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UBE2S mediates tumor progression via SOX6/β-Catenin signaling in endometrial cancer

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ABSTRACT

Dysregulation of ubiquitin-conjugating enzyme E2S (UBE2S) contributes to tumor progression. However, its clinical significance and biological function in endometrial cancer (EMC) remain unclear. Here, we show that UBE2S is upregulated in EMC and exhibits oncogenic activities via activation of SOX6/β-Catenin signaling. High expression of UBE2S is significantly associated with poor prognosis in two independent cohorts consisting of a total of 773 patients with EMC. in vitro studies demonstrate that ectopic expression of UBE2S promotes cell proliferation and migration, whereas knockdown of UBE2S results in opposite phenotypes. Overexpression of UBE2S in EMC cells enhances the nuclear translocation of β-Catenin, and subsequently induces the expression of c-Myc and Cyclin D1. Inhibition of β-Catenin by XAV-939 markedly attenuates UBE2S-promoted cell growth. Mechanistically, UBE2S suppresses the expression of SOX6 to trigger β-Catenin signaling. Re-expression of SOX6 in UBE2S-expressing EMC cells abolishes the nuclear localization of β-Catenin. Collectively, these data suggest UBE2S may serve as a promising prognostic factor and function as an oncogene in EMC. The newly identified UBE2S/SOX6/β-Catenin axis represents a new potential therapeutic target for EMC intervention.

1. Introduction

The incidence and mortality of gynecologic cancers have been increasing, despite of efforts on new strategies for cancer management. In the developed countries, endometrial carcinoma (EMC) becomes the most common cancer of the female reproductive organs (Siegel et al., 2017; Bray et al., 2018). About 60,000 newly diagnosed EMC cases and about 11,000 EMC-related deaths were identified in 2017 in the United States (Siegel et al., 2017). Precision medicine may bring flash hope to the patients with EMC. As a result, more and more attentions have been paid to identify new biomarkers with potential of EMC intervention.

Ubiquitination, a reversible biochemical process that attaches ubiquitin to substrate proteins, is responsible for the post-translational modification of proteins to participate in multiple cellular functions, such as cell proliferation, differentiation and migration (Popovic et al., 2014). This process is controlled by multi-step catalytic reactions mediated by ubiquitin-related enzymes, including E1, E2 and E3. During the first step, ubiquitin-activating enzyme E1 covalently binds to and activates ubiquitin. Sencond, the activated ubiquitin is transferred to ubiquitin-conjugating enzyme E2, and then attaches to substrate protein via ubiquitin protein ligase E3 (Foot et al., 2017). There are approximately 40 E2 family members in the human cells. They play pivotal roles in the formation of lysine-specific chains (Hormaechea-Agulla et al., 2018). Ubiquitin-conjugating enzyme E2S (UBE2S, also known as E2-EPF) is an E2 enzyme that elongates K11-linked polyubiquitin chain on substrates (Wu et al., 2010). UBE2S, coupled with ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C), modulates the cell division via interaction with Emi2 and CDC20 (Sako et al., 2014; Craney et al., 2016), which implies its involvement of tumorigenesis. UBE2S is overexpressed in several human cancers, such as cervical (Dong et al., 2018), renal (Roos et al., 2011) and breast cancers (Ayesha et al., 2016; Tedesco et al., 2007). Increased expression of UBE2S in breast cancer is associated with poor post-surgical survival (Ayesha et al., 2016). Functionally, decrease of UBE2S hinders the tumor growth and reduces chemo-resistance in glioblastoma (Hu et al., 2017). Overexpression of UBE2S facilitates cell proliferation and migration in hepatocellular carcinoma via down-regulation of p53 (Liu et al., 2017; Pan et al., 2018), and promotes colorectal cancer via stabilizing β-Catenin through directly interaction with β-Catenin to ubiquitinate its K19 residue via K11 linkage (Li et al.,

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2018). However, the clinical significance of UBE2S and its biological functions in EMC remain unknown.

In this study, the expression of UBE2S and its correlation with clinical outcome were determined in two independent cohorts consisting of a total of 773 patients with EMC. The role of UBE2S in the progression of EMC and the underlying mechanism were investigated. Our data suggest UBE2S serve as a prognostic factor and exert oncogenic activities towards EMC via SOX6/β-Catenin signaling pathway.

2. Materials and methods

2.1. Patients and tissue specimens

A cohort containing 232 paraffin-embedded tissues and follow-up data were collected from EMC patients who received surgical resection at The First Affiliated Hospital of Sun Yat-sen University, between January 2008 and December 2010, for construction of tissue microarray (TMA) (FH cohort). Another 28 pairs of fresh EMC tissues and the corresponding adjacent nontumorous tissues were obtained for qRT-PCR and western blot. All patients did not received chemotherapy or radiotherapy before surgery. Informed written consents were obtained for the use of retrospective tissue samples from the patients within this study. This study was approved by the Institute Research Medical Ethics Committee of The First Affiliated Hospital of Sun Yat-sen University.

2.2. Cell culture

EMC cell lines HEC-1, ECC-1, KLE, HEC-1B, Ishikawa and RL-95-2 were purchased from the Cell Resource Center, Chinese Academy of Science Committee (Shanghai, China). Cells were aintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT) in a humidified incubator at 37 °C and 5% CO_2. The cells were transfected with UBE2S overexpression vector or shRNAs by Lipofectamine 2000, according to the instruction, and then selected by G418 for 4 weeks to establish stable cells.

2.3. Quantitative real-time PCR (qRT-PCR) and RNAi

Total mRNA extracted from the fresh samples was reversed to cDNA by M-MLV Reverse Transcriptase (Promega Inc., USA). Levels of FOXK2 and β-actin were measured by SYBR green-based real-time PCR using the Stratagene Mx3000 P Real-Time PCR system. Primers were designed as follows: UBE2S, forward: 5′- GACCGAAGAACGCAGGAAG-3′ and reverse: 5′- GTGCGGGGGTAGGTTCTC-3′; β-actin, forward: 5′-TGGCA CCCAGCACAATGAA-3′ and reverse: 5′-CTAAGTCATAGTCCGCCTAGA AGCA-3′. The shRNA plasmids for UBE2S were obtained from Santa Cruz Biotechnology (sc-97109-SH). shRNA Plasmids generally consist of a pool of three to five lentiviral vector plasmids each encoding target-specific 19–25 nt (plus hairpin) shRNAs designed to UBE2S. The control shRNA plasmids were also obtained from Santa Cruz Biotechnology (sc-108060).

2.4. Tissue microarray (TMA) construction

TMA containing 232 EMC and nontumorous tissues were constructed. Briefly, all specimens were fixed in 4% formalin and embedded in paraffin. The corresponding histological H&E-stained sections were reviewed by a senior pathologist to mark out

representative areas. Using a tissue arraying instrument (Beecher Instruments, Sliver Spring, MD), each tissue core with a diameter of 1.0 mm was punched from the marked areas and re-embedded. The blocks were sectioned into 4 μm slides for the detection of UBE2S.

3. Gene microarray

Human HT-12 v4 Expression Bead Chip (Genergy, Shanghai, China) was used to determine the gene alterations in EMC cells transfected with UBE2S-shRNA.

3.1. Western blot

Total proteins extracted from EMC fresh tissue were fractionated by SDS-PAGE, transferred to PVDF membrane, and then incubated with primary specific antibodies for UBE2S (1:1000, #11878, Cell signaling technology), c-Myc (1:1000, Cell signaling technology), Cyclin D1 (1:1000, #2978, Cell signaling technology), and β-actin (1:1000, #4970, Cell signaling technology) in 5% of non-fat milk, followed by a horse radish peroxidase (HRP)-conjugated anti-rabbit second antibody. ECL detection reagent (Amersham Life Science, Piscataway, NJ, USA) was used to show the results. Quantitation of western blot data was performed using grey value measurement by imagine J.

3.2. Immunohistochemistry (IHC) and scoring

TMA sections with a thickness of 4 μm were dewaxed in xylene and graded alcohols, hydrated, and washed in phosphate buffered saline (PBS). After pretreatment in a microwave oven, endogenous peroxidase was inhibited by 3% hydrogen peroxide in methanol for 20 min, followed by avidin-biotin blocking using a biotin-blocking kit (DAKO, Germany). Slides were then incubated with anti-UBE2S antibody (1:500, #11878, Cell signaling technology), overnight in a moist chamber at 4 °C, washed in PBS, and incubated with biotinylated goat anti-rabbit antibody. Slides were developed with the Dako Liquid 3,' 3-diaminobenzidine tetrahydrochloride (DAB) + Substrate Chromogen System and counterstained with hematoxylin. IHC evaluation was determined by semi-quantitative IHC detection, using the H-score method. The percentage of positively-stained cells was scored as "0″ (0%), "1″ (1%–25%), "2″ (26%-50%), "3″ (51%–75%), "4″ (76%–100%). Intensity was scored as "0″ (negative staining), "1″ (weak staining), "2″ (moderate staining), and "3″ (strong staining). The percentage score was multiplied by the staining intensity score. For each case, 1000 cells were randomly selected and scored. The scores were independently decided by 2 clinical doctors. The median of UBE2S IHC score, which was 5.5, was chosen as the cutoff value to identify high and low expression groups.

3.3. MTT

Stable cells were cultured in 96-well plates for 5 days. 20 ul of MTT (5 mg/ml) was added into the wells for 3 h. The formazan crystals were dissolved in DMSO (150 μl/well). The absorbance at 490 nm of each sample was measured. The cell growth rate was calculated.

3.4. Colony formation

Stable cells were constructed. Cells were collected and seeded in 6-well plates uncoated with matrigel at a density of 1.0×10^3 per well and then incubated at 37 °C for 10 days. Colonies were fixed with methanol and stained with 0.1% crystal violet and counted.

3.5. EdU cell proliferation assay

The impact of UBE2S on EMC cell proliferation was assessed by the KeyFluor488 Click-iT EdU Imaging Kit, according to the manufacturer's instructions (KeyGEN BioTECH, Nanjing, China).

3.6. Migration assay

Cells re-suspended in 200 μl of serum-free medium were placed in

the upper compartment of an uncoated Transwell chamber (Corning; 24-well insert, pore size: 8 mm). The lower chamber was filled with 15% fetal bovine serum as a chemoattractant followed by incubation for 48 h. The cells on the lower surface were fixed with methanol and stained with 0.1% crystal violet and counted.

3.7. Immunofluorescence

Cells were fixed for 20 min in PBS containing 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 two times, 5 min each, incubated in blocking buffer (3% donkey serum in TBS) for 1 h, and then incubated with antibody for 2 h at room temperature. After washing in PBS three times, 8 min each, cells were incubated with the appropriate fluorochrome-conjugated secondary antibody for 1 h, and observed under a fluorescence microscope.

3.8. Statistical analysis

Statistical analyses were performed using the SPSS 19.0 software (SPSS, Chicago, IL, USA). Continuous variables were expressed as a mean with SEM and analyzed using the *Student t* test (2-tailed). Kaplan-Meier analysis (log-rank test) was utilized for survival analysis. $P < 0.05$ (two-tailed) was considered statistically significant.

4. Results

4.1. UBE2S is upregulated in endometrial cancer and correlated with poor outcome

The expression of UBE2S in EMC was examined in clinical samples from 28 patients. Results of qRT-PCR showed that UBE2S mRNA expression in EMC was much higher than that in adjacent nontumorous tissues (Fig. 1A). Consistently, the protein expression was noticeably increased by 4-fold in cancer tissues, compared to the corresponding adjacent endometrial tissues (Fig. 1B). To further evaluate the upregulation of UBE2S in EMC, 232 pairs of paraffin-embedded tissues were collected for the construction of TMA. According to the TMA-based IHC assays, UBE2S was mainly localized in the cytoplasm. Nuclear distribution of UBE2S was observed in a small portion of cancer cells (Fig. 1C). Compared to the nontumor cells, expression of UBE2S in EMC cells was markedly increased (Fig. 1D). More UBE2S in EMC were depicted in 80.6% (187/232) of the cases. Furthermore, patients with tumor metastasis were likely to express more UBE2S than those with nonmetastatic tumors (Fig. 1E). Association analyses of clinicopathological features and UBE2S mRNA expression in TCGA database revealed that tumors at higher pathological grade were accompanied with higher expression of UBE2S (Supplementary Fig. 1).

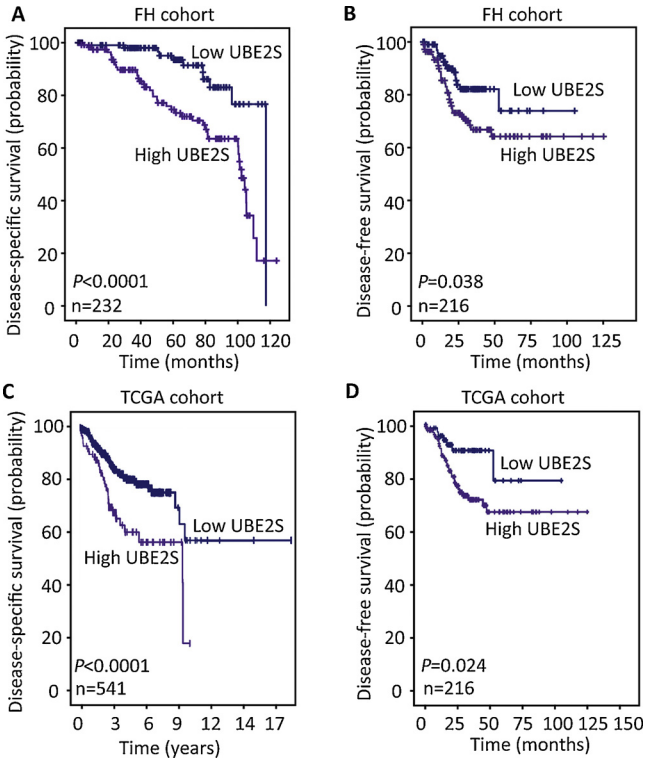

Fig. 2. High expression of UBE2S is correlated with poor prognosis in endometrial cancer. Kaplan-Meier analyses were conducted to evaluate the relationship between UBE2S expression and the prognosis in terms of disease-specific survival (A,C) and disease-free survival (B,D) in both FH and TCGA cohorts.

According to the median of UBE2S IHC score, patients in FH and TCGA cohorts were divided into two groups: low or high UBE2S. Kaplan-Meier analyses revealed that patients with high UBE2S expression frequently lived a shorter life, supporting by the data that high UBE2S expression was correlated with poor disease-specific survival

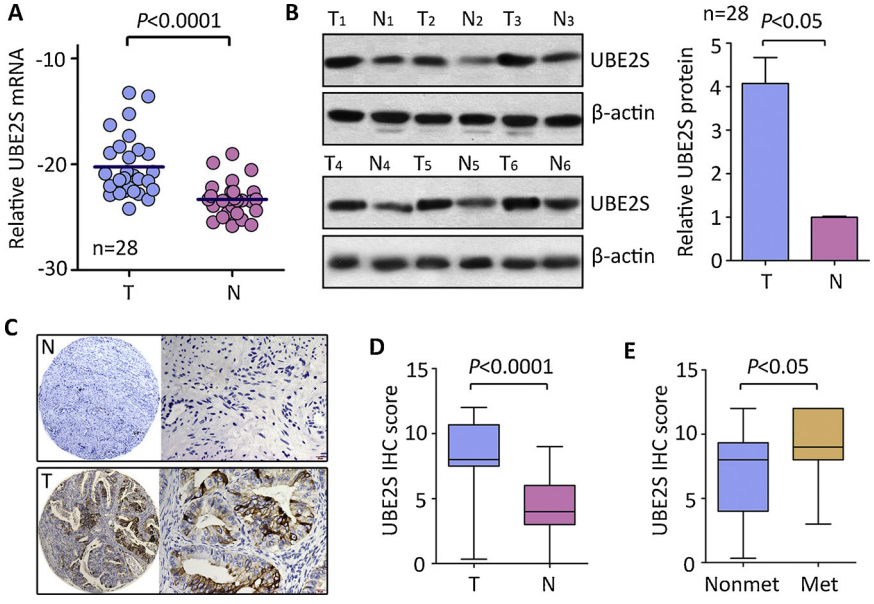

Fig. 1. UBE2S expression is increased in endometrial cancer. A. The mRNA expression of UBE2S in 28 pairs of EMC (T) and nontumorous (N) tissues was determined by qRT-PCR *(Matched Paired t Test)*. B. Proteins from clinical samples were subjected into western blot to examine the protein expression of UBE2S. The related UBE2S protein expression was indicated by histogram. C. IHC was performed to evaluate the expression of UBE2S in 232 patients with EMC in FH cohort. Representative IHC images of UBE2S expression were presented. D,E. IHC scores of UBE2S in EMC and nontumorous tissues (D), and in cases with (Met) or without (Nonmet) tumor metastasis (E) were indicated and compared.

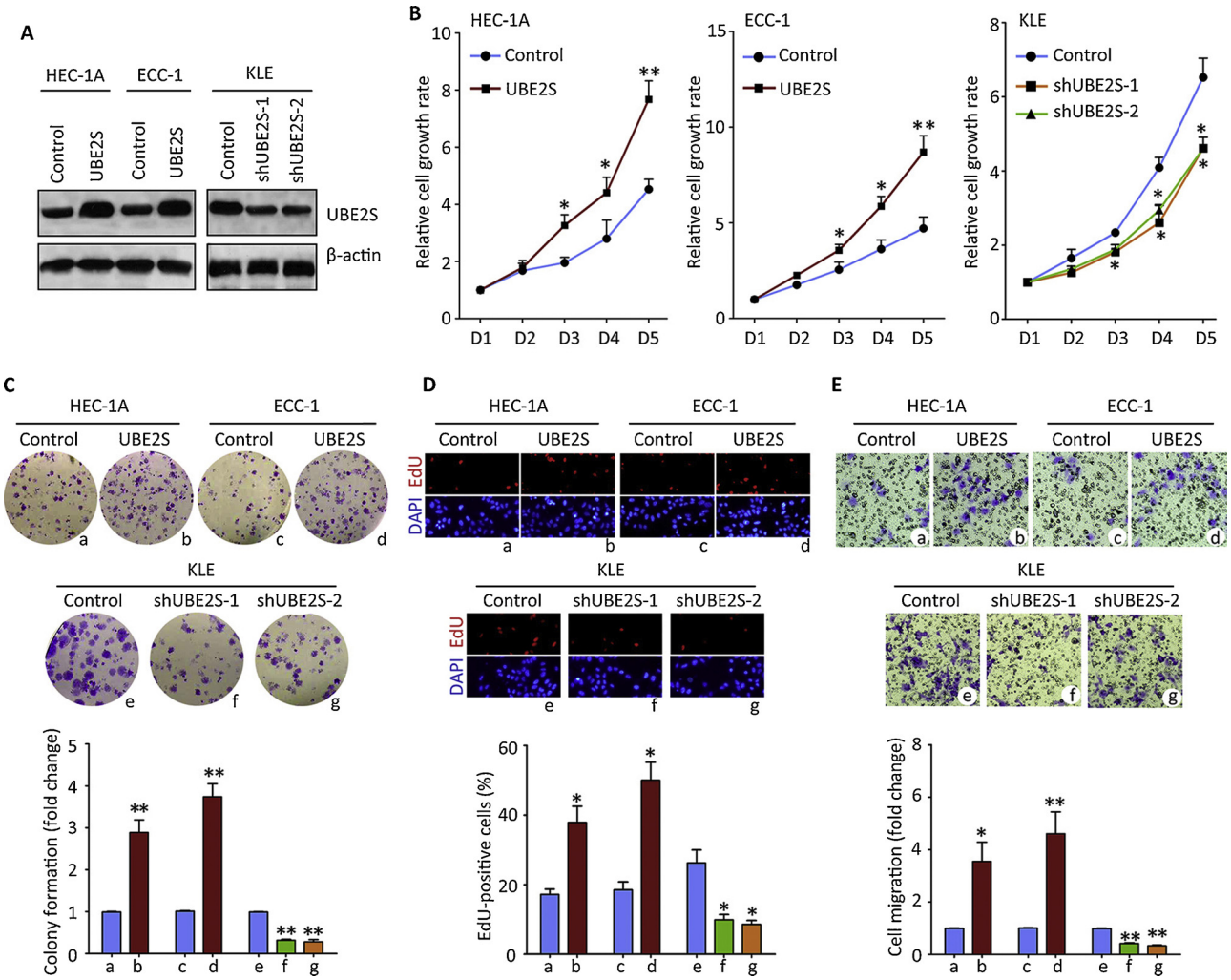

Fig. 3. UBE2S promotes cell proliferation and migration in endometrial cancer. A. Cells were transfected with UBE2S overexpression vector or shRNAs for the establishment of stable cell lines. B. Stable cells were cultured into 96-well plates for 5 days. The cell viabilities were determined by MTT assays. $^{*}P < 0.05$, $^{**}P < 0.01$. C. Cells with or without UBE2S were cultured with 300 mg/L of G418 for 10 days. Colonies were counted and indicated. $^{*}P < 0.05$, $^{**}P < 0.01$. D. Cells with ectopic UBE2S expression or depletion were stained with EdU. The percentage of EdU-positive cells were counted and shown by histogram. $^{*}P < 0.05$. E. Transwell assays were performed to evaluate the effect of UBE2S on the cell migration. Representative data and statistical analyses were shown. $^{*}P < 0.05$, $^{**}P < 0.01$.

and disease-free survival (Fig. 2A&B). The prognostic value of UBE2S was validated in the TCGA cohort, showing that cases with low UBE2S expression were usually accompanied with better prognosis (Fig. 2C& D). Collectively, these data indicate that UBE2S serves as a potent prognostic factor in EMC.

4.2. UBE2S promotes cell proliferation and migration in endometrial cancer

To investigate the biological function of UBE2S in EMC, gain-of-function and loss-of-function assays were performed. UBE2S was either overexpressed in HEC-1 A and ECC-1 cells or knocked down in KLE cells, according to the basal UBE2S expression in 6 EMC cell lines (Fig. 3A and data not shown). in vitro experiments were used to determine the role of UBE2S in EMC progression. As indicated by MTT assays, ectopic expression of UBE2S increased, whereas silence of UBE2S decreased the cell viabilities (Fig. 3B). EdU assays showed that more EdU-positive cells were observed in cells with UBE2S overexpression, and less proliferative cells were depicted in the knockdown group (Fig. 3C). Colony formation assays were applied to confirm the effect of UBE2S on cell growth. Results showed that UBE2S overexpression enhanced, whereas UBE2S knockdown attenuated the ability of clonogenicity of EMC cells (Fig. 3D). Since UBE2S expression was higher in EMC patients with tumor metastasis (Fig. 1E), the impact of UBE2S on cell migration was next investigated. Transwell assays demonstrated that UBE2S overexpression induced more migrated cells, while UBE2S silence suppressed the cell movement (Fig. 3E). Collectively, these findings suggest UBE2S exerts oncogenic activities in EMC.

4.3. UBE2S exhibits oncogenic activities by activating SOX6/β-Catenin signaling

The underlying mechanism via which UBE2S exerted oncogenic function was next investigated. We examined the role of UBE2S in the activation of the β-catenin signaling pathway. Strikingly, the nuclear localization of β-catenin was remarkably increased by ectopic expression of UBE2S in HEC-1 A and ECC-1 cells (Fig. 4A). Protein fraction showed that the expression of nuclear β-catenin was elevated by UBE2S overexpression, but decreased by UBE2S knockdown. Consequently, the downstream effectors of β-catenin, such as c-Myc and Cyclin D1, were upregulated by UBE2S (Fig. 4B). Treatment of XAV-939, a specific inhibitor of β-catenin, significantly attenuated the UBE2S-mediated cell proliferation (Fig. 4C). The cell migration was also suppressed by XAV-939 in UBE2S-expressing EMC cells (Fig. 4D). These data indicate that UBE2S functions as an oncogene in EMC via β-catenin activation.

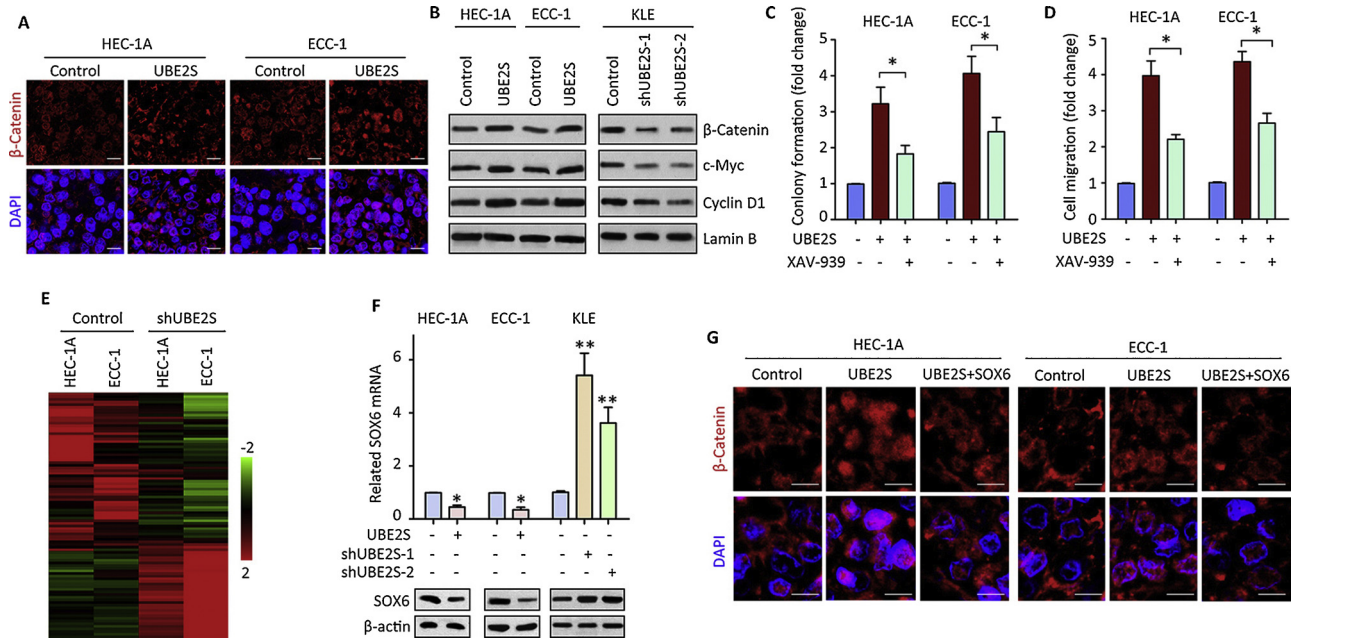

Fig. 4. UBE2S exhibits oncogenic activity via SOX6/β-Catenin signaling. A. The cellular localization of β-Catenin in EMC cells with UBE2S overexpression was indicated by immunofluorescence. Scale bar: 50 μm. B. Nuclear proteins obtained from cells with UBE2S overexpression or knockdown were subjected to western blot to detect the expression level of β-Catenin, c-Myc and Cyclin D1. Lamin B serves as the loading control. C. Cells with UBE2S overexpression vector were treated with XAV-939, a specific inhibitor of β-Catenin pathway. The cell growth was determined by colony formation. D. The effect of XAV-939 on UBE2S-promted cell migration was examined by Transwell assay. All data are mean ± SEM of three independent experiments. All $^*P < 0.05$. E. Gene microarray was used to determine the effect of UBE2S knockdown on the gene expression profile in HEC-1 A and ECC-1 cells. The heatmap showed the altered expression profile. F. The regulation of SOX6 by UBE2S was determined by qRT-PCR and western blot. G. The effect of SOX6 overexpression on the UBE2S-mediated activation of β-Catenin was evaluated by immunofluorescence. Scale bar: 20 μm.

In order to explore how UBE2S activated the β-catenin signaling, gene microarray was used to determine the gene profile altered by UBE2S knockdown in HEC-1 A and ECC-1 cells (Fig. 4E). Forty-five genes were upregulated and 69 genes were downregulated by the treatment of UBE2S shRNA in both EMC cell lines. Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses indicated that UBE2S participated in several pathways including Wnt/β-catenin, MAPK, RNA degradation and PPAR signaling pathways. Among the genes that were involved in β-catenin signaling and upregulated by UBE2S shRNA, SOX6 was chosen for further investigation. Upon the overexpression of UBE2S, SOX6 expression was decreased at both mRNA and protein levels. In cells with UBE2S knockdown, SOX6 was significantly upregulated (Fig. 4F). However, dual luciferase report assays showed no effect of UBE2S on the activity of SOX6 promoter (data not shown), indicating that UBE2S may not directly transcriptionally modulate the expression of SOX6 in EMC cells. Rescue experiments demonstrated that re-expression of SOX6 in UBE2S-expressing cells markedly abrogated the nuclear accumulation of β-catenin (Fig. 4G). These data suggest UBE2S triggered β-catenin signaling via suppressing SOX6 in EMC cells.

5. Discussion

Literatures report that dysregulation of proteins involved in ubiquitination contributes to the progression of human cancers (Liu et al., 2017; Yang et al., 2017). Understanding the roles of ubiquitin-conjugating enzymes in the malignant process of human cancers helps to develop new strategies for clinical management. Here we identify UBE2S as an oncogene in EMC. The expression of UBE2S is upregulated and associated with unfavorable prognosis of patients with EMC. Overexpression of UBE2S activates β-catenin signaling via down-regulation of SOX6 to promote cell proliferation and migration (Fig. 5).

Activation of the β-catenin pathway is frequently observed in EMC and synergistically works with loss of PTEN to trigger the initiation and progression of EMC (van der Zee et al., 2013). Canonically, β-Catenin is

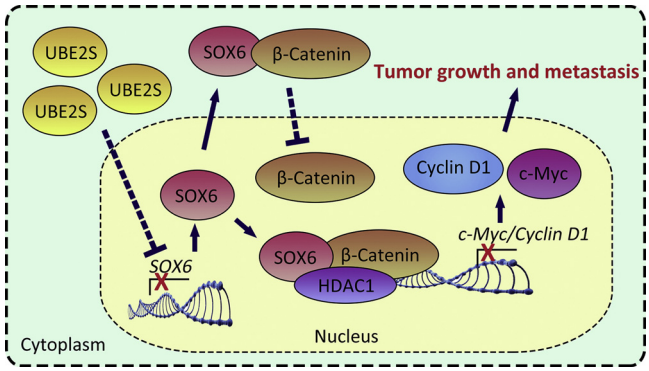

Fig. 5. Schematic diagram of UBE2S-mediated tumor growth and metastasis. Overexpression of UBE2S activates β-catenin signaling via down-regulation of SOX6 to promote cell proliferation and migration.

activated by Wnt/Frizzled signaling. Glycogen synthase kinase-3β (GSK-3β), coupled with Axin1/2 and adenomatosis polyposis coli (APC), phosphorylates the cytoplasmic β-Catenin for proteosomal degradation (Nusse and Clevers, 2017). Once Wnt signaling is activated by Frizzled, β-catenin is dissociated from the degradation complex, leading to its translocation into the nucleus to form the transcriptional complex with TCF/LEF to start the transcription of several downstream genes implicated in carcinogenesis (Nusse and Clevers, 2017). Under certain circumstance, β-catenin pathway could be activated by the CBX8-induced phosphorylation of AKT at Ser552 (Zhang et al., 2018). Our data demonstrated that β-catenin signaling was activated by the overexpression of UBE2S. Further investigations showed that ectopic expression of UBE2S inhibited the expression of SOX6 at both mRNA and protein levels. Previous studies reported that SOX6 exerted anti-tumor activities towards human cancers. Downregulation of SOX6 was found in esophageal squamous cell carcinoma (Qin et al., 2011),

pancreatic cancer (Jiang et al., 2018) and hepatocellular carcinoma (Wang et al., 2016a), and was capable of promoting tumor progression. Furthemore, SOX6 was identified as the upstream regulator of PD-L1 and Cyclin D1 (Dong et al., 2018). During the pancreatic β-cell proliferation, SOX6 suppressed cyclin D1 activities by interacting with β-catenin (Iguchi et al., 2007). SOX6 pysically bound to the armadillo repeats 1–4 of β-catenin to inhibit its oncogenic activities (Iguchi et al., 2007). Based on the above findings, we assume that SOX6 binds to β-catenin to dissociate β-catenin from the transcriptional complex and prevent its nucleus localization, which is abolished by the overexpression of UBE2S. However, more data should be obtained to explore the detailed molecular process.

Strikingly, our findings showed SOX6 was transcriptionally repressed by UBE2S. However, data from luciferase reporter assays indicated that UBE2S did not affect the transcriptional activity of SOX6 promoter. As a member of ubiquintation proteins, UBE2S exhibits functions usually via enhancing the ubiquitation of the substrate to modulate the protein stability. For example, UBE2S functions as an oncogene in hepatocellular carcinoma through increasing the ubiquitination of p53 for its degradation (Pan et al., 2018). UBE2S interacts with Ku70 to participate in nonhomologous end-joining (NHEJ)-mediated DNA repair process (Hu et al., 2017). Ube2S mediates K11-linked polyubiquitin chain formation at the Sox2-K123 residue for proteasome-mediated degradation of SOX2 to repress Sox2-mediated embryonic stem cell differentiation (Wang et al., 2016b). As a result, it is probably that the suppression of SOX6 may be due to UBE2S-mediated ubiquitinylation followed by proteasome-dependent degradation, besides of the transcriptional modulation of SOX6 via UBE2S-mediated mechanism. However, the detailed mechanism via which UBE2S modulates the expression of SOX6 requires further investigations. Collectively, these data suggest UBE2S serves a promising prognostic factor in EMC and exerts oncogenic activities via activation of SOX6/β-catenin pathway. The newly identified UBE2S/SOX6/β-catenin axis may represent a new potential therapeutic target for EMC intervention.

Conflict of interest

Authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.01.014.

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