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14. ABSTRACT The purpose of this proposal is to test the hypothesis that SOX9 contributes to prostate cancer (PCa) progression by supporting the cancer stem cells (CSC). The results have demonstrated that SOX9 is enriched in prostate CSC. SOX9 silencing leads to reduced CSC population. Cells with higher SOX9 activity (as indicated by an activity reporter) have greater tumoreigenecity. Overall, it has been established that SOX9 plays critical role in prostate progression by supporting the CSCs and may serve as a new biomarker and therapeutic target.					
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INTRODUCTION:

Prostate Cancer (PCa) is the most common cancer in North America and a leading cause of cancer-related death in men. Despite improved outcomes through early detection and treatment of localized PCa, many patients still die of metastatic disease. Although androgen ablation therapy remains the most effective management option for patients with advanced disease, most patient will progress to castration-resistant prostate cancer (CRPC). CRPC progression is a complex process by which cells acquire the ability to survive and proliferate despite the castration level of circulating testosterone. Although enthusiasm for androgen receptor targeting approaches remains high, resistance inevitably develops due to prostate tumor heterogeneity and adaptive responses via alternative mechanisms. Additional strategies to kill CRPC cells are critically needed (Yuan et al., 2013). Improved understanding of PCa biology will facilitate the identification of pathways, aside from the androgen receptor, that drive CRPC progression. Accumulating evidence implicates SOX9 (Sry-related HMG box 9) is a critical factor in CRPC progression that supports tumor survival, growth, invasion or therapeutic resistance. The objective of this proposal is to test the hypothesis that SOX9 promote PCa progression by supporting the cancer stem cells (CSC) in PCa cell lines. The results will provide a strong base for future clinical trials to assess the critical role of SOX9 in disease progression and discover new therapeutic means targeting SOX9 signal pathways.

BODY:

Task 1. Do PCa CSCs express high levels of SOX9?

Cancers can be perpetuated by a small population of CSCs (also termed tumor initiating cells, TICs) that are stem-cell like tumor cells capable of self-renew and regenerating the heterogeneous lineages of cancer cells of the parental tumor (Dean et al., 2005). Importantly, surviving CSCs after conventional treatment have been implicated in treatment failure, cancer relapse and subsequent metastasis. SOX9 maintains stem cell populations in many adult tissues, such as intestine, liver, and pancreas (Blache et al., 2004; Furuyama et al., 2011; Seymour et al., 2007; Vidal et al., 2005). Consistently, in human adult prostate, SOX9 expression is restrictively limited to the basal cells, which support epithelium repair/renewal and may contain the prostate stem cells (Wang et al., 2007). This task is to test the hypothesis that SOX9 expression is enriched in the CSC cells compared to non-CSC cells.

In the PC3 PCa cell line (which expresses high levels of endogenous SOX9), the CSCs are enriched in clones that form tight clusters of small cells (holoclone) in contrast to clones that forms loose clusters of larger cells (mero- and paraclones) (Li et al., 2008). The CSC features in the holoclones include the lack of cellular senescence; the capability to indefinite propagate and self-renewal; and enhanced tumor initiation and serial transplantation in mice. Importantly, we have found that SOX9 protein expression level is much higher in the holoclones compared to paraclones. Immunoblotting has shown that SOX9 protein level is much higher among the holoclones compared to the un-selected parental

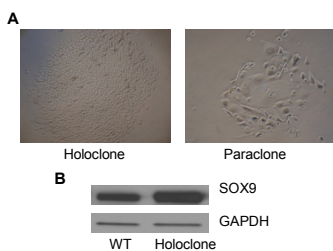


Fig.1 SOX9 is expressed at higher levels in PCa CSC. (A) phase contrast image of holo- and paraclones from PC3 cells. (B) Immunoblot of SOX9 protein in parental (WT) or holoclones.

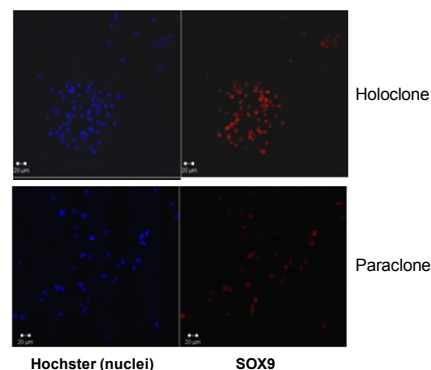


Fig.2 Higher SOX9 expression in PCa CSC. Immunofluorescence demonstration of SOX9 positive cells in a holoclone (upper panels) or a paraclone (lower panels).

cell line (Fig.1). Immunofluorescence staining also demonstrated a higher percentage of cells in the holoclone expressing greater level of SOX9 than those in the paraclones (Fig. 2). These results strongly support the hypothesis that SOX9 plays a role and is preferentially expressed in the PCa CSCs.

We have also attempted to isolate the CSCs from PCa cell lines with FACS (using stem cell markers, such as CD44 or CD133) or with sphere formation. The results were not as conclusive as using the holoclone method. Similarly, our trial of examining the SOX9 expression in CSCs in PCa samples and xenografts by co-immunohistochemical staining of SOX9 and stem cell markers (such as CD133, OLIG2) has been limited due to the nonspecific staining patterns of the stem cells markers.

Task 2. To examine the effect of SOX9 level modulation on CSCs population.

We next proceeded to directly examine the SOX9 regulation of PCa CSC populations by silencing SOX9 expression with shRNA. With the lentiviral shRNA mediated down regulation of SOX9 protein, the level of CD44 protein, a stem cell marker was reduced (Fig. 3).

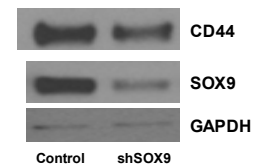


Fig.3 Reduced stem cell markers after SOX9 silencing in PC3 cells.

Similarly, SOX9 silencing also lead to a reduction in the holoclone formation and the size of holoclones (Fig. 4). These results strongly support the conclusion that SOX9 can function to support the CSC populations in PCa cell lines.

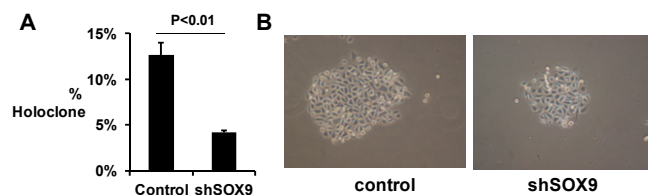


Fig. 4 SOX9 silencing causes a reduction in PCa CSCs.

(A) SOX9 knock down with lentivirus mediated shRNA (shSOX9) lead to a significant reduction in the holoclone population compared to cell infected with control nontargeting shRNA. (B) Smaller size of the holoclones from SOX9 shRNA infected cells compared to control.

In a parallel study, we have discovered that SOX9 may regulated the CSC population by modulating the Wnt/ β -catenin signals, a known pathways intimately involved with stem cells. SOX9 is required for cancer cell response to Wnt stimulation by maintaining two key components of the Wnt/ β -catenin signal pathway, ie. TCF4 and LRP6. These results are summarized in the recent publication (Wang et al., 2013). Together, we have discovered a direct regulation of SOX9 in PCa CSC, at least in part mediated by its influence on the Wnt/ β -catenin CSC signaling.

Task 3. To test the tumorigenicity of SOX9 activity-high cells.

By introducing a SOX9 activity reporter construct into LNCaP PCa cells (which express low levels of SOX9 and contains a small population of SOX9 active cells), we have isolated cells with high SOX9 activity (SOX9-high cells) (Fig. 5A-C). These cells collected after isolation with puromycin

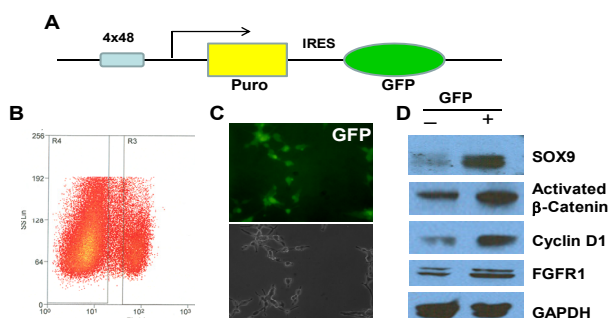


Fig.5 Isolation of SOX9 high activity cells. (A) schematic demonstration of the SOX9 activity reporter with SOX9 dependent Puro resistance and GFP expression. (B) FACS plot of isolation of GFP positive vs negative populations. (C) Fluorescence image of SOX9 high cells (LNCaP). (D) Immunoblot of cell lysates from SOX9 high vs low cells.

selection and FACS, shown much higher SOX9 protein level than the GFP negative population (Fig.5D). These SOX9-high cells also contain a higher Wnt/ β -catenin activity, as indicated by the greater protein levels of activated β -catenin and cyclin D1 (a known Wnt/ β -catenin regulated gene) (Fig. 5D). Interestingly, the SOX9-high cells contain higher FGFR1 expression level, indicating these cells may respond to FGF to grow. Together, these results strong indicate the success of isolation of SOX9 high activity cells with potential mechanisms driving cell proliferation and stem cells activities.

We then proceed to test the tomorigenicity of these SOX9 activity high cells. As shown in Table

	Implanted Cells			
	1.E+06	1.E+05	1.E+04	1.E+03
GFP (+)	6/6	6/6	5/6	2/6
GFP (-)	6/6	1/6	0/6	0/6

Table1. Elevated tumorigenicity in SOX9 activity high PCa cells. Equal number of SOX9-high (GFP+) or SOX9-low (GFP-) LNCaP cells were injected subcutaneously into nude mice. The ratio are listed of the number of mice successfully formed xenograft over the total number of mice that were injected.

1, the SOX9 high cells (GFP+) formed tumors with as little as 1000 cells, while the SOX9 low cells (GFP-) required more than 10,000 cells to form tumors. These results strong support the hypothesis that SOX9 activity high cells are enriched form CSC, which is capable of tumorigenesis with 10 fold less number of cells.

In support of the above finding, we have recently published a paper showing SOX9 is required for xenograft formation in another PCa cells line (VCaP) and SOX9 critically mediates ERG directed tumor growth and invasion (Cai et al., 2013).

KEY RESEARCH ACCOMPLISHMENTS:

- Established a critical function of SOX9 in PCa progression to support CSC populations.
- SOX9 supports PCa CSC is mediated at least in part through its regulation of Wnt/ β -catenin signaling.
- SOX9 therefore can serve as a biomarker for aggressive disease, which more likely to progress , metastasize and be resistant to therapy.
- SOX9 becomes an attractive target for future design for therapy (such as therapies focusing on blocking Wnt/ β -catenin activity; or therapies which specifically degrading SOX9 proteins).

REPORTABLE OUTCOMES:

- A manuscript on SOX9 regulation of Wnt/ β -catenin signaling is published in JBC.
- LNCaP cell clones with characterized SOX9 activity have been generated.
- A post-doctoral fellow supported by this grant has found a factuality position in a Chinese Medical University.

CONCLUSION:

The goals of the original proposal have been successfully achieved. We have demonstrated a critical function of SOX9 in PCa progression, which is the maintenance of CSCs. SOX9 is not only

highly expressed by the CSCs, its expression is critically required for their survival and propagation. SOX9 activity is strongly correlated with the tumoreigenecity of PCa cells.

The results of this project have shed new light onto the important biology of PCa progression and opens new window of development of novel biomarkers and therapeutic modality. This project has paved a strong basis for future studies to further elucidate the molecular mechanism of SOX9 functions and discover new drugs which would specifically block SOX9 activity.

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SOX9 Regulates Low Density Lipoprotein Receptor-related Protein 6 (LRP6) and T-cell Factor 4 (TCF4) Expression and Wnt/ β -catenin Activation in Breast Cancer^{*,§}

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Background: Mechanism of enhanced Wnt/ β -catenin activation in breast cancer (BCa) is not fully characterized.

Results: SOX9 was highly expressed in basal-like BCa. SOX9 maintained and enhanced LRP6 and TCF4 transcription and Wnt/ β -catenin activation *in vitro* and *in vivo*.

Conclusion: SOX9 supports a positive feedback loop to sustain Wnt/ β -catenin signal.

Significance: The results reveal a new mechanism of Wnt/ β -catenin pathway activation in BCa.

Gene expression profiling has identified breast cancer (BCa) subtypes, including an aggressive basal-like (BL) subtype. The molecular signals underlying the behavior observed in BL-BCa group are largely unknown, although recent results indicate a prevalent increase in Wnt/ β -catenin activity. Our immunohistochemistry study confirmed that SOX9, one of the BL-BCa signature genes, was expressed by most BL-BCa, and its expression correlated with indicators of poor prognosis. Importantly, BCa gene expression profiling strongly associated SOX9 with the expression of Wnt/ β -catenin pathway components, LRP6 and TCF4. In cancer cell lines, SOX9 silencing reduced cell proliferation and invasion, LRP6 and TCF4 transcription, and decreased Wnt/ β -catenin activation. SOX9 expression was also increased by Wnt, indicating that SOX9 is at the center of a positive feedback loop that enhances Wnt/ β -catenin signaling. Consistently, SOX9 overexpression in BCa cell lines and transgenic SOX9 expression in breast epithelium caused increased LRP6 and TCF4 expression and Wnt/ β -catenin activation. These results identify SOX9-mediated Wnt/ β -catenin activation as one of the molecular mechanisms underlying aberrant Wnt/ β -catenin activity in BCa, especially in the BL-BCa subgroup.

Breast cancer (BCa)⁴ comprises a remarkably diverse group of diseases in terms of presentation, morphology, and biological or clinical behavior. Gene expression profiling has been used to examine BCa heterogeneity and identified sets of genes (signatures) whose expression can be used to classify BCa into multiple “intrinsic subtypes,” each associated with a different survival rate. The subgroups include luminal subtypes, a subtype with high expression of the HER2 proto-oncogene (Her2), and a basal-like subtype (BL) (1, 2). The BL subgroup (comprising between 17 and 37% of human BCa) is poorly differentiated and is enriched for tumors lacking hormone receptors or HER2 overexpression, so it does not respond to targeted therapies available for receptor-positive cancers (reviewed in Refs. 3 and 4). This subgroup is associated with an early age of onset, a distinct pattern of metastasis, and short times to relapse and disease progression, reflecting its aggressive nature.

The critical signal pathways responsible for the BL phenotype and its aggressive behavior are largely unknown, but recent data support a role for the Wnt/ β -catenin signaling pathway (5–7). Activation of the canonical Wnt/ β -catenin pathway involves stabilization of cytoplasmic β -catenin through the binding of Wnt ligands to their cell surface receptors, the Frizzled family receptors and the low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6. In the absence of Wnt ligands, cytoplasmic β -catenin is phosphorylated by a multi-protein degradation complex that marks it for ubiquitination and degradation by the proteasome. Wnt ligand binding inhibits this complex, allowing the stabilized β -catenin to accumulate in the cytoplasm, to translocate to the nucleus, and to coactivate with the T-cell factor/lymphoid enhancer binding factor (TCF/LEF) transcription factors, which regulate crucial target genes that promote cell proliferation, differentiation, and

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§ This article contains supplemental Methods, table, and data.

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⁴ The abbreviations used are: BCa, breast cancer; BL, basal-like; HMG, high-mobility-group; LRP, low-density lipoprotein receptor-related protein; TCF/LEF, T-cell factor/lymphoid enhancer binding factor; SOX9, Sry-related HMG box 9; TetO, tetracycline operator; tTA, tetracycline transactivator; MMTV, murine mammary tumor virus.

development of multiple tissues including breast (8). Significantly, enhanced cytoplasmic and nuclear β -catenin staining has been reported in human BCa, indicating that aberrant activation of this pathway may contribute to mammary carcinogenesis (9–11). Moreover, recent reports indicate that the BL-subtype in particular is enriched for tumors with increased levels of LRP6 and cytoplasmic and nuclear β -catenin staining (5–7, 12, 13). Although the Wnt/ β -catenin pathway may be activated in BCa, mutations that activate this pathway frequently found in other cancers (including APC truncation, stabilizing mutations in β -catenin, or Axin loss found in colon cancers) are rarely detected in human BCa (7, 14–16). Therefore, the underlying causes of aberrant Wnt/ β -catenin activation in BCa, and its correlation with the BL subgroup, remain unexplained.

SOX9 (Sry-related HMG box 9) is a member of the BL signature genes used in classifying BCa subgroups (1, 2, 17). SOX9 belongs to the SOX family of transcription factors that share a homologous high-mobility group (HMG) box DNA binding domain and regulate many developmental processes (18). SOX9 mutations are the cause of the human autosomal dominant disease campomelic dysplasia, which is characterized by extreme cartilage and bone malformation, frequent XY sex reversal, and multiple defects in other organs, supporting SOX9 as a key mediator of fate determination in developmental processes (19, 20). The identified targets of SOX9 include particular collagen genes during chondrogenesis and the Mullerian inhibiting substance during male sex differentiation (21, 22). However, SOX transcription factors function in a context-dependent manner (23), and the role of SOX9 and its regulated genes in normal and neoplastic breast has not been fully determined. A recent report indicated that SOX9, in cooperation with Slug, plays a critical role in supporting mammary epithelial stem cells and enhancing BCa cell metastasis (24).

In this study, we show that SOX9 protein is expressed at intermediate or high levels in the majority of BL subgroup BCa. Significantly, SOX9 expression in BCa was positively correlated with expression of LRP6 and TCF4, which are two major components of the canonical Wnt/ β -catenin pathway. We show that SOX9 regulates LRP6 and TCF4 expression and supports Wnt/ β -catenin activity. Moreover, we observe an increase in LRP6 and TCF4 levels and in the cytoplasmic and nuclear β -catenin staining in transgenic mice overexpressing SOX9 in mammary epithelium. These data indicate that SOX9-dependent Wnt/ β -catenin pathway activation may contribute to BCa pathobiology, particularly in the majority of BL-BCa expressing high levels of SOX9.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—MCF-7, T47D, Au656, SKBR3, HCC1937, MDA-MB231, and 293T cells were from ATCC. The MCF10A-DCIS.com, SUM149, and SUM1315 were from Asterand. The cells were maintained under conditions recommended by the providers. Recombinant mouse Wnt3A and Dkk1 were from R&D System.

Tissue Microarrays and Immunohistochemical Analyses—A cohort of 129 patients with invasive BCa and their sub-classification by gene expression array were described previously (25,

26). Tissue microarrays were prepared from archived tissue blocks containing representative tumor tissues of 114 cases of this cohort (26, 27). SOX9 expression was detected by standard immunohistochemical methods using a SOX9-specific antibody (O9-1) as described (28). The SOX9 expression in tumor cells was blindly scored by a study pathologist (Xin Yuan) and was categorized according to the percentage of the tumor cells showing distinctively positive nuclear staining: <2% (SOX9 negative), between 2–30% (SOX9 intermediate), and >30% (SOX9 high). The Fishers Exact Test was used to statistically analyze the association of the SOX9 immunostaining score among BCa subtypes, p53 staining patterns, and Bloom-Richardson tumor grades. The antibodies used in the immunohistochemical analyses of BCa xenografts or mouse mammary tissues are detailed in the [supplemental data](#).

Meta-analysis of Gene Expression—SOX9 mRNA expression levels (relative expression units) of the 114 cases were determined from the Affymetrix U133 plus 2.0 gene expression array data (NCBI GEO accession no. GSE5460) using dChip software (29). The analysis of variance function in dChip identified gene probes with significant correlation to two SOX9 probes (202936_s_at and 202935_s_at) using the $p < 1e^{-6}$ as a cut-off (The p value is testing the null hypothesis that the correlation is 0). The selected genes were ranked according to the correlation coefficient.

Immunoblotting—The analyses were performed as described in Ref. 30. The primary antibodies are listed in the [supplemental data](#).

Transfection and Viral Infection—The SOX9 transactivation reporter, Col 2 α 1 4X48, was a gift from B. de Crombrughe (University of Texas M. D. Anderson, Houston, TX), and the pTCF4 promoter reporter was a gift from K. Engeland (Universität Leipzig, Leipzig, Germany). The 8 $\times$ Topflash was acquired from Addgene (plasmid 12456), which was made by Ajamete Kaykas in the Moon laboratory (31). The LRP6-pCS2 was a gift from X. He (Children's Hospital, Boston, MA) (32). The cells were transfected with mixtures of DNA and Lipofectamine 2000 (Invitrogen). Cells were analyzed for luciferase activity using the Dual-Luciferase measurement system (Promega).

The siRNA transfection and retrovirus-mediated shRNA transduction were described (28, 30). Lentiviruses expressing SOX9-specific or luciferase shRNA were generated by transfecting 293T cells with hairpin-pLKO.1 plasmids, together with packaging plasmids. The virus-containing culture media were collected every 24 h for 3 days. The HCC1937 cells were incubated with the lentivirus-containing culture media plus 4 μ g/ml polybrene for 48 h and then selected for 72 h in 1.5 μ g/ml puromycin. The pLKO.1 luciferase hairpin plasmid was a gift from Gregory Finn (Beth Israel Deaconess Medical Center, Boston, MA) and the pLKO.1 SOX9 lentiviral plasmids were purchased from Open Biosystems (shSOX9-L1: RHS3979–9587794).

Real-time RT-PCR—Methods are described in Ref. 30. The primer/probes sets are detailed in the [supplemental data](#).

Cell Proliferation and Invasion—50,000 cells were plated in each well of a 12-well plate, and the cells were counted on the next (day 0) and following days (days 1 and 2).

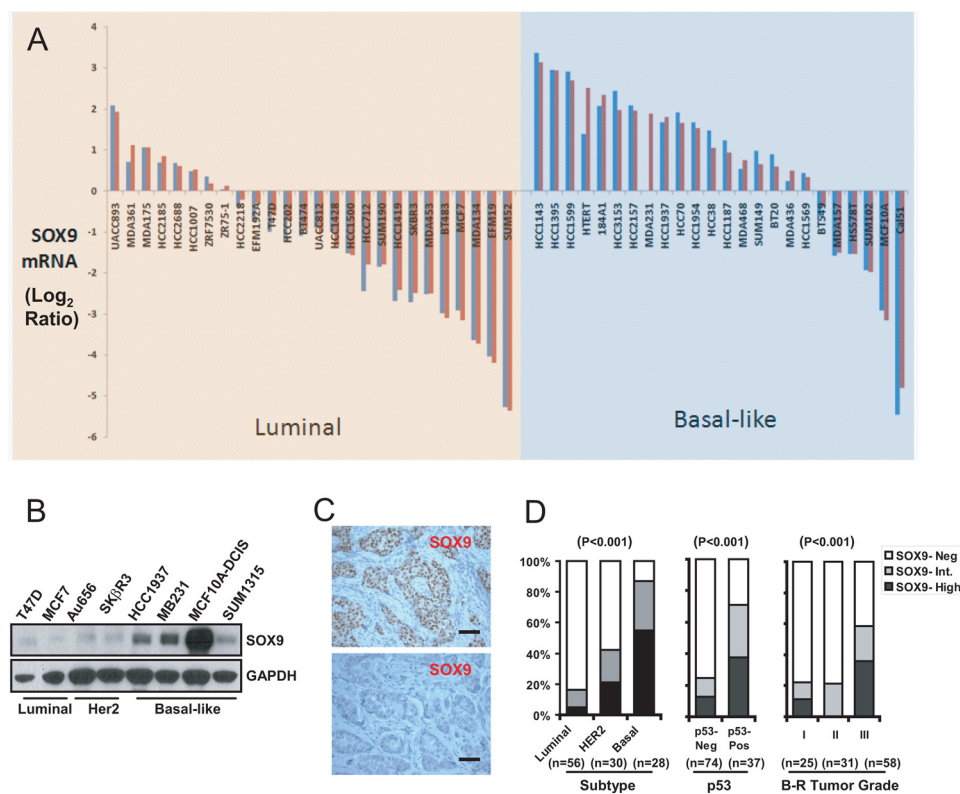


FIGURE 1. SOX9 expression in BCa. *A*, a waterfall chart of SOX9 expression in various BCa cell lines was adapted from a published database (35). The SOX9 mRNA expression level is shown as the $\log_2$ ratio of R/G , where R is the red probe signal of RNA from each individual cell line and G is the green probe signal of pooled RNA from 11 human cell lines. SOX9 mRNA levels measured by one or two independent probes were plotted. *B*, immunoblotting of SOX9 in BCa cell lines subgrouped according to published gene profiling, with GAPDH as a protein loading control. *C*, representative immunohistochemical staining of SOX9 in primary BCa tissue microarrays, demonstrating SOX9 high (upper panel) or negative (bottom panel) staining patterns. Bars, 50 μ m. *D*, the percentage of SOX9-negative (Neg), intermediate (Int.), and high cases were compared among BCa subtypes (left panel); between negative or positive p53 immunostaining groups (middle panel); or among Bloom-Richardson (B-R) tumor grade groups (right panel). The p values of the difference analysis (Fisher's Exact Test) are indicated.

Cell invasion was measured using the QCM™ 24-well collagen-based cell invasion assay kit (Millipore). 15,000 cells were seeded in a well insert, and after 48 h, the invaded cells were stained and directly counted.

ChIP—The experiment was performed as described in Ref. 33. The following anti-SOX9 antibodies were used: Ab-1, sc-20095; Ab-2, sc-17341 (Santa Cruz Biotechnology) and Ab-3, O9-1. The primer sequences are detailed in the supplemental data.

Tetracycline-inducible MCF7 Cell Lines—The 3 $\times$ FLAG-SOX9 fragment was cloned between the BamHI and EcoRI sites of pLVX-Tight-Puro vector (Clontech), which was cotransfected with pLVX-Tet-On Advanced (Clontech) into 293T cells to generate lentiviruses as described above. The inducible SOX9-expressing MCF7 cell lines were generated by lentiviral infection, followed by selection with puromycin (1 μ g/ml) and G418 (1 mg/ml).

Transgenic Mice—A 3 $\times$ FLAG-SOX9 cDNA fragment was cloned into the HindIII-EcoRV site of pTet-splice vector, which contains seven copies of the Tet operator (TetO, Invitrogen). To generate TetO-SOX9 transgenic mice, the XhoI-NotI fragment containing the TetO-3 $\times$ FLAG-SOX9 and SV40 intron/polyadenylation signal was gel purified and injected into the pro-nuclei of fertilized mouse eggs at the Beth Israel Deaconess Transgenic Core Facility. The founders and their offspring mice were genotyped by PCR. The MMTV-tetracycline trans-

activator (tTA) mice (34) were kindly provided by M. Kelliher (University of Massachusetts). The sequences of the genotyping primers are listed in the supplemental data.

RESULTS

SOX9 Protein Expression in BCa—SOX9 is one of the signature genes of the BL-BCa based on mRNA expression array analyses (1, 2). We first compared SOX9 mRNA expression in various BCa cell lines of the luminal or the BL subgroups, based on a published gene expression data set (35). SOX9 expression was much more prevalent in the BL subgroup, compared with its levels in the luminal subgroup (Fig. 1A). We next examined SOX9 protein expression in a series of BCa cell lines that were sub-typed according to the above and another published gene expression-profiling classifications (35, 36). SOX9 levels were higher within the BL subgroup (HCC 1937, MDA-MB231, MCF10A-DCIS.com, and SUM 1315) compared with HER2 (Au656 and SKBR3) or luminal (T47D and MCF7) subtypes (Fig. 1B). Immunohistochemistry was then used to assess SOX9 protein expression *in vivo* in previously sub-typed BCa tissue microarrays (25, 26). Among these tumors, SOX9 immunoreactivity ranged from absent (<2%; Fig. 1C, lower panel), to intermediate (2–30%) and high level expression (> 30% of tumor cells positive; Fig. 1C, upper panel). SOX9 protein expression was statistically different among the BCa subtypes, with the BL subgroup containing the highest percentage of

SOX9 positive (intermediate or high) samples (86%), followed by the Her 2 (43%) and the luminal subgroups (18%) (Fig. 1D, left panel).

Among all cases, SOX9 staining was significantly associated with positive p53 immunostaining, an indicator of p53 mutation and consequently impaired p53 activity and a marker of poor prognosis. SOX9 was expressed in 71% of the p53 positive samples, compared with 25% of the p53 negative cases (Fig. 1D, middle panel). Furthermore, SOX9 expression varied significantly among BCa of different pathological grades, with expression in 57% of the high grade (Bloom-Richardson grade III) compared with 21 and 22% in intermediate and low grades, respectively (Bloom-Richardson grade II or I, Fig. 1D, right panel). These results indicate that SOX9 is prevalently expressed in BL-BCa and is associated with more aggressive diseases, although larger data sets are needed to determine whether SOX9 is a predictive biomarker of aggressive behavior independent of subtyping.

SOX9 Regulates LRP6 and TCF4 Expression in BCa Cells—We first examined the biological functions of SOX9 in BCa cell lines. We examined the effect of SOX9 down-regulation in MCF10A DCIS.com (MCF10A-DCIS) cells, which express the highest level of endogenous SOX9 protein among the examined cell lines (Fig. 1B). MCF10A-DCIS is a BCa line established by *in vitro* adaptation of xenografts generated by v-Ras transformed MCF10A cells (38). Stable SOX9 silencing (shown in Fig. 2D) using retrovirus-transduced shRNA reduced cell proliferation and invasion (Fig. 2A, B).

Being a transcription factor, SOX9 is likely to carry out its biological functions in BCa through regulating the expression of its target genes. We examined genes whose mRNA levels were positively correlated with SOX9 expression among the BCa samples. The top ranked gene was TCF4 (detected by multiple probes with correlation coefficients of 0.51–0.63), whereas another gene strongly associated with SOX9 was LRP6 (correlation coefficient of 0.57) (Table 1). Interestingly, TCF4 and LRP6 are critical components of the canonical Wnt/ β -catenin pathway, where LRP6 is a co-receptor for Wnt and TCF4 is a major TCF family member that co-activates with nuclear β -catenin. The correlation would be consistent with SOX9 being regulated by the Wnt/ β -catenin pathway (30, 37). However, this would also be consistent with SOX9 functioning upstream of the Wnt/ β -catenin pathway by regulating expression of its key components.

SOX9 in MCF10A-DCIS cells was transcriptionally active, as indicated by the transactivation of a transfected SOX9 reporter carrying multiple SOX9-specific enhancer elements from the *Col2a1* gene (39), which was markedly reduced after cotransfection of two independent SOX9 siRNAs (Fig. 2C). To directly test whether LRP6 or TCF4 were SOX9-regulated, we used immunoblotting to first confirm the down-regulation of SOX9 protein in a stable MCF10A-DCIS line expressing a SOX9-specific shRNA (stable) or by transient transfection of two SOX9 siRNAs, compared with their respective controls (Fig. 2D). Importantly, LRP6 and TCF4 protein levels were also reduced in response to stable or transient SOX9 down-regulation. Moreover, LRP6 and TCF4 mRNA levels, as measured by quantitative real-time RT-PCR, were similarly reduced in response

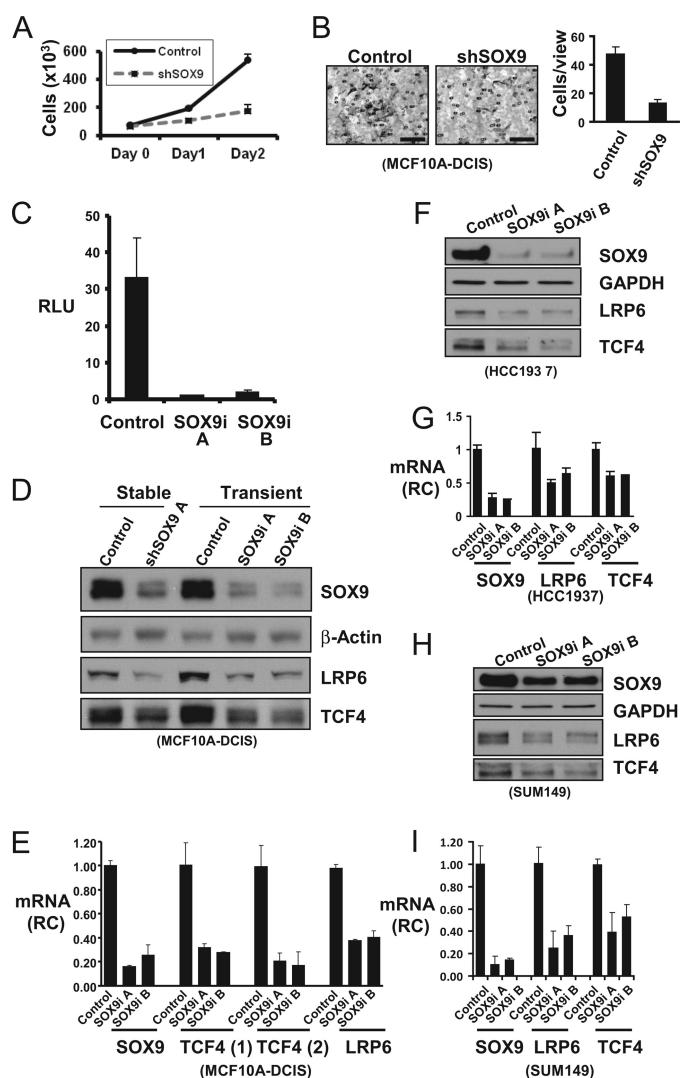


FIGURE 2. SOX9 supports cell proliferation, invasion, and LRP6 and TCF4 expression in BL-BCa cell lines. A, cellular proliferation measured by direct counting was plotted for control or SOX9-specific shRNA (shSOX9) expressing MCF10A-DCIS cells. B, cell invasion through collagen gel was measured at 48 h post-seeding. Representative staining of the invaded cells were shown in the left panels (bars, 100 μ m). The average number of invaded cells per high power view (400 $\times$) was shown in the right panel. C, SOX9 transcriptional activity in MCF10A-DCIS cells was assessed by transfection of a SOX9 transcription reporter plasmid (Col2a1 4 $\times$ 48-luc) together with two SOX9-specific siRNA (SOX9i A and B) or a non-targeting control siRNA (Control), and a control *Renilla* luciferase reporter. The ratio of firefly to *Renilla* luciferase activity (relative light units, RLU) was measured 72 h after transfection. D, MCF10A-DCIS cells were infected with retroviruses expressing either SOX9-specific shRNA (shSOX9 A) or control shRNA and selected with puromycin for 72 h. Alternatively, the MCF10A-DCIS cells were transfected with SOX9-specific siRNA (SOX9i A or B) or control, and the cell lysates were collected at 72 h post-transfection. SOX9, LRP6, and TCF4 were immunoblotted, with β -actin as a protein loading control. E, total RNA from siRNA transfected MCF10A-DCIS cells were collected at 72 h post-transfection, and the RNA levels of SOX9, LRP6, and TCF4 (using two distinct primer/probe sets) were measured by quantitative real time RT-PCR. Relative change (RC) is calculated by comparing to control siRNA transfected cells. F and G, HCC1937 were transfected with control or SOX9-specific siRNA. The protein and mRNA levels were measured at 72 h after transfection. H and I, SUM149 cells were examined similarly as in F and G. Statistical analysis was performed with Student's *t* test (*n* = 3). Error bars, S.E.

to SOX9 silencing, indicating that SOX9 is regulating LRP6 and TCF4 at the transcriptional level (Fig. 2E).

SOX9 regulation of LRP6 and TCF4 expression was further validated using SOX9 siRNAs in two other BL-BCa cell lines,

TABLE 1

The SOX9-correlated genes in BCa

The gene expression arrays for 114 BCa samples were analyzed, and the genes whose expression levels were significantly associated with SOX9 ($p < 1 \times 10^{-6}$) were ranked according to the correlation coefficient (correlation). Genes with a correlation coefficient ≥ 0.50 were listed.

	Accession no.	Correlation	p value
<i>TCF4</i>	AI375916	0.63	4.30×10^{-14}
<i>TCF4</i>	AI703074	0.62	1.72×10^{-13}
<i>TCF4</i>	AV721430	0.58	1.83×10^{-11}
<i>LRP6</i>	NM002336	0.57	2.97×10^{-11}
<i>COL27A1</i>	AK021957	0.56	1.14×10^{-10}
<i>GATA 6</i>	D87811	0.55	1.55×10^{-10}
<i>CITED4</i>	AI858001	0.53	1.08×10^{-9}
<i>CRIM1</i>	AW243081	0.53	1.39×10^{-9}
<i>TCF4</i>	AA664011	0.52	2.13×10^{-9}
<i>BAP28</i>	NM018072	0.52	3.85×10^{-9}
<i>COL27A1</i>	AI949136	0.52	3.92×10^{-9}
<i>TCF4</i>	AI949687	0.52	4.52×10^{-9}
<i>TCF4</i>	AJ270770	0.51	5.61×10^{-9}
<i>COL27A1</i>	AU145229	0.51	9.90×10^{-9}
<i>BAP28</i>	NM018072	0.50	1.19×10^{-8}
<i>NF1X</i>	AI817698	0.50	1.21×10^{-8}
<i>TLR5</i>	AF051151	0.50	1.88×10^{-8}
<i>CRIM1</i>	BG546884	0.50	2.03×10^{-8}

HCC1937 and SUM149. In each cell line, SOX9 down-regulation led to reduction of LRP6 and TCF4, both at the protein (Fig. 2, *F* and *H*) and mRNA levels (Fig. 2, *G* and *I*). Together, these results strongly support a SOX9 function in maintaining LRP6 and TCF4 levels in BCa.

SOX9 Associates with the TCF4 Promoter—To further characterize the molecular basis for SOX9 regulation of TCF4 expression, we performed ChIP assays in MCF10A-DCIS cells to examine SOX9 interaction with the TCF4 immediate promoter region (directly or through chromatin looping with distal enhancers). Using three independent SOX9-specific antibodies, we detected binding of endogenous SOX9 to the TCF4 gene promoter region (detected by the P1 and P2 primer pairs) but not to the exon or intron regions using two primer pairs (C1 and C2) (Fig. 3A). SOX9 transactivation of the TCF4 promoter was further examined by transiently transfecting a TCF4 reporter containing 1.3 kb of the TCF4 promoter sequence (TCF4 1.3 kb-Luc) (40) into MCF7 with increasing amounts of a SOX9 expression vector, which resulted in a dose-dependent transactivation of the reporter (Fig. 3B). Although further studies are needed to pinpoint the precise SOX9 binding sites, these results strongly support the conclusion that SOX9 is a direct transcriptional regulator of the TCF4 gene. Similar studies of the LRP6 gene did not show evidence of SOX9 binding to the promoter region (data not shown), suggesting that SOX9 may bind to distant sites or indirectly influence LRP6 transcription.

SOX9 Regulates Wnt/ β -Catenin Pathway Activation—To determine whether endogenous SOX9 was required for Wnt/ β -catenin pathway activation, we next examined the effect of SOX9 down-regulation on cellular responses to Wnt stimulation. SOX9 regulation of Wnt signaling was first demonstrated by the markedly reduced Wnt 3A stimulation of a β -catenin/TCF4-regulated reporter (Topflash) after SOX9 silencing in two BL-BCa cell lines (MCF10A-DCIS and HCC1937; Fig. 4, *A* and *D*). In control cells, Wnt3A caused a dose-dependent increase in the protein levels of active and total β -catenin, as well as of Axin2 (a canonical Wnt/ β -catenin pathway downstream target protein) (Fig. 4, *B* and *E*). It is noteworthy that

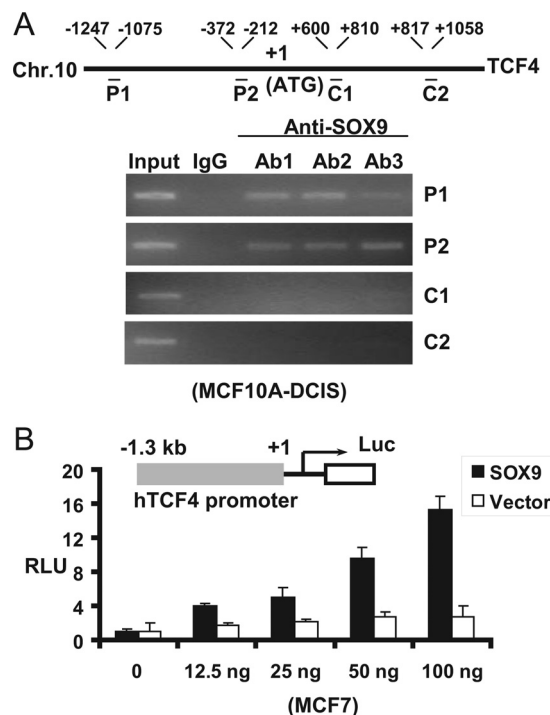


FIGURE 3. SOX9 transcriptionally activates TCF4 by binding to the TCF4 promoter. A, ChIP assay using three independent SOX9-specific antibodies (Ab1–3) was performed in MCF10A-DCIS cells. P1 and P2 are PCR fragments of the promoter region, whereas C1 and C2 are PCR fragments of exon or intron region (numbered from the translation initiation site). B, MCF7 cells were transfected with a TCF4 promoter-driven firefly luciferase reporter and a CMV-driven *Renilla* luciferase reporter together with increasing quantities of SOX9 expression vector or empty vector control. The luciferase activities were assessed at 72 h posttransfection. RLU, relative light unit (the ratio of firefly over *Renilla* luciferase light units; $n = 3$). Error bars show S.E.

SOX9 protein levels also increased in response to Wnt3A, consistent with previously published data that SOX9 is a downstream target of the Wnt signal (30, 37). In contrast, the Wnt3A stimulation of β -catenin and Axin2 protein accumulation was diminished by SOX9 loss in SOX9 shRNA cells. Wnt3A stimulation of the Axin2 mRNA expression was also similarly hampered by SOX9 down-regulation (Fig. 4, *C* and *F*). We next tested whether exogenous LRP6 expression can rescue the Wnt/ β -catenin activation in SOX9 shRNA cells. Transient transfection of a LRP6 expression vector partially rescued Wnt3A stimulated Axin2 mRNA expression in the shSOX9 cells to about half of the level in mock transfected control cells, further supporting that SOX9-mediated Wnt signaling is at least in part mediated through its regulation of LRP6 (Fig. 4G). Overall, these results indicate that SOX9 is both upstream and downstream of Wnt/ β -catenin, resulting in the formation of a SOX9-regulated positive feedback loop that can enhance Wnt/ β -catenin signaling.

SOX9 Overexpression Induced LRP6 and TCF4 Expression and Enhanced Wnt/ β -Catenin Pathway Activation—We next addressed whether SOX9 overexpression in cells expressing low levels of endogenous SOX9 was sufficient to induce LRP6 or TCF4 expression and to enhance Wnt/ β -catenin pathway activation. For this purpose, we generated MCF7 BCa cell lines with tetracycline-regulated expression of exogenous FLAG-tagged SOX9. The exogenous SOX9 is increased in response to

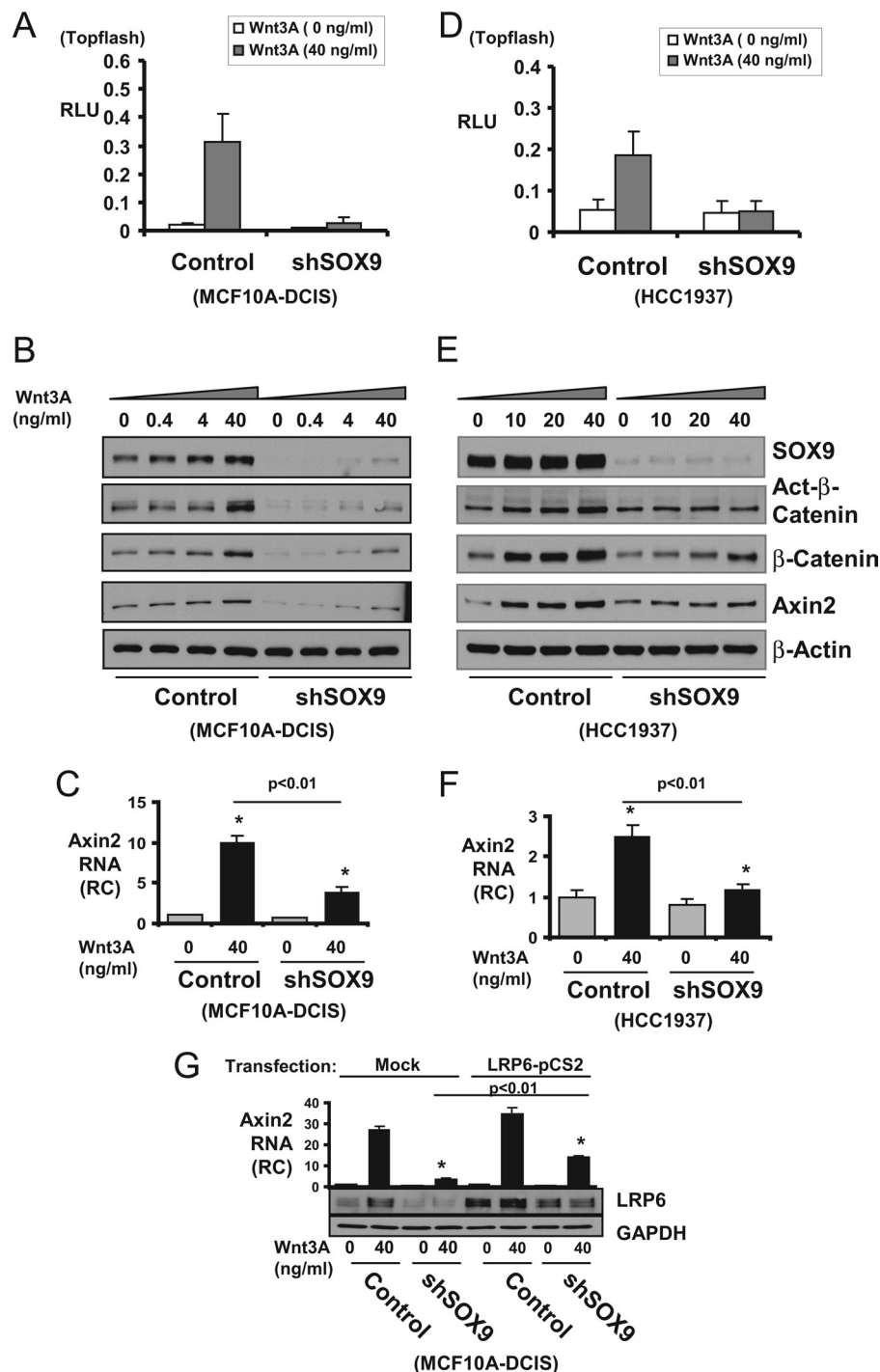


FIGURE 4. SOX9 enhances Wnt3A stimulation of Wnt/ β -catenin activity. *A* and *D*, MCF10A-DCIS (retroviral transduced) or HCC1937 (lentiviral transduced) cells stably expressing control or SOX9-targeted shRNA were transfected with Topflash-firefly-luc together with CMV-*Renilla*-luc reporters for 72 h. The cells were treated with mouse Wnt3A during the last 8 h. The activity was presented as relative light unit (RLU) with firefly corrected for *Renilla* luciferase activity. *B* and *E*, MCF10A-DCIS or HCC1937 cells stably expressing control or SOX9-targeted shRNA were treated with mouse Wnt3A for 8 h, and the cell lysates were immunoblotted for SOX9, active (Act-) or total β -catenin and Axin2. *C* and *F*, cells were similarly treated as described in *B* and *E*. Total RNA was collected, and Axin2 mRNA was measured by quantitative real time RT-PCR. RC, relative change compared with the level in untreated control cell. *G*, MCF10A-DCIS cells expressing control or SOX9-specific shRNA (shSOX9) were transfected with either mock (pcDNA3) or LRP6 expression vectors (LRP6-pCS2) for 48 h. Carrier or Wnt3A (40 ng/ml) were added during the last 8 h. The LRP6 protein levels were measured by immunoblotting (bottom panels), and Axin2 mRNA levels were measured by quantitative real time RT-PCR. Statistical analysis was performed with Student's *t* test (*n* = 3). Error bars, S.E.

doxycycline (Fig. 5A). Importantly, LRP6 and TCF4 proteins were also increased correspondingly, supporting that SOX9 is an upstream activator of these genes.

We next examined SOX9 effect on Wnt3A stimulated β -catenin cytoplasmic and nuclear redistribution in these cells

with induced SOX9 expression. The 6-h-induced or uninduced MCF7-SOX9 cells were treated with Wnt3A during the last hour. Measured by cell fractionation, both cytoplasmic and nuclear β -catenin was accumulated at higher levels in the SOX9-induced cells compared with uninduced cells (Fig. 5B).

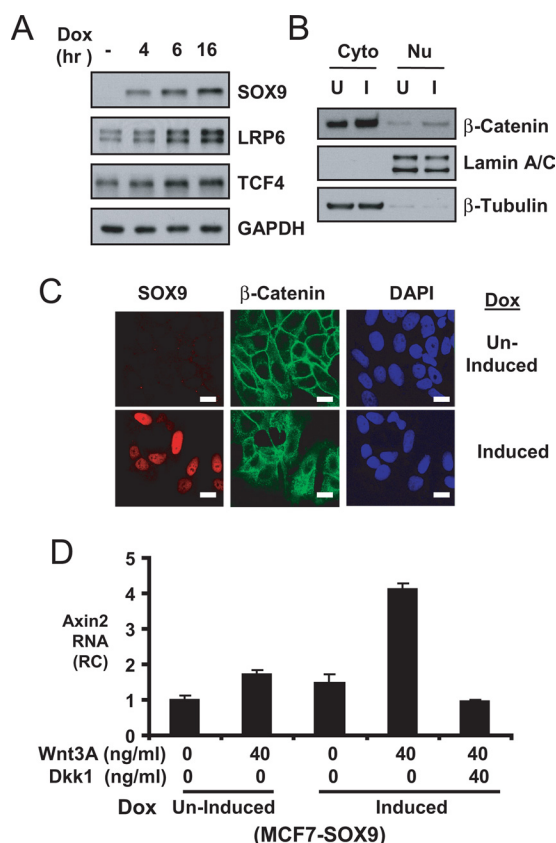


FIGURE 5. SOX9 *in vitro* overexpression leads to increased LRP6 and TCF4 expression and enhanced Wnt/ β -catenin signaling. *A*, inducible SOX9 overexpressing MCF7 cells (MCF7-SOX9) were induced to express SOX9 with 30 ng/ml doxycycline for indicated times and blotted for SOX9, LRP6, and TCF4. GAPDH was also blotted for protein loading. *B*, MCF7-SOX9 were treated with 0 (uninduced, U) or 30 ng/ml doxycycline (induced, I) for 6 h. Both uninduced and induced cells were treated with mouse Wnt3A (40 ng/ml) during the last hour. Cell fractionation analysis and immunoblotting of β -catenin are shown. Lamin A/C and β -tubulin were blotted to demonstrate the effective separation of nuclear (Nu) and cytoplasmic (Cyto) fractions. *C*, the uninduced and induced cells were also examined by SOX9 and β -catenin immunofluorescence analysis with a confocal microscope. 4',6-diamidino-2-phenylindole (DAPI) was used to stain nuclei. Error bars, 20 μ m. *D*, MCF7-SOX9 cells were treated without/with 30 ng/ml doxycycline (uninduced or induced) for 24 h. The cells were then treated with Wnt3A or Dkk1 as indicated during the last 8 h. The Axin2 mRNA levels were measured by quantitative real time RT-PCR.

Immunofluorescence staining further demonstrated an increased Wnt3A-stimulated cytoplasmic and nuclear β -catenin accumulation in the induced MCF7-SOX9 cells (Fig. 5C). Wnt3A-stimulated Axin2 mRNA expression was also increased in induced *versus* uninduced MCF7-SOX9 cells, and the induced Axin2 level was suppressed by concomitant treatment with a LRP6 inhibitor, Dkk1 (Fig. 5D). Together, these results show that SOX9 is capable of enhancing LRP6 and TCF4 expression and boosting Wnt/ β -catenin activation.

Transgenic Expression of SOX9 in Mammary Epithelium Increases Ductal Branching, LRP6 and TCF4 Expression, and Wnt/ β -catenin Activity—Wnt signaling has been implicated in playing an important role during mammary development (reviewed in Ref. 41). To assess the SOX9 effect on Wnt signaling in normal breast, we generated mice with a TetO-regulated 3xFLAG-SOX9 transgene. These were crossed with mice expressing an MMTV-directed tTA to obtain double trans-

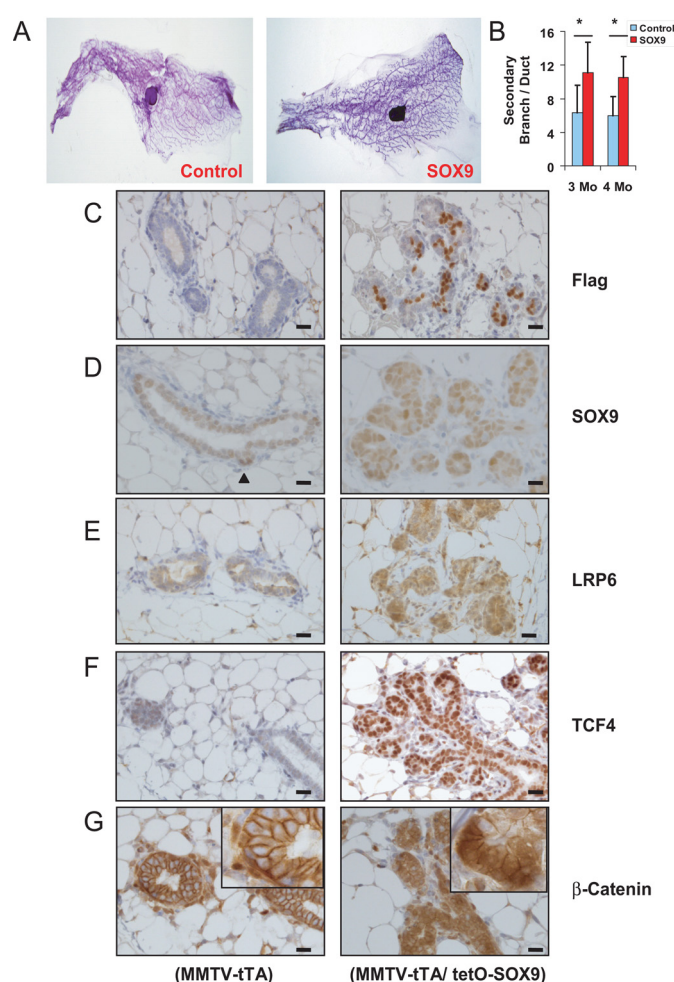


FIGURE 6. SOX9 *in vivo* overexpression leads to increased mammary ductal branching, increased LRP6 and TCF4 expression, and enhanced Wnt/ β -catenin signaling. *A*, whole mount staining of 4th pair mammary tissues of 4-month-old wild type FVB (control) or MMTV-tTA/TetO-SOX9 (SOX9) mice. *B*, the secondary branching was counted under low power view (40 $\times$). Two mice from control or SOX9 groups were examined in either age group. In the 3-month-old groups (3 Mo), a total 39 and 37 ducts were examined in the control and SOX9 group, respectively. Similarly, 39 and 35 ducts were examined in the 4-month-old group (4 Mo). Student's *t* test (two-sided) was performed to determine statistical significance (*, *p* < 0.01). *C-G*, mammary tissues from 4-month-old single (MMTV-tTA) or double (MMTV-tTA/TetO-SOX9) transgenic virgin mice were examined by immunohistochemistry for FLAG-tagged SOX9 transgene (C), SOX9 (D), LRP6 (E), TCF4 (F) and β -catenin (G). Arrowhead in D indicates increased endogenous SOX9 expression at a site that appears to be branching. Insets, higher power views. Error bars, 20 μ m.

genic mice that constitutively express SOX9 in the absence of tetracycline (Tet-off). In 3- or 4-month-old virgin mouse mammary glands, the MMTV/SOX9 double transgenic mice showed modest, but significant increase in the number of secondary branching (Fig. 6, A and B). Expression of the FLAG-tagged SOX9 transgene was evident in a subset of the epithelial cells by anti-FLAG immunostaining (Fig. 6C). SOX9 immunostaining showed increased SOX9 expression in a similar fraction of epithelial cells (Fig. 6D). Interestingly, expression of endogenous SOX9 in control MMTV-tTA alone mice was higher in a small number of cells, especially in areas that appeared to be undergoing ductal branching (arrowhead in Fig. 6D, left panel). LRP6, TCF4, as well as cytoplasmic and nuclear β -catenin levels were increased in the epithelium of double

transgenic mice compared with the single transgenic mice (Fig. 6, E–G). These data showed that SOX9 can increase LRP6 and TCF4 *in vivo* and can enhance Wnt/ β -catenin signaling and ductal proliferation in non-neoplastic breast.

DISCUSSION

SOX9 is one of the signature genes that define the BL subgroup of BCa (1, 2), but the molecular functions of SOX9 and many other BL signature genes have not been fully established in normal breast or in BCa. We found that SOX9 protein was expressed at intermediate or high levels in the majority of BL BCa and in a smaller proportion of HER2 and luminal subtypes and that its expression was associated with indicators of more aggressive disease. Significantly, SOX9 strongly correlates with LRP6 and TCF4, critical components of the canonical Wnt/ β -catenin pathway. Although SOX9 expression was increased by Wnt/ β -catenin pathway activation in BCa cells, we found that SOX9 also functions upstream to increase expression of LRP6 and TCF4 and showed that *Tcf4* is a direct SOX9-regulated gene. SOX9-dependent Wnt/ β -catenin pathway activation was demonstrated *in vitro* by loss of Wnt-stimulated Wnt/ β -catenin activation after SOX9 silencing. Moreover, we found increased LRP6 and TCF4 expression and Wnt/ β -catenin activation, as well as increased ductal branching, in transgenic mice overexpressing SOX9 in mammary epithelium. These data reveal a positive feedback loop between SOX9 and the Wnt/ β -catenin signaling pathway.

The role of Wnt signaling in mammary morphogenesis is suggested by the differential expression of Wnt ligands at distinctive development stages and at different locations within the mammary ductal tree, supporting potential non-redundant Wnt functions in tissue patterning and homeostasis (42–45). Knock-out of *Wnt4*, *Lrp5*, or *Lrp6* genes severely block mammary ductal branching and terminal bud formation (12, 46, 47). Conversely, increased Wnt signaling through ectopic expression of Δ N89- β -catenin (a constitutively active β -catenin) or LRP6 can induce side branching in virgin mice, similar to that seen in early pregnancy (48, 49). Our finding of increased ductal branching in virgin SOX9 transgenic mice is consistent with its mediation of Wnt signal activation.

Enhanced Wnt signaling has also been implicated in increasing mammary stem cells, especially through loss-of-function studies of LRP5/6 *in vivo* and Wnt-mediated long term clonal expansion of mammary stem cells in culture (12, 24, 47, 50, 51). SOX9 has similarly been implicated in supporting stem cells in small intestine and hair follicle (52–54). Recent lineage tracing studies found that SOX9 marks the adult stem cell population that contributes to self-renewal and repair of the liver, exocrine pancreas, and intestine (55). Therefore, we suggest that SOX9 may also participate in normal breast homeostasis and renewal during puberty, estrous cycling, and pregnancy, probably taking part in mammary stem cell maintenance by controlling LRP6 and TCF4 expression and supporting Wnt/ β -catenin activity.

Interestingly, β -catenin can also directly interact with SOX9, so this positive feedback system may reach equilibrium when high levels of SOX9 result in sequestration of nuclear β -catenin and decreased β -catenin/TCF4 activity (56, 57). Indeed, SOX9

in chondrocytes has been reported to suppress nuclear β -catenin activity. In addition, SOX9 may have distinct biological activities in certain cell or tissue contexts due to differences in the spectrum of SOX9-regulated genes. With respect to BCa, previous studies have shown that retinoic acid can strongly induce SOX9 expression in luminal subtype cells expressing lower basal SOX9, but not in BL type cells with high basal SOX9 expression. Significantly, SOX9 induction contributes to the retinoic acid-mediated cell differentiation and growth suppression in luminal BCa cells, with one mechanism appearing to be SOX9-stimulated expression of a transcriptional repressor, HES-1 (58, 59).

Mutations in *Axin*, *Apc*, and β -catenin mediate Wnt/ β -catenin pathway activation in certain types of human cancers such as colon and hepatocellular cancers (60), but these mutations are typically absent in human breast tumors. Nonetheless, aberrant nuclear and cytoplasmic localization of β -catenin in human BCa specimens has been observed, with the BL subtype in particular being enriched for tumors with higher expression of LRP6 and cytoplasmic and nuclear β -catenin (5, 6, 61). The results of this study indicate that aberrant or persistent expression of SOX9 may be an event in carcinogenesis that renders cells hyper-reactive to physiological Wnt signals by up-regulating LRP6 and TCF4 expression. Conversely, as SOX9 expression is also increased by Wnt/ β -catenin signaling, aberrant Wnt activation may be an initiating event driving Wnt/ β -catenin activity and SOX9 expression. In any case, this study indicates that SOX9 expression identifies a subset of tumors with increased Wnt/ β -catenin pathway activation and aggressive behavior and may be predictive of responsiveness or resistance to particular therapeutics. It is reasonable to predict that newly developed Wnt/ β -catenin pathway-targeted therapies will be more effective in the subgroup of BCa expressing high levels of SOX9 and its regulated Wnt/ β -catenin activity.

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