

## ORIGINAL ARTICLE

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

Frank H. C. Cheng<sup>1,2</sup> | Hon-Yi Lin<sup>3,4</sup> | Tzy-Wei Hwang<sup>5</sup> | Yin-Chen Chen<sup>1,2</sup> |  
 Rui-Lan Huang<sup>6</sup> | Chia-Bin Chang<sup>1,2</sup> | Weiqin Yang<sup>7</sup> | Ru-Inn Lin<sup>3</sup> | Ching-Wen Lin<sup>1,2</sup> |  
 Gary C. W. Chen<sup>1,2</sup> | Shu-Yuan Mai<sup>1</sup> | Jora M. J. Lin<sup>1,2</sup> | Yu-Ming Chuang<sup>1,2</sup> |  
 Jian-Liang Chou<sup>1,2</sup> | Li-Wei Kuo<sup>1,2</sup> | Chin Li<sup>1</sup> | Alfred S. L. Cheng<sup>7</sup> | Hung-Cheng Lai<sup>6</sup> |  
 Shu-Fen Wu<sup>1,2</sup> | Je-Chiang Tsai<sup>8</sup> | Michael W. Y. Chan<sup>1,2,9,10</sup> 

<sup>1</sup>Department of Biomedical Sciences, National Chung Cheng University, Chia-Yi, Taiwan

<sup>2</sup>Epigenomics and Human Disease Research Center, National Chung Cheng University, Chia-Yi, Taiwan

<sup>3</sup>Department of Radiation Oncology, Buddhist Dalin Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Chia-Yi, Taiwan

<sup>4</sup>School of Medicine, Tzu Chi University, Hualien, Taiwan

<sup>5</sup>Department of Mathematics, National Chung Cheng University, Chia-Yi, Taiwan

<sup>6</sup>Department of Obstetrics and Gynecology, School of Medicine, College of Medicine and Shuang Ho Hospital, Taipei Medical University, Taipei, Taiwan

<sup>7</sup>School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong, China

<sup>8</sup>Department of Mathematics, National Tsing Hua University, Hsin-Chu, Taiwan

<sup>9</sup>Center for Innovative Research on Aging Society (CIRAS), National Chung Cheng University, Chia-Yi, Taiwan

<sup>10</sup>Research 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

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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

Cheng and Lin contributed equally to this work.

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cancer stemness, by alleviating DNA methylation and repressive chromatin. Finally, 118 ovarian cancer patients with *miR-193a* promoter hypermethylation had poorer survival than those without hypermethylation. These results suggest that an estrogen-mediated E2F6 ceRNA network epigenetically and competitively inhibits microRNA-193a activity, promoting ovarian cancer stemness and tumorigenesis.

#### KEYWORDS

ceRNA, E2F6, epigenetics, miR-193a, ovarian cancer

## 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.<sup>1</sup> Most ovarian cancer patients are diagnosed in late stages, with poor prognosis and short life span,<sup>2-4</sup> 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,<sup>5</sup> and then others.<sup>6,7</sup>

Estrogen, an essential steroid nuclear hormone intimately involved in the growth and differentiation of normal ovaries,<sup>8</sup> has also been implicated in ovarian tumorigenesis.<sup>9</sup> However, antiestrogen therapies have shown only moderate efficacy against ovarian cancer,<sup>10</sup> 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.<sup>11-13</sup> Furthermore, both we and others previously reported aberrant promoter DNA hypermethylation of tumor suppressor genes in numerous human cancers, including ovarian.<sup>14-16</sup> Specific repressive histone “marks” likewise silence tumor suppressor genes, and the histone methyltransferases *EZH2* and *G9a* have both been found to be oncogenes.<sup>17</sup>

To link anomalous epigenetic modifications to estrogen-mediated ovarian carcinogenesis, we recently developed a mathematical model of the progression of this malignancy.<sup>18</sup> 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,<sup>19,20</sup> 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.<sup>18</sup> Second, binding of E2F6 to the *miR-193a* promoter recruits the epigenetic transcriptional repressive

*EZH2*<sup>21</sup> and *DNMT3b*,<sup>22</sup> 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 (IOSE) cells, originally derived by transducing the catalytic subunit of human telomerase and the papilloma virus subunit E7 into primary ovarian epithelial cells,<sup>23</sup> 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,  $1 \times 10^6$  cells were seeded into 90-mm plates and treated with 0.5  $\mu$ M of the DNA-demethylating agent, 5'-aza-2'-deoxycytidine (5azaDC; Sigma) for 72 hours, the *EZH2* inhibitor GSK126 (10  $\mu$ M; Cayman Chemicals, Ann Arbor, MI, USA), the *EZH2* inhibitor GSK343 (10  $\mu$ M; Sigma) for 72 hours, and/or the histone deacetylase inhibitor trichostatin A (TSA, 0.5  $\mu$ M; Sigma), for 12 hours. For 5azaDC, 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  $\mu\text{m}$  pore size; Merck Millipore, Burlington, MA, USA) were first coated with 25  $\mu\text{L}$  Matrigel (BD Biosciences, San Jose, CA, USA). Cells ( $2 \times 10^4$ ) 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  $1 \times$  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 (CB17/1cr-Prkdc<sup>scid</sup>/1cr1coCrIBltw) were obtained from the Taiwan National Laboratory Animal Center (Taipei, Taiwan) or BioLASCO 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 ( $1 \times 10^6$ ) 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 ( $L \times W^2/2$ ). For i.p. tumor growth,  $1 \times 10^6$  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 bisulfite pyrosequencing

Genomic DNA (0.5  $\mu\text{g}$ ) was bisulfite-modified using EZ DNA Methylation Kits (Zymo Research, Orange, CA, USA), according to the manufacturer's protocol. For combined bisulfite restriction analysis assays, bisulfite-modified DNA was subjected to PCR amplification, and 8  $\mu\text{L}$  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 bisulfite pyrosequencing analysis, bisulfite-modified DNA was subjected to a PCR amplification strategy using a tailed reverse primer in combination with a biotin-labeled universal primer, as previously described.<sup>16</sup> 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](http://diana.imis.athena-innovation.gr)), and miRanda ([www.microrna.org/microrna/home.do](http://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.

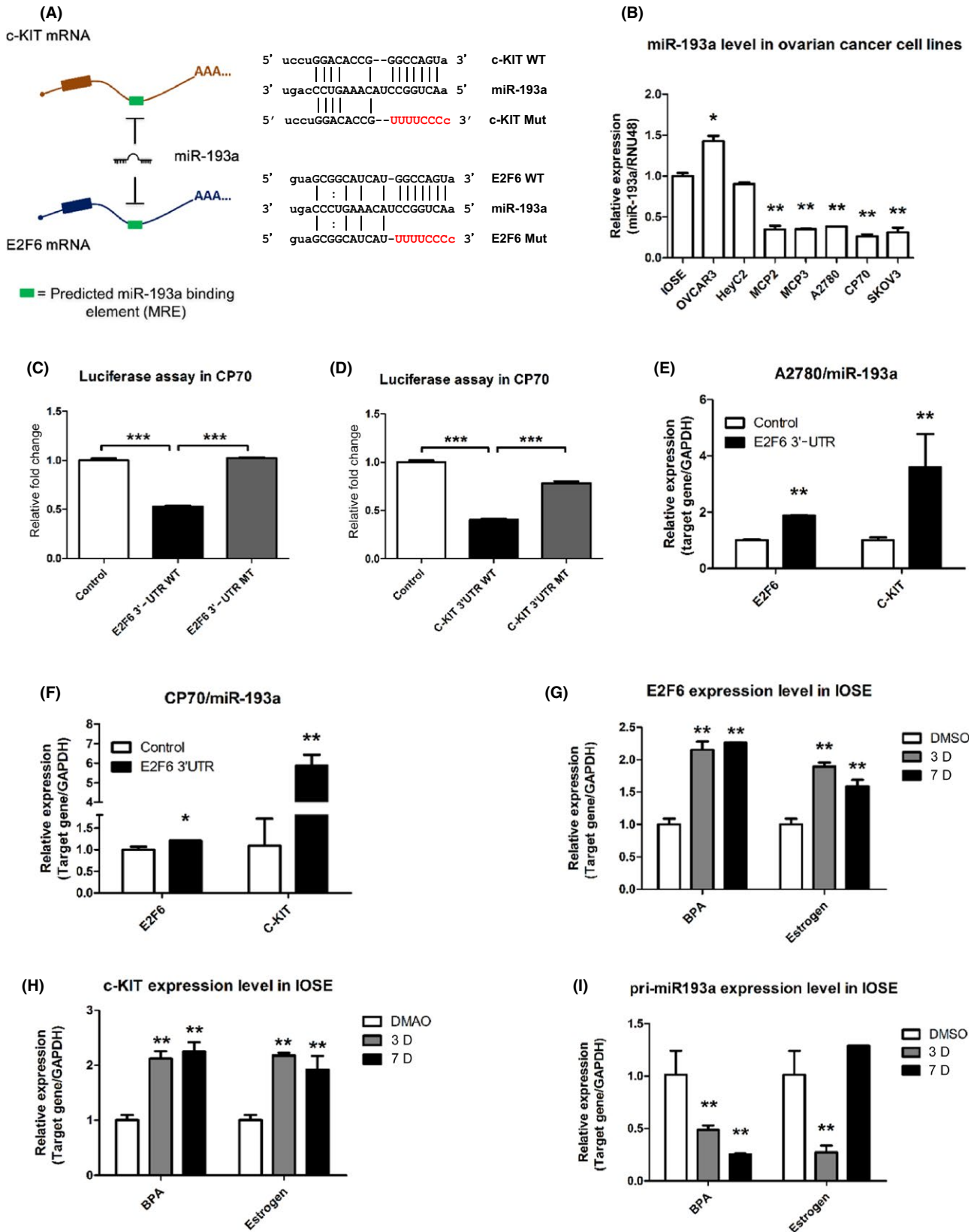
Additional Material and Methods can be found in Document S1.

## 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.<sup>18</sup> 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*.

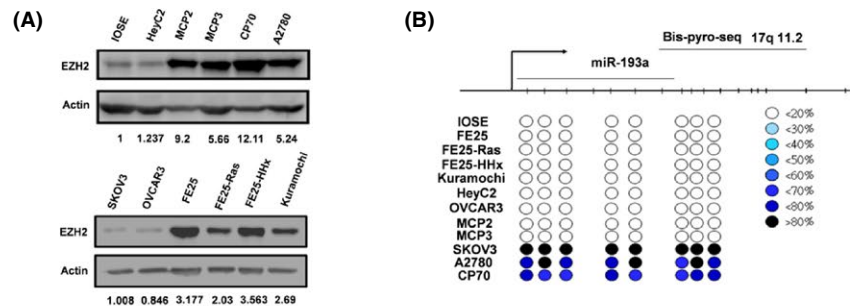
Previously, we posited that *E2F6* is an estrogen receptor (ER) target gene, such that estrogen-mediated upregulation of *E2F6* might eventually upregulate *c-KIT* through a ceRNA mechanism.<sup>18</sup> This



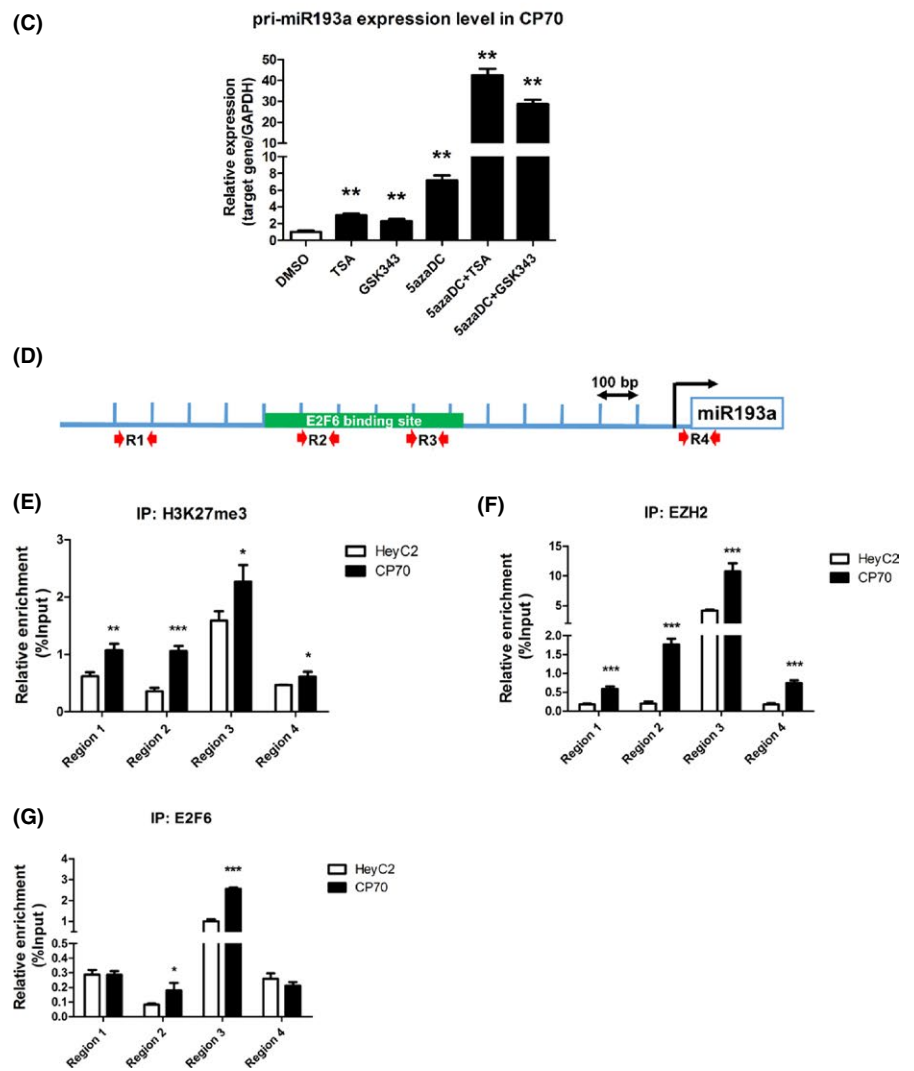
model was confirmed, as overexpression of *E2F6-3'-UTR* in *miR-193a*-overexpressing A2780 and CP70 ovarian cancer cells upregulated both *E2F6* and *c-KIT* (Figure 1E,F; Figure S2C,D). Conversely, overexpressed *c-KIT-3'-UTR* in *miR-193a*-overexpressing HeyC2

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.

**FIGURE 1** Estrogen receptor signaling leads to upregulation of *E2F6* and *c-KIT* through a competing endogenous RNA mechanism. A, Schematic diagram of *E2F6* and *c-KIT* mRNAs showing microRNA (miR)-193a-binding sites (green, left panel), and their mutated sequences (red, right panel), in their respective 3'-UTRs. B, Expression of mature miR-193a in immortalized ovarian surface epithelial (IOSE) and a panel of ovarian cancer cell lines, with RNU48, a small nucleolar RNA, as an internal control. C, D, Reduced luciferase activities confirmed that miR-193a binds to the *E2F6* and *c-KIT* 3'-UTRs in CP70 ovarian cancer cells. E, F, Transfection of the *E2F6*-3'-UTR, including the miR-193a-binding site, into A2780/miR-193a or CP70/miR-193a ovarian cancer cells leads to upregulation of *E2F6* and *c-KIT* mRNA. G–I, Treatment with bisphenol-A (BPA) or estrogen (E2) increases expression of (G) *E2F6* and (H) *c-KIT*, but (I) decreases pri-miR193a expression, in IOSE cells, after 3–7 days (\* $P < .05$ ; \*\* $P < .01$ ). Each bar represents the mean  $\pm$  SD (error bar) of duplicate experiments. GAPDH, internal control



**FIGURE 2** *miR-193a* is epigenetically silenced by DNA methylation and repressive histone modifications in ovarian cancer cell lines. A, EZH2 protein levels in immortalized ovarian surface epithelial (IOSE), fallopian tube epithelial (FE-25), and various ovarian cancer cell lines, as determined by western blot analysis. B, *miR-193a* promoter methylation in various IOSE, untransfected or oncogene-transfected FE-25, and ovarian cancer cell lines, as determined by bisulfite pyrosequencing (Bis-pyro-seq). C, Treatment with the DNA methyltransferase inhibitor 5'-aza-2'-deoxycytidine (5zaDC), histone deacetylase inhibitor trichostatin A (TSA), or EZH2 inhibitor (GSK343), alone or in combination, restored pri-*miR-193a* expression in CP70 ovarian cancer cells. D, Schematic diagram depicting the positions of putative *E2F6* binding sites with respect to the transcriptional start site of *miR-193a*. Red arrows indicate the regions (R1–R4) for ChIP-PCR amplification. E–G, Enrichment of (E) H3K27me3, (F) EZH2, and (G) *E2F6* at the indicated regions (R1–R4) within the *miR-193a* promoter in CP70 and HeyC2 ovarian cancer cells, as determined by ChIP-PCR. CP70 cells possessed more repressive marks (and more *E2F6* and EZH2 protein) in the *miR-193a* promoter regions compared to HeyC2 cells. IP, immunoprecipitant



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.<sup>24,25</sup> 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.<sup>18</sup>

### 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 occupation by E2F6, in association with Polycomb repressive complex-2 (PRC2).<sup>18</sup> 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.<sup>22</sup> 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.<sup>26,27</sup> 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).

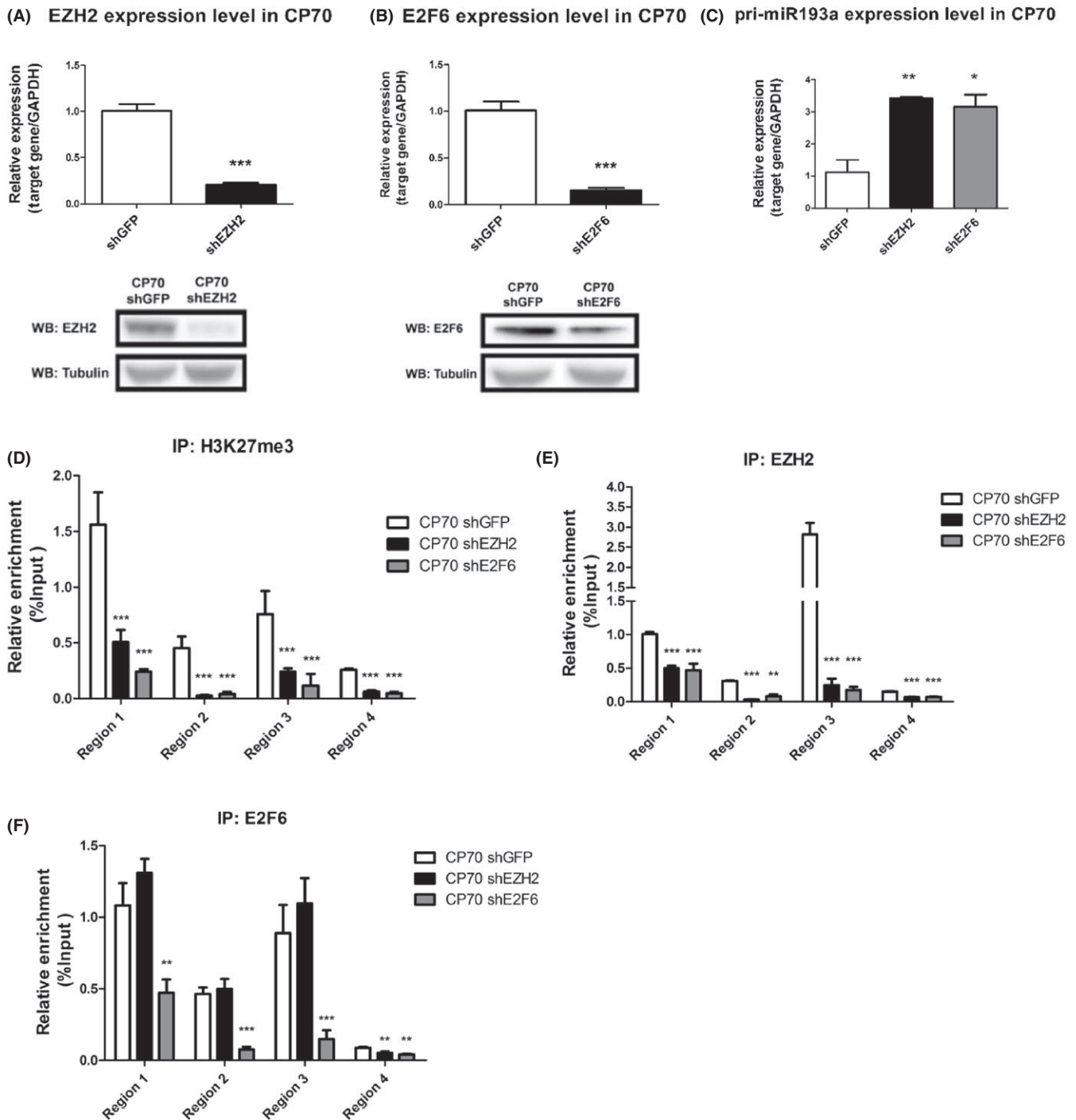
Finally, mechanistic assessments of ceRNA downregulation of *miR-193a* showed that overexpression of the *E2F6*-3'-UTR, containing a wild-type *miR-193a* MRE, but not an MRE mutant, significantly increased CP70 cell colony numbers (Figure S4F). Together, these results strongly suggest a tumor suppressor 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.<sup>28,29</sup> 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

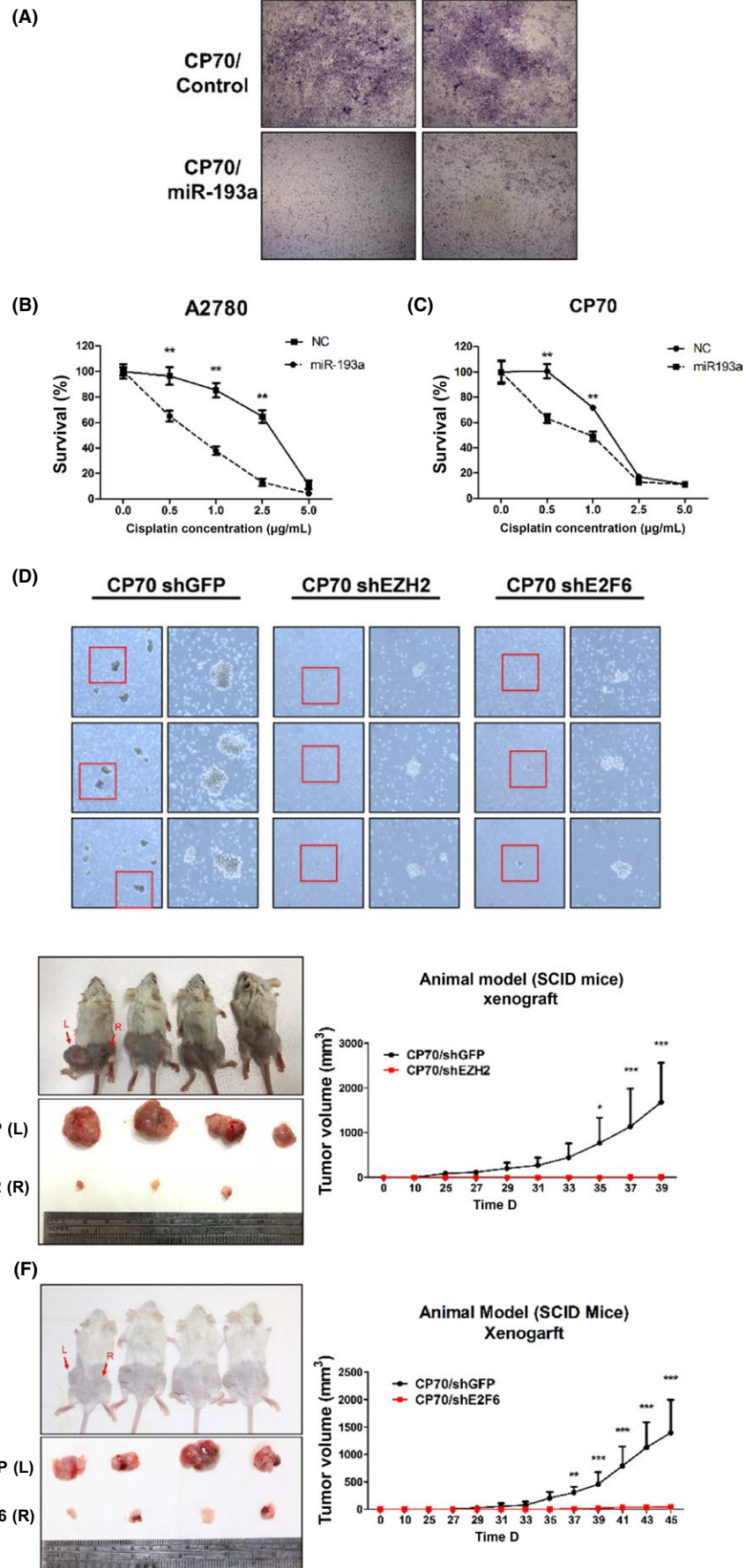


**FIGURE 3** A-C, RT-PCR shows that CP70 cells depleted of (A) EZH2 or (B) E2F6 restored (C) pri-miR-193a expression. D-F, ChIP-quantitative PCR showing enrichment of (D) H3K27me3, (E) EZH2, and (F) E2F6 proteins within the *miR-193a* promoter in CP70 cells depleted of EZH2 or E2F6. (\* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .005$ ). E2F6 depletion resulted in decreased occupancy of EZH2 and its product, H3K27me3. Each bar represents the mean  $\pm$  SD of duplicate experiments. Tubulin, western blot control. IP, immunoprecipitant

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.<sup>18</sup> Taken together, these results indicate that *miR-193a* is epigenetically silenced in ovarian cancer, and the *miR-193a* targets, *E2F6* and *c-KIT*, show a ceRNA relationship.

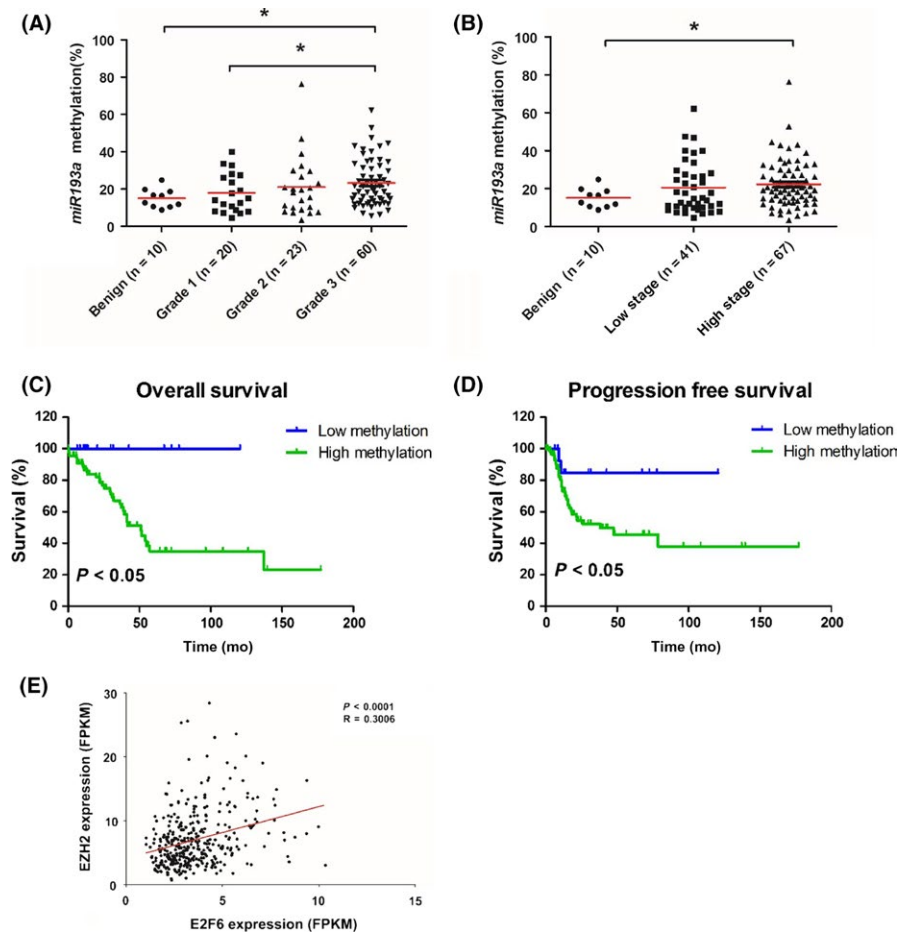
## 4 | DISCUSSION

Although several studies now suggest that activation of the estrogen receptor (ER)- $\alpha$  signaling pathway is intimately involved in ovarian carcinogenesis, its precise role remains controversial. For example, epidemiological studies found that women treated with hormone

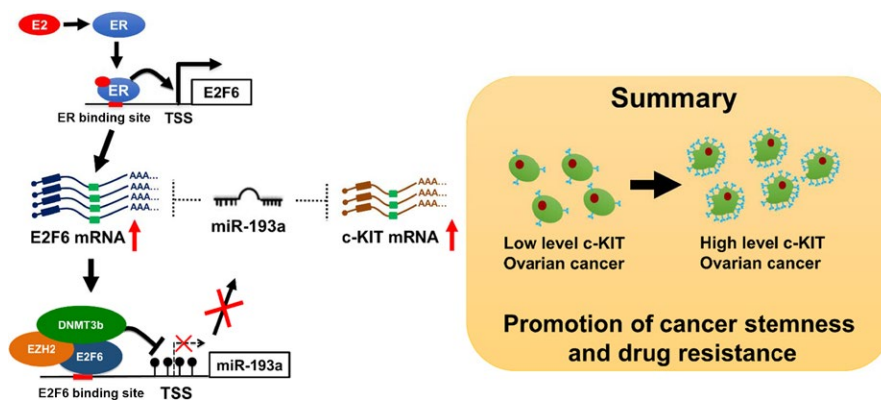




**FIGURE 4** Role of microRNA (miR)-193a as a tumor suppressor in ovarian cancer. A, Overexpression of miR-193a in CP70 cells decreased cell invasion and enhanced chemosensitivity to cisplatin in (B) A2780 and (C) CP70 cells. NC, normal control. D, Depletion of either EZH2 or E2F6 reduced sphere formation 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  $\pm$  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.<sup>30,31</sup> However, antiestrogen therapy was only somewhat effective for a subset of ovarian cancer patients,<sup>10,32</sup> regardless of ER- $\alpha$  expression status.<sup>31,33</sup>

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.<sup>18</sup> 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.<sup>19,20</sup> 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”.<sup>5,34</sup>

To confirm this mathematic model, in this study, we undertook biological experiments to show that E2F6 is upregulated following treatment with E2 or BPA (a “xenoestrogen”)<sup>25</sup> 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- $\alpha$  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.<sup>21,35</sup> This finding agrees with previous reports that, for genes other than *miR-193a*, E2F6's transcriptional repressive activity requires EZH2 and a DNMT.<sup>22</sup> 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,<sup>36</sup> and “side population” cell formation,<sup>37</sup> 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,<sup>19,20</sup> depending on EZH2 expression. E2F6 upregulation 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.<sup>38</sup> However, it is also noteworthy to point out that EZH2 has recently been found to be an important factor in promoting T-cell differentiation.<sup>39</sup> 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.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ORCID

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

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