Low SOX17 expression is a prognostic factor and drives transcriptional dysregulation and esophageal cancer progression

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The transcriptional network of the SRY (sex determining region Y)-box 17 (SOX17) and the prognostic impact of SOX17 protein expression in human cancers remain largely unclear. In this study, we evaluated the prognostic effect of low SOX17 protein expression and its dysregulation of transcriptional network in esophageal squamous cell carcinoma (ESCC). Low SOX17 protein expression was found in 47.4% (73 of 154) of ESCC patients with predicted poor prognosis. Re-expression of SOX17 in ESCC cells caused reduced foci formation, cell motility, decreased ESCC xenograft growth and metastasis in animals. Knockdown of SOX17 increased foci formation in ESCC and normal esophageal cells. Notably, 489 significantly differential genes involved in cell growth and motility controls were identified by expression array upon SOX17 overexpression and 47 genes contained putative SRY element in their promoters. Using quantitative chromatin immunoprecipitation-PCR and promoter activity assays, we confirmed that MACC1, MALAT1, NBN, NFAT5, CSNK1A1, FN1 and SERBP1 genes were suppressed by SOX17 via the SRY binding-mediated transcriptional regulation. Overexpression of FN1 and MACC1 abolished SOX17-mediated migration and invasion suppression. The inverse correlation between SOX17 and FN1 protein expression in ESCC clinical samples further strengthened our conclusion that FN1 is a transcriptional repression target gene of SOX17. This study provides compelling clinical evidence that low SOX17 protein expression is a prognostic biomarker and novel cell and animal data of SOX17-mediated suppression of ESCC metastasis. We establish the first transcriptional network and identify new suppressive downstream genes of SOX17 which can be potential therapeutic targets for ESCC.

Key words: esophageal squamous cell carcinoma, SOX17, transcriptional network, tumor suppressor, prognosis

Additional Supporting Information may be found in the online version of this article

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The prognosis of esophageal squamous cell carcinoma (ESCC), one of the most common cancers worldwide, is extremely poor because of difficulties in early diagnosis and high rate of cancer recurrence.1,2 Carcinogenesis is a multifactorial process with the accumulation of activation of oncogenes and inactivation of tumor suppressor genes (TSGs).3,4 Hypermethylation of CpG islands in promoter and exon 1 regions is an important mechanism in TSGs inactivation4,5 and its association with prognosis have been documented for FHIT, p14ARF, p15INK4b, p16INK4a, E-cadherin, RAR-β and RASSF1A genes in ESCC.6–10 Therefore, characterization of hypermethylated genes and their protein expression levels in ESCC may lead to the identification of new prognostic markers and therapeutic targets.

The human SRY (sex determining region Y)-box 17 (SOX17) gene is hypermethylated in human cancers,11–16 but the low expression of SOX17 in ESCC metastasis and its association with poor prognosis has not been explored. In normal gastrointestinal tract, SOX17 mRNA is preferentially expressed in esophagus, stomach and small intestine than in colon and rectum,
suggesting that SOX17 plays key roles in esophagus tissue.\textsuperscript{17} Human SOX17 gene encodes a member of the SOX (SRY-related high mobility group box) family of transcription factor, a 414 amino acid polypeptide containing a high-mobility group DNA-binding domain (HMG-box), which binds to the target promoters at SRY elements 5'- (A/T)(A/T)CAA(A/T)G-3'.\textsuperscript{11} Sox17 can transcriptionally activate endodermal target genes for the formation of endoderm and vascular endothelium in the Xenopus and mouse model.\textsuperscript{18–21} Moreover, hematopoietic stem cells of conditional mice with deletion of Sox17 in hematopoietic/endothelial cells undergo cell death within days of Sox17 deletion, which leads to expression of Dkk1, a negative regulator of Wnt pathway, in neonatal and embryonic hematopoietic progenitors.\textsuperscript{22} In cultured rat oligodendrocyte progenitor cells, Sox17 antagonizes the Wnt/\(\beta\)-catenin pathway by suppressing the transcriptional activity of \(\beta\)-catenin/TCF complex to control cell cycle and differentiation.\textsuperscript{23} It has also been reported that nuclear SOX17 inhibits the Wnt signaling pathway by degradation of the \(\beta\)-catenin/TCF complex. Inactivation of Sox17 promotes the Wnt/\(\beta\)-catenin signaling pathway and leads to carcinogenesis.\textsuperscript{11,24} However, the transcriptional deregulation of SOX17 in addition to target genes of \(\beta\)-catenin/TCF complex in the somatic cancer cells remains largely unclear. In this study, we discovered a new transcriptional network and verified several novel transcriptional targets of SOX17 in ESCC model. The expression pattern of SOX17 and its biological functions and clinical implications were analyzed in ESCC patient, cell and animal models.

**Material and Methods**

**Clinical samples of ESCC patients**

A total of 154 surgically resected ESCC patients were recruited from Cancer center and Pathology Department, National Cheng Kung University Hospital, Tainan, Taiwan, after obtaining appropriate institutional review board permission and informed consent from the patients. Tumor typing and disease staging were performed according to the World Health Organization classification and the TNM classification system, respectively. Information on the sex, age, and smoking history of the patients were obtained from hospital records. Overall survival was calculated from the day of surgery to the date of death or the last follow-up. The end of the follow-up period was defined as October 2012. The mean follow-up period for all patients was 24.2 months (range, 0.5–212.0 months).

**Immunohistochemistry staining**

The protein expression level of SOX17 was evaluated by immunohistochemistry (IHC) of tumor tissues from 154 ESCC patients. The anti-SOX17 monoclonal antibody (dilution 1:100; clone 2G8; OriGen Technologies) was used to detect SOX17 protein. The evaluation of the IHC was conducted blindly without knowledge of the clinical and pathological characteristics of the patients. The surrounding nonneoplastic stroma served as an internal control for each slide. The staining “intensity” measurements were translated into the two-tier system as positive “1,” or negative “0” staining. The staining “percentage” measurements were graded using a four-tier system and was scored as “3” if >66% tumor cells were immunostaining-positive; “2” for 33–66%; “1” for 1–33% and “0” if <1% were positive. The IHC data were defined by “percentage \times intensity.” SOX17 protein expression level was graded as “low expression” if nuclear staining “percentage \times intensity” was “0.” The same IHC condition was used to perform tissue array of 48 patients for SOX17 and fibronectin (FN1) and tumor tissue of animal xenografts.

**Cell lines and culture**

Japanese ESCC cell lines KYSE70, KYSE150 and KYSE510 were purchased from the DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), where they were characterized by DNA-fingerprinting and isozyme detection. The luciferase-expressing KYSE170-luc cell line was generously provided by Dr. Li-Wha Wu at the Institute of Molecular Medicine, National Cheng Kung University. Cells were cultured in RPMI1640 medium (Gibco, Invitrogen, Carlsbad, CA), Taiwanese ESCC cell lines CE48T, CE81T and CE146T, previously described,\textsuperscript{25} were maintained in DMEM medium (Gibco). Normal human esophageal squamous cell line HET-1A was purchased from the American Type Culture Collection, where they were characterized by DNA-fingerprinting and isozyme detection. The HET-1A cell line was cultured in BEBM (Bronchial/Tracheal Epithelial Cell Basal Medium; CELL Applications). All medium were supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco).

**Tumor growth and experimental metastasis assays in animal model**

Five-week-old male SCID mice were obtained from National Cheng Kung University Laboratory Animal Center and raised...
in pathogen-free conditions after obtaining appropriate institutional review board permission. KYSE170-luc cells were harvested in Hanks' balanced salt solution (HBSS) 24 hr after transfection with plasmid. The mice were implanted subcutaneously with $1 \times 10^7$ KYSE170-luc cells in 100 μl HBSS for tumor growth assay of SOX17 overexpression. Mice were sacrificed by cervical dislocation and then tumor samples were resected and weighed. For the experimental metastasis assay, CE81T cells were harvested in HBSS 24 hr after transfection. About $5 \times 10^7$ cells/200 μl were injected intravenously into SCID mice via tail vein. All mice were sacrificed after 8 weeks of cell injection, and the lung tissues were excised and weighted prior to 4% formaldehyde fixation. The excised tissues were embedded and sectioned for histological H&E staining.

Additional methods
Detailed descriptions of expression array and pathway analyses, expression vector constructs and transfection, pyrosequencing methylation assay, quantitative reverse transcription-polymerase chain reaction (qRT-PCR), chromatin immunoprecipitation (ChIP)-qPCR, re-ChIP, luciferase reporter assay, Western blot analysis, foci formation assay, colony formation assay, wound healing assay, transwell migration, invasion assays, and statistical analyses used in this study are available in Supporting Information Materials and Methods. The antibodies and blotting information are listed in Supporting Information Table S1. The primer sequences and PCR condition for qRT-PCR are listed in Supporting Information Table S2. The primer sequences and PCR conditions for ChIP-qPCR are listed in Supporting Information Table S3. The primer sequences and PCR conditions for promoter constructs are listed in Supporting Information Table S4.

Results
Low protein expression of SOX17 correlates with poor survival outcome in ESCC patients
To date, the expression level of SOX17 protein has only been reported in 72 gastric cancer, 69 colon cancer and 39 ESCC specimens. Therefore, we investigated the prognostic effect of SOX17 protein expression in 154 resected tumors from ESCC patients. IHC staining was used to analyze the protein expression. The SOX17 protein expression was scored as low and normal judging by comparing the stained intensity × percentage between tumor and surrounding normal tissues (Fig. 1a). The same IHC condition was performed on tumor tissue of animal xenografts with SOX17 overexpression (SOX17) group as a positive control and the empty vector (EV) group as a negative control. The evaluation indicated that SOX17 staining was specific (Supporting Information Fig. S1). IHC results demonstrated that 47.4% (73 of 154) of patients showed SOX17 low protein expression in tumor tissues (Table 1). An inverse correlation of SOX17 DNA methylation to protein expression was found in 61 ESCC patients whose DNA samples are available (Supporting Information Fig. S2). Notably, the overall survival curves of Kaplan–Meier method indicated that patients with low SOX17 protein expression had significantly poorer survival than patients with normal expression ($p < 0.0001$, Fig. 1b). Therefore, we performed univariate and multivariate Cox regression analyses of the 154 ESCC patients to define the hazard ratio (HR) of risk of cancer death for variables including SOX17 expression, tumor stage, lymph node status, distant metastases status, sex, age and cancer recurrence (Table 1). The univariate analysis showed that SOX17 protein low expression, TNM staging and recurrence were associated with a significantly increased risk of cancer-related death. Importantly, SOX17 protein low expression was an independent risk factor of cancer-related death (HR = 2.134; 95% CI = 1.424–3.198; $p < 0.001$) even after adjusting for other variables in the multivariate model. Therefore, SOX17 is a potential prognostic biomarker for ESCC patients with either early or late stage of the disease.

SOX17 overexpression suppresses colony formation of ESCC cells and decreases ESCC xenograft growth in animals
Since our clinical data suggested that SOX17 may play a suppressive role in ESCC tumorigenesis, we examined the effects of SOX17 on control of growth and migration in cell and xenograft models of three ESCC cell lines KYSE150, KYSE170-luc and CE81T which showed low SOX17 expression in comparison with the HET-1A normal esophageal squamous cell line (Supporting Information Fig. S3). Figure 2a illustrated that the size and number of foci grown were significantly smaller and fewer in number in the SOX17 transfected KYSE510 cells than in the EV control cells ($p < 0.0001$). However, si-knockdown of SOX17 in the KYSE510 cells resulted in a significant increase in foci formation ($p < 0.01$, Fig. 2b). In addition, anchorage independent growth on soft agar and foci formation ability were significant enhanced in the si-SOX17 group compared to the si-control group of the HET-1A normal esophageal cells (Supporting Information Fig. S4), suggesting that SOX17 expression suppresses colony formation of esophageal cells.

To investigate the effect of SOX17 overexpression on tumor growth in vivo, KYSE170-luc cells expressing SOX17 were subcutaneously injected into SCID mice. SOX17 overexpression decreased ESCC tumor size (Fig. 2c) and tumor weight (Fig. 2d) compared with EV control group. SOX17 overexpression decreased growth of ESCC xenograft in vivo which was confirmed by the luciferase signal assay (Supporting Information Fig. S5). In addition, the results of IHC staining in the tumor xenograft tissues with Ki-67 proliferation marker and Caspase 3 apoptosis marker suggested that the decreased tumor size upon SOX17 overexpression was mainly due to reduced tumor cell proliferation (Supporting Information Fig. S6).

SOX17 overexpression decreases cell motility of ESCC cells and attenuates metastatic potential to lungs in animals
To further verify the effects of SOX17 expression on motility of ESCC cells, we carried out transwell-migration and
invasion assays in cells ectopically expressing SOX17. KYSE170-luc and KYSE510 cells overexpressing SOX17 migrated at a much slower rate compared with the EV control cells using transwell-migration assay ($p < 0.001$ for both cells, Fig. 2e). The migration suppression by SOX17 overexpression was confirmed by wound healing assay ($p < 0.0001$ for both cells, Supporting Information Fig. S7). Furthermore, transwell-invasion assay showed significantly less SOX17 overexpressing cells invaded the Matrigel in comparison with EV control cells ($p < 0.0001$ for both cells, Fig. 2f). These data indicated that SOX17 overexpression significantly decreases migration and invasion potential of ESCC cells.

Figure 1. Low protein expression of SOX17 correlates with poor prognosis and inversely correlates with FN1 protein expression in ESCC patients. (a) Representative IHC of SOX17 protein in four ESCC patients. SOX17 nuclear negative immunoreactivity (−) is found in patient 1 and 2, whereas patient 3 and 4 show SOX17 expression (+) in nuclei of tumor tissue. A fourfold enlarged image of tumor area indicated by arrow is shown in lower left inset for each patient (original magnification: 200×). (b) Kaplan–Meier curves showing ESCC patients with SOX17 protein low expression had significantly poorer overall survival than those with normal expression. (c) IHC of SOX17 and FN1 protein expression level in ESCC tumor tissue array. The representative IHC figures for SOX17 and FN1 protein are shown for 10 ESCC patients. (d) An inverse correlation between SOX17 and FN1 protein expression was found in 48 ESCC patients analyzed. y axis, percent of cases; x axis, type of comparison. Positive (+) and negative (−) expression status of protein are noted. The percentage in the inverse correlation group (gray columns) and noninverse correlation group (white columns) is indicated above. $p$ values are shown as indicated.
ESCC cell line CE81T has been shown to form tumor nodules in lungs using the experimental metastasis model. Therefore, in vivo extravasation and colonization assay was performed by tail-vein injection of the CE81T cells transfected with SOX17 expression vector or EV into SCID mice. Representative H&E stain images showed many metastatic tumor colonies in lungs of mice injected with EV control transfected cells but not in lungs of mice injected with SOX17 transfected cells after 8 weeks postinjection (Fig. 2g). The average number of tumor nodule in lungs was significantly decreased in SOX17 overexpression group compared with EV group (Fig. 2h). These cell and animal results indicated that overexpressed SOX17 inhibits cell migration and metastatic potential of ESCC cells.

Novel transcriptional target genes of SOX17 in ESCC

SOX17 is a transcriptional factor binding to its target genes at SRY elements. To build the transcription network of SOX17 and to reveal novel transcriptional targets for SOX17 involved in ESCC tumorigenesis, we performed Affymetrix HG-U133 plus2.0 GeneChips expression array analyses at 12 hr post-transfection in SOX17 overexpressing and EV control ESCC cells (GEO accession number 35975). A total of 489 significantly change probes were selected (fold change ≥ 1.5 vs. EV transfected cells) and further analyzed for pathway association and disease biomarkers. The results showed that top common altered biological processes of gene ontology (GO) included cell adhesion/extracellular matrix, DNA damage/DNA repair, signal transduction, cell cycle regulation/cell differentiation, RNA splicing process/regulation of transcription, cell adhesion, and metabolic process (Fig. 3a and Supporting Information Fig. S8). Among them, 47 candidate genes contained putative binding sites of SRY element in their promoter (Supporting Information Table S5).

Interestingly, the GO analyses revealed that downregulated genes were mostly related to cancer, in which was not the case for upregulated genes (Supporting Information Fig. S9). Since the current study aimed to explore the regulation of SOX17 in ESCC tumorigenesis, we validated the expression array results of the downregulated genes in the KYSE170-luc and KYSE510 cells by qRT-PCR. Seven genes, MACC1 (metastasis associated in colon cancer 1), MALAT1 (metastasis associated lung adenocarcinoma transcript 1), NBN (nibrin/NBS1), NFAT5 (nuclear factor of activated T-cells 5), CSNK1A1 (casein kinase 1, α1), FN1 (fibronectin) and SERPB1 (serpin peptidase inhibitor member 1 mRNA binding protein 1) were selected because they were downregulated by SOX17 overexpression in

### Table 1. Univariate and multivariate analyses of SOX17 protein expression and pathologic parameters in ESCC patients

|                       | Number of samples | HR (95% CI) | \( p \) | HR (95% CI) | \( p \) |
|-----------------------|-------------------|-------------|---------|-------------|---------|
| **SOX17 protein**     |                   |             |         |             |         |
| Normal expression     | 81                | 1.000       |         | 1.000       |         |
| Low expression        | 73                | 2.116 (1.515–2.954) | <0.001 | 2.134 (1.424–3.198) | <0.001 |
| **TNM stage**         |                   |             |         |             |         |
| T stage               |                   |             |         |             |         |
| T1/2                  | 28                | 1.000       |         | 1.000       |         |
| T3/4                  | 126               | 2.138 (1.361–3.358) | <0.01 | 1.684 (1.009–2.811) | NS (0.05) |
| N stage               |                   |             |         |             |         |
| N0                    | 55                | 1.000       |         | 1.000       |         |
| N1                    | 96                | 2.103 (1.459–3.032) | <0.001 | 2.061 (1.325–3.208) | <0.01 |
| M stage               |                   |             |         |             |         |
| M0                    | 124               | 1.000       |         | 1.000       |         |
| M1                    | 27                | 1.929 (1.256–2.961) | <0.01 | 1.424 (0.843–2.407) | NS (0.186) |
| **Sex**               |                   |             |         |             |         |
| Female                | 7                 | 1.000       |         |             |         |
| Male                  | 147               | 2.266 (0.919–5.590) | NS (0.076) |             | ND      |
| **Age**               |                   |             |         |             |         |
| <57                   | 78                | 1.000       |         |             |         |
| >57                   | 75                | 0.967 (0.697–1.343) | NS (0.841) |             | ND      |
| **Recurrence**        |                   |             |         |             |         |
| No                    | 41                | 1.000       |         | 1.000       |         |
| Yes                   | 83                | 1.799 (1.181–2.737) | <0.01 | 1.717 (1.107–2.664) | <0.05 |

Abbreviations: HR, hazard ratio; NS, not significant; ND, not done.
Figure 2. Overexpression of SOX17 attenuates ESCC cell growth and metastasis in vitro and in vivo. (a) Overexpression of SOX17 attenuates foci formation ability, whereas (b) knockdown of SOX17 promotes foci formation ability in the KYSE510 cells. Western blots showing SOX17 overexpression and si-knockdown in KYSE510 cell. β-actin was used as an internal control. The relative foci formation ability was normalized to EV or si-control group. Data represent mean ± SD from three independent experiments. (c) SOX17 overexpression decreases tumor growth in vivo in ESCC xenograft model. KYSE170-luc cells transfected with EV or SOX17 expression vector were subcutaneously injected into SCID mice and observed for tumor growth. Xenograft images of tumor are shown (n = 4 mice per group). (d) SOX17 effectively suppressed tumor weight compared with EV in SCID mice. (e, f) Transwell migration and invasion assays in two ESCC cells transfected with SOX17 or EV control. The quantitative results showed that SOX17 overexpressing cells migrated or invaded less than vector control cells. Data represent mean ± SD from three independent experiments. (g) SOX17 overexpression decreases in vivo extravasation and colonization in ESCC xenograft animal model. The representative lung tissue images of SCID mice intravenously injected with EV and SOX17 expression CE81T cells via tail vein are shown. The red arrows indicate the sites of tumor nodules in the lung tissues (40×). Tumor boundaries (red lines) of selected areas are shown (100×). (h) Quantification of lung metastatic nodules in EV and SOX17 overexpression groups (n = 5 mice per group). p values were calculated by two-tailed t test.
our dataset and had been reported to promote either cell growth or migration. The qRT-PCR results showed that the expression of MACC1, MALAT1, NBN, NFAT5, CSNK1A1, FN1 and SERBP1 was significantly downregulated in wild-type SOX17 (SOX17-WT) overexpressing cells, while the downregulation of these genes was attenuated in cells overexpressing the HMG-box deleted SOX17 (SOX17-DHMG), which has the HMG-box domain, a conserved motif for DNA recognition, deleted (Figs. 3b and 3c). The results suggested that these genes are transcriptionally suppressed by SOX17.

SOX17 binds at promoter region of novel target genes containing SRY sites via HMG box

SOX17 is a HMG box-containing transcription factor that binds to the SRY site. We thus conducted ChIP-qPCR assay...
to verify whether SOX17 binds to the predicted SRY sites in the promoter of target genes via the HMG box in KYSE170-luc cells. The regions amplified in ChIP-qPCR of MACC1, MALAT1, NBN, NFAT5, CSNK1A1, FN1, and SERBP1 genes are shown in Supporting Information Figure S10. The results of ChIP-qPCR indicated that SOX17-WT could bind to the SRY putative sites of all target genes analyzed, whereas the SOX17-DHMG attenuated the binding activity to the target promoters (Fig. 4a).

We further performed luciferase promoter activity assay to investigate whether these novel target genes are regulated by transcriptional activity of SOX17. The promoter regions with the SRY sites examined are shown in Supporting Information Figure S10. The KYSE170 and KYSE510 cells were transiently transfected with EV, SOX17-WT or SOX17-DHMG vector, then cotransfected with pGL3-renilla and either FN1 or MACC1 promoter. The results indicated that SOX17-WT suppressed FN1 and MACC1 promoter activities, while this suppressive effect was attenuated in the SOX17-DHMG group (Fig. 4b). Collectively, these results demonstrated that SOX17 suppresses the expression of the novel genes identified in our study, and that the HMG

Figure 4. SOX17 binds to the region containing SRY sites on target promoters and represses their promoter activity. (a) ChIP-qPCR showed that wild-type SOX17 (SOX17-WT, black bars) bound to promoter region of target genes (MACC1, MALAT1, NBN, NFAT5, CSNK1A1, FN1, and SERBP1) in KYSE170-luc cells, whereas the binding was attenuated by HMG box-deleted SOX17 (SOX17-DHMG, gray bars), “IgG” is a negative control. (b) Luciferase promoter activity assay showed that SOX17-WT (black bar) decreased the promoter activity of target genes (FN1 and MACC1) compared to EV, whereas the SOX17-DHMG group (gray bar) restored the promoter activity in KYSE170 and KYSE510 cells. (c) Western blots showing the transfection efficiency of SOX17 and HDAC1 (upper right). Luciferase promoter activity assay was done after cotransfection with HDAC (gray bar) or both HDAC and SOX17 (black bar) of target genes (SERBP1, CSNK1A1, and NFAT5) compared to EV control in KYSE-170 cells. p values were calculated by two-tailed student t test and are shown as indicated. (d) Model of SOX17 molecular functions in esophageal cell. SOX17 exerts tumor suppressive effect through control of cell growth, inhibition of cell proliferation, and suppression of cell invasion/metastasis via, in part, transcriptional downregulation of genes shown in black circle.
domain-mediated transcriptional regulation is required for this effect.

Motility regulation of the novel SOX17 transcriptional targets FN1 and MACC1 in ESCC

FN1 is an extracellular matrix protein that mediates the activation of focal adhesion kinase that promotes cell motility through ERK or PI3K/Akt signaling to upregulate MMP9/calcinulin 2 or MMP9/RhoA activity.35 MACC1 is an upstream transcriptional activator of the c-Met oncogene, which contributes to colorectal cancer metastasis and progression.28 Since SOX17 overexpression significantly decreased migration and invasion potentials of ESCC cells and FN1 and MACC1 could be transcriptionally suppressed by SOX17, we therefore examined whether FN1 or MACC1 overexpression could reverse cell migration and invasion ability in the SOX17-WT overexpressing KYSE170-luc and KYSE510 cells. The cotransfection of SOX17-WT and FN1 abolished the suppression of migration and invasion observed in SOX17-WT cells (Figs. 5a and 5b). Similar results were obtained by cotransfection of MACC1 with SOX17-WT (Figs. 5c and 5d), indicating that FN1 and MACC1 are functionally downstream to SOX17 in regulation of migration and invasion of ESCC. In addition, we investigated the SOX17 and FN1 protein expression in 48 resected tumors from ESCC patients by IHC. The results demonstrated an inverse correlation between SOX17 and FN1 proteins (Figs. 1c and 1d). The clinical correlation results further strengthened our conclusion that FN1 is a transcriptional repression target gene of SOX17.

Discussion

In this study, we performed a comprehensive clinical, cell, and animal model study of SOX17 to evaluate the mechanisms involved in ESCC tumorigenesis (Figs. 2). Our clinical studies demonstrated for the first time that low SOX17 protein expression in resected tumors is an effective prognostic marker (Table 1). We provide new evidence for metastasis suppression of SOX17 in the ESCC cell and in animal models. Importantly, we discover the first SOX17 transcriptional network in ESCC and confirmed that overexpression of SOX17 decreases tumor growth and metastasis of ESCC via, in part, disruption of its transcriptional regulation of downstream genes (Figs. 5). The negative regulation of the downstream genes expression of the Wnt/β-catenin signaling pathway by SOX17 was also validated in our ESCC cell model (Supporting Information Fig. S11).

Despite the important and specific role of transcriptional control of SOX17 in embryonic development, few direct transcriptional target genes of SOX17 in addition to those in β-catenin/TCF complex in cancer progression are known. In this study, we performed expression array, qRT-PCR, ChIP-qPCR, and promoter analyses to reveal transcriptional targets of SOX17 in ESCC model. We showed for the first time that NBN, NFAT5, MACC1, MALAT1, CSNK1A1, FN1 and SERBP1 genes were downregulated upon SOX17 overexpression but this effect could be attenuated by HMG-deleted SOX17. Notably, our reconstitution experiments by overexpressing FN1 or MACC1 along with SOX17-WT expression abolished the migration and invasion suppression of SOX17-WT verifying the notion that FN1 and MACC1 are downstream to SOX17 and their overexpression promotes ESCC cell motility. Although FN1 and MACC1 belong to different regulatory pathways,28,35 our results demonstrated that both could be subjected to SOX17 transcriptional repression. These results unveiled the molecular mechanism by which SOX17 inhibited cancer cell migration and invasion through transcriptional regulation (Fig. 4d).

Differential recruitment of transcriptional co-repressors or co-activators is one of the mechanisms by which SOX transcription factors mediate suppression or activation of gene transcription. For example, previous studies show that SOX6 binds β-catenin and recruits histone deacetylase 1 (HDAC1) to suppress cyclin D1 promoter activity, and the N-terminal 139 amino acids of HDAC1 physically interacts with the HMG box of SOX6.36,37 We speculated that the HDAC1 may also be a corepressor with SOX17 to suppress expression of some of the SOX17 target genes. Therefore, we carried out a luciferase promoter assay of SERBP1, CSNK1AI and NFAT5 genes in KYSE170 cells cotransfected with HDAC1 and SOX17. The results indicated that HDAC1 could indeed repress the promoter activity of SERBP1, CSNK1AI and NFAT5 genes and the repression effects on SERBP1 and NFAT5 genes were further augmented by the coexpression of HDAC1 and SOX17 (Fig. 4c). Re-ChIP assay also verified that SOX17 and HDAC1 formed a transcriptional inhibitory complex at the promoter region of SERBP1 and NFAT5 genes (Supporting Information Fig. S12). SOX17-HDAC1 interactions on chromatin in the vicinity of promoters of SOX17 downstream targets warrant future studies.

Our data suggested that SOX17 is a tumor suppressor that transcriptionally downregulates NBN, NFAT5, MACC1, MALAT1, CSNK1AI, FN1 and SERBP1 gene expression. NBN overexpression has been shown to induce epithelial-mesenchymal transition through the Snail/MMP2 pathway in head and neck carcinoma.30 NBN also promotes cell proliferation via the Ras/Raf/MEK/ERK cascade.38 In addition, NFAT5 activity enhances cell migration by promoting the expression of α6β4 integrin.31 NFAT5 is also needed for cell cycle progression.39 MACC1 acts as a transcriptional activator of Met promoter and is a prognostic factor for colon cancer metastasis.28 MALAT1 encodes a long noncoding RNA, which is overexpressed in non-small cell lung cancer patients at high risk for metastasis.29 A previous study indicated that the nuclear form of CSNK1A1 plays a critical role in vascular cell proliferation tied to cancer cell migration.32 FN1 is known to be a metastasis-related matrix protein that promotes cellular growth and migration in vitro.33 SERBP1 is significantly overexpressed in tumor epithelial cells which are involved in tumor invasion and metastasis.34 The novel SOX17 transcriptional repression targets validated in the

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present study are warranted for further interrogations of both biological and clinical implications in ESCC. In addition, the novel SOX17 transcriptional repression targets such as TCF3 are worthy of future investigations.

ESCC is one of the most aggressive cancers, and the outcome is fatal for the majority of patients regardless of the disease stage. SOX17 gene encodes a transcription factor important for esophagus tissue development. However, the transcriptional deregulation of SOX17 in ESCC remains largely unclear. Our cell, animal, and clinical results showed compelling evidence that SOX17 overexpression suppresses tumor growth and metastasis and that SOX17 low expression...
is a prognostic biomarker of ESCC. This study discovered the first transcriptional network of SOX17 in cancer and verified the novel transcriptionally suppressive genes of SOX17 which involved in regulation of migration and invasion of ESCC. Further characterization of these novel oncogenic genes regulated by SOX17 could help to dissect the mechanism of ESCC tumorigenesis and reveal potential therapeutic targets for ESCC.

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