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Inhibition of *SOX17* by MicroRNA 141 and Methylation Activates the WNT Signaling Pathway in Esophageal Cancer

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In this study, we explored the possibility of *SOX17* promoter region methylation as an esophageal cancer detection marker, the regulation of SOX17 expression, and the function of SOX17 in the WNT signaling pathway in esophageal cancer. Eight esophageal cancer cell lines, 9 normal esophageal mucosa samples, 60 cases of dysplasia, and 169 cancer tissue samples were included. Methylation-specific PCR, semiquantitative reverse transcription–PCR, immunohistochemistry, luciferase reporter assay, colony formation, and Western blot analysis were used to analyze methylation and function of SOX17 in esophageal cancer. MicroRNA-related detection methods were performed to evaluate microRNA regulation of *SOX17*. *SOX17* methylation was found in progression tendency with 0% of normal mucosa, 39% of grade 1 dysplasia, 48% of grades 2 and 3 dysplasia, and 65% of primary cancer. *SOX17* methylation is related to esophageal cancer patients' history of alcohol use and may induce β-catenin expression and redistribution. Loss of SOX17 expression is correlated to promoter region hypermethylation, and re-expression was activated by 5-aza-2′-deoxycytidine treatment in esophageal cancer cell lines. Restoration of SOX17 expression suppresses TCF/β-catenin–dependent transcription and colony formation. MicroRNA 141 was also found to down-regulate SOX17 expression and activate the WNT signal pathway. *SOX17* is frequently methylated in esophageal cancer and in a progression tendency during esophageal carcinogenesis. Loss of SOX17 removes the normal inhibition of WNT signaling and promotes esophageal tumorigenesis. ***(J Mol Diagn 2012, 14:577–585; http://dx.doi.org/10.1016/j.jmoldx.2012.06.004)***

Esophageal cancer is the eighth most common malignant tumor and the sixth most fatal disease all over the world.[1–3] Although multiple genetic and epigenetic changes have been associated with esophageal cancer, its underlying molecular mechanism remains incompletely understood.[4,5]

Carcinogenesis is a multifactorial process with the accumulation of genetic and epigenetic changes involving oncogenes and tumor suppressor genes. Methylation patterns are different in esophageal adenocarcinoma and squamous cell carcinoma. *APC* is frequently methylated in human esophageal adenocarcinoma, and *CDX2* is a feature of human esophageal squamous cell carcinoma.[4,6,7] Increasing evidence has shown that constitutive aberrant activation of the WNT/β-catenin signaling pathway is associated with human cancers, including esophageal tumorigenesis.[8,9] SRY-box containing gene 17 (*SOX17*) is a member of the SRY-related high-mobility group (HMG)-box transcription factor superfamily. SOX17 is structurally related to T-cell transcription factor (TCF)/lymphoid enhancer factor (LEF), binding to target DNA sequence 5′-(A/T)(A/T)CAA(A/T)G-3′ and promoting degradation of β-catenin/TCF via a GSK3β-independent mechanism in the WNT signaling pathway.[10] *SOX17* was reported to be frequently methylated in human cancers, but the function and epigenetic

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changes in esophageal cancer and early lesions were not studied before.[10,11]

Materials and Methods

Human Tissue Samples and Cell Lines

A total of 169 cases of primary esophageal squamous cell cancer were studied in this study, including 127 male cases and 42 female cases. Snap-frozen, fresh tissue samples were collected by surgical resection. All samples were classified by TNM (Union for International Cancer Control, 2009) staging, with 5 cases of stage I, 110 cases of stage II, 53 cases of stage III, and 1 case of stage IV. Sixty of these cases had alcohol and smoking use records, and 121 cases had differentiation, tumor size, or metastasis description. Thirty-nine cases of low-grade dysplasia, 12 cases of intermediate-grade dysplasia, and 9 cases of high-grade dysplasia were collected as paraffin-embedded samples. Samples from 9 cases of normal esophageal epithelia were collected by endoscopy biopsy with snap-frozen tissue. Matched adjacent tissue was available in 39 cases, with paraffin blocks for 169 primary cancer samples. All samples were collected under the guidelines approved by the Chinese PLA General Hospital's institutional review board and with written informed consent from patients.

All esophageal cancer cell lines were previously established from primary esophageal cancer, and maintained in 90% RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum.[4,12]

5-Aza-2′-Deoxycytidine Treatment

Esophageal cancer cell lines (SKGT4, YSE2, KYSE30, KYSE70, KYSE140, KYSE150, KYSE180, and KYSE450) were split to low density (30% confluence) 12 hours before treatment. Cells were treated with 5-aza-2′-deoxycytidine (DAC; Sigma, St. Louis, MO) at a concentration of 2 μmol/L. Growth medium, conditioned with DAC at 2 μmol/L, was exchanged every 24 hours for a total of 96 hours of treatment.

RNA Isolation and Semiquantitative Reverse Transcription–PCR

Total RNA was isolated by Trizol reagent (Life Technologies, Gaithersburg, MD). First-strand cDNA was synthesized according to the manufacturer instruction's (Invitrogen). PCR amplification of *SOX17* was performed using primers: 5′-GGCTGGCGCAGCAGAATC-3′ (forward) and 5′-AGCCCTGCTCGGGGAACT-3′ (reverse). Amplified products were analyzed on 1.5% agarose gels. A total of 32 cycles of amplification were performed for each of the reverse transcription–PCR (RT-PCR) experiments. As an internal control, GAPDH was amplified with 25 cycles to ensure cDNA quality and quantity for each RT-PCR.

Bisulfite Modification, Methylation-Specific PCR, and Bisulfite Sequencing

DNA was prepared by the proteinase K method. The bisulfite treatment was performed as described in a previous report.[13] Methylation-specific PCR (MSP) and nested MSP were performed as described previously.[6] MSP primers were designed according to genomic sequences flanking presumed translation start sites and are as follows: 5′-GGGGCGTTCGTAGTGTTATTAGGTC-3′ (M-forward); 5′-AAACACTAAAATACCCCGAAAACTACG-3′ (M-reverse); 5′-TTAGGGGTGTTTGTAGTGTTATTAGGTT-3′ (U-forward) and 5′-TAAAACACTAAAATACCCCAAAAACTACA-3′ (U-reverse). The external primer sequences of nested MSP were as follows: 5′-AGAAAAGGTTTYGYGGTTTAGG-3′ (nest-forward) and 5′-TAAAAAATAAAACACTAAAATA-CCC-3′ (nest-reverse). Bisulfite-treated DNA was subjected to PCR using bisulfite sequencing (BSSQ) primers flanking the targeted MSP regions. Sequencing primers were designed as follows: 5′-GTTTTATTGGTTATATTTGTGTAG-3′ (BSSQ-forward) and 5′-ACTACTCATAACRCTCCAAAC-3′ (BSSQ-reverse). Bisulfite sequencing was performed as described in a previous report.[11]

Immunohistochemistry

β-Catenin antibody (ZSGB Biotech, Beijing, China) and SOX17 antibody (OriGene Technologies, Rockville, MD) were used in this study. Immunohistochemistry was performed as previously described.[11] The χ^2 test was used to analyze the correlation between *SOX17* methylation and location of β-catenin and *SOX17*; $P < 0.05$ was considered statistically significant.

SOX17, MicroRNA, 3′-Untranslated Region Vector Construction, and Mutagenesis

Full-length *SOX17* cDNA was cloned as described previously.[10] To generate microRNA (miRNA) expression vector, a genomic fragment of *Homo sapiens* miRNA precursor was amplified and cloned into pcDNA3.0 (Invitrogen). *SOX17* 3′-untranslated region (3′-UTR) was also generated by PCR with primers designed depending on bioinformatics websites. The PCR product was subcloned into pGL3 control vector with XbaI and NdeI sites (Promega) immediately downstream of the stop codon of the luciferase reporter gene. Mutant construct of 3′-UTR of *SOX17* mRNA was generated by PCR to disrupt the microRNA 141 (miR-141) binding site.

Colony Formation Assay

KYSE140 cells were grown in six-well culture plates 24 hours before transfection. Cells were transfected with empty control vector or SOX17 expression construct according to the manufacturer's instructions (Roche Applied Science, Penzberg, Germany). Colony formation assay was performed as reported before.[11]

Luciferase Reporter Assay

KYSE140 cells were seeded at 5×10^4 cells/well in 24-well culture plates 24 hours before transfection. To examine transcriptional activity driven by β-catenin/TCF, KYSE140 cells were transfected with 200 ng/well pGL3-OT (TCF/LEF-responsive reporter),[14] 30 ng/well pRL-TK control vector (Promega) as an internal control reporter, and 600 ng/well β-catenin construct. Basal transcriptional activity in KYSE140 cells was tested only by transfection with empty vectors. KYSE140 cells were then transfected with 100 ng/well pGL3-OT, 10 ng/well pRL-TK, 150 ng/well β-catenin constructs, and 200 ng/well *SOX17* constructs (wild type and mutant). Thereafter, 100 ng/well pGL3-OT, 10 ng/well pRL-TK, 100 ng/well β-catenin expression vector, and increased amounts of pcDNA3.1/V5-*SOX17*-His B wild-type, mutant (30 ng, 90 ng, and 120 ng, *SOX17* constructs 50–414, 135–414, and 1–353, respectively), or empty vector were transfected into KYSE140 cells with FuGENE 6 (Roche Applied Science).

pGL3-*SOX17*-3′-UTR vector, which contains the 3′-UTR of the *SOX17* gene and the firefly luciferase reporter, was cotransfected at 125 ng/well with 1 ng/well pRL-CMV control vector (Promega) and different miRNA expression vectors (pcDNA3.0-miRNA-26a, −26b, −141, −151, −200a, −329, and −362) or empty vector, respectively, at 375 ng/well using FuGENE 6 (Roche Applied Science); 1 ng/well pRL-CMV vector control (Promega) and 125 ng/well wild-type pGL3-*SOX17*-3′-UTR or 125 ng/well pGL3-*SOX17*-3′-UTR mutant were cotransfected with 375 ng/well pcDNA3.0-miRNA-141 in KYSE140 cells by FuGENE 6 (Roche Applied Science).

Relative luciferase activity was measured according to the manufacturer's instructions (GloMax luminometer; Promega) 48 hours after transfection.

Protein Preparation and Western Blot Analysis

KYSE140 cells were transfected with *SOX17* constructs (wild type and mutant). YSE2 cells were transfected with miRNA mimics or miRNA control. Forty-eight hours later, transfected cells were harvested for Western blotting according to the manufacturer's instructions.

miR-141 Expression Detection in Esophageal Cancer Cell Lines

For miRNA detection, total RNA was polyadenylated by poly(A) polymerase according to the manufacturer's protocol (Ambion, Austin, TX). Reverse transcription was performed using 1 μg of poly(A)-tailed total RNA and 1 μg of reverse transcription primer (5′-GCGAGCACAGAATTAATACGACTCACTATAGG(T)18VN-3′) with 1 μL ImPro-II Reverse Transcriptase (Promega) according to the manufacturer's protocol. Quantitative PCR was performed as described in the method of Quantitect SYBR Green PCR Kit (Qiagen, Hilden, Germany) with Mx3000p (Stratagene, La Jolla, CA). GenEX software, version 4.10 (TATAA Biocenter, Munich, Germany) was used to analyze real-time PCR results.

Statistical Analysis

Statistical analysis was performed using the χ^2 test, and $P < 0.05$ was considered statistically significant.

Results

SOX17 Is Silenced by Frequent Methylation in Esophageal Cancer Cell Lines

SOX17 expression level was determined using semiquantitative RT-PCR in esophageal cancer cell lines. SOX17 was expressed in the YSE2 cell line and weakly in the KYSE70, KYSE180, and KYSE450 cell lines. However, no expression was detected in the SKGT4, KYSE30, KYSE140, and KYSE150 cell lines. *SOX17* gene promoter region was completely methylated in the SKGT4, KYSE30, KYSE140, and KYSE150 cell lines, which did not express this gene. Partial methylation and weak expression were detected in KYSE70, KYSE180, and KYSE450 cells. *SOX17* is unmethylated and strongly expressed in the YSE2 cell line (Figure 1, A and B).

The *SOX17* promoter region CpG island distribution and methylation detection location are shown in Figure 1C, transcription start site is located in the CpG island. Two sets of primers were designed encompassing the transcription start site to perform MSP and BSSQ. The representative BSSQ of *SOX17* was performed in the SKGT4, KYSE140, YSE2, and KYSE70 cell lines. Consistent with MSP results, BSSQ of 10 individual clones from SKGT4 and KYSE140 revealed dense methylation of

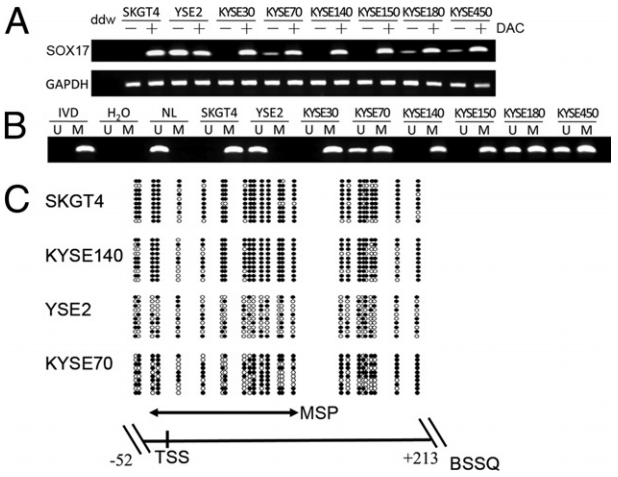

Figure 1. Expression of *SOX17* was silenced by DNA methylation in esophageal cancer cell lines. **A:** Expression of *SOX17* was analyzed by semiquantitative RT-PCR in esophageal cancer cell lines with absence or presence of treatment with 2 μmol/L DAC for 96 hours. (−) denotes untreated; (+) denotes DAC treated. ddw, double-distilled water. GAPDH was used as internal control. **B:** MSP results of *SOX17* in esophageal cancer cell lines (SKGT4, YSE2, KYSE30, KYES70, KYSE140, KYSE150, KYSE180, and KYSE450). Primer efficiency was verified by positive control (*in vitro* methylated DNA, IVD) and negative control (normal lymphocyte DNA, NL). M, methylated alleles; U, unmethylated alleles. **C:** Bisulfite sequencing of *SOX17* in SKGT4, KYSE140, YSE2, and KYSE70 cell lines. The region amplified by MSP is indicated by a **double-headed arrow** and spans 136 bp. Filled circles represent methylated CpG sites and open circles denote unmethylated CpG sites. Bisulfite sequencing focused on a 265-bp (−52 bp to +213 bp) CpG island across the *SOX17* transcription start site. TSS, transcriptional start site.

CpGs within the promoter region. The mixed methylated and unmethylated results by bisulfite sequencing in KYSE70 may represent both methylated and unmethylated alleles, or both methylated and unmethylated clonal subpopulations within cultured cells. Result from YSE2 shows unmethylation status of CpG in the promoter region of *SOX17*.

To further validate whether SOX17 expression is regulated by promoter region methylation, the effect of DAC on SOX17 expression was determined using semiquantitative RT-PCR in esophageal cancer cell lines. DAC is a DNA methylation transferase inhibitor.[15,16] After DAC treatment for 96 hours, SOX17 was re-expressed in the SKGT4, KYSE30, KYSE140, and KYSE150 cell lines. Increased expression of SOX17 was also detected in the KYSE70, KYSE180, and KYSE450 cell lines, which were partially methylated and had reduced expression compared to YSE2 (Figure 1A). These results suggest that SOX17 expression is silenced by promoter region methylation in esophageal cancer.

SOX17 *Is Frequently Methylated in Esophageal Dysplasia and Primary Cancer*

To explore whether *SOX17* is methylated during esophageal carcinogenesis, we examined 9 cases of normal esophageal mucosa, 60 cases of different grades of dysplasia, and 169 cases of primary esophageal cancer. A total of 109 of 169 (65%) cases of primary esophageal cancer were methylated (Figure 2A), and this appears to be a neoplasia-specific alteration, since all 9 cases of normal esophageal mucosa were unmethylated (Figure 2B). There was slightly more frequent methylation of *SOX17* in late (stage III and IV) versus early stage esophageal cancer. To determine the place in carcinogenesis that methylation of *SOX17* occurs, we examined dysplastic lesions. Twenty-five of 60 (42%) cases were methylated in esophageal dysplasia (Figure 2C), more frequently in grades 2 and 3 dysplasia (48%) than in grade 1 (39%), suggesting that methylation of *SOX17* is associated with the progression of esophageal cancer and is a frequent and early event. In the 169 cases of esophageal cancer, 127 patients were male and 42 patients were female. The ratio of male/female is similar to the ratios observed in other high-incidence areas in the world.

It was reported that alcohol is a major cause of squamous cell carcinoma in the United States and Europe.[17–19] *SOX17* methylation is significantly correlated with alcohol history in 60 patients with alcohol and smoking records in this study (χ^2 test, $P = 0.0057$). No significant difference was found when grouped by differentiation, tumor size, or metastasis for the 121 cases with detailed descriptions. There were no significant differences among age, sex, smoking history, and TNM stages for *SOX17* methylation (Table 1). As shown in Figure 2D, the *SOX17* methylation rate increases with the progression of esophageal carcinogenesis from normal esophageal epithelia, grade 1, grade 2, and grade 3 dysplasia to advanced cancer (χ^2 test, $P = 0.0002$). These

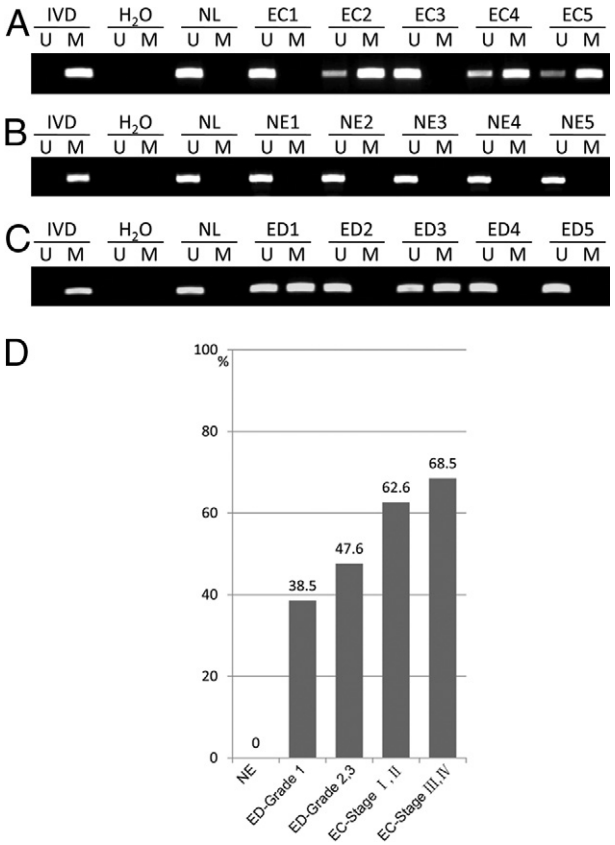

Figure 2. Methylation status of *SOX17* CpG islands in esophageal cancer, esophageal dysplasia, and normal esophageal mucosa. **A:** Representative MSP results of *SOX17* in esophageal primary cancer (EC). **B:** Representative MSP results of *SOX17* in normal esophageal mucosa (NE). **C:** Representative MSP results of *SOX17* in esophageal dysplasia (ED). **D:** Frequency of *SOX17* methylation in grade 1 dysplasia (ED1), grade 2 dysplasia (ED2), grade 3 dysplasia (ED3), and advanced esophageal cancer (EC). The frequency of methylated *SOX17* is plotted according to histological grade. No *SOX17* methylation was found in normal esophageal mucosa. Grade 2 dysplasia and grade 3 dysplasia were combined into one group because of small case numbers. Stages I and II, and stages III and IV, respectively, of esophageal cancer were merged to two different groups.

results suggest that *SOX17* gene promoter region methylation is a potential early detection marker of esophageal cancer.

SOX17 *Methylation Promotes β-Catenin Expression in Nucleus and Cytoplasm*

β-Catenin is reported to be widely distributed in cellular membrane, cytoplasm, and nucleus. Transcription of the WNT signaling pathway downstream genes may be activated by stabilized β-catenin accumulation in the cytoplasm and translocation to the nucleus. To explore the possible interaction of SOX17 and β-catenin, IHC was performed on 39 pairs of available paraffin samples (esophageal cancer and adjacent tissue). *SOX17* methylation status was also already analyzed in these 9 cases of esophageal cancer. Thirty-three samples were methylated, and 6 samples were unmethylated. In 39 samples of adjacent tissue, 7 samples were methylated and 32 samples were unmethylated. Nuclear SOX17 staining was found in 28 samples of unmethy-

Table 1. Clinicopathological Characteristics and *SOX17* Methylation Status of 169 Patients with Esophageal Cancer

Clinical parameter	No.	*SOX17* methylation status: Methylated $n = 109$ (64.5%)	*SOX17* methylation status: Unmethylated $n = 60$ (35.5%)	*P* value*
Age (years)				
<50	22	16 (72.7%)	6 (27.3%)	0.3870
≥50	147	93 (63.3%)	54 (36.7%)	
Sex				
Male	127	85 (66.9%)	42 (33.1%)	0.2506
Female	42	24 (57.1%)	18 (42.9%)	
Alcohol abuse†				
Negative	31	18 (58.1%)	13 (41.9%)	0.0057
Positive	29	26 (89.7%)	3 (10.3%)	
Smoking†				
Negative	33	21 (63.6%)	12 (36.4%)	0.0604
Positive	27	23 (85.2%)	4 (14.8%)	
Tumor size (cm)‡				
<5	86	62 (72.1%)	24 (27.9%)	0.6985
≥5	35	24 (68.6%)	11 (31.4%)	
Differentiation‡				
Poorly	37	27 (73.0%)	10 (27.0%)	0.3247
Moderately	74	50 (67.6%)	24 (32.4%)	
Well	10	9 (90.0%)	1 (10.0%)	
Tumor stage				
I	5	4 (80%)	1 (20%)	0.6387
II	110	68 (61.8%)	42 (38.2%)	
III	53	36 (67.9%)	17 (32.1%)	
IV	1	1 (100%)	0 (0%)	
Metastasis‡				
Negative	74	51 (68.9%)	23 (31.1%)	0.5117
Positive	47	35 (74.5%)	12 (25.5%)	

SOX17 methylation is significantly correlated with alcohol history in 60 cases with alcohol and smoking records in this study (χ^2 test, $P = 0.0057$).
*P values are obtained by the χ^2 test. Statistical significance is indicated by $P < 0.05$.
†In 169 patients, 60 cases had alcohol and smoking records.
‡In 169 patients, 121 cases had pathological descriptions of tumor size, metastasis, and differentiation.

lated adjacent esophageal tissue, but only 3 cases of esophageal cancer showed positive SOX17 nuclear staining (Figure 3A). Nuclear SOX17 staining inversely correlated to *SOX17* methylation (χ^2 test, $P = 0.0359$). In 33 samples of esophageal cancer, 10 cases positive for methylated *SOX17* nuclear β-catenin were found, and cytoplasmic β-catenin staining was found in 26 cases (χ^2 test, $P = 0.0105$) (Figure 3B). In six cases of *SOX17* unmethylated esophageal cancer, β-catenin staining was different: two cases were cell membrane positive, three cases had cytoplasmic staining, and two cases were negative for staining. The results indicate *SOX17* methylation is correlated with β-catenin expression in the nucleus and cytoplasm in esophageal cancer. β-Catenin was predominantly expressed in membrane, but no expression was found in the nucleus for the unmethylated adjacent noncancerous tissues (Figure 3A). SOX17 and nuclear β-catenin expression were significantly different in the matched esophageal cancer and adjacent tissues (χ^2 test, $P = 0.00001$, $P = 0.0011$, respectively) (Figure 3B). SOX17 was mainly expressed in unmethylated adjacent tissues, whereas nuclear and cytoplasmic β-catenin was mainly expressed in methylated esophageal cancer tissues. These results indicate that the WNT/β-catenin signaling pathway may play an important role in esophageal cancer.

Restoration of SOX17 Expression Inhibits Clonogenicity in KYSE140 Cells

To evaluate the inhibitory effect of SOX17 on clonogenicity of esophageal cancer cells, colony formation was assayed in KYSE140 cells. Re-expression of SOX17 was confirmed by Western blot after transfection of wild-type SOX17 expression vector. Colony formation was inhibited by restoration of SOX17 expression (Figure 4), suggesting that SOX17 may serve as a potential tumor suppressor during esophageal cancer development.

SOX17 Suppresses Transcriptional Activation of β-Catenin in KYSE140 Cells

In this study, the TCF/LEF luciferase reporter system was used to explore SOX17's effect on WNT/β-catenin and downstream genes. Relative luciferase activity was increased sharply in KYSE140 cells transfected with β-catenin expression vector compared with control cells that were transfected with empty control vector (Figure 5A). A schematic diagram of different structural deletions on the *SOX17* gene is shown in Figure 5B. In the luciferase reporter assay, pGL3-OT vector is a TCF/LEF-responsive reporter containing three consensus TCF binding sites, and pRL-TK vector is used as system control.[14,20] After transfection with pGL3-OT, pRL-TK, β-catenin, and SOX17 ex-

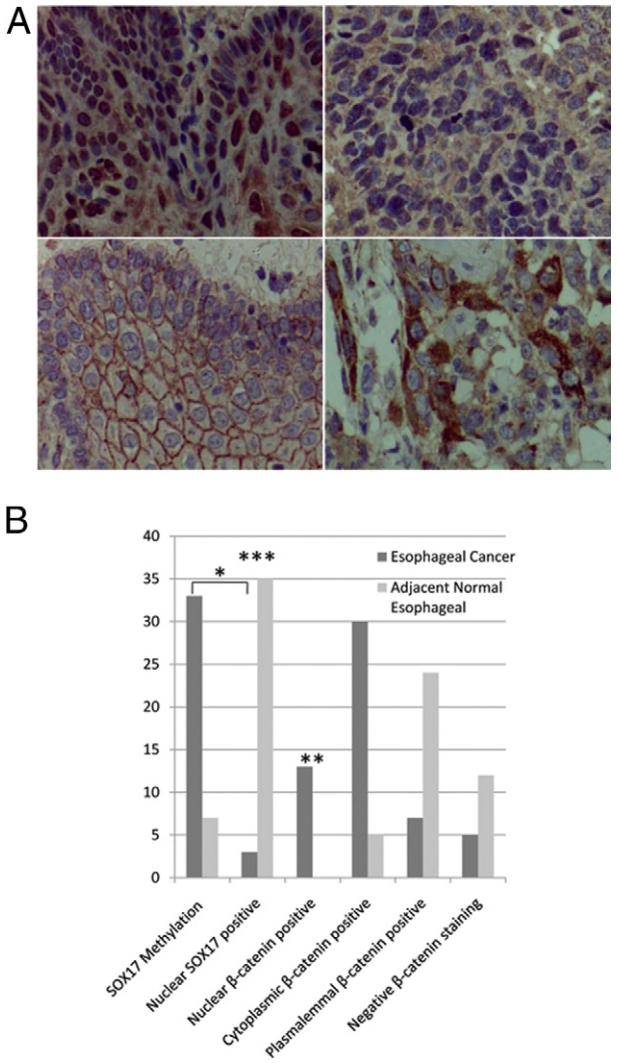

Figure 3. Immunohistochemistry analysis of SOX17 and β-catenin in esophageal cancer and adjacent tissue. **A:** Esophageal cancer and adjacent tissue were immunohistologically analyzed by anti-SOX17 (1:80 dilution) and anti–β-catenin (1:200 dilution) staining (×400). SOX17 was nuclear positive in adjacent esophageal tissue (**upper left**); SOX17 was negative in esophageal cancer tissue (**upper right**); β-catenin was plasmalemmal positive in adjacent esophageal tissue (**lower left**); nuclear and cytoplasmic β-catenin staining was found in esophageal cancer tissue (**lower right**). **B:** Number of positive SOX17 and β-catenin staining cells in esophageal cancer and in adjacent tissue is shown as a bar graph. Statistical analysis was performed using the χ^2 test, and $P < 0.05$ was considered statistically significant. $^*P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$.

pression vector or its different deletion segments, *SOX17* fragment 135–414 with deletion in the HMG domain, showed no suppressive effect on β-catenin/TCF-LEF transcriptional activity. By contrast, the results of using other deletion segments, including *SOX17* fragment 50–414 and fragment 1–353, were similar to wild-type *SOX17* (Figure 5C). To further explore this inhibition of the regulatory effect of *SOX17* HMG, increasing amounts of mutant or wild-type *SOX17* vectors were transfected into KYSE140 cells separately. In groups that were transfected with *SOX17* constructs with a HMG region fragment, the downstream transcriptional activity of β-catenin/TCF-LEF was suppressed in a dose-dependent manner (Figure 5D). Furthermore, Western blotting was performed to test the expression of downstream genes such as *cyclinD1*. CyclinD1 expression was detected in KYSE140 cells transfected with the *SOX17* construct including fragment 135–414; however, expression of cyclinD1 was decreased in other groups (Figure 5E).

SOX17 Is Down-Regulated by miR-141 in YSE2 Cells

Increasing evidence indicates that miRNAs play important roles during human cancer development. MicroRNAs negatively regulate gene expression by targeting mRNA 3′-UTR and degrading mRNA or repressing translation.[21] To clarify whether SOX17 expression is regulated by miRNAs, the TargetScan and miRBase bioinformatics databases were used.[22] As predicted by these databases, the 3′-UTR of *SOX17* harbors potential binding sites of several miRNAs, including miR-26a, miR-141, and miR-200a. Luciferase reporter assay was used to screen candidate miRNAs targeting on 3′-UTR of *SOX17* mRNA in the KYSE140 cell line. Relative luciferase activity was sharply decreased in miR-141–, miR-26a–, and miR-200a–transfected cells in comparison with the control group (Figure 6A). To further validate the inhibitory effect of these miRNAs, Western blotting was used in this study. Expression of SOX17 was down-regulated by miR-141. Furthermore, *MYC* (alias *c-myc*) and *cyclinD1*, downstream genes of the WNT signaling pathway, were simultaneously up-regulated by miR-141 indirectly. By con-

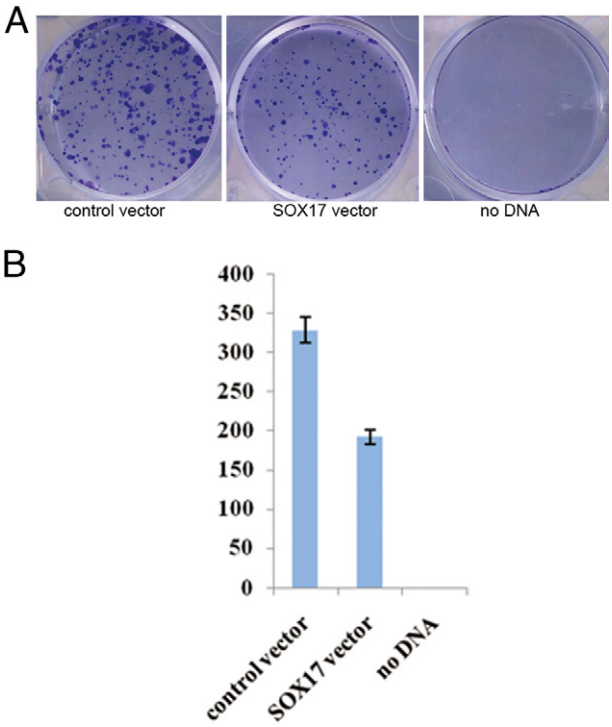

Figure 4. *SOX17* inhibited colony formation of KYSE140 cells. **A:** Empty control vector and *SOX17* expression vector were separately transfected into KYSE140 cells. Clone number is reduced by *SOX17* expression. No clone was found in the absence of anti-neomycin vector after G418 screening. **B:** Density of colonies after G418 selection. Each experiment was repeated in triplicate, and the average number of colonies is presented in the bar graph.

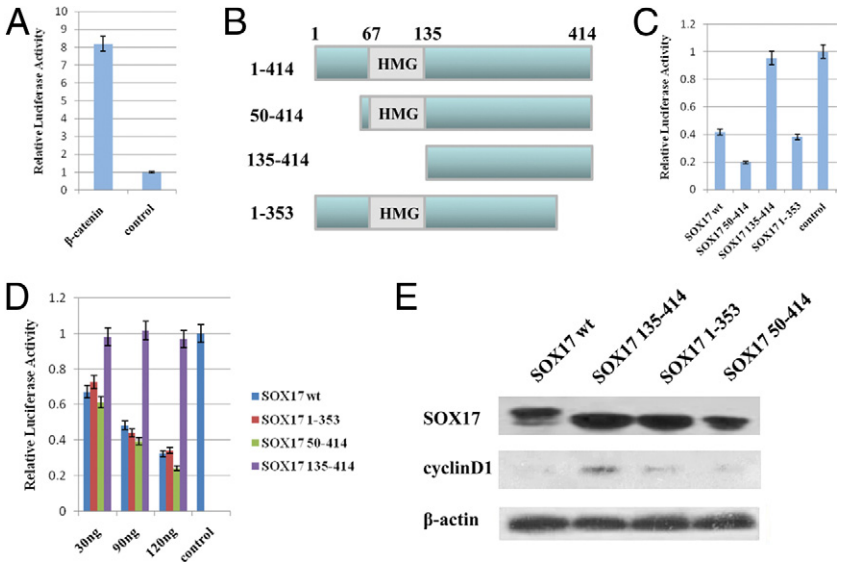

Figure 5. *SOX17* influence on β-catenin and downstream genes transcription. Results of luciferase reporter assay, normalized and shown as relative luciferase activity (a ratio of firefly luciferase to renilla luciferase). These experiments were repeated three times. Average level is shown in the bar graph. **A:** KYSE140 cells were transfected with β-catenin expression vector. The basal transcriptional activity was tested by transfection of empty vectors. **B:** Architecture of *SOX17* with HMG box is depicted in schematic form. Different deletion mutants (*SOX17* constructs 50–414, 135–414, and 1–353) were generated by PCR. **C:** *SOX17* inhibits the WNT signaling pathway by the NH2-terminal HMG. KYSE140 cells were transfected with β-catenin and *SOX17* expression vectors (wild type or mutant). **D:** *SOX17* influence of β-catenin in a dose-dependent manner. Different amounts of wild-type or mutant *SOX17* were transfected into KYSE140 cells. **E:** WNT signaling pathway downstream gene *cyclinD1* was inhibited by wild-type and mutant *SOX17* with HMG box.

trast, the expression levels of SOX17 and downstream genes (*cyclinD1* and *MYC*) were not different between miR-26a, miR-200a, and control groups (Figure 6B). The putative binding sites of *SOX17* 3′-UTR were analyzed by TargetScan. Only one miR-141 binding site was discovered in the 3′-UTR of *SOX17* mRNA. The binding sites are also conserved among *H. sapiens*, *Mus musculus*, *Rattus norvegicus*, and *Oryctolagus cuniculus* (Figure 6C). To validate the interaction of miR-141 and the 3′-UTR of *SOX17* mRNA, the 3′-UTR of *SOX17* mutant was generated by PCR (Figure 6C). Fifty-five percent of relative luciferase activity was reduced in miR-141–transfected cells, in comparison with the control group. No significant changes was found in the 3′-UTR of the *SOX17* mutant group related to miR-141 (Figure 6D). These results indicate that miR-141 may effectively interact with the 3′-UTR of *SOX17* mRNA, but no effective interaction was found in the mutant group. To explore the relationship of miR-141 and SOX17 expression, miR-141 expression was detected by real-time PCR in eight esophageal cancer cell lines. SOX17 expression in these cell lines was described as above. The results indicated miR-141 and SOX17 expression were inversely correlated in these cell lines (Figure 6E). miR-141 expression level was higher in the KYSE30, KYSE140, KYSE150, and SKGT4 cell lines, but was lower in the KYSE70, KYSE180, and KYSE450 cell lines. The expression level of miR-141 was very low in the YSE2 cell line. SOX17 was expressed in the YSE2 cell line and weakly in the KYSE70, KYSE180, and KYSE450 cell lines. No expression was detected in the KYSE30,

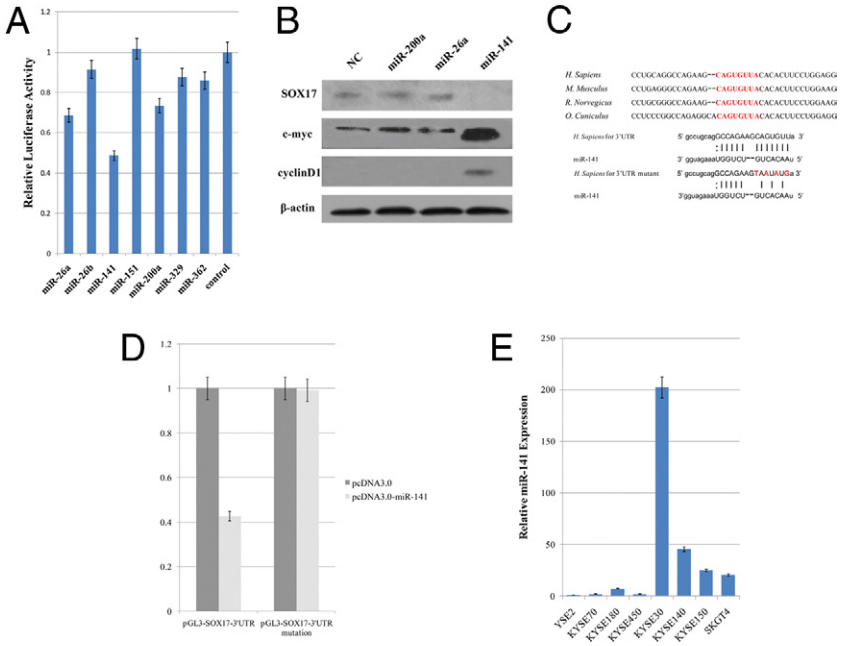

Figure 6. *SOX17* is the target of miR-141 and was down-regulated in esophageal cancer. **A:** pGL3-*SOX17*-3′-UTR reporter plasmid and different pcDNA3.0-miRNA vectors were cotransfected into KYSE140 cells separately. Each experiment was repeated three times. The average level of relative luciferase activity is shown in the bar graph. **B:** YSE2 cells were transfected with miRNA mimics or miRNA control; *SOX17*, *MYC*, and *cyclin D1* expression levels after 48 hours transfection are shown. β-Actin was used as control. **C:** The conserved sequence of *SOX17* 3′-UTR region (**upper panel**, red) is predicted as the target of miR-141 by TargetScan. Wild-type and mutational 3′-UTR of *SOX17* (**lower panel**, red) were distinguished by four bases. **D:** Wild-type or mutational pGL3-*SOX17*-3′-UTR reporter plasmid and pcDNA3.0-miR141, respectively, were transfected into KYSE140 cells. Each experiment was repeated three times. The average level of relative luciferase activity is shown in the bar diagram. **E:** miR-141 expression level in eight esophageal cancer cell lines; Real-time PCR results were normalized relative to the YSE2 expression level. These experiments were repeated twice. The average levels are shown in the bar graph.

KYSE140, KYSE150, and SKGT4 cell lines. These studies suggest that miR-141 may be involved in the silencing of SOX17. All the above results demonstrate that SOX17 expression may be regulated by both DNA methylation and miR-141 in esophageal cancer cell lines.

Discussion

In this study, 8 cancer cell lines, 9 normal esophageal mucosa, 60 dysplasia, and 169 cases of esophageal primary cancer were analyzed for *SOX17* methylation. *SOX17* methylation was correlated to loss of gene expression in esophageal cancer cell lines, and SOX17 expression was restored in SKGT4, KYSE30, KYSE140, and KYSE150 cells after DAC treatment (Figure 1). These results indicate that SOX17 expression is regulated by promoter region methylation. Bisulfite sequencing was performed to confirm the MSP results and further to analyze the distribution and density of methylation in the promoter region in esophageal cancer cell lines. The results indicate that the selected location and primer sequences are proper. *SOX17* is frequently methylated in human primary esophageal cancer (65%) and dysplasia (42%), but not in normal esophageal mucosa. As shown in Figure 2, *SOX17* methylation is in progression tendency during esophageal cancer carcinogenesis from normal epithelia, different grades of dysplasia, and advanced cancer. The above results indicate that *SOX17* methylation may serve as an esophageal cancer early detection marker. Immunohistochemistry results hint that SOX17 expression is regulated by promoter region methylation, and further affects β-catenin expression and distribution. These results suggest that *SOX17* plays an important role in the WNT signaling pathway in esophageal cancer. As shown in Table 1, *SOX17* methylation is related to alcohol history in esophageal cancer. This suggests that alcohol may cause *SOX17* methylation and further lead to esophageal cancer. Reduction of alcohol consumption may help prevent esophageal cancer by hindering *SOX17* gene methylation.

The canonical WNT signaling pathway is involved in many biological processes, including embryogenesis and carcinogenesis.[23,24] It was reported that free cytoplasmic β-catenin enters the nucleus, forms a complex with members of the TCF/LEF transcription factor family, and then up-regulates target gene expression.[25,26] The activated WNT/β-catenin signaling pathway may induce *MYC*, *cyclinD1*, and other downstream genes expression and, finally, promote cell proliferation and cause carcinogenesis. The SOX gene family plays different roles during development and carcinogenesis. Nuclear protein SOX17 may inhibit the WNT signaling pathway by degradation of the β-catenin/TCF complex. Inactivation of SOX17 may promote the WNT/β-catenin signaling pathway and lead to carcinogenesis. The *SOX17* promoter region was frequently methylated, with silenced expression, in esophageal cancer cell lines. Restoration of SOX17 expression reduced colony formation and inhibited β-catenin/TCF–dependent transcriptional activity in KYSE140 cells. As shown in Figure 5, loss of the HMG box of *SOX17* may promote β-catenin and its downstream gene transcription. These results suggested that *SOX17* is an important negative regulator of the canonical WNT signaling pathway in esophageal cancer. Reversion of SOX17 expression may inhibit esophageal carcinogenesis. The above results lead us to consider further studies of targeting therapy in esophageal cancer by demethylation of *SOX17* in the future.

MicroRNAs are endogenous, 18- to 25-nucleotide, non-coding RNAs that mainly serve as regulators of gene expression by binding to the 3′-untranslated region of targeting mRNAs. The miR-141/200 family was reported to take part in maintenance of epithelial phenotype, and loss of its expression may lead to epithelial to mesenchymal transition.[27] Recent publications indicate that miR-141 may stimulate cell proliferation.[28] A high level of miR-141 in plasma is associated with a short survival time in colorectal cancer patients.[29] As shown in Figure 6, our screening results demonstrate that miR-141, miR-200a, and miR-26a may interact with the 3′-UTR of *SOX17* mRNA, but only miR-141 was found to effectively down-regulate SOX17 expression on further study. Up-regulation of MYC and cyclinD1 were also induced indirectly by miR-141 in this study; and the expression of miR-141 was inversely correlated with SOX17. All the above results indicate that *SOX17*, the WNT signal pathway gene, is regulated by both promoter region methylation and miR-141 in esophageal cancer.

In conclusion, *SOX17* is frequently methylated in esophageal cancer. The progression tendency of *SOX17* methylation during esophageal carcinogenesis indicates it may serve as an esophageal cancer early detection marker. *SOX17* methylation is significantly correlated with alcohol history. *SOX17* promoter region methylation may induce β-catenin up-regulation and redistribution. SOX17 expression was regulated by both promoter region methylation and miR-141 in esophageal cancer. *SOX17* is probably a tumor suppressor and involved in the WNT signaling pathway.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ: Cancer statistics, 2009. CA Cancer J Clin 2009, 59:225–249
2. Kamangar F, Dores GM, Anderson WF: Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol 2006, 24:2137–2150
3. Pohl H, Welch HG: The role of overdiagnosis and reclassification in the marked increase of esophageal adenocarcinoma incidence. J Natl Cancer Inst 2005, 97:142–146
4. Guo M, House MG, Suzuki H, Ye Y, Brock MV, Lu F, Liu Z, Rustgi AK, Herman JG: Epigenetic silencing of CDX2 is a feature of squamous esophageal cancer. Int J Cancer 2007, 121:1219–1226
5. Guo M, Liu S, Lu F: Gefitinib-sensitizing mutations in esophageal carcinoma. N Engl J Med 2006, 354:2193–2194
6. Guo M, Ren J, House MG, Qi Y, Brock MV, Herman JG: Accumulation of promoter methylation suggests epigenetic progression in squamous cell carcinoma of the esophagus. Clin Cancer Res 2006, 12: 4515–4522
7. Brock MV, Gou M, Akiyama Y, Muller A, Wu TT, Montgomery E, Deasel M, Germonpre P, Rubinson L, Heitmiller RF, Yang SC, Forastiere AA, Baylin SB, Herman JG: Prognostic importance of promoter

hypermethylation of multiple genes in esophageal adenocarcinoma. Clin Cancer Res 2003, 9:2912–2919
8. Kudo J, Nishiwaki T, Haruki N, Ishiguro H, Shibata Y, Terashita Y, Sugiura H, Shinoda N, Kimura M, Kuwabara Y, Fujii Y: Aberrant nuclear localization of beta-catenin without genetic alterations in beta-catenin or Axin genes in esophageal cancer. World J Surg Oncol 2007, 5:21
9. Salahshor S, Naidoo R, Serra S, Shih W, Tsao MS, Chetty R, Woodgett JR: Frequent accumulation of nuclear E-cadherin and alterations in the Wnt signaling pathway in esophageal squamous cell carcinomas. Mod Pathol 2008, 21:271–281
10. Zhang W, Glockner SC, Guo M, Machida EO, Wang DH, Easwaran H, Van Neste L, Herman JG, Schuebel KE, Watkins DN, Ahuja N, Baylin SB: Epigenetic inactivation of the canonical Wnt antagonist SRY-box containing gene 17 in colorectal cancer. Cancer Res 2008, 68:2764–2772
11. Jia Y, Yang Y, Liu S, Herman JG, Lu F, Guo M: SOX17 antagonizes WNT/beta-catenin signaling pathway in hepatocellular carcinoma. Epigenetics 2010, 5:743–749
12. Guo M, House MG, Akiyama Y, Qi Y, Capagna D, Harmon J, Baylin SB, Brock MV, Herman JG: Hypermethylation of the GATA gene family in esophageal cancer. Int J Cancer 2006, 119:2078–2083
13. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996, 93:9821–9826
14. Shih IM, Yu J, He TC, Vogelstein B, Kinzler KW: The beta-catenin binding domain of adenomatous polyposis coli is sufficient for tumor suppression. Cancer Res 2000, 60:1671–1676
15. Qin T, Jelinek J, Si J, Shu J, Issa JP: Mechanisms of resistance to 5-aza-2′-deoxycytidine in human cancer cell lines. Blood 2009, 113: 659–667
16. Griffiths EA, Gore SD: DNA methyltransferase and histone deacetylase inhibitors in the treatment of myelodysplastic syndromes. Semin Hematol 2008, 45:23–30
17. Eslick GD: Epidemiology of esophageal cancer. Gastroenterol Clin North Am 2009;38:17–25
18. Glickman JN: Section II: pathology and pathologic staging of esophageal cancer. Semin Thorac Cardiovasc Surg 2003, 15:167–179
19. Freedman ND, Abnet CC, Leitzmann MF, Mouw T, Subar AF, Hollenbeck AR, Schatzkin A: A prospective study of tobacco, alcohol, and the risk of esophageal and gastric cancer subtypes. Am J Epidemiol 2007, 165:1424–1433
20. Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H: Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science 1997, 275:1784–1787
21. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004, 116:281–297
22. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB: Prediction of mammalian microRNA targets. Cell 2003, 115:787–798
23. Klaus A, Birchmeier W: Wnt signalling and its impact on development and cancer. Nat Rev Cancer 2008, 8:387–398
24. Clevers H: Wnt/beta-catenin signaling in development and disease. Cell 2006, 127:469–480
25. Sinner D, Rankin S, Lee M, Zorn AM: Sox17 and beta-catenin cooperate to regulate the transcription of endodermal genes. Development 2004, 131:3069–3080
26. Sinner D, Kordich JJ, Spence JR, Opoka R, Rankin S, Lin SC, Jonatan D, Zorn AM, Wells JM: Sox17 and Sox4 differentially regulate beta-catenin/T-cell factor activity and proliferation of colon carcinoma cells. Mol Cell Biol 2007, 27:7802–7815
27. Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, Brabletz T: A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep 2008, 9:582–589
28. Hyun S, Lee JH, Jin H, Nam J, Namkoong B, Lee G, Chung J, Kim VN: Conserved microRNA miR-8/miR-200 and its target USH/FOG2 control growth by regulating PI3K. Cell 2009, 139:1096–1108
29. Cheng H, Zhang L, Cogdell DE, Zheng H, Schetter AJ, Nykter M, Harris CC, Chen K, Hamilton SR, Zhang W: Circulating plasma MiR-141 is a novel biomarker for metastatic colon cancer and predicts poor prognosis. PLoS One 2011, 6:e17745