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 p21Cip1 and p27Kip1 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-stem cells (CSCs). Although representing only a small proportion of the tumor, CSCs are believed to constitute a reservoir of cancer-stem cells (CSCs). 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
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. Interestingly, SOX2 has been linked to EMT in colorectal cancer and SOX2 knockdown reduces the expression levels of Snail, ZEB1, ZEB2, and TGB1 genes, which are known to drive EMT. 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. 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 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 p21CIP1 and p27KIP1
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 (Figure 3b). No difference in senescence-associated β-galactosidase staining was seen between shControl and shSOX2 cells (Figure 3b). No difference in senescence-associated β-galactosidase staining was seen between shControl and shSOX2 cells (Figure 3b).

| PDAC subtype | Patients | Negative | Positive |
|--------------|----------|----------|----------|
| Adenocarcinoma | 175 (80.7%) | 42 (19.3%) |
| Adenosquamous | 0 | 6 (2.8%) |
| Undifferentiated (anaplastic) | 0 | 1 (0.4%) |

| Histological grade | Well differentiated | Moderately differentiated | Poorly/undifferentiated |
|--------------------|-------------------|-------------------------|-----------------------|
| Patients           | 14 (6.5%)         | 78 (35.9%)              | 83 (38.2%)            |

Table 1. SOX2 Staining in TMA

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.

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evaluated the role of SOX2 in self-renewal capacity of CSCs using

Given its key role in maintaining stem cell properties, we next

SOX2 is expressed in pancreatic CSCs through the repression of

SOX2 can regulate cell cycle control in pancreatic cancer cells

immunoprecipitation (ChIP) in L3.6 cells. Interestingly, we detected

shown). To determine whether SOX2 could directly have an impact

due to a perturbation of cell cycle or increased apoptosis. We found

that sphere-forming cells are highly enriched in the expression of

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SOX2 maintains self-renewal capacity of pancreatic CSCs

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. 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

tered 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. 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 ± 5% of L3.6 adherent cells are

positive for CD44, whereas 96 ± 3% of them are positive after

sphere formation. Similarly, 12 ± 2% of adherent cells were

positive for ALDH1 and 30 ± 3% for ESA, and this percentage

increased in sphere cells to 80 ± 5 and 50 ± 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-forming 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 was 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.

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SOX2 regulates pancreatic cancer stemness

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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.

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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<sup>Cip1</sup> and p27<sup>Kip1</sup>. (e) Quantitative RT–PCR showing p21<sup>Cip1</sup> and p27<sup>Kip1</sup> mRNA expressions in shControl and shSOX2 Pan0403 and L3.6 cells. (f) ChIP analysis showing SOX2 binding to specific regions on p21<sup>Cip1</sup> and p27<sup>Kip1</sup> promoter/enhancer regions in L3.6 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 p21Cip1 and p27Kip1 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 ± 4% of control cells were in the S-phase compared with 66 ± 5% for SOX2-overexpressing cells. After 5 h, 55 ± 3% of cells overexpressing Sox2 had reached G2/M phase, whereas this number was 30 ± 4% in the control cells (Figure 7e). These results demonstrate that SOX2 expression facilitates cell cycle progression in the bulk population.

As SOX2 suppression induced p21Cip1 and p27Kip1 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 ± 4% of control cells were in the S-phase compared with 66 ± 5% for SOX2-overexpressing cells. After 5 h, 55 ± 3% of cells overexpressing Sox2 had reached G2/M phase, whereas this number was 30 ± 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, 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 p21Cip1/p27Kip1 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. 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-over-expressing 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
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 Iα 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 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.
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 p21Cip1 and p27Kip1 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.
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, Coll1A and DDR2 expressions in L3.6 spheres versus adherent 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,\(^{35-37}\) 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\(^{\text{Cip1}}\) and p27\(^{\text{Kip1}}\), two key cyclin/CDK inhibitors. Consistently, SOX2 overexpression induced G1/S-specific cyclin D3 expression. Importantly, we identified p21\(^{\text{Cip1}}\), p27\(^{\text{Kip1}}\) 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\(^{\text{Cip1}}\)-pathway is also a target of SOX2 and serves as a barrier in pluripotent stem cell generation,\(^{8,39}\) it would be interesting to determine whether SOX2 regulation of p21\(^{\text{Cip1}}\) 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 iPSCs, 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.\(^{34,41,42}\) 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 vivo model. 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.\(^{2,23}\) EMT has a central role in embryogenesis and is well recognized for its close connection to cancer metastasis also in PDAC.\(^{34-36}\) 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 Snai1/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 Snai 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.\(^{12}\)

The present work identifies SOX2 as a CSC marker, 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 TMA slides containing 349 patient samples, of which 140 are unsélected 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, Sub6B6, BxLa 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, Mannassas, VA, USA). To propagate the CSC-like fraction of the tumor cells, culture conditions favoring proliferation of undifferentiated cells were adopted. 

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 10^5 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-low-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 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'-CAGTCTCGAGACCTATCATGA-3' and 5'-TGAGGACATTGCGC- 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 reported 48 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^(-ΔΔCt) 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 included β-actin (Sigma), SOX2 (Epitomics), ALDH1, E-Cadherin and p27Kip1 (BD, San Jose, CA, USA), p21Waf1 (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 ×100/1.4 Oil Plan-Apochromat objective using Zen Software (Carl Zeiss, Thornwood, NY, USA). Antibodies used included 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 ALDEFLUOR assay kit (Stem Cell Technologies) as described by the manufacturer. Samples were analyzed using FACS Canto II (Becton Dickinson, San Jose, CA, USA) and data analyzed by BD FACS Diva 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 described 47 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. 49 Synchronization of HeLa cells was carried out using double thymidine block. 50 Briefly, cells were treated with 2 mmol/l 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 mmol/l 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 FlowJo 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. 49 Precleared 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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