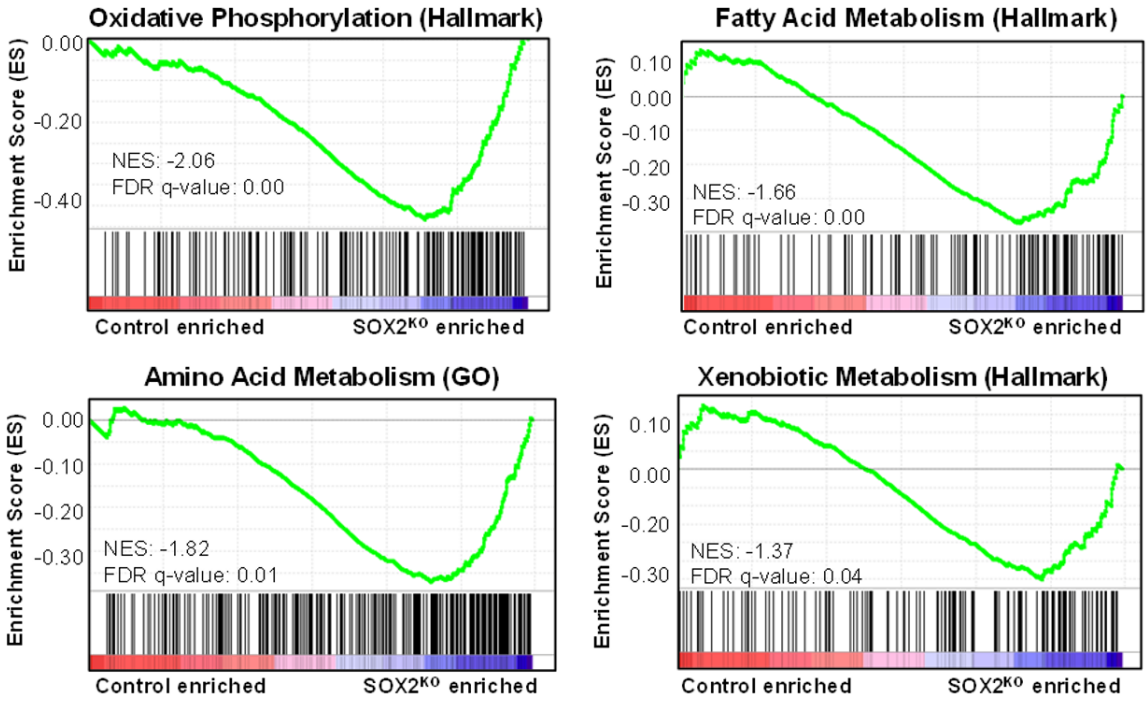

Figure 5.12: Pathways involved with metabolism are altered when SOX2 is deleted. Gene set enrichment analysis (GSEA) demonstrates differences between control and $SOX2^{KO}$ cells for pathways involved with different types of metabolism.

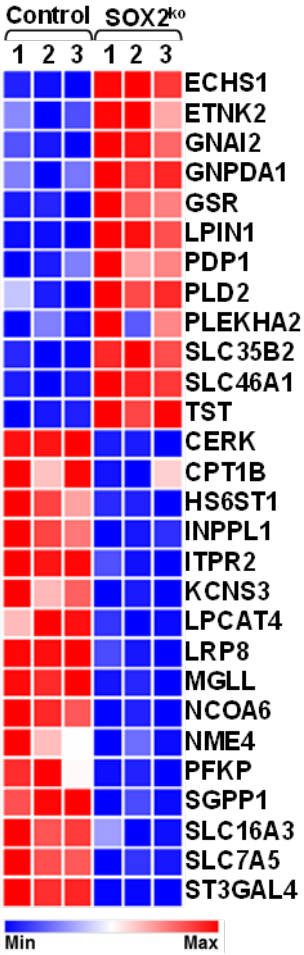

Figure 5.13: Key candidate genes involved with metabolism are differentially regulated by SOX2. Leading edge analysis of GSEA metabolic pathways was used to identify genes involved in multiple pathways, and which are bound by SOX2.

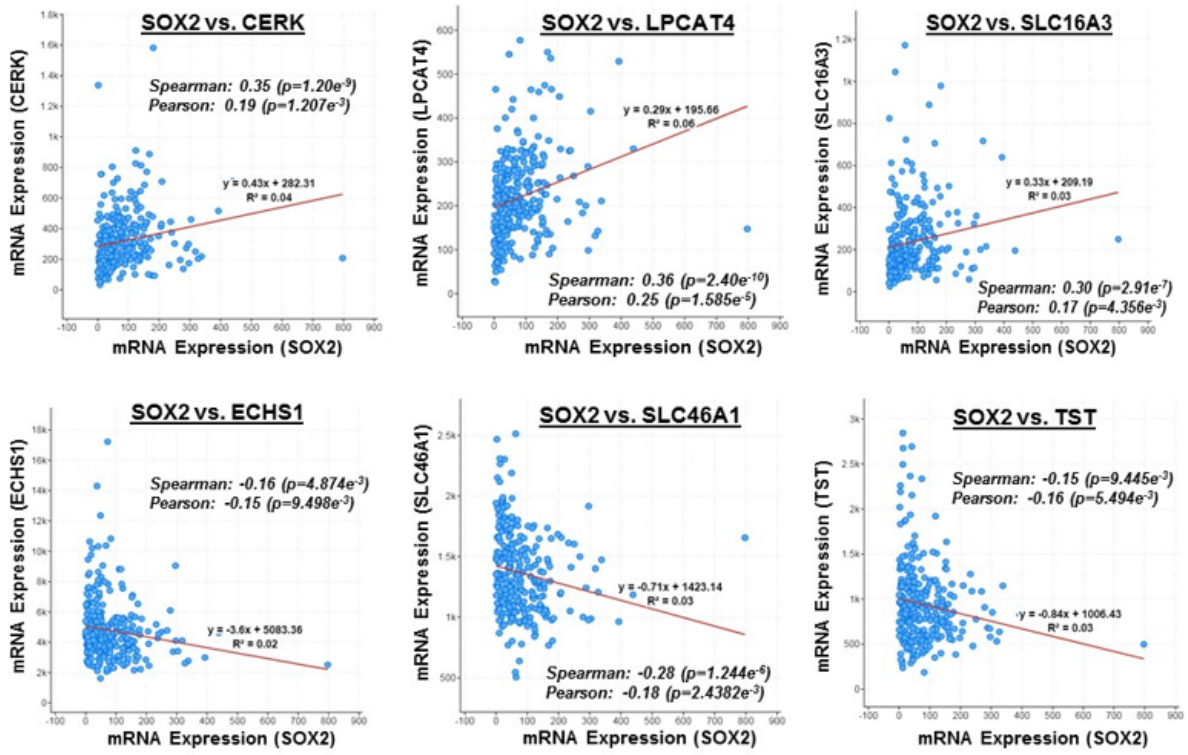

Figure 5.14: SOX2 correlates with several key metabolic genes in primary tumours. SOX2 bound and differentially expressed metabolic genes show correlation with SOX2 expression in primary prostate tumours from TCGA (The Cancer Gene Atlas).

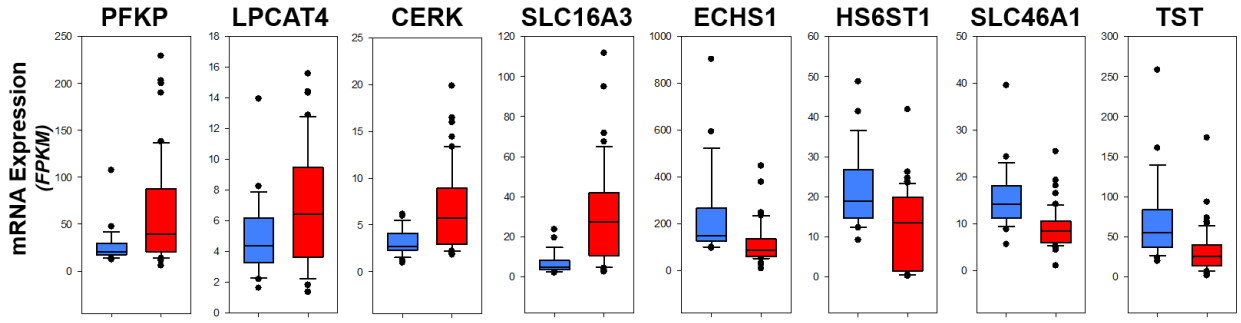

Figure 5.15: Key metabolic genes identified from RNA-sequencing are also differentially expressed between primary prostate tumours and metastases. Several key metabolic genes identified as SOX2 bound and differentially expressed show differential expression between primary tumours (blue) and metastases (red), and follow the same directionality as seen from $SOX2^{KO}$ RNA-sequencing.

5.2.3 SOX2 regulates glycolysis and oxidative phosphorylation in castration-resistant prostate cancer

Prostate cancer metabolism varies from the generally accepted Warburg phenotype, in that primary prostate cancer cells tend to rely on oxidative phosphorylation as the main energy source, while prostate cancer metastases shift towards relying on glycolysis for ATP production [99]. To define metabolic changes when SOX2 is lost (CWR-R1-SOX2^{KO}) or gained (LAPC4-SOX2), we measured and compared mitochondrial respiration and glycolysis between CWR-R1-Control vs. CWR-R1-SOX2^{KO} and LAPC4-Control vs. LAPC4-SOX2 cells. To measure glycolysis and glycolytic capacity, we utilized the Seahorse *Glycolysis Stress Test* which measures key parameters of glycolytic flux such as glycolysis, glycolytic capacity and glycolytic reserve. Upon saturating amounts of glucose, the SOX2-expressing cells were able to undergo higher rates of glycolysis compared to the SOX2^{KO} or SOX2-negative cells (Figures 5.16 and 5.17). Interestingly, in the CWR-R1s, there was not a large increase in glycolytic capacity, and thus a low glycolytic reserve (Figure 5.16), when mitochondrial ATP production was inhibited with the addition of oligomycin, suggesting that these cells are operating at close to maximum glycolytic potential in the presence of glucose. We identified PKFP as a SOX2 target gene (Figure 5.18) that is increased in metastases. It is particularly interesting, due to its role as a key regulator of glycolysis, and SOX2 could be modulating glycolysis through PFKP.

To measure mitochondrial respiration, we utilized the Seahorse *Mitochondrial Stress Test*, whereby cells are treated with oligomycin to inhibit ATP synthase, and the decrease in OCR provides a measurement of mitochondrial respiration associated with cellular ATP production. The subsequent injection of FCCP collapses the proton gradient and disrupts mitochondrial membrane potential, leading to the uninhibited flow of electrons through the electron transport chain, and maximal oxygen consumption by complex IV. The difference between the basal level of oxygen consumption and the maximal oxygen consumption after FCCP treatment is the spare capacity, which measures the ability of a cell to respond to increased

energy demand. These analyses revealed that SOX2-expressing cells have a higher level of maximal respiration and spare respiratory capacity, demonstrating that SOX2-expression leads to higher basal respiration, maximal respiration, and respiratory capacity compared to cells lacking SOX2 (Figures 5.19 and 5.20). This could in part be due to alterations in mitochondria, where there may be increased mitochondrial respiration due to an increase in the number of mitochondria in the SOX2-expressing cells. Quantification of mitochondrial mass through qPCR for mitochondrial genes, $tRNA^{Leu(UUR)}$ and 16S rRNA subunit, reveals an increased amount of mitochondria in SOX2-expressing cells, compared to those without SOX2 (Figure 5.21). Metabolite analysis indicates there is a decrease in metabolites in the glycolysis pathway when SOX2 is lost, while there is an increase in the metabolites involved with the TCA cycle and offshoot pathways from glycolysis (Figure 5.22). This suggests that loss of SOX2 leads to a less glycolytic phenotype, and potentially reprograms the cells to a less metastatic form.

Based on these assays, the SOX2-expressing cells have a higher capacity for both mitochondrial respiration and glycolysis, and this increase in metabolic capacity and energetic reserves potential could enable enhanced proliferation, metastasis, therapy resistance, and plasticity of SOX2-positive prostate cancer cells.

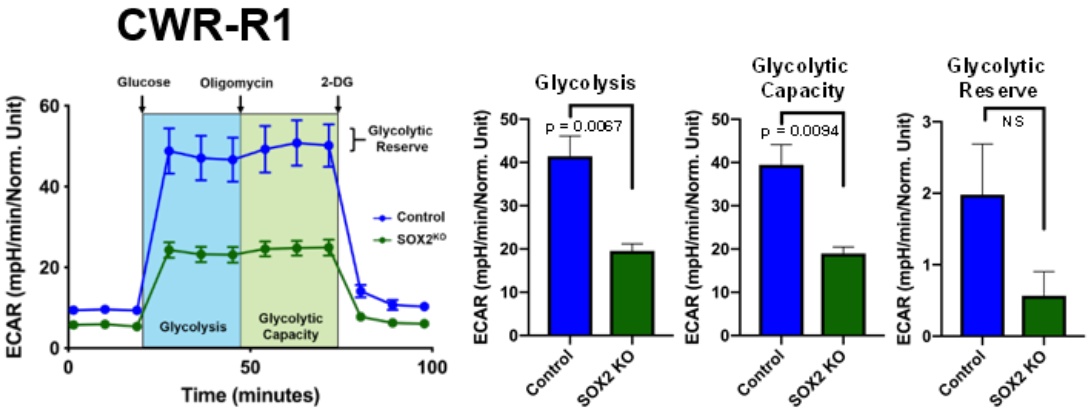

Figure 5.16: Loss of SOX2 leads to decreased glycolysis and glycolytic capacity in CWR-R1 cells. Seahorse Glycolysis Stress Test was used with CWR-R1 control and $SOX2^{KO}$ cells. Extracellular acidification rate (ECAR) was measured as glucose, oligomycin and 2-DG was added.

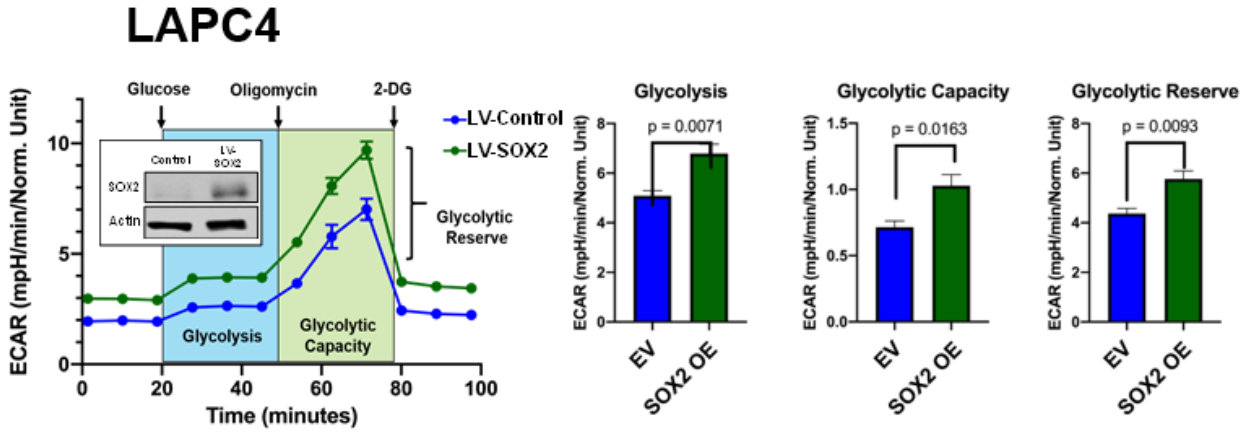

Figure 5.17: Overexpression of SOX2 leads to increased glycolysis, glycolytic capacity, and glycolytic reserve in LAPC4 cells. Seahorse Glycolysis Stress Test was used with LAPC4 empty vector and SOX2 overexpression. Extracellular acidification rate (ECAR) was measured as glucose, oligomycin and 2-DG was added.

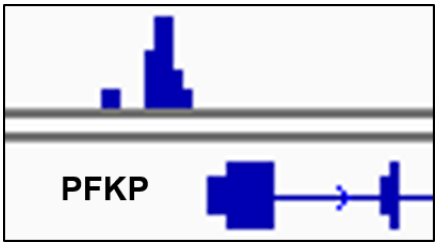

Figure 5.18: PFPK is a SOX2 target gene. in IGV, a SOX2 peak is seen in the PFKP promoter upstream of the transcription start site.

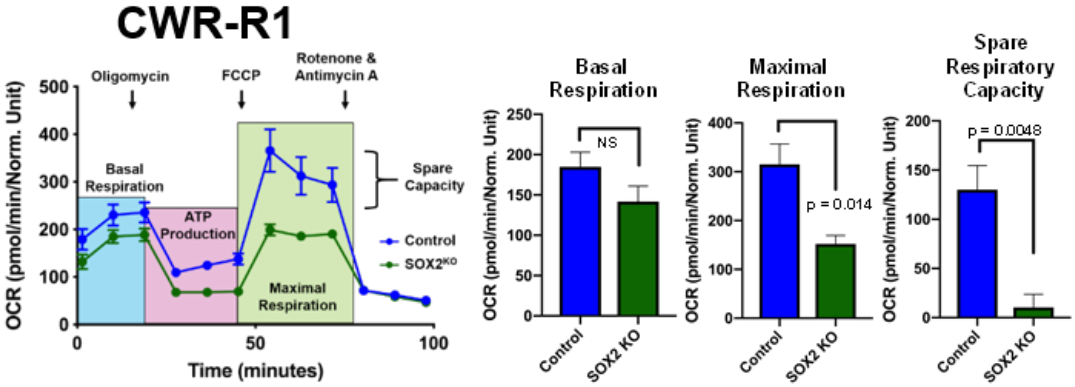

Figure 5.19: Loss of SOX2 leads to decreased maximal respiration, and spare respiratory capacity in CWR-R1 cells. Seahorse Mito Stress Test was used with CWR-R1 control and $SOX2^{KO}$ cells. Oxygen consumption rate (OCR) was measured upon addition of oligomycin, FCCP, and rotenone and antimycin A.

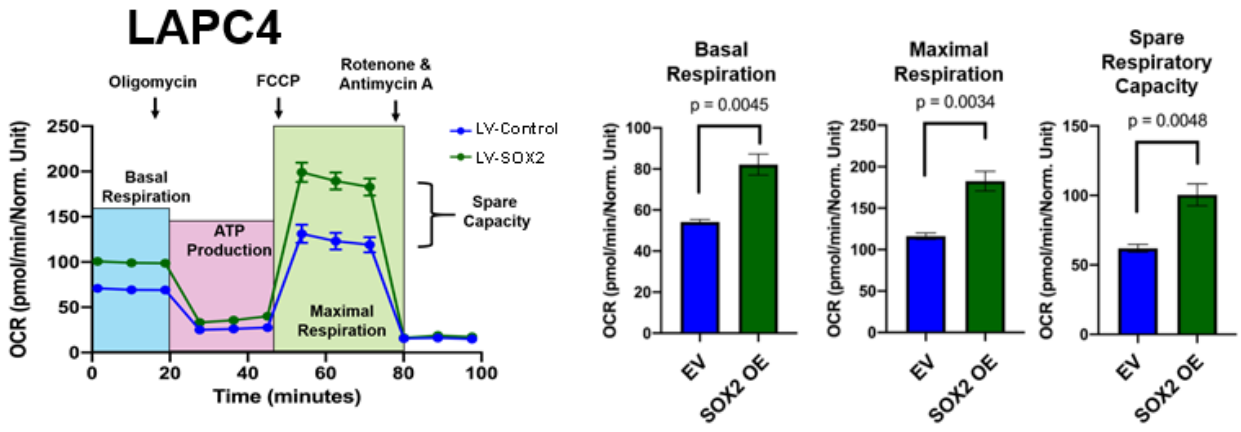

Figure 5.20: Overexpression of SOX2 leads to increased basal respiration, maximal respiration, and spare respiratory capacity in LAPC4 cells. Seahorse Mito Stress Test was used with LAPC4 empty vector and SOX2 overexpression. Oxygen consumption rate (OCR) was measured upon addition of oligomycin, FCCP, and rotenone and antimycin A.

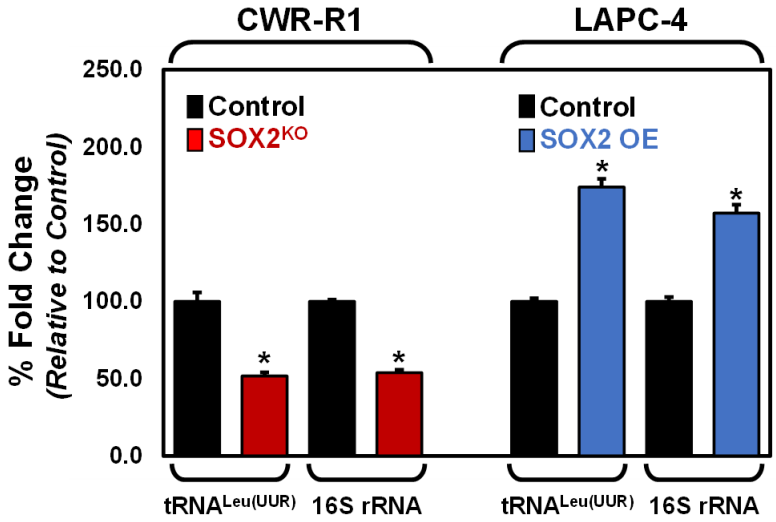

Figure 5.21: SOX2 expressing cells had increased number of mitochondria. PCR was performed for two mitochondrial genes, $tRNA^{Leu(UUR)}$ and 16S rRNA subunit. Decreased levels of these genes are seen in the CWR-R1 cells with the $SOX2^{KO}$, and increased levels of both of these genes are seen in the LAPC4 SOX2 overexpression cells. Levels of both the $tRNA^{Leu(UUR)}$ and 16S rRNA subunit genes were normalised to the nuclear gene nuclear β2-microglobulin (β2M).

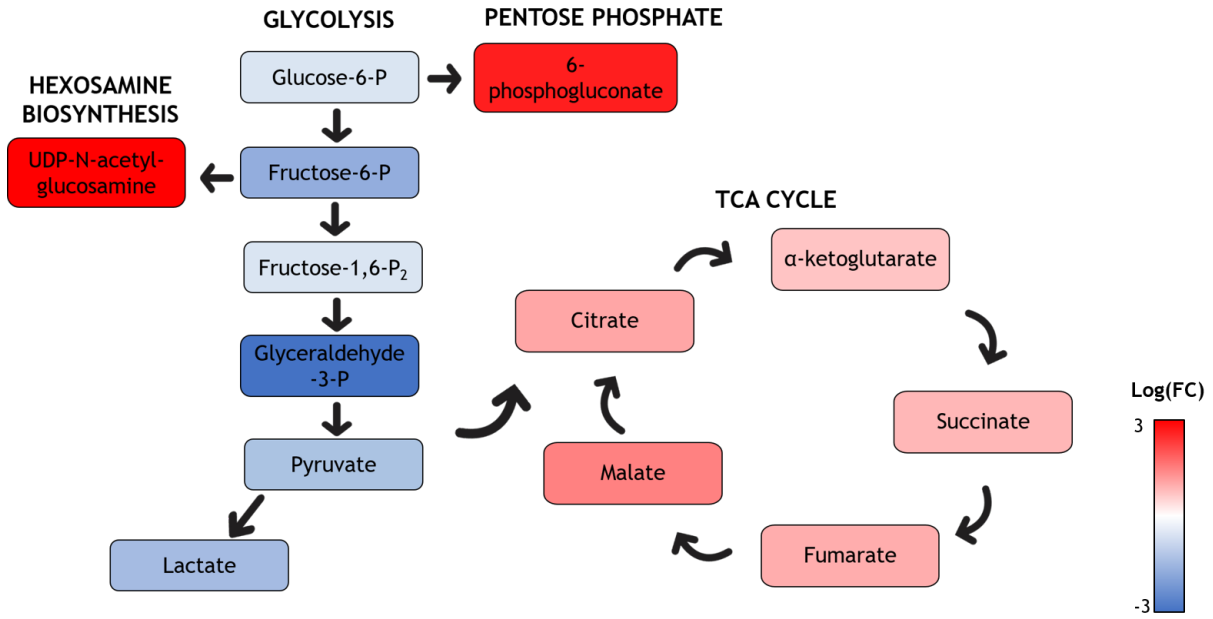

Figure 5.22: Glycolysis metabolites are reduced in cells without SOX2. Intracellular metabolite analysis reveals metabolites in glycolysis are lower in $SOX2^{KO}$ cells, and metabolites in the TCA cycle and alternative pathways out of glycolysis are higher.

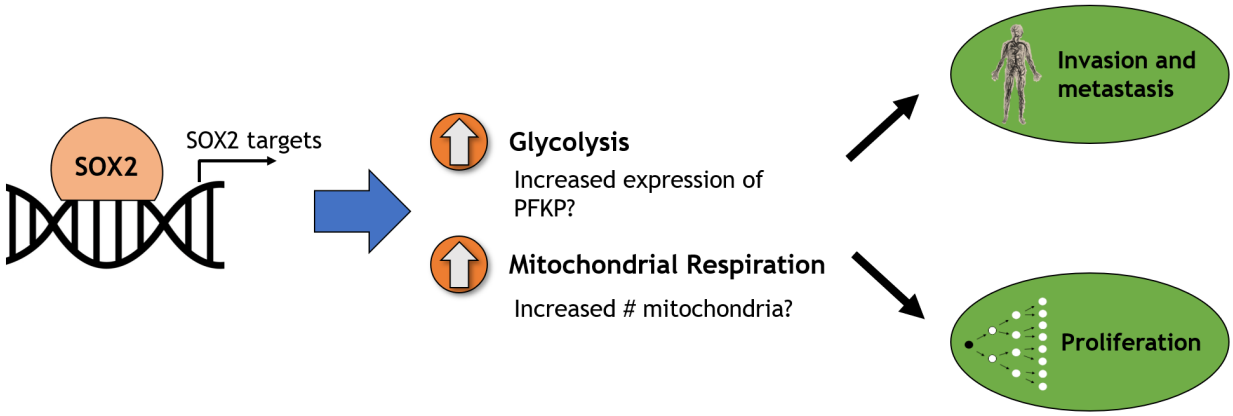

Figure 5.23: Proposed model for the role of SOX2 in metabolism. SOX2 is able to increase both glycolysis and mitochondrial respiration, leading to invasion, metastasis, and proliferation. The mechanisms by which SOX2 is increasing glycolysis and mitochondrial respiration are unclear, but may be due to increased expression of PFKP, a key regulatory enzyme in glycolysis, and an increase in the number of mitochondria.

5.3 Discussion

In this chapter we examined the effect of SOX2 loss on CRPC. We showed that SOX2 loss led to reduced cell growth over time, but this was not due to decreased cell cycle progression or through increased apoptosis. Examination of pathways that were altered upon SOX2 loss reveals pathways involved with various kinds of metabolism, and clinical datasets also indicated modulation of metabolic pathways by SOX2. Further examination showed SOX2 regulates both glycolysis and mitochondrial respiration in prostate cancer, and that there was an increase in the mitochondrial mass in SOX2-expressing cells.

Generation of the $SOX2^{KO}$ clones allowed for a better understanding of how SOX2 regulates the phenotype of CRPC cells. Previous reports on SOX2 in cancer indicated a role for SOX2 in many oncogenic features, such as growth, invasion, survival and therapy resistance. In this chapter, we showed many of these phenotypes in CRPC as well. SOX2 expression led to increased growth compared to $SOX2^{KO}$ cells, which could be attributed to either increased cell cycling or increased survival. Perplexingly, we did not see differences between SOX2-expressing and $SOX2^{KO}$ cells in regards to cell cycle distribution or apoptosis. These data suggested that SOX2 does not cause the cells to proliferate and progress through the cell cycle more quickly or allow for increased survival by preventing apoptosis. We investigated alternative mechanisms to explain why there was increased proliferation, and regulation of metabolism was pursued. The importance of a cancer cell's ability to adapt its metabolic program was particularly interesting, and offers an explanation for why there may be increased growth without changes in cell cycle or apoptosis.

Prostate cancer metabolism is still an understudied area of metabolism, and the work here shows a novel role for SOX2 in regulating metabolism. Unlike most other cancers, prostate cancer only undergoes the Warburg effect upon metastasis, where there is an increased dependence on glycolysis for the cancer cell proliferation and survival. As such, it is not surprising that we see the CRPC cells having increased glycolysis. We showed in Chapter 3 that SOX2 is associated with an increased risk of metastasis, and SOX2 can be regulating

the metastatic capability of prostate cancer by increasing glycolytic capacity. We show here that SOX2 binds PFKP, a key regulatory enzyme in glycolysis that catalyses the irreversible conversion of fructose-6-phosphate to fructose 1,6-bisphosphate, and its expression is decreased when SOX2 is knocked out. We also see increased PFKP in patient samples, where metastases have increased expression of PFKP compared to primary tumours, again suggesting a role for PFKP in metastasis. Additionally, there is a correlation between SOX2 and PFKP in patient samples, further suggesting a role for SOX2 in regulating PFKP clinically. This could suggest that SOX2 regulates glycolysis in prostate cancer through upregulation of PFKP, and upon SOX2 loss, cells are unable to perform glycolysis as efficiently, and must revert to alternate metabolic pathways for survival. The increase in glycolytic activity likely aids in providing the cells with the building blocks and energy to become invasive and survive in distant areas of the body.

We also see an increase in mitochondrial respiration in SOX2-expressing cells. This was surprising as the gene set enrichment analysis suggested oxidative phosphorylation was enriched in the $SOX2^{KO}$ cells. One explanation for this could be that genes involved in oxidative phosphorylation are upregulated in the $SOX2^{KO}$ condition, in an attempt to increase this metabolic pathway upon a decrease in glycolysis. Alternatively, there may be changes in the mitochondrial mass that allows for greater capacity for mitochondrial respiration in the SOX2-expressing cells despite a reduction in metabolites involved in oxidative phosphorylation. We see that there is an increase in the number of mitochondria in the SOX2-expressing cells, as measured by mitochondrial DNA content, and this increased amount could be due to either an increase in mitochondrial biogenesis or a decrease in mitochondrial turnover. The PPAR-γ co-activator 1 (PGC-1) family of proteins, which include PGC-1α, PGC-1β, and PRC, regulate numerous pathways including mitochondrial biogenesis, adaptive thermogenesis, fatty acid β-oxidation, and hepatic gluconeogenesis [142]. PGC-1β has been shown to stimulate mitochondrial respiration, and cell lines derived from PGC-1β-deficient mice have an altered expression of key mitochondrial genes associated with energy metabolism [143].

In our dataset, we see that PGC-1β has reduced expression when SOX2 is knocked out, and offers a potential mechanism to explain the differences in number of mitochondria as well as the mitochondrial respiration phenotype. Not much is known about the mechanism of how PGC-1 regulates mitochondrial biogenesis or how it is regulated, but our data suggests a possible connection with SOX2.

Altogether, the data presented here indicate a novel role for SOX2 in regulating prostate cancer metabolism and its impact on growth and invasion (Figure 5.23). Further mechanistic experiments will be needed to determine more precisely the mechanism of how SOX2 regulates both glycolysis and mitochondrial respiration, and pathways such as PFKP and PCG-1β can be prioritised.

CHAPTER 6

SOX2 IN DNA DAMAGE AND CELL CYCLE REGULATION DURING ENZALUTAMIDE TREATMENT

6.1 Introduction

Despite initial success of ADT, patients often become castration-resistant. Several mechanisms of resistance emerge, including AR gene mutations leading to promiscuous ligand binding, splice variations lacking the ligand binding domain, amplification, and AR protein over-expression [23]. This continued dependence on the AR signalling axis led to the development of second-line therapies such as enzalutamide and abiraterone, which block AR signalling and production of androgens, respectively [23].

Enzalutamide is unique to other available antiandrogen treatments in that it also inhibits AR's translocation into the nucleus, preventing DNA binding and co-activator recruitment [144]. Enzalutamide treatment also increased the rate of radiographic progression-free survival, as well as delaying initiation of chemotherapy and overall death [145]. However, patients become resistant to enzalutamide, and one hypothesis is the presence of AR splice variant 7 (AR-V7), an alternatively spliced variant which lacks the ligand binding domain but maintains the transactivating N-terminal domain [146]. Patients with AR-V7 in circulating tumour cells had lower PSA response rates, shorter PSA progression-free survival, clinical or radiographic progression-free survival, and overall survival rates [139].

Hormonal therapy is also given in combination with radiation, and has been shown to be a radiosensitiser [147]. The mechanisms behind this are thought to be due to the influence of AR on cell cycle checkpoint inhibition and DNA damage repair pathway factors, as well as transcriptional changes in DNA repair gene expression [113, 148, 121]. Enzalutamide has also been approved for use as a radiosensitiser, but the mechanisms of how it achieves this are still unknown [147]. Despite initial efficacy, resistance to these second-line ADT therapies inevitably arises, and coupled with the low increase in survival for other therapies, highlight

the need to identify mechanisms of resistance in order to develop novel therapies for these patients. Given that SOX2 is an AR-repressed gene, we hypothesise that there may be a role for SOX2 in mediating enzalutamide resistance upon AR suppression.

6.2 Results

6.2.1 Loss of SOX2 leads to decreased cell cycle progression in AR-signalling blocked conditions

We again performed GSEA in the enzalutamide condition, where we saw the SOX2-expressing control cells were enriched for pathways involved in DNA replication, DNA repair and regulation of the cell cycle and its checkpoints (Figure 6.1). Based on this RNA-sequencing data corresponding with the cell cycle and BrdU phenotype data (Figures 5.5 and 5.6), we set out to examine levels of key cell cycle proteins. When SOX2 expression is lost in enzalutamide conditions, expression of p-RB and total RB decrease. Inhibitors of cell cycle progression, p21 and p27, are increased, while cyclins CDK2, CDK4 and CDK6 are decreased. Interestingly, while total p53 is decreased, phosphorylated p53 is increased in the $SOX2^{KO}$ cells (Figure 6.2, right). This is also reflected in the RNA expression of these proteins (Figure 6.2, left). To identify potential regulators of cell cycle that SOX2 regulates, we combined our SOX2-bound ChIP-seq analysis with DEGs involved with cell cycle regulation. From our ChIP-seq analysis, E2F1 is a SOX2 target gene, binding within 149 base pairs of the transcription start site (Figure 6.3). E2F1's protein expression is also decreased in the $SOX2^{KO}$ cells (Figure 5.5, right), leading to the hypothesis that SOX2 is mediating cell cycle progression through E2F1, and loss of SOX2 leads to a decrease in E2F1 expression and subsequent G1 arrest.

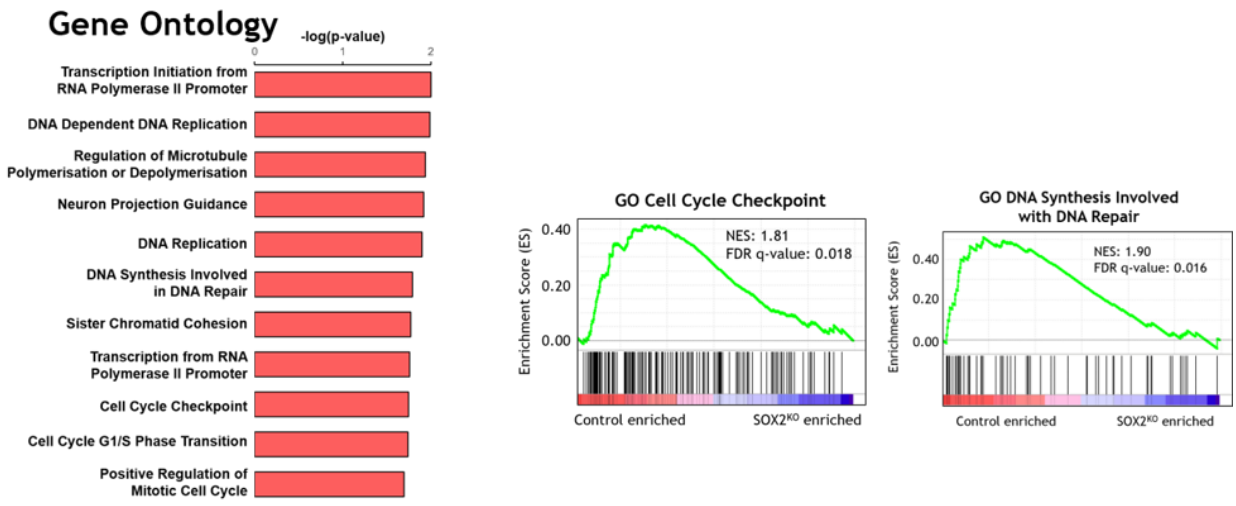

Figure 6.1: Gene set enrichment analysis indicates genes involved with cell cycle and DNA replication and repair are enriched in SOX2-expressing cells when treated with enzalutamide. Top pathways enriched in the SOX2-expressing control cells show pathways involved with DNA replication, cell cycle, and DNA repair during enzalutamide treatment (left). Example plots of pathways indicate these pathways are enriched in the control cells (right).

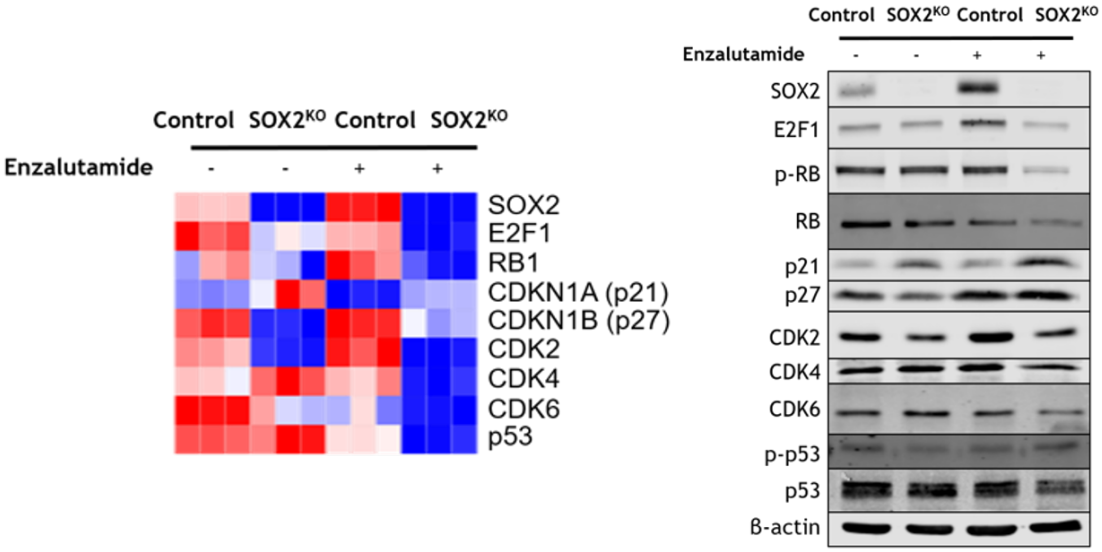

Figure 6.2: Key genes and proteins involved in cell cycle regulation are altered when SOX2 is lost in AR-inhibited conditions. RNA (left) and protein (right) indicate downregulation of several key cell cycle progression genes/proteins, and upregulation of cell cycle arrest proteins.

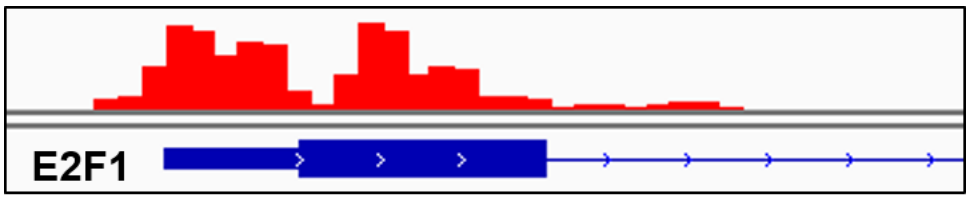

Figure 6.3: SOX2 binds the E2F1 promoter. IGV track of the E2F1 promoter shows peak where SOX2 bind the promoter 149 base pair of the transcription start site.

6.2.2 Loss of SOX2 leads to increased DNA damage in AR-signalling blocked conditions

The second phenotype based on the GSEA results indicated an enrichment of DNA synthesis and DNA repair pathways in the control cells (Figure 6.1), and thus we set out to further elucidate this phenotype in the enzalutamide treated condition. We first examined RNA expression of genes involved in DNA damage signalling, taken from Qiagen's pathway array, and this showed a strong decrease in DNA damage signalling in the $SOX2^{KO}$ cells in the enzalutamide condition, compared to the control cells (Figure 6.4), validating the GSEA data of increased DNA damage in the $SOX2^{KO}$ cells. We next examined key mediators of DNA damage response, such as γ-H2AX, BRCA1, ATR and ATM. As expected from the RNA-sequencing data, there are increased levels of γ-H2AX in the $SOX2^{KO}$ clones compared to the control cells when treated with enzalutamide (Figure 6.5). Interestingly, there are also increased levels of γ-H2AX in the $SOX2^{KO}$ clones compared to the control cells in the vehicle treated condition (Figure 6.5), suggesting there may be DNA breaks when SOX2 expression is lost even with intact AR-signalling. Protein expression of phosphorylated and total BRCA1 is reduced in the $SOX2^{KO}$ clones (Figure 6.5), suggesting that a loss of SOX2 leads to decreased response to DNA damage signalling. Immunofluorescence imaging shows that γ-H2AX is localised in the nucleus of the $SOX2^{KO}$ cells, and there is a significantly higher number of foci in the enzalutamide treated cells than the vehicle treated cells (Figure 6.6). This further supports a role for SOX2 in preventing DNA breaks. Since loss of SOX2 led to a decreased level of BRCA1, one hypothesis is that $SOX2^{KO}$ cells would be more sensitive

to radiation, as these cells would be unable to start the DNA damage signalling cascade. Clinically, the implication of this could be that patients with SOX2-positive tumours may be less sensitive to radiation therapy, and may not benefit from this treatment.

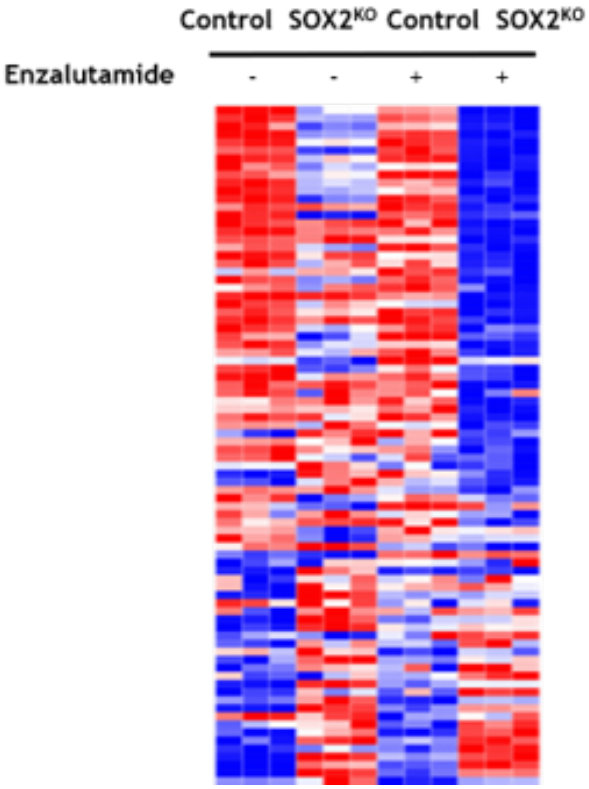

Figure 6.4: Loss of SOX2 during enzalutamide treatment leads to decreased DNA damage signalling. Genes taken from Qiagen's DNA damage signalling gene array was overlaid with our RNA-sequencing data.

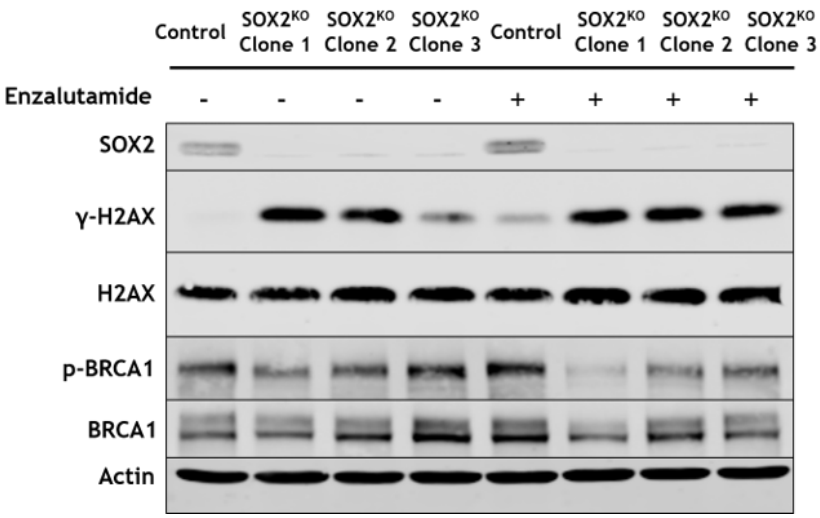

Figure 6.5: Loss of SOX2 leads to increased DNA damage and decreased expression of DNA repair protein BRCA1. Western blot analysis of γ-H2AX shows when SOX2 is knocked out, there is increased expression of γ-H2AX in all clones during both vehicle and enzalutamide treatment. Further, DNA damage repair protein, BRCA1, was downregulated when SOX2 expression was lost, and its phosphorylation decreased, indicating reduced activity.

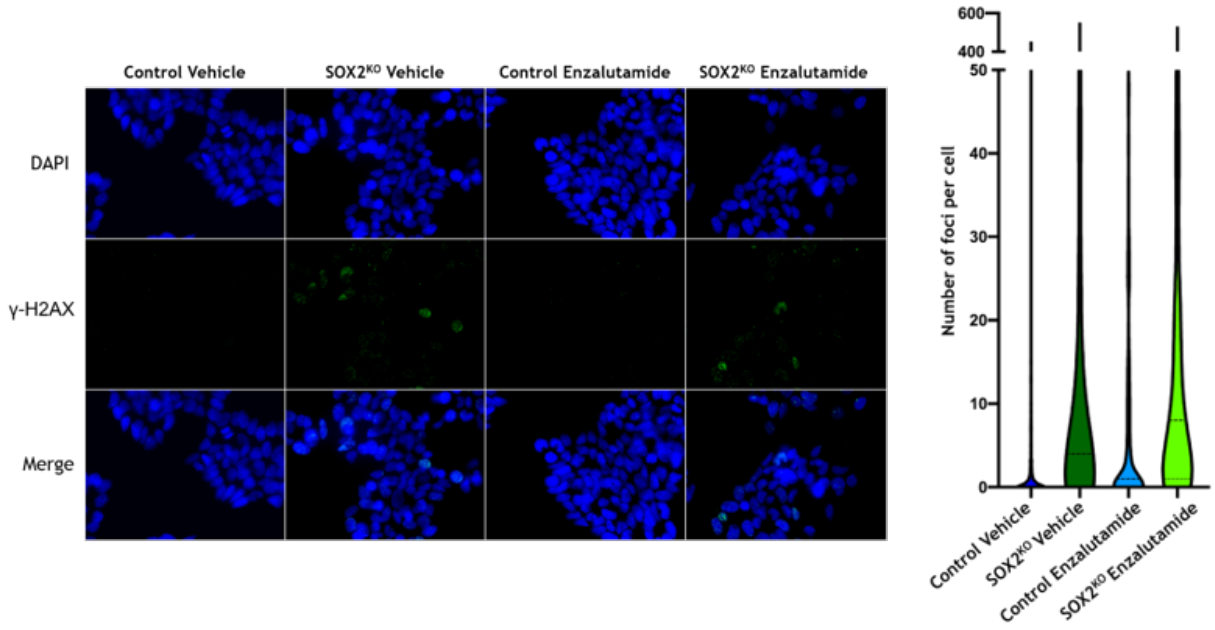

Figure 6.6: Loss of SOX2 leads to increased number of γ-H2AX foci in the nuclei of the cells. Immunofluorescence staining for γ-H2AX shows increased number of foci in the nuclei of $SOX2^{KO}$ cells in the vehicle and enzalutamide treated condition.

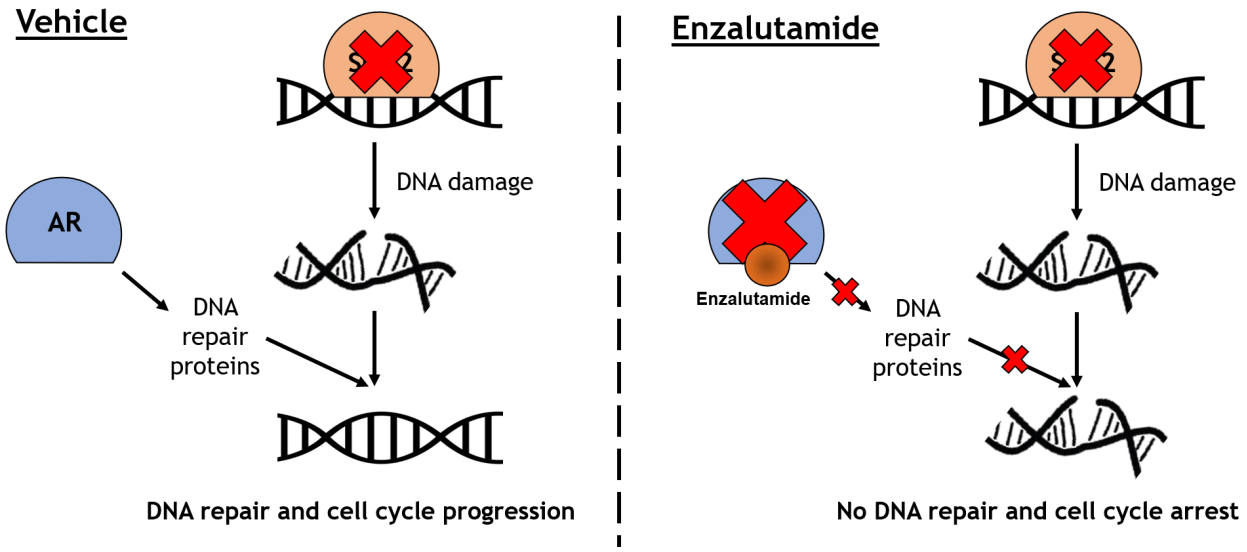

Figure 6.7: Proposed model for the role of SOX2 in DNA damage response and cell cycle progression. Loss of SOX2 causes DNA damage, but under vehicle conditions when AR signalling is intact, AR is able to induce proteins from the DNA damage response and repair DNA, allowing for cell cycle progression (left). When AR signalling is inhibited upon enzalutamide treatment, DNA damage caused by loss of SOX2 cannot be repaired, as AR is unable to induce DNA repair proteins, leading to cell cycle arrest and decreased growth.

6.3 Discussion

In this chapter we examined the role of SOX2 in CRPC in the context of AR-signalling inhibition. We identified pathways involved with cell cycle, DNA damage, and DNA repair as being enriched in SOX2-expressing cells. Subsequent analyses supported this initial data, where $SOX2^{KO}$ cells treated with enzalutamide displayed G1 cell cycle arrest, activation of proteins involved with G1 arrest, and increased DNA damage.

Radiation therapy is used in many different stages of prostate cancer: as a first treatment for low-grade localised disease, in combination with hormone therapy for cancers that have spread locally, and in advanced metastatic cases especially in the palliative setting [149]. Multiple clinical trials examining the combination therapy versus radiotherapy alone has shown to increase disease-free and overall survival in high-risk and intermediate risk patients [150, 151]. Studies examining the mechanisms that allow for increased radiosensitivity have suggested that AR inhibition, especially with second-generation antiandrogen therapies, such

as enzalutamide, lead to a downregulation of DNA repair genes. Further, these repair genes are induced by androgen and represent direct AR targets. Antiandrogen treatments cause increased DNA damage and decreased clonogenic survival [121]. Interestingly, in our CRPC cell line, we only see a small increase in DNA damage, as seen through γ-H2AX expression, when the cells are treated with enzalutamide, and this is much greater in the cells without SOX2. This suggests that while enzalutamide treatment causes some DNA damage, as expected from previous research [121, 152], SOX2 may be able to mitigate some of the damage, and aid in repair of the DNA. As of yet, SOX2 has not been well studied in the context of DNA repair, especially in cancer. However, one may speculate that SOX2 activates similar DNA repair pathways as AR, and can substitute for AR upon AR-signalling inhibition with enzalutamide. It is only when both AR signalling and SOX2 signalling are abrogated, that there is a large amount of DNA damage, as cells are unable to use either mechanism to repair DNA (Figure 6.7). Since we see signs of DNA damage (γ-H2AX) in the SOX2$^{\mathrm{KO}}$ cells in vehicle condition, this could suggest a role for SOX2 in DNA replication, where loss of SOX2 leads to DNA damage. One potential pathway could be due to deficiencies in nucleotides that lead to replication fork collapse. In this context, AR could repair the DNA caused by SOX2 deficiency, but when cells are treated with enzalutamide, there is no repair mechanism available for the damaged DNA. This can cause a G1 arrest as cells are unable to continue replication and need to repair DNA before continuing in the cell cycle (Figure 6.7). Further examination of the exact role of SOX2 in DNA replication and repair is needed, especially in combination with enzalutamide treatment.

CHAPTER 7

SIGNIFICANCE AND FUTURE DIRECTIONS

7.1 Significance

While the survival rate is close to 100% for men who are diagnosed with local disease, the survival rate for advanced stage patients is only about 28% [19]. Approximately 10-20% of men with prostate cancer present with metastatic disease, and many others progress to metastasis after hormone treatment, surgery or radiotherapy [20]. As such, understanding why patients metastasise or become resistant to treatments, as well as identifying biomarkers that will predict response to therapy or propensity for more aggressive disease, is crucial to increasing survival of patients. Our group has previously identified SOX2 as one of the most highly and differentially expressed genes between the prostate and seminal vesicle, an organ with a similar function and dependency on AR-signalling as the prostate, but which is rarely is the site of tumourigenesis [84]. Given these clinical characteristics, we examined the role of SOX2 in prostate cancer, both from a clinical and mechanistic perspective, with the goal of identifying additional clinical outcomes and pathways that lead to prostate cancer growth and treatment resistance.

7.1.1 SOX2 reprograms metabolism in prostate cancer

The work in this dissertation demonstrates a clear role for SOX2 in prostate cancer metabolism. Metabolism in pluripotent stem cells (PSCs), which include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), has been well examined and one hallmark is that PSCs rely heavily on glycolysis compared to differentiated cells. Glycolytic proteins are upregulated and lactate production is higher in PSCs [153, 154], and reprogramming of somatic cells to iPSCs leads to increased lactate production and a decline in oxygen consumption [155]. These data show the importance of high glycolysis levels in maintenance of pluripotency, but the mechanism of how PSCs acquire and maintain high glycolysis levels

is still being examined. One group showed that OCT4 bound several glycolytic genes, such as HK2 and PKM2, and depletion of OCT4 led to a decrease in lactate production [156]. While this suggests that other core factors in pluripotency, such as SOX2 and NANOG, may also regulate glycolysis, this was not examined directly. Furthermore, given that OCT4 and NANOG are not expressed with SOX2 in prostate cancer and our data indicates a non-canonical stem cell role for SOX2 in prostate cancer, additional studies will be required to assess the role of SOX2 in prostate cancer metabolism.

Although not well examined in cancer, SOX2 has been implicated in metabolism in some types of cancers, mostly in terms of glycolysis or oxidative phosphorylation. In squamous cell carcinomas, SOX2 acts with p63 to transactivate the intronic enhancer cluster of SLC2A1, one of the key glucose transporter genes, leading to elevated glucose influx. This glucose influx leads to generation of NADPH from the pentose phosphate pathway (PPP), and provides an anti-oxidative capacity for increased survival and proliferation [100]. Systemic glucose restriction through diet or an inhibitor for SGLT2, led to a decrease in glucose absorption by the kidneys and decreased tumour growth [100]. Knock-down of SOX2 in gastric cancer cells reduced expression of key glycolytic proteins, HK2, GLUT1 and LDHA, and a decrease in glucose uptake and lactate production, suggesting in gastric cells SOX2 also mediates glycolysis [101]. Interestingly, in melanoma, SOX2 has been implicated in modulation of cell metabolism to favour an oxidative phenotype, and knock-down of SOX2 in melanoma cells led to an increase in lactate production and glycolytic genes, further supporting a more oxidative phenotype in melanoma cells with SOX2 expression [102]. The lack of studies examining SOX2 in metabolism show a clear gap in our understanding of SOX2 in cancer, and a complete absence of SOX2's role in prostate cancer metabolism. We show here for the first time that SOX2 has a role in metabolism of prostate cancer cells, by increasing both glycolysis and mitochondrial respiration. However, the exact mechanism of how SOX2 regulates these metabolic pathways is unknown.

A potential mechanism of this phenotype could be linked to the expression of PFKP,

the platelet (P) isoform of phosphofructokinase 1 (PFK1), which catalyses the irreversible conversion of fructose 6-phosphate to fructose 1,6-bisphosphate, and is a key regulatory enzyme in glycolysis [157]. PFKL is the most abundant isoform of PFK1 in the liver and kidneys, whereas PFKM and PFKP are the only forms present in adult muscles and platelets, respectively. Some tissues, such as the brain, have all three isoforms present [158]. PFKP is the predominant isoform of PFK1 in many cancers, and its expression is increased in cancers such as breast cancer, clear cell renal cell carcinoma (ccRCC), lung cancer, and glioblastoma [158, 159, 160, 161]. Suppression of PFKP in ccRCC and oral squamous cell carcinoma (OSCC) also led to decreased tumour growth in vivo [162, 163] and inhibited starvation-mediated glycolysis and autophagy in OSCC cells [162]. Interestingly, in breast cancer, PFKP expression is shown to be increased by KLF4, one of the four Yamanaka factors that SOX2 is also a part of, and may suggest a potential role for these reprogramming factors in metabolism of cancer cells [156, 160]. KLF4 binds directly to the promoter of PFKP, and activates its expression leading to increased glycolysis. PFKP has also been associated with metastasis in many cancers, and participates in metastasis by tightly regulating lactate production, which can stimulate cancer cell migration and metastasis [164, 165]. PFKP is the sole PFK1 isoform that marks metastasis in breast cancer, as PDKL and PFKM expression is unaffected by KLF4, due to a lack of a KLF4 binding site in their promoter [160]. Despite work in other cancers, PFKP and its role as a driver of glycolysis has not been examined in prostate cancer, although it has been found that protein kinase C epsilon (PKCϵ) enhances SMAD2/3-mediated glycolysis, which in turn activates PFKP expression in prostate cancer cells [166]. Examining more closely the role of PFKP in mediating glycolysis in prostate cancer, as well as understanding more clearly how it is regulated is crucial for identification of targets to prevents its activation and offer potential therapeutics to patients with elevated glycolysis in their tumours. Here we suggest a novel mechanism by which SOX2 binds PFKP in prostate cancer and alters its expression, leading to increased glycolysis. Since prostate cancer metastases have increased glycolysis, it is conceivable that metastases in

prostate cancer also have increased expression of PFKP. Indeed, we showed that there is increased expression of PFKP in metastases compared to primary tumour. Altogether, the data presented here suggests a model where patients with SOX2-positive tumours can activate PFKP expression, leading to an increase in glycolysis and allowing for metastasis to occur.

Metabolomics analysis indicates metabolites in the glycolysis pathway are decreased in the SOX2 knockout cells, further supporting that SOX2 leads to a more glycolytic and metastatic phenotype. Interestingly, metabolites involved with the TCA cycle are increased in the SOX2 knockout cells, which is unexpected since we observed that SOX2 expression also leads to increased mitochondrial respiration. A possible explanation is that SOX2 expressing cells have decreased TCA cycle, but since there are more mitochondria, the overall activity is increased. This increase in mitochondrial activity may be due to an increase in the number of mitochondria through increased mitochondrial biogenesis or decreased mitochondrial turnover. One group found that there is an increase in the mitochondrial content from normal prostate epithelial cells to cancer cells, and a further increase was observed with increasing tumour grade and stage, suggesting higher numbers of mitochondria may be necessary for prostate cancer development and progression [167]. Furthermore, a "mitochondrion-rich phenotype", where there are elevated levels of mitochondria, is strongly linked to a poor prognosis [167]. This increase in the number of mitochondria has also been observed in other cancers, for example where oncocytic, mitochondrion-rich modifications were found in breast adenocarcinoma following chemoradiotherapy [168], and offers a potential mechanism to tie together our observed metabolic phenotype with the DNA damage and drug resistant phenotype. The direct link between SOX2 expression and mitochondrial mass has not been well studied, although several studies link increased differentiation (and a decrease in SOX2 expression) with an increase in mitochondrial content [169, 170, 171]. Our work indicates that SOX2 is acting in a non-canonical stem cell manner in prostate cancer, and this fits with the model that increased mitochondrial mass is associated with a less stem-cell like

phenotype. The mechanism by which SOX2 regulates mitochondrial mass is unknown, and will need to be further examined in prostate cancer to determine whether SOX2 acts to increase mitochondrial biogenesis or decrease mitophagy. One avenue to explore is PGC-1β, as it has been shown to stimulate mitochondrial respiration, and cell lines derived from PGC-1β-deficient mice have an altered expression of key mitochondrial genes associated with energy metabolism [143]. In our dataset, we see that PGC-1β has reduced expression when SOX2 is knocked out, and offers a potential mechanism to explain the differences in number of mitochondria as well as the mitochondrial respiration phenotype. Understanding the role of SOX2 in regulating mitochondrial mass offers the potential for therapeutics that can be used in patients with SOX2-positive tumours to reduce tumour burden and metastasis.

7.1.2 Loss of SOX2 leads to DNA damage and cell cycle arrest when treated with enzalutamide

Both embryonic and adult stem cells are adept at preventing the accumulation of genetic lesions, and initiating pathways to repair them in order to prevent propagation to daughter cells. Induced pluripotent stem cells also have a robust DNA damage response, and cancer stem cells are very resistant to DNA damage and do not undergo senescence or cell death upon accumulation of damage to DNA [172]. The mechanisms by which stem cells and tumour initiating cells (TICs) respond to and prevent DNA damage has been well examined, although the exact role of SOX2 in this process has not been elucidated.

The cell cycle in embryonic stem cells (hESCs) is shortened compared to somatic cells, largely due to an abbreviated G1 phase and facilitated G1/S transition by upregulation of CDK4 and cyclin D2 [173]. This rapid cycling exposes ESCs to an increased risk of replicative errors, which lead to double strand breaks (DSBs). Following DNA damage, cells elicit a response that initiates cell cycle arrest, DNA repair, and apoptosis if the damage is too great to be repaired, through activation of ATM-dependent checkpoint signalling, and arrest in the G2/M stage of the cell cycle after irradiation [174]. Interestingly, one

group showed that reprogramming somatic cells to iPS cells led to an altered DNA damage response compared to the parental cells, causing a loss of the G1/S checkpoint, and leading to greater radiosensitivity [175]. In embryonic stem cells, a DNA repair complex consisting of XPC-RAD23B-CETN2, the nucleotide excision repair (NER) complex, is a co-activator with SOX2 and OCT4 to activate expression of NANOG. This complex is also essential for maintaining genome fidelity in stem cells to prevent error propagation to progenitor cells [176].

Tumour initiating cells often have increased expression of SOX2, and tumour cells with a TIC phenotype are resistant to radiation therapy in breast cancer, glioma, and medulloblastoma [177], and in lung cancer cell lines, TIC subpopulations were also more viable and resistant to radiation therapy compared to the bulk cells [178]. It has been suggested stem cells are more resistant to radiation due to a more quiescent and less proliferative state, and that TICs would behave similarly to become resistant to radiation. However, it did not appear as if the TICs proliferated much differently from the bulk lung cancer cells, although they showed continued proliferation and no cell cycle arrest following radiation [178]. Glioma TIC populations showed increased activation of several DNA repair checkpoint proteins, such as ATM, Rad17, Chk1 and Chek2, suggesting these TICs could be primed to respond to DNA damage [116]. All in all, there is much evidence for a strong DNA damage response in cells with a self-renewal phenotype.

Despite the research indicating a role for SOX2 in DNA repair in TICs, our work indicates that SOX2 does not act in a canonical stem cell manner in prostate cancer, suggesting that SOX2 may not necessarily be regulating DNA repair in the same manner as in stem cells and TICs. We show that loss of SOX2 led to DNA damage, as observed by the accumulation of γ-H2AX, as well as a reduction in the expression of some DNA damage response genes, such as BRCA1. We also show that cells without SOX2 that are treated with enzalutamide have an arrest at the G1/S checkpoint. This arrest is different than the DNA damage response in SCs, which tends to cause a G2/M arrest. This suggests potentially a different cause of

damage to the DNA, which could activate a different mechanism of response to DNA damage and cause an arrest at different checkpoints. The G1 arrest tends to be p53-dependent [179], and is also activated by ATM [180]. Studies have shown that a G1 arrest is associated with increased radiosensitivity [181], and this could suggest that the $SOX2^{KO}$ cells would be more sensitive to radiation due to the G1 arrest.

AR signalling has been shown to play an important role in DNA damage response in prostate cancer. AR inhibition, especially with second-generation antiandrogen therapies, such as enzalutamide, leads to a downregulation of DNA repair genes and causes increased DNA damage and decreased clonogenic survival. Furthermore, these repair genes are induced by androgen and represent direct AR targets [121]. It is thought that this is why combining ADT and radiation therapy shows such great efficacy. In our CRPC cell line, we only see a small increase in DNA damage, as seen through γ-H2AX expression, when the cells are treated with enzalutamide, and this is much greater in the cells without SOX2. This suggests that enzalutamide treatment causes some DNA damage, as expected from previous research [121, 152], but SOX2 may be able to mitigate some of the damage, and aid in repair of the DNA. As of yet, SOX2 has not been well studied in the context of DNA repair, especially in cancer. However, one may speculate that SOX2 activates similar DNA repair pathways as AR, and can substitute for AR upon AR-signalling inhibition with enzalutamide. It is only when both AR signalling and SOX2 signalling are abrogated, that there is a large amount of DNA damage, as cells are unable to use either mechanism to repair DNA. Alternatively, SOX2 could be implicated in another facet of DNA replication and repair. We observed DNA damage (γ-H2AX) in the $SOX2^{KO}$ cells in vehicle condition, which could suggest a role for SOX2 in DNA replication, as loss of SOX2 leads to DNA damage. This could be due to several factors, such as deficiencies in nucleotides that lead to replication fork collapse. Replication fork collapse would cause replication perturbation, DNA damage, and genome instability [182], and one group showed that activation of the nucleotide biosynthesis pathways led to an increase in the nucleotide pool, which rescued the replication-induced

instability [182]. In this scenario, AR could repair the DNA caused by SOX2 deficiency, but when cells are treated with enzalutamide, and AR signalling is inhibited, there is no repair mechanism available for the damaged DNA. This can cause a G1 arrest as cells are unable to continue replication and need to repair DNA before continuing in the cell cycle. A better understanding of how SOX2 regulates replication pathways will shed light on how DNA damage is being caused, and how SOX2 leads to its response and repair, especially in combination with AR inhibitors.

7.1.3 SOX2 has potential as a biomarker for metastasis in prostate cancer

Approximately 10-20% of men with prostate cancer present with metastatic disease, and many others progress to metastasis after hormone treatment, surgery or radiotherapy [20]. As such, understanding why patients metastasise or become resistant to treatments, as well as identifying biomarkers that will predict response to therapy or propensity for more aggressive disease, is crucial to increasing survival of patients. Biochemical recurrence (BCR) occurs in approximately 8-20% of patients within 10 years of a radical prostatectomy (RP), but only leads to rapid and aggressive disease in some patients, while in others remains indolent [183, 184, 185]. Recent studies have also found that patient survival rates for patients with and without BCR 10 years after a RP are very similar [186, 187]. Only a few retrospective analyses have evaluated potential predictors of metastasis progression (MP) and prostate cancer specific mortality (PCSM) after a RP and BCR [188, 189, 184], but the ability of the RP to BCR time to predict metastasis has been controversial. Later studies have not been able to show an association between RP-BCR and clinical endpoints such as clinical progression and PCSM [188, 190]. Furthermore, only a small subset of BCR patients had metastatic progression (13.4%) and prostate cancer specific mortality (9.5%) [191]. Patients that experiences MP had other clinical factors that were far more predictive of metastasis, such as high rates of lymph node metastasis, extra-prostatic extension, a higher Gleason Score (GS), and shorter PSA-doubling time (PSA-DT) [191]. Since time to BCR after a RP

is not a good clinical indicator of those patients that will metastasise, additional biomarkers are needed to help inform clinical decisions.

Here, we identified SOX2 as a predictor for increased risk of metastasis, and show that patients with SOX2-positive tumours who undergo BCR metastasise more quickly and have shorter time to PCSM. As such, SOX2 has the potential to become a biomarker for predicting which patients who have BCR will metastasise, and allow physicians to alter clinical decisions. Patients with a SOX2-positive primary tumour might opt to undergo a radical prostatectomy despite a lower Gleason Score (GS), considering the tumour is at a higher risk for metastasis. A patient with a SOX2-positive tumour may also undergo more frequent screening to detect metastasis sooner. Interestingly, we have previously shown that the percentage of SOX2-positive tumours increase with GS [65], and since these clinical studies show a higher GS was associated with an increased MP, this could be further evidence that SOX2 expression can cause metastasis. PFKP, a SOX2 target that is involved with glycolysis, could also act as a biomarker for prediction of metastases, since PFKP is associated with metastasis [164, 165], and there is a higher rate of glycolysis in prostate cancer metastases [99]. Therapies targeting metabolic pathways also have utility with SOX2 as a biomarker.

Patients who are diagnosed with prostate cancer have several treatment options, of which a RP and radiation are two common treatments. A RP, while effective at reducing mortality, has several side effects, including destruction of nerves, blood vessels, and muscular tissues, which has been shown to cause urinary incontinence and erectile dysfunction, among other sexually related side effects [192, 193, 194]. Due to the potential severity of side effects of removing the prostate, some patients may opt to irradiate the prostate, particularly when combined with androgen-deprivation therapy (ADT) [195, 196]. Both treatment options have a similar cancer-specific survival rate, as well as risks of systemic progression and prostate cancer death [188]. Our findings that SOX2-positive cells show increased an DNA damage response have implications for patients who use radiation therapy as a treatment option: patients with SOX2-positive tumours may not respond as well to radiation therapy, and

should opt for other treatments, such as a RP or chemotherapeutics. Additionally, since we have previously shown that SOX2 is an AR-repressed gene, if patients are given neoadjuvant ADT, their levels of SOX2 in the tumour may actually increase, and make their tumour more resistant to radiotherapy, and alternatives to radiotherapy should be selected. As such, knowing before treatment whether a patient's tumour is SOX2-positive may dictate which therapies are given, as well as the order and combinations of these therapies. Once the mechanisms of how SOX2 regulates the DNA damage response is understood more fully, there is the opportunity to treat SOX2-positive cells with therapies against this pathway, which can sensitise the cells to radiation therapy. This may be particularly important for patients who have a RP and have radiation therapy to the surgical margins to completely eliminate SOX2-positive cells that are at a higher risk of metastasis.

In this dissertation, we have identified a role for SOX2 in prostate cancer metastasis and therapy resistance, both from a mechanistic and clinical overview. Further examination of the exact mechanisms by which SOX2 regulates metabolism and DNA damage response will be crucial in identifying potential novel therapies, and aid in a better understanding of how SOX2 can be used clinically as a biomarker for predicting patient response to therapy and clinical outcome.

7.2 Future Directions

The data shown in this dissertation characterises more fully the role of SOX2 in prostate cancer, and its phenotype in prostate cancer growth, invasion, and enzalutamide resistance. Potential mechanisms for driving these phenotypes are alterations in metabolism, cell cycle, and DNA damage response. However, these pathways need to be more fully elucidated if SOX2 is to be used as a biomarker for patient response and treatments.

We demonstrated that SOX2 alters metabolism of prostate cancer cells, and potentially reprograms their metabolic phenotype, but the exact mechanism by which SOX2 does this is still unclear. We showed here that SOX2 expression in prostate cancer cells leads to increased

glycolysis and mitochondrial respiration, and a potential mechanism of this phenotype could be linked to the expression of PFKP, the platelet (P) isoform of phosphofructokinase 1 (PFK1), which catalyses the irreversible conversion of fructose 6-phosphate to fructose 1,6-bisphosphate, and is a key regulatory enzyme in glycolysis [157]. To determine whether PFKP regulation is the mechanism by which SOX2 increases glycolysis and mitochondrial respiration, PFKP expression can be modulated with either knock-down or overexpression and metabolic activity can be tested with the Seahorse assays. Inhibitors for PFKP activity, such as acetylsalicylic acid, 2,5-anhydro-D-glucitol-1,6-diphosphate, or vinblastine, can also be utilised to examine the effect on glycolysis [197]. Our current model suggests that SOX2 regulates PFKP expression to lead to increased metabolic activity in prostate cancer cells, and the experiments above would indicate whether PFKP alteration directly shows changes in glycolysis and downstream mitochondrial activity.

To more clearly delineate intracellular fluxes in metabolism when SOX2 expression is altered, stable isotope tracing can be performed. 13C metabolic flux analysis (13C-MFA) has emerged as the predominant technique for quantifying intracellular fluxes in cancer cells. Due to the redundancy of mammalian metabolic pathways, external rates of metabolites, such as glucose and glutamine uptake and lactate secretion, are not sufficient to draw detailed conclusions about the contribution of each metabolic pathway to the overall metabolism [198]. To examine intracellular fluxes, stable isotopes such as 13C are utilised to label substrates, e.g. [1,2-13C]glucose. Metabolism of these labelled substrates leads to specific labelling patterns in downstream metabolites due to the enzymatic reactions, and can be detected using mass spectrometry of nuclear magnetic resonance [198]. For a well-chosen tracer, different metabolic pathways will produce different labelling patterns, and will reveal the metabolic flux [199]. Use of carbon tracing in our prostate cancer cell system will reveal differences in glycolytic metabolism between SOX2 expressing cells, for example if PFKP is regulating glycolytic activity there may be reduced fructose 1,6-bisphosphate produced when SOX2 is lost, as well as potentially reveal alternative metabolic pathways that are

active when glycolysis is decreased in cells without SOX2 expression.

We have also shown that SOX2-expressing cells have an increased number of mitochondria, offering an explanation for the increase in oxidative phosphorylation in addition to increased glycolysis. Mitochondrial biogenesis has been observed to drive tumour cell proliferation in cancer [200], and SOX2 expression may be causing an increase in the number of mitochondria and allowing for increased proliferation in prostate cancer. Another potential explanation is that loss of SOX2 leads to increased rate of mitochondria turnover, and a decrease in mitochondrial activity and metabolism. One avenue to explore is PGC-1β, as its expression has been shown to stimulate mitochondrial respiration, and cell lines derived from PGC-1β-deficient mice have an altered expression of key mitochondrial genes associated with energy metabolism [143]. In our dataset, we see that PGC-1β has reduced expression when SOX2 is knocked out, and offers a potential mechanism to explain the differences in number of mitochondria as well as the mitochondrial respiration phenotype. Additional analysis of the sequencing dataset with the goal of identifying genes involved with mitochondrial biogenesis can also be performed to identify other potential targets.

Another area that will require more extensive analysis is the role of SOX2 in enzalutamide treated conditions, where loss of SOX2 leads to cell cycle arrest and decreased DNA damage signalling. To understand how SOX2 regulates the DNA damage and repair pathways, we will examine expression of ATM and ATR, both well-known mediators of the DNA damage response pathway. Further experiments to examine protein expression of ATM and ATR will be crucial in narrowing down the type of DNA damage, such as the presence of double stranded breaks (DSBs) or exposure of single stranded DNA (ssDNA), and indicate the mechanism causing DNA damage e.g. replication fork collapse due to nucleotide deficiency. Further, given that a loss of SOX2 leads to a decrease in DNA repair pathways, cells without SOX2 may be more sensitive to therapies that cause DNA damage, such as radiation. This can be experimentally examined through treatment of either direct irradiation or treatment with radiomimetic drugs, such as bleomycin, and subsequent analysis of cell viability and

growth. Clinically, this can also be further tested in patient tumour databases that have data for gene expression and clinical outcome, where SOX2 expression can be correlated with a decreased response to radiation. Our hypothesis is that cells without SOX2 expression may be more sensitive to radiation compared to cells with SOX2, and further understanding of how SOX2 modulates DNA damage response and DNA repair will offer the opportunity for novel targets that could selectively lead to DNA damage in SOX2-positive cells when treated concurrently with radiation.

The androgen receptor (AR) has been well characterised in regulating cell cycle progression and DNA repair [113, 148, 121], and there may be an interplay between SOX2 and AR in allowing for cell cycle progression and DNA repair to continue when either factor is absent; it is only when both factors are missing, either by SOX2 deletion or AR inhibition with enzalutamide, that cell cycle is not able to progress and the DNA damage is not able to be repaired. Teasing out more carefully the role of SOX2 and AR separately in DNA damage response and cell cycle regulation will be key in understanding the role of SOX2 in DNA damage repair and cell cycle progression. E2F1, a key regulator of G1 to S phase, is a SOX2 target gene that only has decreased expression cells are treated with enzalutamide, and offers a potential mechanism of how SOX2 regulates cell cycle progression in the absence of AR-signalling. This mechanism can be tested further by over-expressing E2F1 in the SOX2-knockout cells, and examining the effect on cell cycle progression, through both cell cycle and BrdU incorporation assays. One shortcoming of this work in the enzalutamide treatment condition is that SOX2-bound genes from the vehicle condition were used to identify key target genes that SOX2 regulates in the context of enzalutamide treatment. Given that AR is a key transcription factor in the prostate and prostate cancer, it is possible that the regulation of the transcriptional landscape by SOX2 will be altered when AR is prevented from binding the DNA with enzalutamide treatment, perhaps even binding genes that AR would bind. As such, examining which genes SOX2 binds in the context of AR inhibition will be important to identify additional genes that SOX2 regulates in this signalling context,

and may further clarify the mechanism by which SOX2 regulates cell cycle progression and DNA repair.

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