E2F6 functions as a competing endogenous RNA, and transcriptional repressor, to promote ovarian cancer stemness

Frank H. C. Cheng1,2 | Hon-Yi Lin3,4 | Tzy-Wei Hwang5 | Yin-Chen Chen1,2 | Rui-Lan Huang6 | Chia-Bin Chang1,2 | Weiqin Yang7 | Ru-Inn Lin3 | Ching-Wen Lin1,2 | Gary C. W. Chen1,2 | Shu-Yuan Mai1 | Jora M. J. Lin1,2 | Yu-Ming Chuang1,2 | Jian-Liang Chou1,2 | Li-Wei Kuo1,2 | Chin Li1 | Alfred S. L. Cheng7 | Hung-Cheng Lai6 | Shu-Fen Wu1,2 | Je-Chiang Tsai8 | Michael W. Y. Chan1,2,9,10

1Department of Biomedical Sciences, National Chung Cheng University, Chia-Yi, Taiwan
2Epigenomics and Human Disease Research Center, National Chung Cheng University, Chia-Yi, Taiwan
3Department of Radiation Oncology, Buddhist Dalin Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Chia-Yi, Taiwan
4School of Medicine, Tzu Chi University, Hualien, Taiwan
5Department of Mathematics, National Chung Cheng University, Chia-Yi, Taiwan
6Department of Obstetrics and Gynecology, School of Medicine, College of Medicine and Shuang Ho Hospital, Taipei Medical University, Taipei, Taiwan
7School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong, China
8Department of Mathematics, National Tsing Hua University, Hsin-Chu, Taiwan
9Center for Innovative Research on Aging Society (CIRAS), National Chung Cheng University, Chia-Yi, Taiwan
10Research Center for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

Correspondence
Je-Chiang Tsai, Department of Mathematics, National Tsing Hua University, Hsin-Chu, Taiwan.
Email: tsaijc.math@gmail.com
and
Michael W. Y. Chan, Department of Biomedical Sciences, National Chung Cheng University, Chia-Yi, Taiwan.
Email: biowyc@ccu.edu.tw

Funding information
Ministry of Science and Technology, Taiwan, Grant/Award Number: 102-2320-B-194-006, 103-2320-B-194-002, 104-2115-M-194-011-MY3, 104-2115-M-194-009, 104-2320-B-194-003, 105-2115-M-194-009 and 107-2115-M-007-011-MY2; Buddhist Dalin Tzu Chi Hospital, Grant/Award Number: 105(2)-I-10 and 106(2)-E-18

Ovarian cancer is the most lethal cancer of the female reproductive system. In that regard, several epidemiological studies suggest that long-term exposure to estrogen could increase ovarian cancer risk, although its precise role remains controversial. To decipher a mechanism for this, we previously generated a mathematical model of how estrogen-mediated upregulation of the transcription factor, E2F6, upregulates the ovarian cancer stem/initiating cell marker, c-Kit, by epigenetic silencing the tumor suppressor miR-193a, and a competing endogenous (ceRNA) mechanism. In this study, we tested that previous mathematical model, showing that estrogen treatment of immortalized ovarian surface epithelial cells upregulated both E2F6 and c-KIT, but downregulated miR-193a. Luciferase assays further confirmed that microRNA-193a targets both E2F6 and c-Kit. Interestingly, ChIP-PCR and bisulphite pyrosequencing showed that E2F6 also epigenetically suppresses miR-193a, through recruitment of EZH2, and by a complex ceRNA mechanism in ovarian cancer cell lines. Importantly, cell line and animal experiments both confirmed that E2F6 promotes ovarian cancer stemness, whereas E2F6 or EZH2 depletion derepressed miR-193a, which opposes...
1 | INTRODUCTION

Ovarian cancer is the leading cause of death from gynecologic malignancies, and the second most common cause of all-cancer death, in women. Most ovarian cancer patients are diagnosed in late stages, with poor prognosis and short life span due to drug resistance originating from cancer-initiating cells. Ovarian cancer-initiating cells are characterized by cell surface expression of CD44 and CD117 (ie c-KIT), a phenotype first identified by us, and then others. Estrogen, an essential steroid nuclear hormone intimately involved in the growth and differentiation of normal ovaries, has also been implicated in ovarian tumorigenesis. However, antiestrogen therapies have shown only moderate efficacy against ovarian cancer, and precisely how this hormone contributes to ovarian cancer stemness remains unclear. Consequently, understanding these “missing links” would provide better understanding of the origin of ovarian cancer, and thus, development of better-targeted therapies for this deadly disease.

In addition to altered endocrine signaling, aberrant epigenetic events, including dysregulated DNA methylation, histone modifications, and noncoding RNA translational modulation, are now considered a hallmark of cancer. Furthermore, both we and others previously reported aberrant promoter DNA hypermethylation of tumor suppressor genes in numerous human cancers, including ovarian. Specific repressive histone “marks” likewise silence tumor suppressor genes, and the histone methyltransferases EZH2 and G9a have both been found to be oncogenes.

To link anomalous epigenetic modifications to estrogen-mediated ovarian carcinogenesis, we recently developed a mathematical model of the progression of this malignancy. In this model, we hypothesized a mechanism for estrogen-mediated upregulation of the cell cycle regulator and transcriptional repressor E2F6. Specifically, E2F6 upregulates the ovarian cancer stemness marker c-KIT, by two means. First, a competing endogenous RNA (ceRNA) mechanism, in which overexpressed E2F6 mRNA (ie ceRNA), with sequence homology to microRNA (miRNA)-193a’s seed sequence, competes away miR-193a from binding the 3′-UTR of c-KIT mRNA. Such competition subsequently leads to c-KIT upregulation and increased cancer stemness. Second, binding of E2F6 to the miR-193a promoter recruits the epigenetic transcriptional repressive EZH2 and DNMT3b, resulting in epigenetic silencing of miR-193a and, consequently, derepression of miR-193a targets such as c-KIT. Based on our model, and these previous findings, we herein investigated the role(s) of E2F6 in promoting cancer stemness.

2 | MATERIALS AND METHODS

2.1 | Cell culture and epigenetic inhibitor treatment

Ovarian cancer HeyC2 cells were propagated in DMEM (Gibco, Grand Island, NY, USA) supplemented with 1% MEM NEAA (Gibco), 1% HEPES (Gibco), 5% FBS (Gibco), and 50 units/mL penicillin/streptomycin (P/S; Gibco). All other ovarian cancer cells were propagated in RPMI-1640 (Gibco), supplemented with 10% FBS and 50 units/mL P/S. Immortalized ovarian surface epithelial (OSE) cells, originally derived by transducing the catalytic subunit of human telomerase and the papilloma virus subunit E7 into primary ovarian epithelial cells, were maintained in a 1:1 mixture of MCDB105 (Sigma, St. Louis, MO, USA) and medium 199 (Gibco), supplemented with 10% FBS, 400 ng/mL hydrocortisone (Sigma), 10 ng/mL epidermal growth factor (Sigma), and 50 units/mL P/S. Immortalized fallopian tube epithelial cells (FE-25), and FE-25 cells ectopically expressing the KRAS oncogene (FE-25/RAS), were cultured in MCDB105 and medium 199 (1:1, v/v) supplemented with 10% FBS and 1% P/S.

For epigenetic inhibitor treatment, 1 × 106 cells were seeded into 90-mm plates and treated with 0.5 μM of the DNA-demethylating agent, 5′-aza-2′-deoxycytidine (SazaDC; Sigma) for 72 hours, the EZH2 inhibitor GSK126 (10 μM; Cayman Chemicals, Ann Arbor, MI, USA), the EZH2 inhibitor GSK343 (10 μM; Sigma) for 72 hours, and/or the histone deacetylase inhibitor trichostatin A (TSA, 0.5 μM; Sigma), for 12 hours. For SazaDC, GSK126, GSK343, or TSA treatment, media was changed and new drug added every 24 hours.

2.2 | Patient samples

One hundred and eighteen ovarian tissue samples, including 108 cancer and 10 benign tissues, were obtained from Tri-Service General Hospital, Taipei, Taiwan (Table S1). All studies involving human ovarian cancer tissues were approved by the Institutional Review Board of Tri-Service General Hospital, Taiwan.
2.3  |  In vitro invasion assay

To assess cell invasion, polycarbonate cell culture inserts (8 μm pore size; Merck Millipore, Burlington, MA, USA) were first coated with 25 μL Matrigel (BD Biosciences, San Jose, CA, USA). Cells (2 × 10⁴) were seeded into the upper chambers in medium with 1% FBS, and the inserts then placed into 24-well plates containing medium with 10% FBS. After 48 hours, the cells at the top of the filter were removed by washing with 1× PBS. Cells attached to the membrane bottoms were fixed and stained with Giemsa reagent (Sigma).

2.4  |  In vivo tumorigenicity assay

Eight-week-old, athymic nude (BALB/cByJNarl) or SCID mice (CB17/ lcr-Prkdc.scid/lcrtc2crIbtw) were obtained from the Taiwan National Laboratory Animal Center (Taipei, Taiwan) or BioLASCIO Taiwan (Taipei, Taiwan). All mice were kept under specific pathogen-free conditions, using a laminar airflow rack, with free access to sterilized food and autoclaved water. All animal experiments were approved by the Animal Experimentation Ethics Committee of National Chung Cheng University (Chia-Yi, Taiwan). Cells (1 × 10⁶) in a 1:1 mixture of 0.1 mL medium and Matrigel, were injected s.c. into each flank of each mouse (day 0). Tumor size was measured daily, using calipers, in length (L) and width (W). Tumor volumes were calculated using the formula \( V = \frac{1}{2} \times L \times W^2 \). For i.p. tumor growth, 1 × 10⁶ near-infrared fluorescent protein-overexpressing CP70 cells, with various treatments, were injected i.p. into athymic nude mice. Tumor growth was also observed by fluorescence intensity using an FMT 4000 fluorescence tomography imaging system (PerkinElmer, Waltham, MA, USA). At the end of the experiments, all mice were killed by cervical dislocation, as previously approved.

2.5  |  Combined bisulfite restriction analysis and bisulphite pyrosequencing

Genomic DNA (0.5 μg) was bisulphite-modified using EZ DNA Methylation Kits (Zymo Research, Orange, CA, USA), according to the manufacturer’s protocol. For combined bisulfite restriction analysis assays, bisulphite-modified DNA was subjected to PCR amplification, and 8 μL of the resultant PCR products was then digested by the restriction endonuclease AciI (New England BioLabs, Beverly, MA, USA), at 37°C, for 2 hours. For bisulphite pyrosequencing analysis, bisulphite-modified DNA was subjected to a PCR amplification strategy using a tailored reverse primer in combination with a biotin-labeled universal primer, as previously described.¹⁶ The PCR and sequencing primers were designed using PyroMark Assay Design 2.0 (Qiagen, Hilden, Germany). Pyrosequencing was carried out on a PyroMark Q24 (Qiagen) instrument using Pyro Gold Reagents (Qiagen) according to the manufacturer’s protocol. Methylation percentages for each cytosine were then determined as the fluorescence intensity of cytosines divided by the sum of the fluorescence intensity of cytosines and thymines, at each CpG site. In vitro-methylated DNA (Millipore) was included as a positive control for pyrosequencing. All primer sequences are listed in Table S2.

2.6  |  Bioinformatic and statistical analysis

To identify potential miR-193a targets, results were obtained by the intersection of multiple prediction programs, including TargetScan (http://www.targetscan.org/), DIANA-microT-CDs (diana.imis. athena-innovation.gr), and miRanda (www.microrna.org/microrna/ home.do). Statistical significance was determined using GraphPad Prism Version 5.0 software for Windows (GraphPad Software, La Jolla, CA, USA). Student’s t test or the Mann-Whitney U test was used to compare parameters of different groups. Progression-free survival (PFS) and overall survival were assessed by Kaplan-Meier analysis using the log-rank test. Progression-free survival was defined as the duration from day of diagnosis or chemotherapy to the detection of new lesions or progression of residual lesions. Overall survival was defined as the duration from day of diagnosis to death. Univariate and multivariate survival analyses were determined using a Cox proportional hazards model. A DNA methylation level of miR-193a at 9% (methylation level in IOSE cells) was used as a cut-off for methylation. \( P < .05 \) was considered statistically significant.

Additional Material and Methods can be found in Document S1.

3  |  RESULTS

3.1  |  Competing endogenous RNA regulation of E2F6 and c-KIT through miR-193a

Our previous mathematical model predicted that miR-193a targets c-KIT and E2F6, such that they might act as ceRNAs (Figure 1A) to positively regulate one another.¹⁸ To examine the relationships between miR-193a, E2F6, and c-KIT in ovarian cancer, miR-193a expression was examined in IOSE and a panel of ovarian cancer cell lines. Compared to IOSE and immortalized fimbrial epithelial (FE-25) cells, miR-193a was downregulated in most ovarian cancer cell lines (Figure 1B; Figure S1A). Notably, expression of E2F6 (Figure S1B) and c-KIT (Figure S1C) inversely correlated with the expression of miR-193a in those ovarian cancer cell lines, whereas E2F6 expression was positively correlated with that of c-KIT (Figure S1D). To confirm whether E2F6 and c-KIT are regulated directly by miR-193a, a GFP reporter was fused to the E2F6-3′-UTR, with each containing a miR-193a or mutant miRNA response element (MRE). Overexpression of the E2F6-3′-UTR containing the MRE (but not its mutant) resulted in a lower GFP signal, as indicated by fluorescence microscopy or flow cytometry (Figure S2A,B). Likewise, a similarly constructed luciferase reporter showed significantly reduced luciferase activity in CP70 cells cotransfected with miR-193a and the E2F6-3′-UTR (Figure 1C) or c-KIT-3′-UTR (Figure 1D). No notable alteration of luciferase activity was observed between the control (luciferase empty vector) and E2F6-3′-UTR MRE mutant. These results indicate that both E2F6 and c-KIT are targets of miR-193a.

Previously, we posited that E2F6 is an estrogen receptor (ER) target gene, such that estrogen-mediated upregulation of E2F6 might eventually upregulate c-KIT through a ceRNA mechanism.¹⁸
Model was confirmed, as overexpression of E2F6-3′-UTR in miR-193a-overexpressing A2780 and CP70 ovarian cancer cells upregulated both E2F6 and c-KIT (Figure 1E,F; Figure S2C,D). Conversely, overexpressed c-KIT-3′-UTR in miR-193a-overexpressing HeyC2 cells also upregulated E2F6 (Figure S2E,F) while also increasing chemoresistance to cisplatin (Figure S2G). These results suggest that E2F6 and c-KIT are both ceRNAs for miR-193a, thus facilitating mutual upregulation.
To further examine the role of ER signaling and a ceRNA mechanism in ovarian carcinogenesis, IOSE, an immortalized ovarian surface epithelial cell line, was used. First, IOSE cells were treated with the active form of estrogen, estradiol (E2), or the “environmental estrogen” bisphenol-A (BPA), a compound known to activate ER signaling.24,25 Interestingly, treatment...
with E2 or BPA upregulated both E2F6 (Figure 1G) and c-KIT (Figure 1H); IOSE cells treated with BPA and E2 for 3 days also significantly downregulated miR-193a (Figure 1I). Taken together, these results agree with our previous model that E2F6 might suppress miR-193a transcription through epigenetic modifications.\(^\text{18}\)

### 3.2 MicroRNA-193a is epigenetically silenced in ovarian cancer cell lines

Previously, we modeled that miR-193a may be transcriptionally suppressed by its promoter occupancy by E2F6, in association with Polycomb repressive complex-2 (PRC2).\(^\text{18}\) To test the validity of this model, we first assessed expression of the histone methyltransferase EZH2, an integral member of PRC2, in IOSE and various ovarian cancer cell lines. Interestingly, EZH2 overexpression (Figure 2A) was observed in ovarian cancer cells showing low expression of miR-193a (Figure 1B). Consistently, miR-193a promoter methylation was observed only in SKOV3, A2780, and CP70 ovarian cancer cells, but not in IOSE or FE-25 noncancerous epithelial cells (Figure 2B; Figure S3A). Treatments combining a DNA methyltransferase inhibitor (5azaDC) with a histone deacetylase inhibitor (TSA) or an EZH2 inhibitor (GSK343) in CP70 (Figure 2C) and Kuramochi ovarian cancer cells (Figure S3B) resulted in robust miR-193a re-expression. However, it is noteworthy to point out that SKOV3 with low expression of EZH2 also underexpressed miR-193a but had the highest level of miR-193a promoter methylation. One explanation for this is that E2F6 might be able to directly recruit DNMT3b to epigenetically suppress miR-193a independent of EZH2, as previously reported.\(^\text{22}\)

Indeed, treatment with 5azaDC upregulated miR-193a more than an EZH2 inhibitor (GSK343) in SKOV3 ovarian cancer cells (Figure S3C). Taken together, these results indicate that epigenetic alterations are responsible for miR-193a transcriptional suppression in ovarian cancer cells.

The roles of E2F6 and EZH2 in regulating miR-193 transcription were next examined by ChIP-PCR on CP70 and HeyC2 ovarian cancer cells, showing distinct miR-193 promoter occupancy patterns correlated with its expression. These results showed that E2F6 and EZH2, as well as the repressive histone marker H3K27me3, were all enriched in the miR-193a promoter region in CP70, but not HeyC2, ovarian cancer cells (Figure 2D-G), with HeyC2 cells showing higher miR-193a expression (Figure 1B). Importantly, depletion of EZH2 (Figure 3A) or E2F6 (Figure 3B) restored miR-193a expression in CP70 cells (Figure 3C). Such upregulation was also accompanied by reduced H3K27me3 (Figure 3D) and DNA methylation (Figure S3D) within the miR-193a promoter in CP70 cells. Also noteworthy, E2F6 depletion reduced EZH2 binding to the miR-193a promoter (Figure 3E,F, regions 2 and 3, gray; regions refer to Figure 2D) but not vice versa (Figure 3E,F, region 2 and 3, black). Conversely, overexpression of EZH2 or E2F6, alone or in combination (Figure S3E,F), reduced miR-193a expression in HeyC2 cells (Figure S3G). Taken together, these results confirm our mathematical model that E2F6 recruits EZH2 to silence the promoter of miR-193a, through DNA methylation and repressive histone modifications.

To further investigate the role of miR-193a in ovarian cancer, colony formation and soft agar growth assays were carried out on CP70 or A2780 ovarian cancer cells overexpressing miR-193a or vector alone. Such overexpression dramatically decreased colony numbers (Figure S4A,B) and significantly inhibited anchorage-independent growth (Figure S4C,D) compared to control cells. Furthermore, miR-193a overexpression in CP70 cells decreased cell invasion (Figure 4A). We then tested whether miR-193a overexpression affects chemoresistance, an established hallmark of cancer-initiating cells.\(^\text{26,27}\) Indeed, miR-193a overexpression restored cisplatin chemosensitivity in both A2780 and CP70 cells (Figure 4B,C), and also reduced CP70 xenograft tumor growth in an orthotopic nude mouse model (Figure S4E).

Finally, mechanistic assessments of ceRNA downregulation of miR-193a showed that overexpression of the E2F6-3′-UTR, containing a wild-type miR-193a MRE, but not an MRE mutant, significantly increased CP70 cell colony numbers (Figure S4F). Together, these results strongly suggest a tumor suppressor role for miR-193a, in ovarian cancer.

### 3.3 Roles of E2F6 and EZH2 in ovarian cancer stemness

Next, the roles of E2F6 and EZH2 in ovarian cancer progenitor function were investigated. Genetic depletion of either EZH2 or E2F6 significantly inhibited CP70 cell sphere formation (Figure 4D), and numbers of dye-excluding cells (or side populations) (Figure S5A), two common stemness assays.\(^\text{28,29}\) Similarly, pharmacological inhibition of EZH2, by GSK343 or GSK126, likewise reduced the number of CP70 side population cells (Figure S5B). Finally, EZH2 or E2F6 depletion inhibited in vivo invasion (Figure S5C) and s.c. (Figure 4E,F) and i.p. tumor growth (Figure S5D). Together, the above results indicate that EZH2 and E2F6 contribute to cancer stemness, invasion, and tumor growth in ovarian cancer through epigenetic suppression of miR-193a (Figure 3C).

### 3.4 Clinical significance of miR-193a DNA methylation in ovarian cancer patients

We next assessed the clinical significance of miR-193a methylation in human ovarian cancer (Table S3) by bisulfite pyrosequencing on 108 ovarian cancer patient samples. Specifically, patients with higher disease grade, but not stage, showed greater miR-193a methylation (Figure 5A,B). Kaplan-Meier analysis also showed that miR-193a promoter DNA methylation significantly correlated with decreased overall survival and PFS (Figure 5C,D; Table S4). To further validate these findings, RNA sequencing data from a ovarian cancer dataset (The Cancer Genome Atlas, https://cancergenome.nih.gov/), from 367 ovarian cancer patient samples, showed that higher expression of EZH2, E2F6, and c-KIT was observed in tumor with higher stage and grade (Figure S6). Importantly, E2F6
levels positively correlated with expression of EZH2 (Figure 5E).

Interestingly, our previous study also reported positive correlation between the expression of E2F6 and c-KIT in ovarian cancer patients with low EZH2 levels.\textsuperscript{16} Taken together, these results indicate that \textit{mir}-193a is epigenetically silenced in ovarian cancer, and the \textit{mir}-193a targets, E2F6 and c-KIT, show a ceRNA relationship.

**DISCUSSION**

Although several studies now suggest that activation of the estrogen receptor (ER)-\textalpha signaling pathway is intimately involved in ovarian carcinogenesis, its precise role remains controversial. For example, epidemiological studies found that women treated with hormone
FIGURE 4 Role of microRNA (miR)-193a as a tumor suppressor in ovarian cancer. A, Overexpression of miR-193a in CP70 cells decreased cell invasion and enhanced chemosensitivity to cisplatin in (B) A2780 and (C) CP70 cells. NC, normal control. D, Depletion of either EZH2 or E2F6 reduced sphere formation by CP70 cells. E, F, Depletion of (E) EZH2 or (F) E2F6, in CP70 cells, reduced tumor size in an s.c. xenograft mouse model. Left side (L), knockdown control. Right side (R), shRNA (EZH2 or E2F6) knockdown. (*P < .05; **P < .01, ***P < .005). Each bar represents the mean ± SD of duplicate experiments.

FIGURE 5 Promoter methylation of miR-193a associates with poor survival in ovarian cancer patients. A, B, Ovarian cancer tumors of higher (A) grade and (B) stage associate with higher miR-193a promoter methylation. Red lines denote medians. C, D, Kaplan-Meier analysis showed that patients with higher miR-193a methylation have (C) shorter overall survival and (D) shorter progression-free survival. E, Positive correlation between EZH2 and E2F6 expression in an ovarian cancer RNA sequencing dataset. FPKM, fragments per kilobase of transcript per million mapped reads. (*P < .05; ***P < .005)

FIGURE 6 Model of microRNA (miR)-193/E2F6/c-KIT mediation of ovarian cancer stemness. Estrogen (E2) binding to the estrogen receptor (ER) upregulates E2F6 (upper left). Epigenetic silencing of miR193a by E2F6 recruitment of the transcriptional repressor EZH2, and possibly DNA methyltransferase (DNMT) (bottom left), promotes stemness in ovarian cancer (right panel). Additionally, upregulated E2F6 mRNA, in turn, upregulates c-KIT by a competing endogenous RNA mechanism (through competitive inhibition of miR-193 binding, see proliferation of green binding sites, left and middle panels). Finally, upregulation of c-KIT results in the expansion of ovarian cancer-initiating cells and subsequent drug resistance. TSS, transcriptional start site.
replacement therapy had an increased risk of ovarian cancer. However, antiestrogen therapy was only somewhat effective for a subset of ovarian cancer patients, regardless of ER-α expression status.

To solve this complexity, we previously developed a mathematical model, based on a system of ordinary differential equations, and applied bifurcation analysis to determine the role of estrogen (estradiol, E2) signaling in ovarian cancer. Interestingly, those results showed that at low inhibitory efficiency of E2F6 transcriptional repressor (ie high transcription rate of miR-193a), estrogen-mediated upregulation of E2F6 correlated with upregulation of c-KIT, resembling a "ceRNA" relationship. However, at high inhibitory efficiency of E2F6, upregulation of E2F6 led to epigenetic silencing of miR-193a and, eventually, irreversible overexpression of the oncogene c-KIT, a facilitator of cancer "stemness." 3,4

To confirm this mathematical model, in this study, we undertook biological experiments to show that E2F6 is upregulated following treatment with E2 or BPA (a "xenoestrogen") in IOSE cells expressing miR-193a. In addition to E2F6 upregulation, treatment with E2 or BPA also upregulated c-KIT. Consequently, these results show that ER-α signaling can upregulate the miR-193a targets, E2F6 and c-KIT, in benign ovarian epithelial cells. Notably, overexpression of E2F6-3'-UTR in miR-193a-overexpressing ovarian cancer cells sponged miR-193a and counteracted its suppression of endogenous E2F6 and c-KIT, resulting in their upregulation. Taken together, these experiments confirmed that E2F6 and c-KIT are regulated by an estrogen-mediated ceRNA mechanism, through miR-193a.

We also showed that E2F6 could suppress miR-193a gene transcription by recruitment of the oncoprotein transcriptional repressor EZH2 in CP70, but not HeyC2, ovarian cancer cells, with the former more highly expressing EZH2 than the latter. It is interesting to note that E2F6 depletion reduced EZH2 binding to the miR-193a promoter, but not vice versa, thus confirming that E2F6 is necessary to recruit EZH2 and DNA methyltransferase (DNMT) to the miR-193a promoter. This finding agrees with previous reports that, for genes other than miR-193a, E2F6's transcriptional repressive activity requires EZH2 and a DNMT. However, it is also noteworthy to point out that E2F6 might be able to directly recruit DNMT independent of EZH2 as SKOV3 cells, with low EZH2 expression, showed the highest promoter methylation of miR-193a.

Furthermore, our cell line and animal experiments suggest a tumor suppressor function for miR-193a in ovarian cancer, in agreement with clinical findings that ovarian cancer patients having miR-193a hypermethylation have a poorer prognosis than those lacking such hypermethylation (Figure 5). Importantly, cells depleted of EZH2 or E2F6 had significantly impaired stemness-associated spheres, and "side population" cell formation, due to downregulation of the stemness marker c-KIT by restoration of miR-193a and the ceRNA network.

In conclusion, we propose a model that the tumor suppressor function of miR-193a can be epigenetically silenced, bimodally, in ovarian cancer (Figure 6). As shown, estrogen or xenoestrogens could upregulate the cell cycle-associated transcriptional repressor, E2F6, resulting in upregulation of c-KIT, through E2F6-mediated epigenetic silencing of miR-193a. Alternatively, E2F6 might act through a ceRNA mechanism, depending on EZH2 expression. E2F6 up-regulation might therefore promote cancer stemness in ovarian cancer, thus advancing tumor progression. We therefore propose that epigenetic intervention could be a novel strategy for ovarian cancer patients, especially those not responding to checkpoint inhibitors or traditional chemotherapies. However, it is also noteworthy to point out that EZH2 has recently been found to be an important factor in promoting T-cell differentiation. Therefore, an optimal dosage of EZH2 inhibitor in cancer treatment, without affecting T cell function, should be considered. Overall, these results are significant to suggest the involvement of ceRNA networks in tumor progression and stemness.

ACKNOWLEDGEMENTS

This study was supported by grants from the Ministry of Science and Technology, Taiwan (MOST 107-2115-M-007-011-MY2, 106-2923-B-194-001-MY3, 105-2115-M-194-009, 104-2115-M-194-011-MY3, 104-2115-M-194-009, 104-2320-B-194-003, 103-2320-B-194-002, 102-2320-B-194-006), Buddhist Dalin Tzu Chi Hospital (DTCRD 105(2)-I-10, 106(2)-E-18). This study was also supported by the Center for Innovative Research on Aging Society (CIRAS) and partially supported by MOST, National Center for Theoretical Science (NCTS), and the Brain Research Center (to J.-C. Tsai) from The Featured Areas Research Center Program under the framework of the Higher Education Sprout Project by Ministry of Education, Taiwan.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Michael W. Y. Chan https://orcid.org/0000-0003-1431-322X

REFERENCES

1. Siegel R, Ma JM, Zou ZH, Jemal A. Cancer statistics, 2014. CA Cancer J Clin. 2014;64(1):9-29.
2. Tchagang AB, Tewfik AH, DeRycke MS, Skubitz KM, Skubitz APN. Early detection of ovarian cancer using group biomarkers. Mol Cancer Ther. 2008;7(1):27-37.
3. Schuler S, Ponnath M, Engel J, Ortmann O. Ovarian epithelial tumors and reproductive factors: a systematic review. Arch Gynecol Obstet. 2013;287(6):1187-1204.
4. Jessmon P, Boulanger T, Zhou W, Patwardhan P. Epidemiology and treatment patterns of epithelial ovarian cancer. Expert Rev Anticancer Ther. 2017;17(5):427-437.
5. Zhang S, Balch C, Chan MW, et al. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Res. 2008;68(11):4311-4320.
6. Luo L, Zeng J, Liang B, et al. Ovairanen cancer cells with the CD117 phenotype are highly tumorigenic and are related to chemotherapy outcome. Exp Mol Pathol. 2011;91(2):596-602.
7. Chen J, Wang J, Chen D, et al. Evaluation of characteristics of CD44+CD117+ ovarian cancer stem cells in three dimensional basement membrane extract scaffold versus two dimensional monocultures. BMC Cell Biol. 2013;14:7.
8. Voutsadakis IA. Hormone receptors in serous ovarian carcinoma: prognosis, pathogenesis, and treatment considerations. Clin Med Insights Oncol. 2016;10:17-25.
9. Huang J, Rodriguez GC. Ovarian cancer: etiology, risk factors, and epidemiology. Clin Obstet Gynecol. 2012;55(1):3-23.
10. Smyth JF, Gourley C, Walker G, et al. Antiestrogen therapy is active in selected ovarian cancer cases: the use of letrozole in estrogen receptor-positive patients. Clin Cancer Res. 2007;13(12):3617-3622.
11. Piletic K, Kunej T. MicroRNA epigenetic signatures in human disease. Arch Toxicol. 2016;90(10):2405-2419.
12. Baylin SB, Jones PA. A decade of exploring the cancer epigenome - biological and translational implications. Nat Rev Cancer. 2011;11(10):726-734.
13. Rodriguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. Nat Med. 2011;17(3):330-339.
14. Earp MA, Cunningham JM. DNA methylation changes in epithelial ovarian cancer histotypes. Genomics. 2015;106(6):311-321.
15. Yeh KT, Chen TH, Yang HW, et al. Aberrant TGFbeta/SMAD4 signaling contributes to epigenetic silencing of a putative tumor suppressor, RunX1T1 in ovarian cancer. Epigenetics. 2011;6(6):727-739.
16. Chou JL, Huang RL, Shay J, et al. Hypermethylation of the TGF-beta target, ABCA1 is associated with poor prognosis in ovarian cancer patients. Clin Epigenetics. 2015;7:1.
17. Kelly TK, De Carvalho DD, Jones PA. Epigenetic modifications as therapeutic targets. Nat Biotechnol. 2010;28(10):1069-1078.
18. Cheng FH, Aguda BD, Tsai JC, et al. A mathematical model of bimodal epigenetic control of miR-193a in ovarian cancer stem cells. PLoS ONE. 2014;9(12):e116050.
19. Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature. 2010;465(7301):1033-1038.
20. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta stone of a hidden RNA language? Cell. 2011;146(3):352-358.
21. Attwood C, Oddi S, Cartwright P, et al. A novel repressive EZF6 complex containing the polycomb group protein, EPC1, that interacts with EZH2 in a proliferation-specific manner. J Biol Chem. 2005;280(2):1199-1208.
22. Velasco G, Hube F, Rollin J, et al. Dnmt3b recruitment through EZF6 transcriptional repressor mediates germ-line gene silencing in murine somatic tissues. Proc Natl Acad Sci USA. 2010;107(20):9281-9286.
23. Auersperg N, Maines-Bandiera SL, Dyck HG, Kruk PA. Characterization of cultured human ovarian surface epithelial cells: phenotypic plasticity and premalignant changes. Lab Invest. 1994;71(4):510-518.
24. Weng YI, Hsu PY, Liyanarachchi S, et al. Epigenetic influences of low-dose bisphenol A in primary human breast epithelial cells. Toxicol Appl Pharmacol. 2010;248(2):111-121.
25. Shafei A, Ramzy MM, Hegazy AI, et al. The molecular mechanisms of action of the endocrine disrupting chemical bisphenol A in the development of cancer. Gene. 2018;647:235-243.
26. Hu L, McArthur C, Jaffe RB. Ovarian cancer stem-like subpopulation cells are tumourigenic and chemoresistant. Br J Cancer. 2010;102(8):1276-1283.
27. Steg AD, Bevis KS, Katre AA, et al. Stem cell pathways contribute to clinical chemoresistance in ovarian cancer. Clin Cancer Res. 2012;18(3):869-881.
28. Salcido CD, Larochelle A, Taylor BJ, Dunbar CE, Varticovski L. Molecular characterisation of side population cells with cancer stem cell-like characteristics in small-cell lung cancer. Br J Cancer. 2010;102(11):1636-1644.
29. Nakano A, Tsuji D, Miki H, et al. Glycolysis inhibition inactivates ABC transporters to restore drug sensitivity in malignant cells. PLoS ONE. 2011;6(11):e27222.
30. Beral V, Million Women Study C, Bull D, Green J, Reeves G. Ovarian cancer and hormone replacement therapy in the Million Women Study. Lancet. 2007;369(9574):1703-1710.
31. Modugno F, Laskey R, Smith AL, Andersen CL, Haluska P, Oesterreich S. Hormone response in ovarian cancer: time to reconsider as a clinical target? Endocr Relat Cancer. 2012;19(6):R255-R279.
32. Hurteau JA, Brady MF, Darcy KM, et al. Randomized phase III trial of tamoxifen versus thalidomide in women with biochemical-recurrent-only epithelial ovarian, fallopian tube or primary peritoneal carcinoma after a complete response to first-line platinum/taxane chemotherapy with an evaluation of serum vascular endothelial growth factor (VEGF): a Gynecologic Oncology Group Study. Gynecol Oncol. 2010;119(3):444-450.
33. Andersen CL, Sikora MJ, Boisen MM, et al. Active estrogen receptor-alpha signaling in ovarian cancer models and clinical specimens. Clin Cancer Res. 2017;23(14):3802-3812.
34. Chau WK, Ip CK, Mak AS, Lai HC, Wong AS, c-Kit mediates chemoresistance and tumour-initiating capacity of ovarian cancer cells through activation of Wnt/beta-catenin-ATP-binding cassette G2 signaling, Oncogene. 2013;32(22):2767-2781.
35. Trimarchi JM, Fairchild B, Wen J, Lees JA. The E2F6 transcription factor is a component of the mammalian Bmi1-containing polycomb complex. Proc Natl Acad Sci USA. 2001;98(4):1519-1524.
36. Cao L, Zhou Y, Zhai B, et al. Sphere-forming cell subpopulations with cancer stem cell properties in human hepatoma cell lines. BMC Gastroenterol. 2011:11:71.
37. Tirino V, Desiderio V, Paine F, et al. Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. FASEB J. 2013;27(1):13-24.
38. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12(4):252-264.
39. Zhao E, Maj T, Kryczek I, et al. Cancer mediates effector T cell dysfunction by targeting microRNAs and EZH2 via glycolysis restriction. Nat Immunol. 2016;17(1):95-103.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Cheng FH, Lin H-Y, Hwang T-W, et al. E2F6 functions as a competing endogenous RNA, and transcriptional repressor, to promote ovarian cancer stemness. Cancer Sci. 2019;110:1085-1095. https://doi.org/10.1111/cas.13920