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Yu-Heng Lai Date

THE ROLE OF SOX4 IN PROSTATE CANCER

BY

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THE ROLE OF SOX4 IN PROSTATE CANCER

BY

Yu-Heng Lai
M.S., National Taiwan University, 2004

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EMORY

An Abstract of
A dissertation submitted to the Faculty of the
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ABSTRACT

THE ROLE OF SOX4 IN PROSTATE CANCER

BY

Yu-Heng Lai

SOX4 is a developmental transcription factor that is required for differentiation and proliferation in multiple tissues. SOX4 is overexpressed in many human malignancies, but the precise role of SOX4 in cancer progression is still not well understood.

The first part of my study identified proteins that interact with SOX4 via a one-step affinity purification method that enables rapid purification of SOX4 complexes to perform large-scale proteomics analysis. We discovered that junction plakoglobin (JUP) interacts with SOX4 in both the cytosol and the nucleus, and that the interaction between SOX4 and plakoglobin is significantly increased when prostate and breast cancer cells are stimulated with WNT3A. The SOX4-plakoglobin complex affected the expression of Wnt pathway target genes and SOX4 downstream targets, such as *AXIN2*, *DICER1*, and *DHX9*. In addition, SOX4 DNA binding activity to the promoters of *DICER1*, *AXIN2*, *DHX9* and *SOX4* itself was reduced by conditions that promote SOX4-plakoglobin complex formation. Conditions that enhanced SOX4-plakoglobin interactions resulted in reduced transcriptional activity of β-catenin luciferase reporters. These data suggest that this newly identified interaction between SOX4 and plakoglobin is inhibitory and provides new insights into the role of SOX4.

In the second part of my research, we performed miRNA profiling from 70 tumor samples and identified miRNAs that are regulated by SOX4, including miR-103, miR-339, miR-182 and miR-221. We also discovered that SOX4 regulates miR-16 and miR-196 in response to Wnt signals and also searched for miRNAs that might regulate SOX4. We identified the 1100-2350 nt region of the SOX4 3' UTR as a potential target for miRNAs that translationally inhibit SOX4 expression in prostate cancer cells. These studies provide new insights in to the function of SOX4 and its interaction with the miRNA pathway.

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Chapter I

SOX4: A Transforming Oncogene

In eukaryotic organisms, life begins as a single cell. Careful regulation of gene expression is required for cells to perform appropriate lineage commitment, differentiation, proliferation, and cell death. Much recent research has focused on understanding how cells pass down their inherent genetic materials while maintaining infinite self-renewal capacity. Cell-specific transcription factors function to facilitate cellular decision making to modulate expression of selected gene sets. While a single master transcription factor can be necessary and sufficient to control cell fate decisions and differentiation, it has been demonstrated that sets of transcription factors coordinate the myriad signals to build a cellular network. These transcription factors, include the E-twenty six (ETS), Krüppel-like factor (KLF), PRDI-BF1 and RIZ homology domain containing (Prdm), basic helix-loop-helix (bHLH), homeodomain, forkhead, and Sox families. They can act independently, regulating cell fate and differentiation decisions, or function cooperately, controlling a large variety of lineages. This chapter will first review the SOX family, and then concentrate on SOX4. Each member has the general properties of classical transcription factors, and plays a unique role in the transcriptional symphony.

1.1 The SOX Family

One of the most important properties of a transcription factor is the ability to bind to DNA. Non-histone DNA binding proteins were classified according to the mobility into three unrelated families: the HMG1/2 or HMG domain family, the HMG-I(Y) or AT-hook family, and the HMG- 14/17 or nucleosome-binding protein family [1]. The SOX family is a subset of the HMG domain family. The HMG proteins are well-known for their two arms of the L-shaped HMG domain that bind DNA in the minor groove, with intercalated amino acid side chains between DNA base pairs, inducing a significant bend of the DNA helix. The SOX family and the T-cell factor (TCF)/ lymphoid enhancer binding factor (LEF-1) family, proteins are expressed at a low level in a restricted number of cell types, and harbor a non-canonical HMG domain, called the HMG box domain. This HMG box domain shares only 20% identity to the classical HMG domain, but the ability to alter the DNA conformation with sequence specificity is conserved [2].

1.1.1 Molecular properties of SOX proteins

The first identified SOX protein was SRY (sex-determining region on the Y chromosome) [3, 4]. The *SOX* (Sry-related HMG box) family genes, were later identified and defined as SOX proteins that share high similarity with SRY protein in the HMG box domain. These *SOX* genes fall into eight groups according to sequence comparisons (Fig.1) [5].

SOX proteins within the same group generally share 70-95% identity; however, SOX proteins from different groups share only partial similarity (46%) in the HMG box

domain but not outside this region. HMG boxes are typically 70–80 amino acids in size and can be classified into three subtypes according to their DNA binding preferences. The first class contains the non-histone chromosomal proteins, such as HMG1 and HMG2 [1]. The second class includes the nucleolar and mitochondrial transcription factors, such as UBF, the upstream binding factor in RNA polymerase I transcription [6], and human mitochondrial transcription factor mtTF1 [7]. The last class of HMG box proteins contains sequence-specific transcription factors, such as the sex determining factor SRY [3] and the lymphoid enhancer binding factor LEF-1 [8]. This ability of SOX proteins to bend DNA allows them to function as architectural proteins through changing local chromatin structure and assembling other DNA-bound transcription factors onto promoters. However, the conserved DNA binding ability of SOX proteins in the HMG domain allows them to recognize specific DNA sequences in the promoters of target genes. The consensus motif for the HMG box domain of SOX proteins has been defined as the heptameric sequence 5´-(A/T)(A/T)CAA(A/T)G-3' [9]. A significant number of mutations have been characterized in HMG box domains that interfere with their DNA-binding ability or alter their DNA-bending characteristics [10]. In some subgroups, regions flanking the HMG domain are also conserved, such as two independent nuclear localization signals (NLS) that have been highly conserved in all SOX proteins. A N-terminal bipartite and a C-terminal basic cluster NLSs are identified in SOX group E proteins. SOX proteins completely lose the ability to transport into nucleus only when both nuclear localization motifs are functionally mutated [11]. Also, a 40-amino-acid dimerization domain is shared in SOX subfamily group D and E, and allows

cooperative binding in the presence of target gene promoters. Specific examples include the SOX2–OCT3/4 pairing in cell fate specification and the SOX2–PAX6 pairing in visual development [12]. The two highly conserved leucine-zipper coiled-coil domains, with the major one being associated to a glutamine-rich segment, mediates protein homodimerization in the absence or presence of DNA, resulting in high-efficiency binding to pairs of adjacent recognition sites on DNA [13].

1.1.2 Function and Mechanism

A classical important role for SOX proteins during the initial stages of ontogeny would be sex determination. SRY, the prototype of the SOX protein family, a decisive factor for male sex determination in mammals located on the Y chromosome [4], is expressed for a short period of time (10.5–12 days post-coitum in the mouse), triggering differentiation into Sertoli cells and then initiating testis differentiation from each gonad [14, 15]. SRY most closely resembles SOX3, which is localized on the X chromosome [16]. While SRY and its ancestor SOX3 are present very early during genital development, SOX9 is expressed in both male and female genital ridges up until SRY is expressed [17]. Then, SOX9 expression is restricted in the male gonad, where its expression follows differentiation of Sertoli cells [18], suggesting that SOX9 expression may control the timing of sex-determination. Another important property of SOX proteins is the ability to pair with various types of transcription factors to regulate cell development in early embryogenesis. For example, SOX2 and the POU domain factor OCT3/4 heterodimerize with each other on the *Fgf4* enhancer through their DNA-binding domains in embryonic

stem cells, and to synergize *Fgf4* expression in early blastocyst development [19]. The synergistic effect is not restricted to only one cell type with one partner. In lens cells, SOX2 pairs with PAX6 [20] and also interacts with BRN2 in neural primordium [21]. This suggests that SOX2, in combination with the other three factors, is involved in developmental processes from maintaining pluripotency to programming a fully differentiated cell type.

Nuclear transport is also a highly conserved property of all SOX proteins. The two nuclear localization signals (NLSs) that flank the HMG domain enable SOX proteins to shuttle between the cytoplasm and nucleus. Nuclear translocation constitutes a general mechanism to regulate SOX protein activity *in vivo*. Group E SOX proteins harbor a perfect consensus nuclear export signal sequence in contrast to all other SOX proteins. While the SOX E N-terminal nuclear localization signal binds calmodulin and is potentially regulated by intracellular calcium signaling, the C-terminal nuclear localization signal, which binds importin-β, responds to other signaling pathways such as cyclic AMP/protein kinase A [22]. During early mammalian embryogenesis, SOX9 is found in the cytoplasm of Sertoli cells in both genders, but with SRY expression, SOX9 moves into the nucleus in male embryos [18]. EXP4 (exportin 4) acts as an interaction partner and nuclear import receptor of SOX2 in mouse embryonic stem cells and neural progenitors [23].

Many SOX genes are expressed in the developing adult nervous system. Their expression patterns are overlapping and functionally covered from the neural stem cell stage until terminal maturation of neurons and macroglia. SOXB1 factors (SOX1, SOX2

and SOX3) have been ascribed key roles during proper establishment and maintenance of a functional central nervous system (CNS) by reducing the activity of proneural bHLH transcription factors [24, 25]. The *SOXB1* genes continue to be expressed and to maintain neural stem cells in the adult brain. When the expression of SOX2 is reduced in adult mice, it results in a loss of neuronal precursors and this phenomenon is observed in the Huntington and Alzheimer diseases in neurodegeneration [26]. In contrast, SOX21 is classified as a repressor gene that promotes neurogenesis. The overexpression of SOX21 leads to substantial repression of NGF-induced neurite outgrowth in PC12 cells [27]. This observation, together with its function in early embryogenesis, demonstrates the crucial role of SOX proteins in developmental cell biology.

It is also understood that *SOX* genes control skeletal, hematopoietic, and endodermal derived tissue development. The subfamily C genes, including *SOX4*, are co-expressed in skeletogenic mesenchymal cells throughout the mouse embryo, and their expression is maintained when these cells undergo chondrocyte and osteoblast differentiation[28]. SOX4 is highly expressed in the thymus and promotes pro-B lymphocyte expansion and T lymphocyte differentiation [29, 30]. In addition, induced pluripotent stem [31] cells that have been generated from bone marrow (BM) hematopoietic progenitor cells by ectopic expression of SOX2 may differentiate more efficiently than embryonic stem (ES) cells *in vitro* into hematopoietic cell lineages because of their epigenetic memory [32]. In group F, constitutively expression of SOX7 and SOX17 produces extraembryonic endoderm and defining endoderm progenitors, and can produce endoderm progenitors in human ES cells [33].

Ongoing SOX research (Fig.2) on physiological and molecular roles of SOX proteins will continue to increase our understanding of this distinct and ancient gene family.

1.2 SOX4

Sex-Determining Region Y Box 4 (SOX4) is a 47 K-Da protein encoded by a single exon gene located on human chromosome 6p22. The open reading frame contains an HMG-box, and the remaining sequence is particularly rich in serine residues that has several polyglycine and polyalanine stretches [34]. Homologs of SOX4 have been identified in all sequenced vertebrate genomes including zebra fish and chicken, mouse and humans. Sequences outside the HMG DNA binding domain show little evolutionary conservation while the HMG domain demonstrates little divergence with greater than 95% identity across sequenced species [35]. SOX4 is expressed in a number of tissues during embryonic development, including the heart, central nervous system, lungs and thymus. After cloning the SOX4 gene, a large body of literature has implicated a role for SOX4 in controlling diverse developmental processes, identified cellular regulatory proteins and transcriptional targets, and suggested roles for SOX4 in the regulation of cell death and carcinogenesis.

1.2.1 Developmental regulation

Congenital malformations of the heart are the most common birth defects. Mice genetically deficient in SOX4 die during embryogenesis because of failure in endocardial

ridge development in the heart at embryonic day (ED) 14. The deficiency causes impaired semilunar valve development, and malformations range from infundibular septum defect to complete transposition of the great arteries [36].

SOX4 is expressed in mouse uterus and is regulated by ovarian hormones, suggesting a developmental role of this gene in the female reproductive system. SOX4 transcription is directly induced by progestins, and this is accompanied by an increase in SOX-specific transcriptional activity. On the other hand, estradiol decreased SOX4 mRNA expression in these cells [37].

A very prominent function of SOX4 is regulation of lymphocytes of the B- and T-cell lineages. In fetal liver cells of *SOX4*$^{-/-}$ knockout animals, it was also shown that there is a blockage in early B-cell development at the pro-B-cell stage [29]. Unlike TCF-1 proteins that acts only as T cell-specific enhancers, SOX4 shows high affinity (Kd=3x 10^{-11}M) to the DNA minor groove of the AACAAAG motif in both T and pre-B lymphocyte lines. At the same time, *SOX4* was the first identified transcription factor in the *SOX* gene family with separable DNA-binding (HMG box domain) and transactivation domains at serine-rich C terminus (residues 299-440) in lymphocytes [38].

Besides lymphocytic maturation, SOX4 also plays a role in pancreatic development. SOX4 is detected broadly in the early pancreatic buds and eventually becomes restricted to the nuclei of all islet cells in the adult mouse. SOX4 null mice show normal pancreatic bud formation and endocrine cell differentiation up to embryonic day 12.5; however, they fail to undergo a second round of expansion to form normal islets, resulting in a lack of β-cells that are essential for insulin secretion.

Moreover, other studies have suggested that SOX4 expression must be efficiently down regulated for secretory activation in the mammary gland, as well as in bone development. Also, during prostate development, SOX4 is expressed in prostate buds and surrounding mesenchyme [39]. These studies clearly show that SOX4 is not only required for complete organ development, but also that SOX4 must be tightly regulated during development in specific cell types.

1.2.2 Carcinogenesis

In humans, tight regulation of the levels of transcriptional factors (TFs) is crucial to maintain tissue homoeostasis and many TFs have been found to be oncogenic when their expression is misregulated or when their activity is functionally altered. While SOX4 plays many different roles in cell development, it is not surprising that there is mounting evidence suggesting a role for SOX4 involvement in tumorigenesis. For example, SOX4 has been identified by whole-genome profiling as an oncogene that is overexpressed in several cancers, such as bladder cancer, in which it is 5-fold upregulated compared with normal tissue. Genes involved in signal transduction (*MAP2K5*), angiogenesis (*NRP2*), and cell cycle arrest (*PIK3R3*) are affected by SOX4 overexpression [40]. Another study published by our laboratory indicated that SOX4 is also upregulated at both mRNA and protein levels in prostate cancer and is correlated with higher Gleason score or tumor grade. Downstream target genes of SOX4 were also identified, including *BCL10, CSF1*, *NcoA4/ARA70, TLE-1*, and *BBC3/PUMA*. In addition, stably transfected SOX4 in non-transformed prostate cells enabled colony formation in

soft agar, suggesting that SOX4 can be a transforming oncogene [41]. Not only is overexpression of SOX4 highly correlated with cancer, downregulation of SOX4 leads to malignancy. In melanomas, SOX4 expression was remarkably reduced in metastatic melanoma compared with dysplastic nevi ($P < 0.05$) and primary melanoma ($P < 0.01$). This reduction was correlated with a poorer disease-specific survival of melanoma patients, which suggests SOX4 may serve as a prognostic marker and potential therapeutic target for human melanoma [42].

More examples indicate that aberrant expression of SOX4 leads to cancer progression. By using high-resolution comparative genome hybridization [43] comparing the DNA copy number and mRNA expression levels to identify novel oncogenes altered by gene amplification in lung cancer, SOX4 was identified as an oncogenic target in the chromosome 6p amplicon [44]. Consistent with the concept that SOX4 is an oncogene, studies have found SOX4 to be one of the most common retroviral integration sites, resulting in increased *SOX4* mRNA expression, and leading to neoplastic transformation in murine hematopoietic cells [45, 46]. Also, *SOX4* is activated by viral integration in AKXD-23 tumors and Cas-Br-MuLV-induced myeloid tumors [47]. MicroRNAs can misregulate SOX4 expression in cancer. MiR-335 suppresses metastasis and migration in breast cancer through targeting of SOX4 [48]. SOX4 was overexpressed through the epigenetic silencing of miR-129-2 in gastric cancer which provides another mechanism to emphasize the importance of SOX4 in human homeostasis [49]. In the following chapters, the relationship between SOX4 and microRNAs in prostate cancer will be discussed in greater detail.

1.2.3 Wnt signaling in prostate cancer

Wnt signaling is a key pathway in development and cancer that has been well-characterized in colorectal cancer. When cells are stimulated by Wnt ligands binding to Frizzled-LRP6 co-receptors, the complex that is composed of the tumor-suppressor molecule adenomatous polyposis coli (APC), the scaffold protein axin and glycogen-synthase kinase-3 β (GSK3 β) is disrupted and inactivated. As a result, β-catenin is no longer degraded and it translocates to the nucleus and heterodimerizes with members of the TCF/LEF transcription factor family to activate downstream target genes such as *c-myc* and *cyclin D* [50].

Dysfunction of Wnt signaling has been studied in many aspects in prostate cancer. Aberrant expression of β-catenin has been detected in many clinical prostate cancer tissues [51]. One Wnt inhibitor, dickkopf homolog 1 (DKK1), is reduced in bone metastases and may play a role in the osteoblastic properties of prostate cancer metastases [52]. The expression levels of several Wnt-inhibitory genes have been profiled as potential biomarkers for prostate cancer. Two of these candidates are Wnt inhibitory factor 1 (WIF1) and secreted frizzled related protein (SFRP4) showed the most significant aberrant expression [53], suggesting that regulation of Wnt signaling is crucial for prostate cancer progression.

Additional evidence has shown that SOX4 is involved in Wnt signaling. Evolutionarily related to the TCF/LEF HMG box factor, SOX4 also forms complexes with β-catenin, the key effector of the canonical Wnt pathway. Several members of the SOX

family, SOX17, SOX7, SOX9, and SOX4, have been implicated in regulating β-catenin activity [54-56]. Gain- and loss-of-function analyses have demonstrated that SOX17 and SOX7 proteins antagonize the Wnt pathway by competing with TCFs for β-catenin binding while SOX4 may function to stabilize β-catenin protein to help activate expression of target genes and promote cellular proliferation [57]. In addition, transcriptional profiles of intestinal tumors in APC mutated mice have shown that SOX4 is significantly upregulated in gastrointestinal adenomas indicating that SOX4 might play a role in Wnt pathway signaling [58]. Although these findings have implicated how SOX proteins regulate the transcriptional output of Wnt pathway, the precise role of SOX4 in the Wnt pathway remains unclear.

In the next chapter, I will describe the proteins that interact with SOX4 in LNCaP cells by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This novel interaction between SOX4 and other proteins provides new insights into the role of SOX4 in key pathways in cell proliferation, development, and cancer progression.

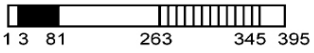
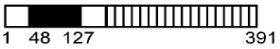
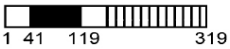
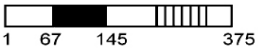
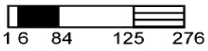
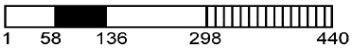
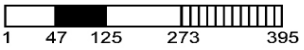
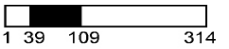
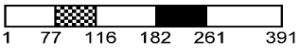
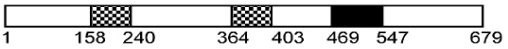
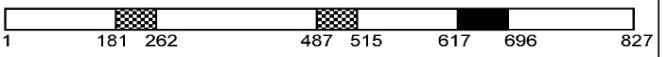
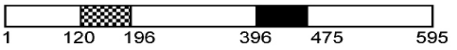
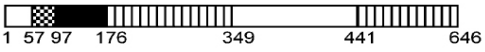
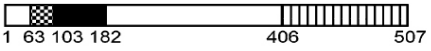
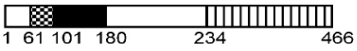
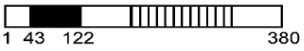
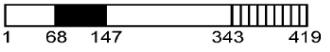
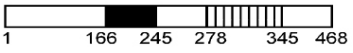
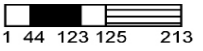
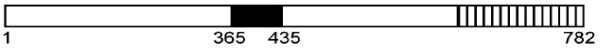
Group	Gene	Schematic
A	*Sry*	
B1	*Sox1*	
	Sox2	
	Sox3	
B2	*Sox14*	
	Sox21	
C	*Sox4*	
	Sox11	
	Sox12	
D	*Sox5*	
	L-Sox5	
	Sox6	
	Sox13	
E	*Sox8*	
	Sox9	
	Sox10	
F	*Sox7*	
	Sox17	
	Sox18	
G	*Sox15*	
H	*Sox30*	

FIGURE 1. SOX family subgroups and protein domain architecture. Black boxes = HMG domain, checkered boxes = dimerization domain, horizontal striped boxes = transrepession domain, and vertical boxes = transactivation domain. Adapted from *Levefbre et. al.* [59].

Group	Factor	Expression pattern	Function
A	SRY	male-specific embryonic: genital ridge preceding differentiation adult: testis, hypothalamus, midbrain	testis determination *mutation in humans leads to XY sex reversal and gonadal dysgenesis*
B	Sox1	embryonic central nervous system (CNS), lens, urogential ridge	lens development induction and maintenance of γ-crystallin gene expression *deletion in knockout mice leads to microphthalmia, cataracts , and spontaneous seizures*
	Sox2	inner cell mass, primitive ectoderm, developing CNS, lens	induction by chordin, repression by BMP-4, activation of FGF4, repression of osteopontin gene expression; induction of δ- and γ-crystallin gene expression modulation of responsiveness to neuralizing signals *deletion in knockout mice is embryonic lethal at implantation*
	Sox3	epiblast, embryonic CNS, urogenital ridge	candidate gene for human X-linked mental retardation syndromes, e.g. Borjeson-Forssman-Lehmann
	ZfSox19	transient; maternal, presumptive CNS in early gastrula, ventral region of embryonic diencephalon, midbrain and hindbrain; retina and lens	
	Sox21	primitve ectoderm, endoderm, developing gut, embryonic CNS: regional, longitudinal stripes in spinal cord	
	Sox70D (Dichaete, fishhook)	entire trunk of syncytial blastoderm, 7 irregular stripes in cellular blastoderm, ventral and cephalic neuroectoderm	*null mutation is lethal, exhibits segmentation defects, CNS defects* regulation of pair-rule genes
C	Sox4 (IRE-ABP)	embryonic heart (endocardial ridges and cushion) developing CNS, lung, tooth buds, mesonephros, gonads, thymus pre-B and T-cells	endocardial ridge development B-cell development *deletion in knockout mice leads to cardiac outflow tract malformation, lack of pro-B cell expansion, minor disturbances of thymocyte development*
	Sox11 (XLS13A, XLS13B)	widespread expression in developing CNS and peripheral nervous system (PNS), nascent somites, developing limbs, facial mesenchyme, somites, kidney, lung, specific postmitotic neuronal subpopulations	neural determination & differentiation events regulation of epithelio-mesenchymal interactions and inductive tissue remodelling

C	Sox22	throughout embryonic CNS and PNS; developing mesenchymal structures, fetal kidney, heart, pancreas, gonads	neural determination & differentiation events
	Sox24	oocytes	
D	Sox5	adult testis, highest in post-meiotic round spermatids	spermatogenesis
	L-Sox5	mesenchymal condensations, chondrocytes, embryonic CNS, notochord, otic vesicle	chondrogenesis
	Sox6 (SoxLZ)	rostrocaudal gradient in embryonic CNS, mesenchymal condensations, chondrocytes, notochord, otic vesicle adult testis	chondrogenesis
	xSox12	ovary	
	Sox13	embryonic: saccular component of inner ear, arterial walls, thymus adult: thymus, ovary, kidney	
	Sox23	brain, ovary, weaker in heart, liver	
E	SoxP1	pituitary, gonads	
	Sox9	mesenchymal condensations chondrocytes, genital ridge and adult testis, notochord, otocysts, vibrissae, tubular heart strucutres, ventricular CNS cells	chondrogenesis, regulator of Col2a1 and aggrecan sex determination, Sertoli cell differentiation factor *mutation leads to campomelic dysplasia in humans*
	Sox10 (SoxM, Sox21)	neural crest, embryonic PNS and CNS, enteric nervous system, melanoblasts, Schwann cells, oligodendrocytes	determination of neural crest & glial cells, *mutation leads to megacolon, pigmentation defects and deafness in humans (Waardenburg-Hirschsprung syndrome) and mice (Dominant megacolon)*
F	xSox7	various adult tissues, e.g. gonads, kidney, lung, brain	
	Sox17	testis: full length form in premeiotic germ cells, truncated form in postmeiotic germ cells	
	xSox17α,β	late blastula & gastrula, exclusively in presumptive endoderm	determination of endoderm; downstream of activin and Mixer; induces endodermin, IFABP, Xhlbox8
	Sox18	lung, cardiac and skeletal muscle	
G	Sox 20	fetal testis	
	SoxD	blastula and gastrula, prospective neuroectoderm, developing CNS	inducer of anterior neural tissues; enhanced expression by chordin, suppressed by BMP-4

FIGURE 2. Summary of expression patterns and biological function of Sox proteins.

Adapted from *Wegner et. al* [60].

Chapter II

Results: SOX4 Interacts with Plakoglobin in a Wnt3a-dependent Manner in Prostate Cancer Cells

2.1 Identification of proteins that interact with SOX4

Mass spectrometry is a highly sensitive technique that enables the rapid identification of proteins and also protein-protein interactions from a variety of biological samples. When combined with affinity purification, whole or targeted protein interaction networks can be elucidated [61]. To investigate the cellular functions of SOX4, we have developed a one-step affinity purification method that enables rapid purification of SOX4 complexes. The pREP4-BLRPwt-IRES-BirA-XL9 plasmid (generous gift of Dr. Jeremy M. Boss, Emory University) contains the *birA* gene of *E. coli* that encodes a biotin holoenzyme synthetase [62]. We cloned the human *SOX4* gene into this vector to generate an amino-terminal fusion to a BirA recognition sequence to produce transiently expressed, intracellularly biotinylated SOX4 protein in LNCaP prostate cancer cells (Fig.3), and purified SOX4 complexes with streptavidin-linked magnetic beads to perform large scale of proteomics analysis. Protein quantity and purity were checked by SDS-PAGE analysis and silver staining prior to mass spectrometry analysis by our collaborator Dr. Jumin Peng (Fig.4A). Seventy proteins were identified in our analysis. These proteins can be grouped into nine categories according to their biological functions (Table 1).

2.2 Plakoglobin interacts with SOX4

Among the SOX4 binding candidates we got from LC-MS/MS analysis, we identified junction plakoglobin (JUP) as one of the SOX4 binding protein via two trypsinized fragments that perfectly matched to human plakoglobin sequences (Fig.4B).

To confirm this interaction, we first repeated the transient transfection of the pREP4-BLRPwt-IRES-BirA-XL9-HASOX4 or vector control plasmid into LNCaP cells, and performed streptavidin-magnetic bead based purification followed by anti-plakoglobin (JUP) immunoblotting. As expected, the result of the immunoprecipitation (IP) and western blot validated the interaction between SOX4 and plakoglobin (Fig.4C). Furthermore, we performed a reverse-IP immunoprecipitating endogenous plakoglobin and probing for transfected HA-SOX4 (Fig.4D) and endogenous SOX4 in untransfected LNCaP cells (Fig.4E). To determine that the interactions were not cell-line specific or DNA-dependent, we repeated the immunoprecipitations in primary human keratinocytes, MDA-MB-231 breast cancer cells, and PC3M prostate cancer cells (Fig. 4F-I) following DNase I digestion of whole cell lysates. Taken together, these data demonstrate that SOX4 directly interacts with plakoglobin and that plakoglobin is a novel SOX4 binding partner.

2.3 Interaction between SOX4 and plakoglobin in the nucleus responds to Wnt signaling

Plakoglobin (JUP), also known as γ-catenin, is a major component of the submembrane of adherens junctions and desmosomes in mammalian cells [63]. It is closely related to the Drosophila segment polarity gene armadillo, which has a role in the transduction of transmembrane signals that regulate cell fate [64, 65]. Plakoglobin shares more than 76% homology with β-catenin, contains a central armadillo repeat domain flanked by the carboxyl and amino terminal domains, and functions in cell–cell

junctions, along with β-catenin and α-catenin when coupled with cadherins [66]. While it is known that β-catenin is essential in the Wnt signaling cascade, plakoglobin also binds to TCF/LEF, and has lower TCF/LEF-dependent transcriptional activity compared to β-catenin when endogenous β-catenin is depleted [67, 68].

When plakoglobin is present in desmosomes, it interacts with desmoglein and desmocollin, and when in adherens junctions it interacts with E-cadherin in the cytoplasmic component [63]. In our mass spectrometry analysis, several proteins, such as plakophilin (PKP3), actin, desmoplakin (DSP), and plectin (PLEC) that makeup the desmosome were identified. Together with plakoglobin, these potential SOX4 binding partners are also key compounds in desmosome structure, which suggests that SOX4 may serve functions other than being only a transcriptional factor in the nucleus.

Recently, additional evidence has suggested that plakoglobin contributes a low level of transcriptional activity to the Wnt signal transduction cascade in the nucleus [67, 68]. Although, it has been confirmed that SOX4 modulates Wnt signaling via interaction with β-catenin [57, 69], the role of plakoglobin in Wnt signaling is still debated. To investigate where and under what conditions SOX4 and plakoglobin interact with each other, we used confocal microscopy to determine whether we could observe subcellular co-localization of HA-SOX4 and plakoglobin (Fig.5A). After treatment of PC3M cells that stably-expressed HA-SOX4 with recombinant human WNT3A, we observed that the interaction between HA-SOX4 and plakoglobin was slightly increased in the nucleus. In contrast, this was not observed in the non-transfected adjacent cells. We observed the same phenomenon in the LNCaP-HA-SOX4 stable cell line (Fig. 5B).

Furthermore, when we treated with nuclear export inhibitor, leptomycin b [70] to inhibit nuclear export of SOX4 and plakoglobin, the co-localization was significantly increased compared to treating the cells with either WNT3A or LMB alone (Fig.5).

To confirm these enhanced interactions, we performed co-immunoprecipitation under the same Wnt-induced conditions in LNCaP-HA-SOX4 cells (Fig. 6A). The interaction in the whole cell lysate was quantified and significantly increased when we treated with WNT3A and LMB together (Fig. 6B). In addition, to determine that SOX4 and plakoglobin interact in the nucleus, we prepared cytosolic and nuclear fractions to confirm the subcellular interaction (Fig. 6C). Immunoblots against AKT and nuclear lamin were used as controls to demonstrate the purity of the nuclear and cytosolic fractions (Fig. 6C). The quantitative results showed that the interaction in the nuclear but not cytosolic fraction was significantly increased in response to the WNT3A and LMB treatment (Fig. 6D). These results show that SOX4 and plakoglobin physically interact in the nucleus of LNCaP cells.

2.4 Wnt signaling and SOX4-associated pathway are affected by SOX4-plakoglobin interaction

To address the functional consequences of modulation of SOX4 transcriptional activity by the SOX4-plakoglobin complex, we tested expression of several genes including the Wnt target gene *AXIN2* [71], as well SOX4 targets *DICER1* and *DHX9* [69]. To characterize if SOX4 DNA binding activity is changed by Wnt-induced interaction with plakoglobin, we performed ChIP assays for *AXIN2*, *DICER1*, *DHX9* and *SOX4* (Fig.7A, B).

Compared to untreated LNCaP HASOX4 cells, the binding of *SOX4* to *AXIN2*, *DICER1* and *DHX9* promoters was increased after Wnt signaling was induced. However, the SOX4 binding was decreased after treatment with both WNT3A and LMB. This difference indicates that increasing the interaction between SOX4 and plakoglobin could inhibit the SOX4 binding activity to downstream targets and may inhibit SOX4 transcriptional activity. Quantitative realtime-PCR (qPCR) analysis of the LNCaP SOX4 stable cell line showed reductions in *AXIN2*, *SOX4* and *DHX9* in RNA expression upon co-treatment with WNT3A and LMB (Fig.7C). To determine whether the effects from combined WNT3A and LMB treatment were dependent on plakoglobin, we targeted plakoglobin by siRNA to determine whether reduced plakoglobin levels could rescue the effects we observed on SOX4 binding to target promoters. Transfection of LNCaP HA-SOX4 cells with plakoglobin siRNA or scrambled control siRNA resulted in approximately 50% knockdown of endogenous plakoglobin protein levels (Fig. 7D). Furthermore, plakoglobin siRNA partially rescued SOX4 binding to target promoters by ChIP assay in the presence of WNT3A and LMB, while scrambled siRNA had no effect (Fig. 7E). These data suggest that SOX4-plakoglobin interactions may interfere with SOX4-mediated transcription in response to Wnt signaling due to reduced promoter occupancy.

2.5 SOX4-plakoglobin complex modulates β-catenin-mediated transcriptional activity

To evaluate whether the SOX4-plakoglobin complex affects the transcriptional activity of β-catenin, we performed luciferase reporter assays with T cell factor (TCF) reporter plasmids containing wild type TCF binding sites (TOP-flash) or mutated TCF

binding site (FOP-flash) [72] (Fig.8A). As expected, the TCF/β-catenin luciferase reporter activity was significantly increased after treatment with recombinant WNT3A when compared to untreated cells. In contrast, co-treatment with WNT3A and LMB, strongly inhibited increases in luciferase activity back to baseline unstimulated levels, suggesting that WNT3A+ LMB-induced SOX4-plakoglobin complexes could compete with and inhibit the transcriptional activity of β-catenin. In addition, we performed anti-β-catenin ChIP assays on the *AXIN2*, *c-Myc*, and *DKK1* promoters, and observed that occupancy of these promoters was stimulated by WNT3A, but that stimulation was inhibited by co-treatment with WNT3A and LMB (Fig. 8B). These results suggest that β-catenin activity was affected by the SOX4-plakoglobin complex and that plakoglobin may compete with β-catenin binding to SOX4 and/or TCF/LEF in the nucleus.

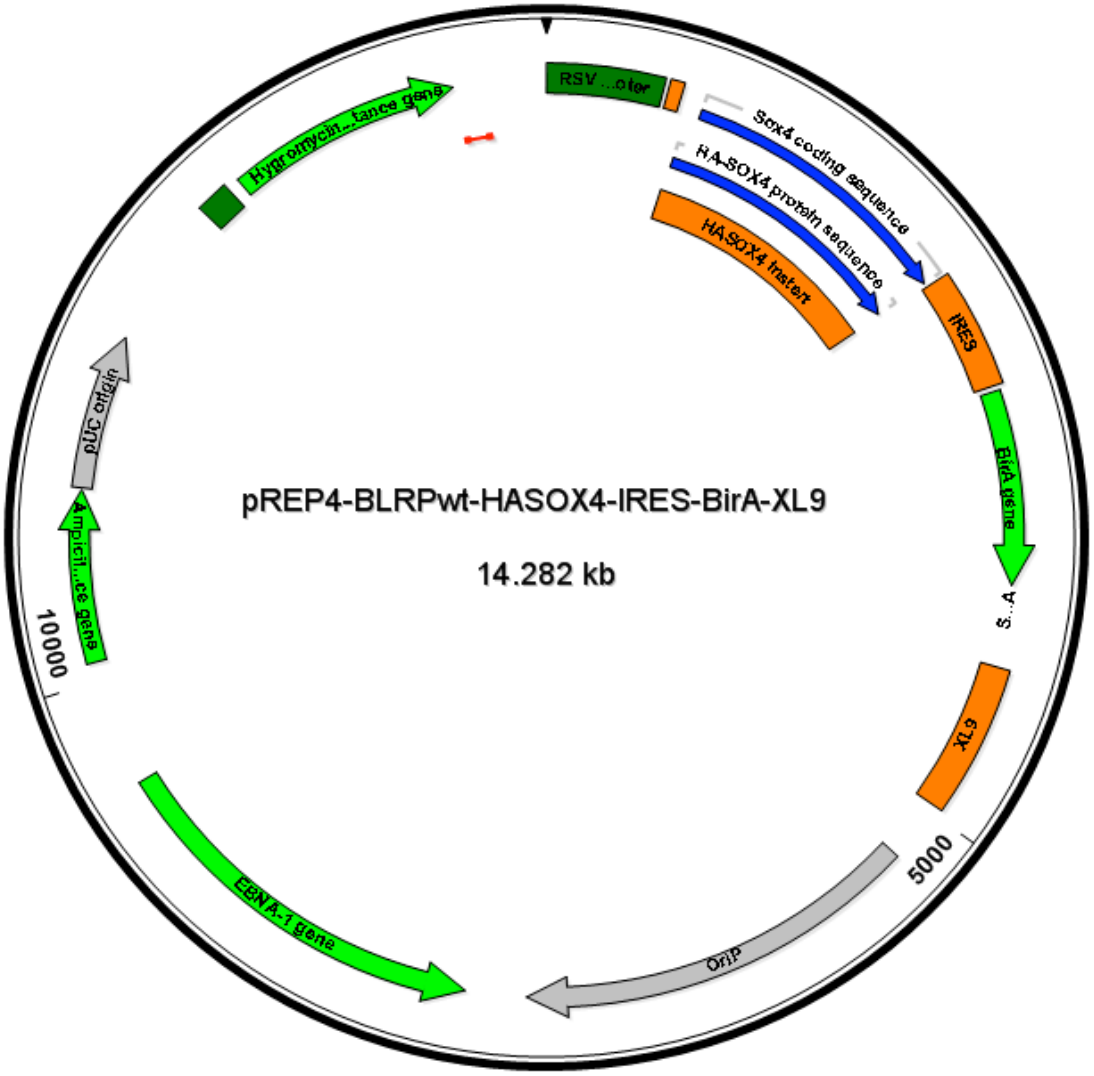


FIGURE 3: The TAP-TAG SOX4 clone. The *birA* gene of *E. coli* encodes a biotin holoenzyme synthetase that can transcribe intracellular biotinylated SOX4 amino-terminus. This a one-step affinity purification provide good quality of protein that can perform LC-MS/MS.

Annotation	Number unique protein	Gene
Cytoskeleton	5	IQGAP2, ACTB, ACTG, MYH9, LMNB1
DNA repair	7	LIG3, SSRP1, SUPT16H, H2AFX, SFPQ, MDC1, XRCC6
Translation/ RNA processing	14	AP2B1, EEF, PABPC1, HNRNPU, HNRNPM, RRBP1, DHX30, RPL, DDX, PRPF8, HLTF, DHX9, ASCC, EFTU
Transcription	7	MATR3, SSRP1, SOX11, SOX12, TRRAP, H2A, H2B
Transport	8	TUBA, TUBB, MAP1B, MYO6, CLTC, GAN, NUP, APO
Cell cycle	3	ILF3, NOLC1, NUMA1
Plasma membrane	4	KCNN, MME, SLC25A, ALDH
Desmosome	9	PKP3, ACTA, ACTB, ACTC, ATCG, DSP, JUP, PLEC1, EPPK1
Mitochondria	7	ATP5, HADHB, GFM1, ACADVL, IDH2, OGDH, DLAT
Others	57	
Total	121	

TABLE 1: LC-MS/MS analysis of SOX4 binding partners in LNCaP cells. Among the peptides we identified, some match more than one protein that we only list out the subgroup or family they belong, such as Actin (ACTA, ACTB, ACTC, ATCG), Tubulin (TUBA, TUBB), and Histone (H2A, H2B). Some proteins are under-investigation that may not fall into any of the functional group and grouped as Others.

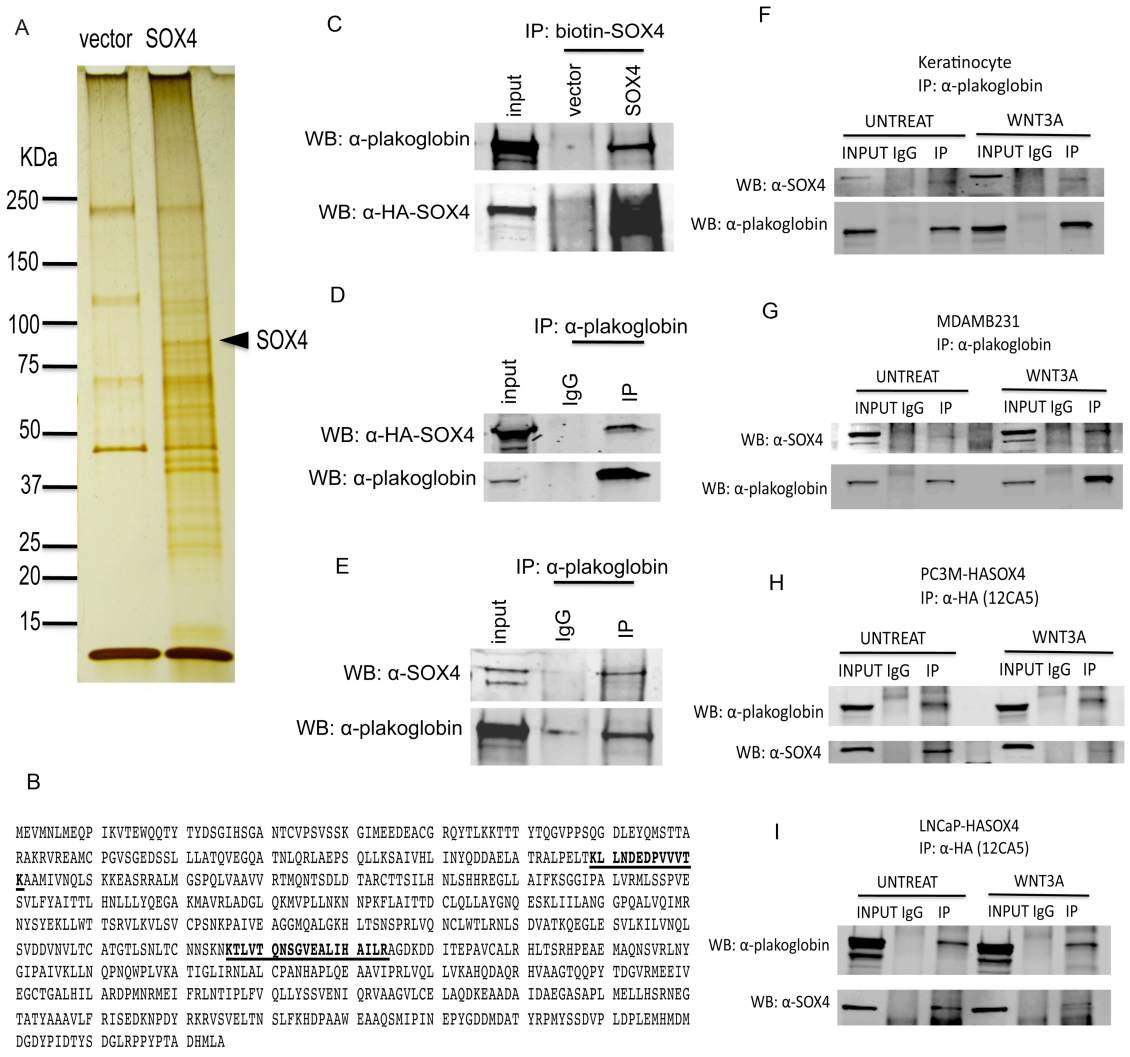
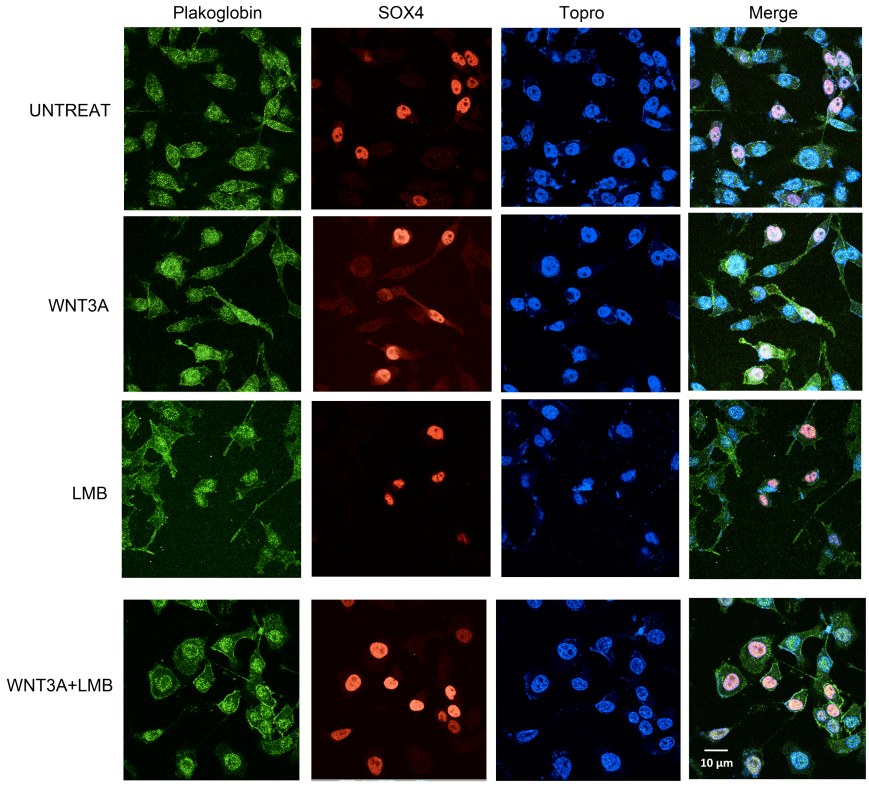


FIGURE 4. Plakoglobin binds to SOX4 in LNCaP cells. *A*, whole cell lysates prepared from LNCaP cells transfected with pREP4-BLRPwt-HASOX4-IRES-BirA-XL9 or vector only were purified using an streptavidin-magnetic beads, and affinity-purified Vector- and SOX4-complexes were visualized by silver staining. SOX4 protein is indicated by an arrow. The molecular weight markers are as shown on the left. *B*, amino acid sequence of plakoglobin with trypsinized peptides from LC-MS/MS analysis indicated in bold and

underlined. *C*, pREP4-BLRPwt-HASOX4-IRES-BirA-XL9 and vector control transfected LNCaP cells. SOX4-associated plakoglobin were analyzed by Western blot with antibodies indicated. Equivalent amounts of the Vector-purified fractions were used to confirm specificity. 5% of transfected LNCaP whole cell lysate was used as Input. *D*, endogenous plakoglobin IPs were analyzed for transfected SOX4 by Western blot with anti-HA 16B12 mAb. *E*, endogenous plakoglobin IPs were analyzed for endogenous SOX4 in untransfected LNCaP cells. *F, G*, endogenous plakoglobin IPs were analyzed for endogenous SOX4 in primary human keratinocytes and breast cancer cell line, MDA-MB-231, respectively. *H, I*, HASOX4 IPs were analyzed for endogenous plakoglobin in stably-expressed SOX4 PC3M and LNCaP cell lines. *F-I*, whole cell lysates were harvested and treated with DNase I for 1 hr at room temperature prior to immunoprecipitation.


Plakoglobin
SOX4
Topro
Merge
UNTREAT
WNT3A
LMB
WNT3A+LMB
10 µm

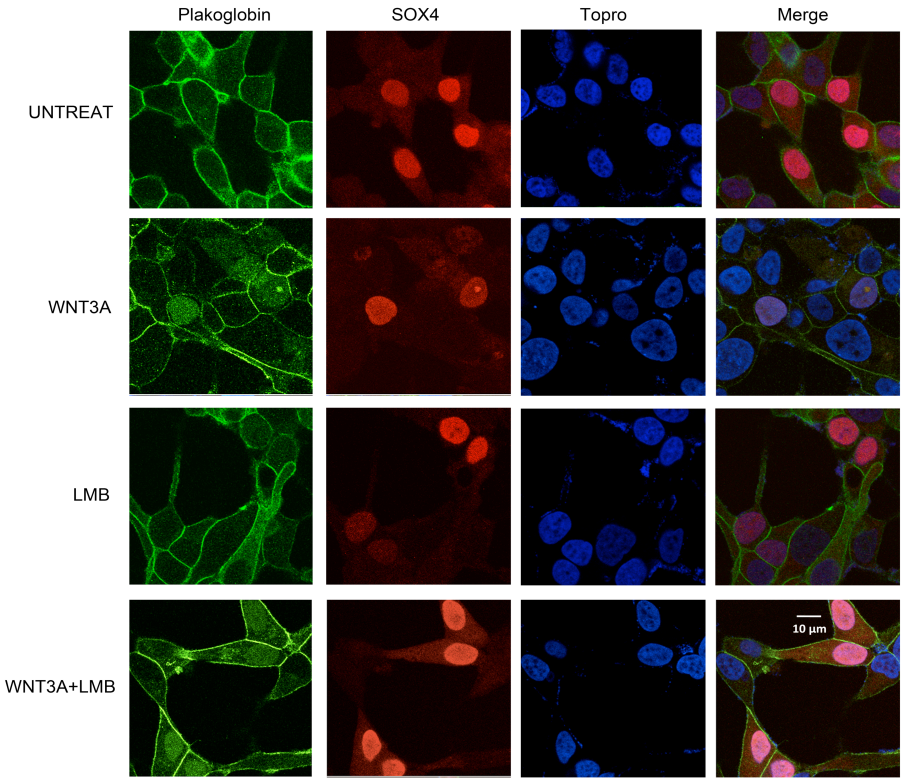

Plakoglobin
SOX4
Topro
Merge
UNTREAT
WNT3A
LMB
WNT3A+LMB
10 µm

FIGURE 5. Wnt signaling induces nuclear colocalization of SOX4 and plakoglobin. Subcellular localization of plakoglobin and SOX4 were examined by confocal microscopy. *A,* PC3M HASOX4 (upper) *B*, LNCaP HASOX4 (lower) stable cell line was treated with 100 ng/ml human recombinant WNT3A or 20 μM leptomycin b [70], or both WNT3A+LMB for 24 hr. The fields shown were visualized independently by confocal microscopy at the appropriate wavelength for plakoglobin (488) and SOX4 (543), and Topro (633) respectively, and then the three images were overlaid (Merge). Strong nuclear localization of plakoglobin was observed in the WNT3A+LMB treated cells that expressed HASOX4. Representative fields from these independent repeated experiments are shown. Plakoglobin localizes to the nucleus following WNT3A treatment, and this effect is strongly enhanced by LMB co-treatment, suggesting shuttling of plakoglobin into and out of the nucleus following WNT3A stimulation. Note that plakoglobin nuclear localization is much stronger in cells expressing HASOX4, suggesting SOX4 may facilitate plakoglobin nuclear import.

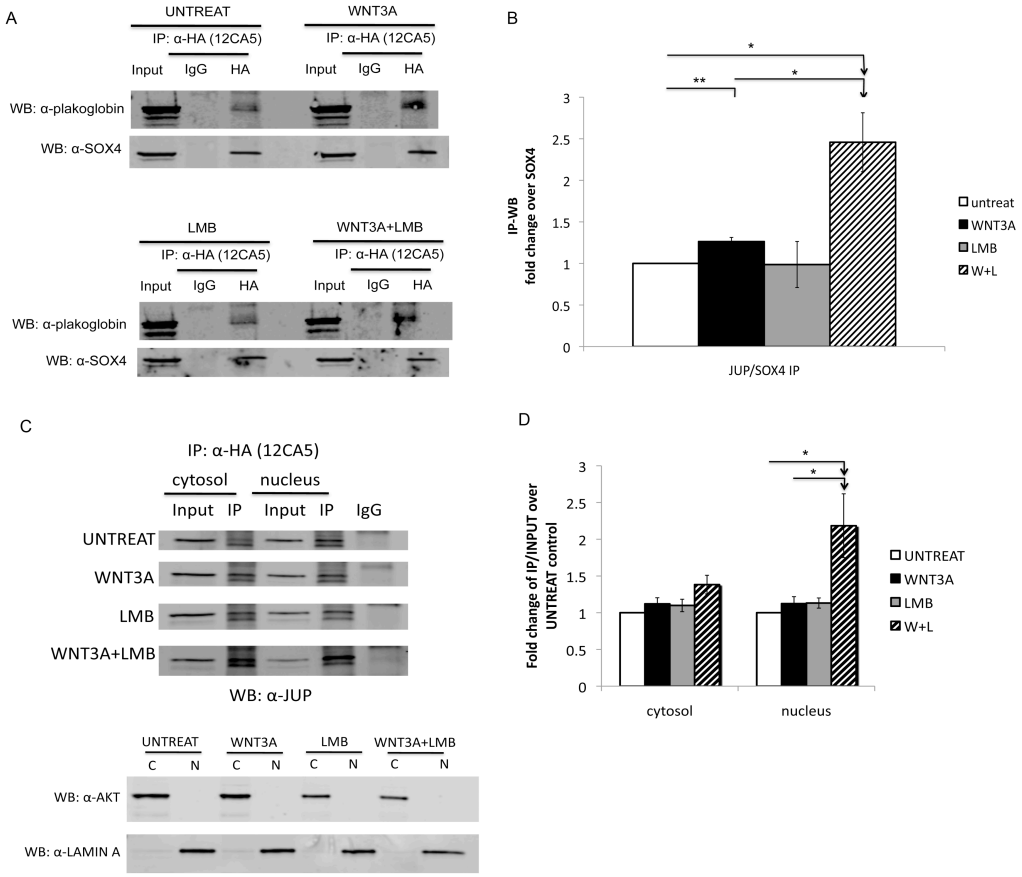


FIGURE 6. Interaction between SOX4 and plakoglobin is affected by Wnt3A signaling. *A,* LNCaP HASOX4 stable cell lines were treated with vehicle, Wnt3A, LMB, or Wnt3A + LMB and anti-HA immunoprecipitations were immunoblotted for the presence of plakoglobin. *B,* quantitative analysis of co-IP and Western blots from panel A using the Odyssey® Infrared Imaging System (Li-Cor Bioscience). Fold changes were calculated as the ratio of immunoprecipitated plakoglobin to SOX4 relative to the vehicle control panel (untreat). (n = 3, independent biological replicates performed on separate days; error bars represent SEM; *, $p < 0.05$; **, $p < 0.01$ for a one-sided paired t-test) *C*, (Upper) LNCaP HASOX4 stable cell lines were treated with vehicle, WNT3A, LMB, or WNT3A + LMB and anti-HA immunoprecipitations were performed using separate

cytosolic and nuclear fractions. (Lower) Anti-AKT and anti-Lamin A antibodies were used as controls to examine markers of cytosolic and nuclear fractions, respectively, to confirm the purity of each fraction. *D,* quantitative analysis of co-IP and Western blots from panel C.

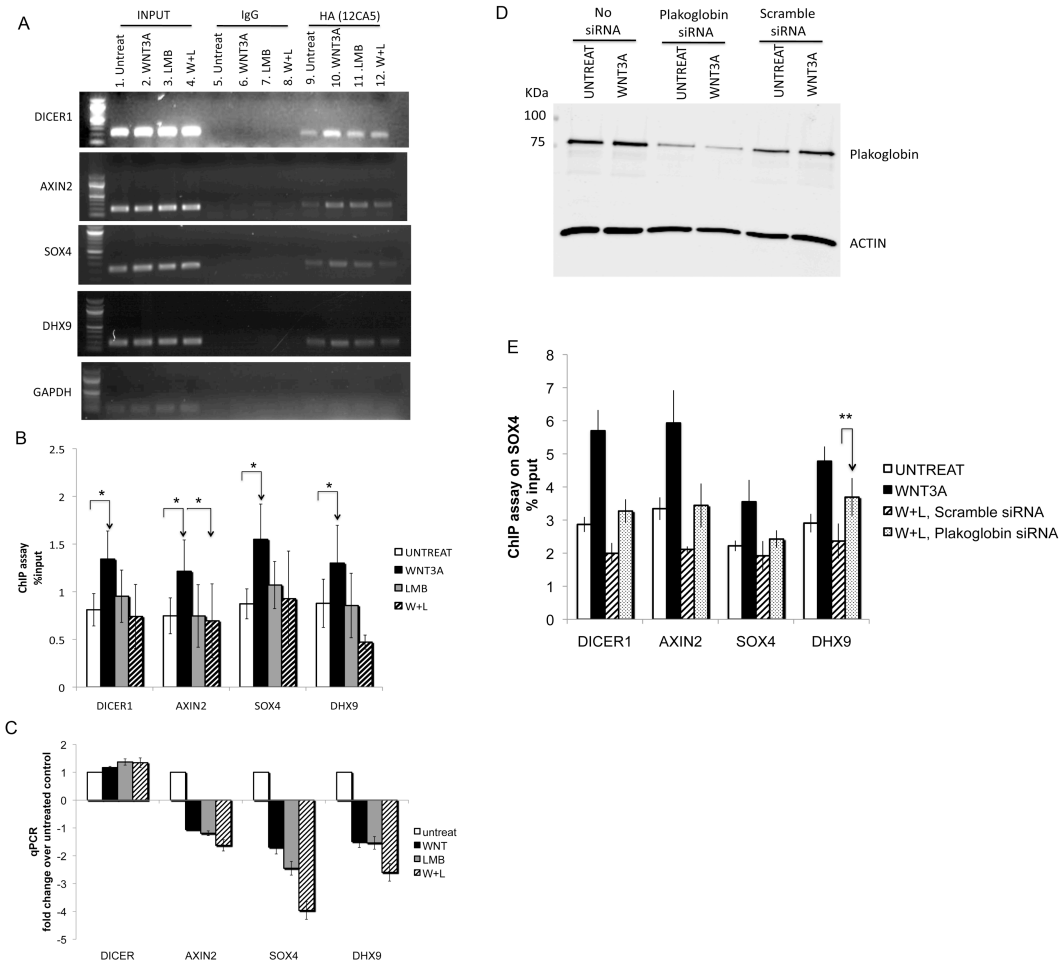


FIGURE 7. SOX4 transcriptional activity is modulated by Wnt-induced interaction with plakoglobin. *A,* ChIP assay of HA-SOX4 bound to the predicted SOX4-binding sites on *DICER1*, *SOX4*, *DHX9*, and *AXIN2* promoters. *GAPDH* is shown as a negative control. *B*, quantitative results of ChIP by realtime-PCR. SOX4 promoter occupancy is increased with Wnt treatment, but is strongly reduced by combined WNT3A+LMB treatment. (n=4; error bars represent SEM; *, $p < 0.05$ for a one-sided paired t-test) *C*, SOX4 targets and Wnt downstream genes are inhibited after Wnt induction and LMB treatment.

Realtime-PCR expression analysis of SOX4 direct targets and Wnt signaling downstream genes after 24 hrs of WNT3A and LMB treatment. *D,* siRNA directed against plakoglobin downregulates protein expression in LNCaP HA-SOX4 cell lines. Cells were harvested 48-hr post-transfection and Western blot were probed with anti-plakoglobin and anti-β-actin antibodies in the presence or absence of WNT3A treatment. *E,*quantitative ChIP-qPCR assay of HA-SOX4 following plakoglobin knockdown. HA-SOX4 promoter occupancy is strongly reduced by combined WNT3A and LMB treatment but is partially restored when plakoglobin is knocked down by siRNA treatment. Control scrambled siRNA had no effect (n=3; error bars represent SEM; *, $p < 0.05$ for a one-sided paired t-test).

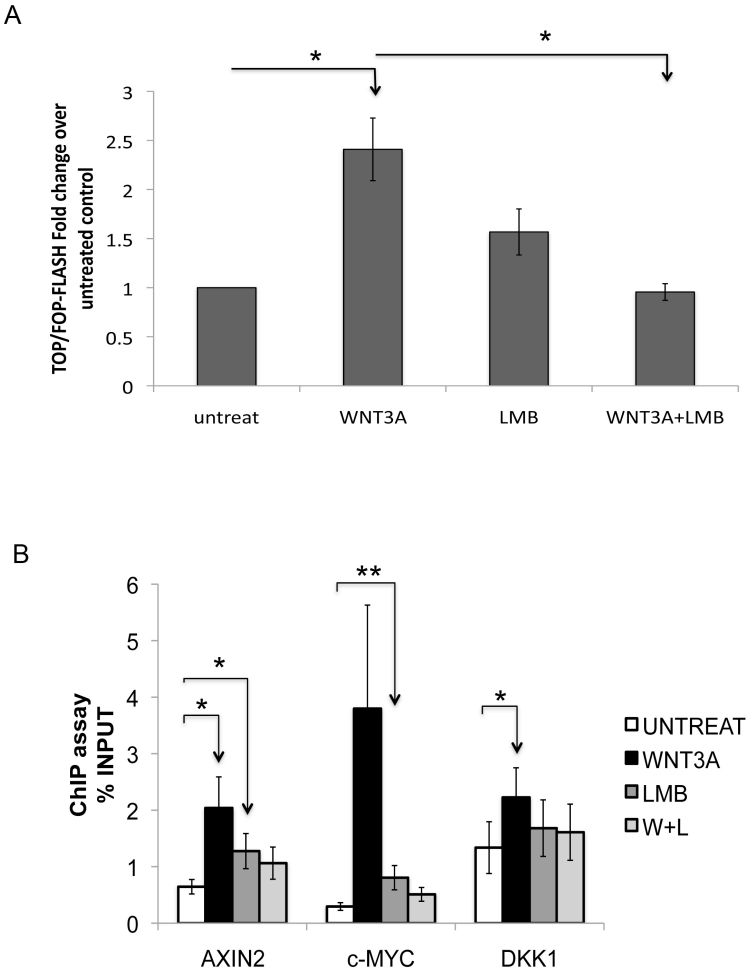


FIGURE 8. Wnt signaling is downregulated by SOX4-plakoglobin complex. *A,* luciferase assay of LNCaP HASOX4 stable cell line co-transfected with 40 ng of TK-Renilla with either 500 ng of TOP-flash or FOP-flash plasmids. Luciferase activity was increased after Wnt inducement, but decreased in combined WNT3A+LMB treatment. (n=3; error bars represent SEM; *, $p < 0.05$ for a one-sided paired t-test) *B*, quantitative results of ChIP of β-catenin by realtime-PCR. β-catenin promoter occupancies of Wnt downstream targets, *AXIN2*, *c-MYC*, and *DKK1*, were increased with Wnt treatment, but is strongly reduced by combined WNT3A+LMB treatment. (n=3; error bars represent SEM; *, $p < 0.05$; **, $p < 0.01$ for a one-sided paired t-test)

Chapter III

Plakoglobin: Discussion

While SOX4 expression is elevated in many malignancies and is tightly correlated with prostate cancer tumor grade, little is known of the mechanism by which SOX4 affects the progression of prostate cancer. Using LC-MS/MS proteomic analysis, we identified a novel SOX4 binding protein, junction plakoglobin, in LNCaP prostate cancer cells. We observed a physical association between SOX4 and plakoglobin with both epitope-tagged and endogenous SOX4. Although the interactions of SOX4 with plakoglobin were enhanced by co-treatment of WNT3A and LMB, we could detect this interaction using four different untransfected and unstimulated cell types (Fig. 1F-I) treated with DNase I. In addition, confocal microscopy and co-immunoprecipitation demonstrated co-localization of SOX4 and plakoglobin in the nucleus when Wnt signaling was induced. ChIP assays showed the SOX4-plakoglobin complex affected the SOX4 DNA binding activity to the *AXIN2*, *DICER1* and *DHX9* promoters that are Wnt signaling downstream genes and SOX4-associated targets. In addition, mRNA expression changes were detected in *AXIN2*, *DICER1*, and *DHX9* by realtime-PCR. These data suggest that SOX4-plakoglobin complex may inhibit Wnt signaling. Indeed, conditions that induced the increased interaction between SOX4 and plakoglobin caused β-catenin TOP-FLASH transcriptional activity to be downregulated and reduced occupancy of the c-Myc promoter by β-catenin.

It is known that SOX4 can directly interact with β-catenin to enhance Wnt signaling [57, 69], but mechanistic data remain very limited. We found that SOX4 also interacts with plakoglobin in a WNT3A-dependent manner in our experimental cancer model. Our model not only supports the hypothesis that SOX4 may stabilize β-catenin

[57], but also suggests a model in which SOX4 can modulate Wnt signaling by binding either β-catenin or plakoglobin (Fig.9). In this model, transcriptional responses to Wnt signaling are increased by SOX4-β-catenin interactions, and subsequently reduced by SOX4-plakoglobin interactions that facilitate nuclear export of SOX4. However, when nuclear export is inhibited, plakoglobin competes with β-catenin for binding to SOX4 and TCF/LEF factors, downregulating Wnt-responsive transcription and reducing SOX4-DNA binding. This model is supported by the observation that combination treatment with WNT3A and LMB enhanced SOX4-plakoglobin interactions, reduced TCF/β-catenin TOP-FLASH transcription, reduced SOX4-DNA binding and β-catenin DNA binding in ChIP assays, and reduced expression of SOX4 downstream targets (Figs. 7 and 8). Consistent with our model, plakoglobin shows little transcriptional activity compared to β-catenin in cell lines that lack β-catenin [67].

Our confocal data show that plakoglobin nuclear localization is strongly enhanced by LMB co-treatment, suggesting shuttling of plakoglobin into and out of the nucleus following Wnt3A stimulation. Although LMB is an artificial inhibitor, others have shown that plakoglobin overexpression can lead to nuclear localization [73, 74], and plakoglobin is overexpressed [75], and amplified [76], in several types of cancer. Moreover, plakoglobin nuclear localization was much stronger in cells expressing HASOX4, suggesting cytoplasmic SOX4 may facilitate plakoglobin nuclear import. Thus, SOX4 may induce nuclear import of plakoglobin in response to WNT3A while plakoglobin destabilizes SOX4 from DNA binding, facilitating nuclear export of SOX4. It is as yet unclear what signals or modifications might tip the balance between nuclear

import and export of plakoglobin-SOX4 complexes, or whether shuttling might be constitutive.

The role of plakoglobin during cancer progression is still controversial. When plakoglobin is overexpressed, it induces cell migration and mobility in HCT116 cells, suggesting that plakoglobin may have some oncogenic effects [73]. In contrast, several reports have demonstrated that plakoglobin has a tumor-suppressive effect that inhibits tumor cell growth [77, 78]. The tumor suppressive activity of plakoglobin may be via nuclear translocation to antogonize β-catenin binding to TCF/LEF proteins in keratinocytes [79], supporting our model that plakoglobin competes with SOX4-β-catenin interactions in the nucleus.

According to our LC-MS/MS data, several desmosomal proteins were identified as SOX4 binding candidates, such as PKP3, DSP, and PLEC1. While SOX4 responds the transforming growth factor (TGF-β)-mediated epithelial-mesenchymal transitions (EMTs) in human keratinocytes [80], it is possible that overexpressed SOX4 promotes metastasis, potentially via interactions with these proteins in our prostate cancer cell model system. This suggests SOX4 may have additional roles in the cytoplasm.

Besides plakoglobin, we have identified a large number of interesting proteins that were co-immunoprecipitated with SOX4 in our mass spectrometry data (Table 1). Several of these proteins are involved in DNA repair, such as DNA ligase III (LIG3), which is one of three ATP-dependent DNA ligases in mammalian cells that is important in DNA replication and repair and is the only ligase that has nuclear and mitochondrial forms. While LIG3 functions with XRCC1 during DNA base repair [81], we also identified XRCC6

(Ku70) as a potential interactor with SOX4. XRCC6 is one of the components in DNA-dependent protein kinase end-joined DNA double strand break repair [82]. These data imply a relationship between LIG3, XRCC6, and SOX4 in DNA repair. Also, one third of the SOX4 protein binding candidates are involved in RNA processing, such as HNRNPU, SFPQ, EFTU, and DHX9, which suggests that SOX4 may function to regulate RNA processing. We are now performing RNP immunoprecipitation-sequencing (RIP-seq) on SOX4 complexes in LNCaP cells and identifying mRNA regions that are bound by SOX4 either directly or indirectly via protein-protein interactions with RNA-binding proteins.

In summary, we have demonstrated that SOX4 interacts with plakoglobin in a Wnt-dependent manner in LNCaP cells. Additional studies will be required to elucidate the detailed mechanisms by which SOX4-plakoglobin interactions may affect Wnt signaling, but the role of the SOX4-plakoglobin complex provides novel insights into the role of SOX4 in Wnt signaling and prostate cancer progression.

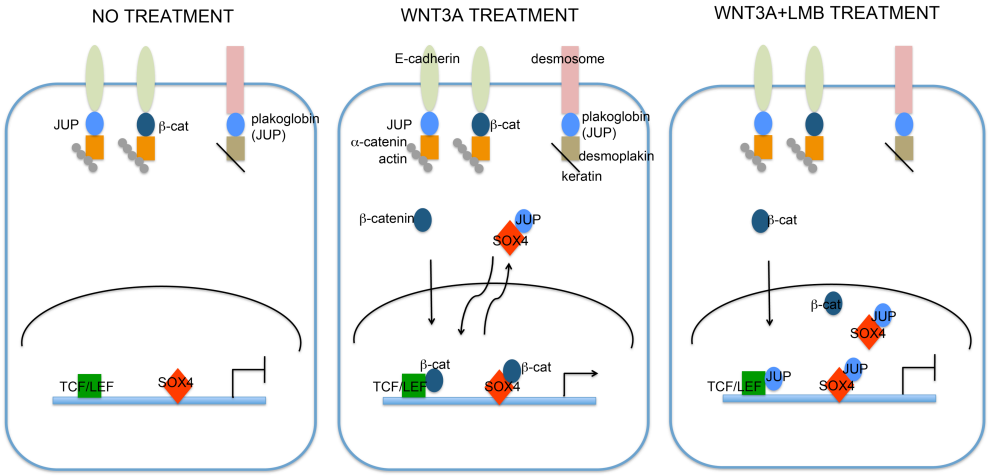


FIGURE 9. Model depicting the role of SOX4 and plakoglobin in Wnt signaling regulation. When cells are stimulated with WNT3A, Wnt-responsive genes are upregulated by nuclear localization of β-catenin and transcriptional activation by the β-catenin-TCF/LEF and β-catenin-SOX4 complexes. Plakoglobin then shuttles into the nucleus and is exported out of the nucleus with SOX4. When nuclear export is inhibited by LMB, plakoglobin competes with β-catenin for SOX4 binding, destabilizes SOX4 binding to target promoters, and inhibits transcription of target genes.

Chapter IV

Network Between SOX4 and MiRNA in Prostate Cancer

4.1 MicroRNA

MicroRNAs (miRNAs) are 22-25 nucleotide single-stranded small non-coding RNAs that regulate translation through targeting messenger RNA (mRNA) 3' untranslated regions (3'UTR), and could be one of the most important newly discovered classes of cellular regulators [83]. MiRNA alterations are involved in a broad spectrum of human diseases. MiRNA expression profiling has been used to identify signatures associated with cancer staging, progression, prognosis and diagnosis. The differential expression of miRNA genes in malignant compared with normal cells can be caused by the location of these genes in genomic regions, by epigenetic alterations, and by miRNA processing misregulation. Also, profiling has been exploited to identify downstream targets of miRNA, which are activated in abnormalities.

Among these miRNAs that were identified as key regulators in development, *SOX* genes have been reported to be miRNA targets. In human embryonic stem cells, miR-145 is upregulated and targets the *SOX2* 3'UTR when cells undergo differentiation [84], which suggests that miRNA-mediated regulation of SOX genes may be an important mechanism in cell programming. To summarize these findings, we can conclude that miRNAs regulate a myriad of genes and have robust effects in diverse aspects of different developmental stages in many species.

4.1.1 Biogenesis

The function and biogenesis of miRNAs was first investigated in *lin-4* and *let-7*, which were originally identified by genetic analysis in *C.elegans* developmental timing

studies. They act as post-transcriptional repressors of target genes when bound to specific sites in the 3´ untranslated region of the target mRNA [85, 86]. The structure of the ~70 nt putative precursor was first discovered as a stem–loop with some bulges by secondary structure prediction [87]. The hairpin-shape pre-miRNAs in the nucleus are then bound and chopped by a series of enzymes (Drosha) into smaller pieces and shuttle out of the nucleus as a complex, which is known as RNA-induced silencing complex (RISC) [88]. Two steps involved in pri-miRNA cleavage are shown in Figure 10. In the first step, the pri-miRNA is processed by the RNaseIII enzyme, Drosha, into pre-miRNA. Then exportin 5 (EXP5) nuclear export factor, which is a member of the karyopherin family of nucleocytoplasmic transport factors, binds the pre-miRNA specifically, but only in the presence of Ran-GTP. Subsequently, Ran forms a nuclear heterotrimer with pre-miRNAs to bring the pre-miRNAs out of nucleus [89].

In the second step, pre-miRNAs transported to the cytoplasm are further cleaved by DICER to yield 20-bp miRNA duplexes. DICER is a 200 k-Da protein containing generally an ATPase/RNA helicase, PAZ domains, DUF283, two catalytic RNase III domains (RIIIa and RIIIb) and a C-terminal dsRBD (RNA binding protein) [90, 91]. DICER also processes dsRNA into siRNAs and thus plays an important role in RNA interference in general. DICER is a highly conserved protein with one homologue in yeast [58], one in human, one in nematode worm (DCR-1), two in Drosophila (DCR-1 and DCR-2), and four in Arabidopsis (DCL1, DCL2, DCL3, DCL4) [92], which suggests its importance from an evolutionary viewpoint. DICER functions as a monomer and bears a single processing center with intramolecular dimerization of the two RNase III domains. The structure and

function of its catalytic center is very similar to that of Drosha [91]. Each RNase domain cuts independently at one RNA strand of the duplex that is produced by Drosha and generates two single-stranded RNA products with 2-nt 3‘ overhangs. The DICER PAZ domain recognizes 3‘ overhangs of pre-miRNAs, and provides a platform for PAZ PIWI domain (PPD) proteins. DICER then excises miRNA from the end of pre-miRNA hairpins that are produced by Drosha. Together with the RNA cleavage products of DICER, the RNA-induced silencing complexes (RISCs) [93], is composed of Argonaute 1 (AGO1) or Argonaute 2 (AGO2), TRBP, and DICER. RISC functions to process miRNAs from pre-miRNAs to their mature form, cleave target mRNAs, and participate in translational inhibition. AGO2 was found to be the catalytic endonuclease in human RISC, and a PIWI domain was identified as the catalytic center [94]. AGO1, another Argonaute protein in fly, is dispensable for cleavage; however, when Drosophila embryos lack AGO2, they still have RNA-cleavage capacity [95]. Another component in RISC is TRBP, which was identified by mass spectroscopy, corresponding to the human immunodeficiency virus [96]-1 transactivating response (TAR) RNA-binding protein (TRBP). TRBP is required for the recruitment of AGO2 to the miRNA bound by DICER and serves as a platform for RISC assembly [97]. After the maturation of miRNA is completed in the cytoplam, RISC uses the siRNA or miRNA as a template for recognizing complementary mRNA [98]. When it finds a complementary strand, it activates RNase and cleaves the RNA. This process is important both in gene regulation by miRNA and in defense against, double-stranded RNA viral infections (Fig. 10).

4.1.2 Developmental regulation

The first miRNA, *lin-4*, was identified in research of genes controlling developmental timing in *C. elegans*. *Lin-4* is essential for the normal temporal control of postembryonic development in *C. elegans* and acts by negatively regulating LIN-14 protein, creating a temporal decrease in LIN-14 protein starting in the first larval stage (L1). Genomic sequence analysis indicated that *lin-4* does not encode a protein product but two small *lin-4* transcripts of approximately 22 and 61 nt products were transcribed. These *lin-4* RNAs are complementary to a repeated sequence on the 3'UTR region of *lin-14*, which suggested a model of an antisense regulation mechanism [85]. The second identified miRNA, *let-7*, is a heterochronic switch gene, and a suppressor of *lin-14* mutants that caused retarded development in the same pathway as *lin-4*, but at later developmental stage. It causes reiteration of larval cell fates during the adult stage in loss of function mutant, whereas increased copy number causes precocious expression of adult fates during larval stages, which suggests *let-7* controls the transition from the fourth larval stage to the adult stage [86].

Developmental processes are also controlled by miRNAs in vertebrates. Several observations have shown that DICER is essential for the normal development of mammals. *Dicer* mutant mouse embryos die before axis formation during gastrulation and showed loss of ES cells [99]. This suggests that the maturation of miRNAs is crucial in early development after depleting maternal DICER and mature miRNAs.

The numbers of detectable miRNAs increases rapidly in tissues derived from all three germ layers (endoderm, ectoderm, and mesoderm). In neural development, miR-

124 and miR-128 are highly expressed in adult brain and preferentially expressed in neurons. In addition, miR-23 is important in neural specification, and its expression is restricted to astrocytes. This finding suggests that lineage specificity in neuron cells may be tightly regulated by miRNAs [100]. In differentiating cardiomyocytes, miR-1 regulates the balance between differentiation and proliferation of cardiomyocytes during heart development in mice by targeting Hand2, a transcription factor that promotes ventricular cardiomyocyte expansion [101]. On the other hand, miR-133, which is clustered on the same chromosomal locus with miR-1, is also transcribed together but in a tissue-specific manner during skeletal muscle development [102]. MiRNA processing is also important in lung development. At embryonic day E11.5, it has been shown that AGO1 and AGO2 are enriched in branching regions. Also, miR-127 overexpression in fetal lung resulted in defective terminal bud formation and uneven development of the lung, showing that unbalanced expression of miRNAs cause severe developmental defects [103]. Recently, it was shown that miR-212 and miR-132 are indispensable for the regulation of epithelial-stromal interactions in mammary stroma that are required for ductal outgrowth during pubertal development of the mammary glands in mouse [104].

4.1.3 Carcinogenesis

A large number of miRNAs have been identified so far and the count is continuously increasing. The diverse expression patterns and the abundance of miRNA targets suggest that miRNAs are likely to be involved in a broad spectrum of human

diseases. One single miRNA can target dozens of genes, and regulation mediated by miRNAs has a large impact on downstream genes. Many studies have shown that miRNAs are aberrantly expressed in cancer, suggesting their role as a novel class of oncogenes or tumor suppressor genes. Studies have pointed out that cancer-associated miRNAs are frequently targeted on fragile sites of the human genome or close to virus integration sites, indicating that the targeting of miRNAs is not a random distribution but a selected mechanism [105].

The first evidence of involvement of miRNAs in cancer came from characterizing the chromosome 13q14 deletion in human chronic lymphocytic leukemia (CLL). Deletion of the 13q14 region occurs in more than half of the cases of B cell chronic lymphocytic leukemias and also in 50% of mantle cell lymphomas, in 16–40% of multiple myelomas and in 60% of prostate cancers, suggesting a high probability of the presence of tumor suppressor genes at this locus [106]. Loss-of-heterozygosity studies were used to characterize the tumor suppressor locus, to define the minimal region of loss, and to identify the critical gene. Two miRNAs, miR-15a and miR-16-1, were soon identified at 13q14.3 within a 30-kb region between two exons of the *LEU2* gene, and it was found that they are down-regulated in more than 60% of CLL cases by Northern blot analyses [107].

Many key pathways in cancer progression are targeted by miRNAs. For example, miR-135a and miR-135b decrease translation of the adenomatous polyposis coli (APC) gene, a tumor suppressor in the Wnt pathway, in early colorectal tumor development, and miR-135a and miR-135b were found to be upregulated *in vivo* in colorectal

adenomas and carcinomas, correlating with low APC levels compared to normal tissues [108]. This observation suggests that alteration in the mir-135 family may be one of the early events in molecular pathogenesis in colorectal cancer. Moreover, the same pathway cascade can be regulated by different miRNAs in other types of malignancies. Downregulation of miR-200a in meningiomas and arachnoidal cells resulted in increased expression of β-catenin and cyclin D1 involved in the Wnt-associated cell proliferation pathway [109]. The epidermal growth factor receptor (EGFR) pathway, which contributes to promotion and progression of broad spectrum of solid tumors and is a promising target for anticancer therapy, is also regulated by miRNAs at many points. Notably, miR-128b is correlated with aberrations at the 3p22 locus and the abundance of EGFR in non-small lung cancer (NSLC) [110]. In addition, NSLC patients with a high risk score of five upregulated miRNAs (let-7a, miR-221, miR-137, miR-372, and miR-182) had an increased relapse rate and shortened survival times, suggesting the potential role of miRNA in predicting clinical response and survival rate after therapy [111]. The *RAS* oncogene is a frequent miRNA target that is part of the EGFR pathway. It has been reported to be a direct target of the let-7 miRNA family in colon cancer [112], whereas miR-18a is potential tumor suppressor in human squamous carcinoma [113].

Altered miRNA expression not only promotes the formation of primary tumors but also promotes metastasis. The epithelial-mesenchymal transition [40] is the conversion of an epithelial cell into a mesenchymal cell. Morphologically, EMT is characterized by a decrease of E-cadherin, loss of cell adhesion, and increased cell motility leading to the promotion of metastatic behavior of cancer cells. The

transcriptional repressor zinc-finger E-box binding homeobox 1 (ZEB1) is a crucial inducer of EMT in various human tumors, and it recently was shown to promote invasion and metastasis of tumor cells. ZEB1 directly suppresses transcription of miR-200 family members, miR-141 and miR-200c that strongly activate epithelial differentiation in pancreatic, colorectal and breast cancer cells in basement membrane components. In addition, the EMT activators transforming growth factor β 2 (TGF β 2) and ZEB1 are the predominant targets downregulated by these miRNAs [114]. These results indicate that ZEB1 triggers a miRNA-mediated feedback loop to perform reciprocal repression that stabilizes EMT and promotes the invasion of cancer cells. Also, a recent study associated the expression of *let-7* with two differentiation stages of a panel of cell lines (with epithelial and a mesenchymal gene signatures) and linked let-7 to EMT [115].

Unlike protein coding genes, miRNAs can function as a polycistronic cluster that contains multiple miRNA components and each has potential target mRNAs. One of the best-characterized oncogenic miRNAs is miR-17-92, a polycistronic miRNA cluster also designated as oncomir-1 [116]. Human miR-17-92 is located at 13q31.3, within intron 3 of the *C13orf25* gene. The precursor transcript derived from the miR-17- 92 gene contains six tandem stem-loop hairpin structures that ultimately yield six mature miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1 [117]. The oncogenic property of miR-17-92 cluster was first identified in human B-cell lymphomas and was later found to be found amplified in diverse cancer types [118] (Fig.11). Misregulated expression or malfunction of c-Myc is one of the most common

abnormalities in human malignancy. C-Myc regulates the transcription of the miR-17-92 cluster and binds directly the genomic locus encoding these miRNAs [116]. Two miRNAs in the miR-17-92 cluster, miR-17-5p and miR-20a, target the important proproliferative /proapoptotic transcription factor E2F1 in aggressive small-cell lung cancers [119].

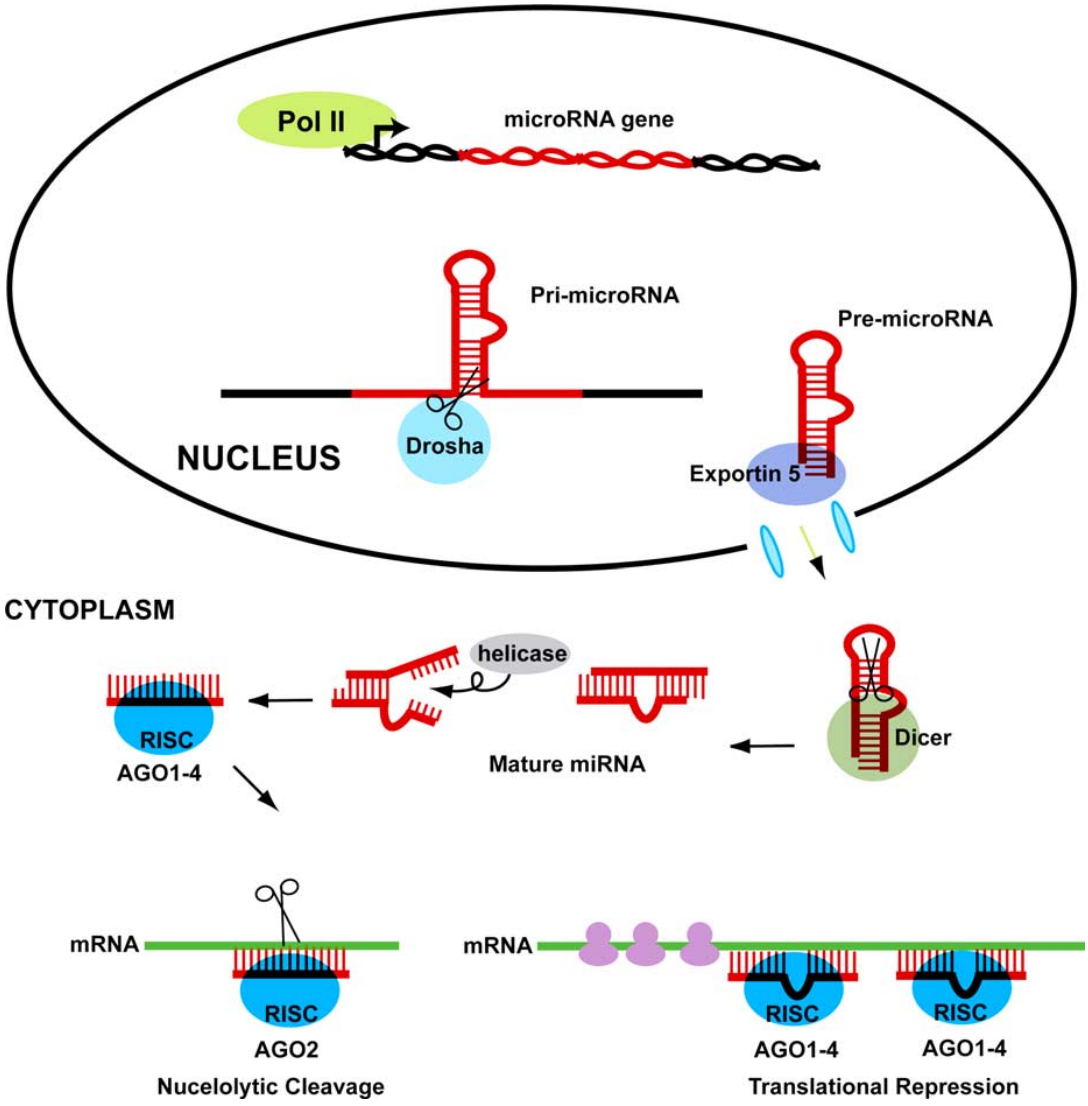


FIGURE 10. The schematic shows the major steps in miRNA processing. Adapted from *Hammond et. al* [98].

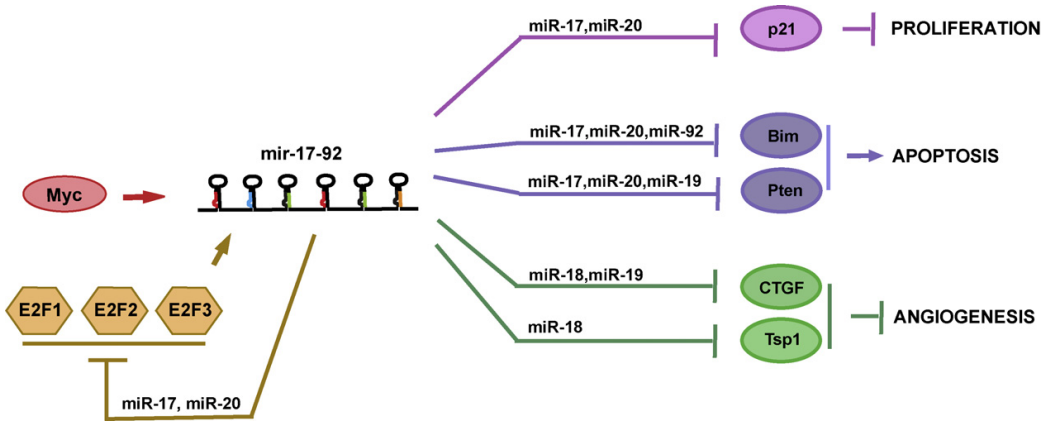


FIGURE 11. The pleiotropic functions of mir-17-92 achieved by repressing specific targets. Depending on both cell type and physiological context, mir-17-92 can promote proliferation, increase angiogenesis, and sustain cell survival through the post-transcriptional repression of a number of target mRNAs. Adapted from *Olive et. al* [120].

Chapter V

Result: The network between SOX4 and miRNA

Recent breakthroughs in high-throughput technologies have enabled development of cancer-specific signatures via miRNA expression profiling. Prostate cancer is the most common noncutaneous neoplasm and second most common cause of cancer in American men. It has been estimated that there were over 217,730 new cases and 32,000 deaths in 2010 [121]. One of the important challenges in current prostate cancer research is to develop effective methods to determine whether a patient is likely to progress to aggressive, metastatic disease after treatment. The current gold standard for pathological evaluation of the status of prostate cancer patients is the Gleason score, which is calculated based on summing the grades of the glandular architecture of the two most prevalent histological components of the tumor. However, it is currently very difficult to predict the outcome of patients with only Gleason scores.

Thus, novel biomarkers could be of great clinical significance. While limited miRNA profiling has been performed in prostate cancer samples along with many other solid tumors, a comprehensive analysis of all miRNAs on a large cohort of samples with defined clinical outcome has not been reported. Several studies have analyzed the miRNA profiling in prostate cancer with different tissue samples to identify novel diagnostic and prognostic tools [122-124]. In this chapter, I will focus on the reciprocal regulation roles of SOX4 and miRNAs in prostate cancer progression, as well as the results from the miRNA expression profiling we performed to identify the clinical relevant miRNA(s) that could be potential biomarkers**.**

5.1 SOX4 mediates the miRNA aberrant expression in prostate cancer

We performed comprehensive miRNA profiling from 70 surgical patients (29 with biochemical recurrence and 41 controls) of different Gleason scores, margin status, ethnic groups, treatments and outcomes. The MicroRNA Expression Profiling Panel (Illumina, Inc) designed for the cDNA-mediated Annealing, Selection, extension, and Ligation (DASL) Assay was used to analyze a set of 70 formalin-fixed paraffin-embedded (FFPE) tumor tissues. The profiling data showed several miRNAs were upregulated in patients with recurrence, including miR-103, miR-339, miR-183, miR-182, while others such as miR-221 and miR-136 were downregulated.

In previous studies published in our lab to identify the SOX4 direct target genes on a global scale, *Scharer et. al* performed ChIP assays on stably transfected HASOX4 LNCaP cells and YFP control cells, and hybridized enriched DNA to a Nimblegen 25K promoter array. Combined QRT-PCR and ChIP-chip analysis of several target genes, including *DICER*, determined that direct binding of SOX4 to their promoters results in increased mRNA levels [69]. This suggests that during cancer progression, SOX4 may play a role in regulating the miRNA pathway though DICER.

In order to test whether SOX4 regulates expression of miRNAs associated with prostate cancer recurrence, SOX4 ChIP assays were performed on the promoter regions of the miRNAs that were up/down regulated in recurrent patient samples. PCR primers were designed based on the predicted SOX4 binding sites from our previous study [69]. The PCR results showed that the promoter regions of miR-103, miR-339, miR-182, and miR-221 were bound by SOX4 in the HASOX4 LNCaP stable cell line compared to control

cells (Fig. 12A). In addition, to investigate the effect of SOX4 on the expression level of these miRNAs, LNCaP cells were transiently transfected with HASOX4 or dominant negative SOX4 (DNSOX4), which contains only the DNA binding domain but is truncated in the activation domain. Combined data from ChIP assays and realtime-PCR, allows us to conclude that SOX4 can directly activate miR-339 and miR-221 expression (Fig. 12B).

To investigate the function of these miRNAs, stably expressed pri-miR-103 and pri-miR-182 precursor cell lines were established to help us understand the mechanism by which they promote recurrence. We adapted miR-express^{TM} lentiviral microRNA system (HMR4842, Open Biosystems) to establish the stable LNCaP cell line (Fig. 13A). Realtime-PCR results showed that while the pri-miRNA constructs were able to process correctly into mature miRNAs, the miR-103 expression was increased with 1.82 fold, and miR-182 was about 40 fold upregulated in the LNCaP cells (Fig. 13B). The LNCaP-miR-182 cell line was sent to our collaborator, Dr. Arun Seth (U. Toronto). Work in his laboratory has defined that cells overexpressing miR-182 have increased tumor growth and metastasis in mouse xenografts (personal communication).

A panel of genes identified as biomarkers of biochemical (PSA) recurrence from the same set of prostate cancer samples by our laboratory (Table 2). In this study, DASL profiling defines the genes that could distinguish potential prognostic difference and give predictive value in FFPE prostate cancer tissues. Ten mRNA and two miRNA genes were validated that could significantly discriminate the patients at higher and lower risk of prostate cancer recurrence [125].

5.2 Wnt-induced SOX4 mediates miRNA expression in prostate cancer cells

Since SOX4 cooperates with β-catenin to activate Wnt downstream gene expression, we hypothesized that Wnt signals may induce miRNAs through SOX4. β-catenin activation of miR-200a was recently proposed in meningiomas [109], whereas PTEN is translationally repressed by miR-21 [126]. One the other hand, β-catenin control of the intensity and spatial pattern of Nodal responds to regulation by miR-15 and miR-16 expression [127]. Additionally, miR-375 is downregulated by β-catenin, although the functional importance is not clear and further investigation is needed. These data suggest that more Wnt-associated miRNAs remain to be identified [128].

To identify the Wnt-induced SOX4-targeted miRNAs, LNCaP HASOX4 and YFP stable cell lines were treated with WNT3A-conditioned media for 4 hrs to induce Wnt signaling. We performed miRNA profiling with these samples using Illumina miRNA microarrays at the Cancer Genomics Shared Resource and identified several miRNAs that are upregulated in response to WNT3A. MiR-16, miR-196a, miR-19a, miR-320a, miR-195, and miR-126 were identified and significantly increased when we induced Wnt signaling (Fig. 14A, 14B). Also, SOX4 was bound to the promoters of miR-16 and miR-196 in ChIP assays (Fig. 14C). Moreover, previous studies have shown that the miR-16/miR-15 cluster is a crucial developmental regulator in Wnt signaling [127]. These data suggest that SOX4 might play a role with β-catenin in regulating miR-15 family expression in Wnt-associated cancer progression.

5.3 The 1100-2350 region of SOX4 3'UTR mediates repression of SOX4 expression

Several retroviral mutagenesis screens to identify oncogenes have been conducted in mouse models [46, 129]. In these reports, the *SOX4* gene is one of the most common retroviral insertion sites for induction of murine leukemias, including several insertion sites in the 3'UTR. Since miRNAs target the 3'UTR region, these studies suggest that miRNAs might repress oncogenic effects of *SOX4* in cancer. To identify the potential miRNAs that target the SOX4 3'UTR region, the SOX4 3'UTR was cloned downstream of a luciferase reporter to investigate whether SOX4 is post-transcriptionally regulated by miRNAs. Deletion analysis identified a region between 1100-2350 nt after the *SOX4* stop codon that mediates 99% repression of SOX4 expression (Fig. 15A). Compared to the empty pGL3 vector, the region of 1-1100 nt and 2350-2836 nt regions of SOX4 3'UTR resulted in more than 8 or 10 fold increased luciferase activity respectively. However, the 1100-2350 fragment showed a significantly downregulation, suggesting a potential miRNA targeted sequence. Sequence analysis of the SOX4 3'UTR by TargetScan [130] prediction revealed that SOX4 may be targeted in this 1100-2350 nt region (Fig. 15C). Consquently, serial deletions of the SOX4 3'UTR were cloned to perform luciferase assays to narrow down the potential region directly regulated by miRNAs (Fig. 15B). Unfortunately, none of these deletions were able to restore the repression of SOX4 expression levels.

5.4 High-throughput selection system for identification of miRNA regulators of SOX4 3'UTR

To identify the miRNAs that bind to the SOX4 3'UTR, another high-throughput screen, MicroRNA Target Selection (MS410A-1, System Biosciences), in a lentivector vector provides us a platform to identify miRNA regulators *in vivo*. A dual reporter system with firefly luciferase (Fire) and a cytotoxic sensor (Ctx) is designed to monitor the direct targeting of a given gene (Fig. 16). The full length of SOX4 3'UTR was cloned into the miR-Selection Fire-Ctx lentivector by Cold Fusion Cloning system (MC100A-1, System Biosciences) and transduced in parallel with empty vector into ARCaPE cells, a prostate cancer cell line that undergoes EMT. When endogenous miRNAs target the SOX4 gene by translated inhibition, cells transduced with the SOX4 3'UTR reporter would survive under Ctx drug selection. In contrast, in the case of no miRNA binding, treatment with Ctx drug would cause cell death due to the actively translated Ctx sensitive protein product.

In our pilot expreiment, after we transduced with miR-selection empty vector or SOX4 3'UTR clones into ARCaPE cells, we found both lentiviral clones were transcribed with same level in the cells (Fig. 17A), but the SOX4 3'UTR clone failed to express luciferase activity compared to empty vector (Fig. 17B). This suggests that the SOX4 3'UTR may have some inhibition though endogenously expressed miRNAs.

However, when we performed miRNA selection with Ctx drug, not only the SOX4 3'UTR clone, but also the empty vector clone has shown resistance to the Ctx drug. This Ctx-resistant property in ARCaPE cells is unfavorable for us to perform selection in the future. Therefore, we have developed both SOX4 3'UTR and empty vector stable cell lines in HEK293 cells that are sensitive to the Ctx drug. In the near future, a miRNA

library will be transfected in these stable cell lines to identify miRNAs targeting the SOX4 3’UTR.

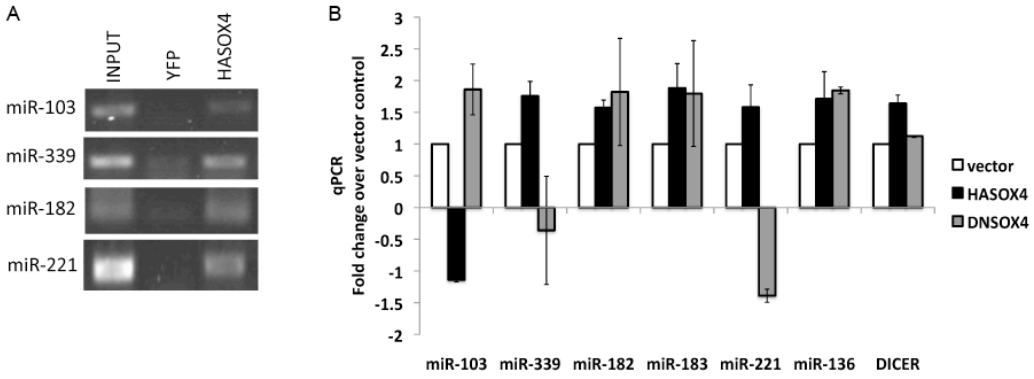


FIGURE 12. Validation of recurrence-associated miRNA. *A*. ChIP assay of HASOX4 bound to the promoter of miR-103, miR-339, miR-182, and miR-221. HASOX4 was bound to the promoter specifically in SOX4 stably expressed LNCaP cells (Lane 3) but not on the YFP expressed control cells. *B*. Realtime-PCR assay of six predicted miRNAs. The cells were transiently transfected with HASOX4, DNSOX4, and empty vector, respectively (n = 3, independent biological replicates performed on separate days).

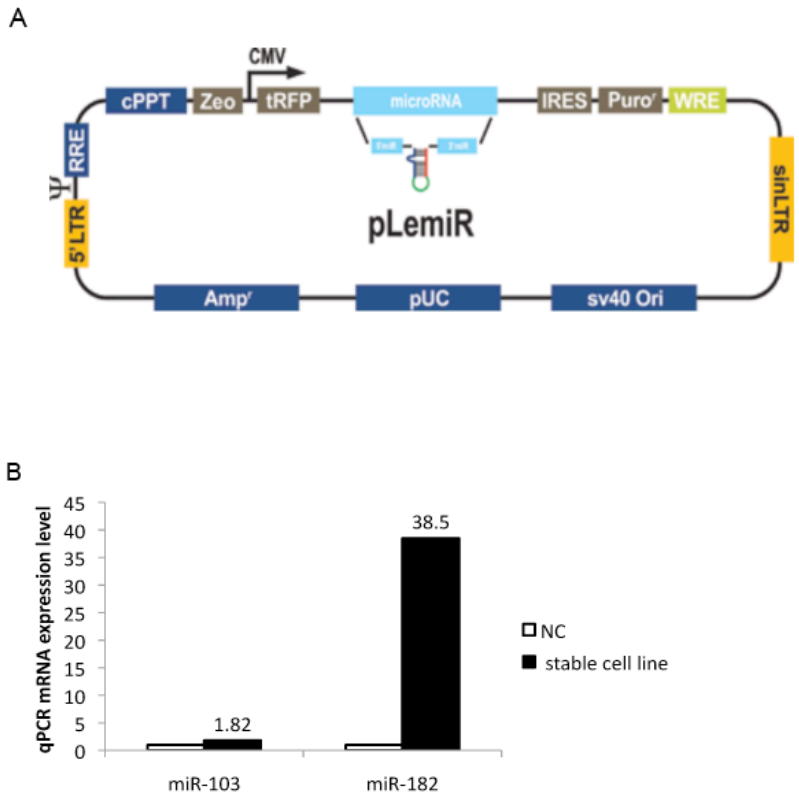
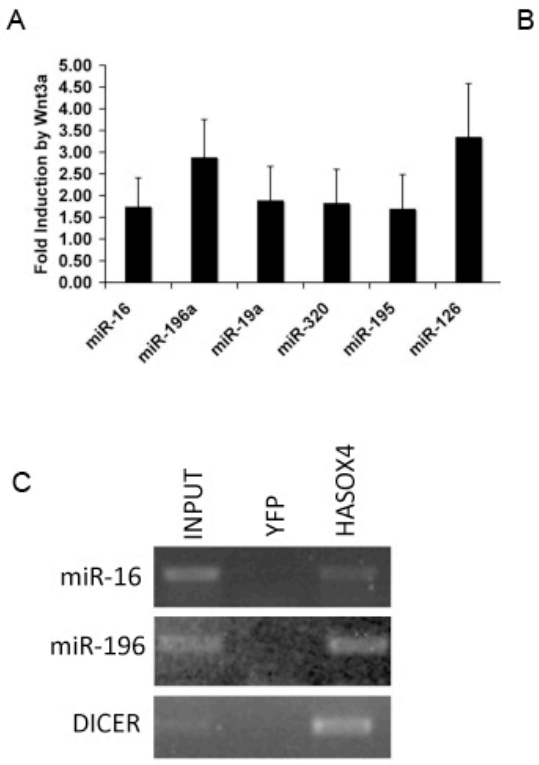


FIGURE 13. Establishment of pri-miRNA-103 and pri-miR-182 stable cell lines. *A*. The 11 Kb pLemir lenitiviral vector detail. Pri-miRNA sequences were cloned into CMV promoter-drived vector and with the puromycin selectable marker on it. *B*. LNCaP cells were transduced and selected with 4ug/ml puromycin. Quantitative realtime-PCR was used to validate the expression level of mature miRNA. The expression levels were compared with the stable cell line transduced with non-silencing negative control construct (NC).



	Fold change	P-value
miR-16	1.7	0.002
miR-196a	2.9	0.01
miR-19a	1.9	0.02
miR-320	1.8	0.02
miR-195	1.7	0.04
miR-126	3.4	0.05

FIGURE 14. SOX4 regulates miRNAs in Wnt signaling. *A*. MiRNA profiling of three independent samples of HASOX4 LNCaP stable cell line compared with control cell line. Six miRNAs were significantly upregulated under WNT3A-condition media treatment. *B*. The fold change and p-value of six upregulated miRNAs. *C*. The ChIP assay of HASOX4 in LNCaP cells. SOX4 bound to promoters of miR-16 and miR-196. The binding of SOX4 on promoter of DICER was performed as positive control.

Symbol	Description	Coefficient
RAD23B	RAD23 homolog B	0.070
FBP1	Fructose-1,6-bisphosphatase 1	0.251
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	-0.588
CCNG2	Cyclin G2	0.008
hsa-miR-647	hsa-miR-647	-0.318
LETMD1	LETM1 domain containing 1	0.063
NOTCH3	Notch homolog 3	0.367
ETV1	ETS variant gene 1 (ETV1)	0.179
hsa-miR-519d	hsa-miR-519d	0.551
BID	BH3 interacting domain death agonist (BID)	0.128
SIM2	Single-minded homolog 2	0.124
ANXA1	Annexin A1	-0.143

TABLE 2. Twelve-gene predictors of prostate cancer recurrence following surgery. Ten mRNAs and two miRNAs were identified. Coefficient is derived from lasso Cox proportion hazard model and was used for computing the predictive score. Positive coefficient indicates a positive association with recurrence, and negative coefficient indicates a negative association with recurrence. Adapted from *Long et. al* [125].

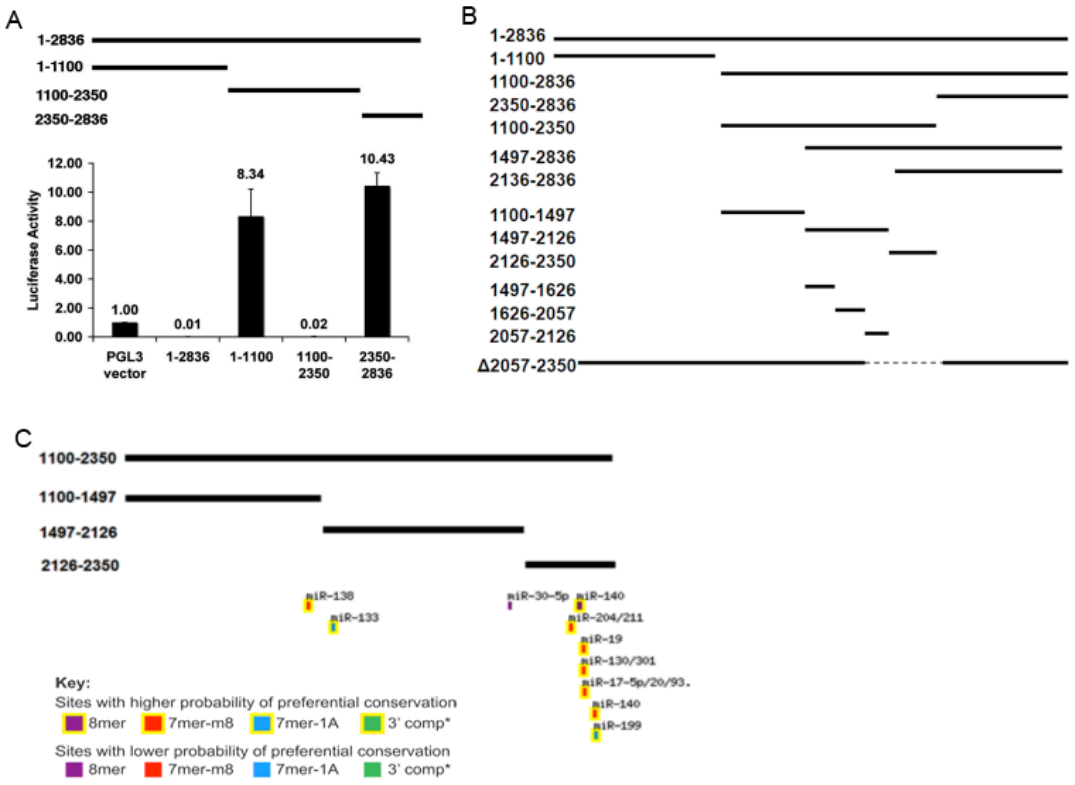


FIGURE 15. Identification of miRNAs targeting the SOX4 3'UTR. *A*. Full length and different portions of SOX4 3'UTR were cloned in to pGL3 luciferase reporter. Luciferase assays showed that both full length (1-2836 nt) and 1100-2350 fragment were severe repressed, suggesting that the 1100-2350 nt region is critical for SOX4 regulation of expression. *B*. The serial deletion diagram of SOX4 3'UTR. Each deletion was cloned downstream to the luciferase reporter to dissect the minimal region that regulated by miRNAs. *C*. TargetScan analysis of 1100-2350 nt region. Candidate miRNA regulators were predicted on SOX4 3'UTR.

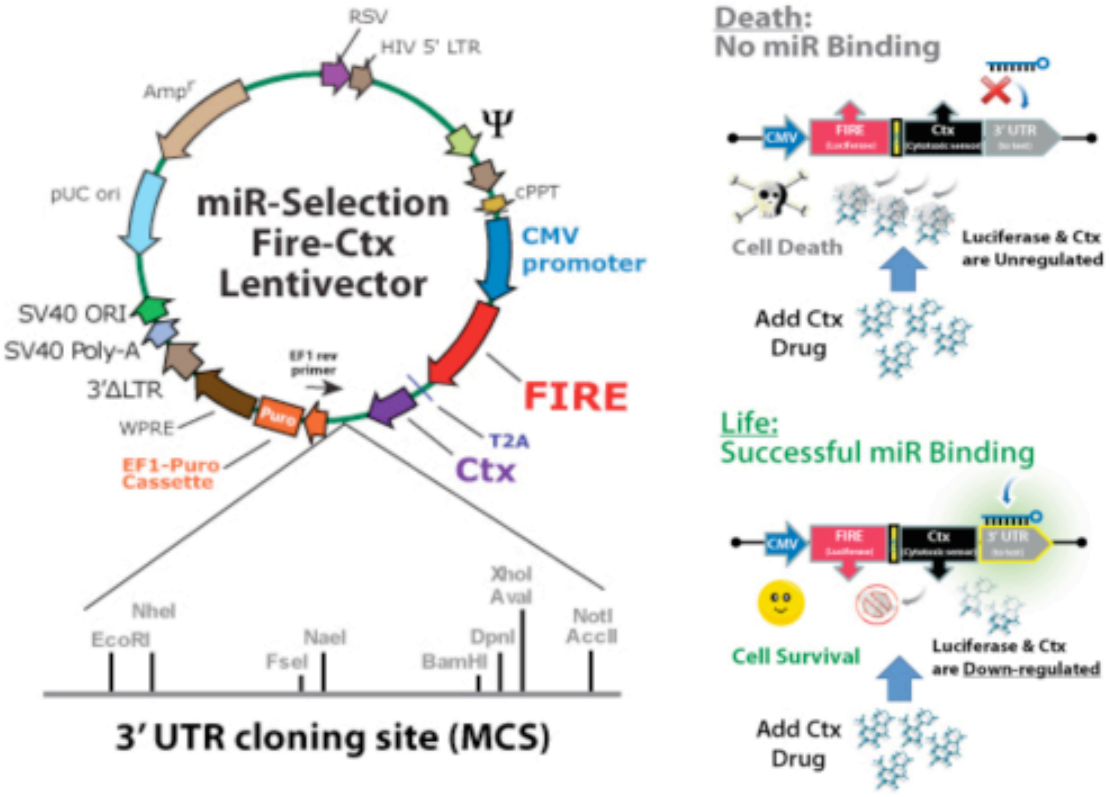


FIGURE 16. The microRNA target selection system. A unique technology for identifying the microRNAs those bind on 3'UTRs by using a cellular selection system. The miR-Selection Fire-Ctx lentivector, a 10.2Kb lentiviral-based plasmid, contains luciferase gene and Ctx to monitor the potential miRNA binding. A selection cassette, puromycin gene, provides a convenience way to make stable cell line to work with. Adapted from System Biosciences Inc.

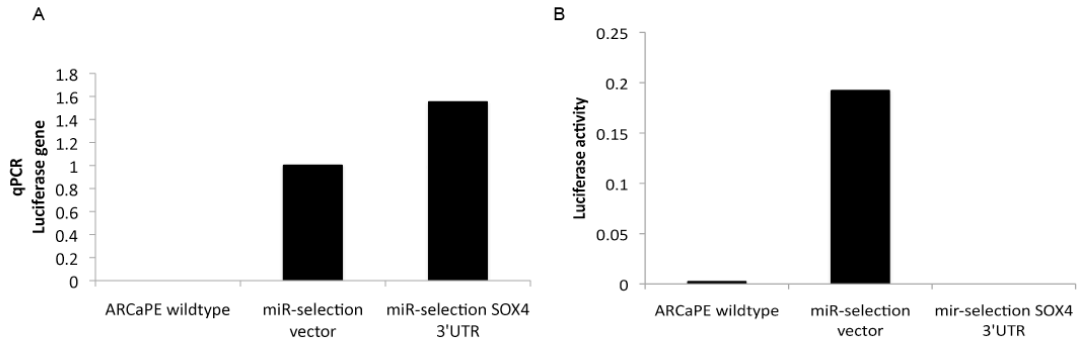


FIGURE 17. Expression of miR-selection lentiviral clones. *A*. Realtime-PCR assay of the luciferase gene in wildtype, miR-selection vector, or SOX4 3'UTR transduced ARCaPE cells. Cells transduced SOX4 3'UTR and empty vector showed expression of luciferase mRNA transcripts. *B*. Luciferase reporter assay of wildtype, miR-selection vector, or SOX4 3'UTR transduced ARCaPE cells. A significant reduction of luciferase activity demonstrated that the miR-selection-SOX4 3'UTR is not translated in ARCaPE cells.

Chapter VI

MiRNA: Discussion

After the discovery of the first miRNAs in *Caenorhabditis elegans*, these short regulatory RNAs have been found to be an abundant class of RNAs in plants, animals, and DNA viruses. About 3% of human genes encode for miRNAs, and up to 30% of human protein coding genes may be regulated by miRNAs. MicroRNAs play a key role in diverse biological processes, including development, cell proliferation, differentiation, and apoptosis. Accordingly, altered miRNA expression is likely to contribute to human disease, including cancer. However, in few cases is the underlying cause of miRNA deregulation in cancer clear. Our data suggest that SOX4 in prostate cancer may regulate expression of miR-15, miR-339, and miR-221.

6.1 MiRNA regulators on SOX4

To identify direct miRNA regultors of SOX4, deletion analysis of the SOX4 3'UTR was performed. First, we dissected the full length of SOX4 3'UTR into three regions according to the miRNA-targeted prediction. Although the central part (1100-2350 nt) of the 3'UTR is the miRNA targeting hotspot, there are still some predicted miRNA binding sites in the 5' (1-1100 nt) or 3' (2350-2836) regions of 3'UTR. There are some data that miRNAs regulate the expression of SOX4 in cancer-associated processes. One study has shown that miR-335 targets the SOX4 3'UTR in breast cancer [48]. However, there is no predicted site for miR-335 to target the SOX4 3'UTR based on TargetScan and Pictar databases. Moreover, this study showed that the miR-335 targets on the 5' end (1-1100 nt) region. According to our reporter data, the 1-1100 nt region showed mild effect on luciferase activity compared to the huge decrease on 1100-2350 region. This suggests

that the targeting of miR-335 might cause minor post-transcriptional repression due to imperfectly hybridized on SOX4 3'UTR, or possibly no effect. In addition, another study showed miR-129 targets SOX4 3'UTR in an epigenetic manner [49], in the 1100-2350 nt region where the most frequently miRNA targeted area in our prediction. To narrow down the 1100-2350 nt region to a single miRNA site that causes a significant repression, the minimum region of the SOX4 3'UTR that can restore the strong inhibition, we performed luciferase reporter assays on serial deletions of the SOX4 3'UTR. Unfortunately we were unable to identify a single region that cause the serious translational inhibition even when narrowed down to few base pairs of potential miRNA target sites. Consequently, we took an alternative approach to adapt a novel system to find the miRNAs. The miR-selection system from System biosciences, may allow us to isolate the endogenous miRNAs that directly bind to the SOX4 3'UTR.

According to sequence analysis, the SOX4 3'UTR contains an AU-rich sequence in the 1100-2350 nt region. Thus the post-transcriptional regulation of RNA stablization of the *SOX4* gene may be mediated by adenine/uridine-rich element (ARE) instead of miRNAs. ARE is an important paradigm for post-transcriptional regulation in the 3' untranslated region of transcripts encoding oncoproteins, cytokines and transcription factors [131]. The involvement of AREs in the decay of mRNA is described as ARE-mediated decay (AMD) [132]. The ARE sequences usually harbor repeats of AUUUA or UUAUUUAUU motifs [133], which we found in three places in the 1100-2350 nt region on SOX4 3'UTR. Recent studies suggest that perhaps the AMD and translational roles of ARE binding proteins (AUBPs) are mediated, or at least influenced, by miRNA. For

example, some data implicate miRNAs in this AUBP interaction, including miR-16 [134]. A model has been proposed that AMD regulates mRNA stability and experiments demonstrated that AMD and miRNA do more than share effectors of degradation (Fig. 18). They operate dependently, and work together in a variety of situations. This provides us with another hypothesis based on the crosstalk between ARE and miRNA that the RNA-binding proteins also play important roles with miRNA in regulating SOX4 mRNA post-transcriptionally.

6.2 MiRNA biomarker in prostate cancer

In many tumor types, biomarkers represent an important index of cancer care. These biologic indicators are increasingly being used to help physicians screen, detect, and diagnose patients. Detection biomarkers help in identifying cancers. One well-known example is prostate-specific antigen (PSA), a marker that has been used in early prostate cancer detection and monitoring for biochemical recurrence. Prognostic biomarkers give a prediction of long-term outcomes, and also may provide information on disease recurrence, and may correlate with clinical outcomes.

Six miRNAs were identified from 70 surgical patients when we performed miRNA profiling analysis. Four miRNAs (miR-103, miR-339, miR-183, and miR-182) were found upregulated, while another two, miR-221 and miR-136, were downregulated. Several crucial targets of these six miRNA are predicted. Among these miRNA downstream targets, the B-cell translocation gene 2 (BTG2) was predicted as a target of miR-103 and BTG1 was a target of miR-183. BTG1 and BTG2 belong to the same BTG family and

functionally relate in the control of the cell cyle. They are antiproliferative factors that are expressed rapidly, but transiently, in response to factors that induce growth arrest and subsequent cell differentiation [135]. Considering studies that showed BTG2 is responsive to p53-assciated DNA damage [136], and induction of SOX4 by DNA damage is critical for p53 stablization [137], it is possible that SOX4 may play a role of regulating miR-103 in the p53-associated DNA damage pathway.

A set of potential biomarkers that was published recently by our research group with the same patient sample sets [125]. In this study, we not only performed DASL expression profiling with a custom-designed prostate cancer panel, but also fit a Cox proportional hazard (PH) model, and then considered the relevant clinical biomarkers including T-stage, PSA and Gleason score, to identify the potential biomarkers for predicting recurrence following radical prostatectomy of prostate cancer. Of the twelve biomarkers, two miRNAs, miR-647 and miR-519d were identified with ten other mRNA genes. Little is known about miR-519d and miR-647 other than miR-519d may be associated with obesity [138].

While we validated the six miRNAs with ChiP assay on SOX4, we may consider performing the ChIP assay on miR-519d and miR-647, which are associated with recurrence in prostate cancer. Such future experiments may help us to elucidate the oncogenic role of SOX4. Also, future analysis of independent samples could improve the accuracy of data analysis, and determine whether these biomarkers are predictive.

6.3 SOX4-mediated miRNAs in Wnt signaling

Six miRNA, miR-16, miR-196a, miR-19a, miR-320a, miR-195, and miR-126 were identified as significantly increased when we induced the Wnt signaling. We validated these miRNAs by quantitative realtime-PCR in wildtype LNCaP cells, and found that the miR-16 and miR-126 were increased 1.8 fold after WNT3A-conditional medium treatment (data not shown). However, we would like to perform quantitative realtime-PCR in stably transfected LNCaP HASOX4 cells following Wnt treatment to confirm the induction of these miRNAs.

Among these miRNAs, the miR-16/miR-15 cluster is a crucial developmental regulator in the Wnt pathway. The most well known downstream target of miR-16 is BCL2, and BCL2 repression by miR-16 induces apoptosis in a leukemic cell line model, which again confirmed that the aberrant expression of miRNAs may serve oncogenic or tumor suppressive function in cancer progression.

To summarize the study above, the miRNAs involved in SOX4-mediated prostate cancer could be a powerful biomarker that provides a novel prognostic tool. Also, to elucidate the connection between SOX4 and miRNA pathway would help us to clarify the oncogenic characteristics of SOX4. Although this study to elucidate the miRNA-SOX4 network is still ongoing, it represents pioneering research to investigate the role of SOX4 in prostate cancer progression.

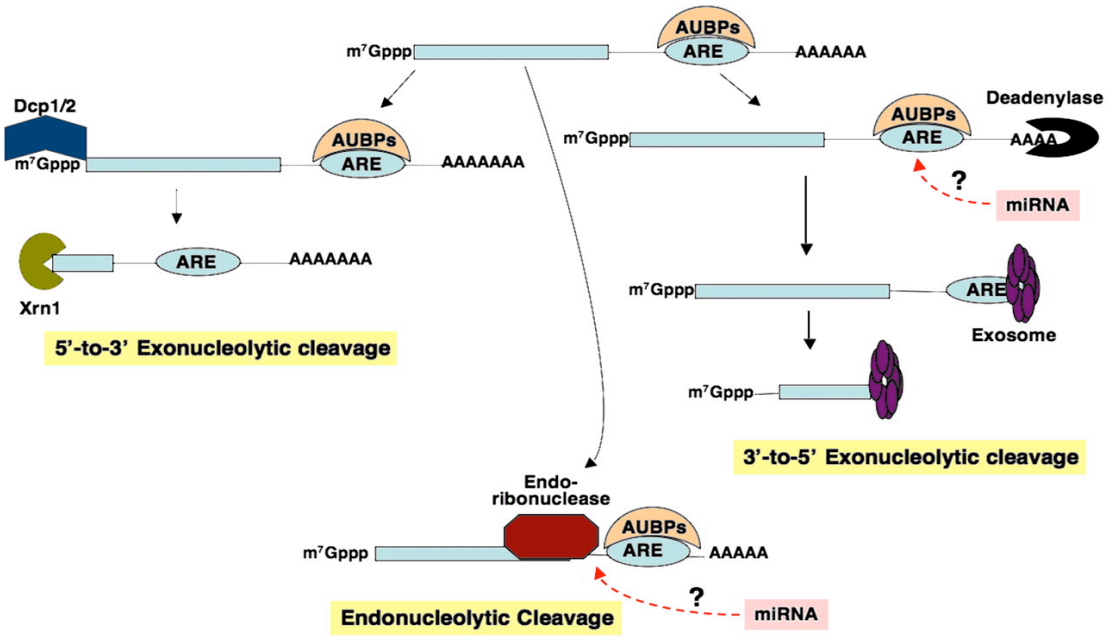


FIGURE 18. Model for AMD. Adapted from *von Roretz et al*. [139].

Appendix: Materials and Methods

I: SOX4 Interacts with Plakoglobin in a Wnt3a-dependent Manner in Prostate Cancer Cells

1.1 *Reagents and cell culture*

LNCaP cells were cultured as described [140] by American Type Culture Collection except using T-medium (Invitrogen). HA-tagged SOX4 was cloned into pHR-UBQ-IRES-eYFP-△U3 lentiviral vector (gift from Dr. Hihn Ly, Emory University), and stable cells were isolated, as previously described [69]. Recombinant Wnt3A was purchased from R & D Systems (5036-WNP) and reconstituted in 0.1% BSA/PBS prior to use. Nuclear export inhibitor leptomycin b [70] was purchased from Sigma (L2913). Cells were treated for 24 hrs with 100 ng/ml WNT3A, 20 uM LMB, or both unless otherwise noted.

1.2 *Biotinlyated HA-tagged SOX4 expression construct*

The pREP4-BLRPwt-IRES-BirA-XL9 plasmid was a gift from Dr. Jeremy Boss (Emory University). The pcDNA3.1-HisA-HASOX4 was constructed as described [41]. The pREP4-BLRPwt-HASOX4-IRES-BirA-XL9 was made by excision on KpnI and XbaI sites of HA-tagged SOX4 from pcDNA3.1-HisA-HASOX4 filled in with Klenow fragment and blunt ligation into the NotI site of pREP4-BLRPwt-IRES-BirA-XL9.

1.3 *Purification of biotinlyated HA-tagged SOX4*

The pREP4-BLRPwt-HASOX4-IRES-BirA-XL9 and control empty vector were transfected into two 90% confluent (8x 10^6 cells) 100mm dishes of LNCaP cells respectively. 48 hrs post-transfection, the plates were then placed on ice and the cells were washed twice with ice-cold PBS, and lysed in 1 ml/plate with ice-cold IP lysis buffer (0.137M NaCl, 0.02M Tris pH8.0, 10% glycerol, 1% NP-40) supplemented with protease inhibitors, and harvested by scraping. Biotinlyated HA-tagged SOX4 complexes were purified by incubating with 50 ul slurry of Dynabeads® M280 Streptavidin (Invitrogen) at 4°C for 2 hrs. The beads ware washed 3 times with IP lysis buffer and eluted by boiling in Laemmli sample buffer.

1.4 *Sample preparation for mass spectrometry*

5% of the proteins resulting from the purification were subject to 4-15% gradient SDS-PAGE and silver staining to analyze sample purity. The rest of the purified proteins were subjected to 4-15% gradient SDS-PAGE and concentrated on a very short distance (~2 mm long), and the protein in those bands were excised for in-gel digestion and Liquid chromatography coupled with tandem mass spectrometry based on an optimized protocol [61].

1.5 *Co-immunoprecipitation and Western blot*

Cells were washed twice with ice-cold PBS, lysed in 1 ml/plate with ice-cold IP lysis buffer supplemented with protease inhibitors, and harvested by scraping. The whole cell

lysates were pre-cleared and then incubated with 25 ul slurry of Dynabeads® M280 Streptavidin or protein G (Invitrogen) at 4°C for 2 hrs. The beads were washed 3 times with IP lysis buffer and eluted by boiling in Laemmli sample buffer before running SDS-PAGE. Western blots were performed as previously described [69]. Antibodies to JUP (13-8500, Nitrogen) and hemagglutinin (HA12CA5) were used in immunoprecipitations [31]. Antibodies to JUP (610253, BD Biosciences), SOX4 (LS-B3520, LifeSpan Biosciences), β-actin (3700S, Cell Signaling), and HA 16B12 (AFC-101P-1000, Covance Research Products) were used in Western blot.

1.6 *Immunofluorescence antibody staining*

Sub-confluent LNCaP HASOX4 stable cells were grown on glass cover slips, and serum starved for 24 hrs with serum-free T-Medium before treating with 100 ng/ml Wnt3a, 20 uM LMB, or both for another 24 hrs. Cells were washed three times with HBSS+ and fixed with 3.7% paraformaldehyde for 20 min at room temperature. Cells were permeablized with 100% ethanol for 20 min at -20°C and blocked in 3% BSA in HBSS+ for 1hr. anti-HA 16B12 (1:500 dilution) and JUP antibodies (1:50 dilution) (sc-H80, Santa Cruz Biotechnology were used as primary antibodies and were diluted in blocking buffer and incubated for 1hr. Then cells were washed in HBSS+ and incubated in fluorescence-labeled secondary antibodies for 1hr at room temperature. Cells were washed in HESS+ and then stained with Topro (T3605, Invitrogen) for 5min at room temperature. Labeled

cells were then washed in HBSS+ and mounted in p-phenylene diamine antifade agent. Confocal fluorescence images were captured using a laser-scanning microscope.

1.7 *Cell fractionation into nuclear and cytoplasmic lysates*

One 100-mm dish of LNCaP HASOX4 stable cells was grown to 80-90% confluency (8x 10^6 cells) and serum starved for 24 hrs with serum-free T-Medium before treating with 100 ng/ml Wnt3a, 20 uM LMB, or both for another 24 hrs. The crude subcellular fractionation was performed as previously described using digitonin, NP40, and RIPA lysis methods [141].

1.8 *Quantitative real-time PCR*

90% confluent cells (8x 10^6 cells) were harvested using the RNeasy kit (Qiagen), and reverse transcription was performed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Quantitative real-time PCR (qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories) on a Bio-Rad iCycler using 18s or β-actin as a control, and data were analyzed using the δCt method[142].

1.9 *Chromatin immunoprecipitation (ChIP) assay*

One 100-mm dish of LNCaP HASOX4 stable cells was grown to 80-90% confluency (8x 10^6 cells). The cells were fixed with 1% formaldehyde, then lysed, and sonicated as

described [143]. Sonicated chromatin was precleared and then immunoprecipitated with 4μg of 12CA5 ascites, or anti-β-catenin (610154, BD Biosciences) or mouse IgG overnight and immunoprecipitated by Dynabeads® Protein G (10004D, Invitrogen) for 2hr at 4°C. The beads were washed and eluted as described [143]. The ChIP DNA was purified and then subjected to PCR amplification.

1.10 *Luciferase reporter assay*

Cells grown in twelve-well tissue culture plates were transfected with 0.5 μg of either TOP-flash or FOP-flash with 0.04μg of TK-Renilla control vector (Promega). At 6 hrs post-transfection, cells were placed in 0.5% FBS medium for recovery and serum starvation. At 24 hrs post-transfection, cells were treated with WNT3A and LMB. Reporter gene activity was measured in a TD-20/20 luminometer (Turner Design) with the DLR Luciferase Assay System (Promega) after 48 hrs and was normalized for transfection efficiency by measuring Renilla activity.

1. 11 *siRNA transfection*

The siRNA sequence for plakoglobin AGTCGGCCATTGTGCATCT was targeted at the 5' end of the gene lacking of homology with other catenin members [78] (Dharmacon RNA Technologies). LNCaP HASOX4 cells were transfected using Lipofectamine 2000 (Invitrogen) with plakoglobin or scramble siRNAs at final concentration of 200 nM. At 6 hrs post-transfection, cells were placed in 0.5% FBS medium for recovery and serum starvation. At 24 hrs post-transfection, cells were treated with WNT3A, LMB, or both for Western blot analysis or chromatin immunoprecipitation assay.

II: The network between SOX4 and miRNAs

2.1 *Reagents and cell culture*

LNCaP cells were cultured as described [140] by American Type Culture Collection (ATCC) except using T-medium (Invitrogen). HA-tagged SOX4 and YFP were cloned into pHR-UBQ-IRES-eYFP-△U3 lentiviral vector (gift from Dr. Hihn Ly, Emory University), and stable cells were isolated, as previously described [69]. ARCaP E cells were purchased from Novicure Biotechnology (Birmingham, AL). ARCaP E were established from bone tumor tissue derived from ARCaP cells. ARCaP E with cobblestone morphology expressed higher E-cadherin and cytokeratin 18, 19, which were associated with typical epithelial cell characters, and concomitant with lower level of vimentin expression. These cells were cultured in Prostate Epithelial Cell Medium (MCaP) supplied by Novicure Biotechnology, and the medium was supplemented with 5% fetal bovine serum (FBS), 50 units/ml Penicillin, and 50 μg/ml Streptomycin. The protocol of preparing Wnt3A-conditioned media was followed by ATCC website. LNCaP YFP or HASOX4 stable cell lines were treated with Wnt3A-conditioned medium for 4 hrs to perform the following assay.

2.2 *Patient samples*

In the initial training set, 70 cases were used (29 with biochemical recurrence and 41 controls), 45 patients from Sunnybrook Health Science Center (Toronto, ON), and 25 patients from Emory University. The 45 cases of paraffin-embedded tissue

samples from Toronto were drawn from men who underwent radical prostatectomy as the sole treatment for clinically localized prostate cancer (PCa) between 1998 and 2006. The clinical data includes multiple clinicopathologic variables such as prostate specific antigen (PSA) levels, histologic grade (Gleason score), tumor stage (pathologic stage category for example; organ confined, pT2; or with extra-prostatic extension, pT3a; or with seminal vesicle invasion, pT3b), and biochemical recurrence rates. For the 25 cases from Emory University, FFPE samples were also selected from a screen of over a thousand patients through an IRB-approved retrospective study at Emory University of men who had undergone radical prostatectomy between 1990 and 1994. Those who were included met specific inclusion criteria, had available tissue specimens, documented long term follow-up and consented to participate or were included by IRB waiver. The cases were assigned prostate ID numbers to protect their identities. These patients did not receive neo-adjuvant or concomitant hormonal therapy. Their demographic, treatment and long-term clinical outcome data have been collected and recorded in an electronic database. Clinical data recorded include PSA measurements, radiological studies and findings, clinical findings, tissue biopsies and additional therapies that the subjects may have received. Adapted from *Long et al* [125].

2.3 *SOX4 3'UTR construct*

The pGL3 control plasmid was from Promega (E1751, Promega). The pGL3 control vector was first excised on XbaI site and cloned in double-stranded oligo containing AgeI and SpeI sites. The SOX4 3'UTR fragment was amplified from human genomic DNA extracted

from LNCaP cells and cloned into pGL3 control vector with AgeI and SpeI sites. Deletion clones of SOX4 3'UTR were generated from pGL3-SOX4 3'UTR full-length clone.

2.4 *Luciferase reporter assay*

Cells grown in twelve-well tissue culture plates were transfected with 0.5 μg of either pGL3 control vector, pGL3-SOX4 3'UTR full length or deletion clones, respectively, with 0.04μg of TK-Renilla control vector (Promega). At 6 hrs post-transfection, cells were placed in 0.5% FBS medium for recovery and serum starvation. At 24 hrs post-transfection, cells were performed luciferase assay. Reporter gene activity was measured in a TD-20/20 luminometer (Turner Design) with the DLR Luciferase Assay System (Promega) after 24 hrs and was normalized for transfection efficiency by measuring Renilla activity.

2.5 *Cold fusion cloning and miR-selection stable cell line establishment*

Full length of SOX4 3'UTR was cloned into miR-selection Fire Ctx lentivector following the protocol provided by System Biosciences (MC010A-1, System Biosciences). Viral packaging of empty vector and SOX4 3'UTR were performed by Viral Vector Core in Emory University. ARCaP E cells were transduced with miR-selection empty vector or SOX4 3'UTR virus for 6hrs in serum-free culture medium and changed to regular culture medium. The cells were then selected by 1ug/ml puromycin for stable cell lines.

2.6 *Chromatin immunoprecipitation (ChIP) assay*

One 100-mm dish of LNCaP HASOX4 and YFP stable cells were grown to 80-90% confluency. The cells were fixed with 1% formaldehyde, then lysed, and sonicated as described[143]. Sonicated chromatin was precleared and then immunoprecipitated with 4μg of 12CA5 ascites or mouse IgG by Protein G agarose beads (16266, Millipore) for 2hr at 4°C. The beads were washed and eluted as described [143]. The ChIP DNA was purified and then subjected to PCR amplification.

2.7 *Quantitative real-time PCR*

90% confluent cells were harvested using the RNeasy kit (Qiagen), and reverse transcription was performed using SuperScript II transcriptase (18064-014, Invitrogen). Quantitative real-time PCR (qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories) on a Bio-Rad iCycler using 18s or β-actin as a control, and data were analyzed using the δCt method [142].

2.8 *pLemiR-miRNA stable cell line establishment*

The pLemiR empty vector, non-silencing negative control, and miR-103 expressed clone were purchased from Open biosystems (HMR4842, Open biosystems). The pLemiR-miR-182 expression clone was made by excision on MluI and NotI sites of pLemiR empty vector filled in with PCR fragment of pri-miR-182 transcript. The viral packaging was followed by company suggested protocol. LNCaP cells were transduced with virus for 6hrs in serum-free culture medium and changed to regular culture medium. The cells were then selected by 4ug/ml puromycin for stable cell lines.

Appendix: Primer lists

Primer for chromatin immunoprecipitation	Sequence
miR-103 FWD	CTCTTCCAAACAAAGAATCCCAACT
miR-103 REV	GACCTGTTCTTCATGATATTTGAGTGGTG
miR-339 FWD	AACACAAGCTCTGGGAAGGTGAGT
miR-339 REV	CCTCCAGCATTCCACTTCATGGAT
miR182 FWD	TGACTCTCTCCTCTCTCTCTGTCT
miR-182 REV	ACACACACACACACACACACACAC
miR-221 FWD	TCCAATGCTATGTTCTCCCGACCT
miR-221 REV	CTTGCACTGGTGAAATTGAGGCCA
miR-16 FWD	ACACACATTCGCGCCTAAAG
miR-16 REV	CTGGGCACAGAATGGACTTCAGTT
miR-196R FWD	GTGTGACAGGGATACAGCAACT
miR-196 REV	GGTGCTTAGGTAAATCAAACACCA
Axin2 ChIP for beta-catenin FWD	CTGGAGCCGGCTGCGCTTTGATAA
Axin2 ChIPfor beta-catenin REV	CGGCCCCGAAATCCATCGCTCTGA
c-myc ChIP for beta-catenin FWD	GCGGGTTACATAC AGTGCACTTCA
c-myc ChIP for beta-catenin REV	TGGAAATGCGGTCATGCACAAA
dkk1 ChIP for beta-catenin FWD	CACATTAGCCCACCACTGAG
dkk1 ChIP for beta-catenin REV	CAGACGCGTGAGATCAAAGT
SOX4 ChIP FWD	CCCTGTTTGGGCTATGCAGGATTT
SOX4 ChIP REV	GTGGTACCTAGACACCTGTCATCA
Axin2 ChIP FWD	CAGGACACTGCTCTCTCAGATTCA
Axin2 ChIP REV	TCACAACAGCCTTTGCAGGG
DHX9 ChIP FWD	TCTTCCTCTGGGCACCACAAACTT
DHX9 ChIP REV	TCACTGCAGAAAGGAAGTCAGCCT

Primer for reltime-PCR	Sequence
hsa-mir-16-1	TAGCAGCACGTAAATATTGGCG
hsa-mir-103	AGCAGCATTGTACAGGGCTATGA
hsa-mir-221	AGCTACATTGTCTGCTGGGTTTC
hsa-mir-339	TCCCTGTCCTCCAGGAGCTCACG
hsa-mir-182	TTTGGCAATGGTAGAACTCACACT
hsa-mir-183	TATGGCACTGGTAGAATTCACT
hsa-mir-19	AGTTTTGCATAGTTGCACTACA
hsa-mir-491	AGTGGGGAACCCTTCCATGAGG
hsa-mir-195	TAGCAGCACAGAAATATTGGC
hsa-mir-126	TCGTACCGTGAGTAATAATGCG
hsa-mir-320	AAAAGCTGGGTTGAGAGGGCGA
hsa-mir-136	ACTCCATTTGTTTTGATGATGGA
mir-rev primer	GCGAGCACACAATTAATACGAC
SOX4 QPCR FWD	CCGAGCTGGTGCAAGACC
SOX4 QPCR REV	CCACACCATGAAGGCGTTC
DHX9 QPCR FWD	TGGCCAATTTCTGGCCAAAGCA
DHX9 QPCR REV	TTAGCCAGGATTCGTCCCAAAGGA

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