Research Article

**SOX15 regulates proliferation and migration of endometrial cancer cells**

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**Introduction**

Endometrial cancer (EC) is recognized as the fourth most widespread gynecologic malignancy in the United States, with an estimated 60000 new cases and 10500 deaths in 2016 [1]. It is the sixth most common cancer amongst women worldwide, with nearly 319600 cases diagnosed in a year [2]. The most susceptible group of this disease is the postmenopausal women. It is also reported that there exists a genetic predisposition in some cases [3], which accounts for above 10% of total EC cases [4]. Generally, EC has been classified into two types [5]. Type I neoplasms like endometrioid adenocarcinomas are the most common type. More aggressive histological variants are Type II neoplasms, occupying approximately 10% of total EC cases [4]. The most frequent symptoms of EC include abnormal uterine bleeding and vaginal discharge. [6]. For female EC patients, lymph node involvement may be the most important prognostic factor [7-10]. Total hysterectomy with bilateral salpingo-oophorectomy is often considered an effective and primary treatment for EC patients, as nearly 75% of women with stage I disease are likely to be cured by surgery alone [4]. Clinicians frequently use multimodality therapy instead of monotherapy for women with intermediate-risk EC as well as advanced-stage disease [11,12]. Considering the prevalence and fatality of EC, it is quite necessary to study its pathogenesis and treatment methods. With the development of technology, the EC studies at molecular level have been emerging [13-16].
Sry-like high mobility group box (SOX) proteins belong to a multigenic family and are characterized by a unique DNA-binding domain, which is known as the high mobility group (HMG) box [17]. Initially, the SOX family evolved and developed based on Sry, Tdy, and TDF. Sry is its initiator, and Tdy and TDF are the determinants of mammalian testis in mouse and human, respectively [18]. Based on HMG box domains, gene structure as well as some functional domains, 20 different SOX proteins have been identified and subdivided into eight groups [18]. In mammals, SOX15 is the only member of the SOX G [19]. The transcription factor of the SOX family encoded by SOX15 is involved in the regulation of the embryonic development. SOX genes, as participants of a wide range of essential biological processes, remain unknown in the pathogenesis of some diseases, especially in genetic diseases and cancers [18]. Therefore, it is crucial for the treatment of EC to investigate the mechanism of SOX genes. Up to now, a multitude of studies have revealed the potential involvement of different SOX genes in human cancer. Some studies revealed that SOX genes are frequently down-regulated and act as tumor suppressors or oncogenes in different tumor types [20]. It was reported that SOX15 is also a candidate tumor suppressor in pancreatic cancer [21]. Relevant studies demonstrated that SOX15, as a tumor suppressor, might lead to an ideal anticancer therapeutic strategy in the future [18]. However, the affects of SOX15 on some cancers are still not fully elucidated.

The purpose of the current study was to investigate the influence of SOX15 on proliferation and migration of EC cells. We hypothesized that SOX15 might act as an anti-oncogene in EC, which could regulate the progression and migration of EC cells.

Materials and methods
EC tissue samples
We collected 60 samples of EC patients who received surgery during the period between June 2015 and June 2016 in Changzhou First People's Hospital, and no patients were given chemotherapy or radiotherapy before surgery. Sixty samples were all classified into neoplasms Type I. Written informed consent was obtained from all the subjects prior to the study. EC tissues and adjacent normal tissues were collected and stored in −80°C refrigerator. Our study was approved by Ethics Committee of Changzhou First People's Hospital.

Immunohistochemistry
Tissue paraffin sections were heated in a 60°C oven for 1–2 h and then dewaxed using dimethyl benzene. H₂O₂ (3%) was incubated with the sections at 25°C for 10 min to inactivate endogenous enzymes. Sections were then washed with sterilized water and immersed in 0.01 mol natrium citricum buffer solution. After that, the sections were heated in a 220-W microwave oven. PBS with 5% BSA was added to the sections and incubated at 25°C for 20 min. Next, rabbit anti-human SOX15 polyclonal antibody (ab55960, 4 μg/ml, Abcam, Cambridge, MA, U.S.A.) was applied and sections were placed at 4°C overnight. After that, PBS was used to wash sections and then biotinylated goat anti-rabbit IgG was applied at 4°C for 30 min. After avidin–biotin complex (SABC) was instilled, the sections were stained with 3,3’-diaminobenzidine (DAB) and counterstained by Hematoxylin. Finally, after mounted using dehydrated jelly neutral mounting medium, sections were observed under an optical microscope. According to the positive-staining intensity in immunohistochemical assay, we set it to be that: colorless is 0 score (−), pale yellow is 1 score (+), palm yellow and above is 2 score (++).

Cell culture, transfection, and grouping
Endometrial adenocarcinoma cell line HEC-1-A (BNCC338711) and Ishikawa (BNCC338693) were bought from BeNa Culture Collection (Beijing, China). HEC-1-A (BNCC338711) cells were cultured in 90% McCoy’s 5A and 10% FBS, Ishikawa (BNCC338693) cells were cultured in 90% EMEM and 10% FBS, which were all placed in an incubator with 5% CO₂ at 37°C and 95% humidity. Double-digested carrier pCDH (System Biosciences, Mountain View, CA, U.S.A.) was ligated with target gene segment SOX-cDNA and amplified with PCR. Then, pCDH plasmid, lentivirus shuttle plasmid, and its additive original packaging vector plasmid (pCDH-CMV-MCS-EF1-copGFP) were transfected into HEC-1-A and Ishikawa cells. After 4–6 h, cells were placed in complete medium, and cell supernatant with lentivirus particles was collected, and high titer lentivirus concentrate could be obtained after 48 h. Within 24 h before transfection, HEC-1-A and Ishikawa cells were collected in logarithmic phase, digested by trypsin. Cells were then plated in a six-well plate for 5 × 10⁵ per well to ensure the consistency of and even distribution of cells in each well. Within 3 h before transfection, the original medium was removed and cells were placed in fresh basal medium without serum and antibiotics. Next, lentivirus transfection solution was added to the cells in each group and cells were incubated at 37°C with 5% CO₂ saturation humidity. After 4–6 h, transfected mixed solution was removed and
the cells were placed in fresh Dulbecco's modified Eagle's medium (DMEM) complete medium and cultured at 37°C in a CO2 incubator for 24-48 h.

HEC-1-A cells and Ishikawa cells were divided into four groups: cells with no transfection were the control group, cells transfected with sh-SOX15 negative control were sh-NC group, cells transfected with SOX15-cDNA were the SOX15 group, and cells transfected with sh-SOX15 were the sh-SOX15 group.

Reverse transcription and real-time PCR
After cells were lysed, RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, U.S.A.) based on manufacturer's instructions and quantitated using NanoDrop 2000 (Thermo Fisher Scientific Inc, U.S.A.). Two hundred nanograms RNA was reverse transcribed using ReverTra Ace qPCR RT Kit (Toyobo, Japan) following the manufacturer's protocol. THUNDERBIRD SYBR® qPCR Mix Kit (Toyobo, Japan) was used to determine the relative RNA expression. The instrument used in this experiment was CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, U.S.A.). The reaction condition was: pre-denaturation at 94°C for 3 min, degeneration at 94°C for 30 s, annealing at 60°C for 1 min, extension at 72°C for 1 min (30 cycles), and extension again at 72°C for 5 min. When measuring mRNA level, GAPDH acted as the internal control. Primer sequences used were: SOX15 sense: 5′-GAACAGGTTGGAAGCAAAAGGC-3′ and antisense 5′-GAACAGGTTGGAAGCAAAAGGC-3′; GAPDH sense: 5′-ACCACAGTCCATGCCATCAC-3′ and antisense 5′-TCCACCACCTGTTGCTGTA-3′.

Western blot
Cells were prepared in a six-well plate for 1 × 10^6 cells per well and cultured for 3 days. Then cells were centrifuged at 4°C for 10 min at 2000 rpm and the supernatant was collected. After measuring protein concentration using BCA, protein electrophoresis was performed in SDS/PAGE. The PVDF membrane was washed with TBS containing 20% Tween20 (TBST) for 5 min three times, and then blocked at 4°C overnight. After incubation with 4 ml primary antibody diluent (SOX15 antibody, 1:500) at room temperature for 2 h, the membrane was washed with TBST for four times, and then incubated with second antibody for 1 h and washed with TBST for four times. After 1–2 min, ECL developing solution (GE Healthcare, Amersham, United Kingdom) was added on to PVDF membrane. Samples were exposed, photographed, and observed under a microscope.

Cell proliferation assay
Colony formation assay was performed in a six-well plate with 200 cells in each well. After 2 weeks' regular culture, cells were washed with PBS and fixed with 4% paraformaldehyde, and stained with 1% Crystal Violet. Cell culture was washed three times using PBS. Colony numbers in each group were counted under a microscope. For MTT assay, cells were plated into a 96-well plate for 1×10^4 cells per well, taken out on 1, 2, 3, 4, 5, 6, and 7 days. Ten microliters of MTT was added to each well to present colors. After cultivating the cells for 4 h, 150 μl DMSO was added to dissolve and crystallize them, 10 min later, optical density (OD) value was detected with 450-nm wave length. Each group was performed in triplicates and the experiment was repeated three times. For 5-ethyl-2′-deoxyuridine (EdU) assay, cells were seeded in 96-well plate for 1×10^4 cells per well and stained with EdU. After incubation at room temperature for 15 min, cells were washed with 1 ml PBS containing 3% BSA. After being added with 200 μl PBS containing 0.5% Triton X-100, cells were incubated at room temperature for 20 min and supernatant was discarded. Later, staining reaction solution was prepared and 100 μl solution was added in every tube, after which cells were incubated at 25°C in dark environment for 30 min.

Cell cycle analysis
Cells were collected in logarithmic phase, plated in six-well plates at 1×10^5 per well, and incubated for 12 h. We added lentivirus transfected cells and cultivated them for 24–48 h. After that, all the cells were digested with 0.25% trypsin, and then cells were centrifuged at 1000 rpm for 5 min and the supernatant was discarded. Remainders were washed by PBS twice. One milliliter of 70% ethanol was added and after that samples were placed at 4°C for 24 h. Later, samples were washed and centrifuged again. Staining buffer, propidium iodide (PI) staining solution (20×), and RNase A (50×) were added to sample and incubated for 30 min at room temperature in dark. PI fluorescence was detected using a BD FACSAria flow cytometry system (Beckman Coulter FC500, Gallios, Beckman Coulter, U.S.A.) and analyzed using FlowJo 7.6 (Tree Star, San Carlos, CA, U.S.A.)
Table 1 SOX15 expression in tissue samples

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>SOX15, n (%)</th>
<th>(\chi^2)</th>
<th>P</th>
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<tbody>
<tr>
<td>Tumor</td>
<td>60</td>
<td>0</td>
<td>6 (10%)</td>
<td>54 (90%)</td>
</tr>
<tr>
<td>Adjacent</td>
<td>60</td>
<td>23 (38.3%)</td>
<td>18 (30%)</td>
<td>19 (31.7%)</td>
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Transwell migration assay
The migration assay was performed on Transwell plates. For cell migration assay, 2 \(\times 10^5\) cells were seeded on a polycarbonate membrane insert in a Transwell apparatus (Corning, Tewksbury, MA, U.S.A.) and cultured in DMEM without serum. DMEM containing 10% FBS was added to the lower chamber. After incubation for 24 h at 37°C in a CO\(_2\) incubator, the insert was washed with PBS, and cells on the top surface of the insert were removed using a cotton swab. Cells that migrated to the bottom surface of the insert were fixed with methanol, stained with 0.4% Crystal Violet, and counted in five random fields at 200×.

Statistical analysis
All experimental data were represented as mean ± S.D., and results were analyzed using SPSS 21.0 software (IBM Corp., Armonk, NY, U.S.A.). Measurement data were compared by Student’s t test or one-way ANOVA. Nonparametric chi-square test was used to analyze categorical data. P < 0.05 was considered statistically significant.

Ethics approval and consent to participate
All procedures were in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Changzhou First People’s Hospital. All patients participated have given consent for the present study.

Consent for publication
All patients participated have given consent forms for publication of the present study.

Results

The expression of SOX15 in tumor tissues was lower than that in normal tissues
The immunohistochemistry (IHC) results (Figure 1A) showed that SOX15 was mainly expressed in cells rather than in intracellular substances. The expression of SOX15 in tumor tissues was significantly lower than that in adjacent normal tissues. The positive rates of the strong expression of SOX15 in two groups were 90% (54/60) and 31.7% (19/60), the average score of tumor was 1.9, and the score of adjacent normal tissues was 0.933 \((P<0.01)\). (Figure 1B), respectively \((\chi^2 = 50.50, P<0.01, \text{Table 1})\). In addition, the mRNA (Figure 1C) and protein expression (Figure 1D) of SOX15 in tumor tissues were also lower compared with adjacent normal tissues \((P<0.01)\).

Successful construction of SOX15 overexpression and SOX15 down-regulation of EC cell line
According to reverse-transcription quantitative real-time PCR (RT-qPCR) results, both in HEC-1-A and Ishikawa cells, the expression of SOX15 mRNA in SOX15 group was conspicuously higher than the control group \((P<0.01)\). Besides, the expression of SOX15 mRNA in the sh-SOX15 group was lower than sh-NC group \((P<0.05, \text{Figure 2A})\). In addition, Western blot results displayed that the expression of SOX15 was higher in SOX15 group than in control group \((P<0.05, \text{Figure 2B})\) and the expression in sh-SOX15 group was lower than sh-NC group \((P<0.05)\). All the above results confirmed the successful construction of SOX15 overexpression in EC cells and the SOX15 down-regulation in EC cells.

SOX15 regulated EC cell proliferation, viability, and promoted apoptosis
The colony formation results indicated that the number of cell clone in SOX15 group was significantly smaller compared with NC group both in HEC-1-A and Ishikawa cells \((P<0.05)\) and the number of sh-SOX15 group was higher than that in sh-NC group both in HEC-1-A and Ishikawa cells \((P<0.05, \text{Figure 3A,B})\).

The Hoechst 33342 staining results demonstrated that the cell apoptosis was much more intense in SOX15 group than in NC group, and the cell apoptosis rate decreased in sh-SOX15 than in sh-NC group. The EdU staining results
displayed that proliferative cells were fewer in SOX15 group than in control group, in addition, the proliferative cells were more in sh-SOX15 group compared with sh-NC group (Figure 3C).

The results of MTT assay showed that the growth of cells in different groups all presented an increasing trend. The OD value of cells in control group was obviously higher than that of cells in SOX15 group. The OD value of cells in sh-SOX15 group was the highest than other groups both in HEC-1-A and Ishikawa cells (P<0.05, Figure 3D).

**SOX15** **effected cell cycle and migration**

According the flow cytometry results, the percent of control group, sh-NC, SOX15, and sh-SOX15 group cells in G1 stage were 51.18, 52.08, 61.91, and 41.39% in HEC-1-A cells, in addition, the percent in G1 stage in Ishikawa cells were 53.72, 51.35, 67.80, and 40.53%. Flow cytometry analysis results revealed that the number of cells in G1 stage in SOX15 group was significantly larger in comparison with the control group (P<0.05, Figure 4A), whereas the number of cells in S and G2 stages in SOX15 group was smaller than that in control group, besides, the number of cells in G1 stage in sh-SOX15 group was larger than sh-NC group and was the largest compared with the other groups both in HEC-1-A and Ishikawa cells. In addition, Transwell migration assay results showed that the number of migrated cells in SOX15 group was significantly bigger than that in control group after 12 h of transfection (P<0.01, Figure 4B), and the number of migrated cells in sh-SOX15 group was higher than sh-NC group. As a whole, overexpression of SOX15 hindered cell cycle and weakened migration, but in the meantime, low expression of SOX15 promoted these.

**Discussion**

EC is a huge potential menace to the female group. It is reported that the incidence of this disease has increased to 21% from 2008 to 2012. Moreover, the mortality of EC has also continuously risen in the past 20 years [4]. Therefore,
Figure 2. Relative mRNA and protein expression of SOX15 in HEC-1-A and Ishikawa cells of different groups detected by RT-qPCR and Western blot

(A) The expression of SOX15 mRNA in SOX15 group was obviously higher than that in the control, and the expression of sh-SOX15 was lower than the sh-NC group both in HEC-1-A and Ishikawa cells. (B) Western blot results showed that the expression of SOX15 was higher in SOX15 group than in control group and the expression of SOX15 in sh-SOX15 was lower than that in sh-NC group both in HEC-1-A and Ishikawa cells. Data were presented as mean ± S.D. for three independent experiments; *P < 0.05, **P < 0.01 compared with control group; #P < 0.05, ##P < 0.01 compared with sh-NC group.

...it is imperative to find effective treatment of EC by studying the key oncogenes and tumor suppressors during EC development.

The roles of SOX family members in transcription process may be different. It is either transcriptional activators or suppressors that depends on their specific amino acid sequences and corresponding binding partners [22]. Over the past years, members of SOX family have emerged with increasing importance in pathogenesis of multiple cancers [18,23-32]. Hong et al. [23] reported that the up-regulation of SOX9 promotes cell proliferation as well as oncogenesis for esophageal squamous cell carcinoma (ESCC). Up-regulation of SOX3 was reported in ESCC as well and was associated with poor overall survival and disease-free survival [24,26]. On the other side, SOX17 was demonstrated to be a tumor suppressor, which was down-regulated in ESCC patients [25]. Qin et al. [28] found that the expression of SOX6 was reduced in ESCC cell lines. In our study, we confirmed the different expression levels of SOX15 in EC tissues and adjacent normal tissues, and substantiated the inhibitive function of SOX15 on the proliferation, viability, cell cycle, and migration in EC cell line. In fact, the expression level of SOX15 has already been reported in several other human cancers such as gastric carcinoma, colon cancer, and thyroid cancer [20]. Thu et al. [21] found that the low expression of SOX15 also existed in pancreatic ductal adenocarcinoma (PDAC) cell lines and tumors [22].
Figure 3. Effects of SOX15 on EC cell proliferation, viability, and apoptosis in HEC-1-A and Ishikawa cells

(A, B) Colony formation assay results showed that the number of cell clone in SOX15 group was significantly smaller compared with the control group in HEC-1-A and Ishikawa cells (both $P<0.05$), and the number of cell clone in sh-SOX15 group was larger than the sh-NC group in HEC-1-A ($P<0.01$) and Ishikawa cells ($P<0.05$); $^\#P<0.05$ compared with control group, $^\#P<0.05$ compared with sh-NC group. $^\star P<0.05$; $^\star\star P<0.01$ (C) The cell proliferation was evaluated 48 h after transfection by EdU-incorporation assays. Case Viewer, 20x. Hoechst 33342 staining results demonstrated that the cell apoptosis was much more intense in SOX15 group than in control group. The EdU staining results displayed that proliferative cells were fewer in SOX15 group than in control group both in HEC-1-A and Ishikawa cells. The results in the sh-SOX15 compared with sh-NC group were opposite. $^\star P<0.01$; $^\star\star P<0.05$; $^\#P<0.01$ (D) The growth curves of cells were measured after transfection with indicated vectors by MTT assays. Cells in control group, sh-NC group, SOX15 group, and si-SOX15 group all presented an increasing trend. The OD value of cells in control group was obviously higher than that of cells in SOX15 group, and the OD value of cells in sh-SOX15 was higher than that in sh-NC group both in HEC-1-A and Ishikawa cells. $^\star P<0.05$. $^\star\star P<0.01$ compared with control group, $^\#P<0.05$, $^\#\#P<0.01$ compared with sh-NC group. Data were presented as mean $\pm$ S.D. for three independent experiments.
Figure 4. Overexpression of SOX15 suppressed cell cycle and migration

(A) Flow cytometry results showed that cells in SOX15 group was significantly stuck in G1 stage, and the number of cells in G1 stage in sh-SOX15 group was larger than sh-NC group and was the largest compared with the other groups both in HEC-1-A and Ishikawa cells. *P < 0.05 compared with control group, #P < 0.05 compared with sh-NC group. (B) Migration of cells was assessed by Transwell assay. The results demonstrated that the number of migrated cells in SOX15 group was significantly larger than that in control group after 12 h of transfection, and the number of migrated cells in sh-SOX15 group was higher than sh-NC group both in HEC-1-A and Ishikawa cells. *P < 0.05, **P < 0.01 compared with control group,  #P < 0.05, ##P < 0.01 compared with sh-NC group. Data were presented as mean ± S.D. for three independent experiments.
Besides, the IHC and qRT-PCR results indicated that the expression of SOX15 in EC tissues was significantly lower than that in normal tissues, which might suggest that SOX15 inactivation could be selected to treat EC.

We constructed a stable SOX15 overexpression cell line and a SOX15 low-expression cell line in HEC-1-A and Ishikawa cells, and then we observed the effects of SOX15 overexpression or low expression on EC cells through monoclonal culture, EdU staining, and MTT assay. These consistent experiment results indicated that overexpression of SOX15 suppressed EC cell proliferation to some extent. Moreover, overexpression of SOX15 could also induce a cell-cycle arrest in G0/G1 stage and suppress the migration of EC, revealed by flow cytometry analysis and Transwell assay, respectively; in addition, the low expression of SOX15 had the opposite results both in HEC-1-A and Ishikawa cells. These results demonstrated the role of SOX15, as a tumor suppressor, might be conducive to the treatment for EC patients. The tumor-suppressed ability of SOX15 was also clarified in some other studies [18,22]. The public datasets of the Cancer Genome Atlas data portal revealed that SOX15 might function as tumor suppressor in colon cancer [21]. According to a recent review article, the aberrant expression of SOX factors is regulated by copy number alteration, methylation modulation, miRNAs, transcription factors, and post-translational modification [33], and in addition, the blockage of the Wnt/β-catenin signaling pathway proves to be the main activity downstream of SOX regulation, besides, a newly published study indicated that the inhibitive effect of SOX15 might be associated with the cross-talk between SOX15 and Wnt signaling pathway [20]. Wnt signaling has been identified as a key pathway in multiple human cancers, and the development of therapeutic Wnt inhibitors has been widely applied in numerous malignancies such as colorectal cancer, liver cancer, lung cancer, and PDAC [34-37]. Indeed, there has been several studies demonstrating that SOX was a key modulator of the Wnt/β-catenin signaling pathway, which directly binds β-catenin and TCF and prevents the interaction between them [37-40]. SOX17 has already been shown to be antagonistic to Wnt signaling [22]. As for SOX15, Thu et al. [21] found that it was negatively related to Wnt pathway in PDAC [22]. However, despite its crucial role in muscle development [41,42], little has been known regarding the function of SOX15 in some human cancers.

Our study investigated and demonstrated the role and function of SOX15 in EC, which not only laid the foundation for the intensive study of EC, but provided some valuable evidence for the therapeutic strategies for EC. Nevertheless, there were still some limitations in our study. Although we showed the impact of SOX15 in EC cells, the specific mechanism or underlying pathways remain unknown. Given the complex cross-talk between SOX family and Wnt signaling, it could be a meaningful topic for future study to explore relationship between SOX15 and Wnt signaling. In addition, in vivo experiments can also help further prove the study results and assess the function of SOX15 in a more precise way. Due to the limitations of the conditions, the functional test of SOX15 and the in vivo experiments will be demonstrated later.

Conclusion
The expression of SOX15 in EC tissues was significantly lower than that in adjacent normal ones. The overexpression of SOX15 contributed to inhibition of EC cell proliferation, and inducing a cell-cycle arrest in G1 stage as well as suppressing the migration of EC cells.

Competing interests
The authors declare that there are no competing interests associated with the manuscript.

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Author contribution
X.R. and Y.X. designed and drafted this research. X.R. and J.J. analyzed and interpreted the patients’ data. Y.X., X.J., and C.G. conducted statistical analysis. J.J. critically revised the manuscript. All authors read and approved the final manuscript.

Abbreviations
DMEM, Dulbecco’s modified Eagle’s medium; EC, endometrial cancer; EdU, 5-ethynyl-2'-deoxyuridine; ESCC, esophageal squamous cell carcinoma; HMG, high mobility group; IHC, immunohistochemistry; OD, optical density; PDAC, pancreatic ductal adenocarcinoma; PI, propidium iodide; RT-qPCR, reverse-transcription quantitative real-time PCR; SOX, Sry-like high mobility group box; TBST, TBS containing 20% Tween-20.
References