SOX2 regulates apoptosis through MAP4K4-Survivin signaling pathway in human lung cancer cells

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Previous studies have implicated cancer stem cells in tumor recurrence and revealed that the stem cell gene SOX2 plays an important role in the tumor cell resistance to apoptosis. Nonetheless, the mechanism by which SOX2 regulates apoptosis signals remained undefined. Here, we demonstrated the surprising finding that silencing of the SOX2 gene effectively induces apoptosis via the activation of death receptor and mitochondrial signaling pathways in human non-small cell lung cancer cells. Unexpectedly, reverse transcription–PCR analysis suggested that downregulation of SOX2 leads to activation of MAP4K4, previously implicated in cell survival. Evaluation of the apoptotic pathways revealed an increased expression of key inducers of apoptosis, including tumor necrosis factor-α and p53, with concurrent attenuation of Survivin. Although p53 appeared dispensable for this pathway, the loss of Survivin in SOX2-deficient cells appeared critical for the observed MAP4K4 induced cell death. Rescue experiments revealed that SOX2-silencing-mediated killing was blocked by ectopic expression of Survivin, or by reduction of MAP4K4 expression. Clinically, expressions of Survivin and SOX2 were highly correlated with each other. The results reveal a key target of SOX2 expression and highlight the unexpected context-dependent role for MAP4K4, a pluripotent activator of several mitogen-activated protein kinase pathways, in regulating tumor cell survival.

Introduction

Several studies have demonstrated that cancer stem cells (CSCs) are tumor-initiating cells that exhibit greater resistance to apoptosis than differentiated cancer cells (1–3). This antiapoptotic nature makes CSCs resistant to programed cell death, providing a mechanism of chemotherapy resistance that may promote cancer recurrence. Mechanisms to increase apoptosis or to induce differentiation among CSCs have been touted as a strategy for cancer therapy (4). In this regard, the stem cell marker gene SOX2 has been found to contribute to tumorigenesis and recurrence of certain cancers (5,6). Targeting SOX2 was reported to be effective for cancer therapy; however, the functions of SOX2 in the apoptosis-resistant nature of cancer cells and its underlying mechanisms still need to be explored.

Apoptosis is a programmed cell death process, which plays key roles in numerous physiological and pathophysiological processes, including organ development, tissue homeostasis and immunoregulation (7,8). Apoptosis has been broadly classified to occur via ‘extrinsic’ and ‘intrinsic’ apoptotic signaling pathways (9). The latter pathway, which is also known as the mitochondrial pathway (10), undergoes two major changes upon activation by apoptotic signals of mitochondria. These include permeabilization of the outer membrane and reduction of the inner membrane potentials, resulting in release of cytochrome c, which interacts with apoptotic protease activating factor-1, leading to the recruitment of procaspase 9 to form a complex termed the apoptosome (11). The apoptosome, in turn, cleaves caspase 3, resulting in activation of the caspase cascade and progression of apoptosis. In contrast, the extrinsic pathway of apoptosis (also known as the death receptor pathway), is activated upon binding of extracellular ligands, such as tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand, to specific death receptors on the plasma membrane. This is followed by recruitment of adaptor molecules that assemble into a ‘death-inducing signaling complex’ where TNF-receptor-associated death domain protein or Fas-associated protein with a death domain recruits procaspase 8. The procaspase is activated at the death-inducing signaling complex, dimerizing and promoting cell death either via direct cleavage of caspase 3 or via an amplification step via cleavage of t-BID and activation of the intrinsic pathway. A common terminal process in either apoptotic pathway is the activation of DNasease, which cleaves interhistone segments of DNA, producing a characteristic DNA ‘laddering’ (12).

Survivin belongs to the inhibitor family of apoptosis proteins that counteract the function of apoptotic proteins (13), thus contributing to the antiapoptotic properties of tumor cells (14,15). Previous studies indicated that Survivin is specifically overexpressed by almost all human tumors but undetectable in normal adult tissues, suggesting its possible contribution to tumorigenesis (16–21). The expression of Survivin in non-small cell lung cancer (NSCLC) is significantly elevated in adenocarcinomas, squamous cell carcinomas and large cell carcinoma subtypes of lung cancer (20). Survivin may be localized in both the cytoplasm and nucleus of tumor cells (21–23), with cytoplasmic localization considered a predictor of poor prognosis of cancer patients (21). The Survivin transcription is activated by mitogen-activated protein kinase (MAPK) signaling pathways or by c-Myc (24,25), whereas p53, Runx2 and RB/EF2 all have inhibitory effects on the activation of its promoter (26–28). Since Survivin plays such an important role in regulating the apoptosis resistance of tumors, targeting of Survivin maybe a potential promising strategy for lung cancer therapy.

Here, a lentivirus system was used to deliver shRNA to NSCLC cells—resulting in silencing of SOX2 gene expression and effectively induced apoptosis in vitro and in vivo. Surprisingly, cell death required MAP4K4 activation, which has been previously associated with survival activity. In this case, apoptosis was context dependent, requiring the loss of Survivin caused by downregulation of SOX2. Together, these findings revealed a significant mechanism by which SOX2 regulates apoptosis signals, and further identify Survivin as a key downstream molecule that contribute to the antiapoptotic properties of SOX2.

Materials and methods

Ethics statement

The animal experiments were approved by the Institute Research Ethics Committee at the Nankai University and performed strictly under the guidelines...
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on laboratory animals of Nankai University. All efforts such as anesthetized with a mixture of oxygen/isoflurane were made to minimize the suffering of mice.

**Cell culture**

Wild type of A549, H1299 and H460 cells were obtained from ATCC. A549 cells with stable overexpression of FL (A549-FL) and A549, H460, H1299 or A549-FL cells with stable expression of Doxycycline (Dox)-inducible shRNA-SOX2—A549/H1tet-FL/shRNA-SOX2 (A549-sh-SOX2), H460/H1tet-FL/shRNA-SOX2 (H460/sh-SOX2), H1299/H1tet-FL/shRNA-SOX2 (H1299/sh-SOX2) or A549-FL/H1tet-FL/shRNA-SOX2 (A549-FL/sh-SOX2) and their control cell lines A549/H1tet-FL/shRNA-scrumble (A549-S55), H460/H1tet-FL/shRNA-scrumble (H460-S55), H1299/H1tet-FL/shRNA-scrumble (H1299-S55) or A549-FL/H1tet-FL/shRNA-scrumble (A549-FL/S55) were generated following the protocols described previously (29). Stable polyclonal cells of A549 or H460/H1tet-FL/shRNA-SOX2/H1tet-FL/shRNA-MAP4K4 (A549 or H460/sh-SOX2/sh-MAP4K4) and their scramble control cells A549 or H460/H1tet-FL/shRNA-scrumble (A549 or H460/sh-SOX2/sh-scrumble) were established separately by infecting A549 or H460/sh-SOX2 cells with lentivirus carrying Lv-H1tet-FL/shRNA-MAP4K4-Bsd and Lv-H1tet-FL/shRNA-scrumble-Bsd (Biosettia, San Diego, CA) and selected by 10 μg/ml Blasticidin (Bsd). Stable polyclonal cells of A549/H1tet-FL/shRNA-scrumble/ Survivin (A549/S55/Survivin), A549/H1tet-FL/shRNA-SOX2/Survivin (A549/ sh-SOX2/Survivin) and their control cells A549/H1tet-FL/shRNA-scrumble/Con (A549/S55/Con), A549/H1tet-FL/shRNA-SOX2/Con (A549/sh-SOX2/Con) were established separately by infecting A549-S55 or A549-sh-SOX2 cells with lentivirus carrying Lv-EF1-Fluxin-ires-Bsd and empty vector control Lv-EF1-Fluxin-ires-Bsd (Biosettia, San Diego, CA), followed by clonal selection using 10 μg/ml Bsd. The shRNAs used in this study are summarized in Table I. Stable A549-sh-SOX2 cells with overexpression of SOX2 gene (A549-sh-SOX2/Con) and its control cell line (A549-sh-SOX2/Con) were established by infecting A549-sh-SOX2 cells with lentivirus carrying Lv-EF1-Fluxin-SOX2/Con-Bsd and empty vector control Lv-EF1-Fluxin-ires-Bsd (Biosettia, San Diego, CA), followed by clonal selection using 10 μg/ml Bsd.

**Cell treatment and time-dependent survival curve**

Cells were plated on six-well culture plates at 25–30% confluency and incubated with Dox; they were washed with phosphate-buffered saline twice for removal of unattached dead cells. Propidium iodide (Sigma–Aldrich, Shanghai, China) was then added to the culture medium to 1 μg/ml for visualization of the remaining cells undergoing apoptosis. The cells were monitored at indicated time point in five fixed fields of culture dish around the center region under a microscope by a ×10 objective for each test; the number of living cells was defined as attaching cells minus those positive for propidium iodide staining in each image field and then averaged. For all the following in vitro experiments, cells were treated with 1 μg/ml Dox for 96 h to induce expression of shRNA prior to each experiment.

**Western blotting**

Cell lysates were prepared from A549, H460 and H1299 cells lines with radioimmunoprecipitation assay buffer in the presence of Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail 2 and 3 (P8340, P5726 and P0044, Sigma–Aldrich, Shanghai, China) as described previously (30). Protein (40 μg) was loaded onto 5–12% Tris–Acrylamide gels and blotted with antibodies that included anti-SOX2, TNF-α, Ki67 and Survivin (Cat. # ab95797, ab9739, ab16667 and ab16667, Abcam, Cambridge, UK) antibody at 1:200 and 1:100 dilutions respectively, and ≥50% was judged as strong immunostaining intensity. Specifically, ≤10% was negative, 11–30% was weak, 31–49% was moderate, and ≥50% was judged as strong immunostaining intensity.

**Flow cytometry analysis**

A549, H460 and H1299 cells were harvested after 96-h treatment with Dox, which induced SOX2 knock down. Cells were washed three times with phosphate-buffered saline and then prepared for annexin-V and propidium iodide staining according to the manufacturer’s directions (Cat. # KG850, KeyGEN Biotech, China), then subjected to Western blotting to detect the expression of Cytochrome C with the antibody (Cat. # 10993-1-AP, Proteintech Group, Chicago, IL). Hematoxylin and eosin staining

Tissue sections were deparaffinized with xylene and nuclei were stained with hematoxylin, rinsed in running tap water, differentiated with 1% HCl in 70% alcohol. This was followed by counterstaining with eosin, then dehydrated and rendered transparent in xylene, to be finally coverslipped with mounting media.

**Immunohistochemistry and tissue microarrays**

Expressions of SOX2 and Survivin in high-density tissue microarrays (Cat. # LC952, LC2085b, LC2161, Alenabio, Xi’an, China PR) and xenograft tumors from lung tissues of nonobese diabetic/severe combined immunodeficiency mice were detected by standard biotin–avidin complex method with antibody against SOX2 (Cat. # ab75485, Abcam Inc, Cambridge, UK) at a 1:200 dilution. Flow cytometry analysis

**Real-time reverse transcription–PCR**

Total messenger RNAs were isolated from A549, H460 and H1299 cells and reverse transcribed into complementary DNAs with Moloney Murine Leukemia Virus reverse transcriptase (Cat. # D2640, TaKaRa, Dalian, China). Primers used for this experiment are summarized in Table I. Real-time reverse transcription–PCR was performed following the procedure described previously (29).

**Tumor xenograft and bioluminescence imaging**

Male NOD/SCID mice at 6–8 weeks of age were grafted with 1 × 10⁶ A549-FL/SS or A549-FL/sh-SOX2 cells through tail vein injection; the bioluminescence signals were monitored as described previously (29). All mice were fed 0.2 mg/ml Dox plus 0.05% sucrose in their drinking water from day 42 after inoculation.

**Statistical analysis**

Values were expressed as means ± standard error of the mean. Significance in Figure 6 (Correlation between Survivin and SOX2) was determined by the non-parametric method ( spearman’s rho, two tailed), and others were determined by Student’s t-test. A value of P < 0.05 was used as the criterion for statistical significance: * indicates significant difference with P < 0.05 and ** indicates highly significant difference with P < 0.01.

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Table I. shRNAs designed to downregulate the expression of corresponding genes

<table>
<thead>
<tr>
<th>sh-SOX2</th>
<th>sh2-SOX2</th>
<th>sh1-MAP4K4</th>
<th>sh2-MAP4K4</th>
<th>Scramble control</th>
</tr>
</thead>
<tbody>
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<td>AAAAGGGCACATGACGATGGATCGATCAGCATGTATTGGATCCAATACATGCTGATCATGTCCC</td>
<td>AAAAGCTACACTATCGAGCAATTTTGGATCCAAAATTGCTCGATAGTGTAGC</td>
<td>AAAAGGGCACATGACGATGGATCGATCAGCATGTATTGGATCCAATACATGCTGATCATGTCCC</td>
<td>AAAAGCTACACTATCGAGCAATTTTGGATCCAAAATTGCTCGATAGTGTAGC</td>
<td>AAAAGGGCACATGACGATGGATCGATCAGCATGTATTGGATCCAATACATGCTGATCATGTCCC</td>
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Regulation of lung cancer cell death

Results

Silencing of SOX2 gene induces apoptosis in human lung cancer cells

SOX2 regulates cell cycle and contributes to antiapoptosis properties of tumor cells (31) and is critical for maintaining the stem cell population during normal cell growth (29). To elucidate the function of SOX2 in regulating cell survival and apoptosis, we used a lentivirus system in which insertion in the chromosome created a system of Doxycycline (Dox)-inducible shRNA expression. Using a cultured tumor cell model of human lung cancer (A549 cells), we observed that lentivirus infection resulted in stable cell lines in which exposure to Dox resulted in suppression of SOX2 relative to untreated cells (29). We then tested whether exposure to Dox resulted in changes in the pattern of cellular proliferation. Dox treatment of A549 cells harboring a ‘scrambled’ shRNA construct resulted in sustained cell growth, whereas exposure to Dox in A549 cells harboring either two different shRNAs to SOX2 resulted in suppression of SOX2 expression and of cell growth over a period of 96 h (Figure 1A). In fact, cell numbers actually declined during the final 24 h following SOX2 suppression.

To test whether the decline in cell number was due to apoptosis, we performed annexin-V staining after 96 h of Dox treatment (Figure 1B). Among the cells harboring shRNA-SOX2 constructs, significant apoptosis was observed (~45%). Although the scrambled cells grew to a higher concentration, they were still remarkably healthy, with only about 3% of cells undergoing apoptosis. Thus, suppression of SOX2 expression in A549 cells results in apoptosis within a period of 3–4 days.

To determine whether this was unique to the A549 cell line, we established two additional cell lines in which the SOX2 gene could be silenced in an inducible manner: the human lung adenocarcinoma cell line H1299 and the human large cell carcinoma cell line H460 (Figure 1C). Relative to A549 cells, an almost similar efficiency of SOX2 suppression was observed following exposure to Dox in these cell lines (~77% in H1299 and 82% in H460). Importantly, suppression of cell growth was observed as early as 48 h after exposure to Dox, and an apoptotic response was confirmed by annexin-V assay (Figure 1D and Supplementary Figure S1A, available at Carcinogenesis Online). The results suggest that SOX2 may be a critical gene for the survival of human lung cancer cells since suppressing SOX2 compromises cell survival and vigor. To further exclude the off-target effect of shRNA, we overexpressed SOX2 gene in A549 with SOX2 silencing (Supplementary Figure S1B, available at Carcinogenesis Online) and found that it can significantly rescue the apoptotic effect induced by SOX2 silencing, which was reflected by the reduced amount of cytochrome c presented in the cytosol and the reduced cleavage of caspase 3 (Supplementary Figure S1B, available at Carcinogenesis Online).

Silencing of SOX2 gene induces apoptosis through both mitochondria and death receptor signaling pathways

Once a role for SOX2 in suppressing the apoptosis of human lung cancer cells was identified, the underlying mechanism by which SOX2 regulates this process was explored. Initial proteomic array suggested significant alterations of key proteins in the mitochondrial apoptosis signal pathway, including p53, Bax and Bad (Supplementary Figure S2, available at Carcinogenesis Online). We extended these initial screens by direct immunoblot for these proteins. As shown in Figure 2A, the expression of p53 (and its phosphorylated form), as well as that of the proapoptotic proteins—Bax, Bad and cytosol cytochrome c, were dramatically increased following the silencing of SOX2, whereas the expression level of the prosurvival proteins XIAP and Survivin was decreased.

In contrast, no significant changes were observed in the expression of receptors or adaptors specific to the extrinsic pathway (Supplementary Figure S2, available at Carcinogenesis Online), providing at least a superficial indication that this apoptotic pathway was not enhanced. However, we could not exclude this pathway completely since we had observed that key downstream negative modulators of both the intrinsic and extrinsic pathways (i.e. XIAP, Survivin) were diminished following suppression of SOX2. Investigating this somewhat further, we determined that the expression of TNF-α, a death receptor ligand, was dramatically upregulated. Concomitantly, the downstream target of death receptors, caspase 8, was observed to be undergoing maturation cleavage selectively among cells in which SOX2 was suppressed (Figure 2A). Similar results were observed following SOX2 downregulation in H460 and H1299 cells (Supplementary Figure S3, available at Carcinogenesis Online). Thus, our data altogether supported the notion that downregulation of SOX2 induces apoptosis by activating elements in both the mitochondrial and death receptor signal pathways. By checking the protein expression levels of cell-cycle-related proteins, we found decreased Cyclin A2, B1, D1, E2 and Ki67 in two A549-sh-SOX2 cells (Figure 2B). The almost similar results were also observed in H460 and H1299 cells (Supplementary Figure S3, available at Carcinogenesis Online) even though the expressions of Cyclin A2 in H460 cell and Cyclin B1 in H1299 cell did not show significant changes after SOX2 silencing. These results indicate suppressed cell proliferation and reduced amount of cells in the cell cycle upon SOX2 silencing in these lung cancer cell lines.

Silencing of SOX2 activates MAPK signaling, altering the expression of TNF-α, p53 and Survivin

MAPK signaling pathways regulate inflammation and apoptosis in part by coordinated controlling expression of TNF-α and p53. Specifically, MAPK4 is a key mediator of TNF-α signaling (32) that also has been implicated in the activation of the TNF-α promoter (33,34), thus creating a ‘signal amplification’ circuit. The expression of both SOX2 and MAPK4 has been independently associated with accelerated NSCLC tumor progression (35,36). Since MAPK4 has been strongly associated with the survival of a number of different carcinoma cells (36–38), we initially presumed that the proapoptotic loss of SOX2 would result in a concordant decrease in expression of MAPK4.

Surprisingly, however, we detected significantly increased MAPK4 messenger RNA expression (~2.5-fold) that was associated with the cell death following SOX2 suppression (Figure 3A).
Fig. 1. Silencing of SOX2 gene induces apoptosis in human lung cancer cell lines. (A) A549 cells with stable expression of Dox inducible expression of shRNA-SOX2—A549-sh-SOX2, and A549-SS controls were incubated with Dox for 4 days and the downregulation of SOX2 was tested by western blotting (left panel). Right panel showed the time-dependent survival curve of A549 cell lines with or without silencing of SOX2 (n = 3). (B) Representative flow cytometry analysis of apoptotic cells in the A549 after 4 day addition of Dox to their culture medium (left panel) and the corresponding statistical evaluation (n = 3). (C) Upper panel: western blotting results of SOX2 in H1299-sh-SOX2, H460-sh-SOX2 cells and their controls (SS) after incubation of Dox for 4 days. Lower panel: time-dependent survival curve of H1299 and H460 cells after incubation with Dox (n = 3). (D) Statistical results of the apoptotic cells from flow cytometry results shown in Supplementary Figure S1A, available at Carcinogenesis Online, n = 3.
were also observed in these three cell lines (Supplementary Figure S4A, available at Carcinogenesis Online). To test this unexpected proapoptotic role of MAP4K4 following SOX2 silencing, lentiviral knockdown of MAP4K4 was performed in A549-sh-SOX2 and H460-sh-SOX2 cells (Figure 3B and Supplementary Figure S4B and C, available at Carcinogenesis Online). As shown, the cosuppression of MAP4K4 prevented the elevation of TNF-α in response to silencing of SOX2 (Figure 3B and Supplementary Figure S4C, available at Carcinogenesis Online) and rescued cell survival following knockdown of the SOX2 gene (Figure 3C and D, Supplementary Figure S4C, available at Carcinogenesis Online) in both cells.

MAP4K4 has been implicated in suppressing the insulin response in muscle tissue (39) and in predicting chemotheraphy efficacy (36). However, it has not been previously assigned a role in promoting apoptosis and in fact has been associated with increased tumor malignancy in some cases (37,38). Given this, we considered the possibility that MAP4K4-triggered apoptosis might only occur in a context-dependent manner. We hypothesized that if key downstream mediators were not in place to support MAP4K4 signaling, the signals might be channeled into ‘negative’ roles.

Among cells in which SOX2 expression was silenced, MAP4K4 signaling was implicated in the induction of p53 since suppression of MAP4K4 expression prevented p53 expression (Figure 3E and Supplementary Figure S4C, available at Carcinogenesis Online). Similarly, suppression of MAP4K4 permitted maintenance of Survivin expression in SOX2-suppressed A549 and H460 cells (Figure 3E and Supplementary Figure S4C, available at Carcinogenesis Online). Together, the data support the notion that upregulation of MAP4K4 played a key proapoptotic role following suppression of SOX2 and was required for both the coordinated induction of apoptotic pathways (p53) and for the depletion of prosurvival factors (Survivin).

**Elevated Survivin expression inhibits apoptosis induced by SOX2 suppression**

All three of the cell lines examined expressed Survivin, whereas only the A549 and the H460 cells expressed wild-type p53. We therefore focused on Survivin as a key target suppressed by MAP4K4 since Survivin also has been identified as a key prognostic indicator in lung cancer (40). We hypothesized that Survivin was a functional activator that facilitated the prosurvival activity of SOX2. To test this hypothesis, we ectopically expressed Survivin in A549-sh1-SOX2 cells (Figure 4A) to bypass the MAP4K4-induced downregulation, then evaluated whether this was sufficient to rescue cells in which SOX2 expression was suppressed. As shown, ectopic expression of the Survivin gene in A549-sh1-SOX2 cells could significantly rescue cell survival induced by SOX2 silencing (Figure 4B and 4C), suggesting that Survivin is a key downstream protein that contributes to the prosurvival properties of SOX2.

**Silencing of SOX2 induces apoptosis in vivo**

To extend these studies, we next evaluated the function of SOX2 in an orthotopic model of lung tumor progression. Luciferase-labeled A549-sh1-SOX2 cells were followed for 6 weeks, then suppression of SOX2 was induced by introduction of Dox into the drinking water of mice (Figure 5A). As we demonstrated previously, most of the lung tumor cells were trapped in lung capillaries within 1 day of injection (29,41) (Figure 5B). Attenuation of bioluminescence signals was detected during the subsequent 7 days as successful tumor cells were selected, followed by increasing signals as the tumor progressed to week 6. The mice were fed with Dox for 1 week to induce the expression of shRNA. The induction of shRNA-SOX2 resulted in decreased tumor size, whereas induction of a control shRNA-scramble resulted in continued tumor growth (Figure 5C). This observation was also supported by hematoxylin and eosin staining of tumor colonies in lung tissues of the mice, which revealed reduced tumor area (Figure 5D). Together, these data support the notion that suppression of SOX2 induces apoptosis, whereas induction of shRNA-scramble resulted in continued tumor growth.

This was not specific to the A549 cells since it was recapitulated in both H460 and H1299 cells (Figure 3A). The upregulations of its downstream molecules, including MAP2K4, JNK1 and ERK1 (39)
SOX2 regulates expressions of TNF-α, p53 and Survivin through MAP4K4 signaling pathway. (A) Real-time reverse transcription–PCR was used to detect messenger RNA changes of MAP4K4 in human lung cancer cells induced by SOX2 silencing. (B) Real-time reverse transcription–PCR results of MAP4K4 (upper panel) and TNF-α (lower panel) in A549 cells with or without MAP4K4 silencing. (C) Representative fluorescence-activated cell sorting analysis of apoptotic cells among A549 cells with MAP4K4 silencing and their controls. (D) Statistical results of apoptotic cells in A549 cells (n = 3). (E) Western blotting was used to detect expression changes of p53, P-p53 and Survivin in A549 cells after MAP4K4 silencing.
Fig. 4. Overexpression of Survivin rescues the apoptosis effects induced by SOX2 silencing. (A) Western blotting of Survivin in A549 cells with Survivin overexpression and their controls. (B) Representative fluorescence-activated cell sorting analysis of apoptotic cells in A549 cells. (C) Statistical results of apoptotic cells in the A549 cell lines from fluorescence-activated cell sorting (n = 3).
Fig. 5. Silencing of SOX2 induces apoptosis and regulates expression levels of Ki67, p53, Survivin and TNF-α in vivo. (A) The schematic diagram of experimental protocols. (B) A549 cells were injected via tail vein into NOD/SCID mice and bioluminescence images of xenograft tumors were taken at the times indicated. (C) Statistical results of the bioluminescence intensity from each treatment group measured at different days, n = 5. (D) Hematoxylin and eosin staining was used to detect xenograft tumors in lung tissues of NOD/SCID mice. Tumor regions were circled by black dashed lines. (E) The percentage of tumor areas in the lung.
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Fig. 6. Correlated expressions of SOX2 and Survivin in human lung tumors. (A) Immunohistochemical staining of Survivin in human lung tissue. (B) Statistical analysis of the relationship between SOX2 and Survivin in human lung tissues of normal, non-small-cell lung cancer and small-cell carcinoma. Plots represent the percentage of tissue samples with different expression level of Survivin (y-axis) in each group that was defined as SOX2 negative, weak, moderate or strong (x-axis).

- Normal/Paracarcinoma
  - Correlation coefficient = 0.915
  - p < 0.0001
  - SOX2: Negative n=29, Weak n=6, Moderate n=1, Strong n=0

- Non-small cell lung cancer
  - Correlation coefficient = 0.752
  - p < 0.0001
  - SOX2: Negative n=7, Weak n=36, Moderate n=84, Strong n=250

- Small cell carcinoma
  - Correlation coefficient = 0.997
  - p < 0.0001
  - SOX2: Negative n=1, Weak n=1, Moderate n=2, Strong n=31

- Tissues of NOD/SCID mice (n = 5).
- (F) Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (green) of the apoptotic cells in xenograft tumors from lung tissues of NOD/SCID mice (upper panel) and the statistical results of TUNEL-positive cells in the xenograft tumor tissues are depicted in the lower panel, n = 5.
- (G) Immunohistochemical staining of SOX2, Ki67, Survivin, TNF-α and p53 (all shown in brown color) of xenograft tumor in lung tissues of NOD/SCID mice in situ. The images in B, D, F and G represent 1 of 5 mice separately.
the control (SS) group. Apoptosis of the tumor cells was confirmed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining of tumor xenografts from the lung tissues of NOD/SCID mice (Figure 5F). As previously observed in in vitro data, increased apoptosis was seen in the shRNA-SOX2 group relative to the control shRNA (scrambled) group, and this correlated with increased TNF-α and p53, as well as suppressed expression of Ki67 and Survivin (Figure 5F and 5G). Together, these data demonstrated a key in vivo role of SOX2 in regulating tumor progression by modulating crucial downstream regulators of programed cell death and cell proliferation.

Expression of SOX2 correlates with that of Survivin in human lung cancer tissues

To determine whether SOX2 regulates expression of Survivin in the development of human lung carcinomas, immunohistochemical analyses were performed with SOX2 and Survivin-specific antibodies in tissue microarrays containing 448 samples of human normal lung tissues, and tissues with different clinical degrees of human lung carcinoma. As shown in Figure 6A, strong expressions of Survivin were only detectable in human lung cancer tissues. Statistical analysis showed that expression of Survivin strongly correlates with that of SOX2 (Figure 6B), indicating a transcriptional regulation of Survivin by SOX2, suggesting a possible functional interaction between SOX2 and Survivin in lung carcinoma patients. These results suggest the possibility that SOX2 and Survivin could be developed as double markers to improve accuracy in the diagnosis and prognosis of human lung cancer.

Discussion

SOX2 is a key epigenetic regulator that has been reported to be a downstream target of TNF-α (42), suggesting context-dependent coexpression with MAP4K4. This is supported by observations that SOX2 expression helps define basal breast cancer status (5). SOX2 contributes to the antianapoptotic property of cancer cells; since targeting SOX2 with agents that downregulate its expression increases the sensitivity of cancer cells to apoptotic stimuli, this may be a promising approach to decrease tumor chemoresistance and recurrence (31). Here, we found that silencing of SOX2 gene via a lentivirus system is sufficient to induce apoptosis of human lung cancer cells. This is consistent with reports on tumor and normal cells (43-45) and supports the view that SOX2 is an ‘addictive’ oncogene, the depletion of which rapidly induces apoptosis.

Importantly, our most unexpected finding was the identification of MAP4K4 as a key protein inducing the apoptotic cascade following SOX2 silencing. In contrast, recent studies demonstrated that MAP4K4 contributes to growth and migration properties of tumor cells (46,47) and showed its expression level to closely correlate with clinical progression and poor prognosis among various tumor types, including hepatocellular carcinoma (46), colorectal cancer (38) and pancreatic ductal adenocarcinoma (37). However, here we demonstrated the contribution of MAP4K4 to the induction of programed cell death. This appeared to involve coordinated induction of elements of both the mitochondria and death receptor signaling pathways and also required suppression of Survivin. Prior reports indicated that p53 upregulates the c-jun N-terminal kinase (JNK) signaling pathway to drive apoptosis (48). This may occur in two of the cell lines studied; however, it can be bypassed in the H1299 cells, which are p53 deficient (49,50).

Small GTPases, such as RAS and RHO protein family members, play an important role in connecting MAPK signals to cell surface receptors or external stimuli. Upon activation, MAPKs translocate from the cytoplasm to the nucleus and activate certain key proteins or transcriptional factors such as ATF2, c-Myc, c-Jun and c-Fos to regulate the expression of related genes, including key apoptosis-related proteins, such as TNF-α, p53 and Survivin (33,51-53) and thus may play an important role in conducting apoptosis signals. Our data raise the notion that Survivin acts as a homeostatic checkpoint molecule, permitting survival in the face of aberrant MAPK activation. From the RNA-seq results of our previous study, the upstream activator, such as epidermal growth factor was significantly upregulated upon SOX2 silencing (29), thus may contribute to the activation of MAPK signal pathway. Previous study demonstrated the direct upregulation effect of SOX2 on the expression of Survivin (54). In addition, MAP4K4 was disclosed to be an important upstream mediator of TNF-α action on the beta cell (55). However, our study showed the contribution of MAP4K4 to attenuation of Survivin and the upregulation of TNF-α upon SOX2 silencing. The molecular mechanisms for SOX2 to regulate the expression of MAP4K4 and TNF-α are still unknown, revealing of these mechanism may deepen the understanding on these genes’ function in apoptosis.

The link between SOX2 and Survivin levels is borne out among clinical samples of human lung tumor. Indeed, our results demonstrate that SOX2 may functionally interact with Survivin to increase its antianapoptotic effect on lung cancer cells, particularly since Survivin overexpression rescued death induced by SOX2 silencing. It remains possible that cotargeting both Survivin and SOX2 could synergize with other therapeutic effects and thus reduce the recurrence of lung tumors. In contrast, care should be taken not to combine small molecules that inhibit MAP4K4 (either directly or as an off-target effect), with SOX2 suppression as these may well compromise therapeutic efficacy and enhance tumor cell escape. This unexpected mechanism by which apoptosis is regulated in non-small cell lung cancer provides both cautionary and instructive lessons with which to plan future molecular intervention.

Supplementary material

Supplementary Figures S1–S4 can be found at http://carcin.oxfordjournals.org/

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References

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