

ORIGINAL ARTICLE

SOX2 promotes dedifferentiation and imparts stem cell-like features to pancreatic cancer cells

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SOX2 (Sex-determining region Y (SRY)-Box2) has important functions during embryonic development and is involved in cancer stem cell (CSC) maintenance, in which it impairs cell growth and tumorigenicity. However, the function of SOX2 in pancreatic cancer cells is unclear. The objective of this study was to analyze SOX2 expression in human pancreatic tumors and determine the role of SOX2 in pancreatic cancer cells regulating CSC properties. In this report, we show that SOX2 is not expressed in normal pancreatic acinar or ductal cells. However, ectopic expression of SOX2 is observed in 19.3% of human pancreatic tumors. SOX2 knockdown in pancreatic cancer cells results in cell growth inhibition via cell cycle arrest associated with p21^{Cip1} and p27^{Kip1} induction, whereas SOX2 overexpression promotes S-phase entry and cell proliferation associated with cyclin D3 induction. SOX2 expression is associated with increased levels of the pancreatic CSC markers ALDH1, ESA and CD44. Importantly, we show that SOX2 is enriched in the ESA⁺/CD44⁺ CSC population from two different patient samples. Moreover, we show that SOX2 directly binds to the Snail, Slug and Twist promoters, leading to a loss of E-Cadherin and ZO-1 expression. Taken together, our findings show that SOX2 is aberrantly expressed in pancreatic cancer and contributes to cell proliferation and stemness/dedifferentiation through the regulation of a set of genes controlling G1/S transition and epithelial-to-mesenchymal transition (EMT) phenotype, suggesting that targeting SOX2-positive cancer cells could be a promising therapeutic strategy.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most chemoresistant tumors, with a survival rate of <5%.¹ PDAC is characterized by a heterogeneous population of cancer cells surrounded by stroma and a distinct subpopulation of cancer stem cells (CSCs). Although representing only a small proportion of the tumor, CSCs are believed to constitute a reservoir of cancer-initiating cells, also called tumor-propagating cells.² Pancreatic CSCs were first characterized by Li *et al.*³ and were shown to be not only highly tumorigenic but also to harbor the ability to self-renew and produce differentiated progeny that reflected the heterogeneity of the patient's primary tumor. Further studies from different groups have linked CSCs to aggressive growth, metastasis and resistance to conventional therapy,^{4–8} suggesting that CSCs have a pivotal role in PDAC biology and therapy. Therefore, understanding the mechanism(s) underlying CSC maintenance and regulation may lead to novel therapeutic strategies specifically targeting this subpopulation of cells.

SOX2 (Sex-determining region Y (SRY)-Box2) is a member of the SOX family of transcription factors responsible for coordinating disparate functions such as maintaining stem cell properties and differentiation restriction.^{9,10} In particular, SOX2 is involved in the

regulation of stem cell fate during embryonic development and its expression levels need to be tightly regulated to ensure normal embryonic development.^{11,12} SOX2 depletion by RNA interference promotes embryonic stem cell differentiation into multiple cell types.¹³ Seminal work by Takahashi *et al.*^{14,15} showed that SOX2 is a key factor capable of inducing pluripotency in somatic cells along with KLF4, Oct3/4 and c-Myc. SOX2 is also one of the four transcription factors capable of reprogramming human somatic cells to pluripotent stem cells with characteristics of embryonic stem cells.¹⁶ In fact, SOX2 and Oct3/4 together are sufficient to generate pluripotent stem cell from human cord blood cells.¹⁷ These data suggest that SOX2 is a key factor conferring 'stemness' characteristics and maintaining stem cell identity. The stemness program can also have an important role in cancer because self-renewal is a hallmark for cancer-initiating cells/tumor-propagating cells. Indeed, recent studies have shown SOX2 deregulation in different human cancer types.^{18–24} Several studies present evidence for the presence of SOX2 in stem cell-like progenitor cells in the adult human pancreas,^{25,26} but the function of SOX2 in pancreatic cancer remains unknown.

CSCs have also been linked to epithelial-to-mesenchymal transition (EMT) in various solid tumors including PDAC. Cancer

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cells that undergo EMT lose epithelial polarity and acquire invasive properties and stem cell-like features, which are believed to prelude metastasis. Indeed, circulating pancreatic cancer cells underwent EMT prior to dissemination in a genetically engineered mouse model, as identified by expression of mesenchymal markers.²⁷ Interestingly, SOX2 has been linked to EMT in colorectal cancer²⁸ and SOX2 knockdown reduces the expression levels of *Snail*, *ZEB1*, *ZEB2* and *TGB β 2* genes, which are known to drive EMT.^{28,29} Therefore, SOX2 could be a key protein mediating properties shared by CSCs and EMT.

Currently, very little is known regarding SOX2 expression in PDAC and its role in carcinogenesis or progression of carcinogenesis. Sanada *et al.*³⁰ performed immunohistochemical analysis on 14 cases of PDAC, and observed weak expression of SOX2 in PanIN-3 lesions and relatively high and frequent expression in invasive and poorly differentiated PDAC. It was therefore suggested that SOX2 might be involved in invasion and metastasis, and not in the early progression of the disease. Here, we undertake a more detailed analysis of SOX2 expression and its clinical relevance in a cohort of pancreatic cancer tissue microarray (TMA) samples, and characterize the role of SOX2 in regulating cell proliferation, stemness and the expression of genes involved in these processes.

RESULTS

SOX2 is aberrantly expressed in primary PDAC and cancer cell lines. To investigate the expression and distribution of SOX2 in PDAC, 10 TMAs containing 349 patient samples, of which 140 were unselected for treatment and 209 have been treated with gemcitabine were stained for SOX2 expression. Notably, we routinely observed the staining of nuclei within nerve bundles, which is consistent with the known expression of SOX2 in neurons (Figure 1). Of these 349 cases, 454 TMA cores representing 217 cases were evaluable. Of these 217 cases, 175 (80.7%) were negative and 42 (19.3%) were positive for SOX2 protein expression (Table 1). Although SOX2 was not observed in normal pancreatic acinar or ductal cells, we did observe SOX2 nuclear staining in premalignant PanIN lesions and PDAC of varying grades (Figure 1 and Table 1). As PDAC is a very heterogeneous disease, we observed SOX2 staining in areas of well-differentiated PDAC, but these tumors also contained areas of either moderate or poorly differentiated cancer. Although no statistically significant correlation of SOX2 expression was seen with tumor grade, age of onset or other clinical features, we did note that SOX2 expression was only observed in high-grade cancer (Figure 1 and Table 1). Additionally, we did note that six adenocarcinoma cases with

areas of adenosquamous differentiation stained strong positive for SOX2, as did the only anaplastic tumor represented on the TMA.

We next assessed SOX2 expression in a panel of pancreatic cancer cell lines by qRT-PCR and immunoblotting. Compared with the HPDE non-transformed epithelial cell line with no significant SOX2 expression, SOX2 was overexpressed in several pancreatic cancer cell lines (Figures 2a and b). The highest level of SOX2 was detected in L3.6, followed by CFPAC and BxPC3. In addition, we found SOX2 expression in 5 of 14 primary cell lines (unpublished observation). As expected, SOX2 is nuclear localized in these cells as demonstrated by immunoblotting of cytosolic/nuclear fractions (Figure 1b) and immunofluorescence (Figure 2c). Taken together, these data suggest that SOX2 is ectopically expressed in pancreatic cancer and can be found in high-grade diseases.

SOX2 regulates cell growth in pancreatic cancer cells via downregulating p21^{Cip1} and p27^{Kip1}

To assess the role of SOX2 in pancreatic cancer cell proliferation, we performed MTT assays on control and SOX2-suppressed cells. SOX2 was efficiently reduced in all four cell lines tested as confirmed using immunoblot and qRT-PCR (Figure 3a and data not shown). Significantly, the depletion of SOX2 reduced cell proliferation compared with shControl cells (Figure 3b). No difference in senescence-associated β -galactosidase staining was seen between shControl and shSOX2 cells (Supplementary Figure S1), indicating that senescence induction is not the cause of growth inhibition. We therefore analyzed whether changes in cell proliferation were

Table 1. SOX2 Staining in TMA

	SOX2 staining	
	Negative	Positive
<i>Patients</i>	175 (80.7%)	42 (19.3%)
<i>PDAC subtype</i>		
Adenocarcinoma	175 (80.7%)	35 (16.1%)
Adenosquamous	0	6 (2.8%)
Undifferentiated (anaplastic)	0	1 (0.4%)
<i>Histological grade</i>		
Well differentiated	14 (6.5%)	0
Moderately differentiated	78 (35.9%)	28 (12.9%)
Poorly/undifferentiated	83 (38.2%)	14 (6.55%)

Abbreviations: PDAC, pancreatic ductal adenocarcinoma; SOX, Sex-determining region Y (SRY)-Box2; TMA, tissue microarray.

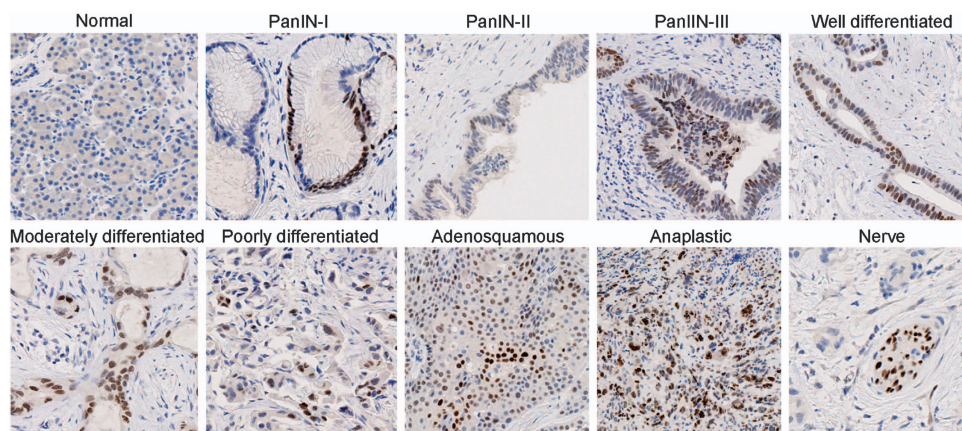


Figure 1. Expression of SOX2 in human pancreatic tissues. Representative immunohistochemistry images for SOX2 staining in human pancreatic cancer tissues of various histological and differentiation status as specified.

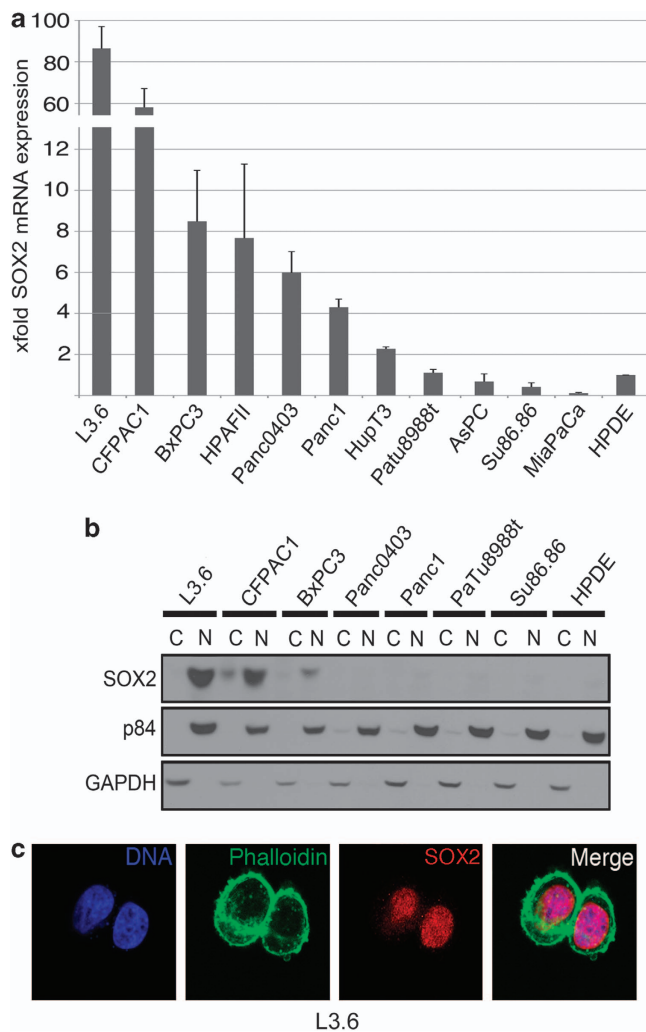


Figure 2. SOX2 expression in human pancreatic cancer cell lines. (a) Quantitative RT-PCR showing SOX2 expression in different pancreatic cancer cell lines and immortalized HPDE. (b) Cytosol (C) and nuclear (N) extracts were prepared from the indicated cell lines and immunoblotted for SOX2. (c) L3.6 cells were stained with Hoechst to detect DNA, phalloidin to detect F-actin and SOX2.

due to a perturbation of cell cycle or increased apoptosis. We found that SOX2 suppression did not induce apoptosis (Supplementary Figure S2) but did cause a decrease of cells in the S-phase and an increase of those in the G0/G1 phase (Figure 3c), which prompted us to examine the expression of proteins that regulate the cell cycle. Significantly, we observed increased mRNA as well as protein expression levels for the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ (Figures 3d and e and data not shown). We did not observe an increase in p16 or p57 (data not shown). To determine whether SOX2 could directly have an impact on the transcription of these two genes, we examined SOX2 binding at the $p21^{Cip1}$ and $p27^{Kip1}$ promoters by chromatin immunoprecipitation (ChIP) in L3.6 cells. Interestingly, we detected SOX2 binding at both the $p21^{Cip1}$ and $p27^{Kip1}$ promoters or enhancers (Figure 3f). Taken together, these data suggest that SOX2 can regulate cell cycle control in pancreatic cancer cells through the repression of $p21^{Cip1}$ and $p27^{Kip1}$ gene expression.

SOX2 is expressed in pancreatic CSCs

Given its key role in maintaining stem cell properties, we next evaluated the role of SOX2 in self-renewal capacity of CSCs using

the sphere-formation assay.⁵ Interestingly, we could successfully obtain spheres only in those cell lines that express the highest levels of SOX2 (L3.6, CFPAC and BxPC3), whereas other cell lines formed only small irregular aggregates or stayed as single cells that died after 2–3 days in the sphere-culture medium (Figure 4a and data not shown). Importantly, spheres formed by L3.6, CFPAC and BxPC3 could be serially passaged to form secondary (also referred as P2) and tertiary (P3) spheres (data not shown).

As the sphere-forming process is intended to enrich the potential CSC subpopulations, we characterized spheres for the expression of pancreatic CSCs markers. Spheres and control adherent cells were analyzed for the expression of previously described CSC markers CD44, ALDH1, ESA and Nestin.⁵ We found that sphere-forming cells are highly enriched in the expression of these CSC markers (Figures 4b–e). Cell quantification using flow cytometry indicated that $85 \pm 5\%$ of L3.6 adherent cells are positive for CD44, whereas $96 \pm 3\%$ of them are positive after sphere formation. Similarly, $12 \pm 2\%$ of adherent cells were positive for ALDH1 and $30 \pm 3\%$ for ESA, and this percentage increased in sphere cells to 80 ± 5 and $50 \pm 4\%$, respectively. These data indicate that pancreatic cancer cell lines harboring high levels of SOX2 contain cells with stem cell-like properties that can be enriched following sphere formation.

As SOX2 expression appeared to predict sphere-forming capacity, we next analyzed the expression of SOX2 in the spheres. As shown in Figure 4f, SOX2 protein could be visualized in the nucleus of L3.6 sphere-forming cells. Moreover, the percentage of SOX2-positive cells increased during the sphere-formation process (Figures 4g and h). Additionally, we found strong coexpression of CSC markers with SOX2 expression in sphere-forming cells (Figure 5a), and the expression of SOX2 and these markers were lost following replating of the cells in normal growth medium on adherent culture dishes (Figures 5b and c). To determine whether SOX2 was similarly enriched in primary patient-derived CSCs, we examined the expression of SOX2 in the $CD44^+/ESA^+$ population obtained from two patient xenografts. As can be seen in Figure 5d, SOX2 expression was found in $> 50\%$ of the $CD44^+/ESA^+$ population. Taken together, these data indicate that SOX2 expression pattern changes according to the enrichment of pancreatic CSC and this is a reversible process.

SOX2 maintains self-renewal capacity of pancreatic CSCs

As sphere formation is considered a selection method that enriches CSC-like cells, and SOX2 expression increases in spheres, we hypothesized that SOX2 is not only a marker for CSCs but might also have an impact on CSC properties such as self-renewal. To analyze whether SOX2 is necessary to maintain stem cell-like properties, we performed sphere-formation assays in SOX2 knock-down cells. We found that SOX2 suppression in adherent cells prevents BxPC3 and L3.6 sphere formation (Figure 6a). In fact, all SOX2-suppressed cells stay as single cells without forming spheres (Figure 6b), lose CSC markers (Figure 6c) and ultimately undergo apoptosis over the 4–5-day time frame (Supplementary Figure S3).

To address the question of whether SOX2 is required for the self-renewal of pancreatic CSCs, we used lentivirus-mediated short-hairpin RNAs (shRNAs) to suppress SOX2 in primary spheres and analyzed their ability to form secondary spheres. Significantly, we observed a reduction in the number as well as the size of spheres following SOX2 knockdown (Figures 6d and e). Additionally, single cells derived from primary spheres were replated and evaluated for secondary sphere formation. Moreover, we found that SOX2 suppression dramatically impairs secondary sphere formation (Figure 6f) and has diminished the expression of CSC markers (Figure 6g), whereas $p21^{Cip1}$ and $p27^{Kip1}$ expression levels increased, leading to a reduction in cell growth in the cells forming spheres (Figure 6h). Altogether, these data indicate that SOX2 is necessary for sphere formation, maintenance of CSC marker expression and the self-renewal capacity of pancreatic CSCs.

SOX2 expression induces sphere-forming capacity and contributes to accelerated cell cycle progression

Given the essential role of SOX2 in sphere formation, we wanted to further test whether the overexpression of SOX2 could impart

sphere-forming capacity to cells that can only form small aggregates such as HeLa or cannot form spheres at all such as PaTu8988t. Using HeLa and PaTu8988t cells stably overexpressing SOX2 (Figure 7a), we found that SOX2 expression led to a

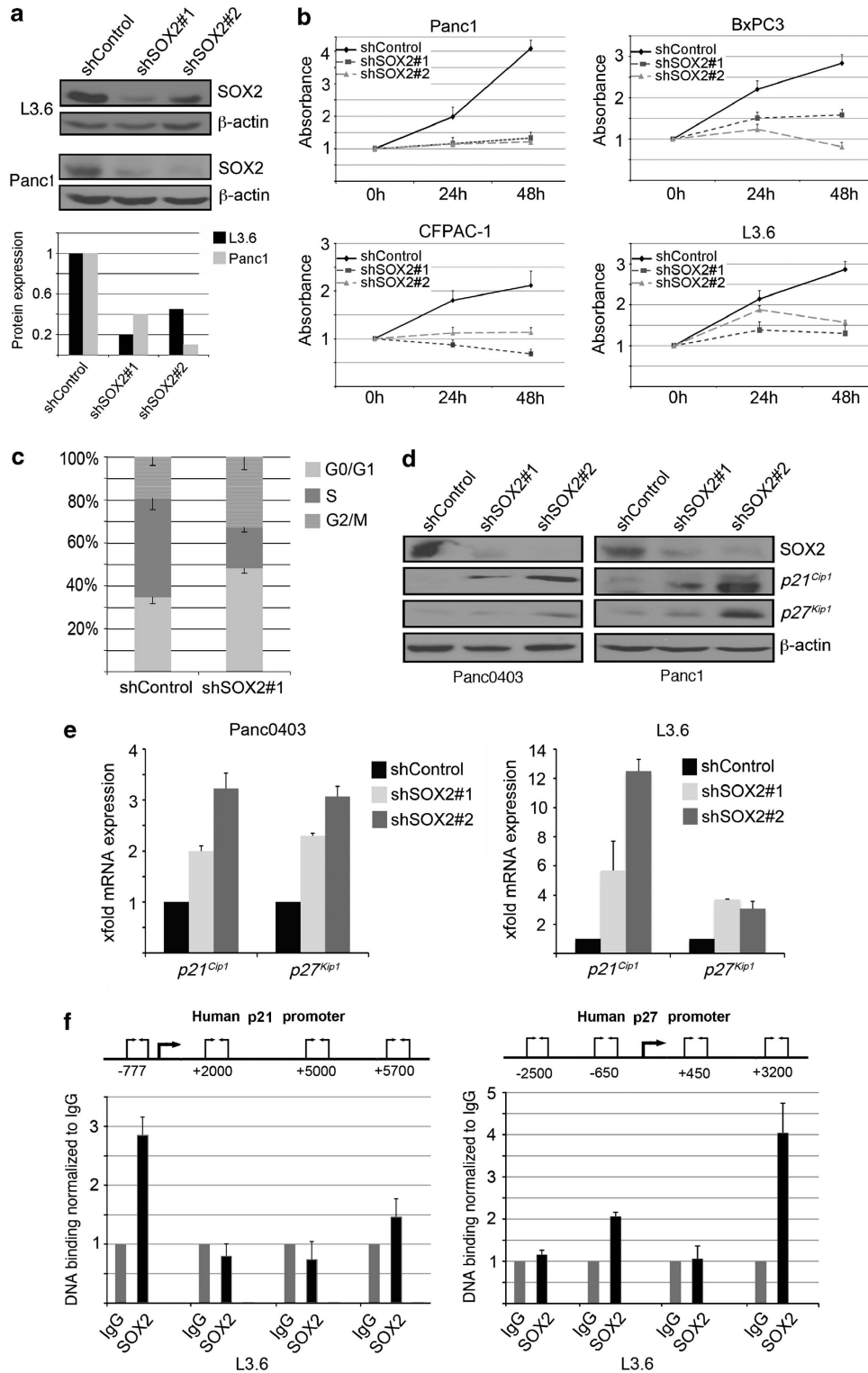


Figure 3. SOX2 regulates pancreatic cancer cell proliferation. **(a)** Immunoblot showing efficient SOX2 knockdown by Lentivirus-mediated shRNA in L3.6 and Panc1 cells (upper panel) and densitometry (lower panel). **(b)** Results of MTT assays showing effect of SOX2 knockdown on cell proliferation in the indicated pancreatic cancer cell lines. **(c)** Cell cycle analysis of L3.6 cells infected with Lenti-shControl and Lenti-shSOX2. **(d)** Immunoblot analysis of lysate from Panc1 and Panc0403 cells showing shSOX2-induced expression of *p21^{Cip1}* and *p27^{Kip1}*. **(e)** Quantitative RT-PCR showing *p21^{Cip1}* and *p27^{Kip1}* mRNA expressions in shControl and shSOX2 Pan0403 and L3.6 cells. **(f)** ChIP analysis showing SOX2 binding to specific regions on *p21^{Cip1}* and *p27^{Kip1}* promoter/enhancer regions in L3.6 cells.

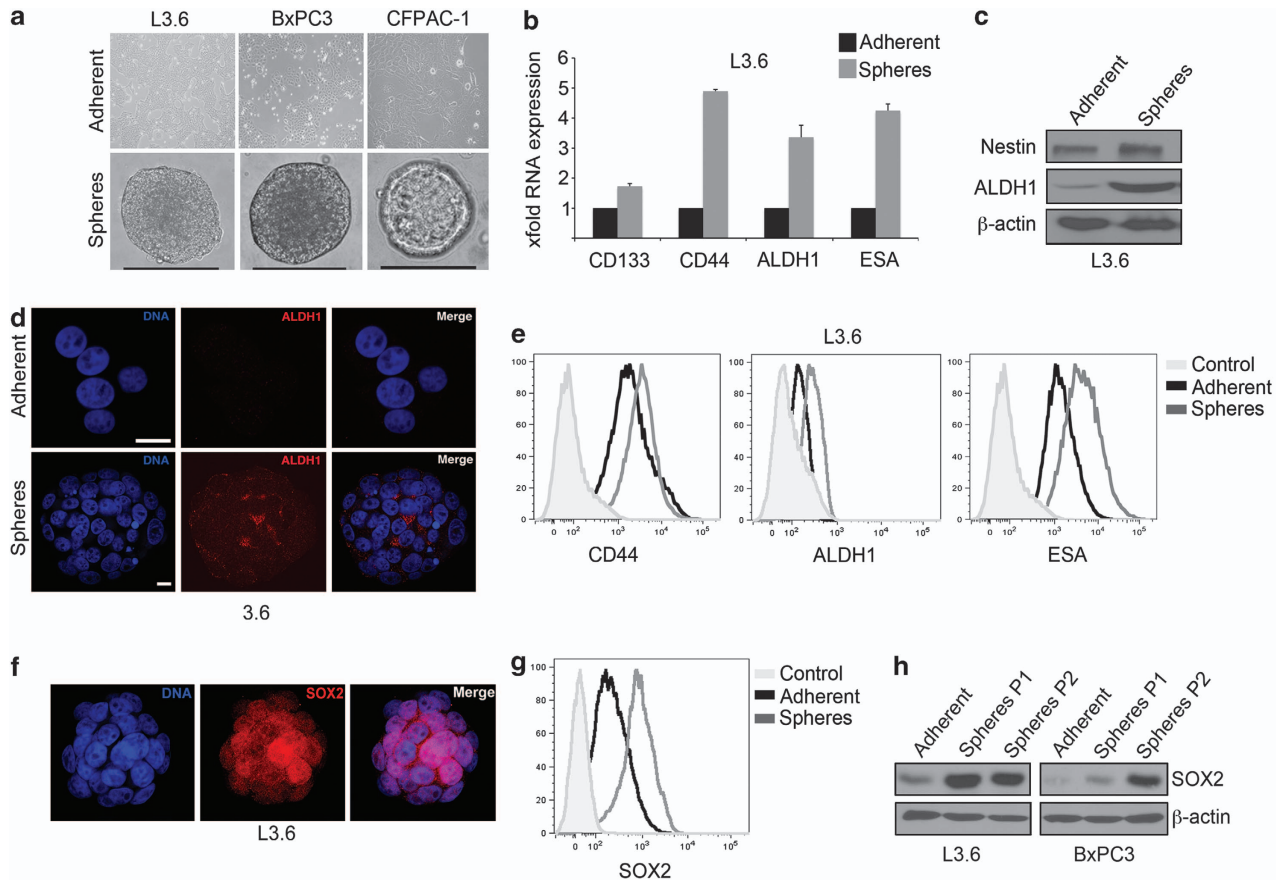


Figure 4. Characterization of CSCs in pancreatic cancer cell lines. **(a)** Bright-field microscopy images of adherent cells and corresponding spheres in L3.6, BxPC3 and CFPAC-1 cells; Scale bar 100 μ m. **(b)** Quantitative RT-PCR showing mRNA expression of CD133, CD44, ALDH1 and ESA in L3.6 cells (adherent versus spheres). **(c)** Immunoblot showing Nestin and ALDH1 protein expressions during L3.6 sphere formation. **(d)** Immunofluorescence staining and confocal imaging for ALDH1 in L3.6 adherent versus spheres; Scale bar 10 μ m. **(e)** Flow cytometry analysis for CD44, ALDH1 and ESA in L3.6 adherent cells and spheres. **(f,g)** Immunofluorescence and flow cytometry analyses showing SOX2 expression in L3.6 spheres after 7 days in culture. **(h)** Immunoblot showing increased SOX2 expression in L3.6 spheres relative to adherent cells.

significant increase in the number and size of spheres formed (Figures 7b and c). Moreover, these spheres demonstrated an increased mRNA expression of the CSC markers CD133, CD44 and ALDH1 (Figure 7d).

As SOX2 suppression induced *p21^{Cip1}* and *p27^{Kip1}* expression levels, we further tested the effect of SOX2 expression on cell proliferation. Interestingly, we did not observe cell cycle changes as a consequence of SOX2 overexpression (data not shown) in non-synchronized cells. However, using G1/S-phase-synchronized HeLa cells with double thymidine block, we observed that SOX2-overexpressing cells progressed significantly faster through the S-phase compared with the control cells (Figure 7e). At 2.5 h post release, 58 \pm 4% of control cells were in the S-phase compared with 66 \pm 5% for SOX2-overexpressing cells. After 5 h, 55 \pm 3% of cells overexpressing Sox2 had reached G2/M phase, whereas this number was 30 \pm 4% in the control cells (Figure 7e). These results demonstrate that SOX2 expression facilitates cell cycle progression in the bulk population. Interestingly, we found that SOX2 induced the expressions of *cyclin D3* and its complex partner *CDK6* (Figures 7f and g), which might contribute to faster cell cycle progression and confer a growth advantage to these cells. Furthermore, H3K4 trimethylation, a mark of active transcription localized along with SOX2 at the *cyclin D3* promoter (Figure 7h). As expected, we also observed more robust SOX2 binding to the *cyclin D3* promoter/enhancer in spheres compared with the adherent cells (Figure 7i). Consistent with SOX2-regulated expression, *cyclin D3* levels decrease gradually upon replating of the

sphere cells on adherent plates in regular media (Figures 7j–k). Taken together, these data indicate that SOX2 may facilitate cell cycle progression in pancreatic cancer cells via the regulation of cyclin D3 and CDK6 activation and *p21^{Cip1}/p27^{Kip1}* gene repression.

Overexpression of SOX2 induces dedifferentiation and EMT marker expression

Accumulating evidence has pointed to a causal relationship between CSCs and EMT in pancreatic tumors, in which EMT is suggested to have a role in the generation as well as maintenance of CSCs.³¹ To determine whether SOX2 could affect this important process, we next examined PaTu8988t cells stably overexpressing SOX2 for expression of epithelial markers. We found that SOX2 overexpression significantly reduced the expression of the epithelial markers E-cadherin and ZO-1 (Figures 8a and b). The repression of E-cadherin and ZO-1 gene expression during EMT involves several transcription factors including *Twist*, *Slug*, *Snail*, *ZEB1* and *ZEB2*. We therefore investigated their regulation by SOX2. Indeed, we observed an increased expression of *Twist*, *Snail* and *Slug*, but not *ZEB1* and *ZEB2*, in SOX2-overexpressing PaTu8988t cells compared with SOX2-depleted L3.6 cells (Figure 8C and data not shown). SOX2-induced Snail expression was further confirmed using immunoblot and immunofluorescence analyses (Figures 8d and e).

The close correlation of SOX2–Snail expression during EMT induction raised the possibility that Snail is a direct transcriptional

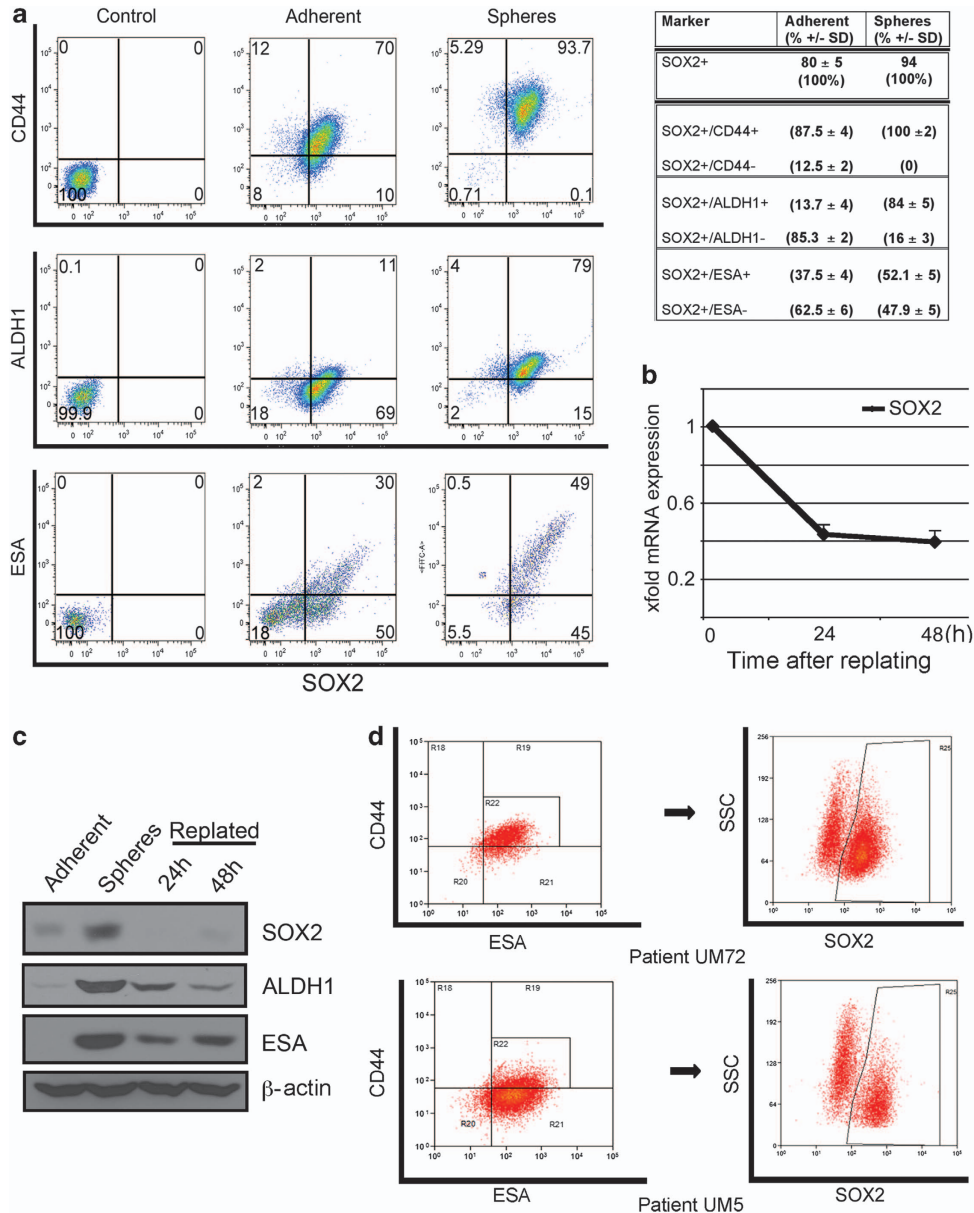


Figure 5. SOX2 expression in pancreatic cancer stem cells. **(a)** Flow cytometry analysis showing CD44, ALDH1, ESA and SOX2 expressions in L3.6 cells (adherent and spheres). **(b)** Quantitative RT-PCR showing decreased SOX2 expression in L3.6 sphere cells after replating and grown as monolayer culture. **(c)** Immunoblot for SOX2, ALDH1 and ESA in L3.6 sphere cells replated and grown under adherent conditions. **(d)** Flow cytometry analysis for SOX2 expression in the CD44⁺/ESA⁺ CSC population obtained from two different primary pancreatic cancer xenografts, UM72 and UM5.

target of SOX2. We therefore examined SOX2 loading onto the *Snail* promoter/enhancer by ChIP. Interestingly, we detected enriched SOX2 binding at both the promoter (especially at +250 after start site) and the 3'-enhancer regions. Concomitant binding of Tri-methylated H3K4 (H3K4me3) and RNA pol-II confirmed the active transcription of the locus (Figure 8f and Supplementary Figure S4A). Accordingly, we also observed an increased SOX2 binding to the *Snail* promoter/enhancer in L3.6 spheres compared with adherent cells (Figure 8g and data not shown). Increased SOX2 binding to *Slug* or *Twist* promoter and enhancer activity were also observed (Supplementary Figures S4B-C). As expected, when L3.6 sphere cells were replated and grown under adherent conditions, *Snail*, *Slug* and *Twist* expression levels were decreased (Figure 8h). Together, these data suggest that SOX2 can directly bind and regulate the expression of genes involved in EMT in pancreatic cancer cells.

Our data suggest that SOX2 overexpression drives cancer cell dedifferentiation from epithelial (E-Cadherin⁺ and ZO-1⁺) to an EMT-like phenotype, as reflected by increased *Snail*, *Twist* and *Slug* expression levels. To further understand whether SOX2 regulates only certain aspects of the dedifferentiation process or induces a full EMT phenotype, we examined additional progenitor markers of epithelial and mesenchymal lineages. We found that L3.6 sphere cells and SOX2-overexpressing PaTu8988t cells maintain the expression of progenitor markers for epithelial cells such as *FoxA2* and *Pdx1*, whereas the mesenchymal markers *Desmin*, *collagen IA* or *DDR2* (Discoidin domain Receptor 2) are decreased (Figures 8i and j). Together, our data suggest that SOX2 drives dedifferentiation of cells toward EMT but not to a complete mesenchymal phenotype. This is consistent with partial overlapping transcriptional programs underlying EMT and CSCs.³²

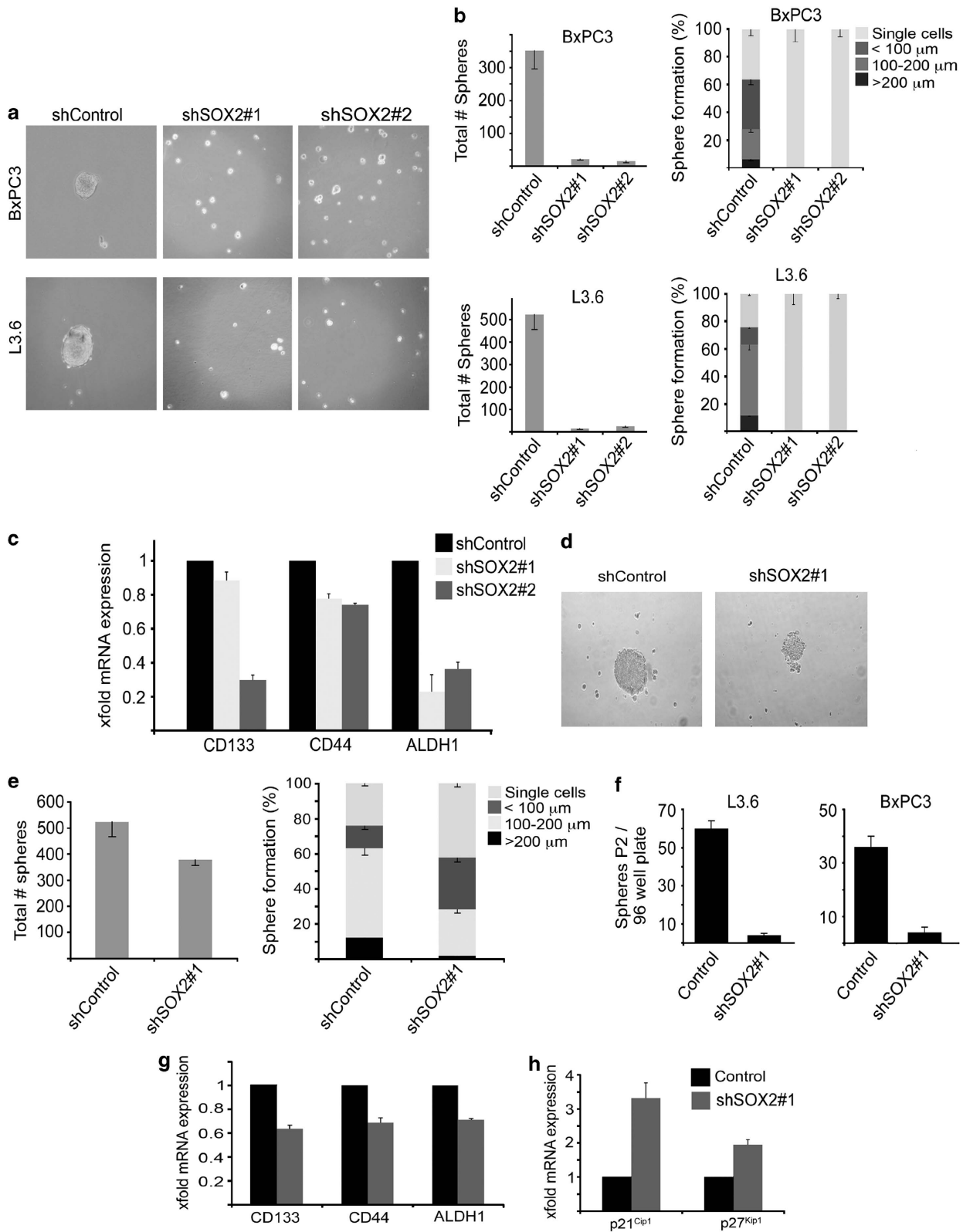


Figure 6. SOX2 is necessary for the maintenance of CSC properties. (a) Bright-field microscopy images showing spheres, small aggregates and single cells after knockdown of SOX2 in BxPC3 and L3.6 cells. (b) Quantification of total number of spheres and percentage of spheres based on size in shSOX2 BxPC3 and L3.6 cells compared with shControl cells. (c) Quantitative RT-PCR showing CD133, CD44 and ALDH1 expressions in parental L3.6 cells and SOX2-suppressed cells. (d) Bright field microscopy images of spheres generated by shControl and shSOX2 L3.6 cells. (e) Quantification of total spheres (left panel) as well as quantification of spheres in different size groups (right panel) in control and SOX2 knockdown L3.6 cells. (f) Quantification of secondary spheres formed in self-renewal assay. (g) Quantitative RT-PCR showing CD133, CD44 and ALDH1 mRNA expressions in spheres generated from shControl and shSOX2 L3.6. (h) Quantitative RT-PCR showing p21^{cip1} and p27^{kip1} mRNA expression in shControl and shSOX2 L3.6 spheres.

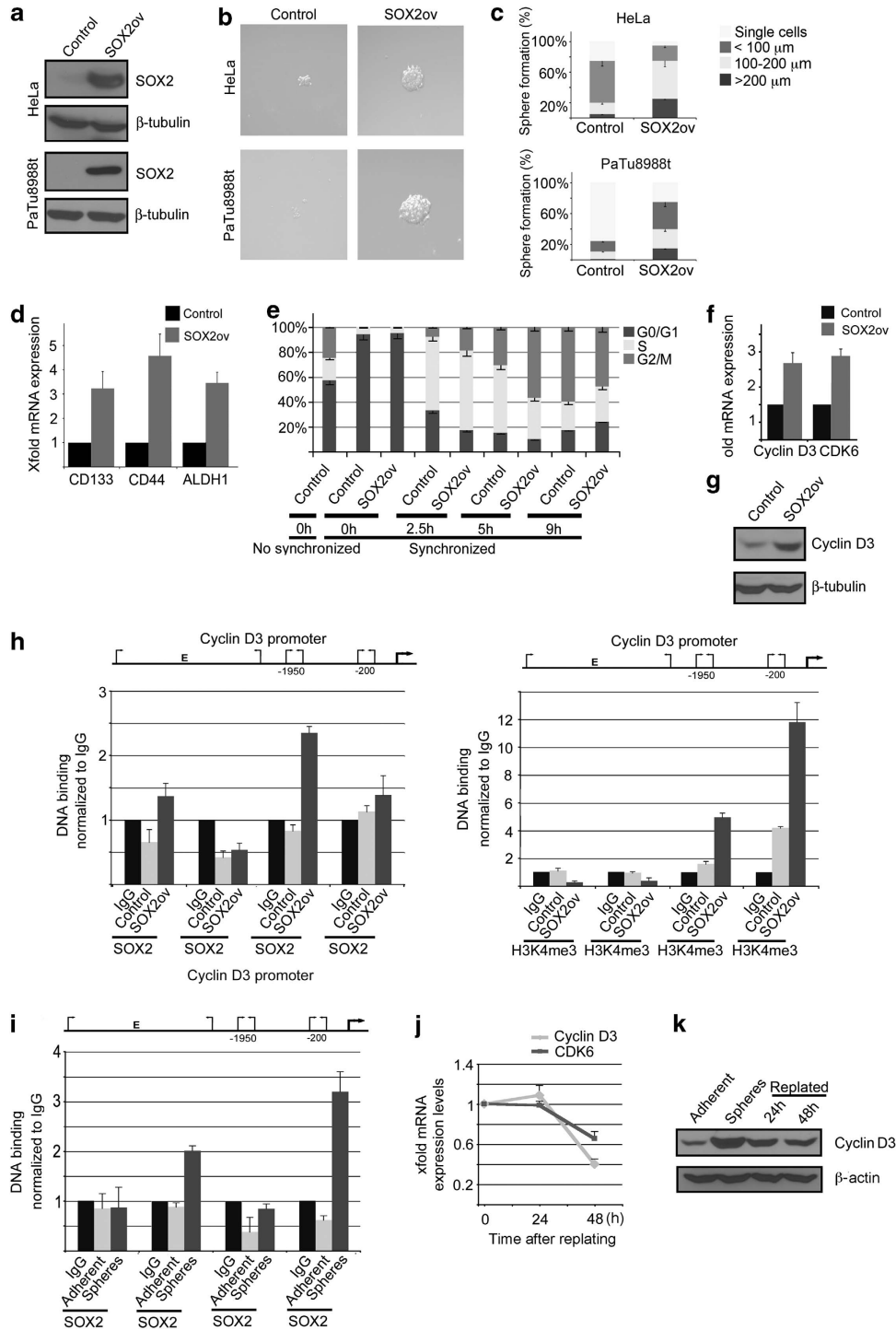


Figure 7. SOX2 overexpression induces self-renewal capacity and a dedifferentiated phenotype in pancreatic cancer cell lines. **(a)** Immunoblot for SOX2 overexpression in HeLa and PaTu8988t cell lines. **(b)** Bright-field microscopy images showing aggregates and spheres in HeLa and PaTu8988t control and SOX2-overexpressing (SOX2ov) cells. **(c)** Quantification of number and size of spheres formed in control versus SOX2ov HeLa and PaTu8988t cells. **(d)** Quantitative RT-PCR for CD133, CD44 and ALDH1 in control versus SOX2ov PaTu8988t cells. **(e)** Cell cycle analysis of HeLa cells synchronized at the G1/S-phase boundary at different time points after thymidine removal. **(f)** Quantitative RT-PCR for *cyclin D3* and *CDK6* expression in control and SOX2ov PaTu8988t cells. **(g)** Immunoblot showing cyclin D3 expression in control and SOX2ov PaTu8988t cells. **(h)** ChIP assay shows SOX2 binding to a specific region on the *cyclin D3* promoter along with H3K4 trimethylation mark in PaTu8988t cells. **(i)** ChIP assay shows SOX2 binding to specific regions on the *cyclin D3* promoter in adherent L3.6 compared with spheres. **(j)** Quantitative RT-PCR showing *cyclin D3* and *CDK6* mRNA expression in sphere-forming cells replated and grow in adherent conditions. **(k)** Immunoblot showing cyclin D3 and CDK6 expressions in spheres replated and grown in adherent conditions.

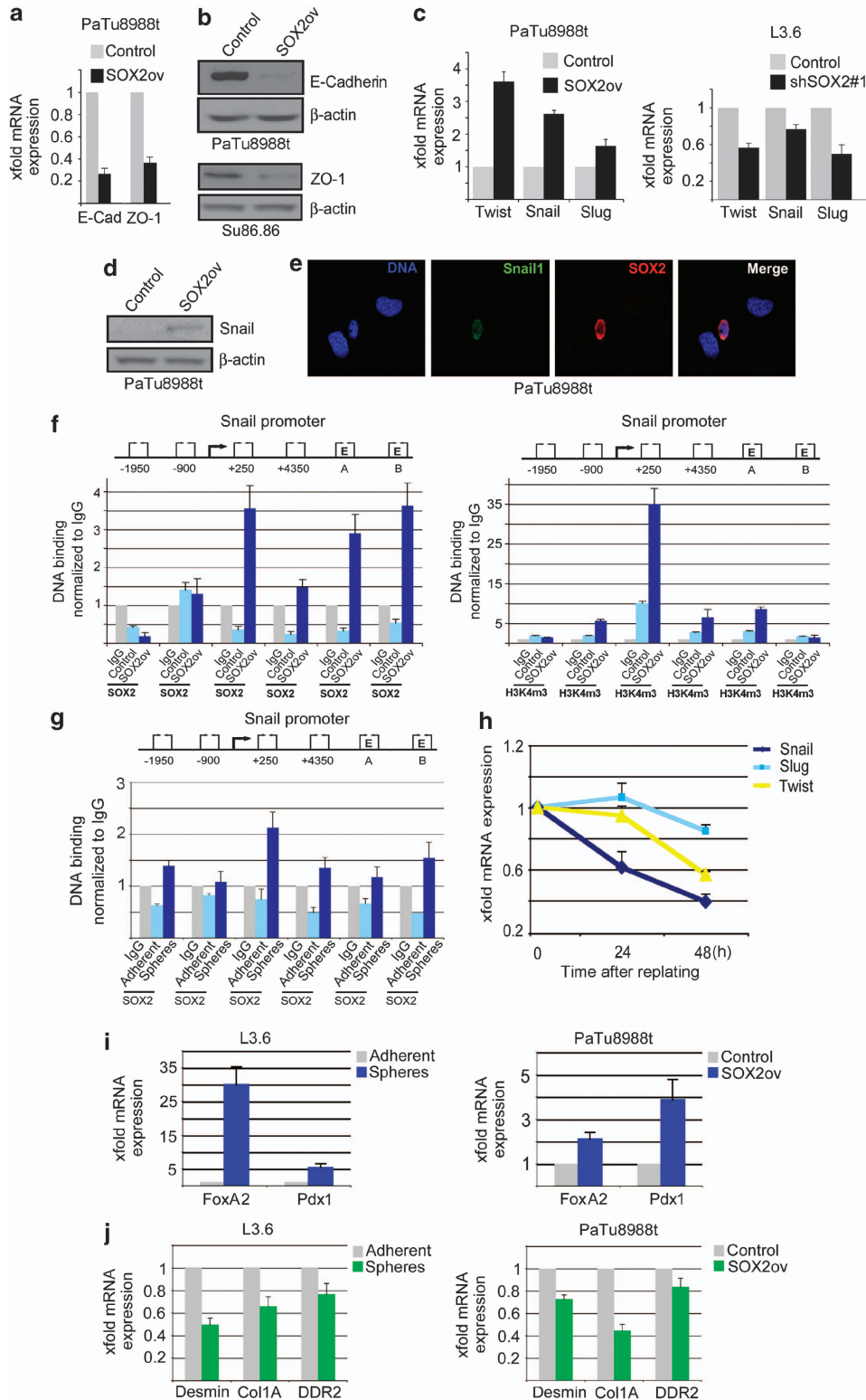


Figure 8. SOX2 regulates EMT-related marker genes in pancreatic cancer cells. **(a)** Quantitative RT-PCR analysis for E-Cadherin and ZO-1 in control versus SOX2ov PaTu8988t cells. **(b)** Immunoblot showing E-Cadherin or ZO-1 expression in control versus SOX2ov cells. **(c)** Quantitative RT-PCR analysis for Twist, Snail and Slug in control versus SOX2ov PaTu8988t cells and control versus shSOX2 L3.6 cells, respectively. **(d)** Immunoblot showing Snail expression in control versus SOX2ov PaTu8988t cells. **(e)** Immunofluorescence staining and confocal imaging showing nuclear localized Snail expression in SOX2ov cells. **(f)** ChIP assay shows SOX2-binding H3K4me3 mark at the Snail promoter/enhancer regions in PaTu8988t cells. **(g)** ChIP assay shows SOX2 binding to Snail promoter in L3.6 spheres. **(h)** Quantitative RT-PCR showing expressions of Snail, Slug and Twist in L3.6 sphere-forming cells replated and grown under adherent conditions. **(i)** Quantitative RT-PCR analysis showing markedly increased expressions of *FoxA2* and *Pdx1* in L3.6 spheres compared with adherent cells, and SOX2ov versus control PaTu8988t cells. **(j)** Quantitative RT-PCR analysis of *Desmin*, *Col1A* and *DDR2* expressions in L3.6 spheres versus adherent cells and SOX2ov versus PaTu8988t control cells.

DISCUSSION

SOX2, a key factor in maintaining the stemness of embryonic stem cells/pluripotent stem cells, is overexpressed in several types of human tumors.^{18,19,21–24} Our immunohistochemical analysis of human TMA confirmed the aberrant expression of SOX2 in PDAC. Significantly, SOX2 immunoreactivity in PanIN lesions was rarely detected in contrast to more widespread and robust staining in PDAC, particularly in moderately and poorly differentiated tumors as well as all adenocarcinomas. Overall, our results agree with previous reports suggesting that SOX2 is mainly involved in later events of carcinogenesis.³⁰ Both epigenetic and genetic factors, particularly gene amplification, have been identified as frequent causes of SOX2 overexpression in several tumors.^{33,34} Although the molecular mechanism driving aberrant SOX2 expression in PDAC is unknown and remains a subject of further study, our functional characterization demonstrates a pleiotropic effect of SOX2 in regulating cell proliferation and stemness in PDAC. Moreover, our findings demonstrate an important and novel role for SOX2 independent of its association with OCT3/4-Nanog, as we have been unable to demonstrate the expression of these factors in the SOX2-expressing cell lines (data not shown). Our data are also consistent with several recent reports that have shown an enrichment of SOX2 in pancreatic CSCs,³⁵ as well as its decreased expression as a consequence of anti-CSCs therapies.^{36,37}

We uncovered a critical role for SOX2 in PDAC cell proliferation showing that SOX2 knockdown arrests cells at the G1 phase and SOX2 overexpression alone is sufficient to drive cell proliferation by facilitating G1/S transition. Mechanistically, G1 arrest in SOX2 knockdown cells is associated with a marked induction of p21^{Cip1} and p27^{Kip1}, two key cyclin/CDK inhibitors. Consistently, SOX2 overexpression induced G1/S-specific cyclin D3 expression. Importantly, we identified p21^{Cip1}, p27^{Kip1} and cyclin D3 as *bona fide* SOX2 targets as demonstrated by mRNA/protein expression and ChIP. These results together suggest that SOX2 can have an impact on pancreatic cancer cell proliferation by directly targeting cell cycle checkpoint genes. As it has been shown that the TP53-p21^{Cip1} pathway is also a target of SOX2 and serves as a barrier in pluripotent stem cell generation,^{38,39} it would be interesting to determine whether SOX2 regulation of p21^{Cip1} has a role in stemness in PDAC. Of note, whereas several studies showed that SOX2 suppression inhibits tumor cell proliferation and induces apoptosis,^{22,24,40} our data from different pancreatic cancer cell lines suggest that SOX2 affects only cell proliferation, but not apoptosis, except in the CSC population.

Consistent with its role in ES or iPS cells, we found that SOX2 expression contributes to stemness in PDAC. We discovered a strong correlation of sphere-forming capacity with SOX2 expression level. Knockdown of SOX2 in high-expressing cells abolished sphere formation and decreased CSC marker expression. Strikingly, although SOX2 generally functions in concert with other stem cell factors, we found that SOX2 overexpression alone is sufficient to drive CSC features including sphere-formation and expression of CSC markers.^{5,41,42} Detailed analysis of SOX2 along with other pancreatic CSC markers suggested that SOX2 expression mainly coincided with CD44⁺ and ALDH1⁺ populations. This is particularly true in sphere cells in which these genes are all highly enriched. Considering that sphere-generating cells are highly aggressive (proliferation and metastasis) *in vivo* when compared with adherent cells,⁴³ we propose SOX2 as a functional pancreatic CSC marker and that SOX2⁺ cells could define a subpopulation of CSC cells, with an increased propensity of invasiveness and metastasis. Further investigations are necessary to corroborate this in *in vivo* models. In addition, we show that >50% of the CD44⁺/ESA⁺ CSC population derived from two primary patient xenograft samples is SOX2 positive. Clearly, the CSC populations are also heterogeneous, and thus it will be of interest to examine the

in vivo tumor-forming capacity of the CD44⁺/ESA⁺/SOX2⁺ and CD44⁺/ESA⁺/SOX2⁻ populations.

Emerging data have highlighted shared molecular characteristics of CSCs and EMT cells.^{2,32} EMT has a central role in embryogenesis and is well recognized for its close connection to cancer metastasis also in PDAC.^{44–46} EMT is also believed to enhance metastasis due to the increased migratory capacity of mesenchymal cells. Our data found that SOX2 regulates cellular dedifferentiation, and overexpression of SOX2 dramatically reduced the expression of epithelial markers (E-Cadherin and ZO-1), which is suggestive of EMT. In fact, the loss of the epithelial phenotype coincided with the increased expression of members of the Snail/Slug family of zinc-finger transcription factors, well-known EMT drivers responsible for downregulation of E-cadherin and ZO-1. Silencing of SOX2 has been shown to downregulate Snail and induce mesenchymal-to-epithelial transition in colorectal cancer and adenocystic carcinoma,²⁸ which is consistent with our observations. However, we could not detect reproducible and significant induction of the key mesenchymal markers in these cells, suggesting that SOX2 overexpression was insufficient to complete EMT, but resulted in a dedifferentiation process toward a cell with stem-like pluripotent qualities. Consistent with this idea, we observed an induction of *Pdx1* and *FoxA2*, two genes products involved in epithelial developmental pathways including pancreas development. These observations are consistent with the notion that the occurrence of EMT in pancreatic cancer is often accompanied by re-activation of developmental pathways. We conclude that SOX2 is capable of driving dedifferentiation, inducing the expression of certain EMT markers, but is unable to confer a full mesenchymal phenotype in PDAC, therefore supporting the partial overlapping transcriptional programs underlying CSCs and EMT.³²

The present work identifies SOX2 as a CSC maker, which defines a subpopulation of PDAC cells that largely overlap with CD44- and ALDH1-positive cells. More importantly, we provide the first experimental evidence that aberrantly expressed SOX2 contributes to PDAC proliferation, stemness and dedifferentiation through the regulation of some EMT gene drivers. Owing to the critical nature of these attributes in PDAC progression, we propose SOX2 as a promising target to eliminate CSCs, the root cause of cancer progression, drug resistance and recurrence.

MATERIALS AND METHODS

Immunohistochemistry

All studies carried out on human specimens were approved by the Mayo Clinic Institutional Review Board (Rochester, MN, USA). Ten adenocarcinoma TMAs containing 349 patient samples, of which 140 are unselected for treatment and 209 have been treated with gemcitabine, were stained for SOX2 expression in the Pathology Research Core. TMA slides were placed in the BOND III (Leica Biosystems, Chicago, IL, USA) stainer for online processing. They were treated with Epitope Retrieval 2 solution for 20 min, stained with SOX2 (Epitomics, Burlingame, CA, USA, clone EPR3131 1:300) for 15 min and detection was achieved using the Polymer Refine Detection kit as per the manufacturer's instructions (Leica Biosystems). Counter staining was performed for 5 min with Hematoxylin. Slides were dehydrated through increasing concentrations of alcohol, cleared in xylene and coverslipped in xylene-based mounting media. Data analysis: For this study, 454 TMA cores from 217 unique patients with pancreatic adenocarcinoma were evaluated for the final analysis. The TMAs were evaluated for SOX2 expression by a trained pancreatic pathologist and were scored as positive or negative. Information across the multiple evaluable cores per patient was reduced to one observation per unique subject by using the core, which stained with the highest expression. Demographic variables are presented as mean (s.d.) for continuous variables and frequency (percentage) for categorical variables.

Cell culture

Panc0403, BxPC3, CFPAC-1 PaTu8988t, Panc1, Su86.86, HeLa and HPDE cell lines were obtained from ATCC. They were maintained in RPMI or DMEM

medium supplemented with fetal bovine serum, except HPDE that was cultured in keratinocyte serum-free medium supplemented with bovine pituitary extract (Life Technologies, Grand Island, NY, USA). Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). L3.6 cells were maintained in MEM medium (Invitrogen, Mannasas, VA, USA). To propagate the CSC-like fraction of the tumor cells, culture conditions favoring proliferation of undifferentiated cells were adopted.^{5,41} We cultured the cells in serum-free DMEM-F12 medium containing insulin (Gibco, Grand Island, NY, USA), Albumin Bovine Fraction V (Sigma, Billerica, MA, USA), N-2 Plus media (Gibco), B-27 (Gibco), EGF and FGF (Preprotech, Rocky Hill, NJ, USA) at a density of 10^4 cells/ml in low-attachment dishes (Corning, Corning, NY, USA). For quantification purpose, round aggregates containing six or more cells were considered as 'spheres'. For single-cell assays, single cells from primary spheres were seeded in 96-well ultra-attachment plates (Corning). The number of secondary spheres formed following a 1-week incubation was counted. β -galactosidase staining was carried out as per the manufacturer's protocol (Cell Signaling Technologies, Danvers, MA, USA). Patient tumor xenografts maintained in NOD/SCID mice were harvested and single-cell suspensions were created as previously described.⁴⁷

Plasmids, lentiviruses and transfections

For lentivirus-mediated suppression of SOX2, two shRNA expression vectors were generated in pLKO.1 vector (Sigma) with the target sequences: 5'-CAGCTCGCAGACCTACATGAA-3' and 5'-TGGACAGTTACGCG-CACATGA-3'. The scrambled vector (Sigma) was obtained from the Mayo Clinic RNA Interference Shared Resource. Lentivirus packaging, cell infection and selection of puromycin-resistant cell were performed as previously described.⁴⁸ Pooled resistant clones were used after validation of successful SOX2 suppression by qRT-PCR and immunoblotting. To generate SOX2 expression vectors, full-length SOX2-coding sequences were obtained by RT-PCR from L3.6 cells and cloned into pCMV-Tag2B (Stratagene, La Jolla, CA, USA) and pLenti6.3 vector (Invitrogen) in frame with an N-terminal FLAG tag. All complementary DNA and shRNA expression plasmids were verified using direct sequencing at the Mayo Molecular Biology Core Facility.

RNA extraction and qRT-PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription and qRT-PCR were performed as previously reported⁴⁸ using the primers indicated in Supplementary Table 1. Experiments were performed in triplicate using three independent complementary DNAs and the results were calculated following the $2^{-\Delta\Delta C_t}$ method.

Protein analysis

Cells were lysed with radio-immunoprecipitation assay buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mmol/l NaCl, 50 mmol/l Tris/HCl (pH 7.2), 10 mmol/l EDTA and 10 mmol/l EGTA). Cleared lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting as described.⁴⁹ Antibodies used include β -actin (Sigma), SOX2 (Epitomics), ALDH1, E-Cadherin and p27^{Kip1} (BD, San Jose, CA, USA), p21^{cip1} (Calbiochem, Billerica, MA, USA), Snail and cyclin D3 (Cell Signaling Technologies, Beverly, MA, USA).

Immunofluorescence

Sphere cells were resuspended in pre-warmed media and allowed to adhere to poly-L-lysine-coated coverslips at 37 °C and then fixed with 4% paraformaldehyde. Images were obtained with an LSM-710 laser scanning confocal microscope with the $\times 100/1.4$ Oil Plan-Apochromat objective using Zen Software (Carl Zeiss, Thornwood, NY, USA). Antibodies used include SOX2 (Epitomics), ALDH1 (BD) and Snail (Cell Signaling Technologies).

Flow cytometry analysis

The following antibodies were used for flow cytometry analysis: CD133/1-PE (Miltenyi Biotec, Auburn, CA, USA), SOX2-PE (R&D Systems), CD44-FITC/APC (Becton Dickinson, Auburn, CA, USA), ESA-FITC (StemCell Technologies, Vancouver, British Columbia, Canada) and ALDH1-PE (Miltenyi Biotec). ALDH activity was detected using the ALDEFUOR assay kit (Stem Cell Technologies) as described by the manufacturer. Samples were analyzed

using FACSCanto II (Becton Dickinson, San Jose, CA, USA) and data analyzed by BD FACSDiva software V6.1.3 (BD Biosciences, San Jose, CA, USA) or FlowJo software (TreeStar, Stanford, CA, USA). The analysis for SOX2 expression in primary pancreatic CSCs was carried out as previously described⁴⁷ with the addition of SOX2-PE.

Cell proliferation, cell cycle and apoptosis analysis

Cell growth was measured by MTS assay (Promega, Madison, WI, USA) as previously described.⁴⁸ Synchronization of HeLa cells was carried out using double thymidine block.⁵⁰ Briefly, cells were treated with 2 mM thymidine (Sigma) in DMEM containing 10% fetal bovine serum for 18 h, washed twice with phosphate-buffered saline and then cultured in fresh thymidine-free medium for 9 h. The cells were then treated again with 2 mM thymidine for additional 17 h. The block was released by incubating cells in thymidine-free medium. Cells were harvested at the indicated time points and cell cycle analysis was performed using propidium iodide staining and flow cytometry. The DNA content was analyzed and the fraction of cells in the G0/G1, S and G2 phases were calculated using ModFit (Verity Software House, Topsham, ME, USA). The fraction of apoptotic cells was analyzed after staining with Annexin-V-FITC antibody (BD) and PI (Sigma) using FloJo 887 Software (Ashland, OR, USA).

Chromatin immunoprecipitation assay

ChIP was carried out using the EZ ChIP kit (Upstate Biotechnology, Temecula, CA, USA) following the manufacturer's instructions as described.⁴⁸ Preleared chromatin was immunoprecipitated with specific antibodies using normal mouse or rabbit IgG as control and antibodies for SOX2 (Epitomics), RNA polymerase-II (Upstate Biotechnology) and H3K4me3 (Millipore, Temecula, CA, USA). The specific primers used for qPCR for ChIP samples are indicated in Supplementary Table II.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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