Sohlh2 inhibits ovarian cancer cell proliferation by upregulation of p21 and downregulation of cyclin D1

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Spermatogenesis and oogenesis basic helix-loop-helix (bHLH) transcription factor 2 (Sohlh2) functions as a bHLH transcription factor to regulate mouse germ cell differentiation. Our previous data showed that Sohlh2 was highly expressed in human normal tissues, but low level of Sohlh2 was observed in many cancer cell lines, suggesting a possible role of Sohlh2 in tumorigenesis. In this study, we examined this possibility by using immunohistochemistry, RT–PCR, 5-bromo-2-deoxyuridine, clonogenic assay and tumor xenograft techniques. Our results showed that the expression of Sohlh2 was decreased in epithelial ovarian carcinoma (EOC) tissues compared with benign ovarian tumors and ovarian tumors with low malignant potential. Forced expression of Sohlh2 led to a significant reduction in cancer cell proliferation in vitro and tumorigenesis in nude mice. Conversely, silencing of Sohlh2 enhanced ovarian cancer cell proliferation. Furthermore, Sohlh2 had opposite effects on its two direct targets p21 and cyclin D1: overexpression of Sohlh2 upregulated p21 but downregulated cyclin D1 expression. p21 knockdown could reverse the effects of Sohlh2 overexpression on inhibiting cell proliferation, and cyclin D1 knockdown could reverse the effects of Sohlh2 ablation on promoting cell proliferation. Thus, our data indicate that Sohlh2 likely functions as a tumor suppressor in EOCs, which is achieved by inducing p21 expression but repressing cyclin D1 expression.

Introduction

Ovarian cancer is the leading cause of gynecological cancer deaths. Epithelial ovarian carcinoma (EOC) accounts for ~90% of all ovarian malignancies. More than 70% of diagnosed cases are in advanced stage (1–4). Currently, the standard treatment for EOC is the combination of cytoreductive surgery and chemotherapy, but most patients with advanced EOC eventually relapse with a median progression-free survival of 18 months (5). Thus, new strategies are urgently required to improve the survival rate. Recently, two major molecularly targeted agents were applied to ovarian cancer treatment: anti-vascular endothelial growth factor (VEGF) antibody bevacizumab and poly (ADP-ribose) polymerase (PARP) inhibitor Olaparib (6,7). Nevertheless, the survival rate of patients has not been improved significantly. The heterogeneity and poor prognosis of EOC prompt the researcher to develop new therapeutic targets.

Abbreviations: BrdU, 5-bromo-2-deoxyuridine; ChIP, chromatin immunoprecipitation; EGFP, enhanced green fluorescent protein; EOC, epithelial ovarian carcinoma; LMP, low malignant potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT–PCR, reverse transcription–PCR; Sohlh2, spermatogenesis and oogenesis basic helix-loop-helix (bHLH) transcription factor 2.

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The spermatogenesis and oogenesis basic helix-loop-helix (bHLH) transcription factor, spermatogenesis and oogenesis basic helix-loop-helix (bHLH) transcription factor 2 (Sohlh2), is one of important members in bHLH family (8). bHLH proteins are a large superfamily of transcription factors that play critical roles in many physiological processes including cellular differentiation, cell cycle arrest and apoptosis (9). The conserved bHLH domain is involved in homo- or hetero-dimerization to form a functional transcriptional unit that binds to the canonical E-Box response element (CANNTG) in the promoter of many genes (9). In mouse, Sohlh2 expression is restricted in undifferentiated and differentiated spermatogonia, oocytes of primordial follicle and early development of primary follicle (8). Sohlh2 is essential for spermatogenesis and oogenesis as revealed in Sohlh2-null mice. Sohlh2 induces cell differentiation from type A spermatogonia to type B spermatogonia and the transition of primordial to primary follicle (10–12). In human, Sohlh2 protein is not restricted in human reproductive organs. High Sohlh2 expression is observed in many normal tissues, especially in epithelial tissues. However, low level of Sohlh2 expression is detected in many cancer cell lines (13,14). These preliminary observations prompt us to investigate the roles of Sohlh2 in tumorigenesis.

In the present study, we examined Sohlh2 expression in ovarian cancer. Our data showed that Sohlh2 expression was significantly decreased in EOC tissues compared with benign ovarian tumors and ovarian tumors with low malignant potential (LMP). Overexpression of Sohlh2 inhibited EOC cell growth in vitro and tumor growth in vivo. We further demonstrated that Sohlh2 induced p21 expression but repressed cyclin D1 expression. Moreover, Sohlh2 downregulation was correlated with poor prognosis of patients with EOCs, suggesting that Sohlh2 might serve as a tumor suppressor in the development and progression of EOCs.

Materials and methods

Reagents

Polyclonal rabbit anti-human Sohlh2 antibody (ab101402) was purchased from Abcam (Cambridge, MA). Monoclonal mouse anti-human cyclin D1 antibody (sc-20044), polyclonal rabbit anti-rat cyclin E antibody (sc-481), polyclonal rabbit anti-mouse cyclin A antibody (sc-596), polyclonal rabbit anti-human p21 antibody (sc-397), polyclonal rabbit anti-human p27 antibody (sc-7767), monoclonal mouse anti-human Ki-67 antibody (sc-101861) and mouse monoclonal anti-avian β-actin antibody (sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Trizol was purchased from Invitrogen (Grand Island, NY). Reverse transcription–PCR (RT–PCR) kit was purchased from Thermo Fisher Scientific (Waltham, MA).

Cell culture

Human immortalized superficial epithelial cell lines (HOSE6-3 and HOSE11-12) were generously presented from Prof. George SW Tsao (Hong Kong University, China). All human ovarian cancer cell lines (HO8910, OVCAR3, A2780, SKOV3, 3AO and Caov-3) were obtained from the cell center of the Chinese Academy of Science. All cell lines were maintained in Dulbecco’s modified Eagle’s medium containing 1% penicillin/streptomycin and 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Plasmids and shRNA

The human Sohlh2 cDNA was cloned into the pEGBP-N1 (Clontech, CA) vector to generate stable cell lines. The luciferase reporter plasmids pGL4-P21 and pGL4-cyc D1 were constructed by cloning the promoter regions of human p21 and human cyclin D1. The shRNAs were cloned into the GV428 (Genechem, Shanghai, China), using the targeted sequences as follows: Sohlh2-5′-GCT CCAAATCTGCAG TATATC-3′ (pShS1) and 5′-TCT CCAAGGC TGTCCGAGATTA-3′ (pShS2), p21-5′-GACCATGGACCTGTCAC-3′ (pShP21), cyclin D1-5′-GGGAAACAAACAGATCATC-3′ (pShyc D1). All constructs were verified by sequencing.

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Transfections and Sohlh2 stable cell lines
Cancer cells (HO8910, OVCAR3, A2780, SKOV3) and HOSE cells were seeded at 25% confluence in 24-well plates the day before transfection. The pSohlh2 or shRNA plasmids against Sohlh2 were transfected into the cells using Lipofectamine 2000 (Invitrogen). Forty-eight hours later, transfected cells were selected with 800 µg/ml G418 or 1 µg/ml puromycin (Sigma–Aldrich, St Louis, MO) for 2 weeks to obtain overexpression or knockdown stable cell lines.

Mouse xenograft model
Female NOD-SCID mice (5–6 weeks old) were purchased from Vital River Company (Beijing, China) and maintained in a barrier facility on a 12:12 h light: dark cycle. Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Animal Care and Use Committee of Shandong University. HO8910 (5 x 10⁶) cells were subcutaneously injected into both sides of the dorsal thigh of ten female NOD-SCID mice in each group. Tumor size was monitored twice a week for 5 weeks and tumor weight was measured at the end of the experiment.

Cell proliferation
For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 1.0 × 10⁴ cells were seeded into 96-well plates in regular culture medium, and cell growth was monitored every day over the course of 5 days by MTT assay following the manufacturer’s instructions (Sigma–Aldrich).

For 5-bromo-2-deoxyuridine (BrdU) staining, cells were labeled with 15 µg/ml BrdU for 4 h in regular culture medium, then washed three times with PBS. The treated cells were fixed in 4% formaldehyde/PBS for 20 min, treated with 2N HCL/1% Triton-X100 for 60 min, washed three times with 0.1% NP-40/PBS; incubated with mouse anti-BrdU (B8434, 1:100 dilution; Sigma–Aldrich) at 4°C overnight in a humidified chamber and then incubated with fluorescein isothiocyanate-conjugated secondary antibody (1:100 dilution) for 1 h at 37°C. Nuclei were counterstained with 1 µg/ml 4′,6-diamidino-2-phenylindole. BrdU-positive cells were counted under a fluorescence microscope (Olympus, Tokyo, Japan).

For cell cycle analysis, ovarian cancer cells in each group were trypsinized and fixed in 75% ethanol for 1 h at −20°C. Fixed cells were incubated with the solution containing 20 mg/ml propidium iodide, 10 mg/ml RNase A and 0.1% Triton X-100 for 30 min at 4°C before analysis. The cell cycle was assessed on

![Fig. 1. Downregulation of Sohlh2 expression in human EOC tumors. (A) Immunohistochemical staining of Sohlh2 in paraffin-embedded tissues. Strong signals of Sohlh2 were observed in ovarian superficial epithelial cells (a), oviduct epithelial cells (b), benign ovarian tumor (c), LMP-tumor (d), negative (e), weak (f), moderate (g) and strong (h) positive staining of Sohlh2 in EOC tissues. Scale bar = 50 µm. (B) EOC tumors exhibited significant reduction in Sohlh2 score in comparison with benign tumors (P < 0.001) or LMP-tumors (P < 0.01). (C) Real-time PCR analysis of Sohlh2 expression in immortalized human ovarian superficial epithelial cell lines HOSE 6-3 and HOSE 11-12 and indicated human EOC cell lines. (D) Kaplan–Meier analysis of the correlation between the Sohlh2 level and the OS and progression-free survival (PFS) rate in EOC patients with positive (Sohlh2 immunoreactive score: 4–12) and negative (Sohlh2 immunoreactive score: 0–3) Sohlh2 expression. (E) Statistical analysis of Sohlh2 expression in poor, moderate or highly differentiated EOC samples. Data are shown as mean ± SD from three experiments. **P < 0.01; ***P < 0.001.](image-url)
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Colonies were visualized after staining with 0.04% crystal violet in methanol for 1–2 h.

Patient samples and immunohistochemistry

A total of 83 cases of primary EOC tissues, 18 cases of benign ovarian tumors, 25 cases of ovarian tumors with LMP, 11 cases of normal ovary and 8 cases of normal human fallopian tube specimens were collected from patients at Qilu Hospital and Weifang People’s Hospital from 2009 to 2012. The histological characterization and cell differentiation-based EOC tumor grading were determined by the criteria from Union for International Cancer Control (UICC). The written consent was obtained from all patients and this study was approved by the Medical Ethics Committee of Shandong University. The clinical information of samples is presented in Supplementary Table S1, available at Carcinogenesis Online.

The tissues, including normal tissues, benign ovarian tumors and EOCs, were stained according to the standard immunostaining procedure (5). Briefly, formalin-fixed and paraffin-embedded tissues were sectioned (5 μm). After deparaffinization, sections were immunostained using anti-Sohlh2, anti-p21, anti-cyclin D1 and anti-Ki-67 at a 1:100 dilution, respectively. The slides were counterstained with hematoxylin and mounted. Control experiments were performed by using non-immune immunoglobulins. The immunostaining images were captured by using the Olympus computerized image analysis system.

Each sample of Sohlh2 immunostaining was scored according to the intensity (no staining = 0; weak staining = 1; moderate staining = 2; strong staining = 3) and the percentage (0% = 0; 1–10% = 1; 11–50% = 2; 51–80% = 3; 81–100% = 4) of stained cells. Final immunoreactive scores were determined by multiplying the intensity score with the positivity score, which ranged between 0 and 12 (15,16). Sohlh2-positive expression was defined for tumors with Sohlh2 immunoreactive scores from 4 to 12, while Sohlh2-negative expression was defined for tumors with Sohlh2 immunoreactive scores from 0 to 3.

Quantitative real-time PCR

Total RNA was prepared using Trizol reagent (Invitrogen) following the manufacturer’s instructions. Five micromgrams of total RNA was reverse-transcribed by using the SuperScript II Reverse Transcriptase Kit (Invitrogen). A 25 μl volume reaction consisted of 1 μl reverse transcription product and 10 pM of each primer. The specific primers for RT–PCR were as follows: Sohlh2: 5'-CAACAGTCTCCCTCAACACTG-3' and 5'-GCC CATTGCCATCCCTTAAAG-3'; p21: 5'-TGTG ACTGCTCTTACC TTG-3' and 5'-GGCGTTTTGAGTGTAGAAG-3'; cyclin D1: 5'-CA TCTAACCTCACA GGCAAACTCCATC-3' and 5'-TC TGGCATTT TGGAAGAGAAG-3'. GAPDH: 5'-ATCTTCCAGGAGCAGACCC-3' and 5'-TCC ACAATGCCA AAGTGTCA TGG-3'.

Western blot

Cells were lysed in RIPA lysis buffer (Santa Cruz). Proteins (10 μg) were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel, and then transferred onto polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA). The membranes were probed with appropriate primary antibody at 4°C overnight, followed by incubation with peroxidase-conjugated anti-rabbit (or anti-goat) IgG (isotype-matched immunoglobulin) antibody for 1 h at room temperature. The interaction was monitored with an electrochemiluminescence kit (Amersham Life Sciences, GE Healthcare Life Sciences, Pittsburgh, PA). Anti-β-actin antibody was used to monitor the loading amount.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) analysis was performed as previously described (17). HO8910 cells expressing Sohlh2 or control vector were prepared using a ChIP assay kit (Millipore) following the

**Fig. 2.** Overexpression of Sohlh2 suppresses EOC cell proliferation. (A) MTT assays revealed cell growth curves of HO8910 transfected with control pEGFP or pSohlh2 and of A2780 and HOSE6-3 cells transfected with vector control, pShS1 or pShS2. (B) Representative micrographs (left) and relative quantification (right) of BrdU-positive cells in EOC cell lines transfected with pSohlh2 or Sohlh2 shRNAs. Quantitative analysis of EOC cell proliferation is indicated by the percentage of BrdU-positive cells. BrdU-positive and 4′,6-diamidino-2-phenylindole staining cells were counted at 200× magnification in 10 randomly selected fields, total 400–500 cells were counted in each experiment. (C) Representative micrographs (left) and relative quantification (right) of crystal violet-stained colonies analyzed by clonogenic formation. Data are shown as mean ± SD from three experiments. **P < 0.01; ***P < 0.001.
manufacturer’s guidelines. Chromatin solutions were precipitated with 30 µl Sohlh2 antibody-conjugated agarose beads at 4°C overnight. Rabbit IgG was used as a negative control. Precipitated DNAs were analyzed by PCR. The specific primers for human p21 and cyclin D1 promoter were as follows: p21: 5'-TAAGCCCTCGGAGCTA-3' and 5'-GGGCCTCAGGAGGTCTCA-3'; cyclinD1: 5'-AACCTCCGTGTGCCTTCCCTA-3' and 5'-GGCTGCGCTTCCAACTTGA-3'.

Luciferase reporter assay
HO8910 (5 x 10⁴) cells were plated in 24-well plate and transiently transfected with 200 ng of Sohlh2 expression plasmid, 100 ng of p21 or cyclin D1 promoter firefly luciferase reporter construct and 17 ng of Renilla luciferase plasmid (Promega) by Lipofectamine 2000 (Invitrogen). Luciferase activities were determined at 24 h after transfection by a dual-luciferase reporter assay system (Promega). Results were represented as the ratio of firefly to Renilla luciferase activity and normalized to vector control.

Statistical analysis
Quantitative data are expressed as mean ± SD. GraphPad Prism (GraphPad Software, San Diego, CA) was used for data analysis. The Student t-test or one-way ANOVA was used to identify significant differences between groups. The chi-square test was used to analyze the relationship between categorical variables. Survival curve was plotted by using the Kaplan–Meier method and compared by the log-rank test. *P < 0.05 was considered statistically significant in all cases.

Results
Sohlh2 is lowly expressed in primary human EOCs
Sohlh2 expression was examined in 83 cases of primary human EOC specimens, 18 cases of benign ovarian tumors, 25 cases LMP-tumors and 11 cases of normal ovary. Sohlh2 expression was observed in all (100%) ovarian superficial epithelium, benign ovarian tumors and LMP-tumors and in 51 of 83 (61.4%) of ovarian cancers. Additionally, recent evidence suggested that a proportion of high-grade serious EOC may arise from distant fallopian tube epithelium (18). Thus, we also included eight cases of normal human fallopian tube specimens. In normal ovarian and oviduct tissues, Sohlh2 was present predominantly in ovarian superficial epithelial cells and oviduct epithelial cells, as well as in smooth muscle cells of blood vessels. Sohlh2 immunostaining was both nuclear and cytoplasmic (Figure 1A).

Sohlh2 expression was significantly lower in EOCs compared with the benign tumors or LMP-tumors with a median IHC-score 8 for benign tumors, 6.7 for LMP-tumors and 3 for ovarian cancer tissues (P < 0.01; Figure 1B)

We sought to examine the expression of Sohlh2 mRNA in human EOC cell lines and primary human ovarian surface epithelial (HOSE) cells by quantitative RT–PCR analysis. As shown in Figure 1C, Sohlh2 mRNA was lowly expressed in human EOC cell lines compared with those in HOSE cells. These data strongly suggest that Sohlh2 expression is significantly suppressed in EOC.

A total of 65 EOC patients were followed up for 24 months. We found that Sohlh2-positive and Sohlh2-negative patients exhibited significant difference in overall survival (OS). The 2-year overall survival rate was 27.3% for Sohlh2-negative patients and 53.5% for Sohlh2-positive patients, respectively (P = 0.017, Figure 1D).

Univariate analysis showed that Sohlh2 expression was associated with the survival of EOC patients, as shown in Supplementary Table S2, available at Carcinogenesis Online. Moreover, Sohlh2 expression was significantly higher in well-differentiated EOC tissues (86.7%) than that in moderately (69%) or poorly differentiated (44.7%) ones (Figure 1E, P = 0.0082). Sohlh2 expression was not associated with the histological types in low-grade EOCs (Supplementary Table S3, available at Carcinogenesis Online). Thus, low Sohlh2 expression seems to be a risk factor associated with high mortality rate. These data suggest that Sohlh2 may play a role in EOC pathogenesis and its expression represents a potential biomarker for the disease.

Sohlh2 inhibits the proliferation of ovarian cancer cell lines both in vitro and in vivo
To explore the biological function of Sohlh2, we analyzed the effects of Sohlh2 expression on the cell growth by overexpressing or silencing Sohlh2 in ovarian cancer cells and HOSE cells. The ovarian cancer cell lines HO8910 and OVCAR3 had low endogenous Sohlh2 levels. In both cell lines, Sohlh2 overexpression inhibited the proliferation

![Fig. 3](image-url). Overexpressed Sohlh2 inhibits tumor growth of EOC cell xenografts in vivo. (A) Xenograft tumor growth was monitored over a 30-day period. (B) At the end of experiment, tumors formed by HO8910 cells transfected with pEGFP or pSohlh2 were photographed. (C) Weight of xenografted tumors. (D) Relative quantification of Ki-67-positive cells in pEGFP- and pSohlh2-xenografted tumors. Data are presented as mean ± SD from five mice in each group. *P < 0.05; **P < 0.01; ***P < 0.001.
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Fig. 4. Sohlh2 induces cell cycle arrest and regulates p21 and cyclin D1 expression. The cell cycle in HO8910 cells (A) transfected with pEGFP or pSohlh2 or A2780 cells (B) and HOSE6-3 cells (C) transfected with control, pShS1 or pShS2 vector was analyzed by flow cytometry. A representative plot from one experiment is shown. (D) Representative micrographs of the protein expression of Sohlh2, cyclin D1, cyclin A, cyclin E, p21, p27 and β-actin in indicated transfected ovarian cancer cell lines. (E) Real-time RT–PCR analysis of Sohlh2, p21 and cyclin D1 mRNA levels in HO8910 cells transfected with pEGFP or pSohlh2, and in A2780 cells transfected with shRNAs against Sohlh2 (pShS1 and pShS2). Data are shown as mean ± SD from three experiments. *P < 0.05; **P < 0.01.
of EOC cells over a 5 day period (Figure 2A). Conversely, Sohlh2 ablation by pShS1 and pShS2 in ovarian cancer cell lines A2780 and SKOV3 and HOSE6-3 that have higher endogenous Sohlh2 expression significantly promoted cell proliferation (Figure 2A). Moreover, BrdU assay revealed that Sohlh2 overexpression dramatically attenuated the EOC cell proliferation, as evidenced by less BrdU-positive cells than that of vector control. Sohlh2 knockdown resulted in more BrdU-positive cells than that of vector control (Figure 2B). The inhibitory properties of sohlh2 were also examined using colony formation assay. Sohlh2 overexpression in both HO8910 and OVCAR3 cell lines led to a significant decrease in the number of colonies after fourteen days culture, while Sohlh2 knockdown in A2780 and SKOV3 cell lines significantly increased the number of colonies (Figure 2C). These results indicate that Sohlh2 suppresses the proliferation of EOC cell lines.

To further investigate the effects of Sohlh2 on tumorigenicity in vivo, HO8910 cells overexpressing Sohlh2 or vector were subcutaneously injected into nude mice. Thirty days after injection, tumors were excised from tested mice for further analysis. Sohlh2 overexpression significantly inhibited tumor growth in the course of the experiment (Figure 3A). Consistent with this finding, the tumor weight in pSohlh2-injected mice was significantly less than that in pEGFP-injected mice (Figure 3B and C). We next sought to determine whether cell proliferation was suppressed by Sohlh2 overexpression in dissected tumors. Towards this goal, we examined the expression of Ki-67 by IHC. There was a significant decrease in the number of Ki-67-positive cells in Sohlh2-overexpressed tumors compared with control tumors (Figure 3D), indicative of reduced proliferation. Overall, these in vitro and in vivo studies indicate that Sohlh2 functions as a tumor suppressor to inhibit cell growth.

To confirm this, we tested whether overexpression of Sohlh2 resulted in cell cycle arrest by flow cytometry analysis. Sohlh2 overexpression in HO8910 cells and OVCAR3 cells (data not shown) increased the fraction of cells in the G0/G1 phase (49.1 ± 2.14% of 2
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Figure 4A. In a reciprocal experiment, Sohlh2 knockdown in A2780 cells (Figure 4B), SKOV3 cells (data not shown) and HOSE6-3 decreased the proportion of cells in G0/G1 phase (Figure 4C). To further dissect the molecular mechanisms underlying the cell proliferation regulation, we investigated the expression of some genes related to cell cycle progression. Sohlh2 overexpression in HO8910 cells increased p21 expression but decreased cyclin D1 expression (Figure 4D). This effect appears to be specific, as the knockdown of Sohlh2 by shRNA had the opposite effects (Figure 4D). Real-time RT–PCR analysis showed that p21 mRNA level was dramatically increased, while cyclin D1 mRNA level was significantly decreased when Sohlh2 was highly expressed in HO8910 cells; the opposite effects on p21 and cyclin D1 expression were observed when Sohlh2 was ablated in A2780 cells (Figure 4E). These data suggest that Sohlh2 influences cell cycle progression by regulating p21 and cyclin D1 expression.

Sohlh2 blocks cell proliferation by upregulation of p21 and down-regulation of cyclin D1 transcription

To further explore the regulation of Sohlh2 on p21 and cyclin D1 expression, the effects of Sohlh2 on p21 and cyclin D1 promoters were analyzed by luciferase reporter assay. The results demonstrated that Sohlh2 activated p21 promoters, but repressed the activity of cyclin D1 promoter (Figure 5A). To determine if Sohlh2 binds to these promoters, ChIP assays were performed in Sohlh2-overexpressed HO8910 cells. As shown in Figure 5B and C, Sohlh2 was associated with the promoter regions of p21 and cyclin D1, suggesting that Sohlh2 regulates the expression of p21 and cyclin D1 by interacting with their promoters. These unique dual roles of Sohlh2, i.e. upregulating p21 and downregulating cyclin D1, may serve as a dominant regulator in the process of cell proliferation.

To test the roles of p21 and cyclin D1 in the growth inhibition of Sohlh2, p21 was knocked down by shRNA in the Sohlh2-overexpressed cells, while cyclin D1 was ablated by shRNA in the Sohlh2-ablated cells. According to colony growth and cell proliferation assays, we found that the decreased colony growth and cell proliferation in Sohlh2-overexpressed HO8910 cells were reversed by p21 ablation, cyclin D1 knockdown abrogated the accelerated colony growth and cell proliferation in Sohlh2-ablated A2780 (Figure 5D and F) and SKOV3 (data not shown) cells. These data provide evidences that Sohlh2 inhibits ovarian cancer cell proliferation by elevating p21 expression and attenuating cyclin D1 expression.

Correlation of Sohlh2 expression with p21, cyclin D1 and Ki-67 expression in ovarian tumors

Here, we stained Sohlh2, p21, cyclin D1 and Ki-67 in continuous tissue sections from same patients. Consistent with our in vitro data from ovarian cancer cell lines, there was a markedly positive correlation between Sohlh2 and p21 expression and an inverse correlation between Sohlh2 and cyclin D1 expression (Figure 6A and B). In addition, increased
Sohlh2 expression was accompanied by decreased Ki-67 expression (Figure 6C), suggesting that Sohlh2 inhibits tumor growth in vivo.

Discussion

In this report, we found that Sohlh2 inhibited ovarian cancer growth in vitro and in vivo. In addition, our data showed that the expression of Sohlh2 was significantly downregulated in primary human EOC samples compared with ovarian superficial epithelial cells and oviduct epithelial cells. Moreover, low expression of Sohlh2 was associated with malignant ovarian tumors. Whereas, it has been reported that Sohlh2 is not expressed in EOC (19). In the manuscript, only the mRNA levels of sohlh2 were assayed in the samples of EOC (19). The negative results may be due to the primers and PCR condition. Based on our experiments, sohlh2 expression at the protein level was very low or negative in some samples (38.6%), but relatively high in most of samples (61.4%). Collectively, these results reveal a novel function of Sohlh2 in tumor suppression.

Basic helix-loop-helix (bHLH) proteins play critical roles in tumorigenesis including cellular differentiation, cell cycle arrest and apoptosis (9,20). It has been reported that bhlh transcription factors function as a negative regulator for cell proliferation in normal, immortalized and cancer cell lines (21). Consistent with this, overexpression of Sohlh2 in ovarian cancer cells leads to G1 arrest and inhibits cell proliferation. Protein p21 is a cyclin-dependent kinase inhibitor, a key regulator of the cell cycle (22–25). In the nucleus, p21 binds to the promoters of the cyclin-dependent kinases Cdk1 and Cdk2 and inhibits their expression, which subsequently blocks the transition from G1 phase into S phase (25,26). Thus, in the present study, the G1 cell cycle arrest caused by ectopic Sohlh2 expression is due to upregulation of p21. The result that p21 abolished the inhibitory effects of Sohlh2 on ovarian cancer cell proliferation further verifies this conclusion. Using luciferase reporter genes and CHIP assay, we found Sohlh2 could bind with p21 promoter, suggesting p21 is a transcriptional target of Sohlh2. Furthermore, positive correlation between Sohlh2 and p21 expression was observed in our clinical relevance study, suggesting that Sohlh2 functions as a tumor suppressor.

The reduction in cyclin D1 abundance induced by forced expression of Sohlh2 and subsequent reduction in cell proliferation were in agreement with the previous report that the G1/S cell cycle transition and proliferation of human ovarian cancer cells are dependent upon the abundance of cyclin D1 (27). Cyclin D1 is essential for the regulation of the G1-S phase transition in normal cell cycle, and the alterations or mutations of cyclin D1 are found in most human cancers (28). The oncogenic properties of cyclin D1 in ovarian cancer have been established in various studies (29–31). In ovarian cancer cell lines, the induction of cyclin D1 results in a shortened G1 and increased number of cells progressing through G1/S cell cycle checkpoint (32). Inhibiting cyclin D1 expression prevents the entry into S phase (33,34). Using luciferase reporter genes and CHIP assay, we found Sohlh2 could bind with cyclin D1 promoter and inhibit its expression. It has been shown that there is an E-box/E2F binding site on the promoter region of cyclin D1, which is involved in the cyclin D1 transcriptional regulation (35,36). Evidence shows that bhlh transcription factor DECI directly binds to the E-box in the cyclin D1 promoter and negatively regulates cyclin D1 expression (21). Several potential bhlh DNA binding sites (E-box, CANNTG) are present in the cyclin D1 promoter. Further studies will be required to clarify the specific binding sites of Sohlh2 on the cyclin D1 promoter.

In summary, our data demonstrated, for the first time, that Sohlh2 was lowly expressed in human EOCs compared with benign ovarian tumors and LMP-tumors. A lower level of Sohlh2 expression correlated with tumor progression and shorter overall survival in EOC patients. The ectopic expression of Sohlh2 inhibited the growth of human EOC cells both in vitro and in vivo. Importantly, Sohlh2 induced G1 arrest in human EOC cells and resulted in upregulation of p21 but downregulation of cyclin D1. Together, our data provide insights into the molecular mechanisms for the suppressive role of Sohlh2 in EOC and suggest that Sohlh2 can be considered as a potential novel target for developing EOC therapeutics.

Supplementary material

Supplementary Tables S1–S3 can be found at http://carcin.oxfordjournals.org/

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