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The Role of SOX4 in Bladder Cancer Cell Lines

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An abstract of
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Abstract

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By Josue D. Moran M.S.

In 2018, 81,190 patients will be diagnosed with bladder cancer and 17,240 will die of the disease. As many as 23% of Bladder cancer patients harbor focal amplification of chromosome 6p.22, which contains several genes included the gene encoding SOX4. SOX4 is a developmental transcription factor that is overex- pressed in a variety of tumors including lymphomas, breast, and prostate cancers but is most highly altered in bladder cancer. Despite the high frequency of alterations, no clear consensus exists regarding SOX4's role in bladder cancer. In order to determine the mechanisms by which SOX4 drives tumorigenesis, we have investigated SOX4 in two separate contexts. First, by way of determining novel SOX4 protein-protein interactions and second, determining high confidence SOX4 target genes as po- tential mechanisms to drive different hallmarks of bladder cancer. Our lab's co-immunoprecipitation data demonstrate novel endogenous protein-protein interactions between CDKN2A, a tumor suppressor, and SOX4. We hypothesize SOX4 interacts with CDKN2A to promote cell cycle progression and tumorigenesis. Moreover, our knockdown of SOX4 using CRISPR interference (CRISPRi) highlights a number of high-confidence SOX4 regulated genes. We have specifically identified, a novel mechanism whereby SOX4 elicits tumor promoting ability by way of inhibiting, directly or indirectly, the tumor-suppressive arm of Wnt5a. Wnt5a is highly expressed in SOX4 knockdown cells and is positively correlated with decreased in- vasion. Restoring SOX4 levels drives down Wnt5a expression and concomitantly increases the invasiveness of T24 bladder cancer cell lines. In summary, our re- search suggests that SOX4 could promote various aspects of tumorigenicity via two distinct but not mutually exclusive pathways. The long term goal of this re- search could implicate SOX4 or it's putative target genes as potential druggable targets or biomarkers for novel therapeutic approaches for bladder cancer patients.

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List of Abbreviations

BLCA Bladder cancer. 2, 5, 31

CIS Carcinoma in situ. 5

FGFR3 Fibroblast Growth Factor Receptor 3. 7

MIBC Muscle Invasive Bladder Cancer. 5, 7, 12

NMIBC Non-Muscle Invasive Bladder Cancer. 5, 7, 12

TCGA The Cancer Genome Atlas. 7

TNM Tumor-Node-Metastasis. 2, 5

Chapter 1 Introduction

1.1 Bladder Cancer

1.1.1 Overview

The bladder, and relevant genitourinary systems, represent a unique type of epithelial tissue referred to as transitional epithelium that is not found anywhere else in the body. This unique cell type is designed to withstand repeated distension & relaxation of kidney-filtered fluids throughout a person's life [1]. When the bladder is empty, the transitional cells appear columnar but when full and distended, up to a capacity of 600ml, the cells are squamous in appearance – a unique chracteristic found in the genitourinary system. [2]. Such a distinct tissue-type merits a basic description that can provide a foundation for understanding the pathology of this organ as it relates to cancer.

The bladder is organized into three distinct layers out from the bladder lumen: 1) epithelium, 2. lamina propria, and 3. the outer musclel. The bladder lumen is lined with transitional epithelial cells that are at the forefront of a lifetime's exposure to carcinogens. These cells are followed by the inner muscle and outer muscle. The bladder urothelium is comprised of three cell types, basal, intermediate and umbrella cells. The urinary bladder acts both as holding vessel for ready-to-be excreted urine as and the urothelium acts as a protective barrier to the underlying layers of transitional epithelium. This blood-urine barrier of urothelium is thought to be the most impermeable barrier in the human body system [1]. There is also

evidence that suggests the bladder wall layer of cells functions beyond a protective layer and relays signaling messages about the chemical state of the urine [1]. However, despite all the protective urothelium and it's tight junctions, the bladder is not protected from cancer due to the likely accumulation of carcinogenic urine content over time. This chapter will take a deeper look at the epidemiology, cancer genomic landscape and molecular characterization – especially as it relates to my dissertation work with aberrant SOX4 transcription factor expression - and finally the treatment of bladder cancer and what my work could mean for bladder cancer patients today and beyond.

1.1.2 Bladder Cancer Epidemiology

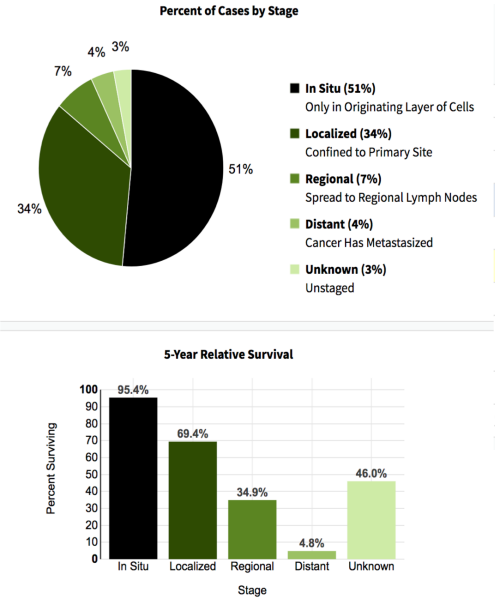
Bladder cancer (BLCA) is the 6th most common cancer in the United States (4.7% of all new cancer cases) and accounting for 79,030 new cases and 16,870 deaths in 2017 alone [3], with a median age of onset of 72 years old. Bladder cancer grading and staging follows the Tumor-Node-Metastasis system Tumor-Node-Metastasis (TNM) (Figure 1.2). Overall five year survival is 77.3% across all tumor stages and decreases with increasing tumor stage at presentation [3]. A breakdown of 5-year survival by stage is provided in (Figure 1.1A). Briefly, 51% of patients present with carcinoma in situ and have a 5 year survival of 95.4%. About 34% present with localized disease with a 5 year survival of 69.4%, while regional and distant (metastatic) disease present in 7% and 4% of newly diagnosed cases with 34.9% and 4.8% 5-year survivals rates, respectively.

Interestingly, in the United States, BLCA afflicts white males more than any other race, but no racial disparity is apparent in women diagnosed with bladder

cancer. Moreover, death rates from BLCA reflect no racial disparities (Figure 1.1B) [3]. It is often stated that cancer, in general and absent genetic/hereditary components, is a disease of old age. This is most certainly the case with BLCA. The greatest association with BLCA is age: ages 65-74 and 75-84 represent nearly 60% of all new BLCA cases in the US [3].

Risk factors for bladder cancer include various carcinogens related to occupational exposures to polycyclic hydrocarbons, aromatic amines, arsenic contaminated water, or even exposure to ionizing radiation [4]. Nonetheless, the single greatest lifestyle risk factor is tobacco smoke [5]. Initial diagnosis usually occurs as an incidental finding after patients present with blood in the urine (hematuria) [4, 5]. Usually, the first signs of a malignant state in the bladder appear in the basal cell layer and as a result of incidental findings [1]. Unfortunately, the lack of a well-established active screening protocol for bladder cancer presents an unmet medical need as patients with microscopic hematuria are sometimes not adequately diagnosed until they present with macroscopic hematuria – which is usually suggestive of an advanced disease state [5].

A.



B.

	Common Types of Cancer	Estimated New Cases 2018	Estimated Deaths 2018
1.	Breast Cancer (Female)	266,120	40,920
2.	Lung and Bronchus Cancer	234,030	154,050
3.	Prostate Cancer	164,690	29,430
4.	Colorectal Cancer	140,250	50,630
5.	Melanoma of the Skin	91,270	9,320
6.	**Bladder Cancer**	**81,190**	**17,240**
7.	Non-Hodgkin Lymphoma	74,680	19,910
8.	Kidney and Renal Pelvis Cancer	65,340	14,970
9.	Uterine Cancer	63,230	11,350
10.	Leukemia	60,300	24,370

C.

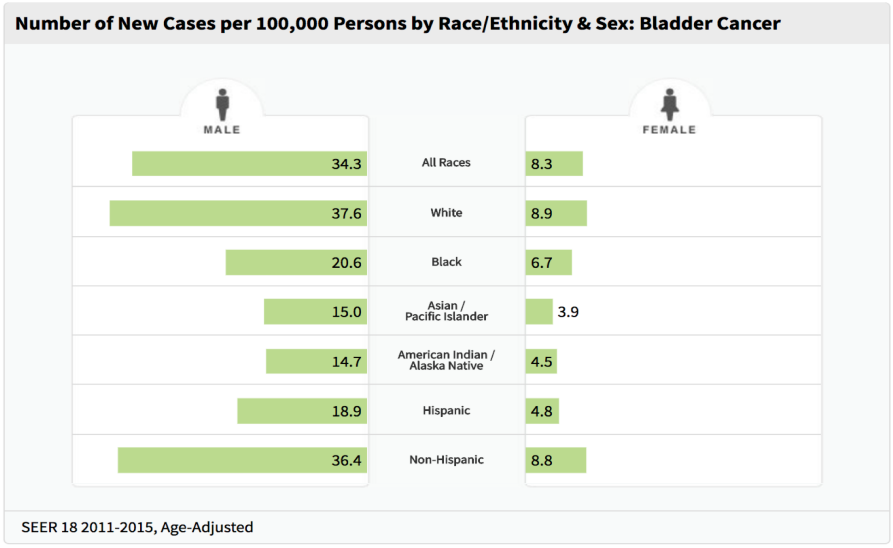


Figure 1.1: Adapted from [3]. SEER A.) Percentage of cases at diagnosis and 5-year survival by state B.) Bladder cancer is 6th most common cancer in the U.S. C.) Number of bladder cancer cases by gender and race.

1.1.3 Bladder Cancer Staging

Bladder Cancer is grouped into two main pathological classes based on histology: Non-Muscle Invasive Bladder Cancer (NMIBC) and Muscle Invasive Bladder Cancer (MIBC) and follows the TNM classification system. The extent of local invasion (i.e. how far the tumor has grown into the bladder wall) is described by "T". "N" denotes if and to what extent the tumor has spread to nearby lymph nodes and "M" indicates the degree of metastasis to other organs, if any. NMIBC usually begins with carcinoma *in situ* Carcinoma in situ (CIS), also referred to as Tis. Carcinoma *in situ* is usually flat, non-invasive and growing only in the bladder urothelium (i.e. inner lining of the bladder), Ta indicates a papillary non-invasive tumor that has grown outward toward the lumen with finger like projections but is confined to the urothelium, T1 indicates a Ta tumor that has invaded the lamina propria or connective tissue, T2a or T2b tumors have grown into the top muscle layer or deeper muscle layer, respectively. Finally, T3 and T4 tumors have invaded through the muscle of the bladder and spread to the uterus or prostate respectively [4, 5] (Figure 1.2). A tumor that has invaded the pelvic wall or abdominal wall is denoted T4a and presents the most severe local invasion. Other types of bladder cancer are known such as adenocarcinoma, squamous cell carcinoma, small cell anaplasia, and sarcoma's that originate in the muscle or fat layers but all of these are less common and not within the scope of this dissertation. Molecular characterizations of BLCA will be discussed in the next section.

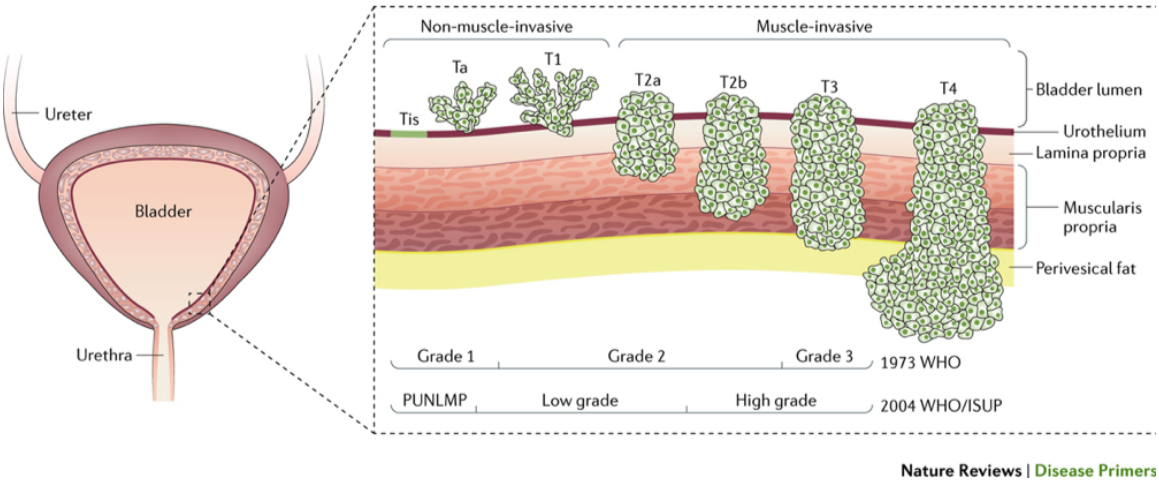


Figure 1.2: Adapted from [4, 5]. A cross sectional view of the bladder to illustrate bladder cancer stumor staging Tis, Ta, T1, T2a, T3 and T4.

1.1.4 Genomic Landscape of Bladder Cancer

Over the last 10 years, with the advent of microarrays and later RNAseq methods, researchers have been able to assess bladder cancer tumors beyond the visual/histological classifications. Such characterization of the genomic landscape of bladder cancer has helped us better understand this disease based on amplifications, deletions, and mutations. These results have also elucidated potential druggable targets to treat this disease. Data from The Cancer Genome Atlas (TCGA) have revealed as many as three different molecular subtypes of in NMIBC and five different subtypes in MIBC [6–8]. The topic of these mutations as potential drivers of tumorigenesis, proliferation, aberrant growth processes and stratification markers for treatment are discussed in the following sections.

1.1.4.1 Mutational Spectrum in NMIBC

Of all bladder cancer diagnoses, approximately 75% are of the NMIBC variety [9], typically arising from an epithelial hyperplasia on the bladder lumen [4]. One of the most urgent unmet clinical goals in the NMIBC setting is to identify patients with Ta, low-grade, or CIS patients who might progress to a more aggressive disease state. Currently, the scientific consensus is that papillary urothelial hyperplastic lesions, which carry deletions in chromosome 9, such as deletion of CDKN2A, as well as point mutations in Fibroblast Growth Factor Receptor 3 (FGFR3), are histological precursors of NMIBC [5, 10]. Approximately 80% of low-grade Ta tumors display FGFR3 upregulation that often co-occurs with PIK3CA activating mutations. Also on chromosome 9 is the tumor suppressor gene TSC1 [5]. One recent study by Hedegaard et al., conducted a molecular characterization of 460 early stage NMIBC via RNA-seq [8]. By way of an unsupervised consensus

clustering, this study revealed three very important subclasses of NMIBC; - each of which stratified a unique clinical prognosis – a finding much needed in this field [8] (Figure 1.3). These data show that NMIBC subclasses have a distinct set of expression signatures that correspond to biologically annotated features such as early cell cycle, late cell cycle, keratins, Uroplakins, cancer stem cell (CSC) markers, epithelial to mesenchymal transition (EMT) and differentiation. Briefly, class 1 exhibited luminal-like differentiation, no EMT changes, some CSC activity, early cell cycle activity and FGFR3 mutations. Class 2 also showed signs of luminal-like differentiation, high EMT transcription factor activity (ZEB1, ZEB2, etc), late cell cycle gene activity, and interestingly, it was the only class that had a high TP53 mutation rate. Class 3 are more basal-like, do not have EMT markers, have neither early or late cell cycle activity, mutations in FGFR3, or an RNA-editing signature (long non-coding RNAs or circular RNAs).

Perhaps the most important contribution from Hedegaard et al., is in elucidating a gene signature for stratification of NMIBC patients who might progress to MIBC. Hedegaard et al., propose that normal urothelium progresses through two branches to either Ta or CIS pathways. Tumors in the Ta stage, which are usually high in FGFR3 mutations, diverge to either Class 1 or Class 3. Class 1 progress to MIBC, whereas Class 3 shifts to a Class 2 and then MIBC. Alternatively, Normal urothelium can start in the CIS pathway and lead immediately to Class 2, followed by MIBC (Figure 1.4).

Interestingly, SOX4 was not identified as being significantly overexpressed in this dataset. Indeed, this in-depth analysis provides strong evidence against high

SOX4 expression being an early event in tumorigenesis. Instead, SOX4 might be a later event in MIBC tumorigenesis as described below.

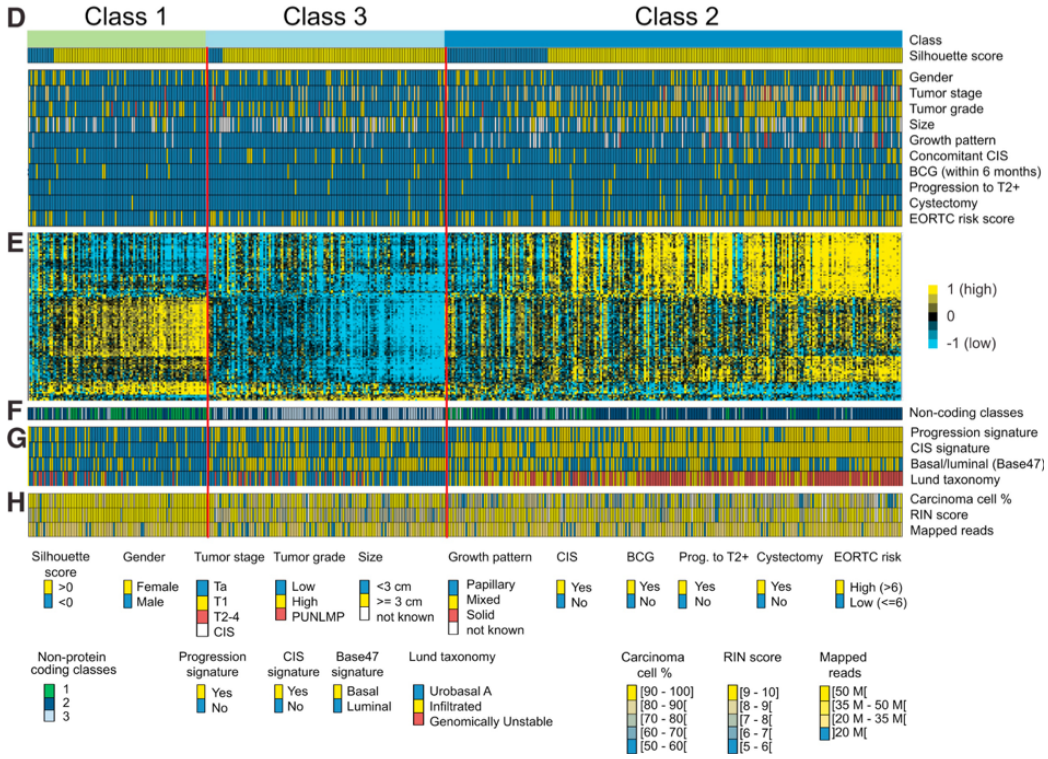
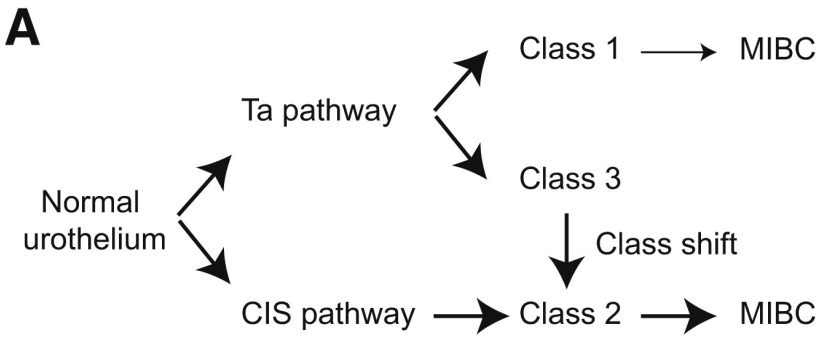


Figure 1.3: Adapted from [8].D-E Stratification of Classes 1 - 3 based on histopathology, clinical outcome, 117 gene classifiers, clustering, other publically available signatures, RNA quality/sequencing data.



B

	Luminal-like Differentiation	Basal-like	EMT TF activity	CSC activity	Cell cycle activity	Molecular signature	Mutations
Class 1	UPKs PPARG GRHL3 BAMBI SPINK1			SHH RPSA ALDH1A3 ITGA6	*Early:* CCND1 ID1 RBL2		*FGFR3*
Class 3	GATA3	KRT5 KRT14 KRT15 CD44		ITGA6		BASE47+	*FGFR3* RNA-editing signature
Class 2	UPKs PPARG KRT20 GRHL3 BAMBI SPINK1	KRT14	SOX9 TWIST1 FOXF1 ZEB1 ZEB2 GATA6	PROM1 ALDH1A1 ALDH1A2 ALDH1A3 NES THY	*Late:* CDC20 CDC25A CDKs PLK1	CIS+ Prog.+	*TP53* *ERCC2* APOBEC mutation signature

Figure 1.4: Adapted from [8]. A.) Novel pathway for progression from NMIBC to MIBC B.) Summary of features specific to Classes 1 -3.

1.1.4.2 Mutational Spectrum in MIBC

As mentioned previously, approximately 25% of patients present with MIBC. Approximately half of all MIBCs will metastasize, whereas metastasis in NMIBC is rare [4]. MIBC has been more extensively studied over the years than NMIBC, likely owing to the fact that there have not been new treatment options in over 20 years for the later metastatic stages, although new immunotherapy treatment options are showing promise [5, 11]. The most recent study by Roberston et al [7], which layered-on genetic information as well as validated the previous TCGA data [6], built upon the original TCGA bladder dataset from 2014 [6]. Here, Robertson et al., molecularly characterized 412 chemotherapy-naïve MIBC patients by way of somatic copy number alterations (SCNAs), DNA Methylation, RNA-seq, and whole exome sequencing of all 412 tumors/matched normal samples. These data revealed a treasure-trove of mutational signatures that would go unnoticed based on histopathological classifications. In general, MIBC typically has more mutations than NMIBC [6, 7]. Specifically, the TP53/cell cycle pathway was inactivated in approximately 89% of tumors, and 17% had RB1 mutations, many of which were inactivating. FGFR3, represented the second most significantly mutated gene after TP53 mutations and mutational burden correlated with poor 5-year survival [7]. MIBC generally contains a greater number of genomic insults in the form of copy number alterations such as deletions, amplifications and even rearrangements than NMIBC, and many loss of function mutations in tumor suppressor genes are seen in MIBCC [7]. In summary, the Robertson et al., analysis combined integration of pathway information, EMT/CIS signatures, immune infiltration data and mRNA subtype-data that yielded a stratified frame-

work of as many as five different subtypes underlying the well-established luminal and basal/squamous types (Figure 1.5). This framework predicts therapeutic approaches to help further inform clinical trial design. I believe this approach represents a way forward for clinical trial enrollment, as evidenced by basket trials, whereby we no longer treat a single cancer based on organ type, but based on genetic mutations/drivers that might be inherent across various cancer tissue types.

SOX4 is amplified in many bladder cancer patients but the precise functional consequences have not yet been elucidated. I will discuss amplifications, as they relate to SOX4, in more detail in the next section and then propose a possible functional consequence of SOX4 that leads to increased invasiveness in Chapter 3 (paper section).

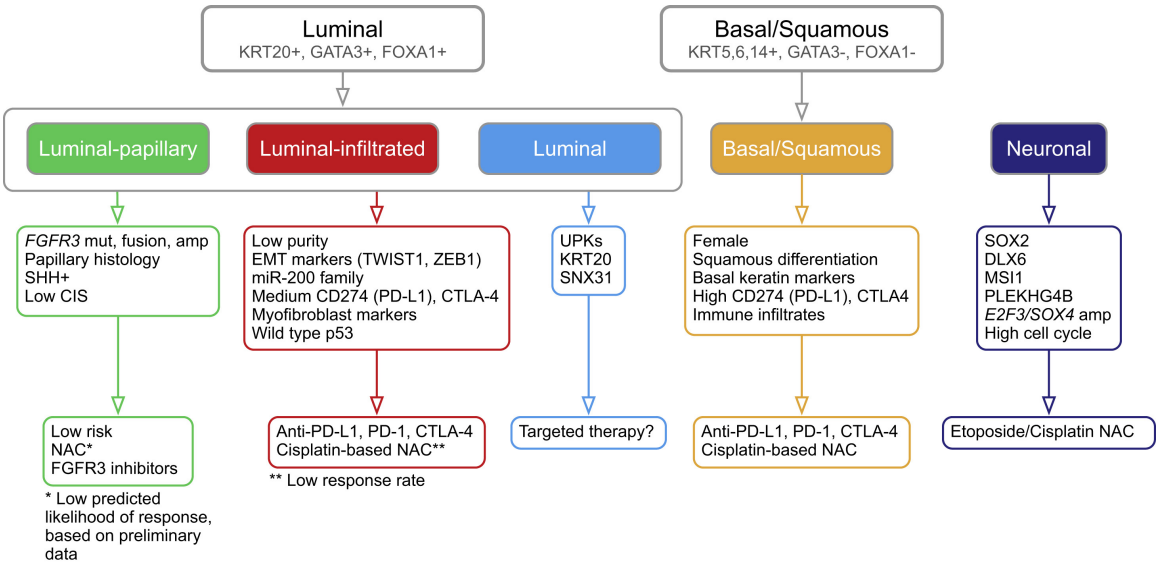


Figure 1.5: Adapted from [7]. Analysis of 412 MIBC tumors revealed 3 new luminal subtypes and carved out 2 distinct subtypes from Basal/Squamous groups - including a neuronal subtype which includes the SOX4 amplificaiton. In all, the analysis revealed 5 new subtypes that could aid to drive treatment decisions.

1.1.5 The chr6p22.3 Amplification and CDKN2A Deletion

Published RNAseq, whole genome and whole-exome sequencing data from bladder cancer patients have revealed new genomic alterations and potential molecular mechanisms for tumorigenesis that merit further investigation [6, 7]. Understanding how specific downstream perturbed signaling proteins go awry to upregulate growth and deregulate the cell cycle holds promise for targeted cancer therapies. These may be especially important in bladder cancer where no targeted therapies currently exist. The chr6p22.3 amplification and CDKN2A deletion are the most common focal amplification/deletions in bladder cancer patients. Here we will discuss some of the main genes involved in this amplification/deletion including SOX4, E2F3, CDKN2A and how they converge on elements such as cell cycle and TP53.

1.1.5.1 SOX4 and Bladder Cancer – a brief introduction

SOX4 and E2F3 genes encode transcription factors and are found on the 6p22.3 amplification. SOX4 is a 46kD transcription factor related to the Sex-Determining Region on the Y-Chromosome gene, or SRY for short. SOX4 is one of 20 different SOX family genes that are implicated in a host of developmental and differentiation process such as cardiogenesis, lymphopoiesis, pancreas formation and neuronal maturation [12–15]. In one meta-analysis, SOX4 is included in a list of 64 cancer signature genes as determined by relative overexpression in normal vs cancerous tissue [16].

Data from The Cancer Genome Atlas (TCGA) revealed SOX4 as amplified or overexpressed in as many as 24% of bladder cancer patients. Molecular analysis

from Robertson et al., [7] stratified 412 chemotherapy naive bladder cancer patients into five distinct subtypes: Luminal-papillary, Luminal-infiltrated, Luminal, Basal/Squamous and Neuronal 1.5. Patients with SOX4/E2F3 amplifications are part of the neuronal subgroup and are also characterized as highly proliferative [7, 17]. However, while SOX4 overexpression is associated with bladder cancer, it remains to be determined if SOX4 is a driver of oncogenesis. Moreover, there has not been an association between worsening of tumor stage and increased SOX4 expression. Conflicting immunohistochemical studies have shown that SOX4 expression confers both poor patient survival [18] and improved survival, suggesting a tumor protective effect of SOX4 [19]. Data on SOX4 expression in other tumor types, such as prostate cancer, have shown that SOX4 has transforming ability. Additionally, 6p22.3 amplifications were most frequently observed in MIBC compared to NMIBC [18]. As a result SOX4 could be partly implicated in the progression from NMIBC to MIBC [18, 20]. We will discuss the SOX family of transcription factors with an emphasis on SOX4 in more detail in chapter 2.

1.1.5.2 CDKN2A, RB, and E2F3 in Bladder Cancer

The same RNAseq analysis from TCGA revealed that 42% of bladder cancer patients exhibit misregulation of cyclin-dependent kinase inhibitor 2A (CDKN2A), also known as p16 or p16INK4A, either via homozygous deletion or truncating/missense mutations [6, 21, 22]. CDKN2A is a well- characterized tumor suppressor involved in cell cycle arrest by complexing with cyclin-dependent kinases 4 (CDK4) and 6 (CDK6) and inhibiting these kinases from phosphorylating the retinoblastoma tumor suppressor protein (RB1) – the first tumor suppressor gene ever discovered. Unphosphorylated RB1 functions to negatively regulate E2F transcription

factors, preventing cell cycle progression [23–26]. RB1 is also implicated in bladder cancer with approximately 22% of patients harboring deep deletions or missense/truncating mutations. Because RB1 and CDKN2A are in the same pathway, these mutations tend to be mutually exclusive in most patients.

Similar to SOX4, E2F3 is overexpressed in 20% of bladder cancers, usually as a result of 6p22.3 amplification. E2F3 is downstream of the CDKN2A/RB1 pathway and is not typically mutated, as is its RB1 binding partner. Deregulation of this pathway via mutations and copy number changes that combine E2F3/SOX4 and RB1 mutations are common events in BLCA.

1.2 Scope of this Dissertation

For years SOX4 overexpression and cancer have been merely correlative across a number of organ-specific cancers. Pinpointing a universal role of SOX4 across various cancer tissue types has proven inconsistent and may not be possible. This fact is likely due to a number of factors, including the fact that SOX4 is a transcription factor and it's function is dependent on 1) access and availability of SOX4 binding sites, which are driven by changes in chromatin structure and organization, and 2) availability of binding partners and transactivators that are likely tissue specific. We therefore aim to understand SOX4 in a context-specific/tissue-specific manner.

The Moreno lab has previously shown that SOX4 is a transforming oncogene in prostate cancer cell lines and that SOX4 levels increase with worsening tumor

grade in patient samples [27]. As discussed earlier, this is not the case for bladder cancer. In this dissertation, we will discuss results that suggest SOX4 drives invasion of T24 bladder cancer cell lines. We will further evaluate 173 high-confidence SOX4 regulated genes as a result of SOX4 knockdown and re-expression within the same cell line. These data revealed SOX4 as a putative negative regulator of Wnt5a, and high Wnt5a expression in SOX4-knockdown cell lines correlated with decreased invasive capability. Invasive capability was restored by re-expressing SOX4 and concomitant decreased expression of Wn5a was observed. We therefore, propose that Wn5a is tumor protective and that SOX4 could negatively regulate Wn5a in T24 bladder cancer cells. Furthermore, we will discuss ongoing protein-protein interaction studies in support of our hypothesis that SOX4 might bind and sequester CDKN2A (p16) as a mechanism for inhibiting CDKN2A's function in its tumor suppressive role. Taken together we show that SOX4, as a transcription factor, functions in various capacities outside of its canonical transcription factor binding capacity to regulate processes that drive tumorigenesis.

Chapter 2 SOX4 and the SOX gene Family

2.1 Introduction

Tracing back the discovery of SOX4, and all other SOX genes, first begins with the discovery of a different gene responsible for male sex organ differentiation. For decades the genes and mechanisms that drive male sexual differentiation remained elusive. The Sex-Determining Region on the Y Chromosome (SRY) gene, also known as the Testis Determining Factor (TDF) codes for a transcription factor first discovered in the 1990s [28, 29]. This landmark discovery of Sry revealed a transcription factor containing a High-Mobility Group DNA-binding domain with no other apparent functional domains [30]. Sry was the first of a subfamily of genes that code for proteins that contain this HMG-Box DNA binding domain and set the stage for the discovery for what now accounts for 20 genes in the SOX gene subfamily with various roles related to cell fate and terminal differentiation [31]. Here we will briefly discuss the overall grouping of SOX genes at a high level and then narrow our focus to SOX4, its role in development, signaling pathways and cancer.

2.1.1 Grouping of SOX Proteins gene family

The 20 SOX genes, all share at least 46% identity to Sry in the HMG Box domain[32]. They are further subcategorized into 8 different groups (A-H) based on sequence identity of their respective HMG-Box domains. SOX genes that share at least 80% sequence identity in their HMG-Box domains are classified together into

groups labelled A-H. [31, 33, 34] see figure (Figure 2.1). Whereas all SOX proteins harbor HMG-Box domains, not all of them contain true transactivation domains. SOX proteins display a wide array of biological functions based on each domain from transactivation, repression and even dimerization (e.g. SOX9).

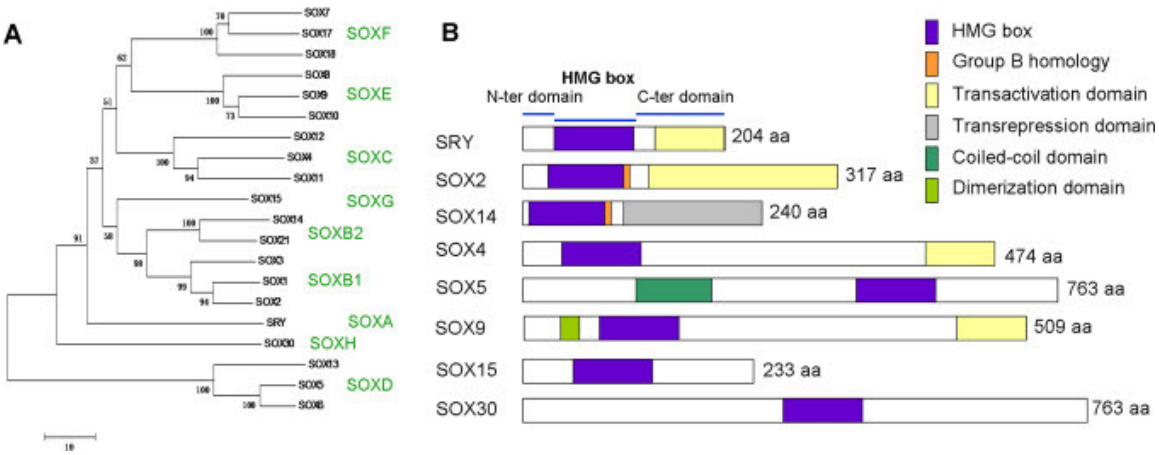


Figure 2.1: Adapted from [35]. A.) Phylogenetic origin and evolution of all 8 SOX transcription factor groups B.) Representative primary and tertiary structure of one SOX protein from each of 8 SOX groups.

SOX4, or more formally known as Sry (sex-determining region on the Y chromosome)-related HMG-box 4, is a single exon gene that codes for the SOX4 transcription factor protein on chromosome 6p22. SOX4 is one of 20 different SOX proteins whose DNA binding capabilities and structure closely resemble SRY. SOX4 is grouped together with SOX11 and SOX12 to make up the SOXC group of SOX proteins that share approximately 84% identity in their DNA binding domains and a high degree of identity in the C-terminal domain in all vertebrates [32, 35] (Figure 2.2). Like SOX4, SOX11 and SOX12 are also intron-less genes and these three proteins share some redundancy. As a whole the SOXC group of transcription factors are implicated in various developmental processes such as cardiac and neuronal. SOX4 is unique in certain respects and is the only SOXC transcription factor within the scope of this dissertation.

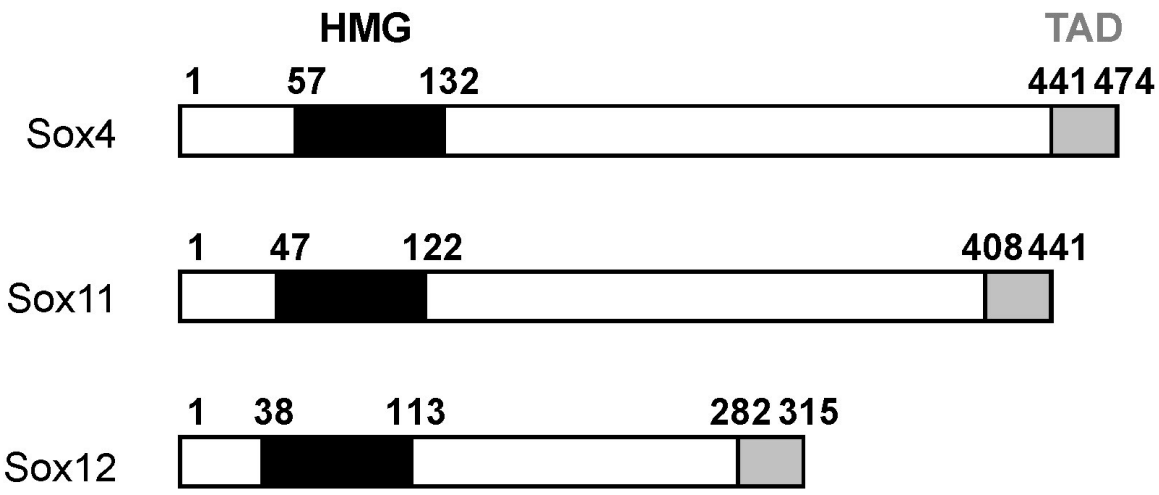


Figure 2.2: Adapted from [35]. The SOXC group of transcription factors include SOX4, SOX11 and SOX12. These proteins share most of their identity within the HMG-Box domain. All SOXC proteins contain both an HMG-Box domain and a Transactivation domain with varying degrees of transactivation capabilities.

2.2 SOX4

SOX4 was first discovered in lymphocytes in an attempt to uncover T-Cell specific genes that drive T-cell differentiation and bind to DNA elements similar to TCF/LEF1 proteins [36]. Full length SOX4 is a 474 amino acid (46kDa) protein, and unlike SRY, contains 4 distinct functional domains: a HMG-box DNA binding domain (aa 59-138), a glycine rich region (GRR, aa 152-227), a serine rich region (SRR, aa 333-397), and a transactivation domain (TAD, aa 441 – 474) [33] (Figure 2.3). The crystal structure of SOX4 revealed an HMG Box domain composed of three alpha helix domains forming a distinct L-shaped structure that binds, like other SOX proteins do, to DNA through the minor groove [37]. This mechanism of binding forces a kink or bend in the DNA that likely induces changes in chromatin structures [37]. SOX4 binds primarily to motifs of AACAAAG and secondarily to AATTGTT sequences as demonstrated by Electrophoretic Mobility Shift Assay (EMSA). [32, 36, 37].

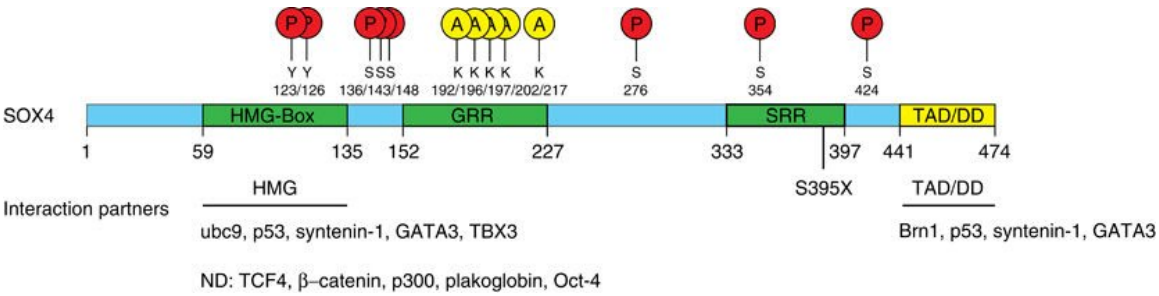


Figure 2.3: Adapted from [33]. The SOX4 sequence is composed of single exon gene that translates into a 474 amino acid protein sequence with 4 distinct functional domains: the HMG-Box, Glycine Rich Region (GRR), Serine Rich Region (SRR) and Transactivation/Death Domain (TAD/DD).

Carefully calibrated levels of SOX4 expression in cells are responsible for crucial roles in developmental pathways during embryogenesis and differentiation that normally are turned off or dramatically decreased after embryogenesis. However, if for some reason pathway activity levels spike again in specific organs, cancer can arise. Nevertheless, whether SOX4 is an initiating factor in tumorigenesis is not yet clear. While the Moreno lab has made the case that SOX4 is a transforming oncogene in non-transformed prostate cell lines, this has not been confirmed in other tissues and further studies are needed in tissues such as breast and bladder cancer.

2.2.0.1 SOX4 and Development

The SOX4 protein is a transcription factor responsible for various developmental and differentiation processes such as cardiogenesis, lymphopoiesis, pancreas formation and neuronal maturation [12–15, 31]. SOX4 is expressed in many additional organs including the bladder, brain, liver, and breast [27, 31, 33, 38]. As a transcription factor, SOX4's target genes are involved in a variety of cellular processes including microRNA processing, control of cell cycle and even apoptosis [27, 39–41].

Much of what we know about tissue specific SOX4 expression levels during development comes from mouse models [32]. Knockout of SOX4 in the mouse leads to embryonic lethality and developmental defects [42]. SOX4 is essential in a variety of different organ and cell types including thymocyte differentiation, formation of endocrine islet cells, osteoblast development, and neural cell development [13–15]. SOX4 is crucial for heart tract development since transgenic mice with ho-

mozygous mutations of SOX4 die during embryogenesis as a result of improper formation of semilunar valves [12, 32]. Although it is true that SOXC family proteins exhibit similar functions, it is usually the case that each of the SOXC proteins are co-expressed [32]. Areas of differential expression include eyelid primordium, palatal shelf (Sox11 and SOX12 only), heart endocardial cushions (Sox4 and Sox12 only), and thymus/hair follicles (SOX4 only).

2.2.0.2 SOX4 and Cancer

In addition to its normal functions in development and organogenesis, SOX4 can lead to unintended consequences of aberrant growth and proliferation in a variety of organ types. SOX4 is over expressed in at least 17 different cancer subtypes including leukemia, medulloblastoma, melanoma, lung and bladder cancer [18, 43–46]. Although SOX4 overexpression is associated with as many as 17 different cancers [33] we will focus our discussion of SOX4 in the prostate – specifically castration resistant prostate cancer and of course, in bladder cancer.

2.2.0.3 SOX4 - The Oncogene

Aside from regulating progenitor development, SOX4 is also involved in crucial pathways of cell proliferation and development of cancer [47]. SOX4 expression in cancer was first observed in the context of breast cancer by [48]. The authors observed not only normal levels of SOX4 expression in healthy breast tissue but also increased expression levels in breast cancer cells [48] that correlated with progesterone levels. Over the last 20 years SOX4 expression levels have been positively correlated with cancer. Few databases have demonstrated this as vividly as cBioportal (www.cbioportal.). The below image shows the relative SOX4 ex-

pression levels across the major organ types (Figure 2.4A) as well as data from oncomine [49] showing 107 published cancer vs. normal analyses with increased SOX4 expression by cancer type (Figure 2.4B).

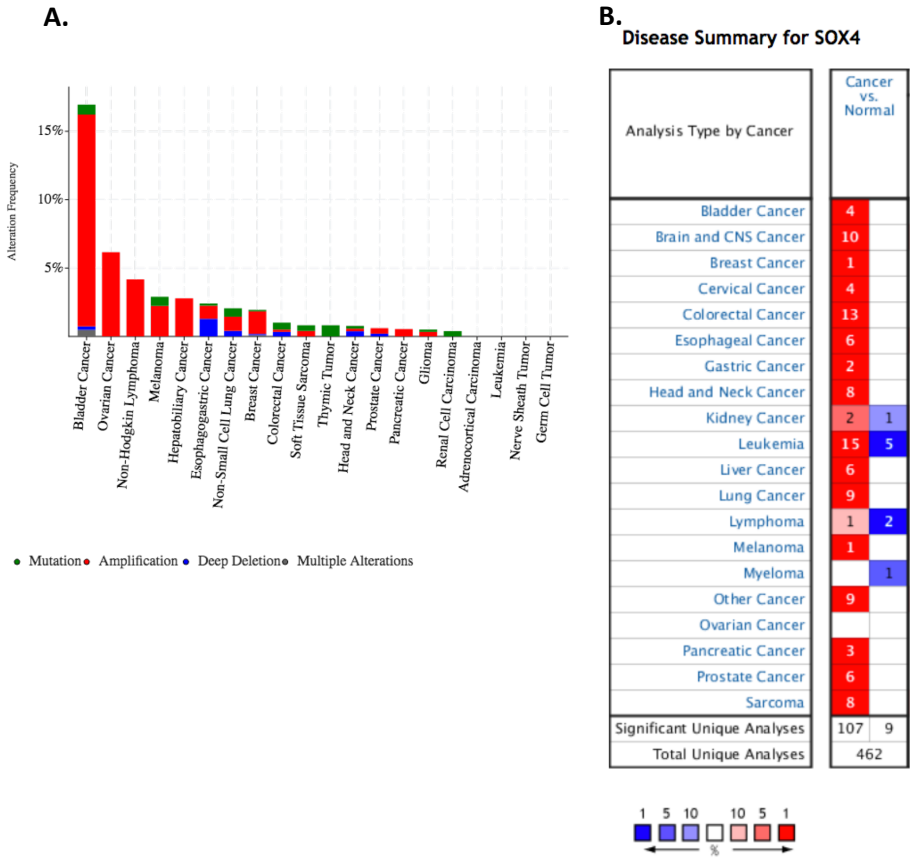


Figure 2.4: A.) Queried from cBioportal [21, 22]. Sox4 expression across various cancer subtypes show bladder cancer with the greatest alteration frequency. B.) Data from oncomine showing the number of cancer vs. normal analyses available by cancer type for SOX4 [49].

One of the earliest studies of SOX4's association with malignant transformation comes from McCracken et al., [50], in which SOX4 cooperated with Ets-1 to drive lymphoid-specific protein tyrosine kinase (p56lck). In 2004, a meta-analysis of many large scale microarray datasets across 36 tumors compared to normal tissue established a meta-signature of neoplastic transformation relative to normal healthy tissue. This meta-signature included 64 genes deemed "cancer signature genes", including SOX4 [16]. The Moreno lab was the first to show increased SOX4 levels with worsening Gleason score in prostate cancer [27]. While the evidence in the literature suggests that SOX4 expression is positively correlated with cancer, the mechanism of SOX4 in tumorigenesis is not well understood, and there are no small molecules that target SOX4 function.

2.2.0.4 SOX4 in Prostate Cancer

Our laboratory has extensively characterized SOX4 and its overexpression in prostate cancer patients compared to normal tissues [27]. SOX4 overexpression correlates with worsening prostate tumor grades and Gleason scores [27]. Additionally, our lab identified SOX4 as a transforming oncogene via over expression of SOX4 into non-neoplastic prostate cells and visualizing growth in soft agar [27]. Furthermore, siRNA silencing of SOX4 induced apoptosis in prostate cancer cell lines, further establishing SOX4 as an oncogene [27].

2.2.0.5 SOX4 in Bladder Cancer

As mentioned previously, according to data from TCGA, bladder cancer patients have some of the highest frequency of SOX4 aberrations. This is a direct consequence of either increased mRNA expression or amplification of the locus at

chromosome 6p22.3 [6, 18, 46]. Since the late 1990's researchers have attempted to identify all of the target genes on the 6p22.3 locus with the hope of finding a cancer driver gene amplification [51–53].

The Aaboe group were one of the first studies to look at SOX4 expression in clinically annotated BLCA samples [19]. Using a tissue microarray containing 2360 patients samples they found that SOX4 expression levels correlated with increased patient survival. In addition they overexpressed SOX4 in a SOX4 null bladder cancer cell line followed by a time course microarray experiment. They observed that increased SOX4 expression lead to lower cell viability and apoptosis. They identified approximately 130 SOX4 regulated genes involved in various pathways such as angiogenesis (*NRP2*) and cell cycle arrest (*PIK3R3*). These data suggest a tumor-protecting role of ectopic SOX4 expression.

However, data from Shen et al., in 2015 showed that knockdown of SOX4 in 5637 cells induced MET as indicated by increased E-Caherin, and decreases in both N-Cadherin and Vimentin [18]. RNA-seq data from siSOX4 in RT-112 bladder cell lines showed a down-regulation of cell cycle genes, chromatin remodeling genes, and DNA replication genes compared to siControl cells. The authors also analyzed 309 tissue microarray samples and showed that patients with muscle invasive bladder cancer have the highest SOX4 expression and a worse overall survival.

The conflicting data between Aaboe et al., and Shen et al., [18, 19] demonstrate the lack of consensus regarding the role of SOX4 in bladder cancer and merits further research. In chapter 3 we will look at the effects of SOX4 knockdown in

bladder cancer cell lines and establish our model for the role of SOX4 in bladder cancer.

2.2.1 SOX4 and EMT

2.2.1.1 History of EMT

The concept of epithelial to mesenchymal transition (EMT) was first described by Elizabeth Hay in her work studying the developing chick embryo [54]. To better understand what we mean by EMT first requires a basic understanding of the two main tissue types in vertebrates: Epithelium and Mesenchyme. Epithelium is classified as cells that form a single cell layer that lines the lumen of many tissues, express E-Cadheren proteins, and are joined together by tight junctions of adherens such as desmosomes. These epithelial cells sit atop an extracellular matrix, also known as the basement membrane, and are said to have distinct apical-basal polarity. In contrast, mesenchymal cells typically do not express E-Cadherin and as a result are not bound to one another and are highly motile and invasive. Instead, these cells are typically elongated and exhibit a trailing pseudopodium and a leading front edge. Interestingly, the leading edge contains the golgi apparatus that secretes proteins for the filopodia's locomotion through the extra cellular matrix [54, 55]. The precise method of movement and mechanisms herein are beyond the scope of this dissertation but suffice it to say that the locomotion is well documented. As such, the Boden International Conference on EMT defined the mesenchymal cell on four criteria 1) elongated morphology 2) front end=back end polarity 3) observed filopodia and 4) invasive motility.

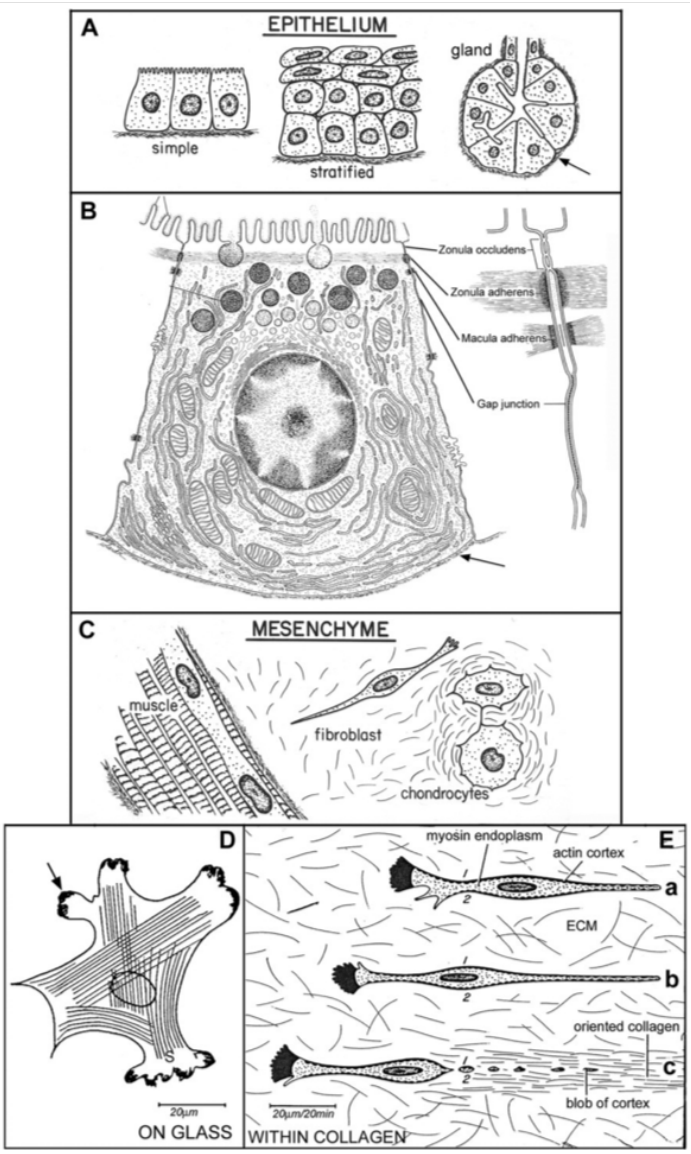


Figure 2.5: Adapted from [55]. A.) Overview of various forms of epithelial cells. B: Magnified structure of epithelia show enrichment of E-Cadherin at junctions linked to catenins at the cytoskeleton. C.) Depiction of mesenchymal cell (fibroblast) D.) Deption of how mesenchymal cells produce stress fibers upon loss of polarity. E.) Complete mesenchymal transformation shows elongated cells with filopodia at the leading edge. The trailing edge detaches and moves forward with the leading edge.

Within the cancer space EMT is ascribed a pernicious role, but EMT is actually a normal process with roots in embryonic development and wound healing; however highjacking of the EMT program outside of these two contexts contributes to metastatic disease - the cause of 90% of all cancer deaths [56].

2.2.1.2 EMT in Embryonic Development and Wound Healing

In the cancer biology field EMT has acquired a negative connotation due to its association with invasion and metastasis. However, EMT is a normal and required transformation of cells during development and even tissue repair [57]. In fact, many cells require several iterations back and forth between EMT and MET – which is the reversion of mesenchymal cells to epithelial cells [57].

In the embryo, the first EMT process occurs during gastrulation which creates the mesoderm. Here, a tightly controlled sequence of events drives cells out of epithelial residence and into the mesenchymal state. Depending on the specific organism, β-catenin levels can rise, and TGF-β can drive SNAIL1 and SNAIL2 gene expression, which in turn are crucial for altered cell shape, adhesion, and movement [57].

In addition to development, EMT is crucial for wound healing. Keratinocytes, cells of the skin, are able to induce a mesenchymal state whereby they cells migrate in between other keratinocytes. Work by Arnoux et al., showed that cells at the leading edge of this migration express SNAIL2 which is driven by Erk5 to facilitate wound healing [58].

2.2.1.3 The Role of SOX4 in EMT

Portions of the remaining chapter are adapted from [59] in which I am a co-author and wrote sections of the manuscript, edited the manuscript and designed de novo the main figure

SOX4 and EMT have been well studied in the context of breast and prostate cancer. Typically, prostate cancer mortality is related to metastasis to the bone, adrenal gland, liver and lung [60]. The epithelial to mesenchymal transition (EMT) is a major step in the metastatic process. To metastasize, cancer cells need to acquire migratory and invasive capabilities, a process that involves EMT [61]. EMT encompasses vast molecular changes including gain of mesenchymal markers such as vimentin and N-Cadherin, and loss of epithelial markers such as E-Cadherin, mediated by aberrant developmental signaling pathway activation that allows epithelial cells to discard differentiated characteristics and acquire migratory and invasive capabilities typical of mesenchymal cells [61]. These changes include the loss of cell-cell adhesion, planar and apical-basal polarity, increased motility, and resistance to apoptosis and anoikis (cell death due to the detachment from the extracellular matrix) [61, 62]. Among the developmental signaling pathways that are aberrantly activated during EMT is the TGF-β signaling pathway, a highly studied major inducer of EMT [63]. The canonical TGF-β pathway is stimulated via TGF-βinduced receptor complex activation, leading to phosphorylation of SMAD 2/3. Subsequently, these SMADs form a trimer with SMAD4, translocate to the nucleus and associate with other transcription factors to transcribe EMT-inducing genes [64].

Recently, it was found that SOX4 is a master regulator of TGF-β induced EMT via induction of EZH2 in breast cancer [65]. Tiwari et al., demonstrated that SOX4 directly activates EZH2 expression upon TGF-β treatment and that forced expression of EZH2 can overcome SOX4 knockdown and restore TGF-β induced EMT [65]. Moreover, Wang et al., found that, in prostate cancer cells, SOX4 knockdown inhibited TGF-β induced EMT, while SOX4 over expression promoted adoption of the mesenchymal phenotype [66]. They also demonstrated that TMPRSS2-ERG is critical for TGF-βinduction of SOX4 expression [66]. Tiwari et al. and Zhang et al. both demonstrated that ectopic expression of SOX4 could induce EMT by increasing the expression of mesenchymal markers and decreasing the expression of epithelial markers [65, 67]. In addition, SOX4 knockdown was sufficient to cause a reversion from a mesenchymal to epithelial phenotype after a 15-day TGF-β treatment [65].

Another SOX family factor, SOX9, has also been implicated in prostate cancer progression. Deletion of SOX9 in two different mouse models (TRAM and Hi-Myc) inhibited prostate cancer initiation [68]. ERG redirects AR to a cryptic enhancer of SOX9 to activate SOX9 expression, and knockdown of SOX9 inhibits invasion and growth of VCaP cells *in vitro* and *in vivo* [69]. SOX9 cooperates with PTEN deletion to drive prostate tumorigenesis [70], and it activates expression of Wnt pathway components such as LRP6 and TCF4 [71]. Like SOX9, SOX4 also plays an important role in Wnt signaling via direct interaction with β-catenin [40, 72]. SOX4 can act as an oncogene in prostate cells [27], and activates expression of additional Wnt pathway components such as FZD3, FZD5, and FZD8 [38, 40].

2.2.1.4 SOX4 – The Tumor Suppressor

Despite the vast amount of data supporting SOX4 as tumor promoting or driving different aspects of tumorigenesis, there are a few studies that suggest SOX4 has tumor protective effects or that high expression correlates with better patient outcomes. Pan et al., described a mechanism by which SOX4 can drive apoptosis and cell cycle arrest by binding and stabilizing p53, thus blocking MDM2-mediated p53 degradation in HCT116 colon cancer cell lines [73]. Interestingly, these data stand in stark contrast to Hur et al., who found that SOX4 overexpression can inhibit p53 mediated apoptosis in hepatocarcinogenesis [74]. As discussed previously Aaboe et al., [19] found that increased SOX4 expression correlated with better survival outcomes from a clinically annotated tissue microarray of 2360 bladder cancer patient samples. While we cannot discount these data, it should be noted that conflicting data from Shen et al., [18] using a different SOX4 antibody to analyze another bladder cancer tissue microarray demonstrated that patients with high SOX4 expression had worse overall survival.

2.3 Conclusion

The SOX family of transcription factors is of great interest as we continue to elucidate transcriptional network differences amongst the SOX genes. Although the roles of SOX4 in embryonic development in different tissues are fairly well understood, it's precise role in cancer and tumorigenesis is less clear.

Of all the cancers, by organ type, SOX4 is altered with greatest frequency in bladder cancer, which underscores the need for further investigation into the functional consequences of SOX4 amplification and overexpression in this cancer type. In the

following chapters we will discuss novel contributions as we elucidate functional consequences and putative SOX4 targets as a consequence of SOX4 knockdown in bladder cancer cell lines.

Chapter 3 SOX4 Regulates Invasion of Bladder Cancer Cells Via Repression of WNT5A

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3.1 Abstract

SOX4 is a developmental transcription factor that is overexpressed in as many as 23% of bladder cancer patients, but the role of SOX4 in bladder cancer tumorigenesis is not well understood. Given SOX4's many roles in embryonic development and context-dependent regulation of gene expression, we sought to understand SOX4's contribution to bladder cancer and to elucidate SOX4 regulated genes that might contribute to tumorigenesis. We employed a CRISPR interference (CRISPRi) method to transcriptionally repress SOX4 expression in T24 bladder cancer cell lines, rescued these cell lines with lentivirally expressed SOX4, and performed whole genome expression profiling. SOX4 knockdown cells exhibited decreased invasive capabilities but no changes in migration or proliferation, while rescue with SOX4 lentiviral vector restored the invasive phenotype. Gene expression profiling revealed 173 high confidence SOX4 regulated genes, including Wnt5a as a potential target of repression by SOX4. Treatment of T24-SOX4-KD cells with a Wnt5a antagonist restored the invasive phenotype seen in T24-scrambled control cells and SOX4 lentiviral rescued cells. High Wnt5a expression tracked with decreased invasion and was inversely correlated with SOX4 expression, suggesting that SOX4 could negatively regulate Wnt5a levels either directly or indirectly and that Wnt5a likely contributes a protective role against invasion in bladder cancer cells.

3.2 Introduction

Urothelial Carcinoma of the Bladder is the 6th most common cancer in the United States. Bladder Cancer disproportionately affects more men than women

and risk factors include smoking, certain environmental and occupational exposures [75, 76] and to a lesser extent alcoholism. There is also data to suggest that diets poorly supplemented with fruits and vegetables are linked to bladder cancer incidence but are not necessarily risk factors [77].

Bladder cancer is typically grouped into two main pathological classes; non-muscle invasive (NMIBC) and muscle invasive (MIBC). While histological and pathological grading and staging systems have existed for some time, recent advances in genomic sequencing have lent insight into molecular characterizations that stratify patients into various subtypes based on genetic markers such as amplifications, mutations and deletions [7, 17, 78, 79]. It is hoped that these new classifications might lend insights into prognosis or new subtype-specific treatment regimens.

One of the most commonly amplified and overexpressed genes in bladder cancer is the Sry-Related HMG-BOX-4 (SOX4) transcription factor. SOX4 is responsible for regulating a number of genes implicated in cellular development and differentiation [31]. SOX4 has both transcriptional activation and repressive roles, either alone or in combination with other transcription factors, that vary according to tissue type and context [72, 80–82]. Some of the most well established SOX4 target genes include DICER, TEAD2, TUBB3, and TNC TNC [15, 32, 37, 39, 40, 44]. SOX4 is on chromosome 6p22 – a genomic locus that also represents one of the most significant focal amplifications in bladder cancer and affects a number of different genes including SOX4, ID4, CDKAL1, E2F3, and MBOAT1 [46, 52].

Nevertheless, the exact role of SOX4 expression in various tumors, including bladder cancer, has not been determined and a comprehensive model of SOX4 function remains elusive. Increased SOX4 expression is associated with many other cancer types [33] and in some cases expression levels increase with worsening tumor grade [27]. Most in vitro studies associate aberrant SOX4 induction with transformation ability of cell lines, tumorigenicity, and inducing a mesenchymal phenotype. However, contradictory data exists showing higher SOX4 levels associated with increased apoptosis, stabilizing p53 induction and cell cycle arrest, suggesting a possible context-specific tumor suppressive arm of SOX4 [73, 74, 83, 84]. Although SOX4 over expression has been implicated in a variety of different cancers [27, 33], the downstream targets, mechanisms, functional consequences, and clinical prognosis vary amongst tumor subtypes [19, 39, 73] and in some cases studies within the same tissue type can show conflicting results [18, 19]. As a result, there is growing consensus that the role of SOX4 is context dependent, and the role of SOX4 in bladder cancer, like other tumor types, is thus not well defined.

In this study we have investigated the role of SOX4 expression in the T24 bladder cancer cell line by transcriptionally repressing SOX4 expression using a CRISPR-interference (CRISPRi) approach [85] to assess functional effects on migration, invasion, and proliferation. We also re-established SOX4 expression in the SOX4 knockdown T24 cell lines and identified a set of 173 high-confidence SOX4 regulated genes. Specifically, we show that SOX4 knockdown induces Wnt5a expression and that high Wnt5a expression in T24-SOX4-KD cells correlates with decreased invasion of bladder cancer cells.

3.3 Materials and Methods

Cell Culture, Cell Lines and Reagents

Bladder cancer cell lines 5637 (HTB-9), HT1376(CRL-1472), TCCSUP (HTB5), T24 (HTB-4), and SW780(CRL-2169) were obtained from American Type Culture Collection. 5637 cells were maintained in RPMI, T24, HT1376 and SW780 cells in DMEM, and TCCSUP cells in MEM growth media. All media were supplemented with 10% FBS, 1% L-Glutamine, and 1% Penicillin-Streptomycin. Cells were cultured in a 37C incubator with humidified atmosphere of 5% CO2. Parental T24 cells and subsequent cell lines used to generate stable T24 cells were genetically authenticated by Bio Synthesis (Lewisville, TX), an Accredited Human Cell Line Genotyping Service company. Wnt5a antagonist, BOX5, was purchased from EMD Millipore (Cat #681673) and used as described [86].

Generation of Stable T24-SOX4 Knockdown and T24-YFP-HA-SOX4 re-expression Cell Lines

Plasmid pHR-SFFV-KRAB-dCas9-P2A-mCherry was a gift from Jonathan Weissman (Addgene plasmid #60954). SOX4 specific small guide RNAs (sgRNAs) were generated using Zhang Lab's CRISPR design tool (http://crispr.mit.edu/) and validated using NCBI BLAST for non-specific targets. Scrambled or SOX4-TSS targeted sgRNAs were designed, annealed and ligated into the lentiviral construct pLKO.1-puro U6 sgRNA BfuAI large stuffer (a gift from Scot Wolfe - Addgene plasmid #52628). T24 cells were seeded at a density of $2x10^5$/well in a 6-well plate and 24 hours later spinfected at 500g for 90 minutes at 32°C with pHR-SFFV-KRAB-dCas9-P2A-mCherry and grown for one week in a 37°C incubator with humid-

ified atmosphere of 5% CO2. Cells were then sorted for pure mCherry positive cells at Emory's Flow Cytometry Core on a BD FACSAria II to establish our stable T24-KRAB-dCas9-P2A-mCherry cell line. These stable cells were seeded into a 6-well plate and transduced via spinfection as described above with either scrambled sgRNA pLKO.1-puro U6 sgRNA BfuAI large stuffer or pooled seven sgRNAs targeting SOX4 transcription start site (TSS), and selected with puromycin (2 µg/ml) for 48 hours after infection to create stable T24-KRAB-dCas9-P2A-mCherry-SOX4-sgRNA and stable T24- KRAB-dCas9-P2A-mCherry-Scrambled-sgRNA, hereafter referred to as T24-SOX4-KD and T24-Scr respectively.

Re-expression of SOX4 was performed in the T24-SOX4-KD background as described above. Briefly, we used our pHR-UBQ-HA-SOX4-IRES-eYFP-LlU3 lentiviral vector as previously described [40, 47] to transduce T24-SOX4-KD cells. We performed a dual sort for pure mCherry-positive and YFP-positive cells at Emory's Flow Cytometry Core on a BD FACSAria II to create stable T24- SOX4-KD+YFP-HA-SOX4 cells, hereafter referred to as T24-SOX4-Rescue.

Cell Migration and Invasion Assay

Cell invasion was evaluated using the Boyden Chamber assay. $1.25x10^5$ cells were seeded in 2 ml of serum-free and antibiotic free DMEM media in the top Boyden chamber containing Matrigel-coated 8µm pore membranes (Corning Cat # 354481) and 2.5 ml of complete DMEM media (supplemented with 10% FBS, 1% Penicillin-Streptomycin, 1% L-Glutamine) in the bottom chamber as a chemoattractant. After incubation for 24 hours at 37°C, non-invaded cells in the upper chamber were aspirated and membranes then fixed and stained in 0.5% crystal vi-

olet for 5 minutes, then washed 3x for 1 min in ddH20 and washed for 3 minutes on a shaker at room temperature. ddh20 was aspirated and membranes allowed to dry for 2 hours in cell culture hood. Membranes were then visualized under an upright confocal microscope using 40x on a Nikon Eclipse Ti-S inverted microscope. Representative images from 2-3 random fields were taken for each chamber. Cells were counted using Fiji open source analysis software (https://fiji.sc/). Each sample was assayed in triplicate in three independent experiments.

Cell migration assay was evaluated using a scratch-wound assay. $1.25x10^5$ cells were seeded in each well of a 12 well plate and allowed to grow to confluency. Media was aspirated and a scratch was made using a sterile 200µl pipette tip and fresh media was added. Images were taken at time zero, 6 hours, 12 hours, 18hours and 24 hours on a Biotek Lionheart widefield microscope.

MTT Assay

To evaluate proliferation of T24 cells expressing KRAB-dCas9-P2A-mCherry with SOX4 sgRNAs and scrambled controls, an MTT assay (ATCC Cat # 30-1010K) was performed by seeding $5x10^3$/well into 96-well plates. Cells were analyzed daily per the manufacturer's protocol for 5 consecutive days. Plates were read daily on a Biotek SYNERGY HT microplate reader. Each sample was assayed in triplicate in three independent experiments.

RNA extraction and Microarray Analysis

Total RNA was isolated from cultured cells using Qiagen RNAeasy kit as per manufacturer's protocol. RNA concentrations were measured using NanoDrop

Spectrophotometer (Mode # ND-1000). RNA samples were sent to Emory Integrated Genomics Core for quality control analysis and analyzed using Affymetrix Clariom D Genechips platform. Total RNA from four independent control samples T24-KRAB-dCas9-P2A-mCherry- Scrambled sgRNA, three independent SOX4 knockdown samples expressing T24-KRAB-dCas9-P2A-mCherry-SOX4-sgRNA, and three independent SOX4-Rescue samples expressing T24-KRAB-dCas9-P2A-mCherry-SOX4-sgRNA+YFP-HA-SOX4 cell lines were analyzed. Gene level signal was generated by RMA normalization. Differential gene expression was determined using the samr package [87, 88] in R Bioconductor [89] with 500 permutations, minimum fold change of 1.5 fold, and median FDR <0.05. Samples are available on GEO (ascension number #PENDING).

Immunoblotting

Cells were washed twice with 1X PBS and harvested with RIPA lysis buffer (Sigma Cat # R0278) containing protease inhibitors (Sigma Cat # P8340) and phosphatase inhibitors (Roche Cat # 4906845001). Whole cell lysates were centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatants were transferred to fresh tubes and protein concentration was quantified using Pierce Bradford protein assay (Thermo Fisher Cat # 23225). Thirty µg of protein was analyzed on a 10% SDS-polyacrylamide gel for SDS-PAGE electrophoresis and transferred to a PVDF membrane (Biorad Cat #1620177). Membranes were blocked in 1X TBS buffer containing 5% BSA and 0.001% Tween for 1 hr at room temperature, and then incubated with primary antibody (SOX4 polyclonal 1:1000 Abcam Cat # 80261, ZEB1 rabbit polyclonal 1:1000 Cell Signaling Cat # 3396s, E-Cadherin rabbit polyclonal 1:1000 Cell Signaling Cat # 3195s, N-Cadherin rabbit polyclonal 1:1000 Santa Cruz Cat #, CRISPR/Cas9 mono-

clonal 1:500 Cat #A-9000-100 , B-Actin rabbit polyclonal 1:3000 Cell Signaling Cat# 3700s , GAPDH rabbit polyclonal 1:1000 Cell Signaling Cat # 2118s) overnight at 4°C. Blots were washed with TBST three times for 5 minutes each and incubated with secondary antibodies (anti-mouse IgG - Cell Signaling Cat # 7076S 1:2000, or anti-rabbit IgG - Abcam Cat # ab6721 1:3000) for 1 hour at room temperature. Signals were visualized using SuperSignal West Pic PLUS chemiluminescence substrate (Pierce Cat #34580).

Quantitative RT-PCR

Cells were harvested by treatment with 0.25% Trypsin. Total RNA was isolated as described above using Qiagen RNAeasy kit as per manufacturer's protocol and treated with on-column DNAse digestion to remove possible contaminating genomic DNA. All RNA was converted to cDNA using iScript cDNA Sythesis Kit containing a mixture of RNase H + MMLV reverse transcriptase (Cat # 1708891). qRT-PCR were performed on a Biorad (Model CFX Connect Real-Time System). Primer sequences for SOX4, TM7SF2, DHCR7, MVD, Wnt5a, TNC, IDO1, and 18s are listed below. Relative expression levels were normalized to 18s. 5'- 3'

TM2SF2 FWD 'CTGCCTCATCAATGGGCTTG' REV 'GAGGTAGAAGTAGGGCAGCAG'

DHCR7 FWD 'GAGGTGTGCGCAGGACTTTA' REV 'TGGCTTTGGGAATGTTGGGT'

MVD FWD 'ATCAAGTACTGGGGCAAGCG' REV 'TTCAGCCAAATCCGGTCCTC'

Wnt5a FWD 'CGCCCAGGTTGTAATTGAAG' REV 'GCATGTGGTCCTGATACAAGT'

TNC FWD 'AGCATCCGGACCAAAACCAT' REV 'CCGATGCCATCCAGGAAACT'

IDO1 FWD 'TTGCTAAAGGCGCTGTTGGA' REV 'GTCTGATAGCTGGGGGTTGC'

SOX4 FWD ' CCGAGCTGGTGCAAGACC' REV 'CCACACCATGAAGGCGTTC'

3.4 Results

3.4.1 Expression of SOX4 in bladder cancer patients and bladder cancer cell lines

We queried The Cancer Genome Atlas (TCGA) bladder cancer dataset via cBioPortal (http://www.cbioportal.org) for SOX4 using the TCGA *Cell 2017* dataset [7] and observed that the SOX4 gene has either copy number amplifications or increased mRNA expression in 23% (93/404) of bladder cancer patients (Figure 3.1 A). To understand how representative SOX4 levels are in bladder cancer cell lines, we performed western blot analysis of 5637, HT1376, TCCSUP, T24, and SW780 cells (Figure 3.1 B). These data indicate that these bladder cancer cell lines recapitulate the range of genetic alterations and SOX4 expression levels observed in bladder cancer patients.

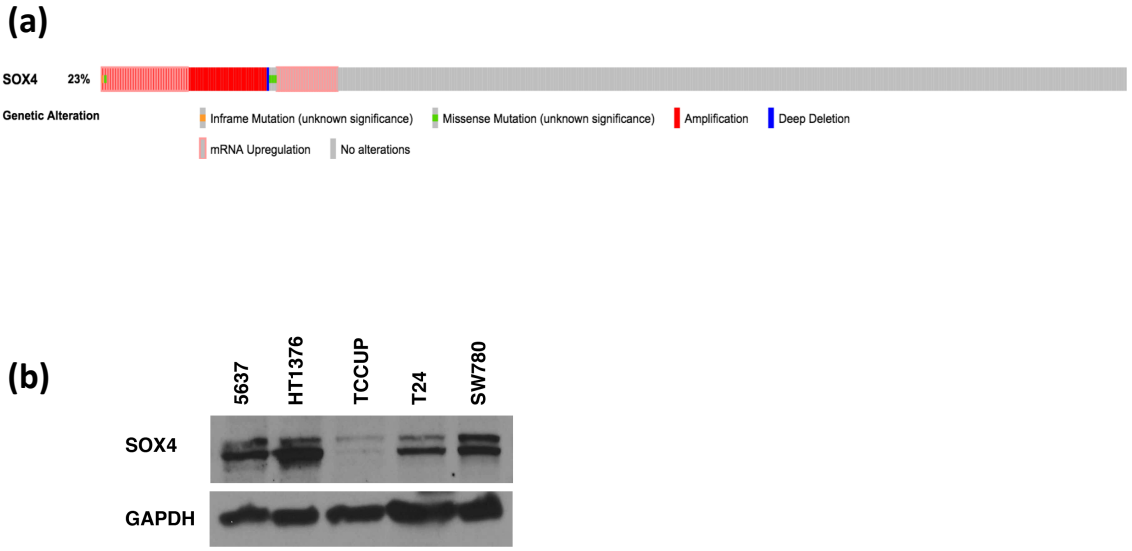
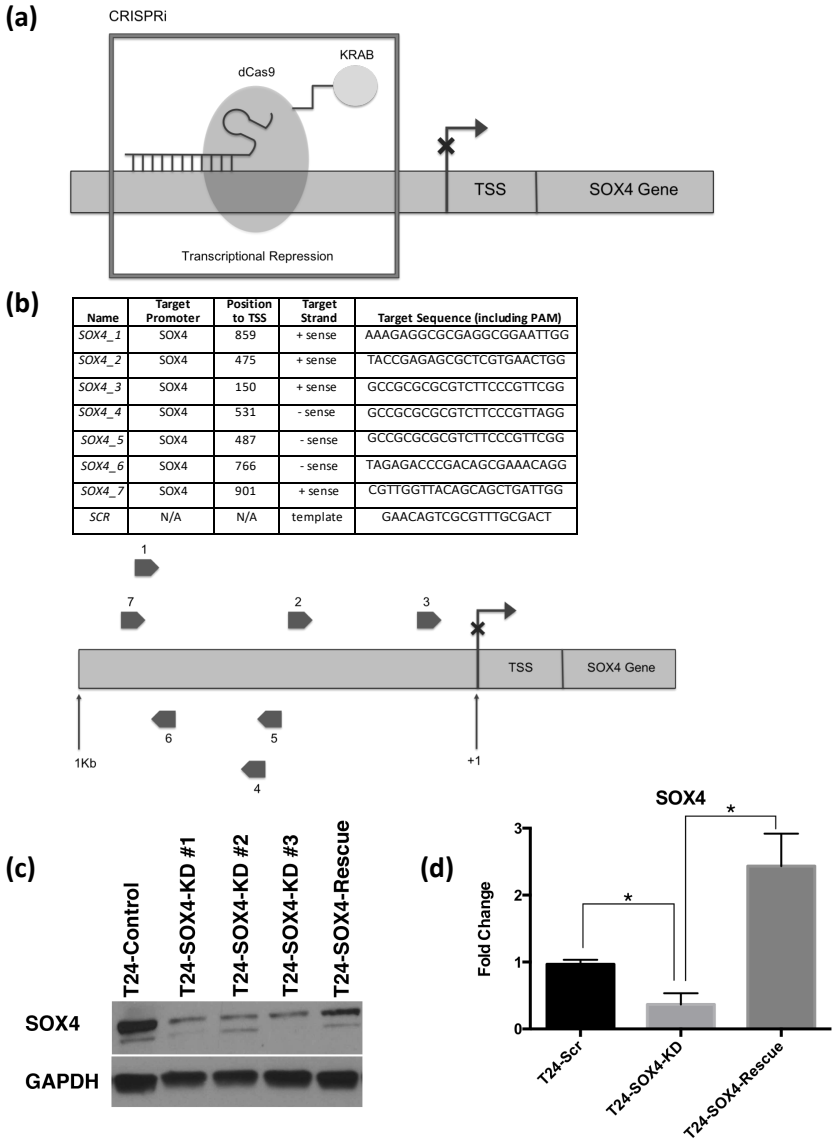


Figure 3.1: (A) cBioPortal data from the *Cell, 2017* [7] dataset showing copy number alterations and mRNA expression levels (+2.0) in 93 out of 404 (23%) patients. (B) Immunoblot of bladder cancer cell lines from ATCC showing varying degrees of SOX4 protein expression.

Generation of stable SOX4 knockdown in T24 bladder cancer cells using CRISPRi

To better understand the function of SOX4 in bladder cancer cells with high expression and/or amplification of SOX4, we performed CRISPR interference (CRISPRi) as previously described [90–92] to induce stable repression of SOX4 mRNA expression by targeting sgRNA's upstream of the SOX4 transcription start site (TSS) (Figure 3.2 A.) This approach uses a catalytically inactivated Cas9 enzyme (dCas9) fused to the KRAB repressor domain (KRAB-dCas9). In this way, the sgRNAs and KRAB-dCas9 act as an RNA-guided DNA binding domain that can both block RNA polymerase and also induce heterochromatin at the SOX4 TSS. Briefly, we stably transduced T24 cells, followed by flow cytometry sorting to enrich for positive mCherry subpopulations to produce T24-SOX4-KD cells (see Methods). Cas9 expression in T24 cell lines was confirmed by western blot using Cas9 antibody (Figure 3.6). We designed seven sgRNAs targeting both the sense and antisense strands corresponding to sites 150 bp-901 bp upstream of the SOX4 TSS (Figure 3.2 B). We prepared lentivirus containing a scrambled sgRNA to create stable T24-Scr negative control cells (Figure 3.2 B). In addition, we transduced T24-SOX4-KD cells with YFP-HA-SOX4 lentiviral constructs to produce stable T24-SOX4-Rescue cells that express SOX4 in the presence of the KRAB-dCas9 and sgRNAs. To confirm SOX4 knockdown and overexpression at the protein levels, we performed western blot on T24-Scr control, T24-SOX4-KD, and T24-SOX4-Rescue cells Figure 3.2C). We further confirmed chanages in SOX4 mRNA via with qRT-PCR (Figure 3.2D).



Name	Target Promoter	Position to TSS	Target Strand	Target Sequence (including PAM)
SOX4_1	SOX4	859	+ sense	AAAGAGGCGCGAGGCGGAATTGG
SOX4_2	SOX4	475	+ sense	TACCGAGAGCGCTCGTGAACTGG
SOX4_3	SOX4	150	+ sense	GCCGCGCGCGTCTTCCCGTTCGG
SOX4_4	SOX4	531	- sense	GCCGCGCGCGTCTTCCCGTTAGG
SOX4_5	SOX4	487	- sense	GCCGCGCGCGTCTTCCCGTTCGG
SOX4_6	SOX4	766	- sense	TAGAGACCCGACAGCGAAACAGG
SOX4_7	SOX4	901	+ sense	CGTTGGTTACAGCAGCTGATTGG
SCR	N/A	N/A	template	GAACAGTCGCGTTTGCGACT

Figure 3.2: (A) CRISPRi model with KRAB effector domain to transcriptionally silence SOX4 at sites upstream of the transcription start site (TSS). (B) Seven sgRNAs targeting the SOX4 TSS at both sense and anti-sense strands and a scrambled sgRNA control are shown. (C) Western blot confirms SOX4 knockdown and SOX4 overexpression at the protein level. (D) q-RT-PCR data confirms decreased SOX4 mRNA expression in T24-SOX4-KD cells and increased SOX4 mRNA in T24-SOX4-Rescue cells compared to T24-Scr controls.

Knockdown of SOX4 in T24 cells do not have altered proliferation rates but are more invasive.

Loss of SOX4 in a variety of cell lines has been shown to decrease proliferation [93, 94]. To investigate the role of SOX4 in proliferation of T24 bladder cancer cell lines, we tested the hypothesis that T24-SOX4-KD would proliferate slower than T24-Scr control. We performed an MTT assay to assess proliferation changes in T24-Scr, T24-SOX4-KD, and T24-SOX4-Rescue cells and observed that SOX4 KD in T24 cells resulted in no significant changes in proliferation compared to controls (Figure 3.3A). Moreover, re-expression of SOX4 by transducing T24-SOX4-KD with lentiviral SOX4 did not alter proliferation rates compared to controls. These data indicate that SOX4 expression levels do not have a substantive effect on proliferation in T24 cells, which is consistent with previous findings in 5637 bladder cell lines [46].

SOX4 has been shown to induce various cellular changes related to invasion, migration and EMT in other cell types [45, 67]. However, western blot analysis indicated no changes in the canonical EMT markers, ZEB1, E-Cadherin, and N-Cadherin, (Figure 3.7) as a result of SOX4 knockdown in T24 cells. We nevertheless investigated the effects of SOX4 knockdown on cellular migration and invasion. Although we observed no changes in migration by scratch-wound assay in T24-SOX4-KD cells (Figure 3.3B), we did observe that T24-SOX4-KD cells exhibited significantly decreased invasion compared to T24-Scr control cells (Figure 3.3 C). Moreover, re-expression of SOX4 in T24-SOX4-Rescue cells restored invasive capabilities to levels similar to T24-Scr controls, but had no effect on migration

(Figure 3.3 B,C).

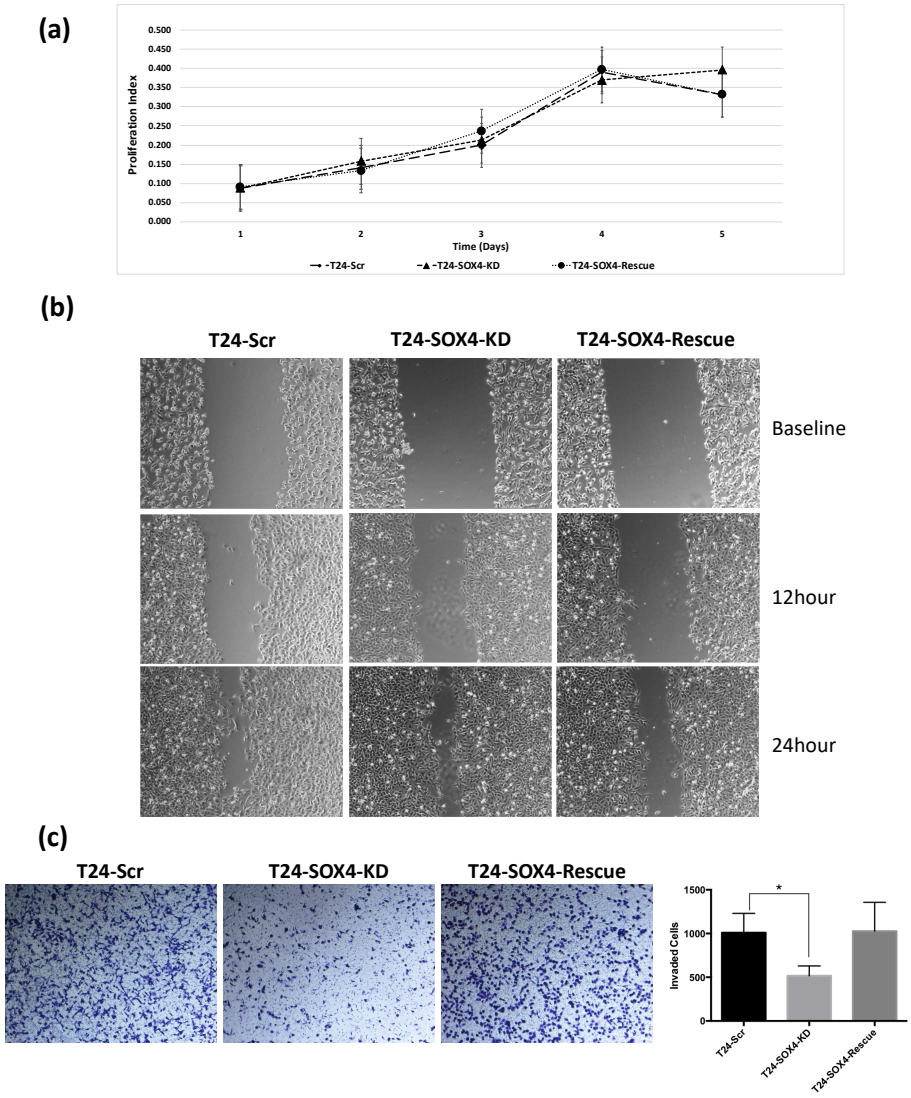


Figure 3.3: (A) Five day MTT assay shows no changes in proliferation as a result of SOX4 knockdown or overexpression compared to scrambled control. (B) Scratch wound assay indicates no change in migratory pattern across all three cell lines. (C) Boyden chamber invasion assay shows de- creased invasive ability in T24-SOX4-KD cells and increased invasion in T24-SOX4-KD-Rescue.

CRISPRi Knockdown of SOX4 and Gene Expression Analysis

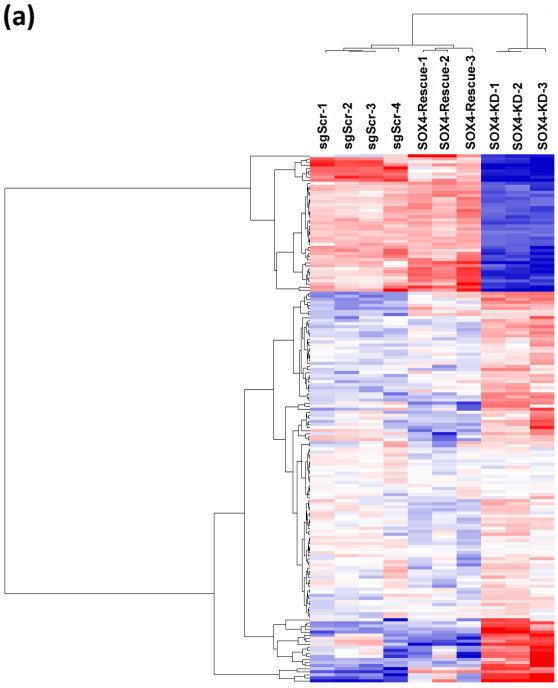
To further understand global transcriptome changes as a result of SOX4 knockdown, we analyzed total RNA from T24-SOX4-KD cells, T24-Scr, and T24-SOX4-Rescue cells using Affymetrix Clariom D microarrays. Whole genome expression profiling analysis identified 1487 genes significantly affected by SOX4 knockdown (FDR 0.05) compared to T24-Scr cells, and 561 genes significantly impacted by SOX4 re-expression (GEO accession number-pending). Ingenuity Pathway Analysis (IPA) between T24-Scr and T24-SOX4 knockdown showed significantly upregulated Osteoarthritis and Wnt/β-catenin signaling (Figure 3.8A). Interestingly the most significantly downregulated pathways in this analysis were associated with cholesterol metabolism (Figure 3.8A). Our qRT-PCR validation of a selection of these cholesterol related genes confirmed the microarray data (Figure 3.9B). To our knowledge SOX4 has not been previously associated with cholesterol biosynthesis pathways and thus this remains an area for future investigation.

We also observed Tenascin C (TNC) as the most significantly upregulated gene in SOX4-KD cell lines and validated this finding via qRT-PCR (Figure Supplemental 3.9C). This potential regulation is supported by our prior data showing TNC as a SOX4 target gene in LNCaP prostate cancer cells [40], although in those cells we observed that SOX4 positively regulates TNC expression rather than represses TNC. This context-dependent difference in SOX4 activity suggests an opposite form of regulation in bladder cancer cell lines that could be due to the availability of other binding partners at the TNC promoter. Additionally, we observed significant decreases in IDO1 mRNA levels upon SOX4 knockdown (Figure 3.9C). IDO1

is critical for immune system evasion in many cancers [95] and recent clinical trials of IDO1 blockers have shown promise in bladder cancer [96].

In order to identify genes regulated by SOX4 with high confidence, we compared the gene expression patterns of T24-SOX4-KD, T24-Scr, and T24-SOX4-Rescue cells. We identified 173 high-confidence genes regulated in opposite directions by SOX4 knockdown and re-expression (Figure 3.4A, complete list see Table 3.2). The top 10 up-regulated and down-regulated genes are shown in (Figure 3.4B). Some of the most significantly up-regulated pathways via IPA analysis were also in the Wnt/β-catenin Signaling and Osteoarthritis pathways (Table 3.1).

(a)



(b)

	Fold Change	
	SOX4-KD vs. SCR	SOX4-Rescue vs. SOX4-KD
Top 10 Up		
TNC	176.67	-24.13
stawswu	78.54	-24.85
STC1	58.52	-38.40
MMP1	50.28	-18.91
IL13RA2	38.79	-6.72
WNT5A	36.39	-25.22
MAN1A1	33.22	-2.90
TNFRSF11B	24.55	-29.00
DNER	24.32	-14.22
PRLR	24.11	-22.05
Top 10 Down		
LYPD1	-23.03	11.09
ZFHX4-AS1	-17.24	20.81
PKP2	-15.58	8.37
FOXA1	-14.36	4.34
SCDP1	-11.33	3.27
LOC101928161	-10.20	3.51
HTR1D	-9.69	3.43
RBPMS2	-8.58	3.85
RGS4	-8.26	19.71
DUSP1	-6.34	4.33

Figure 3.4: (A) Heatmap of 174 genes regulated as a result of SOX4 knockdown and re-expression in T24 cell lines (B) List of Top 10 up-regulated and down-regulated genes from the 174 gene data set.

p-value	Ingenuity Canonical Pathways
2.29E-06	Axonal Guidance Signaling
1.78E-04	Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis
4.07E-04	Human Embryonic Stem Cell Pluripotency
1.41E-03	Basal Cell Carcinoma Signaling
1.45E-03	Colorectal Cancer Metastasis Signaling
1.78E-03	Role of Wnt/GSK-3Beta Signaling in the Pathogenesis of Influenza
2.24E-03	Inhibition of Matrix Metalloproteases
2.88E-03	Ovarian Cancer Signaling
3.09E-03	Osteoarthritis Pathway
6.17E-03	Wnt/Beta-catenin Signaling
7.94E-03	PCP pathway
8.71E-03	Wnt/Ca+ pathway
8.91E-03	Regulation of the Epithelial-Mesenchymal Transition Pathway
9.12E-03	Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency

Table 3.1: IPA analysis of statistically significant upregulated and downregulated pathways in T24 SOX4-KD cell lines compared to T24-SOX4-Rescue.

3.4.2 Wnt5a antagonist restores invasive ability in T24-SOX4-KD Cell line

Among the top 10 putative SOX4-regulated genes was Wnt5a, a non-canonical Wnt pathway ligand and a component of both Wnt/β-catenin Signaling and Osteoarthritis pathways. Moreover, Wnt5a is one of the most statistically significant upregulated genes as a result of SOX4 knockdown and significantly downregulated upon re-expression of SOX4. Wnt5a expression has been shown to decrease the migratory or invasive characteristics in FTC-133 thyroid cell lines and EJ bladder cancer cell lines [97, 98]. We confirmed high expression of Wnt5a in T24-SOX4-KD cell lines compared to T24-Scr and T24-SOX4-Rescue by qRT-PCR (Figure 3.5 A), and hypothesized that Wnt5a might mediate SOX4's effects by inhibiting cellular invasion of T24 cells. We tested this hypothesis by treating T24-SOX4-KD cells with a Wnt5a peptide antagonist, BOX5, for 24 hours as previously described [86]. Treatment of T24-SOX4-KD cells with Wnt5a antagonist significantly increased invasiveness to levels comparable to T24-Scr (Figure 3.5 B). These data suggest that SOX4 may inhibit Wnt5a expression in T24 cells directly or indirectly, and that high Wnt5a levels inhibit invasion in T24 bladder cancer cell lines.

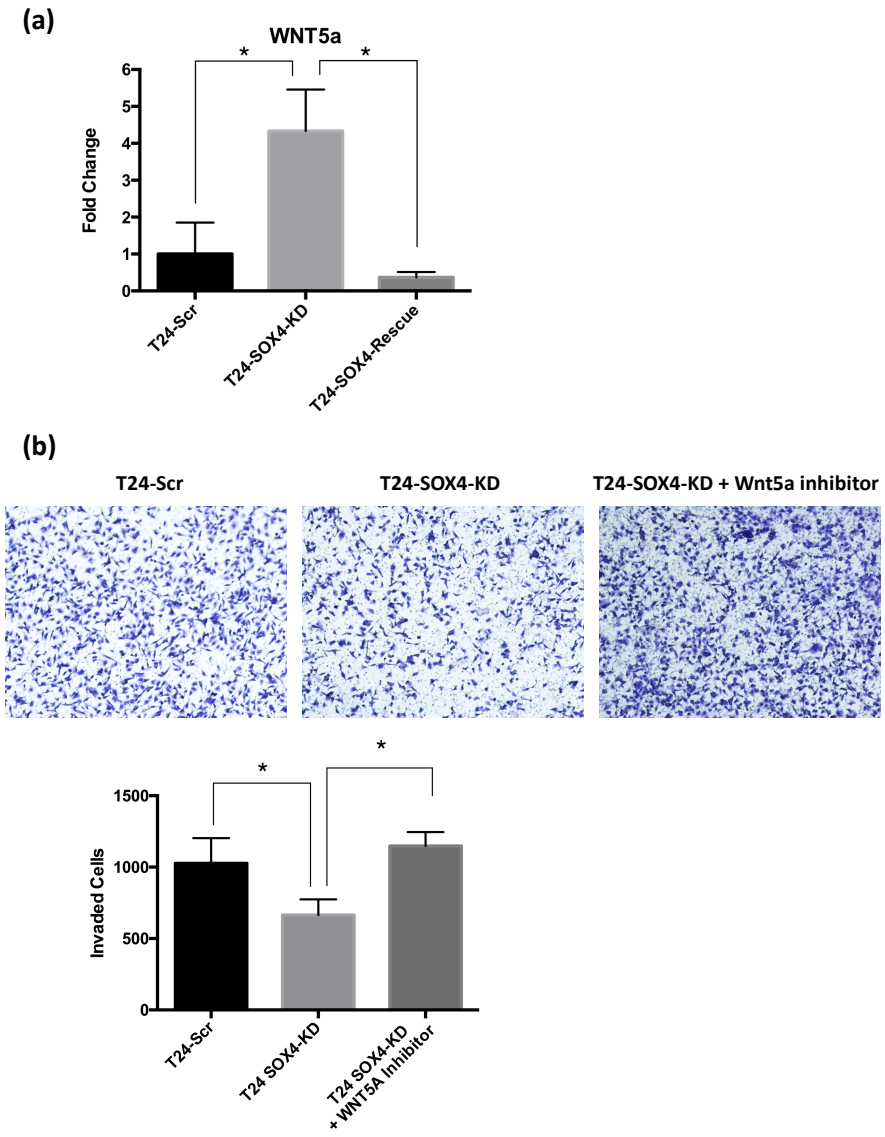


Figure 3.5: (A) qRT-PCR validation of microarray for Wnt5a mRNA expression across our T24 bladder cancer cell line samples (B) Boyden chamber invasion assay shows increased invasive ability in T24-SOX-KD cells treated with 200um of Wnt5a antagonist.

3.5 Discussion

While the precise function of SOX4 in bladder cancer is not yet well understood, the observation that it is overexpressed in as many as 23% of bladder cancer patients strongly supports the case for continued research into the role of SOX4 in bladder cancer. While it is well established that SOX4 expression is increased in many bladder cancer patients, some studies are in disagreement regarding associations between SOX4 expression levels and tumorigenicity, and tumor stage or grade [18, 19], and there is not yet a consensus as to whether SOX4 expression is tumor protective or tumor promoting in bladder cancer patients. Tissue microarray analysis of 309 transitional cell carcinoma supported an oncogenic role for SOX4 since high SOX4 expression tracked with worse patient survival and was enriched in muscle-invasive patients [18]. These data were contradicted by tissue microarray from Aaboe et al., which showed that although SOX4 might be an early event in tumorigenesis, there was no association between SOX4 expression levels and tumor stage [19]. Moreover, patients with strong SOX4 expression, either cytoplasmic or nuclear, exhibited increased survival, suggesting a tumor suppressive role [19]. Discrepancies between these two immunohistochemical studies could be due to the fact that they used different SOX4 antibodies, and the possibility of cross-reactivity with other SOX family proteins.

In this study, we aimed to elucidate the role of SOX4 and to identify high confidence SOX4-regulated genes in T24 bladder cancer cells. We used a CRISPRi + re-expression system to identify 173 high confidence SOX4 regulated genes by whole genome expression profiling. Some of the most significantly altered pathways in-

cluded the basal cell carcinoma, colorectal cancer metastasis, WNT/β-catenin, and Osteoarthritis pathways. Although neither SOX4 knockdown nor re-expression changed proliferation rates in T24 cell lines compared to scrambled controls, we did observe a marked decrease in matrigel invasion as a result of SOX4-KD and a restored invasion upon SOX4 re-expression. Whole genome expression profiling suggested that the non-canonical Wnt5a pathway could play a critical role as mediator of SOX4's effects on invasion.

Surprisingly, IPA analysis of genes significantly changed between the scrambled control and the SOX4 KD group revealed the most significantly downregulated pathway was regulation of cholesterol biosynthesis. The most significantly downregulated genes in this group included TM7SF2, DHCR7 and MVD. However, the expression of this pathway was not rescued in the SOX4 overexpressed groups. There are a number of potential reasons for the lack of rescue for these genes. For example, the HA-tag fused to the amino-terminus of SOX4 could interfere with putative SOX4 binding partners, and the availability of co-activators may be limiting such that the reintroduction of SOX4 alone might not be sufficient to restore expression. This represents a limitation in our study not only for cholesterol biosynthesis pathway genes but also for other genes putatively regulated by SOX4. Interestingly, previous studies support cholesterol biosynthesis in maintaining the mesenchymal state [99, 100], and thus this observation represents an area of great interest for further research.

Previous work with SOX4 in prostate cancer cells identified TNC as a direct target of SOX4 [40]. Interestingly, the microarray data in this study revealed TNC as

the most significantly upregulated gene in response to SOX4 KD, and TNC levels decreased significantly when rescued with SOX4. These findings stand in contrast to our previous observations in LNCaP prostate cancer cells that suggest that SOX4 positively drives TNC expression, but are consistent with the finding that TNC is a target of SOX4. This observation could be due to context/cell line dependent differences in transcriptional networks and availability of co-factors that in one context function as activators and then as repressors in another context.

Interestingly, our data corroborates previous results [19] using transient expression of SOX4 in SOX4-null HU609 bladder cancer cell lines. The most prominent genes upregulated by SOX4 in that study included ZNF195, EFNA4, and CGI-62. Our data confirmed SOX4 positive regulation of both ZNF195 and EFNA4 and repression of NRP2 [19]. However, we did not observe increased cell death as a result of SOX4 expression.

While the effects of SOX4 on canonical WNT/β-catenin signaling have been extensively studied, the effects of SOX4 on non-canonical WNT signaling are less clear. Here we identified Wnt5a as one of the most significantly regulated genes affected in T24 SOX4 knockdown (+36.39 fold) and T24-SOX4-Rescue (-25.21 fold) cell lines, which we confirmed via qRT-PCR. Importantly, treatment of T24-SOX4-KD with Wnt5a antagonist restored the invasive phenotype to levels comparable to T24-Scr cells. The Wnt5a signaling pathway has two well established arms: the planar cell polarity and Ca2+ signaling pathways [101]. Further research will be needed to evaluate which arm of the Wnt5a pathway is active in T24-SOX4-KD cells.

Our observation that Wnt5a may have a tumor suppressive effect by means of decreasing invasion is consistent with previous studies in thyroid carcinoma cells in which Wnt5a inhibited migration, invasion, and proliferation [97]. Similarly, Wnt5a has been shown to impair migration in breast epithelial wcells [102]. Moreover, patients with increased Wnt5a expression in Dukes B colon carcinomas showed improved 5- and 10-year survival rates compared to patients with loss of Wnt5a [103]. Similarly, prostate cancer patients with low-grade localized disease and high Wnt5a expression post-surgery had a much better outcomes than patients with low Wnt5a expression [104]. Consistent with our data in SOX4-KD and SOX4-Rescue cells, siRNA knockdown of Wnt5a increased the invasive ability of LNCAP and 22RV1 prostate cancer cells [104]

In contrast to our results, a previous study showed that the SOXC family of transcription factors (SOX4, SOX11 and SOX12) positively regulate Wnt5a expression in mouse growth plate chondrocytes, although this was mostly driven by SOX11 and no direct regulation was demonstrated [105].Interestingly, our microarray data showed no significant gene expression changes in SOX11 or SOX12, suggesting that SOX4 can regulate Wnt5a without changes in other SOXC family members. Promoter sequence analysis did not identify any obvious SOX4 binding sites in Wnt5a regulatory regions, and thus it is likely that SOX4 may indirectly regulate Wnt5a expression. Further studies will be needed to adequately evaluate the mechanism by which SOX4 regulates Wnt5a either directly or indirectly.

In summary, our findings suggest a mechanism by which SOX4 contributes to

overall tumor aggressiveness in bladder cancer by modulating cellular invasion. Taken together, these data provide further evidence of a tumor promoting role for SOX4 and a tumor suppressive mechanism of Wnt5a and suggest a novel mechanism of SOX4 regulation of non-canonical Wnt signaling. While effects of SOX4 on activated canonical WNT signaling through β-catenin have been well established [106], this is the first study to demonstrate that SOX4 might repress non-canonical Wnt5a signaling in bladder cancer cells.

Supplementary Figures

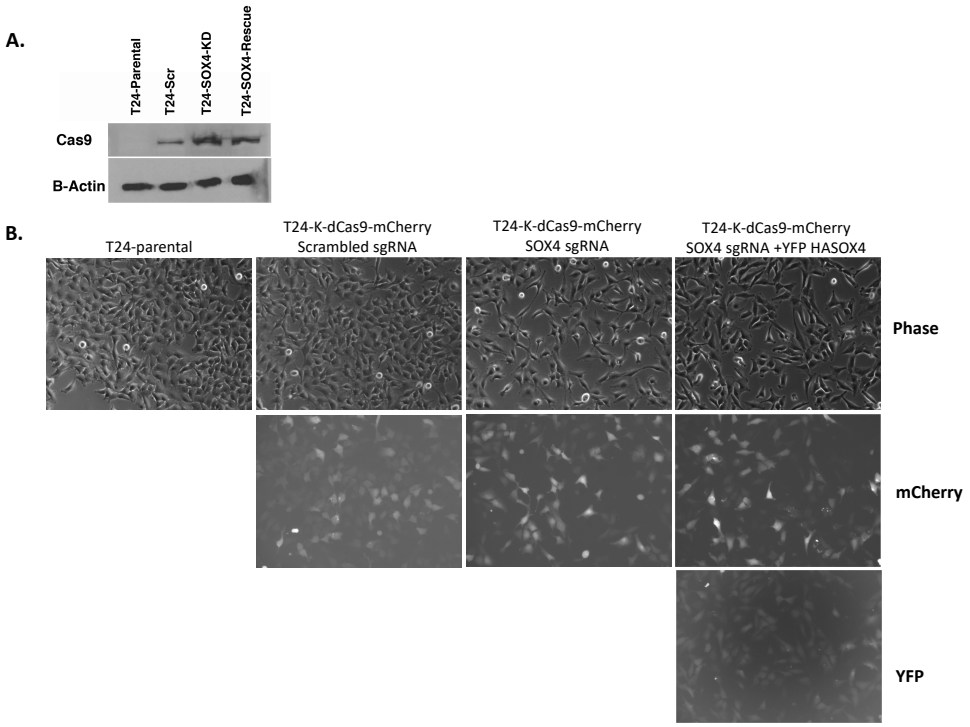


Figure 3.6: **Supplementary Figure 1**(A) Western blot indicating Cas9 expression in T24-Scrambled, T24-Sox4-KD and T24-SOX4-Rescue compared to T24 parental. (B) Phase and fluorescent imaging showing K-dCas9-mcherry expression in T24-Scr, T24-SOX4-KD and dual mCherry +/YFP + in T24-SOX4-Rescue.

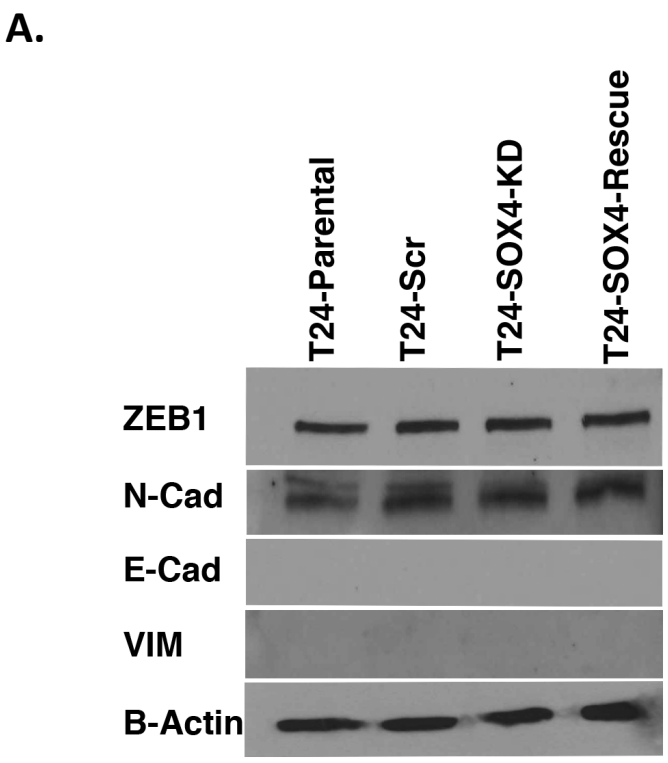


Figure 3.7: **Supplementary Figure 2**(A) SOX4-KD revealed no changes in EMT markers ZEB1 or N-Cadherin. T24 cell lines do no express E-Cadherin.

A.

Figure 3.8: **Supplementary Figure 3A** IPA analysis reveals cholesterol genes most significantly deregulated along with Wnt/β-catenin and Osteoarthritis Pathways.

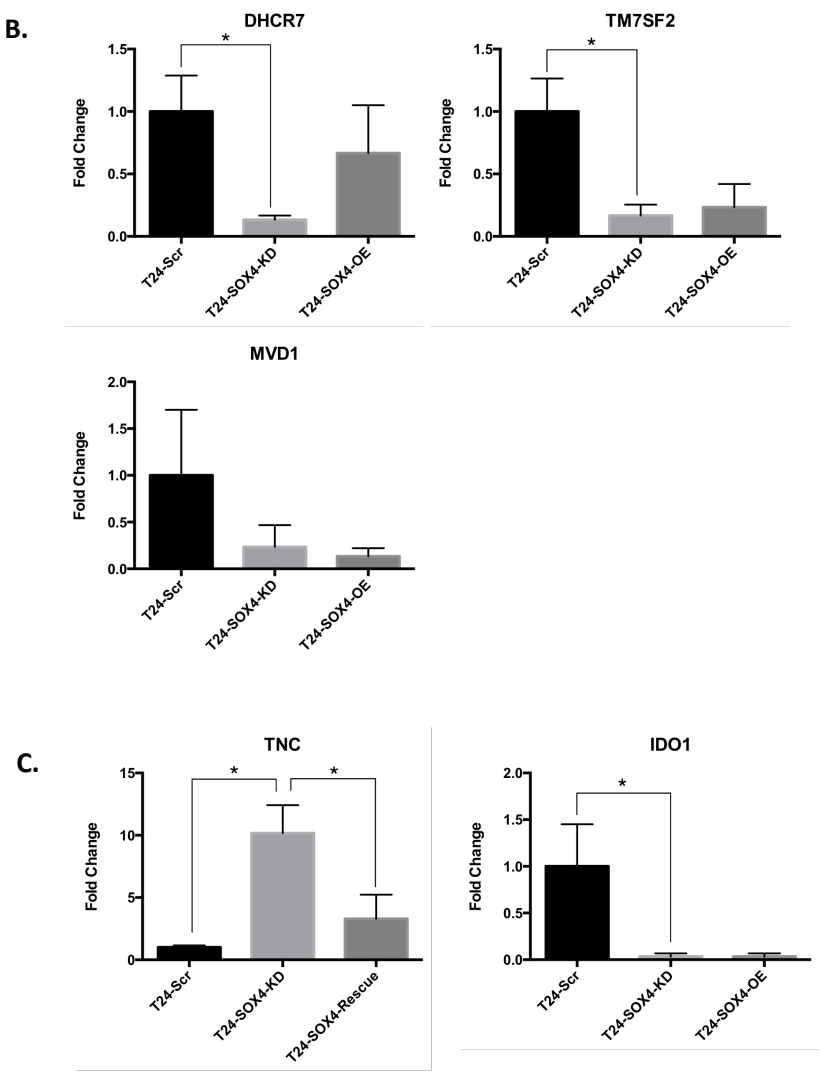


Figure 3.9: **Supplementary Figure 3B,C** (B)qRT-PCR validation of Clariom D microarray for selected number of cholesterol related genes confirms repressed mRNA in T24-SOX4-KD and confirms no changes in mRNA expression of cholesterol genes in T24-SOX4-Rescue. (C) qRT-PCR of TNC and IDO validates microarray data. * = p-value ¡0.05.

Table 3.2: 173 High-Confidence SOX4 Regulated Genes.

Gene Name	Probe ID	Fold Change SOX4-KD vs SCR	q-value(%)	Fold Change SOX4-Rescue vs SOX4-KD
LYPD1	TC0200014361.hg.1	-23.0266266	0	11.09380218
ZFHX4-AS1	TC0800012431.hg.1	-17.23853179	0	20.8106819
PKP2	TC1200010341.hg.1	-15.58371723	0	8.372696519
FOXA1	TC1400008981.hg.1	-14.35617325	0	4.338394761
SCDP1	TC1700007240.hg.1	-11.33267211	0	3.272656082
LOC101928161	TC0200009393.hg.1	-10.1967854	0.333076753	3.510756654
HTR1D	TC0100013272.hg.1	-9.691857324	0	3.431253383
RBPMS2	TC1500009756.hg.1	-8.579926764	0	3.849607433
RGS4	TC0100010393.hg.1	-8.264178028	1.607801004	19.70895893
DUSP1	TC0500012842.hg.1	-6.341829832	0.333076753	4.327658523
CYFIP2	TC0500009211.hg.1	-5.227514227	0	4.788383383
SCD5	TC0400011180.hg.1	-4.753833913	0	2.804597361
MEST	TC0700009145.hg.1	-4.588190629	0.333076753	12.83282595
ADGRG6	TC0600009669.hg.1	-4.249182084	0.333076753	3.775569654
RASSF2	TC2000008267.hg.1	-4.057536295	0	10.03625644
SMAD6	TC1500007619.hg.1	-4.014352346	0	3.706947458
TLE4	TC0900007680.hg.1	-3.588439672	0	10.18814858
RNF122	TC0800010081.hg.1	-3.375607506	0.333076753	5.154682852
SCARB1	TC1200012327.hg.1	-3.367382642	0	4.09312956
RP11-80B9.1	TC0100012158.hg.1	-3.179099674	0.333076753	6.175143516
NETO1	TC1800009011.hg.1	-3.150802517	3.845686185	6.110978195
EVL	TC1400008193.hg.1	-2.867143607	0.407942629	2.85417079
GP1BB	TC2200006614.hg.1	-2.824292806	1.037101959	4.86570549
CYP2S1	TC1900008141.hg.1	-2.820970019	1.037101959	8.586429464
kleylaw	TC0900007683.hg.1	-2.583232804	1.037101959	7.334657843
PTGER4	TC0500007231.hg.1	-2.566190016	1.607801004	3.46415624
ZFHX4	TC0800008011.hg.1	-2.50232527	0.664908359	4.166533751
GAB2	TC1100011744.hg.1	-2.493958283	0.333076753	2.52447665
GARNL3	TC0900012173.hg.1	-2.492062066	0.333076753	2.882514769
TCF7L1	TC0200008236.hg.1	-2.392933686	3.845686185	2.717667582
EPHB4	TC0700012016.hg.1	-2.378166679	1.607801004	3.606314791
FNBP1L	TC0100009064.hg.1	-2.295552881	0.664908359	3.409026939
CTD-2022H16.3	TSUnmapped00000551.hg.1	-2.281694938	2.472534784	3.104655086
41698	TC0100011665.hg.1	-2.275536667	3.845686185	3.812203146
PTX3	TC0300009301.hg.1	-2.208610733	1.607801004	2.765301272
ANKRD1	TC1000011400.hg.1	-2.185797751	3.845686185	18.27652646
FAM60BP	TC1800008335.hg.1	-2.18189549	3.845686185	2.346307336
FZD3	TC0800007137.hg.1	-2.169402879	0.407942629	3.184249393
ZSCAN2	TC1500008154.hg.1	-2.133709841	0.407942629	3.335953857
LMO7	TC1300009980.hg.1	-2.06197356	2.472534784	3.780376698
SMO	TC0700009082.hg.1	-2.035855854	2.472534784	5.492855476
PRR3	TC0600007534.hg.1	-1.945878772	2.472534784	2.365397534
KIAA1958	TC0900008467.hg.1	-1.934780184	2.472534784	3.771417847
LBR	TC0100017488.hg.1	-1.91663307	1.037101959	2.159698617
\	TC1100008409.hg.1	-1.711243427	2.472534784	3.356932047
EIF5A2	TC0300013104.hg.1	1.737414119	2.472534784	-2.400586076
—	TC0900008537.hg.1	1.937475857	1.607801004	-2.599042094
TSPAN4	TC1100006495.hg.1	1.93859643	1.607801004	-2.198358488
sugyby	TC1100006732.hg.1	1.986699032	1.607801004	-3.818960505
kuchoybu	TC0700010064.hg.1	2.001569207	2.472534784	-2.855411492
DSE	TC0600014177.hg.1	2.010910794	1.037101959	-2.734369734
NRP1	TC1000010273.hg.1	2.081530936	0.664908359	-2.627984318
ACSL5	TC1000008926.hg.1	2.102203743	2.472534784	-3.550420739
ZHX3	TC2000009128.hg.1	2.192891097	1.037101959	-2.013859916
FAM225A	TC0900008481.hg.1	2.205574766	3.845686185	-2.490885432
zarvo	TC1400008679.hg.1	2.21819034	1.037101959	-2.428384158
CD109	TC0600008539.hg.1	2.294521513	3.845686185	-3.1373359
LAPTM5	TC0100013534.hg.1	2.351031985	0.263891671	-2.627365054
RP11-20B7.1	TC0300007852.hg.1	2.375454517	0.664908359	-2.714266415
SNAI2	TC0800010382.hg.1	2.384711928	0.407942629	-2.961674997
RFX8	TC0200013669.hg.1	2.397950287	0.664908359	-3.817518989
IL4R	TC1600007312.hg.1	2.400136156	0.407942629	-3.810310764
PDE4DIP	TC0100009837.hg.1	2.414403273	1.037101959	-2.1064534
SETBP1	TC1800007186.hg.1	2.444351905	0.664908359	-3.752495478
MCOLN3	TC0100014769.hg.1	2.447356466	1.607801004	-3.534188525
HMGN5	TC0X00010172.hg.1	2.458818367	0.664908359	-2.521055712
POF1B	TC0X00010207.hg.1	2.489043453	0	-2.463035918
HOMER2	TC1500010251.hg.1	2.49540532	1.607801004	-3.354660261
P2RY2	TC1100008378.hg.1	2.552079168	1.037101959	-2.982607217
POPDC3	TC0600012709.hg.1	2.603757338	0.263891671	-3.799992752
TMOD1	TC0900008150.hg.1	2.672898863	3.845686185	-3.589540893
SLC37A2	TC1100009408.hg.1	2.691633533	2.472534784	-9.340574801
ADTRP	TC0600010825.hg.1	2.839546263	3.845686185	-3.963853565
CPQ	TC0800008300.hg.1	2.847823036	0	-2.232919127
NRP2	TC0200010545.hg.1	2.885496338	1.607801004	-5.574613049
TESPA1	TC1200010850.hg.1	2.959086254	1.607801004	-2.421906775
KCNK3	TC0200007042.hg.1	2.9604714	2.472534784	-6.693236539
IL31RA	TC0500007432.hg.1	2.980985794	3.845686185	-4.135283771
FAM198B	TC0400012245.hg.1	2.999820479	2.472534784	-3.773827118
EPHA4	TC0200015815.hg.1	3.081912353	2.472534784	-4.873789696
TFAP2C	TC2000007830.hg.1	3.110193126	3.845686185	-2.657126046
SLIT2	TC0400007016.hg.1	3.115184441	0	-6.251138642
RNU6-917P	TC2000008163.hg.1	3.150359566	2.472534784	-3.352858658
SLC16A2	TC0X00007655.hg.1	3.239534263	0	-5.520350997
MLPH	TC0200011251.hg.1	3.251494725	3.845686185	-9.51435375
OSBPL6	TC0200016571.hg.1	3.316747456	0	-4.542692549
TMEM255B	TC1300010007.hg.1	3.329144135	0.263891671	-2.689522391
RP11-752L20.3	TC0400008944.hg.1	3.383256363	0.263891671	-4.351993293

Continued on next page

Table 3.2 – continued from previous page

Gene Name	Probe ID	Fold Change SOX4-KD vs SCR	q-value(%)	Fold Change SOX4-Rescue vs SOX4-KD
CYP4F11	TC1900009896.hg.1	3.383972279	0.664908359	-5.003227312
TPK1	TC0700012917.hg.1	3.397318945	1.607801004	-4.415715835
MMP14	TC1400006659.hg.1	3.458736242	0	-3.700602046
MYO1D	TC1700010358.hg.1	3.515270803	0.263891671	-3.710751353
PRKG2	TC0400011159.hg.1	3.549754701	0.263891671	-2.901666404
ANKH	TC0500010169.hg.1	3.571234823	0	-3.184100781
LAMB3	TC0100017167.hg.1	3.577594809	0	-5.333613683
EPHB1	TC0300013877.hg.1	3.690665535	0	-2.961139929
RP5-875H18.9	TC1700008231.hg.1	3.755191765	0.407942629	-3.100149237
SH3PXD2A	TC1000011726.hg.1	3.755557057	1.607801004	-4.208104894
PHACTR1	TC0600006976.hg.1	3.774805996	0	-2.997393165
ATP2B4	TC0100011267.hg.1	3.931021261	1.037101959	-4.072857485
BCL2A1	TC1500010184.hg.1	3.934076468	0.263891671	-6.55176828
NTNG1	TC0100009307.hg.1	3.934092375	0	-5.340743756
CLMP	TC1100012615.hg.1	4.015123415	1.037101959	-5.549412324
CADPS2	TC0700012444.hg.1	4.0976618	1.037101959	-4.102303793
ATP2B1	TC1200011474.hg.1	4.245077326	0.263891671	-3.699524877
CR1L	TC0100011417.hg.1	4.26752469	1.037101959	-6.779162249
KCND2	TC0700008918.hg.1	4.274053104	1.607801004	-5.918228229
CD68	TC1700012191.hg.1	4.28349083	0	-5.502073199
PREX1	TC2000009357.hg.1	4.2897146	0	-3.858793924
MME	TC0300009258.hg.1	4.341583758	0.407942629	-3.484983116
MTSS1	TC0800011713.hg.1	4.393003808	0	-5.16911154
GLT8D2	TC1200011752.hg.1	4.42430241	0.263891671	-13.02776774
SEL1L3	TC0400010282.hg.1	4.473816758	1.037101959	-5.025555244
PPP4R4	TC1400008058.hg.1	4.499254318	0	-4.385510122
CSTA	TC0300008550.hg.1	4.541076214	0	-4.451465373
STEAP2	TC0700008293.hg.1	4.55836121	1.037101959	-4.360839823
PROX1	TC0100011566.hg.1	4.643514318	0	-4.491378582
DPP4	TC0200014764.hg.1	4.789068817	1.607801004	-5.917287532
—	TC0600009216.hg.1	4.881460984	0.263891671	-3.078614728
KCNAB2	TC0100006675.hg.1	4.909964465	0	-2.097048168
STEAP1B	TC0700010443.hg.1	5.329541475	0	-4.007269857
PAG1	TC0800010926.hg.1	5.414377269	0	-3.330535901
CAMK2N1	TC0100013182.hg.1	5.532449332	0	-7.234500113
PPP1R14C	TC0600009831.hg.1	5.582812099	0	-4.254693874
EDNRA	TC0400008943.hg.1	5.744239226	0.407942629	-11.22627189
SIRPA	TC2000006501.hg.1	5.881272484	0	-3.48892681
TRIML2	TC0400012696.hg.1	5.921229752	0	-3.138032585
—	TC1900011214.hg.1	6.16400237	0	-4.450466804
G0S2	TC0100011453.hg.1	6.23476781	0	-3.556266234
SIRPD	TC2000009969.hg.1	6.292151175	0.407942629	-10.36716363
CD82	TC1100007394.hg.1	6.422328677	0	-23.59947729
VDR	TC1200010559.hg.1	6.424627978	1.607801004	-10.46185422
MAGEC2	TC0X00011001.hg.1	6.54786427	0	-1.935215192
—	TC0300011389.hg.1	6.721417021	0	-6.022566144
RAB27B	TC1800007360.hg.1	6.74721827	0	-8.690087278
BACE2	TC2100007198.hg.1	6.974796702	0	-6.06058196
XDH	TC0200012163.hg.1	7.272799228	0	-4.21040788
—	TC0900008601.hg.1	7.421623167	0.407942629	-4.96236894
STEAP1	TC0700008292.hg.1	8.129718603	0	-6.031375262
SIRPB1	TC2000009970.hg.1	8.146530884	0.263891671	-31.66886666
RIMS2	TC0800008487.hg.1	8.212628278	0	-5.471987748
THBS2	TC0600013998.hg.1	8.610560089	0	-7.779556975
LINC00973	TC0300008123.hg.1	8.62080843	0	-3.695842644
SLC16A6	TC1700011558.hg.1	8.637526111	1.607801004	-26.92650976
PTPRU	TC0100007584.hg.1	8.655144064	0	-10.12716179
FEZ1	TC1100012708.hg.1	9.233914239	0	-8.743379382
NTRK2	TC0900007752.hg.1	9.533549806	0.263891671	-11.02260259
reniru	TC2000008164.hg.1	9.888974482	0	-8.346515341
SLC14A1	TC1800007198.hg.1	10.41461005	0	-86.26066901
DUSP10	TC0100017420.hg.1	10.4417968	0	-8.818196751
starawbo	TC0100013053.hg.1	11.28481477	0	-3.637540556
NOV	TC0800008667.hg.1	11.93727181	2.472534784	-10.97107254
AOX1	TC0200010421.hg.1	13.0955296	0	-6.792429407
ADAMTS9	TC0300011391.hg.1	13.83054481	0	-17.27061238
WFDC2	TC2000007492.hg.1	14.3234521	0.664908359	-16.04767068
ITGA11	TC1500009861.hg.1	15.8731476	0.263891671	-35.12455396
—	TC2000009931.hg.1	16.00313241	0	-4.557135707
SSX1	TC0X00007176.hg.1	16.66698218	0	-7.074420624
TSPAN18	TC1100007400.hg.1	17.20424588	0	-16.16090405
PARM1	TC0400007868.hg.1	17.7883721	0	-10.60940615
CTSS	TC0100015752.hg.1	18.93459511	0	-33.90806768
—	TC2000009329.hg.1	19.19431053	0	-5.900531854
SEMA5A	TC0500010043.hg.1	22.73633009	0.407942629	-21.15614212
PRLR	TC0500010480.hg.1	24.11042901	0	-22.05171058
DNER	TC0200015958.hg.1	24.31982878	0	-14.22025075
TNFRSF11B	TC0800011611.hg.1	24.55307678	0	-28.99905535
MAN1A1	TC0600013010.hg.1	33.21966225	0	-2.903634776
WNT5A	TC0300011247.hg.1	36.39312838	0	-25.21837948
IL13RA2	TC0X00010557.hg.1	38.79299479	0	-6.721115362
MMP1	TC1100012131.hg.1	50.28268382	0	-18.91144023
STC1	TC0800009891.hg.1	58.5209824	0	-38.39723889
stawswu	TC0900008530.hg.1	78.5390731	0	-24.85262495
TNC	TC0900011305.hg.1	176.6674056	0	-24.13134349

Author Contributions Author Contributions: Conceptualization, J.D.M. and C.S.M.; Methodology, J.D.M.; Software, J.D.M. and C.S.M.; Validation, J.D.M., Z.L., C.S.M.; Formal Analysis, J.D.M., Z.L., and C.S.M.; Investigation, J.D.M., H.K. and Z.L.; Data Curation, J.D.M.,H.K., Z.L .; Writing-Original Draft Preparation, J.D.M.; Writing-Review & Editing, J.D.M. and C.S.M.; Visualization, J.D.M. and H.K.; Supervision, C.S.M.; Project Administration, J.D.M. and C.S.M.,, please turn to the CRediT taxonomy for the term explanation.

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Chapter 4 Novel Protein-Protein Interactions in Bladder Cancer Cell Lines

This chapter is centered around the novel SOX4 and P16 interaction and represents the extent of my unpublished work

4.1 Abstract

Identification of oncogenic drivers – the genetic changes that provide a cancer with an important selective advantage to survive and proliferate – is of pivotal importance in cancer research. Identifying and validating oncogenic drivers in cancer can provide a means for developing targeted therapies. This method has already seen successes for breast and lung cancers. However, scientific advances to find targeted therapies in bladder cancer have not yet been successful. Analysis of The Cancer Genome Atlas (TCGA) data revealed that the single most significant focal amplification in bladder cancer patients encompasses the SOX4 and E2F3 genes. SOX4 is a transcription factor that is overexpressed in a variety of tumors including lymphoma, prostate and bladder cancer. E2F3 is also a transcription factor that, when activated, promotes progression through the cell cycle. However, the effects of overexpressed SOX4 and E2F3 alone or in combination are not fully understood and while they are both located on the same 6p22 amplified locus it is not known if both are necessary for tumorigenicity. Our recent collaboration with the Fu lab at Emory University revealed novel SOX4 Protein-Protein Interactions Protein-Protein Interaction (PPI) with CDKN2A [107] – a cyclin-dependent kinase inhibitor and tumor suppressor protein. Interestingly, TCGA data shows

that SOX4 amplification and CDKN2A deletions exhibit near perfect mutual exclusivity, suggesting that these proteins act in the same pathway, and that they may negatively regulate each other. Here we demonstrate a novel endogenous SOX4 co-immunoprecipitation with CDKN2A. This novel PPI may lead to deregulation of the cell cycle and permit growth and uncontrolled proliferation. In addition to elucidating novel SOX4 PPI's in bladder cancer cell lines, we sought to establish tumorigenicity of a bladder cell line *in vivo* using an orthotopic mouse models injected with SW780 cell lines stably expressing luciferase. Additionally, we demonstrated in initial cell cycle experiments that SOX4 might sequester CDKN2A to inhibit G1/S arrest, thereby deregulating cell cycle checkpoints and promoting tumorigenesis. These data suggest a novel role for SOX4 that is independent of its transcription factor ability to promote tumorigenesis. While further experiments and validation are necessary, this research could implicate SOX4 as a drug target for novel treatment approaches for bladder cancer patients.

4.2 Introduction

Advanced bladder cancer represents an unmet medical need in which new molecular targets could serve as stratification markers and consequently improve patient outcomes. In the United States there are an estimated 79,030 new cases of bladder cancer each year resulting in 16,870 deaths annually [3]. Fortunately, combination chemotherapy regimens can be successful in many bladder cancer patients who present at an early stage. However, absent a few emerging immunotherapy options, few novel small molecule treatments exist in the metastatic or relapse/refractory setting and as a result mortality rates have not improved

over the last few years [5]. Perturbed transcription factor signaling is a pivotal step in the etiology of many cancers [108, 109]. The SOX4 transcription factor is overexpressed in a variety of tumors including prostate, leukemia, pediatric medulloblastoma, melanoma, lung, and bladder cancers [6, 16, 43]. Our laboratory has extensively characterized SOX4 as a transforming oncogene in non-transformed prostate cell lines [27]. However, the direct role of SOX4 in bladder cancer has yet to be defined. Thus, understanding downstream perturbed signaling networks that lead to unregulated growth and cancer cell survival is of great importance in bladder cancer.

Recently, The Cancer Genome Atlas (TCGA) research network released a comprehensive genomic analysis of 412 urothelial carcinomas [7]. This TCGA data on muscle invasive bladder cancer revealed that SOX4 is altered in 23% of primary bladder carcinomas through either copy number aberrations (CNA) via focal amplification of 6p22 or increased mRNA expression as shown from RNA-seq analysis, thus implicating SOX4 as a major contributor to bladder cancer tumorigenesis.

CDKN2A, E2F3 and Cancer

The cyclin-dependent kinase inhibitor 2A (CDKN2A), also known as p16 or p16INK4A, is a small 16kD protein that promotes cell cycle arrest, and restricts cell growth and proliferation by binding to cyclin-dependent kinases 4 (CDK4) and 6 (CDK6). This binding blocks CDK4/CDK6 from complexing with Cyclin D. CDK4/CDK6 and Cyclin D form an active kinase complex capable of phosphorylating Retinoblastoma protein, RB [110]. RB is a tumor suppressor protein

that when phosphorylated cannot bind E2F3 [111]. E2F3 is part of the E2F family of transcription factors that, when not bound to RB, promote G1 phase cell cycle progression by binding to DNA promoters of cell cycle target genes critical for DNA replication, DNA synthesis, and mitosis [112]. Unphosphorylated RB negatively regulates E2F3 and thereby causes G1 arrest [113]. The role of E2F3, RB and CDKN2A in cancer is well understood and this pathway is disrupted in a variety of ways. Overexpression of E2F3 is found in many cancers including ovarian and uterine cancer [21, 22]. In bladder cancer E2F3 is amplified or overexpressed in 25% of tumors. Additionally, viral oncoproteins such as E1A are known to disrupt the interaction of a non-mutated RB (RB-positive) with E2F3 by binding to RB [114]; this interaction can also be disrupted via deleted/mutated RB [115]. If CDKN2A is deleted or sequestered by another protein, this allows CDK4/CDK6 and Cyclin D to phosphorylate RB, thus permitting E2F3 to up-regulate target genes for cell cycle progression. Because of CDKN2A's ability to halt the cell cycle, it is considered a tumor suppressor protein, and deregulation of this delicate pathway can result in tumorigenesis [116, 117]. In some cancers, such as head and neck cancer, patients who are positive for CDKN2A expression often exhibit improved survival [118]. Furthermore, CDKN2A is mutated or deleted in a variety of cancers including stomach, pancreas, head & neck, and melanoma [21, 22].

Here we establish a novel protein-protein interaction between SOX4 and CKDN2A. Additionally, we show that SOX4 and CDKN2A PPIs could represent a new mechanism for SOX4 to deregulate the cell cycle and drive tumorigenesis outside of SOX4's canonical transcription factor function.

How SOX4, CDKN2A and E2F3 converge on Bladder Cancer

The SOX4, E2F3 overexpression and CDKN2A deletions are some of the most significant copy number alterations (amplifications and deletions) in bladder cancer patients [7]. These data suggest that SOX4, E2F3 and CDKN2A could play an important role in bladder cancer tumorigenesis. In collaboration with the Fu lab, we have recently discovered a novel PPI via a high-throughput Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) screen [107]. TR-FRET assays are an appropriate method to detect direct protein-protein interactions because the detected interactions are limited to a distance of only 10nm between proteins [119]. The top protein interacting with SOX4 via the TR-FRET assay was CDKN2A (Figure 4.2). TCGA data indicates that SOX4 amplification and CDKN2A deletion are mutually exclusive, indicating they likely act in the same pathway (Figure 4.3). These data suggest that SOX4 could be an oncogenic driver in bladder cancer, but the connection between SOX4 overexpression and tumorigenesis has not yet been definitively demonstrated.

In this study we show that SOX4 interacts with CDKN2A, and in shSOX4 cells, CDKN2A can increase the percentage of cells in G1, suggesting a SOX4 inhibits canonical CDKN2A tumor suppression. These data could support a role for SOX4 in bladder cancer that is independent of its transcription factor binding activity. Devising small molecule inhibitors of SOX4 could thus represent a new therapeutic approach in bladder cancer.

4.3 Methods

4.3.1 Cell Lines , Reagents and Transfections

Bladder cancer cell lines (5637, HT1376, and SW780) were obtained from American Type Culture Collection. 5637 cells were maintained in RPMI, T24 cells in McCoy's 5A, HT1376 and SW780 cells in DMEM, and TCCSUP and UMUC3 cells in MEM growth media. All media were supplemented with 10% FBS, 1% L-Glutamine, and 1% Penicillin-Streptomycin. Cells were cultured in a 37°C incubator with humidified atmosphere of 5% CO2. Plasmid pLenti-CMV-p16-Neo (w111-1) for stable CDKN2A (p16) expression was obtained from Addgene (Plasmid # 22260). Parental SW780 cells and subsequent cell lines used to generate stable SW780 cells were genetically authenticated by Bio Synthesis (Lewisville, TX), an Accredited Human Cell Line Genotyping Service company.

Endogenous Co-Immunoprecipitation

Cells were washed twice with 1X PBS and harvested with IP lysis buffer (0.5% NP-40 lysis buffer)) containing protease inhibitors (Sigma Cat # P8340) and phosphatase inhibitors (Roche Cat # 4906845001), scrapped and transferred to and 1.5ml tube and incubated on a rotator for 30 minutes. Whole cell lysates were then centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatants were transferred to fresh tubes and split between Biotinylated-SOX4 antibody (Santa Cruz Cat # 154C4a was incubated with MACS Miltenyi Biotec Biotylated kit Cat #130-093-385 for 24 hours at room temperature) and Biotinylated-Angi-IgG Cat# 8887S) and incubated on a rotator at 4°C overnight. Streptavidin magnetic beads (invitrogen Cat # 11205D)

were then added to lysate and incubated on a rotator at 4°C for 3 hours. Beads were then washed three times in 500ul of 0.5% NP-40 lysis buffer (0.5% NP-40) and eluted from the complexes with addition of 20ul of 2x SDS loading buffer and boiled for 5 minutes along with input controls. The lysate was then analyzed on a 10% SDS-polyacrylamide gel for SDS-PAGE electrophoresis and transferred to a PVDF membrane (Biorad Cat #1620177). Membranes were blocked in 1X TBS buffer containing 5% BSA and 0.001% Tween for 1 hr at room temperature, and then incubated with primary antibody (SOX4 Santa Cruz monoclonal antibody 1:1000 Santa Cruz Cat # 154C4a) overnight at 4°C. Blots were washed with TBST three times for 5 minutes each and incubated with secondary antibodies (anti-mouse IgG - Cell Signaling Cat # 7076S 1:2000 - Abcam Cat # ab6721 1:3000) for 1 hour at room temperature. Signals were visualized using SuperSignal West Pic PLUS chemiluminescence substrate (Pierce Cat #34580).

4.3.2 High Throughput PPI Screening

The methods for high-throughput PPI screening and fold over change statistics were performed as described in [107].

4.3.3 In vivo mouse models

Ten female NSG mice were purchased from Jackson Labs (Bar Harbor, ME). Mice were kept in cages at Emory's DAR animal facility. Prior to inoculation with SW780-Luc bladder cancer cell lines or PBS controls, bladders were pretreated with 100µl of 01.mg/ml poly-L-lysine (Sigma) as previously described [120]. Mice were anesthetized & injected with D-Luciferin to detect luciferase activity and tumor growth using the IVIS Imaging Spectrum at the Winship Cancer Animal Models

Core to monitor tumor formation. All standards and procedures were followed according to IACUC and DAR at Emory University.

4.3.4 Cell Cycle Analysis

Cells were grown in 60mm plates to confluency and harvested with 0.05% trypsin. Cells were spun down at 1500rp for 5min at 4°C. Cells were then washed with 5ml PBS and gently vortexed. Cells were spun down as described above. Supernatent was discarded and cell pellet was gently vortexed. Cells were then washed with 5ml of 70% ethanol at 4°C for 2 hours or overnight. The next day cells were spun down and ethanol discarded. Cells were washed with 5ml of cold PBX and vortexed briefly. Cells were spun down as described above, then washed with 5ml of staining bufffer (BD Pharmingen Cat # 554656) and then centrifuged at 2000rpm for 5min at 4°C. Supernatant was asprirated followed by addition of 500 µL of RNase and propidium iodide staining buffer (BD Pharmingen Cat # 550825). Cells were placed in the dark for 30 minutes. Cells were then transferred to meshed blue capped falcon tubes (Cat #352235) to avoid clumped cells. Cells were then analyzed in the flow cytometry core facility on a Becton Dickinson LSR II analyzer.

4.4 Results

4.4.1 TR-FRET data Reveal Novel SOX4 Protein-Protein Interactions

We recently discovered putative novel SOX4 protein-protein interactions (PPI) via a high-throughput Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) screen and identified SOX4 as a potential PPI hub (Figure 4.1) [107]. From the putative SOX4 hub we chose only those PPIs with a fold-over control

change greater than 1.5. This resulted in a list of the most significant high-confidence SOX4 protein-protein interactions. Of these the most significant PPI was SOX4 and the tumor suppressor protein CDKN2A (Figure 4.2). We then sought to evaluate copy number and mutation information of the six high confidence PPIs in relation to SOX4 expression in cBioPortal from the *Cell, 2017* dataset [7]. These data revealed SOX4 and E2F3 as the most significant amplification (23% and 25% respectively) and CDKN2A as the most significant deletion (43%) out of 408 patient samples (Figure 4.3A). We therefore, hypothesized that SOX4 could bind and sequester CDKN2A and inhibit its normal tumor suppressive function.

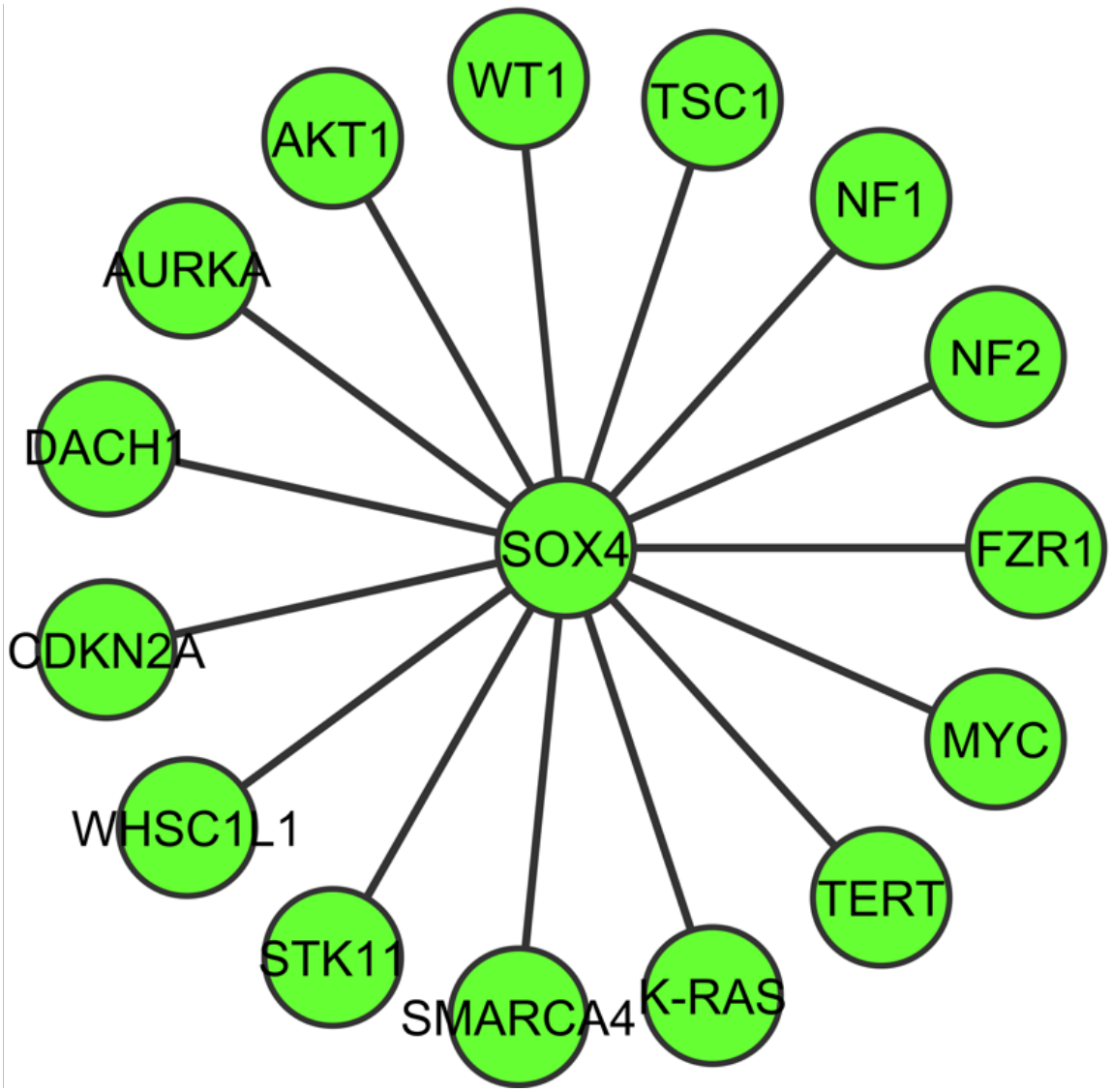
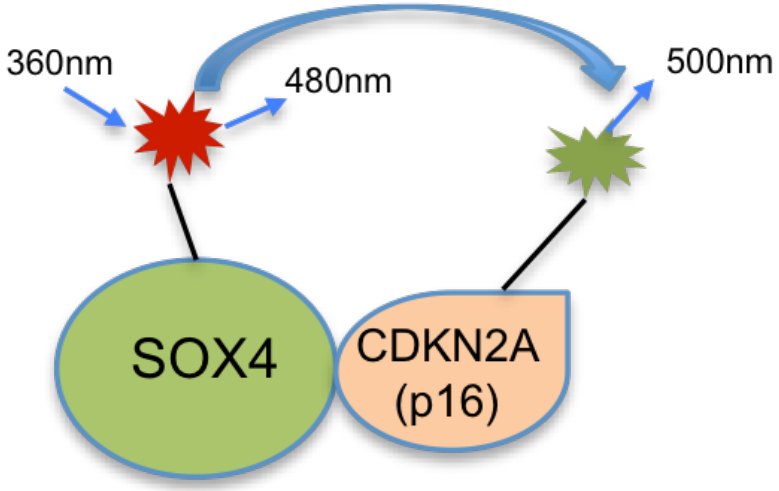


Figure 4.1: List of all the putative SOX4 protein-protein interactions as determined by TR-FRET data [107]



Protein1	Protein2	FOC Avg.
SOX4	CDKN2A(p16)	3.59
SOX4	DACH1	1.89
SOX4	WHSC1L1	1.85
SOX4	AKT1	1.5
SOX4	AURKA	1.5
SOX4	SMARCA4	1.5

Figure 4.2: TR-FRET data from the Fu lab [107] revealed six high confidence SOX4 protein-protein interactions.

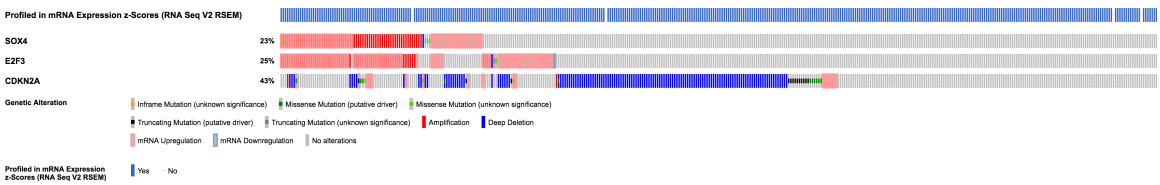


Figure 4.3: Data queried from cBioportal for SOX4, E2F3 and other high confidence SOX4 Protein-Protein Interactions A.) Data from cBioportal show SOX4 and E2F3 amplifications in bladder cancer patients and CDKN2A (p16) deletions B.) Statistical analysis of cbioportal data show statistically significant mutual exclusivity with between both SOX4/E2F3 and CDKN2A, p-value = 0.011, $<$0.001 respectively.

4.4.2 Validating SOX4 Protein-Protein Interactions

I then sought to validate the endogenous interaction at physiological levels in 5637 and HT1376 bladder cancer cell lines (Figure 4.4). We observed that co-immunoprecipitation of SOX4 in 5637 cell lines pulled down p16 compared to anti-IgG control and compared to a negative control cell line, SW780, harboring CDKN2A deletion. These data suggest that SOX4 interacts with CDKN2A and could negatively regulate it. The interaction of SOX4 and CDKN2A suggests a novel tumor promoting activity that is independent of SOX4's activity as a transcription factor. Our data, combined with TCGA data provide a rationale for SOX4 as a novel therapeutic target in bladder cancer. Therefore, disruption of SOX4/CDKN2A interactions may represent a novel therapeutic approach in bladder cancer patients.

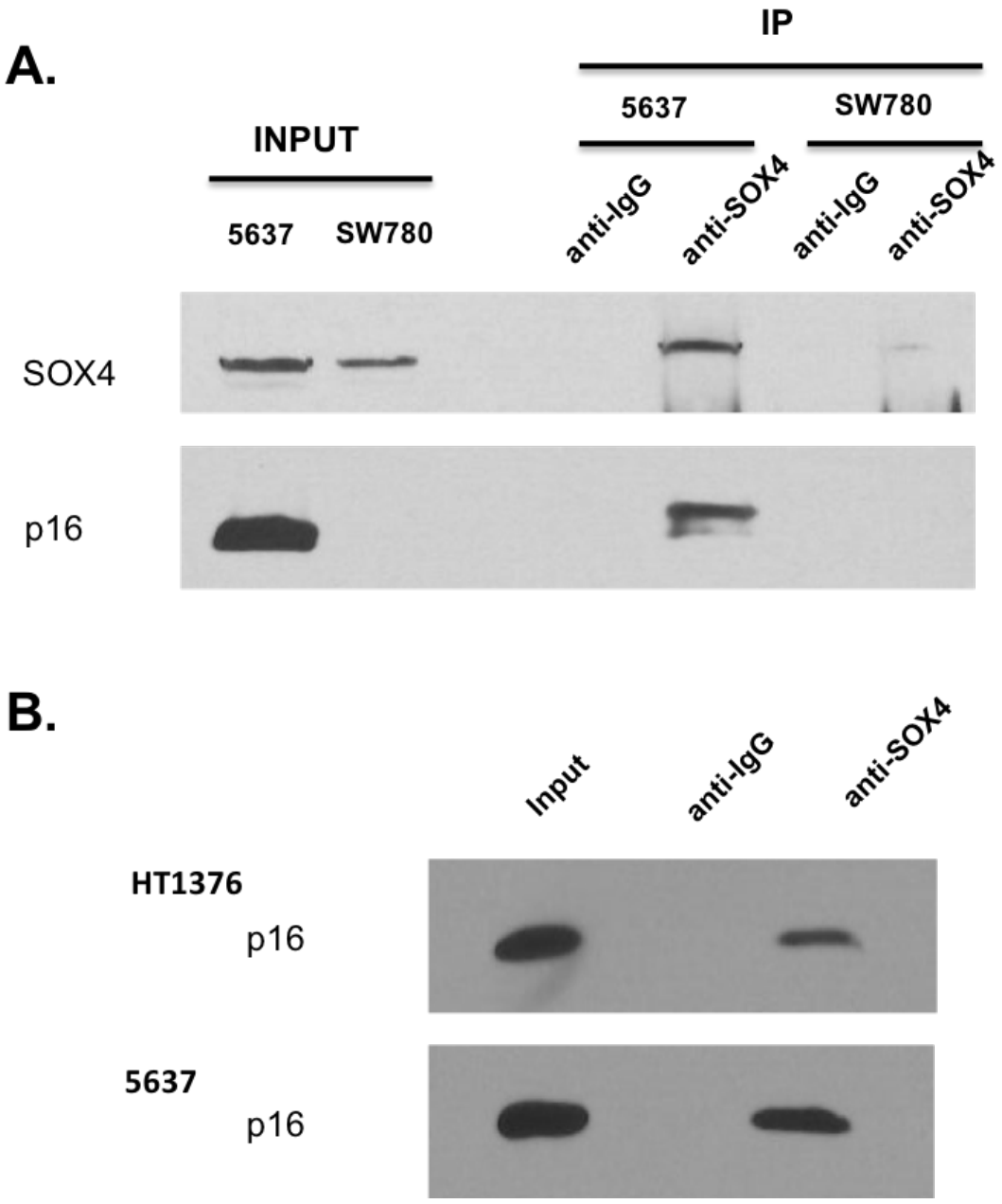


Figure 4.4: A.) Endogenous pulldown of p16 with SOX4 in 5637 cell lines compared to anti-IgG negative control (lanes 3,5) and SW780 negative control harboring deep homozygous deletion of p16 (lane 2) B.) Endogenous pull down of CDKN2A with SOX4 (lane 3) in HT1376 and 5637 cell lines compared to negative control IgG (lane 2).

4.4.3 SOX4 knockdown and re-expression of p16 in shSOX4 cell line alters cellular morphology

We created stable shSOX4 knockdown cell line in SW780 cell lines that have an intact RB pathway with exception of CDKN2A. To test our hypothesis that p16 will exert it's tumor suppressive effects under the context of SOX4 ablation, we ectopically re-expressed p16 in $SOX4^{+/+}$ SW780 cell lines and in SW780-shSOX4 cell lines (Figure 4.6). We observed drastic morphological changes when re-expressing p16 in SOX4-KD cell lines compared to $SOX4^{+/+}$ and with shSOX4 alone (Figure 4.7). These data suggest that CDKN2A might be exerting tumor suppressive effects in the form of apoptosis or cell cycle arrest in the context of SOX4 knockdown but not if SOX4 is present.

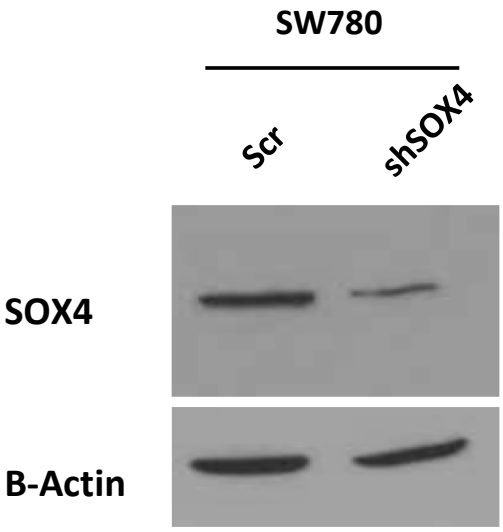


Figure 4.5: Western blot showing partial knockdown of SOX4 in SW780 cells using a lentiviral shRNA against the coding region of SOX4.

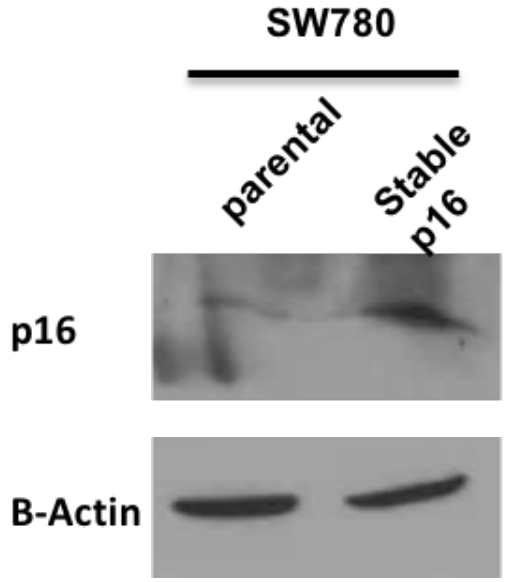


Figure 4.6: Ectopic stable expression of CDKN2A (p16) in SW780 bladder cancer cell line

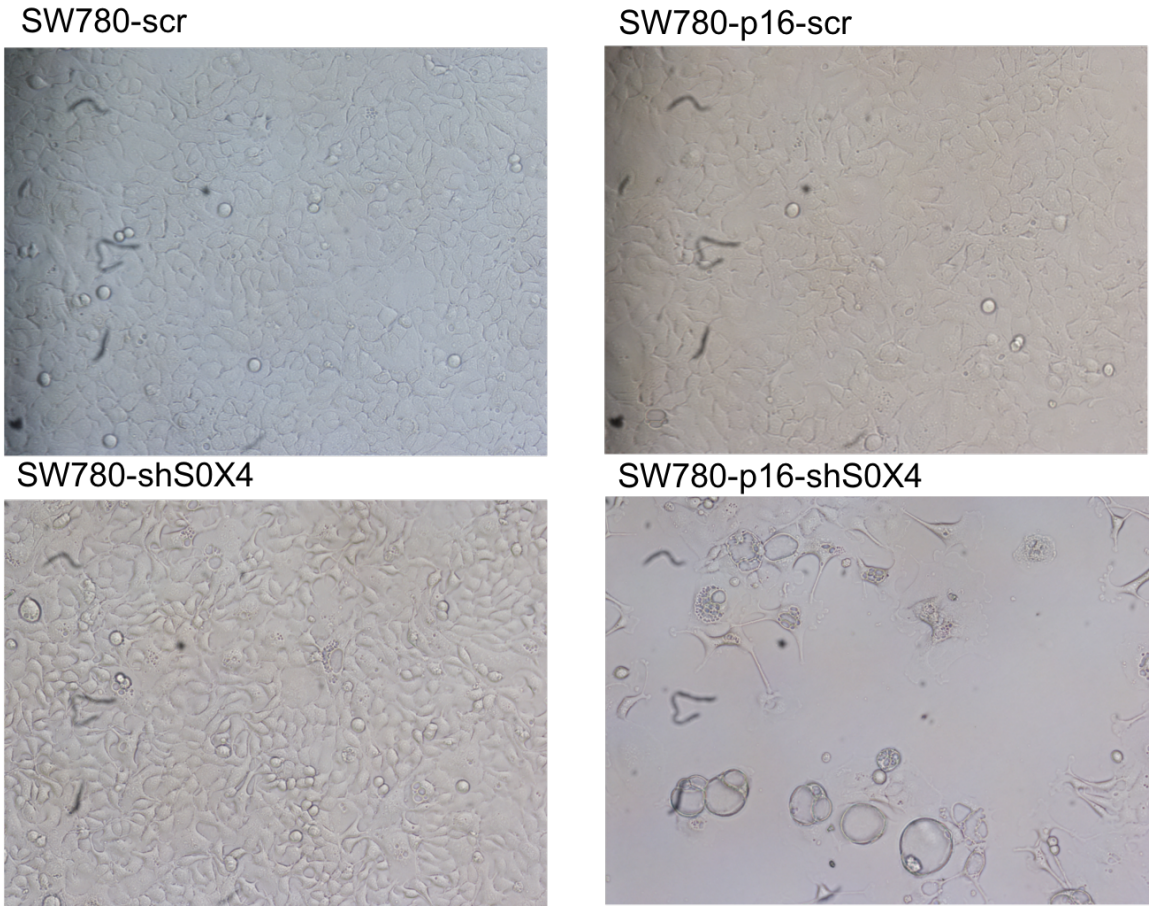


Figure 4.7: SW780-shSOX4 re-expressing p16 showed blebbing morphology compared to control cells with SW780-Scr-p16, suggesting either apoptosis or senescence.

4.4.4 Re-expression of p16 in shSOX4 cell lines induces G1 arrest

Because p16 acts on CDK4/6 and Cyclin-D to block cell cycle progression and SOX4 interacts with p16, we then sought to determine if CDKN2A to could induce G1 arrest in the context of a SOX4 knockdown. We performed cell cycle analysis with propidium iodide in our SW780 cells ectopically expressing CDKN2A in a physiologically relevant SOX4 positive cell line, SW780 (Figure 4.8). We observed that re-expression of CDKN2A in SW780-shSOX4 cells increased the percentage of G1 cells compared to either shSOX4 or re-expression of CDKN2A alone. These data suggest that CDKN2A can induce G1 arrest only in the context of decreased SOX4 expression. These experiments are worth repeating as they were only done one time.

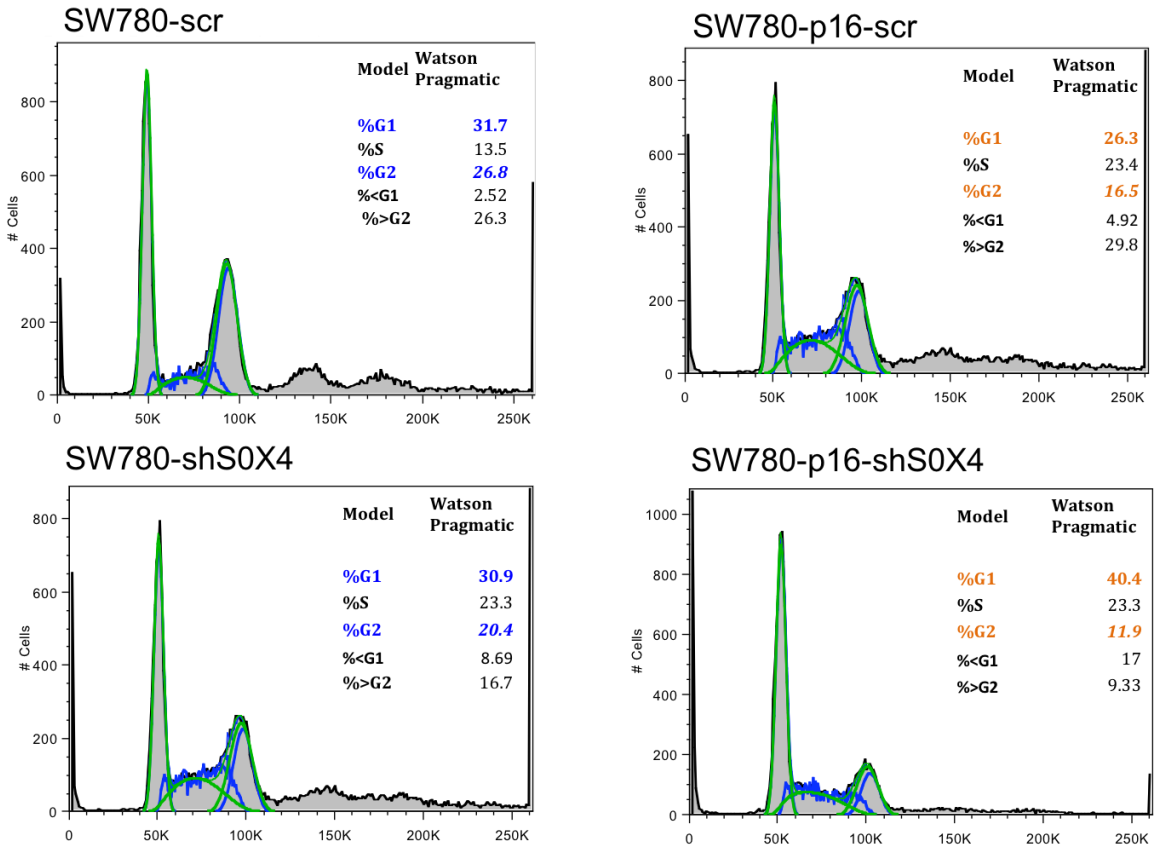


Figure 4.8: Cell Cycle analysis indicates that re-expression of CDKN2A in a shSOX4 knockdown SW780 cells induces G1 arrest compared to re-expression of CDKN2A in a SOX4$^{+/+}$ control cell lines and compared to SW780-scr controls.

4.5 Orthotopic Bladder Injection of SW780 cells form tumors in mice

I conducted a proof of concept experiment whereby I orthotopically injected via a 24-gauge catheter: PBS control, 2.5x10^6, or 5.0x10^6 SW780 cells stably expressing Luciferase into nude mouse bladders at day 0. We used five mice per group and used the IVIS Spectrum whole body *in vivo* optical imaging system to monitor tumor formation for 28 days. This work was performed as a proof-of-principle experiment in preparation of future *in vivo* work comparing tumor size and growth in cells with or without SOX4 knockdown in a CDKN2A$^{+/+}$ background cell lines. Here we have successfully performed a method by which we can establish an orthotopic bladder cancer tumor model.

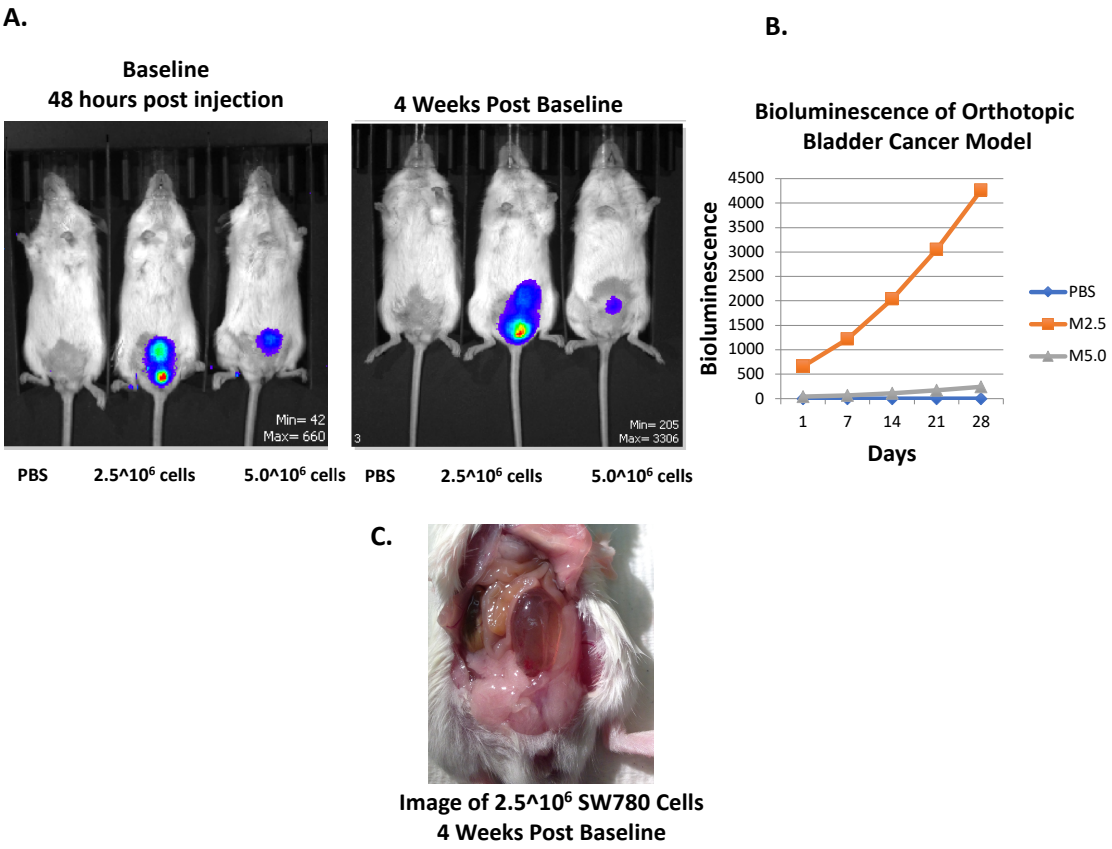


Figure 4.9: A.) Injection of $2.5x10^6$ and to a lesser extent a $5.0x10^6$ SW780-Luc SW780-Luc bladder cancer cells formed tumor 4 weeks post implantation compared to PBS control cells. B.) Bioluminescence of tumor measured over the course of 4 weeks. C.) Anatomical view of large solid tumor formation in mouse injected with $2.5x10^6$ Luc SW780-Luc cells.

4.6 Discussion

In the United States there are an estimated 81,190 new cases of bladder cancer each year resulting in 17,240 deaths annually [3]. Unfortunately, in terms of research dollars per patient, bladder cancer receives the lowest research funding of all cancers and as a consequence bladder cancer remains the most expensive malignancy to treat [121]. Research dollars directed towards prostate and kidney cancer have helped increase the 5-year survival rate in those cancers, but 5-year survival rates have increased by only 1% for bladder cancer [121, 122]. Recently published data of RNAseq, whole genome and whole-exome sequencing of bladder cancer patients from different groups, including The Cancer Genome Atlas (TCGA), revealed new genomic alterations and potential molecular mechanisms for tumorigenesis that merit further investigation [7, 123, 124].

Here we have identified a novel protein-protein interaction between a transcription factor, SOX4, and the tumor suppressor protein CDKN2A in 5637 and HT1376 cell lines. However, these cell lines were not ideal for testing functional effects on proliferation. Because our proposed interaction lies upstream of the RB pathway, we tested our hypothesis across bladder cell lines with a functional RB pathway (RB-Positive) – i.e. non-mutated and non-homozygous deletions of RB. Attempts to abrogate upstream phosphorylation complexes such as CDK4/Cyclin-D are ineffective in cells with compromised RB signaling either from RB mutations or homozygous deletions. RB mutation status is a stratification marker when determining benefit with agents upstream of RB such as CDK4 inhibitors in breast cancer [125]. In other studies SOX4 shRNA knockdown in 5637 bladder cell lines

did not show decreased proliferation, likely because downstream RB is mutated and homozygously deleted in 5637 bladder cancer cell lines and supports the hypothesis drawn from RB-negative cell lines [18, 46, 125–127].

We chose to pursue SW780 as a candidate cell line model because it has a non-mutated and non-deleted RB, but lacks CKDN2A. We therefore chose to re-express CDKN2A in SW780 to create a functional RB-pathway. Here we have shown that re-expressing p16 in SW780-shSOX4 cell lines induces morphological changes that appear apoptotic in nature. Further cell cycle analysis indicates that re-expressing CKDN2A in SW780-shSOX4 correlates with an increased number of cells in G1 residence compared to cells with CDKN2A only or scrambled control. However, further experiments are needed here to assess proliferation rates via MTT assay, as well as determining if the morphological changes are apoptotic, necrotic or a sign of cellular senescence. Additional work is needed to test various apoptotic assays including cleaved Caspase-3 and cleaved PARP. In preparation for future *in vivo* studies we have established an orthotopic bladder cancer mouse model using our SW780-Luc expressing cells. These mice established and maintained tumors for 4 weeks.

Proposed Overall Mechanism of SOX4

SOX4 is a transcription factor but its role in regulating key points in the cell cycle has not been clearly established. Our research could provide a molecular mechanism by which SOX4 deregulates the cell cycle by sequestering CDKN2A to permit aberrant proliferation. However, we cannot rule out the possibility that SOX4-

CDKN2A could drive activation or repression of target genes. Future studies are needed using mutant SOX4 that no longer binds DNA but retains its CDKN2A binding activity. This novel SOX4-CDKN2A interaction may drive malignant transformation in as much as 24% of bladder cancer patients. Understanding how specific downstream perturbed signaling proteins go awry to upregulate growth and deregulate the cell cycle holds promise for targeted cancer therapies and especially bladder cancer – where no targeted therapies exist. In this study we have identified a putative novel protein-protein interaction of SOX4 and CDKN2A in bladder cancer that we hypothesize may have tumor promoting activity independent of its role as a transcription factor (Figure 4.10).

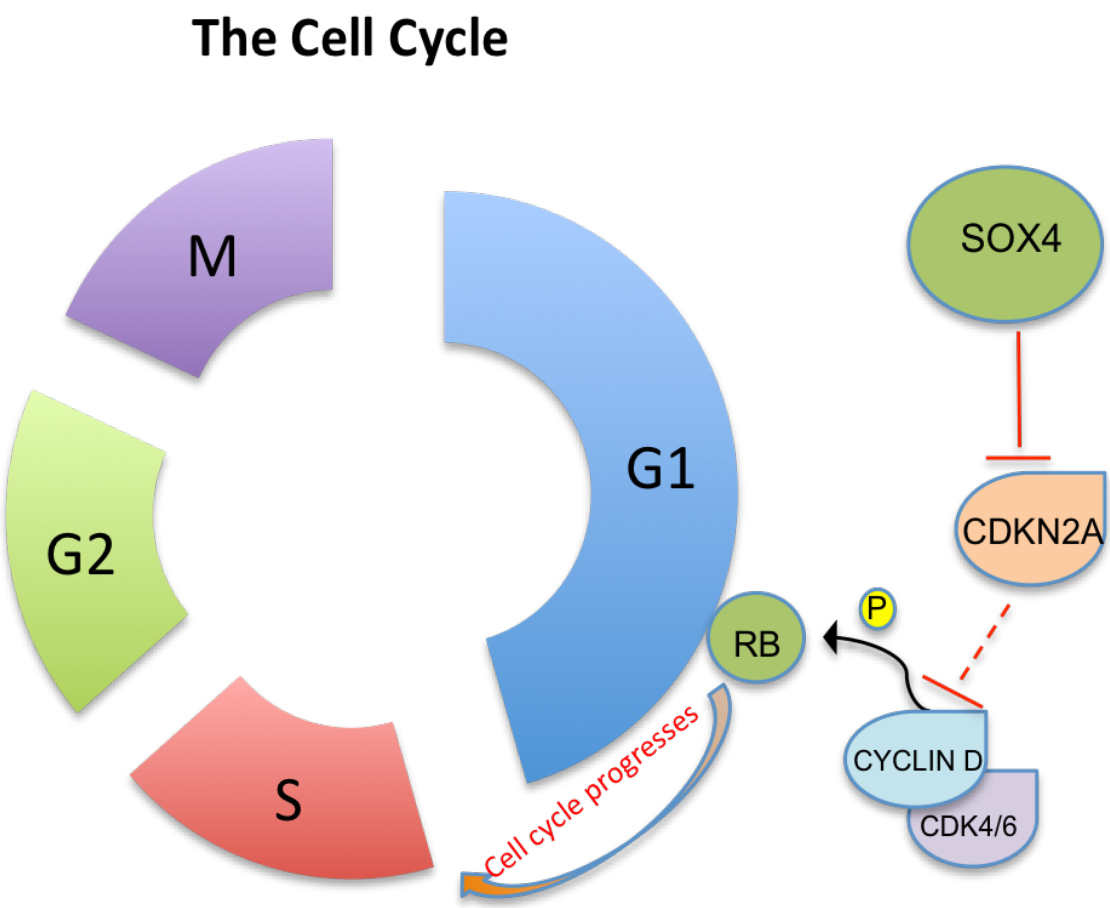


Figure 4.10: SOX4 sequesters CDKN2A. This deregulates the cell cycle and thereby allowing the cyclin-D/CDK4/6 complex to phosphorylate RB and allow cell cycle progression.

Chapter 5 Discussion

The focus of this dissertation was to better understand the role of SOX4 in bladder cancer and on understanding how an overexpressed developmental transcription factor might drive or maintain certain aspects of tumorigenicity. Here I will summarize our findings and reflect on future and ongoing experiments to increase our understanding of the mechanisms by which SOX4 contributes to bladder cancer.

5.1 SOX4/CDKN2A PPI Summary of Findings and Future Directions

Summary of Findings

In this study we used high-throughput TR-FRET data [107] to infer putative novel protein-protein interactions involving the SOX4 transcription factor. These data showed that the most high confidence SOX4 PPI involved a tumor suppressor protein p16 (CDKN2A). I then validated the TR-FRET SOX4/p16 interaction endogenously in two bladder cancer cell lines (5637 and HT1376) compared to a negative control cell line, SW780 that harbors p16 deep deletion. We then chose to re-express p16 in SW780 cell lines that have a wild type RB followed by shSOX4. Although our experiments must be repeated, we observed distinct morphological changes in SW780-p16-shSOX4 cell lines compared to SW780-p16 or SW780-shSOX4 alone.

We hypothesized this might be due to either G1 arrest or apoptosis. We performed cell cycle analysis with propidium iodide and observed increased percentage of G1 cells in SW780-p16-shSOX4 cell lines compared to SW780-p16 or SW780-shSOX4 alone. Combined with our endogenous PPI data, these data suggest that a functional effect to increase the number of cells in G1 occurs only under the context of shSOX4. While these results are promising and support our hypothesis that SOX4 could interact with CDKN2A to facilitate progression through the cell cycle, future studies as outlined below could further support this model of action.

Cell Lines

Central to my hypothesis is that cells must have an intact RB (non-mutated, non-deleted) pathway for SOX4-CDKN2A interactions to impact cell cycle progression. While SW780 cells exhibit this feature, we must repeat all experiments with 5637 or HT1376 that harbor deletions/mutations in RB as a negative controls. Additionally, we will repeat these experiments in T24 cell lines, which exhibit a similar genetic profile to SW780 in that they have an intact RB and silenced CDKN2A. Moreover, pushing this work into *in vivo* mouse models with Luciferase expressing cells, with and without p16, in 5637, HT1376, T24 and SW780 would allow us to better understand the genetic profiles in which SOX4/CDKN2A PPI drive tumorigenesis and functional effects on tumor progression *in vivo*. Additionally, SOX4, CDKN2A and RB do not function in a vacuum. There are 8 other genes on the 6p22 focal amplification that could cooperate to drive tumorigenesis including: E2F3, ID4, CDKAL1 and MBOAT1. Further research is needed to elucidate in what way, if at all, the aforementioned genes cooperate with each other in

the context of cancer.

SOX4/CDKN2A Deletion Domains

While we confirmed endogenous interactions between SOX4/CDKN2A we have not yet identified which domains of SOX4 could interact with CDKN2A. Identification of SOX4-CDKN2A interaction domains may facilitate development of competitive peptide inhibitors, dominant negative mutants, or rationally designed drugs that can interfere with these interactions. Full length SOX4 is a 474 amino acid (aa) protein with at least 4 distinct functional domains. In future studies we may seek to identify the SOX4 domains responsible for SOX4/CDKN2A interactions by creating SOX4 deletion domains as well as CDKN2A deletion domains. We could follow-up on deletions that disrupt interactions by making smaller deletions to identify the minimal domain necessary for interaction. One of our goals is to create a deletion construct that no longer binds CDKN2A but retains its DNA binding activity and vice versa. We will use this as a way to distinguish SOX4's transcriptional role from its role of binding CDKN2A. This could enable development of inhibitors of these interactions using functional domain deletion constructs. Thus, SOX4 could serve as a candidate therapeutic target. Our research could lay the groundwork for scientists to develop small molecules or alternative methods to disrupt the perturbed signaling networks in bladder cancer.

SOX4/CDKN2A as Co-Factors in Transcription

Although we have discussed here ways in which SOX4 could interact with

CDKN2A to inhibit its tumor promoting ability, we must not rule-out the potential for CDKN2A as a SOX4 transcriptional co-factor either together or in combination with other proteins such as TP53. Motivation for this hypothesis lies in piecing together data from Pan et al., [73], in which SOX4 was upregulated as a result of DNA damage, co-localized in the nucleus with TP53, and interacted with TP53 in a manner that enhanced TP53's inherent transcription factor activity. Furthermore, additional studies have shown [128] that CDKN2A complexes with TP53 to enhance transcription of BAX. Additional data from Pan et al. also showed shSOX4 in HCT116 cell lines decreased BAX expression and TP53 downregulation. Taken together, I propose a novel pathway whereby in the presence of DNA damaging agents in wild type TP53 cells, SOX4 stabilizes TP53 expression allowing TP53 to bind CDKN2A to drive BAX expression and leading to apoptosis. Whether or not SOX4 is bound together with CDKN2A and TP53 at the promoter merits further research since a potential SOX4/p16/TP53 protein complex could act together to either promote tumorigenesis or function in a tumor protective manner.

5.2 SOX4 and Wnt5A Summary of Findings and Future Directions

Summary of Findings

In the second part of our SOX4 research in bladder cancer we used CRISPRi of SOX4, followed by SOX4 re-expression in T24 bladder cancer cell lines. Although these initial experiments did not show changes in proliferation, despite having a wild type RB. The lack of altered proliferation is likely due to silencing of the

single copy of CDKN2A in T24 cells through methlyation of its promoter [129]. This is consistent with our central hypothesis discussed above regarding no expected changes in proliferation a result of SOX4 knockdown as long as CDKN2A is deleted or silenced. If re-expressing CDKN2A in T24-SOX4-KD cells also increased G1 arrest this could further support our central hypothesis discussed above. Additionally, no changes were observed in migration but we did observe significantly decreased invasion as a consequence of SOX4-KD, which was re-established upon SOX4-Rescue. Analysis of T24-SOX4-KD and T24-SCR revealed a number of interesting putative targets of SOX4 that included a complete and coordinated downregulation of cholesterol biosythesis genes such as DHCR7, TM7SF2, and MVD1. While there is some literature to support a role for cholesterol proteins in EMT these genes were not upregulated upon SOX4 re-expression and there are no SOX4 binding sites at the promoters [99, 100]. Two other genes of interest to us were TNC and IDO1. The Moreno lab has shown that SOX4 resides at the TNC promoter via CHIP assays and can drive TNC expression. Additionally, IDO1 downregulation as a consequence of SOX4-KD was of interest give the recent paper involving IDO1 blockade and tumor shrinkage in bladder and cervical cancer [96].

However, of greatest interest was the analysis of microarray data between T24-SOX4-KD and T24-SOX4-Rescue that revealed 173 high confidence SOX4 regulated genes. Among these genes we observed Wnt5a as inversely correlated with SOX4 expression and cellular invasion. A review of the literature pointed to a possible tumor suppressive role of Wnt5a expression. Treatment of Wnt5a-high T24-SOX4-KD cells with a Wnt5a antagonist, Box5, restored invasion comparable to T24 controls. These data motivate two areas of interest: 1) How does SOX4 reg-

ulate Wnt5a expression and 2) what receptors/ signaling pathways are activated through Wnt5a signaling? In the following paragraphs we will review some literature on Wnt5a and explore future directions to address the gaps in our knowledge.

Wnt Signaling

At a high level Wnt signaling is grouped into two main categories: 1) canonical Wnt signaling, requiring β-catenin, and 2) non-canonical Wnt signaling that is β-catenin independent. For decades Wnt signaling has been well studied within the context of colon cancer in which Wnt signaling functions downstream to save β-catenin from proteasomal degradation, thus stabilizing and permitting β-catenin translocation to the nucleus where it complexes with TCF/LEF to drive target genes such as Cyclin-D1 and c-Myc [130–132]. On the other hand, non-canonical Wnt signaling does not require β–catenin and in fact, ectopic Wnt5a expression in colorectal carcinoma cancer cells has been shown to antagonize canonical Wnt signaling by driving β-catenin degradation [133, 134]. SOX4 has been implicated as a component of canonical Wnt-signaling by interacting with β–catenin/TCF/LEF[72]. Our study is the first to implicate SOX4 as a putative regulator of non-canonical Wnt signaling in human cancer cell lines.

Wnt5a

Wnt5a signaling is implicated in a tumor-promoting fashion by way of increased invasiveness and metastasis [135, 136], but also in a tumor-suppressive manner by way of decreased metastasis and invasion [137, 138]. The tumor-promoting

aspect of Wnt5a is much better studied and attributed to Wnt5a binding to Ror2 receptor and activating target genes such as AP-1 [139]. However, a mechanism or target genes for the tumor suppressive arm of Wnt5a signaling is much less clear, even though many studies support the tumor-suppressive role of Wnt5a [102, 137, 138, 140, 141]. This polarization of Wnt5a mechanisms may be attributed to varying availability of Wnt5a receptors in different tissue types.

SOX4's Regulation of Wnt5a signaling

We have not yet shown how SOX4 might regulate Wnt5a. Analysis of the Wnt5a promoter did not reveal any SOX4 bindings sites. Additionally, analysis of all putative transcription factors that bind at the Wnt5a promoter were not regulated as a result of SOX4-KD or SOX4-Rescue. This leaves a large question mark about the biology occurring as a result of SOX4 expression. To further test the idea of a possible direct SOX4 regulation of Wnt5a we propose conducting a chromatin immunoprecipitation assay in T24 parental cell lines. Previous attempts in prostate cancer cell lines using antibodies to endogenous SOX4 have been difficult and our lab has used antibodies against HA-tagged SOX4 in prostate cancer cell lines but SOX4 was not found at or near Wnt5a in those experiments [40].

Indirect modulation of Wnt5a expression by SOX4 is also possible through epigenetic regulation. Promoter methylation of Wnt5a is a likely mechanism of indirect regulation. Silencing of Wnt5a through promoter methylation has been observed in thyroid and colorectal cell lines [133]. Although we have not tested this, it would be interesting to perform either bisulfite conversion or whole genome

methylation profiling in the context of SOX4-KD and re-expression. Moreover, SOX4 is known to drive certain aspects of chromatin remodeling, specifically EZH2 and the Polycomb histone methyltransferases for gene repression [65]. It is possible that downregulation of SOX4 drives widespread demethylation of certain genes by decreased activity of Polycomb methyltransferases. This would represent an indirect regulation of WNT5A by SOX4.

A limitation of our study is that we only achieved stable SOX4 knockdown in T24 cell lines. In order to ascribe some generalizability with respect to SOX4's putative regulation of Wnt5a in bladder cancer cells, we would need to replicate our data across other bladder cancer cell lines. However, our CRISPRi method could present with some difficulty in other bladder cancer cell lines (5637, HT1376) that harbor SOX4 amplifications and present a greater number of sites for which sgRNA's to bind. A way around this would be to design a few more sgRNA's, in addition to our seven sgRNAs. Additionally, we did not perform assays involving either recombinant Wnt5a or Foxy5 (a Wnt5a mimetic) applied to T24 parental or T24-Scr in the top chamber of a Boyden chamber migration assays. This assay could suggest if either compound alone will reduce invasive properties of T24 parental cell lines.

Decoding Wnt5a Receptor Binding and Signaling

While our data suggests a putative regulation of Wnt5a by SOX4, it only represents the beginning of what might constitute a complete mechanism. The road ahead is exciting and there is plenty more to discover. Specifically, beyond simply

understanding how SOX4 might regulate Wnt5a, we need to understand the specific Wnt5a receptors inhibiting invasion. One method woud be to knock down some of the putative Wnt5a receptors individually or in combination (e.g. ROR, FZD5, etc.) as well as the downstream signaling proteins (e.g. JNK). We will also need to see what downstream target genes are expressed or repressed a consequence of Wnt5a signaling. One approach to this would be to use shRNA against individual receptors. Moreover, our CRISPRi system positions us well to design sgRNA's to our gene of choice and make use of our T24 cells already expressing KRAB-dCas9.

5.3 Final Thoughts and Mechanism

On the surface it may appear that the putative SOX4/CDKN2A PPI and SOX4 regulation of Wnt5a are independent of each other. However, the reality in cancer is that the dysregulation of any of a number of tumor suppressors or transcription factors can have a multi-pronged mechanism. The regulation of cell division and invasion are finely tuned mechanisms that when uncontrolled or mutated can lead to cancer. One goal of our research is to evaluate how SOX4 could allow cells to continue to divide even in the presence of stop signals.

In the absence of disease like cancer, there is a core principle that each cell in the human body has the exact same underlying DNA sequence – yet cells of the hair, skin, and organs all develop from the same DNA that vary only in their packaging (i.e. chromatin state). With this in mind, it makes sense, that SOX4 or Wnt5a

can have tumor promoting contexts in one tissue type and yet tumor-suppressive roles in another tissue type. Therefore we propose that SOX4 has a cell type specific gene expression pattern that of which can still vary within the same tumor type depending on the availability/mutation state of its co-factors.

Given our current body of work we propose that SOX4 could function through two different pathways, either concurrently or individually, that can both contribute to different hallmarks of cancer (Figure 5.1). One pathway deals with SOX4 as a pure transcription factor that either drives expression of genes involved in proliferation or contributes to repression of tumor-suppressive signaling such as the Wnt5a pathway. The second arm of SOX4 involves novel protein-protein interactions with CDKN2A that inhibit CDKN2A's inherent tumor suppressive role. As mentioned previously, this arm could also function in the form of co-factors required for DNA binding and expression of target genes.

There are currently no targeted therapies in bladder cancer, and our research could potentially reveal a novel therapeutic target for use in the clinic. The SOX4/CKDNA PPI axis and Wnt5a repression by SOX4 may represent an opportunity to develop specific targeted therapies for bladder cancer patients. Ultimately, our research will seek to thoroughly elucidate the mechanisms by which the SOX4 amplification drives bladder cancer both in terms of SOX4's transcriptional role and in manners independent of SOX4's transcription factor activity, such as novel PPI's.

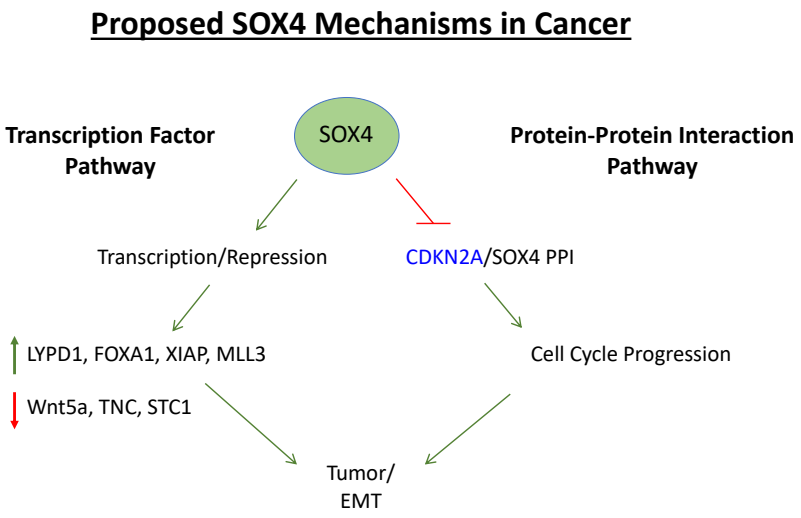


Figure 5.1: SOX4 could function through two separate pathways that converge on cancer either through a pure transcriptional pathway or through protein-protein interactions independent of its transcription factor ability.

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