SOX genes encode a family of high-mobility group transcription factors that play critical roles in organogenesis. The functional specificity of different SOX proteins and the tissue specificity of a particular SOX factor are largely determined by the differential partnership of SOX transcription factors with other transcription regulators, many of which have not yet been discovered. Virtually all members of the SOX family have been found to be deregulated in a wide variety of tumors. However, little is known about the cellular and molecular behaviors involved in the oncogenic potential of SOX proteins. Using cell culture experiments, tissue analysis, molecular profiling, and animal studies, we report here that SOX2 promotes cell proliferation and metastasis and through its transcription regulation of the CCND1 gene in breast cancer cells. In addition, we identified β-catenin as the transcription partner for SOX2 and demonstrated that SOX2 and β-catenin act in synergy in the transcription regulation of CCND1 in breast cancer cells. Our experiments not only determined a role for SOX2 in mammary tumorigenesis but also revealed another activity of the multifunctional protein, β-catenin.

The SOX2 gene family encodes a group of transcription factors that are characterized by a highly conserved high-mobility group (HMG) domain (1–3). These genes are found throughout the animal kingdom, are expressed in a restricted spatial-temporal pattern, and play critical roles in stem cell biology, organogenesis, and animal development (3, 4). For example, overexpression of Sox2 in mouse neural stem cells blocks their differentiation, and inhibition of Sox2 in these cells causes their premature exit from the cell cycle and differentiation into neurons (5). Depletion of Sox2 by RNA interference blocks the proliferation of neural stem-like cells and causes them to differentiate into neurons (6).

Recently, a number of links have been found between SOX transcription factors and human cancers (7). For instance, SOX2 has been found to be an immunogenic antigen in 41% of small cell lung cancer patients (8) and in 29% of meningioma patients (9). Immunohistochemistry results suggest that SOX2 is involved in later events of carcinogenesis, such as invasion and metastasis of pancreatic intraepithelial neoplasia (10). SOX2 may also be involved in gastric carcinogenesis (11) and may be amplified in prostate cancers (12). Furthermore, SOX2 expression has been observed in 43% of basal cell-like breast carcinomas and was found to be strongly correlated with CK5/6, EGFR, and vimentin immunoreactivity, suggesting that SOX2 may play a role in conferring a less differentiated phenotype in these tumors (13).

How SOX2 exerts its oncogenic potential is currently unknown. SOX proteins including SOX2 bind to specific DNA sequences (C(T/A)TTG(T/A)(T/A)) by means of their HMG domains in functioning as transcription factors to activate or repress target gene expression (2, 3). It is currently accepted that SOX proteins themselves do not possess sufficient affinities for DNA binding. Rather, the transcription activity of SOX proteins requires recruitment of other protein partners, which facilitate and stabilize the formation of SOX transcription initiation complexes (2, 3). In fact, the functional specificities among members of the SOX family and the tissue specificity for a particular SOX protein are believed to be determined largely by the differential partnership of SOX proteins with other proteins (2, 3).

Numerous transcription factors from a wide range of families have been found to partner with SOX proteins (2, 3). As stated above, because the partnership of SOX proteins with different partner proteins is a crucial element in determining the functional specificity of SOX proteins, it is of great importance to identify partner proteins for different SOX proteins in different tissues to understand the pathophysiological activities of SOX proteins. Another prominent and related issue is the identification of the downstream targets of SOX proteins. To date, investigation of the functions of SOX proteins has concentrated on embryonic development, and information on the physiological functions of SOX proteins in adult tissues, as well as their potential roles in carcinogenesis, is limited.

In this study, we found that SOX2 overexpression is a frequent event in breast carcinomas and that the levels of SOX2...
expression were strongly correlated with tumor grades and with the levels of cyclin D1 expression in breast carcinoma samples. We demonstrated that SOX2 promoted the proliferation of breast cancer cells through facilitating the G1/S transition and through up-regulation of the CCND1 gene. Furthermore, we identified β-catenin as the transcription partner for SOX2 in breast cancer cells and demonstrated that SOX2 synergized with β-catenin in transactivation of the CCND1 gene.

EXPERIMENTAL PROCEDURES

Tissue Specimens and Cell Lines—Breast carcinoma tissues were obtained from Peking University Oncology Hospital. Samples were frozen in liquid nitrogen immediately after surgical removal and maintained at −80 °C until use. All human tissue was collected using protocols approved by the Ethics Committee of the Peking University Health Science Center. The normal human breast epithelia cell lines and the human breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA).

Recombinant Retroviral Construction—Recombinant Retro-GFP, Retro-SOX2, and Retro-SOX2 RNAi were generated using the BD Retro-X™ Universal Packaging System (BD Biosciences, San Jose, CA) according to the manual provided by the vendor. The cDNA for full-length SOX2 and the specific oligonucleotide for generating SOX2 siRNA were cloned into the pLNCX2 vector. The resultant plasmids were transformed into Escherichia coli DH5α, purified, and co-transfected with the pVSV-G vector into the GP2-293 packaging cell line (BD Biosciences) using Lipofectamine (Invitrogen). Three days after transfection, cell lysates were obtained from the 293 cells. Cell lysates were added to 293 cells again, and when most of the cells had been killed by virus infection and detached, cell lysates were collected and filtered through a 0.45-μm cellulose acetate filter. The viral titers were determined according to the procedure provided by the vendor. For cell infection, cells were incubated with viruses at an MOI (multiplicity of infection) of 10 for 6 h before fresh medium was added, and cultures were continued for another 48 h. For generation of SOX-overexpressing cells to be transplanted into nude mice, infected cells were grown in the presence of 400 μg/ml of G418 for 3 weeks. Individual drug-resistant clones were collected, pooled, and expanded.

Tumor Xenografts—MCF-7 breast cancer cells were plated and infected in vitro with mock, Retro-GFP, Retro-SOX2, or Retro-SOX2 RNAi at an MOI of 100. Forty-eight hours after infection, 5 × 10⁶ viable MCF-7 cells in 200 μl of phosphate-buffered saline were injected into the mammary fat pads of 6–8-week-old female BALB/c mice (Charles River, Beijing, China). Six animals per group were used in each experiment. 17β-Estradiol pellets (0.72 mg/pellet, 60 day release; Innovative Research of America, Sarasota, FL) were implanted 1 day before the tumor cell injection. Tumors were measured weekly using a vernier caliper, and volume was calculated according to the formula: \( V = \frac{1}{2} \times \text{length} \times \text{width}^2 \). Tumors were collected at the time of sacrifice, 8-weeks posttransvirus infection. Deparaffinized sections of tumor tissue were used to assess proliferation using antibodies against Ki-67 (1:200, Zymed Laboratories). Apoptosis was determined using the In Situ Cell Death Detection kit, POD (Roche Applied Science, Germany), according to the manufacturer’s instruction. Nuclei that stained brown were scored as positive for apoptosis and blue as negative. All studies were approved by the Animal Care Committee of Peking University Health Science Center.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed essentially as described previously (14–19). Primers that specifically amplify the CCND1 promoter were used in the PCR amplifications, as listed: for proximal promoter (spanning −86 to +234 bp) forward: 5′-tcgcgggctttgaccttt-3′ and reverse: 5′-cggtctgagggagttggg-3′; for control region (spanning −2598 to −2377 bp) forward: 5′-ttttgattttccttc-3′ and reverse: 5′-gcattttgatttt-3′.

RESULTS

SOX2 Is Overexpressed in Human Mammary Tumors and Its Level Is Correlated with Tumor Grade—Similar to other developmentally regulated genes, the expression of SOX2 is supposed to be switched off in adulthood. The observation that SOX2 is expressed in several types of tumors (7–13) prompted us to investigate whether SOX2 was also expressed in breast cancers. Using immunohistochemistry (IHC), we screened paraffin-embedded mammary tissue sections from 19 normal and 56 breast cancer patients for the expression of SOX2. The results of these experiments indicated that, while normal mammary epithelial cells displayed none or weak SOX2 staining (Fig. 1A, left panel), breast carcinoma cells were strongly positive for SOX2 staining in the nucleus (Fig. 1A, right panel).

To investigate whether there is any association between the expression level of SOX2 and the development and progression of breast tumors, quantitative real-time RT-PCR analysis of primary tumor mRNAs was carried out to determine the relative expression levels of SOX2. We defined the overexpression cutoff value for tumors to be the normalized mean expression of SOX2 in normal tissue plus three times the standard deviation (20). In 19 normal and 56 primary human breast cancer tissues that we examined, we observed a striking difference in the level of SOX2 expression. According to Bloom and Richardson’s classification system (21), SOX2 overexpression was observed in 33.3% (3 of 9) of ductal carcinoma in situ (DCIS) tumors, 42.3% (11 out of 26) of grade II tumors, and 57.1% (12 of 21) of grade III tumors (Fig. 1B). Moreover, Western blotting analysis of immunoreactive SOX2 in established mammary epithelial cell lines indicated that the levels of SOX2 in breast cancer cell lines were considerably higher than those in the two cell lines (MCF-10A, HBL100) derived from normal mammary epithelial cells (Fig. 1C). Collectively, these data suggest that the overexpression of SOX2 might be a frequent event in human breast cancer and that the level of SOX2 expression is correlated with the tumor grade.

SOX2 Promotes Proliferation and Tumorigenesis of Breast Cancer Cells—Because of the crucial role of SOX2 in cell proliferation and differentiation, the deregulation of SOX2 expression in breast carcinomas and breast cell lines may have important pathological relevance. Thus, we next investigate the role of SOX2 expression in cell proliferation and tumorigenesis. First, both gain-of-function and loss-of-function experiments were performed to examine the effect of SOX2 overexpression or knockdown on cell cycle regulation in mammary carcinoma
cells. In these experiments, MCF-7 cells were synchronized at the G0/G1 phases by serum starvation (22–24), at the G1/S boundary by double thymidine blocking (25), or at mitosis (M phase) with nocodazole blocking (26). Cell cycle profiling by FACs indicated that, after release from thymidine and nocodazole blocking, cells with either SOX2 overexpression or with SOX2 knockdown proceeded through the S and G2 phases with efficiencies and kinetics similar to those of control cells (Fig. 2A, upper panel). However, after release of the cells from G0/G1 arrest, overexpression of SOX2 was associated with an increase in the population of cells in the S + G2/M phases and a concomitant decrease in the population of cells in the G0/G1 phases (Fig. 2A, upper panel). Consistently, knockdown of the expression of SOX2 with siRNA resulted in an accumulation of cells in the G0/G1 phases. Similar results were also observed in one of basal-like breast cancer cell lines, MDA-MB-231 (data not shown). To confirm that the phenotypic behavior of cells with SOX2 knockdown was specifically caused by loss of SOX2 protein, we sought to “rescue” the effect of the SOX2 RNAi by constitutively expressing murine Sox2 in MCF-7 cells. As shown in Fig. 2A (lower panel), the expression of Sox2 in cells with SOX2 knockdown resulted in an increase in the population of cells in the S + G2/M phases, similar to the profile of the cells co-transfected with a combination of Sox2 and nonspecific siRNA, suggesting that the G0/G1 accumulation of cells with SOX2 knockdown was specifically associated with the loss of SOX2 protein. Collectively, these results indicate that SOX2 promotes the proliferation of breast cancer cells by facilitating the G1/S transition.

To investigate the role of SOX2 in breast tumorigenesis, retrovirus (retro)-mediated overexpression and knockdown strategy was utilized. Retro-EGFP (retroviruses carrying the EGFP gene), Retro-SOX2 (retroviruses carrying the SOX2 gene), or Retro-SOX2-siRNA (retroviruses carrying an oligonucleotide specific for SOX2 mRNA) were constructed and used to infect MCF-7 cells. Anchor-independent growth assays demonstrated that SOX2-overexpressing cells exhibited an increase both in the size and number of colonies formed on the soft agar, whereas knockdown of SOX2 expression resulted in a substantial reduction in the number of colony formed (Fig. 2B). The level of SOX2 expression in MCF-7 cells infected with Retro-EGFP, Retro-SOX2, or Retro-SOX2-siRNA was monitored by Western blotting (Fig. 2B, right panel).

To assess the role of SOX2 on breast tumorigenesis in vivo, equal numbers of mock-, Retro-EGFP, Retro-SOX2, or Retro-SOX2-siRNA-infected MCF-7 cells were implanted onto the mammary fat pad of athymic BALB/c mice, and the growth of the implanted tumors was measured. The results of these experiments indicated that overexpression of SOX2 was associated with a significant tumor growth over an 8-week period, and knockdown of SOX2 expression resulted in a dramatic reduction in tumor volume (Fig. 2C).

The increase/decrease in tumor volume could either be a reflection of enhanced/inhibited cell proliferation or a manifestation of inhibited/enhanced apoptosis. To further elucidate the mechanism by which SOX2 promotes mammary tumor growth, serial xenograft tumor sections were stained with Ki-67 and TUNEL, markers for cellular proliferation and apoptosis, respectively. As shown in Fig. 2D (upper panel), compared with the control, tumors with SOX2 overexpression showed more Ki-67-positive nuclei, whereas tumors with SOX2 knockdown exhibited significantly fewer Ki-67-positive nuclei. However, the apoptosis assay revealed no statistically significant difference in the percentage of TUNEL-positive cells between the control and experimental groups (Fig. 2D, lower panel), suggesting that SOX2 was able to promote cell proliferation and tumorigenesis in xenograft models. Taken together, these experiments indicate that SOX2 contributed to breast cancer cell proliferation and tumorigenic properties both in vitro and in vivo.

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profile in SOX2-overexpressing MCF-7 cells using Human Genome U133 Plus 2.0 Affymetrix Gene Chip. The microarray analysis identified 145 up-regulated genes and 41 down-regulated genes. The data were analyzed, and genes were classified into cellular signaling pathways that are known to be implicated in cell proliferation and differentiation with the Gene Ontology classification system using DAVID software (supplemental Fig. S1A). These alleles were all up-regulated by SOX2. The microarray results were validated by quantitative RT-PCR analysis of 7 genes selected to represent different levels of induction, including 4 up-regulated genes (CCND1, FGFR2, SMARCA1, and CXCL10) and 3 down-regulated genes (CSTA, MME, and ZNF407) (supplemental Fig. S1B).

Identification of Cyclin D1 as a Downstream Target for SOX2—Among these SOX2-regulated genes, 4 (FGFR2, RhoB, CCND1, and RET), all of which were up-regulated by SOX2, are reported to be related to cell growth and proliferation. To further dissect the signaling pathway leading to SOX2-mediated cell proliferation and tumorigenesis, gain-of-function and loss-of-function experiments were performed in MCF-7 cells in which the expression of SOX2 was silenced while the 4 genes were ectopically individually expressed (gain-of-function) or where SOX2 was overexpressed while the expression of the 4 genes was individually silenced (loss-of-function). The proliferation of MCF-7 cells under these experimental conditions was measured by BrdU incorporation assays. As shown in Fig. 3A, both the gain-of-function and loss-of-function experiments revealed that, while FGFR2, RHOB, and RET had marginal effects on SOX2-promoted cell proliferation, the most profound effect was found with CCND1, indicating that CCND1 is an important downstream target in mediating the cell proliferation activity of SOX2. The expression of
Next we investigated the regulation of endogenous cyclin D1 protein expression by SOX2 in MCF-7 cells. As shown in Fig. 3C, while either overexpression or knockdown of SOX2 had little effect on the expression of other cell cycle-regulated genes including cyclin D3, cyclin E, p21CIP1, and p27KIP1, the expression of cyclin D1 protein was elevated in cells overexpressing SOX2 and inhibited in cells with SOX2 knockdown. These results further support the argument that CCND1 gene is a downstream target for SOX2 in breast cancer cells.

To further explore the role of cyclin D1 in mediating SOX2-promoted proliferation of breast cancer cells, we tested whether overexpression of cyclin D1 could alleviate the effect of G0/G1 accumulation under SOX2 knockdown. For this purpose, MCF-7 cells were co-transfected with SOX2 siRNA and cyclin D1 expression construct or with SOX2 siRNA plus an empty vector as control. FACS analysis revealed that cyclin D1 transfection in cells with SOX2 knockdown resulted in a significant increase in the percentage of cells in the S+G2/M phases (Fig. 4A, upper panel). On the other hand, even with SOX2 overexpression, knockdown of cyclin D1 expression was associated with a failure in the G1/S transition in MCF-7 cells (Fig. 4A, lower panel). The expression of SOX2, cyclin D1, and other cell cycle-related genes under different experimental conditions is shown in Fig. 4B. These results strongly favor the idea that cyclin D1 is a critical downstream mediator of SOX2 in promoting the G1/S transition and cell proliferation.

**Correlation of SOX2 and Cyclin D1 Expression in Breast Tumor Samples**—As stated before, SOX2 is up-regulated in several types of cancers including pancreatic intraepithelial neoplasia, prostate cancer, gastric carcinoma, and breast cancer (7–13). Analogously, CCND1 amplification/overexpression has been documented in ~50% of breast carcinomas (27–29). In light of our observation that SOX2 is capable of upregulating CCND1 expression, it was logical to postulate that the expression of SOX2 is correlated with the expression of CCND1 in primary breast carcinomas. To validate this hypothesis, the expression of CCND1 and SOX2 mRNAs was analyzed by real time RT-PCR in mammary tissues. As shown in Fig. 4C, statistical analysis found a Spearman correlation coefficient of 0.725 (p < 0.0001) between the expression of CCND1 and SOX2 mRNAs in the 26 breast carcinoma samples tested, whereas there was no correlation between CCND1 and SOX2 mRNA expression in 19 normal mammary tissue samples. These experiments further substantiate a functional link between SOX2 and cyclin D1 in breast cancer.

**Transcriptional Regulation of CCND1 by SOX2**—Although cyclin D1 overexpression is found in ~50% of human breast cancers, CCND1 gene amplification accounts for only 10% of the proteins was monitored by Western blotting (Fig. 3B). Similar results were also observed in one of basal-like breast cancer cell lines, MDA-MB-231 (data not shown).

**FIGURE 2. Tumorigenic properties of SOX2.** A, MCF-7 cells were treated under different experimental conditions and were collected for cell cycle profile analysis by flow cytometry. The percentage of cells in G0/G1, S, and G2/M phases is shown (upper panel). Serum-starved MCF-7 cells were transfected with indicated constructs. Twenty-four hours after transfection, cells were released by culturing in medium with 10% fetal bovine serum for another 16 h and were then collected for cell cycle profile analysis by flow cytometry. The percentage of cells in G0/G1, S, and G2/M phases is shown (lower panel). B, MCF-7 cells were infected with retroviruses and assayed for anchorage-independent growth in soft agar by colony formation. Data are means ± S.D. from triplicate experiments. The expression of SOX2 in MCF-7 cells infected with Retro-GFP, Retro-SOX2, and Retro-SOX2-RNAi was examined by Western blotting analysis. C, MCF-7 cells were infected with Retro-GFP, Retro-SOX2, Retro-SOX2-RNAi or mock, cultured for 48 h, and then injected into the mammary fat pad of female athymic nude mice (n = 6 for each experimental condition). Tumors were measured weekly with vernier calipers. The growth curve of implanted tumor cells and representative images of tumor-bearing mice and their tumors are shown. Each time point represents the mean tumor volume ± S.D. D, immunohistochemical staining for TUNEL and Ki-67 in MCF-7 tumor xenografts.

**FIGURE 3. Identification of genes that are responsible for the cell proliferative effect of SOX2.** A, effect of cyclin D1, FGFR2, RET, and RhoB on SOX2-mediated cell proliferation. MCF-7 cells were transfected with the indicated constructs, and cell proliferation was measured by BrdU incorporation. Data are means ± S.D. from triplicate experiments. B, expression of indicated proteins was examined by Western blotting. C, expression patterns of various cell cycle regulatory proteins upon overexpression and knockdown of SOX2. MCF-7 cells were transfected with an empty vector, SOX2 expression construct, control siRNA (con siRNA), or SOX2 siRNA. Total proteins were extracted and examined by Western blotting analysis using antibodies against the indicated proteins.

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To confirm the specificity of SOX2 in the activation of the CCND1 promoter, we mapped the cis-element(s) within the CCND1 promoter that is/are responsible for SOX2 transactivation. A series of 5’ truncated CCND1 promoter-luciferase constructs were transfected into MCF-7 cells with or without co-expression of SOX2, and then luciferase activity was measured (Fig. 5B). The results indicated that a −186 bp to −106 bp deletion resulted in ~2-fold decrease in SOX2-stimulated CCND1 promoter activity, and further deletion to −53 bp led to a complete loss of SOX2-mediated transcriptional activation of the CCND1 promoter. We conclude that the region between nucleotides −186 to −53 in CCND1 promoter harbors essential element(s) responsible for mediating SOX2 transcriptional activity.

Examination of the −186 to −53 region of the CCND1 promoter revealed a sequence CTTTGAT at position −81 to −75, which is compatible with the proposed SOX2 canonical binding site (C(T/A)TTG(T/A)(T/A)) (2, 3). Indeed, deletion mutation of this sequence resulted in a significant reduction in SOX2-stimulated CCND1 gene transcription (Fig. 5C). To determine if SOX2 is indeed associated with this region in vivo, we performed ChIP assays to examine the recruitment of SOX2 on the CCND1 promoter. As shown in Fig. 5C (right panel), SOX2 was found to bind to the proximal region of the CCND1 promoter (−86 to +234 bp), and the binding was specific since negligible binding was detected in the genomic sequence 2.5-kb upstream of the CCND1 gene transcription start site or with a control antiserum. This result suggests that SOX2 indeed physically associates with the CCND1 promoter.

To understand the molecular details involved in the transcriptional regulation of the CCND1 gene by SOX2, we next analyzed the functional domain(s) of SOX2 implicated in the transactivation activity of CCND1. For this purpose, we generated SOX2 mutants in which the HMG domain, the serine-rich domain, and the C terminus were individually or in combination deleted (Fig. 5D). A FLAG tag was added to the N terminus of the deletion constructs to monitor the expression of these SOX2 mutants by Western blotting with an anti-FLAG antibody (Fig. 5D). The experiment revealed that, while SOX2 that lacked the C terminus and/or the serine-rich domain preserved transactivation activity of SOX2, deletion of the HMG domain was associated with a diminished effect of SOX2 on CCND1 promoter activation (Fig. 5D), indicating that the HMG domain is essential for the transactivation activity of SOX2.

The Physical and Functional Interaction between SOX2 and β-Catenin—Because CCND1 is a known target for the Wnt signaling pathway (31), we next investigated whether there is any functional connection between the Wnt pathway and SOX2 in transcriptional activation of the CCND1 gene. For this purpose, the SOX2 expression vector and the β-catenin expression vector were transfected either alone or in combination into MCF-7 cells, and the effect of these protein expressions on CCND1 promoter activity was measured. As shown in Fig. 6A, expression of either SOX2 or β-catenin alone was associated with an elevated CCND1 promoter activity, and co-transfection of SOX2 and β-catenin resulted in a synergistic effect on CCND1 promoter activity. In addition, β-catenin-silenced cells, not only was the β-catenin-enhanced activation of the CCND1 promoter diminished, but the synergy between SOX2 and β-catenin no longer existed. Moreover, when the expression of SOX2 was knocked down, β-catenin-
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stimulated reporter activity was also significantly reduced. These results suggest that SOX2 is functionally connected to β-catenin in the activation of the CCND1 promoter.

The synergistic effect of SOX2 and β-catenin on the transcriptional activation of the CCND1 promoter could be a reflection of signal cross-talk between the Wnt pathway and the SOX2 pathway. Alternatively, the functional connection between SOX2 and β-catenin could be a result of physical interaction between these two proteins. As transcription factors, all SOX proteins, including SOX2, do not stably bind to DNA on their own. Rather, SOX proteins exert their transcription regulation function through interacting with partner proteins (2, 3). To understand the molecular basis behind the synergistic activity of SOX2 and β-catenin and to determine a transcription partner for SOX2 in transactivating the CCND1 gene in breast cancer cells, SOX2 and β-catenin were either transfected alone or co-transfected into MCF-7 cells with overexpression or knockdown of TCF-4, and CCND1 promoter activity under these experimental conditions was measured. We reasoned that if the synergistic activation of the CCND1 promoter by SOX2 and β-catenin in breast cancer cells was a result of Wnt and SOX2 signal cross-talk, then the synergistic effect could be affected by the level of TCF-4 expression. As shown in Fig. 6B, either overexpression or knockdown of TCF-4 expression affected CCND1 promoter activity when there was no ectopical expression of SOX2 or β-catenin. Further, as expected, when β-catenin was overexpressed, overexpression or knockdown of TCF-4 affected β-catenin-stimulated CCND1 promoter activity. Interestingly, however, TCF-4 overexpression or knockdown did not affect SOX2-stimulated CCND1 promoter activity in cells with SOX2 overexpression, and neither did the synergistic effect of SOX2 and β-catenin. These results argue against the idea that

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**FIGURE 5. Transcription regulation of CCND1 by SOX2.** A, SOX2 dose-dependently transactivates CCND1 promoters. MCF-7 (left) and HEK293 cells (right) were co-transfected with CCND1-Luc reporter, a Renilla construct and different amounts of SOX2 expression construct (0.05, 0.2, and 0.8 μg/well). Twenty-four hours after transfection, cells were harvested and assayed for luciferase activity. Data are means ± S.D. from triplicate experiments. B, mapping of cis-acting elements in the CCND1 promoter, which is responsible for SOX2 transactivation. Shown is a schematic representation of the 5′-truncated CCND1 promoter luciferase constructs and their activities in MCF-7 cells. Cells were transiently transfected with the indicated reporter plasmids with or without co-transfection of a SOX2 expression construct (0.8 μg/well). Data are means ± S.D. from triplicate experiments. C, schematic representation of CCND1 reporter construct and a mutant reporter construct in which the sequence between nucleotides −81 and −75 is deleted. MCF-7 cells were transfected with the indicated reporter plasmids with or without co-transfection of a SOX2 expression construct (0.8 μg/well). Twenty-four hours after transfection, cells were collected for assay of luciferase activity. Data are means ± S.D. from triplicate experiments. Right panel shows the recruitment of SOX2 on the CCND1 promoter. Soluble chromatin from MCF-7 cells was immunoprecipitated with anti-SOX2 or a control rabbit normal IgG. The final DNA extractions were amplified by PCR using primers that cover the upstream control region (lower panel) or the proximal promoter region of the CCND1 gene (upper panel). D, functional domain(s) of SOX2 implicated in its transactivation activity of CCND1. Shown are the schematic diagram of SOX2 and SOX2 deletion mutants (0.8 μg/well) (left); the expression ofFLAG-SOX2 fusion protein and FLAG-SOX2 deletion fusion proteins in transfected MCF-7 cells (middle); and the transactivation activity of SOX2 deletion mutants on the CCND1 promoter (right). Data are means ± S.D. from triplicate experiments.
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DISCUSSION

It is widely accepted that many of the molecular pathways that underlie carcinogenesis represent aberrations in the normal processes that are vital for embryogenesis. In many cases, cancer seems to be caused by the deregulation of transcription factors that affect cell fate and proliferation.

The SOX genes encode a group of transcription factors that are expressed in various phases of embryonic development in a manner linked to cell-type specification and cellular differentiation (2, 3). Like many other developmental regulatory factors, the improper functioning of SOX genes has been implicated in a number of severe clinical disorders including human cancers; almost all of the SOX family genes have been found to be deregulated in a diverse range of solid tumors (7). However, except for a report of a causal relationship between SOX3 overexpression and cellular transformation (32), the links to cancers described for other SOX proteins are mostly correlative studies. In fact, the oncogenic behaviors of SOX proteins at the cellular and molecular levels are largely unknown.

Overexpression of SOX2 has been documented in several types of solid tumors (7–13). Through expression analysis of a panel of breast cancer cell lines and primary tumors, we showed that SOX2 is also overexpressed in human breast cancer tissue and cell lines, and its levels are correlated with tumor grade. Both gain-of-function and loss-of-function experiments indicate that SOX2 contributes to breast cancer cell proliferation and tumorigenic properties in vitro and in vivo. At the cellular level, it appears that SOX2 promotes cell cycle progression by facilitating the G1 to S transition.

SOX proteins, including SOX2, are HMG domain-containing transcription factors that exert their biological activities through transcriptional regulation of their downstream target genes (2, 3). Therefore, target identification is essential for the understanding of the oncogenic potential of SOX proteins. Extensive efforts have been invested in studying embryonic stem cells and neural- and lens-related organogenesis where θ-crystallin, fibroblast growth factor 4 (fgf4), undifferentiated embryonic cell transcription factor (LTF1), N-cadherin, and Oct3/4 have been identified as the transcriptional targets of SOX2 (3, 33, 34), but otherwise no strong functional themes have emerged for SOX2 in carcinogenesis. In the current study, microarray analysis and categorization identified the potential SOX2 target genes involved in cell proliferation and tumorigenesis in breast cancer cells. Among those genes, CCND1 has been shown to be transcriptionally regulated by SOX2 and to mediate the effect of SOX2 in promoting cell proliferation and tumorigenesis. The protein product of CCND1, cyclin D1, is one of the critical regulators functioning in the G1/S transition of the cell cycle. Its identification as the downstream target of SOX2 and the ongoing efforts to establish a role for SOX2 in breast cancer cell proliferation and tumorigenesis will provide new insights into the understanding of the molecular mechanism involved in breast carcinogenesis.
CCND1 amplification and/or overexpression is one of the most prevalent alterations in breast carcinomas, occurring in \(\sim 50\%\) of cases (28, 29). In fact, CCND1 is a well recognized oncogene that is also amplified and/or overexpressed in a substantial proportion of other human cancers including parathyroid adenoma, colon cancer, lymphoma, melanoma, and prostate cancer (35). CCND1 oncogenic activity in the mammary gland has been confirmed in genetically modified mouse models where CCND1 overexpression leads to the development of mammary carcinoma (36), while CCND1 ablation results in mice resistant to cancer induced by several oncogenes (37). In addition, CCND1-null mice show defective postnatal mammary development as revealed by a lack of proliferation of alveolar epithelial cells in response to the sex steroid milieu of pregnancy (38, 39).

Another approach to understanding the oncogenic potential of SOX proteins is to decipher their molecular actions, that is, the molecular details involved in their transcription regulation activities. SOX transcription factors bind sequence specifically to DNA by means of an HMG domain (2, 3). However, the binding of SOX proteins to DNA is weaker than for most of the classical transcription factors. As a result, SOX proteins usually require partners to form stable protein-DNA complexes (2, 3). Furthermore, the same SOX protein might regulate a different set of genes and act in a cell-specific fashion by pairing with different cell-specific partner factors (2, 3). In this report, we demonstrated that SOX2 acted synergistically with \(\beta\)-catenin in transcription regulation of cyclin D1 expression, and SOX2 and \(\beta\)-catenin were physically and functionally associated, suggesting that \(\beta\)-catenin might be a transcription partner for SOX2 in mammary carcinomas.

There is abundant evidence that SOX proteins might affect the Wnt/\(\beta\)-catenin pathway. SOX proteins can either antagonize or facilitate \(\beta\)-catenin/TCF-mediated transcription in the context of different SOX species (40–42). In addition to partnership with SOX2 in breast cancer cells, \(\beta\)-catenin has been identified as the transcription partner of XSox17 and XSox3 (43) in Xenopus and Sox17 in mouse embryos (40). It is conceivable that the interaction of \(\beta\)-catenin with different SOX proteins may explain, in part, how the Wnt signaling pathway can elicit diverse transcriptional responses in different cellular contexts.

It remains to be seen if SOX2 and the partnership between SOX2 and \(\beta\)-catenin are physiologically important in normal mammary gland development. In addition, in light of the function of SOX2 in the maintenance of the pluripotent properties of stem cells, it will be intriguing to examine the activity of SOX2 in the tumorigenic population of breast cancer cells. Also relevant to our study, it will be interesting to investigate the mechanism for deregulation of SOX2 expression in breast carcinomas. A growing body of evidence highlights the role of cancer-linked hypomethylation in gene activation and carcinogenesis (17, 44). The greatest Cpg content in the SOX2 gene is centered over the translational initiation codon and immediately upstream of the transcriptional start site. In human embryonic stem cells, the SOX2 gene promoter is not methylated (45), suggesting that epigenetics might play a role in SOX2-related carcinogenesis.

In summary, we have reported here the overexpression of SOX2 in breast carcinomas. We demonstrated that SOX2 is able to promote cell proliferation and tumorigenesis of breast cancer, possibly through facilitating the G1/S transition of the cell cycle. We described the identification of the CCND1 gene as a downstream target and \(\beta\)-catenin as the transcription partner in mediating the tumorigenic effect of SOX2 in breast cancer cells. These results will be important in understanding the oncogenic potential of SOX2. Our experiments also revealed a novel function for \(\beta\)-catenin in breast cancer carcinogenesis.

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