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SOX9 activity is induced by oncogenic Kras to affect MDC1 and MCMs expression in pancreatic cancer

H Zhou^{1,2}, Y Qin^{3,4}, S Ji^{3,4}, J Ling¹, J Fu¹, Z Zhuang^{1,5}, X Fan¹, L Song⁶, X Yu^{3,4}, and PJ Chiao^{1,7}

¹Department of Molecular and Cellular Oncology, the University of Texas MD Anderson Cancer Centre, Houston, TX, USA

²Liver Cancer Institute & Zhongshan Hospital, Fudan University, Shanghai, China

³Department of Pancreatic and Hepatobiliary Surgery, Fudan University Shanghai Cancer Center, Shanghai, China

⁴Pancreatic Cancer Institute, Fudan University, Shanghai, China

⁵Department of General Surgery, Beijing Tsinghua Changgung Hospital, Tsinghua University, Beijing, China

⁶Department of Oncology, The First Affiliated Hospital of Zhengzhou University, Henan Province, China

⁷The University of Texas Graduate School of Biomedical Sciences, Houston, TX, USA.

Abstract

SRY (sex determining region Y)-box 9 (SOX9) is required for oncogenic Kras-mediated acinar-to-ductal metaplasia (ADM), pancreatic intraepithelial neoplasias (PanINs) and ultimately pancreatic ductal adenocarcinoma (PDAC). However, how oncogenic Kras affects SOX9 activity is not yet understood, and SOX9-associated genes in PDAC are also unknown at all. Here, we investigated the mechanistic link between SOX9 and oncogenic Kras, studied biological function of SOX9, and identified SOX9-related genes and their clinical significance in patients with PDAC. Our studies reveal that oncogenic Kras induces *SOX9* mRNA and protein expression as well as phosphorylated SOX9 expression in human pancreatic ductal progenitor cells (HPNE) and pancreatic ductal cells (HPDE). Moreover, oncogenic Kras promoted nuclear translocation and transcriptional activity of SOX9 in these cells. TAK1/I κ B α /NF- κ B pathway contributed to induction of SOX9 by oncogenic Kras, and SOX9 in turn enhanced NF- κ B activation. SOX9 promoted the proliferation of HPNE and PDAC cells, and correlated with minichromosome maintenance complex components (MCMs) and mediator of DNA damage checkpoint 1 (MDC1)

Correspondence: Dr X Yu, Department of Pancreatic and Hepatobiliary Surgery, Fudan University Shanghai Cancer Center, Shanghai 200032, China or Dr PJ Chiao, Department of Molecular and Cellular Oncology, Unit 107, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030, USA. yuxianjun@fudan.edu.cn or pjchiao@mdanderson.org.

AUTHOR CONTRIBUTIONS

HZ: study design, carrying out of experiments, analysis of data and manuscript writing; YQ: carrying out of experiments and technical support; SJ: analysis of data and material support; JL, JF, XF, ZZ, LS: technical support; PJC, XY: study supervision and manuscript writing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

expression. The overexpressive MDC1 was associated with less perineural and lymph node invasion of tumors and early TNM-stage of patients. Our results indicate that oncogenic Kras induces constitutive activation of SOX9 in HPNE and HPDE cells, and Kras/TAK1/I κ B α /NF- κ B pathway and a positive feedback between SOX9 and NF- κ B are involved in this inducing process. SOX9 accelerates proliferation of cells and affects MCMs and MDC1 expression. MDC1 is associated negatively with invasion and metastasis of PDAC.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human cancers in western countries.¹ It has an extremely poor prognosis and lacks early diagnostic and therapeutic modalities.² Hence, PDAC poses one of the greatest challenges in cancer research.

The mutational activation of Kras is an early event in PDAC development and has been detected in 80–95% of PDAC, which plays a critical initiating role in this disease.³ Previous findings reveal that oncogenic Kras mutations induce acinar-to-ductal metaplasia (ADM), pancreatic intraepithelial neoplasia (PanINs) and ultimately PDAC.^{4,5} A recent study demonstrates that SRY-related human motility group box factor 9 (SOX9) is necessary for Kras^{G12D}-mediated ADM and PanIN formation. Deletion of *SOX9* in the presence of oncogenic Kras completely blocked Kras^{G12D}-mediated development of ADM and PanINs.⁶ However, the mechanisms that link SOX9 activation with oncogenic Kras are not yet understood, and what genes are affected by SOX9 to facilitate PanIN formation are also unknown at all.

SOX9 is a member of the SRY-related human motility group-box (SOX) gene family of transcription factors. It has been known that SOX9 interacts with other co-factors to form nucleoprotein complexes to regulate downstream gene transcription, and phosphorylation of serine residues S64 and S181 enhances nuclear localization and transcriptional activity of SOX9.⁷ SOX9 plays a pivotal role in progenitor proliferation and differentiation during human pancreas and liver development.^{8,9} Some studies showed that SOX9 plays an oncogenic role in tumor development, and is upregulated in tumor tissues and associated with poor patient survival.^{10–12} But SOX9 was also reported to be a potential tumor suppressor in cervical cancer, and even high SOX9 correlated with good prognosis in pancreatic cancer.^{13–15} Therefore, functions and clinical significance of SOX9 in pancreatic cancer remain to be further clarified.

In the present study, we verified whether oncogenic Kras induces activation of SOX9 in immortalized pancreatic ductal progenitor cells and ductal cells, as well as PDAC cells, and further investigated the potential signaling pathway by which Kras links SOX9 activity. In addition, we researched biological functions of SOX9 in pancreatic ductal progenitor cells and PDAC cells. Finally, the SOX9-relevant genes and their clinical significances in patients with PDAC were investigated.

RESULTS

Oncogenic Kras induces expression of SOX9

The immortalized HPNE cells have properties similar to that of the intermediary cells produced during ADM, and HPDE cells are immortalized human pancreatic ductal epithelial cells,^{16,17} so we utilized HPNE, HPDE and their derivative cells with mutant Kras (Kras^{G12V}) overexpression (HPNE/Kras and HPDE/Kras) to investigate Kras-induced SOX9 expression in pancreatic ductal progenitor cells and ductal cells. It was observed that both *SOX9* messenger RNA (mRNA) and protein levels in HPNE/Kras and HPDE/Kras were significantly increased when, respectively, compared with control HPNE and HPDE cell lines (Figure 1a). In order to observe more clearly the inducing effect of oncogenic Kras on SOX9 expression, we established doxycycline-induced expressing-Kras^{G12V} HPNE cell line (HPNE/iKras) in this study. It was found that mRNA and protein levels of SOX9 were all elevated significantly in response to induced expression of Kras^{G12V} after third day following doxycycline treatment (Figure 1b). Furthermore, we also saw similar inductive effect of Kras^{G12D} on SOX9 in the mouse inducible Kras^{G12D} p53^{L/+} PDAC cell line (iKras) that was obtained from Dr Ronald DePinho's laboratory.¹⁸ SOX9 was induced in response to induced expression of Kras^{G12D} from 24 h after doxycycline was applied on iKras cells (Figure 1c).

We further determined whether SOX9 expression is different between human PDAC cell lines with wild-type Kras and those with mutant Kras. *SOX9* mRNA and protein expression levels were tested in the human PDAC cell lines with wild-type Kras (BxPc-3) and the PDAC cell lines with mutant Kras (Panc-1, Capan-1, AsPc-1 and MiaPaCa-2). We found that SOX9 protein levels were significantly higher in the PDAC cells with mutant Kras than that in the PDAC cells with wild-type Kras. However, the *SOX9* mRNA levels did not have significant differences between the two kinds of cell lines, suggesting posttranslational modification of proteins might contribute to the increase of SOX9 protein levels (Figure 1d). As well known, elevated protein expression can be caused by increasing protein stability besides activation of transcription. Thus, we tried to figure out whether SOX9 expression were upregulated via increased stability of SOX9 protein. The SOX9 protein levels in BxPc-3 and AsPc-1 were measured at indicated time points following treatment with the protein synthesis inhibitor cycloheximide (Figure 1e). It was found that SOX9 half-life of AsPc-1 was significantly longer than that of BxPc-3, suggesting that SOX9 stability in AsPc-1 cells is better than its stability in BxPc-3 cells (Figures 1e and f). To verify if Kras promotes stability of SOX9 protein, we analyzed the half-life of SOX9 in HPNE, HPNE/Kras, HPDE and HPDE/Kras cells. The results showed that SOX9 half-life times in HPNE/Kras and HPDE/Kras were significantly longer, respectively, than that in HPNE and HPDE (Supplementary Figure S1). We also investigated if SOX9 was induced by Kras in PDAC cell lines. We overexpressed Kras^{G12D} in BxPc-3 cells (BxPc-3/Kras) using Lipofectamine transfection system, and reduced Kras level in AsPC-1 by the Kras siRNA (AsPc-1/siRNA). It was observed that SOX9 expression level was increased in BxPc-3/Kras cells, while decreased in AsPc-1/siRNA cells (Supplementary Figure S2). Taken together, these results indicate that oncogenic Kras can independently induce SOX9 expression in pancreatic ductal progenitor cells, pancreatic ductal cells and PDAC cells.

Oncogenic Kras promotes nuclear translocation and transcriptional activity of SOX9

To determine whether oncogenic Kras facilitates nuclear translocation of SOX9 and enhances transcriptional activity of SOX9, HPNE, HPNE/Kras, HPDE, HPDE/Kras cells were subjected to immunofluorescence staining of SOX9 or phosphorylated SOX9 at Serine 181 (SOX9-p^{S181}). It was observed that HPNE/Kras cells had obvious nuclear expression of SOX9, while few control HPNE cells had SOX9 expression in nucleus, suggesting SOX9 in HPNE/Kras cells underwent nuclear localization (Figure 2a). That was further verified by western blot assay (Supplementary Figure S3). In addition, SOX9-p^{S181} expression in nucleus and cytoplasm of HPNE/Kras cells was increased comparing with the control HPNE cells (Figure 2b). For HPDE and HPDE/Kras cells, they already had nuclear localization of SOX9 (Figure 2c). However, HPDE/Kras cells had higher SOX9 and SOX9-p^{S181} expression in cell nucleus and cytoplasm than the control HPDE cells (Figure 2d). Western blot assays further confirmed that SOX9-p^{S181} expression levels in HPNE/Kras and HPDE/Kras were obviously elevated when compared, respectively, to HPNE and HPDE (Figure 2e). Furthermore, immunofluorescence staining of SOX9 or phosphorylated SOX9 were conducted in PDAC cells. It was found that AsPc-1 and miapaca-2 cells already had nuclear localization of SOX9 (Supplementary Figure S4). All these results suggest that oncogenic Kras promotes nuclear translocation of SOX9 in HPNE cells and enhances expression of phosphorylated SOX9 besides total SOX9 in HPNE and HPDE cells. SOX9 might undergo nuclear translocation and be gradually functionally activated in the process from ADM to PanINs and ultimate PDAC.

We then analyzed transcriptional activity of SOX9 by testing AMH expression, a direct target gene of SOX9. The results showed that both *AMH* mRNA and protein expression levels in HPNE/Kras and HPDE/Kras were significantly higher than that in HPNE and HPDE (Figures 2f and g). To sum up, the data suggest that oncogenic Kras promotes constitutive activation of SOX9 during PDAC development.

SOX9 accelerates the proliferation of pancreatic ductal progenitor cells and PDAC cells

To understand the biological function of SOX9 during PDAC development, we established the HPNE cell line with ectopic expression of *SOX9* (HPNE/*SOX9*) using a lentivirus-mediated overexpression system (Supplementary Figure S5A). The growth characteristics of these cells were examined. As shown in Figure 3a, SOX9 overexpression promoted significantly the proliferation of HPNE cells *in vitro*. However, clone formation assays showed that clone-forming capability of HPNE cells was not facilitated by SOX9.

We next constructed a lentiviral vector expressing *SOX9* shRNA and established the AsPc-1/*SOX9*-shRNA and MiaPaCa-2/*SOX9*-shRNA cell lines which stably express SOX9 shRNA. Successful knockdown of *SOX9* in these cells was confirmed by western blot (Supplementary Figure S5B). As shown in Figures 3b and c, cell proliferation was suppressed significantly by *SOX9* knockdown in the AsPc-1 and MiaPaCa-2 cells *in vitro*. Furthermore, we observed that AsPc-1/*SOX9*-shRNA and MiaPaCa-2/*SOX9*-shRNA cells grew significantly less and smaller colonies when, respectively, compared to their control cells (Figures 3d and e), indicating *SOX9* knockdown attenuated clone-forming capability of PDAC cells. In addition, migration and matrigel invasion assays revealed that *SOX9*

knockdown did not influence migration and invasion ability of PDAC cells (Supplementary Figure S6). We further confirmed the promoting action of SOX9 on tumor growth *in vivo* by nude mouse orthotopic xenograft model. Results showed that AsPc-1/*SOX9*-shRNA cells formed smaller xenograft tumors than their control cells did in nude mouse model (Figures 3f and g).

Since Kras can accelerate cell proliferation, we further investigated whether SOX9 promoted proliferation of HPNE and AsPc-1 through Kras. To this aim, we determined Kras activity in the cell lines with *SOX9*-overexpression (HPNE/*SOX9*) and *SOX9*-knockdown (AsPc-1/*SOX9*-shRNA). The results showed that Kras activity were not affected by SOX9 in these cell lines (Supplementary Figure S7). These findings together suggest that SOX9 positively regulates growth of pancreatic ductal progenitor cells and PDAC cells.

TAK1/I κ B α /NF- κ B pathway contributes to induction of SOX9 by oncogenic Kras and a positive feedback exists between SOX9 and NF- κ B

Accumulated evidences have shown a key role of the NF- κ B signaling pathway in Ras-driven cancers using animal models of cancer.^{19–21} Recent studies suggest that SOX9 is upregulated by NF- κ B in endometrial carcinoma cells and pancreatic cancer stem cells.^{22,23} Consequently, we hypothesized that TAK1/I κ B α /NF- κ B pathway participates in the induction of SOX9 by oncogenic Kras. To confirm this hypothesis, we first tested the effects of TAK1-kinase selective inhibitor LYTAK1 on expression of SOX9 in iKras cells.¹ As described before, SOX9 should have been inductively increased along with Kras from 24 h of doxycycline treatment in iKras cells, but in the presence of LYTAK1, SOX9 could not be completely induced by Kras (Figure 4a). We then conducted similar experiment in HPNE/Kras and HPDE/Kras cell lines. Likewise, we observed that the SOX9 expression was significantly suppressed in presence of LYTAK1, suggesting that TAK1 contributes to inductive increasing of SOX9 by Kras (Figure 4b). Further, we analyzed SOX9 expression in AsPc-1 cell line expressing inducible *TAK1* shRNA (AsPc-1/ish *TAK1*) and mouse pancreatic cancer cell line with *TAK1* knock out (*P-D*, *Kras*^{G12V}, *P53*^{m/+}, *TAK1*^{F/F} cell line), all of which had been established in Dr Chiao's laboratory. It was found that SOX9 expression levels in the AsPc-1/ish *TAK1* apparently decreased along with TAK1 reduction on the third day of doxycycline induction (Figure 4c). Similarly SOX9 expression levels in mouse *P-D*, *Kras*^{G12V}, *P53*^{m/+}, *TAK1*^{F/F} cell lines were visibly lower than that in mouse *P-D*, *Kras*^{G12V}, *P53*^{m/+}, *TAK1*^{+/+} cell lines (Figure 4d). Then, we tested SOX9 expression levels in AsPc-1/mutant-*I κ B α* cell line, which harbors flag-tagged phosphorylation defective mutant of I κ B α (I κ B α S32, 36A) and had been established in Dr Chiao's laboratory.²⁴ Western blot results showed that SOX9 expression levels were remarkably downregulated in AsPc-1/mutant-I κ B α cell line compared with control cell line (Figure 4e), suggesting that inhibition of NF- κ B attenuates expression of SOX9. Furthermore, to clarify if increased NF- κ B activity elevates SOX9 expression in the absence of oncogenic Kras, we treated HPNE cells with TNF- α to activate NF- κ B and then test Sox9 level. As we expected, SOX9 expression levels were elevated once NF- κ B activation was induced by cytokine TNF- α in HPNE cells (Supplementary Figure S8). Since NF- κ B has previously been linked to Kras activity, we investigated if LYTAK1 influences Kras activity. The Kras activity in HPNE/Kras and AsPc-1 cells was tested after these cells were treated with LYTAK1. It was

found that Kras activity was not affected by LYTAK1 in these cells (Supplementary Figure S9). All these data revealed that oncogenic Kras induces SOX9 expression in part through TAK1/I κ B α /NF- κ B pathway.

Interestingly, we also found that NF- κ B subunit p65 and phosphorylated P65 (p-P65) in AsPc-1/sh*SOX9* and MiaPaCa-2/sh*SOX9* cell lines were downregulated significantly when, respectively, compared to their control cell lines (Figure 4f). These findings suggest that inhibition of SOX9 also reduces NF- κ B activation and there may be a feedback mechanism for positively regulating NF- κ B and SOX9 in PDAC cell. To further verify these findings in pancreatic ductal progenitor cells, we tested p65 and p-P65 expression levels in the HPNE and HPNE/*SOX9* cell lines by western blotting. As expected, p65 and p-P65 protein expression levels in HPNE/*SOX9* cells were significantly elevated compared with control cells (Figure 4g). These observations suggest that SOX9 and NF- κ B might promote each other during initiation and progression of PDAC by establishing a positive feedback mechanistic loop.

SOX9 is associated with expression of MCMs and MDC1

In view of important roles of SOX9 in PDAC development, we carried out RNA-seq assay to characterize the SOX9-related genes and pathways using the PDAC cell lines with *SOX9* knockdown. Gene expressions of MiaPaCa-2/*SOX9*-shRNA cells and their control cells were profiled and a number of known and previously unknown SOX9-related gene sets were identified by Gene Set Enrichment analyses using gene ontology, and a list of differential expressed gene sets between MiaPaCa-2/*SOX9*-shRNA and control scramble cells was generated (Supplementary Tables S1 and S2). Twenty-five gene sets were significantly enriched at nominal *P*-value<1% in MiaPaCa-2/scramble cells. three gene sets were significantly enriched at false discovery rate *q* value (FDR)<25% and 43 gene sets were significantly enriched at nominal *P*-value<1% in MiaPaCa-2/*SOX9*-shRNA cells. Gene Set Enrichment analysis revealed that collagen gene set and BMPs were downregulated in *SOX9* knockdown cells, which indicates SOX9's important roles during cartilage and bone development as we understood before. Furthermore, we observed that minichromosome maintenance complex components (*MCMs*), a family of eukaryotic DNA helicase complex required for the process of DNA replication, and some cyclins and *CXCL/CCLs* were differentially expressed between MiaPaCa-2/*SOX9*-shRNA and control scramble cells. Gene Set Enrichment analysis of significant gene downregulation in MiaPaCa-2/*SOX9*-shRNA revealed that they are strongly associated to DNA replication, cell cycle and inflammatory response (Figure 5a), which is consistent with preceding results of SOX9 function analyses. The upregulated and downregulated genes in MiaPaCa-2/*SOX9*-shRNA cells also were classified using Panther Classification System according to molecular function (Figure 5b).

Among the differentially expressed genes, we selected some genes mainly related to DNA replication and DNA damage repair to further confirm the association of *SOX9* with these genes. Quantitative PCR assay validated the PDAC cells with *SOX9* knockdown had lower mRNA levels of *MCM2*, *MCM4*, *MCM5*, *MCM7*, mediator of DNA damage checkpoint 1 (*MDC1*), ubiquitin specific peptidase 2 (*USP2*), proliferating cell nuclear antigen and bone morphogenetic protein 4 (*BMP4*) (Figure 5c). Further, western blot assay demonstrated that

the protein expression levels of MCM2, MCM7 and MDC1 were decreased in PDAC cell lines with SOX9 knockdown compared to the controls (Figure 5c). We then tested the expression of these genes in HPNE/*SOX9* cells and their control. It was found that mRNA and protein expression levels of MCM2, MCM7 and MDC1 in HPNE/*SOX9* cells were higher significantly than that in the control cells (Figure 5d). All together, these data suggest that SOX9 is associated with MCMs and MDC1 expression.

SOX9 positively correlates with MDC1 in pancreatic cancer and tumor-adjacent pancreatic tissues

Given that role of SOX9 in PDAC growth and important functions of MCMs and MDC1 in DNA replication and damage repair progression,^{25–27} we explored clinical significances of SOX9, MCM2 and MDC1 in tissue samples from patients with PDAC.

Immunohistochemical staining for SOX9, MDC1 and MCM2 was performed on primary PDAC tissues and tumor-adjacent pancreatic tissues from a large cohort of PDAC patients ($n = 104$). All the patients have tumors and matched tumor-adjacent pancreatic tissues. SOX9 and MDC1 staining was found in the cytoplasm and nucleus of tumor cells and of tumor-adjacent pancreatic acinar cells, while MCM2 was mainly expressed in nucleus of tumor cells and few MCM2 was stained in tumor-adjacent pancreatic tissues (Figures 6a and b).

We examined the correlation of MCM2 and MDC1 with SOX9 in human pancreatic cancer tissues and tumor-adjacent pancreatic tissues according to immunohistochemistry scoring. The results showed that MDC1 expression levels positively correlated with SOX9 expression levels whether in tumorous tissues or surrounding pancreatic tissues (tumorous tissues, Spearman's $\rho = 0.341$, 95% confidence interval (CI): 0.2–0.5, $P = 0.0004$ and tumor-adjacent pancreatic tissues, Spearman's $\rho = 0.404$, 95% CI: 0.2–0.6, $P < 0.0001$). However, MCM2 expression levels were not significantly associated with SOX9 levels whether in tumorous tissues or in adjacent pancreatic tissues (tumorous tissues, Spearman's $\rho = 0.097$, $P = 0.327$ and tumor-adjacent pancreatic tissues, Spearman's $\rho = 0.1411$, $P = 0.153$).

We then compared, respectively, SOX9, MCM2 and MDC1 expression levels between tumor tissues and tumor-adjacent pancreatic tissues. The results revealed that MCM2 expression levels in tumor tissues were significantly higher than that in tumor-adjacent pancreatic tissues (Figure 6c), which is consistent with previous studies suggesting MCM protein is absent in quiescent cells but abundant in active mitotic cells.^{25,26} In contrast, we did not find any differences of expression levels between tumor tissues and tumor-adjacent pancreatic tissues regarding SOX9 and MDC1 (Figure 6c). To rule out the field effects of immunohistochemistry staining, we investigated if SOX9 and MDC1 levels in tumor tissues were associated with their levels in tumor-adjacent tissues. Both SOX9 and MDC1 levels in tumorous tissues were not associated with their levels in adjacent pancreatic tissues. (SOX9, Spearman's $\rho = 0.099$, $P = 0.319$ and MDC1, Spearman's $\rho = -0.048$, $P = 0.629$).

The associations of SOX9, MCM2 and MDC1 in tumor tissues with clinicopathologic features of patients with PDAC were further analyzed using the median expression levels as the cutoff value. We observed that MDC1 expression levels in tumor tissues were significantly associated inversely with perineural and lymph node invasion of tumors and TNM-stage of patients (Table 1 and Figure 6d), suggesting MDC1 correlates negatively with

invasion and metastasis of PDAC. In contrast, neither SOX9 nor MCM2 expression levels was related to any clinicopathologic features (Table 1).

To investigate the correlation of SOX9 with outcome of PDAC patients, Kaplan–Meier analyses for overall survival were performed using the median tumorous SOX9, MDC1 or MCM2 levels as the cutoff for the definition of subgroups. No significant association was found between their expression levels and overall survival of patients (Supplementary Figure S10).

DISCUSSION

In this study, we first demonstrated that oncogenic Kras induced SOX9 expression by promoting SOX9 gene transcription and SOX9 protein stability. SOX9 is lowly expressed in pancreatic ductal progenitor cells (HPNE) and ductal cells (HPDE), which do not have oncogenic Kras, but highly expressed in PDAC cells with oncogenic Kras. These data indicate that Kras can independently induce SOX9 in progenitor cells of PDAC, suggesting that SOX9 is induced to express once pre-malignant cells get mutation of Kras during initial and developmental stage of PDAC and finally constitutively activated in PDAC cells. Accumulated evidences have indicated that the oncogenic Kras plays a critical initiating role in PDAC.^{3,16} A previous study based on genetically engineered mouse models demonstrated that concomitant expression of SOX9 and oncogenic Kras can induce transformation of acinar cells into duct-like cells and subsequent PanIN formation.⁶ Here, our data indicate that Kras can independently induce SOX9 in progenitor cells of PDAC. Our results increased the understanding of the mechanisms that link SOX9 activation with oncogenic Kras.

On the other hand, we found that there was not SOX9 nuclear localization in HPNE cells; however, SOX9 nuclear localization occurred when oncogenic Kras were overexpressed in these cells. In contrast, HPDE and PDAC cells had already SOX9 nuclear localization even without forced overexpression of Kras. Furthermore, phosphorylated SOX9 was elevated in HPNE/Kras and HPDE/Kras compared to their original cells without oncogenic Kras. It was well known that both translocation of SOX9 from cytoplasm to nucleus and phosphorylation of SOX9 are functionally important for activation of SOX9. In view of that the HPNE cells have properties similar to that of the intermediary cells produced during ADM, and HPDE cells belong to ductal epithelial cells, we suppose that SOX9 undergoes nuclear translocation and functional activation during the course from ADM to PanINs and ultimate PDAC. Importantly, Kras may well promote SOX9 expression, nuclear localization and constitutive SOX9 activation in the initiation and development of PDAC.

Furthermore, our results show that TAK1/I κ B α /NF- κ B pathway are involved in Kras-induced SOX9 activation. That indicates the presence of Kras/TAK1/I κ B α /NF- κ B/SOX9 axis in pancreatic ductal progenitor cells and PDAC cells. Interestingly we also found that SOX9, in turn, promoted NF- κ B activity, suggesting there is a feedback mechanism between SOX9 and NF- κ B pathway. Our previous findings reveal that AP-1 induced by oncogenic Kras^{G12D} initiates feedforward loops of IL-1 α and p62 to induce and sustain constitutive NF- κ B activation.³ Here, we show additional mechanism by which oncogenic Kras

promotes NF- κ B activity, namely Kras/TAK1/I κ B α /NF- κ B/SOX9 axis and activation of feedback from SOX9 to NF- κ B. Accumulating evidence shows that the NF- κ B signaling pathway has a key role in Ras-driven cancers. The inhibition of NF- κ B signaling can suppress tumorigenesis of pancreatic cancer cells and the NF- κ B signaling pathway is a potential target for anticancer agents.^{21,28,29} The feedback between SOX9 and NF- κ B signaling pathway found in this study, will amplify activating of NF- κ B and SOX9. Therefore, the mechanism underlying this feedback remains to be further researched. Strategies designed to disrupt this feedback loop might be developed to prevent partly initiation and progression of PDAC.

In the present study, RNA-seq and Gene Set Enrichment analyses results indicate that the SOX9-regulated genes included mainly the genes related to cell-cycle progression and DNA replication which is consistent with results from SOX9 function research in this study. Our study first demonstrated MCM2, MCM7 and MDC1 are associated with SOX9 in pancreatic ductal progenitor cells and PDAC cells. The MCM proteins are critical for licensing DNA replication in proliferating cells and are obvious markers for proliferation. Accumulating evidences suggest that during early cancer development altered MCM2–7 resulting from oncogene expression leads to a particularly mutagenic form of DNA replication that fuels genomic instability and proliferation.^{25,26,30} Consequently, we assume that dysregulation of MCMs contribute to the SOX9-promoting ADM or PanIN formation. This hypothesis remains to be confirmed in future.

MDC1 is a crucial component in the DNA damage response network. It is chromatin remodeling factors required for the recruitment of DNA repair proteins to the DNA damage sites.^{27,31,32} In this study, we found that MDC1 was correlated with good clinicopathologic features of patients. The patients with high MDC1 in their tumor tissues tend to have less perineural and lymph node invasion and early stage, suggesting it is negatively associated with invasion and metastasis of PDAC. This finding also agrees basically with two recent studies in other solid tumors.^{33,34} However, whether MDC1 functions as an invasion- or metastasis-suppressor in PDAC remains be further studied. In addition, our results showed that SOX9 was positively associated with MDC1 in PDAC cells; however, we did not find that SOX9 affect migration and invasion of PDAC cells. We thought that these seemingly inconsistent data might be because SOX9, as a transcription factor, regulated many genes in PDAC cells as shown in the results from RNAseq in our study. SOX9 has effects not only on potential invasion suppressor (for example, MDC1) but also on possible invasion promoter. As showed in Figure 5c, SOX9 also influences BMP4. BMP4 has been reported to promote metastasis and invasion in breast cancer and hepatocellular carcinoma.^{35,36} Moreover, SOX9 also was associated with several gene sets relevant to tumor metastasis as showed in the RNA-seq data (Supplementary Tables S1 and S2), for example, ZUCCHI_METASTASIS_UP and NAKAMURA_METASTASIS_MODEL_UP. Therefore, SOX9 might not show an obvious effect on invasion and metastasis of PDAC cells.

Although SOX9 is a mediator of oncogenic Kras and plays a positive role in proliferation of pancreatic ductal progenitor cells and PDAC cells, we did not find that SOX9 was associated with the tumor stage or overall survival of PDAC patients in this study. Several aspects may contribute to this discrepancy. First, SOX9 facilitates not only proliferation-promoting genes

but also tumor-suppressing genes (for example, MCMs and MDC1 found in our study), depending on specific context of tumor development. Second, it is also possible that SOX9 is necessary for the initiation of PDAC whereas it may become nonessential in later stage. Third, SOX9 not only is a mediator of oncogenic Kras but also plays a physiological role in the developing and adult human pancreas.³⁷ In addition, SOX9 is acknowledged as a ductal cell maker; however, we observed that SOX9 was also expressed in acinar cells of tumor-adjacent pancreatic tissues in this study. We demonstrated that peritumoral SOX9 did not correlate with tumorous SOX9, suggesting high peritumoral SOX9 level was not caused by field effect of immunostaining. The specificity of the SOX9 antibody used for immunohistochemical staining in this study was confirmed by western blot experiments (Supplementary Figure S11). Kopp *et al.* demonstrated that SOX9 expression had already been initiated before Kras^{G12D}-mediated ADM and PanINs.⁶ We assume that SOX9 was expressed in some tumor-adjacent acinar cells which are at the starting stage of acinar-to-ductal reprogramming, which remains to be further researched.

In this study, we did not see differential expression of SOX9 between tumor and tumor-adjacent pancreatic tissues and MCM2 had low expression in many PDAC tissues. Furthermore, our immunohistochemical data did not show SOX9 and MCM2 were good biomarkers for predicting clinical outcome of PDAC patients. It seems that SOX9 and MCM2 have no vital roles in maintaining of PDAC. But we found that SOX9 was induced independently by Kras and played an important role in proliferation of progenitor cells and PDAC cells. We thought that SOX9 and MCM2 might play critical roles in the initiation and development of PDAC rather than later stage of PDAC. The other two previous studies also support this view point. Grimont *et al.* found that SOX9 correlated with high expression of several members of the EGFR pathway, but SOX9 in tumor tissue was not associated with poor prognosis of PDAC patients.¹⁴ Kopp *et al.* demonstrated that SOX9 was necessary for early stage of PDAC formation.⁶ Our and their results suggest that SOX9 function is required for the initial phase of PDAC development but may become dispensable later. In addition, although we observed that SOX9 affected MCM2 expression in HPNE and PDAC cells *in vitro*, SOX9 did not display a significant association with MCM2 in the immunohistochemistry studies. The inconsistent expression between SOX9 and MCM2 is possibly because MCM2 is also regulated by other signal or molecules from surrounding cells or tissues *in vivo*.

In summary, we report here oncogenic Kras promotes constitutive activation of SOX9 potentially through Kras/TAK1/I κ B α /NF- κ B/SOX9 axis during PDAC development and there is a feedback mechanism between NF- κ B pathway and SOX9; SOX9 is associated with expression of MCMs and MDC1, and MDC1 appears to be negatively correlated with invasion and metastasis of PDAC. Future studies should be encouraged to investigate the mechanism underlying the feedback between NF- κ B pathway and SOX9, verified anti-invasion function of MDC1, and to examine whether dysregulation of MCMs contribute to the PanIN formation promoted by SOX9.

MATERIALS AND METHODS

Cell lines and culture

The hTERT-immortalized human pancreatic epithelial nestin-expressing cells (HPNE) and Kras^{G12V}-expressing HPNE cells (HPNE/Kras) were grown in medium containing one volume of M3 Base F culture medium, three volumes of glucose-free Dulbecco's modified eagle's medium, 10% fetal bovine serum, 5.5 mM glucose and 10 ng/ml epidermal growth factor. human papilloma virus (HPV) E6E7-immortalized HPDE cells and Kras^{G12V}-expressing HPDE cells (HPDE/Kras) were cultured at 37 °C in 5% CO₂ in keratinocyte serum-free medium supplemented with 50 µg/ml bovine pituitary extract and 5.0 ng/ml epidermal growth factor. HPNE/Kras and HPDE/Kras had been established in our previous studies and stably express mutant active Kras.^{16,38} The other cell lines were grown in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO₂.

Patients and samples

A total of 104 PDAC patients who had undergone curative resection in Fudan University Shanghai Cancer Center (China) from January to December 2012 were enrolled in this study. Specimens of PDAC and paired tumor-adjacent pancreatic tissues were collected, all of which were confirmed by their pathology. All patients have complete medical records. Detailed clinicopathological characteristics are summarized in Supplementary Table S3. None of the patients received preoperative chemoradiation treatments. These samples were used for the immunohistochemical analysis.

Plasmid construction, transfection, lentiviral production, infection and establishment of stable cell lines. Detailed methods can be found in Supplementary Table S4.

Western blot and quantitative PCR analyses

Western blot and quantitative PCR assays of cells were performed as described previously.^{3,16,38} Q-PCR primer sequences were shown in Supplementary Table S4.

Tissue microarray and immunohistochemistry

Tissue microarrays were constructed and immunohistochemistry was performed as described previously.³⁹ Evaluation of immunohistochemical variables in the tissues was performed using a Leica CCD camera DFC420 connected to a Leica DM IRE2 microscope (UK). SOX9, MCM2 and MDC1 immunostaining was assessed using the semiquantitative histological score (H-score) approach, which combines the intensity and number of cells positive for each marker expression. Ten random × 400 microscopic fields per slide were evaluated. The mean percentage of positively stained cells was scored as follows: 0% (0); 1–10% (1); 11–25% (2); 26–50% (3); 51–75% (4) and 76–100% (5). Staining intensity was categorized as follows: – (0); + (1); ++ (2) +++ (3) and ++++ (4). The evaluation of immunohistochemical variables was performed without knowledge of the clinicopathologic data by two independent investigators. Where discrepancies were observed, results were jointly assessed by both investigators and the final score was formed by consensus.

Cell proliferation and colony formation assay

For cell proliferation assay, methyl thiazolyl tetrazolium assays were performed in triplicate on 96-well plates as previously described.¹⁶ A total of 500 cells contained in the complete growth media were seeded into six-well plates for colony formation assay. The cells were allowed to grow into colonies for 2 weeks at 37 °C under 5% CO₂. Finally the cells were stained with crystal violet. The representative wells of each experiment were photographed.

RNA-seq and gene-set enrichment analysis

Total RNA was extracted in biological triplicates using Trizol from MiaPaCa-2/SOX9-shRNA cells and control cells. RNA-seq was performed on Ion Proton platform at the MD Anderson Cancer Center Core Facility. The comparison of the gene expression profiles of the two groups was performed by using tophat2 and cuffdiff standard procedure. Genes with at least 2.0 fold changes between the two groups were considered statistically significant differential expression. Gene Set Enrichment Analysis was performed using the Gene Set Enrichment analyses software (<http://www.broadinstitute.org/gsea>).⁴⁰

Nude mouse orthotopic xenograft model

A total of 1×10^6 viable cells in 50 μ l of complete growth medium with 50% growth factor-reduced matrigel were injected into the pancreas of 4–6-week-old female non-obese diabetic/severe combined immunodeficient mice; a total of six mice were used for each experimental condition. The orthotopic injection of cells was performed as described previously.¹

Statistical analysis

Analysis was performed with Prism 6.0 software (GraphPad, Aurora, CO, USA). Comparisons between two groups were conducted using the Student's t-test. The one-way analysis of variance was used when comparing more than two groups. For all immunohistochemical variables, the cutoff for definition of subgroups was the median value. Results were considered significant at a P -value<0.05.

Additional details are provided in Supplementary information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Oncogenic Kras induced SOX9 transcription and protein expression. (a–d) Analyses of *SOX9* mRNA and protein expression levels in the indicated cells by quantitative PCR and western blotting. Fold changes of *SOX9* mRNA expression compared to control were shown. (e) AsPc-1 and BxPc-3 cells were treated with 20 μ g/ml cycloheximide (CHX) followed by analyses of the levels of SOX9 at indicated time points by western blotting. (f) Quantification of the blots was shown. The levels of SOX9 protein were normalized to the levels of β -actin protein. The normalized levels of SOX9 protein at 0 h point of CHX treatment were arbitrarily set to 100%. ** $P<0.01$. Error bars represent $\pm$ s.d from three independent experiments.

Figure 2.

Oncogenic Kras promoted nuclear translocation and transcriptional activity of SOX9. (a–d) Confocal microscopy analyses showed SOX9 staining of cell nucleus and cytoplasm in the indicated cells. Scale bar = 20 μm. (e) Western blot analysis of SOX9-p^{S181} expression in the indicated cells. (f) Analyses of *AMH* mRNA expression levels in the indicated cells by quantitative PCR were performed. Fold changes of SOX9 mRNA expression compared to control were shown. Error bars represent ±s.d. from three independent experiments. (g) Analyses of AMH protein expression levels in the indicated cells by western blotting.

Figure 3.

SOX9 overexpression promotes proliferation of pancreatic ductal progenitor cells, and SOX9 knockdown suppresses growth and clone formation of PDAC cells. (a–c) The cell growth of indicated cells was analyzed with methyl thiazolyl tetrazolium assay ($n=5$). Statistical analyses in (b) and (c) were performed between PDAC cells with first shSOX9 and scramble cells. (d, e) The capability of clone formation of indicated cell lines and quantification of clone number were showed ($n=5$). (f) SOX9 knockdown by shRNA resulted in suppressed tumor growth of AsPc-1 cells *in vivo*. The first sh-SOX9 was used in this experiment. (g) Reduced tumor weights of xenografts generated by AsPc-1 cells transfected with SOX9-shRNA ($n=6$). * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Data are presented as means $\pm$ s.d.

Figure 4.

TAK1/IκBα/NF-κB pathway contributed to induction of SOX9 by oncogenic Kras. (**a**, **b**) SOX9 protein expression in indicated cells and at indicated time points following chemicals treatment was analyzed by western blot. Quantification of the blots was shown in right panel. The levels of SOX9 protein were normalized to the levels of β-actin protein and the normalized levels of SOX9 protein at 0 h point of chemicals treatment were arbitrarily set to 1. (**a**) SOX9 expression in iKras cells at indicated time points following doxycycline treatment with or without addition of LYTAK1. (**b**) SOX9 expression in HPNE/Kras and

HPDE/Kras cells at indicated time points after LYTAK1 addition. (c, d) Western blot analyses of SOX9 and TAK1 expression levels were performed in the indicated cells. (e) SOX9 and flag-mutant $\text{ikB}\alpha$ expression levels were determined by western blot in AsPc-1/mutant-*I κ B α* cells and their control cells. (f, g) P65 and phosphorylated p65 (p-P65) were measured in the indicated cells by western blot. Error bars represent $\pm$ s.d. from three independent experiments.

Figure 5.

Gene set enrichment analyses between MiaPaCa-2/*SOX9* shRNA and MiaPaCa-2/scramble and validation of *SOX9*-related genes. **(a)** GSEA analyses identify the enriched gene sets expressed in MiaPaCa-2/*SOX9* shRNA and MiaPaCa-2/scramble using KEGG pathway gene sets. GSEA plots relevant to *CXCL/CCLs*, *MCMs*, *MDC1* and *PCNA* gene sets for MiaPaCa-2/*SOX9* shRNA versus MiaPaCa-2/scramble were shown. Enriched gene sets were selected based on statistical significance (normalized *P*-value<0.05). NES, normalized enrichment score; Nom *P*-val, nominal *P*-value. **(b)** The molecular function of upregulated and downregulated genes in MiaPaCa-2/*SOX9* shRNA comparing to control scramble cells was classified. PANTHER Bar Chart showed the percent of gene hit against the total. Fold change is above 2. **(c, d)** The mRNA and protein expression of indicated genes in the indicated cells were analyzed by quantitative PCR and western blot. Fold changes of *SOX9*

mRNA expression compared to control were shown. * $P<0.05$; ** $P<0.01$. Error bars represent $\pm$ s.d. from three independent experiments. GSEA, Gene Set Enrichment analysis; PCNA, proliferating cell nuclear antigen.

Figure 6.

Analyses of clinical significances of SOX9, MCM2 and MDC1 in patients with PDAC. **(a)** Expression patterns of SOX9 and MDC1 and MCM2 immunohistochemistry of representative tumorous tissues with high or low expression levels are shown (bar, 50 μ m). **(b)** Expression patterns of SOX9 and MDC1 and MCM2 immunohistochemistry of representative tumor-adjacent pancreatic tissues with high or low expression levels are shown (bar, 50 μ m). **(c)** SOX9, MDC1 and MCM2 expression levels in pancreatic cancer tissues were compared, respectively, with tumor-adjacent pancreatic tissues. **(d)** Analysis of MDC1 expression levels in tumor tissues from PDAC patients with different TNM stages. Data are presented as means $\pm$ s.d.

Table 1.
Association of SOX9, MCM2 and MDC1 in tumor tissues with clinicopathologic features of pancreatic cancer

Variable	SOX9			MCM2			MDC1		
	High (N = 48) No. of patients	Low (N= 56) No. of patients	P	High (N= 54) No. of patients	Low (N= 50) No. of patients	P	High (N= 51) No. of patients	Low (N= 53) No. of patients	P
<i>Age</i>									
>60	26	25		27	24		22	29	
≤60	22	31	0.432	27	26	0.847	29	24	0.248
<i>Sex</i>									
Male	26	34		32	28		28	32	
Female	22	22	0.554	22	22	0.843	23	21	0.692
<i>Tumor size</i>									
>2 cm	39	52		48	43		42	49	
≤2 cm	9	4	0.135	6	7	0.769	9	4	0.146
<i>Vascular emboli</i>									
Yes	8	11		10	9		5	14	
No	40	45	0.801	44	41	1.0	46	39	0.041
<i>Perineural invasion</i>									
Yes	40	44		41	43		38	46	
No	8	12	0.622	13	7	0.221	13	7	0.139
<i>Lymph node</i>									
Yes	25	27		28	24		19	33	
No	23	29	0.844	26	26	0.845	32	20	0.018
<i>TNM stage</i>									
I	9	12		12	9		16	5	
II	35	40		37	38		31	44	
III	4	4	0.929	5	3	0.674	4	4	0.019
<i>Histolog. grade</i>									
G1	3	4		6	1		4	3	
G2	26	29		27	28		29	26	
G3	19	23	0.964	21	21	0.179	18	24	0.569

Variable	SOX9		MCM2		MDC1		P
	High (N = 48) No. of patients	Low (N= 56) No. of patients	High (N= 54) No. of patients	Low (N= 50) No. of patients	High (N= 51) No. of patients	Low (N= 53) No. of patients	
CA19-9							
>37 u/ml	38	45	43	40	40	43	
≤37 u/ml	10	11	11	10	11	10	0.81
OS mean, mo	19.9	18.3	17.3	20.9	19.5	18.5	

Abbreviations: TNM stage, tumor-node-metastasis stage; OS, overall survival; mo, month. Fisher' s exact test for all the analyses.