Abstract. – OBJECTIVE: Long noncoding RNA sex determination region of Y chromosome (SRY)-related HMG-box (SOX) is involved in the development of various cancers. However, the molecular mechanism of SOXOT, an overlapping transcript of SOX, in pancreatic cancer (PC) is still undefined. We aimed to explore the epigenetic function of SOX2OT and its downstream factors in advanced PC.

PATIENTS AND METHODS: The levels of SOX2OT, miRNA, and DEK proto-oncogene (DEK) in pancreatic cancer tissues and cell lines were evaluated by quantitative polymerase chain reaction (qPCR). The log-rank test was applied to evaluate the role of high SOX2OT levels in shortening the overall survival of pancreatic cancer patients. The Chi-squared test was made to assess the relation between SOX2OT expression and clinicopathological features of PC patients. Colony assay tested the cell proliferation of PC cells with SOX2OT knockdown. Flow cytometry and Western blotting were used to determine the stemness of tumor cells in vitro. The underlying regulatory mechanism between SOX2OT and miR-200a/141 was predicted by bioinformatics and verified by RNA transfection, qPCR, and Western blotting. Mice xenograft models were applied to determine the promoting effects of SOX2OT on PC in vivo.

RESULTS: The expression of SOX2OT in PC tissues and cell lines is strongly elevated. High levels of SOX2OT expression are more likely to present in patients with advanced TBM stage, positive CD44, and poor overall survival. SOX2OT overexpression promotes proliferation and stemness maintaining of PC cells in vitro and boosts tumor growth in vivo. Furthermore, SOX2OT upregulates DEK expression by binding to miR-200a/141 as a competing endogenous RNA.

CONCLUSIONS: DEK induced by SOX2OT-miR-200a/141 axis may markedly promote stem cell property of PC, resulting in an advanced stage and inferior survival. These findings suggest the SOX2OT-DEK axis as a novel therapeutic target in PC.

Key Words: Pancreatic cancer, Long noncoding RNA SOX2 overlapping transcript, DEK proto-oncogene, Stemness, MiR-200a/141.

Introduction

Although the incidence of pancreatic cancer (PC) ranks over tenth among all cancers, its new cases (458 918) and deaths (432 242) are so close1, suggesting that PC is a destructive malignancy. Early diagnosis and therapy can effectively reduce the harm of pancreatic cancer. The lack of effective early diagnosis and prognostic markers has led to a lack of treatment options for tumor discovery2. Approximately 50% of patients are diagnosed with metastases (liver, abdominal cavity, etc.), resulting in a 5-year survival rate of less than 8%3,4. Although existing treatments such as surgery and radiation/chemotherapy are known to help prolong survival and relieve symptoms, there is not much improvement in overall patient survival4. Therefore, genetic studies of the aberrant expression underlying PC development will help us fully understand the tumorigenesis of PC, furthering the identification of promising therapeutic targets that may ameliorate the inferior survival of patients.

Low survival rates and tumor stem cell characteristics are mediated by long non-coding RNAs (lncRNAs)5,6. LncRNAs are transcripts with more than 200 bases and cannot encode proteins7 but inhibit microRNA (miRNA) function through

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interactions. Generally, carcinogenic lncRNAs are abnormally expressed in malignant tumors, enhancing cell proliferation, metastasis, and stem cell characteristics. Inhibition of these carcinogenic lncRNAs can impair cellular malignant biological behavior, thereby controlling cancer growth and improving therapeutic outcomes. The dysregulation of lncRNA is linked to the proliferation, metastasis and stem cell characteristics of pancreatic cancer cells. LncRNA actin filament-associated protein 1 antisense RNA 1 (AFAP1-AS1) is found to be related to the stemness features. LncRNA growth arrest-specific 5 (GAS5) not only represses epithelial-mesenchymal transition but also inhibits tumor stem cell-mediated chemotherapy resistance via increasing suppressor of cytokine signaling 3 (SOCS3) expression. Linc-dynein cytoplasmic 2 heavy chain 1(DYNC2H1)-4 plays an important role in the maintenance of pancreatic self-renewal.

Here, we address that the anomalous levels of the LncRNA SOX2 overlapping transcript (SOX2OT) in PC cancerous tissues and explore the underlying biological mechanism. We show that increased lnc SOX2OT is linked with CD44, a marker for stemness, and also a strong predictor for poor survival. All these suggest its pathogenic significance for maintaining the destructiveness of PC. Investigating the downstream epigenetic regulation of lnc SOX2OT may help us in developing a novel therapeutic target for PC.

### Patients and Methods

#### Patients, Corresponding Tissues and Follow-Up

The Ethics Committee of the First Hospital of Jilin University approved the study. All patients gave their written informed consent before this investigation. After a biopsy or surgery, a total of 96 PC patients were collected from April 2014 to October 2014. All patients diagnosed by the biopsy-histology. Any distant clinical metastasis (M1 stage, the 7th AJCC TNM staging system) is confirmed by Magnetic resonance imaging (MRI) or bone scanning. Tissue specimens were frozen in -80°C for further experiments. 5-year-follow-up was implemented to attain overall survival.

#### RNA Extraction and Quantitative PCR

Total RNA of tissue and cell line was extracted using RNAiso Plus (TaKaRa, Beijing, China) according to the instruction. The extracted RNA was synthesized to cDNA by the PrimeScript™ RT reagent Kit (TaKaRa, Beijing, China). Quantitative PCR was done using SYBR® Green Real-time PCR Master Mix (TOYOBO, Shanghai, China) on the Applied Biosystems Veriti Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s protocol. The quantitation of the target RNA expression was assessed using the endogenous control by the 2^(-ΔΔCt) method (glyceraldehyde-phosphate dehydrogenase, GAPDH). Qubit Flex Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to evaluate the quality of the prepared RNA, and cDNA was measured. All sequences for primers were shown in Supplementary Table I.

#### Cell Culture

The cell lines Capan-1, BxPC-3, Hs 766T, and normal pancreatic ductal epithelial cell line HPDE were purchased from the Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Shanghai, China). All the cell lines were cultured in 10% fetal bovine serum-supplementing Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA). All of them were in a 37°C atmosphere with 5% CO2.

#### Gene Overexpression and Cells Transfection

For gene overexpression, lentiviral vectors (pcD-ciR vector, Geenseed Biotech, Guangzhou, China) were used to construct SOX2OT expressing particles (oe-SOX2OT, SOX2OT sequence was shown in Supplementary Table I). SOX2OT-cDNA or NC-cDNA (MOI = 20) with polybrene was transfected into BxPC-3 and Hs 766T. 24 h after transfection, a new medium replacement was done to the culture medium. Stably transfected cells were selected by puromycin (1 μg/ml). The Puromycin (1 μg/ml, 2-3 times) selection was made until green fluorescence was shown in all cancer cells via the fluorescence microscope (Olympus IX71, Japan).

#### Proliferation Ability of Tumor Cells

PC cells were cultured in 96-well plates (5 \times 10^4 cells/well) for 2 weeks. Then, tumor cells were implanted with 500 cells/well for colony formation assay. Finally, 4% paraformaldehyde (5 min) and 1% crystal violet (10 min) were used to fix and stain the colonies. All colonies were observed by a microscope.
Flow Cytometry

The surface markers analysis of the tumor cells was performed by flow cytometry. Fifty thousand cells stained with antibody (Percp-cy5.5-CD44, PE-CD24, BD Biosciences, Franklin Lakes, NJ, USA) were measured by BD Accuri C6 Flow Cytometry (BD Biosciences, Franklin, NJ, USA).

Animal Experiment and Tumor Growth In Vivo

4-week-old BALB/c-nude mice were purchased from the Laboratory Animal Center of Jilin University, fed in an atmosphere with a 12 h light/dark cycle under specific pathogen-free conditions. PC cells (2 × 10^5 cells in 100 ul) transfected with an expression vector (oe-SOX-2OT) or vector control (oe-NC) were respectively incubated subcutaneously into mice (each group n = 5). The tumor sizes of every mouse were collected every week. In the fifth week, tumor weight was measured after the mice were sacrificed. All the procedures of these experiments attained approval from the Institutional Animal Care and Use Committee of the First Hospital of Jilin University.

Dual-Luciferase Reporter Analysis

The wildtype lncRNA SOX2OT sequences containing miRNA-binding sites or mutant sites were cloned to psiCHECK-2 plasmid (Promega, Madison, WI, USA). Then, following the manufacturer’s instruction, the Renilla Luciferase reporter vector was transfected into PC cells along with the synthetic vectors, miRNA, anti-miRNA, or relative controls. The above transfected PC cells were seeded for incubation 48 h. The Luciferase activities of the target gene were tested by a Dual Luciferase-Reporter Assay System (Promega, Madison, WI, USA).

Western Blotting Assay

Radioimmunoprecipitation assay (RIPA) buffer (Solarbio, Beijing, China) was applied to extract total proteins, supplementing with 1% phosphorylation and protease inhibitors (Solarbio, Beijing, China). According to the manufacturer’s protocol, the concentration of the protein samples was tested by the bicinchoninic acid assay (BCA) protein assay kit (Tiangen, Beijing, China). After denatured at 96°C for 10 min, 9% sodium do-
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deoxysulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Solarbio, Beijing, China) used to divide the target proteins. The polyvinylidene difluoride (PVDF) membrane (Solarbio, Beijing, China) was used for transfer. After incubation with 5% non-fat milk for a blockade of non-specific signals, PVDF membranes were incubated with primary antibodies against Nanog (1:4000), OCT4 (1:3000), DEK (1:2000), and GAPDH (1:4000) (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Then, the PVDF membrane was dealt with horseradish peroxidase (HRP) conjugated secondary antibody (1:8000, Cell Signaling Technology, Danvers, MA, USA). The protein blots were photographed using a Western imaging system (General Electric Company, Boston, MA, USA). The density of bands was quantified by Image J software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis
All data were analyzed using GraphPad Prism 6 software (San Diego, CA, USA), and presented as the mean ± standard deviation. Comparison between two groups was carried out by Student’s t-test. Each experiment was done independently three times. \( p < 0.05 \) was defined as a significant difference.

Results

**Elevated SOX2OT Expression Is Highly Related to CD44 and Poor Survival in PC**

By analyzing 96 pairs of tissues from patients, we found SOX2OT expression inclined in PC tissues relative to adjacent normal tissues (Figure 1A, \( p < 0.01 \)). Then, we observed that the expression of SOX2OT was higher in PC...
cells (Capan-1, Hs766T, BxPC-3) than normal pancreatic ductal epithelial cell line (HPDE) (Figure 1B, p < 0.01). Based on Kaplan-Meier analysis, a higher expression of SOX2OT was associated with inferior overall survival (Figure 1C, p < 0.05). Moreover, PC patients with late TNM stage and positive CD44 expression (stemness) exhibited notably increased SOX2OT expression than PC tissues with less advanced TNM stages (Table I, p = 0.037) or negative CD44 expression (Table I, p = 0.003; Figure 1D, p < 0.01), respectively. Altogether, our results indicated that SOX2OT is up-regulated in PC tissues, and it demonstrates an oncogenic role in PC by maintaining stemness.

**SOX2OT Promotes PC Cell Proliferation and Stem Cell Feature In Vitro**

A synthesized lentiviral SOX2OT-overexpressing (oe-SOX2OT) vector was transfected into two cell lines (Hs766T and BxPC-3) to increase SOX2OT expression (Figure 2A, B, p < 0.01). Then, we applied molecular experiments *in vitro* to verify the regulatory function of SOX2OT on cell proliferation and stem cell feature. Figure 2C, D show the proliferative ability and CD24+CD44+ stem cell feature in PC cells transfected with overexpressing vector or vector control measured by colony assay (400×). Figure 2E, F show the percentage of CD24+CD44+ cell (%). **p < 0.01, *p < 0.05.**

**Figure 2.** SOX2OT promotes PC cell proliferation and stem cell feature in vitro. A, B, SOX2OT expression was increased by lentiviral vectors in PC cells. C, Proliferative ability of PC cells transfected with overexpressing vector or vector control measured by colony assay (400×). D, The CD24+CD44+ rate of PC cells transfected with overexpressing vector or control showed by flow cytometry. **p < 0.01, *p < 0.05.**
the biological behavior of tumor cells. Colony formation assays revealed that the proliferation of Hs766T and BxPC-3 (Figure 2C, $p < 0.05$) increased notably after SOX2OT overexpression. Also, the percentage of CD24+CD44+ cells (Figure 2D, $p < 0.05$) and the expression of Nanog, OCT4 (distinctive transcription factors of cancer stem cell) (Figure 3A, B, $p < 0.05$) were inclined after the SOX2OT level was increased. Therefore, these data suggest that lncRNA SOX2OT can mediate the stem-cell-abilities of PC cells in vitro, including proliferation and distinctive surface markers.

**SOX2OT Accelerates PC Tumor Growth In Vivo**
Hs766T and BxPC-3 cells transfected with oe-SOX2OT or oe-NC were incubated in immune-deficient mice to induce xenograft tumor models. The experiment revealed that increased SOX2OT accelerated the growth (Tumor size, figure 4 A, B, $p < 0.05$; Tumor weight, figure 4 C, D, $p < 0.05$) of PC tumor nodes, compared to that in vector control mice. The macroscopic observation of tumor nodes is shown in Figure 4 E, F. Thus, these results suggest that SOX2OT dysregulation is involved in PC tumor formation in vivo.

**SOX2OT Modulates PC Stemness by Targeting MiR-200 Family**
Bioinformatic analysis by miRTarBase predicted the complementary binding potential between SOX2OT and miR-200a/200b/141 (Figure 5A). Assays validated that the Luciferase activity of miR-200a/200b/141 family was significantly repressed by the wildtype SOX2OT rather than the mutant form in PC cells (Figure 5B, C, D, $p < 0.01$). Compared to the oe-NC treated cells, the expression of miR-200a/200b/141 declined in PC cells transfected with oe-SOX2OT (Figure 5E, $p < 0.01$). Further verifying that SOX2OT could target miR-200 family, the expression of miR-200a/200b/141 in PC cells (BxPC-3 and Hs 766T, Figure 5F, $p < 0.01$) and cancerous tissues (Figure 3).

![Figure 3. SOX2OT promotes stem cell feature of PC in vitro. A, B. Western blot assay showed the OCT4 and Nanog protein expression in PC cells transfected with overexpressing vector or control in BxPC-3(A) and Hs 766T(B). * $p < 0.01$, ** $p < 0.05$.](image-url)
5G, \( p < 0.01 \) was markedly lower than normal ones, shown by qPCR. Collectively, our data suggested that SOX2OT interacted with miR-200a/200b/141 as a competing endogenous RNA to modulates stemness in CRC.

**MiR-200a/141 Directly Targets DEK in PC Development**

Based on bioinformatics analysis using TargetScan, we predicted that DEK proto-oncogene (DEK, protein coded by oncogene at 6p22.3), which exhibits increased expression in PC, may be a downstream target for miR-200a and miR-141 but not miR-200b. Their complementary binding relation (Figure 6A) was verified by a Luciferase reporter assay (Figure 5C, D, \( p <0.01 \)). DEK expression was inclined in cancer cells transfected with oe-SOX2OT, compared to that in vector controls (Figure 5E, \( p <0.01 \)). Altogether, these results indicate that DEK is an effector protein targeted by SOX2OT/miR-200 axis in PC.

**Discussion**

Despite extensive research on the molecular mechanisms of tumor cell stem cell characteristics, the underlying causes of malignant tumor invasion, metastasis and drug resistance, there is still a lack of an effective intervention strategy. Exploring the significant molecular characteristics of the stem-cell-phenotype in cancerous cells may lead to the discovery of more effective therapeutic targets. We investigated the molecular mechanism of SOX2OT/DEK regulatory axis
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in the maintenance of stem cell characteristics in pancreatic cancer cells and explored potential new interventions in tumor progression. Previously, researchers have confirmed that SOX2OT can be used as an oncogene for various cancers\(^{15}\). SOX2OT was found to be significantly correlated with overall survival in esophageal squamous cell carcinoma\(^{16}\). Besides, SOX2OT levels in serum were strongly associated with larger tumor size, advanced stage, and positive lymph node of lung squamous cell carcinoma\(^{17}\). Moreover, SOX2OT accelerates the expression of Sox2, thus promoting epithelial-mesenchymal transition and stem cell-like properties of PC in a competitively binding way\(^{18}\). Since Sox2 shares part of the gene sequence with the adjacent Sox2ot gene, most of the current studies believe that the final regulatory target of SOX2OT is Sox2. Little is known about the non-SOX2 regulatory axis mediated by SOX2OT, especially in PC. In this study, we found that the aberrant expression of SOX2OT is strongly related to tumor stem cell characteristics and may be an independent predictor of inferior overall survival of pancreatic cancer (Figure 1). The silencing of SOX2OT in pancreatic cancer cells inhibits the proliferation

Figure 5. SOX2OT modulates PC stemness by targeting miR-200 family. A, The schematic diagram presents the complementary binding sites within SOX2OT and miR-200a/200b/141. B, C, D, Luciferase reporter assay confirmed the molecular binding between SOX2OT and miR-200 family. E, qPCR showed the miR-200a/200b/141 expression in PC cells transfected with overexpressing vector or control. F, MiR-200a/200b/141 expression in PC cells (Capan-1, Hs766T, BxPC-3) and HPDE cells. G, MiR-200a/200b/141 expression in PC tissues and adjacent normal tissues shown by qPCR. *p < 0.05, **p < 0.01.
of *in vitro* cell lines (Figure 2) and the growth of tumors *in vivo* (Figure 4). SOX2OT can alter the biological behavior of tumor cells through non-SOX2 molecules via miRNA regulation. Altogether, these results indicate that SOX2OT does participate in stemness maintaining and poor prognosis in PC patients.

Competitively binding to target microRNAs for post-transcriptional regulation is a distinctive interaction style of IncRNAs. In our study, bioinformatics analysis predicted the interaction between the SOX2OT and miR-200 families (miR-200a/200b/141) (Figure 5A). The Luciferase activity of the miR-200 family was inhibited by wild-type SOX2OT but was not inhibited by the mutant, thus demonstrating the direct-binding between them (Figure 5B, C). Furthermore, our results show that SOX2OT overexpression promotes the increased expression of the miR-200 family in tumor cells (Figure 5E). Therefore, since the oncogenic role of SOX2OT and its competitive repression on the miR-200 family, the miR-200 family is a significant suppressor of tumor development. Diaz-Riascos et al. found that

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**Figure 6.** miR-200a/141 directly targets DEK in PC development. A, Bioinformatics tools reveal the complementary binding sites within miR-200a/141 and DEK 3’-UTR. B, C, Luciferase reporter assay validated the molecular binding between miR-200a (B)/141(C) and DEK 3’-UTR. D, Western blot assay showed the DEK protein expression in PC cells transfected with SOX2OT overexpressing vector or control. **p < 0.01, *p < 0.05.
miR-200a, miR-429, and miR-205 are frequently overexpressed in pancreatic tumors in the mi-200 family, while cadherin-1 (CDH1) is downregulated. Functional validation indicated that miR-141 and miR-429 inhibit the tumorigenic potential of pancreatic cancer cells. The restraining function of miR-200 family members on migration and invasion of pancreatic cancer cells can be mediated by the suppression of the sonic hedgehog (Shh) pathway via modulating the expression of cyclin D1 and Bcl-2. An intensive investigation found that ribonuclease monocyte chemoattractant protein-1 (MCP1) can counteract Dicer1 activity during miRNA-200 family maturation, leading to a dysregulation of this family in pancreatic cancer. Our results represent the novel investigation on the important role of the miR-200 family in stemness maintenance in PC breast, and our observations are in accordance with other researches of the miR-200 family in breast cancer and glioma.

DEK is a structural protein within chromatin, possessing the chaperone activity of histone H3. Several molecular functions in nuclei are regulated by DEK activity, including transcription, DNA repair, and homologous recombination. Besides, DEK was found to be of importance for long-term self-renew of stem cells, including hematopoietic ones and cancerous ones. In mammary tumors, overexpressing miR-489 can decrease progenitor cell populations (CD49fhiCD61hi) by mediating oncogenes including DEK and SHP2, suppressing tumor growth and metastasis. Increased DEK aggravates the mammosphere formation (stem cell characteristic) and motility of breast cancer cells partly via β-catenin activation. Shibata et al. found that the aberrant chromosomal-gain of the DEK oncogene locus (6p22.3) was significantly associated with poor prognosis in high-grade neuroendocrine carcinoma. Silencing of DEK by small hairpin RNA leads to loss of lung cancer stem cell property. Although DEK is addressed to be involved in the metastasis and inferior overall survival of patients with PC, few studies have demonstrated the role of DEK in stemness maintaining in PC cancer cells. Our research has shown that the SOX2OT-miR200 axis increases the endonuclease level of DEK in PC cells with a stem-cell-feature (Figure 6). Altogether, our results suggest that DEK acts as a modulator of stem cell property in PC cancer cells via a lncRNA-miRNA-interaction way.

**Conclusions**

We have demonstrated that high levels of SOX2OT expression are associated with stem cell characteristics in PC. SOX2OT can strongly promote the proliferation and stemness maintaining of PC in vitro. Furthermore, DEK is upregulated by increased SOX2OT via complementary binding to miR-200a/141, resulting in tumor growth of PC. These data suggest that the lncRNA SOX2OT- miR-200a/141-DEK axis may be a promising therapeutic target for PC.

**Conflict of Interest**

The Authors declare that they have no conflict of interests.

**Data Availability Statement**

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

**Disclosure of Financial Arrangements**

The research and manuscript preparation are funded by Yu Fu.

**Authors’ Contribution**

Guarantor of integrity of the entire study: Yu Fu; Study concepts: Yu Fu; Study design: Yu Fu; Literature research: Qiang Zhou; Clinical studies: Yandong Zhang; Animal experiments: Chuishui Liu, Yandong Zhang; Molecular assay: Chuishui Liu; Data acquisition: Chuishui Liu; Statistical analysis: Chuishui Liu, Qiang Zhou; Manuscript preparation: Chuishui Liu; Manuscript editing: Chuishui Liu; Manuscript review: Yu Fu, Yandong Zhang, Qiang Zhou.

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