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Supplemental information

MicroRNA-dependent inhibition of WEE1 controls cancer stem-like characteristics and malignant behavior in ovarian cancer

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Supplementary Material

Materials and methods

Lentivirus production and generation of stable cells

Lenti-miR microRNA precursors for hsa-miR-424 and hsa-miR-503 (System Biosciences, Palo Alto, CA, USA) were used along with a GFP control lentivirus. The Lenti-X HTX Packaging System (Clontech, Mountain View, CA, USA) was used to generate the lentiviral particles. HEK293T cells were transfected with lentiviral constructs using the FuGene HD Transfection Reagent (Promega, Madison, WI, USA) and incubated for 24 h. The viral particles in supernatants were harvested on ice, and the titer was measured using a qPCR Lentivirus Titration Kit (Applied Biological Materials, Richmond, BC, Canada). To generate the miR-424/503 overexpression stable cell line, SKOV3 cells were transfected with lenti-miR miRNA precursors for hsa-miR-424/503 and control, after which they were selected with puromycin (1 μg/mL, Sigma- Aldrich, St. Louis, MO, USA). The stable overexpression of miR-424/503 was confirmed through quantitative real-time PCR and fluorescence microscopy.

Proliferation assay

Cells were seeded at 1×10^4 cells/well in 96-well plates and treated with atorvastatin for 24 h in the monolayer state, and the spheroids were treated with atorvastatin in poly-HEMA-coated 96-well plates for 3 days. Cytotoxicity was measured using the EZ-Cytox WST kit (Daeil Lab Service, Seoul, Korea) according to the manufacturer's instructions. After adding 10 μL of the WST-1 reagent, the cells were incubated for 30 min, following which the absorbance was measured at 450 nm using a microplate reader.

Luciferase reporter assay

The pLightSwitch_WEE1 3′ UTR vector including Human WEE1 3′ UTR was purchased from SwitchGear Genomics (Carlsbad, CA, USA). The seed sequence of the WEE1 gene, TGCTGCTA, was mutated to CGCCGCCA using the Muta-DirectTM Site Directed Mutagenesis Kit (iNtRON Biotechnology, Seongnam, Korea). SKOV3 cells were transfected with the luciferase reporter constructs (containing either the wild-type or mutant WEE1 3′ UTR) and miRNAs (either pre-miR-424/503 mimics or negative control) using Lipofectamine 2000 (Invitrogen). After incubation for 48 h, the cells were lysed using a passive lysis buffer, and luciferase activity was measured using the Dual Luciferase® Reporter Assay System (Promega). The human miR-424/503 promoter luciferase construct was previously described.[1, 2] The SKOV3 cells were transfected with miR-424/503 promoter luciferase construct, renilla luciferase, and NANOG constructs. Luciferase activity was quantified using the Dual-Luciferase Reporter Assay kit (Promega).

Migration assay

Cells were infected with lentiviral miR-424/503 and control GFP and incubated for 24 h. The infected cells were seeded at 2×10^5 cells/well in 12-well plates. The cells were scratched using a 200 μL pipette tip and incubated for 12 h in a starvation medium supplemented with 1% FBS. The cells were observed under an optical microscope at 40× magnification. The differences in the width of the gaps were calculated using the ImageJ software.

Colony formation assay

For the colony formation assay, 1×10^3 cells were plated in 6-well plates with complete medium to culture for 2 weeks after transfection with miRNAs. After growth in the plates for 14 days, the colonies were washed with PBS buffer and stained with 0.5% crystal violet in 20% methanol

for 20 min, after which the number of colonies was determined.

In vitro limiting dilution assay

Cells were seeded in poly-HEMA coated 96-well plates at various concentrations (1–128 cells/well). DMSO and 10 μM atorvastatin were administered simultaneously with seeding. After incubation for 7 days, the number of wells without spheres at each seeding density was determined and plotted against the number of cells/well.

Flow cytometry analysis

For the analysis of individual cells, the spheroids were dissociated using a Cell Dissociation Buffer (Thermo Scientific). The number of cells exhibiting aldehyde dehydrogenase (ALDH) enzyme activity was measured using an Aldefluor kit (Stem Cell Technologies, Vancouver, Canada). For this, 1×10^6 cells were suspended in 1 mL of the ALDEFLOR assay buffer containing the ALDH substrate (BODIPY aminoacetaldehyde), and as negative controls, aliquots of each sample were treated with diethylaminobenzaldehyde, a specific ALDH inhibitor. Samples were incubated for 45 min at 37ºC before analysis. To detect the CSC marker CD133, the cells were labeled with the CD133-PE antibody (1:300, 130-080-801, Miltenyi Biotec, Bergisch Gladbach, Germany) for 1 h in PBS containing 0.1% BSA. All assessments were performed using an FACS CantoII flow cytometer, and the data were analyzed using the FlowJo software.

Bioinformatics analysis

Public data was downloaded from GEO (https://www.ncbi.nlm.nih.gov/geo/) and re-analyzed to validate inverse and/or positive correlation patterns between the expression levels of genes (GSE30161). Correlation was confirmed with correlation coefficiency values and linear

regression. The correlation coefficient between variables was calculated by the Pearson` method of cor() function and the linear regression model was calculated with the lm() function in R language. Progressive-free survival (PFS) and overall survival (OS) analyses were performed using Kaplan-Meier Plotter (Lanczky A, Gyorffy B: Web-Based Survival Analysis Tool Tailored for Medical Research (KMplot).[3]

Supplementary Table

Table S1. List of antibodies used

Application	Antibody	Raised	Manufacturer	Catalog number	Dilution
WB	AGO2	Mouse	Abcam	ab27113	1:1000
WB	Caspase 3	Rabbit	Cell Signaling	#9662	1:1000
WB	GAPDH	Rabbit	Cell Signaling	#2118	1:4000
WB	NANOG	Rabbit	Cell Signaling	#4903	1:1000
WB	WEE1	Rabbit	Cell Signaling	#4936	1:1000
WB	Anti-rabbit IgG-HRP	Goat	Cell Signaling	#7074	1:4000
WB	Anti-mouse IgG-HRP	Goat	Thermo Fisher Scientific	31430	1:4000
FACS	CD133-PE	Mouse	Miltenyi Biotec	130-080-801	1:300
IHC	WEE1	Mouse	Santa Cruz Biotechnology	sc-5285	1:100
IF	DAPI		Thermo Fisher Scientific	62248	1:1000
IF	ALDH1-Alexa 594		Santa Cruz Biotechnology	sc-166362 AF594	1:100
IF	WEE1-Alexa 488		Santa Cruz Biotechnology	sc-5285 AF488	1:100

Supplementary Figures

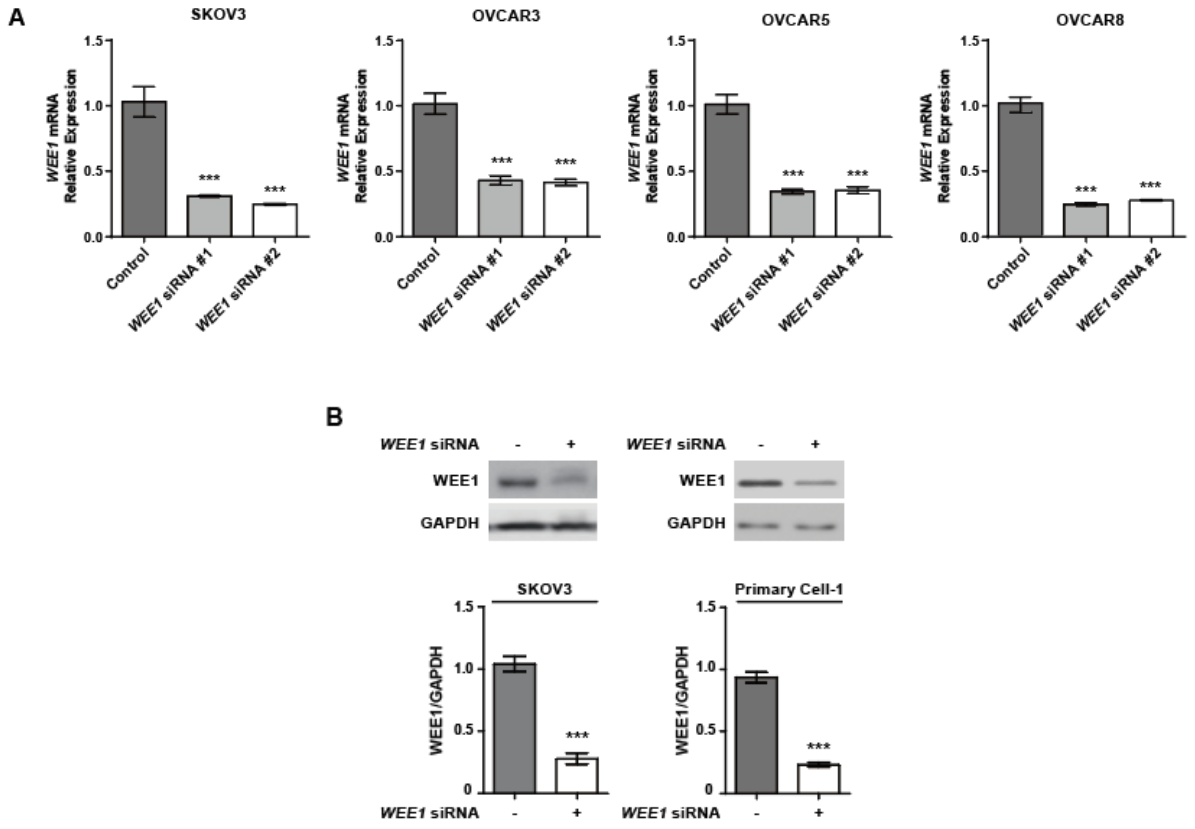

Figure S1. Knockdown efficacy of WEE1 in ovarian cancer cells.

(A) qRT-PCR showing *WEE1* mRNA expression after transfection with two distinct siRNAs (B) Western blot showing WEE1 protein levels. ***$P < 0.001$ by Student's *t*-test.

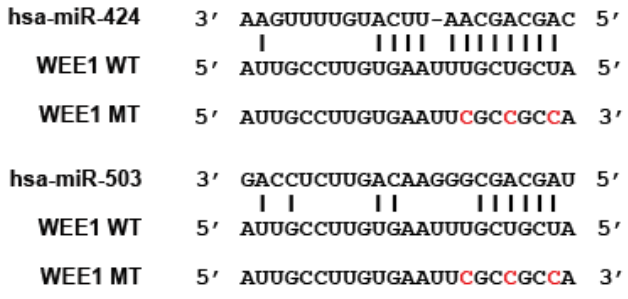

Figure S2. Predicted sequences of the WEE1 3′-UTR targeted by miR-424 and miR-503 and mutated sequences for disruption of the miR-424 and miR-503 binding sequence.

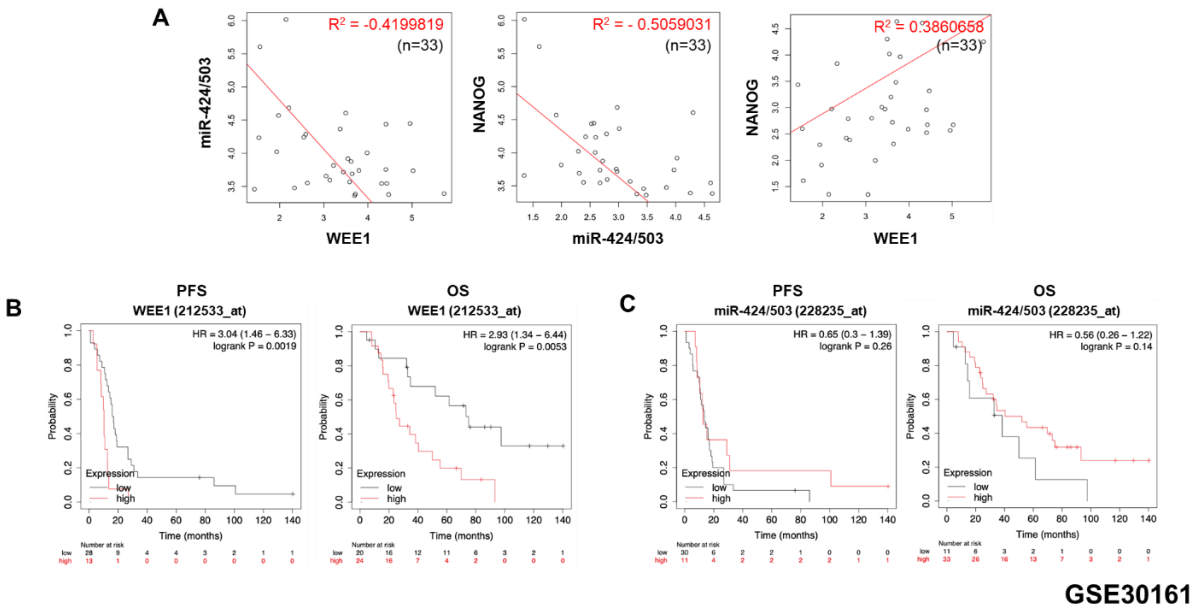

Figure S3. Relationship between miR-424/520, WEE1, and NANOG and patient outcomes in the GSE30161 dataset.

(A) Correlation analysis on the expression of miR-424/503, WEE1 and NANOG in patients with ovarian cancer. As shown, miR-424/503 had a strong inverse correlation with both WEE1 and NANOG, whereas WEE1 and NANOG had a significant positive correlation. (B) Kaplan-Meier survival curves for high and low expression of WEE1 in patients with ovarian cancer. WEE1 high expression is associated with poor PFS and OS survival. (C) Kaplan-Meier survival curves of the high and low expression of miR-424/503 in patients with ovarian cancer. Higher level of miR-424 and miR-503 expression revealed a trend in favor of PFS and OS survival. PFS; Progression-free survival, OS; Overall survival.

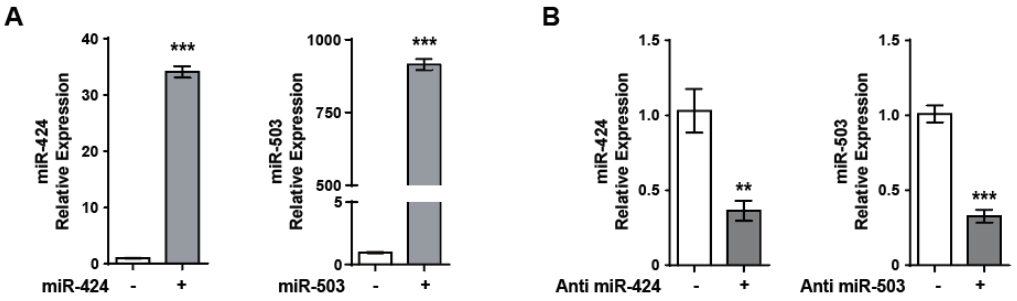

Figure S4. Overexpression knockdown efficacy of miR-424/503 in ovarian cancer cells.

(A) Levels of miRNA achieved with overexpression in ovarian cancer cells. (B) Levels of miRNA achieved with knockdown in ovarian cancer cells. $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test.

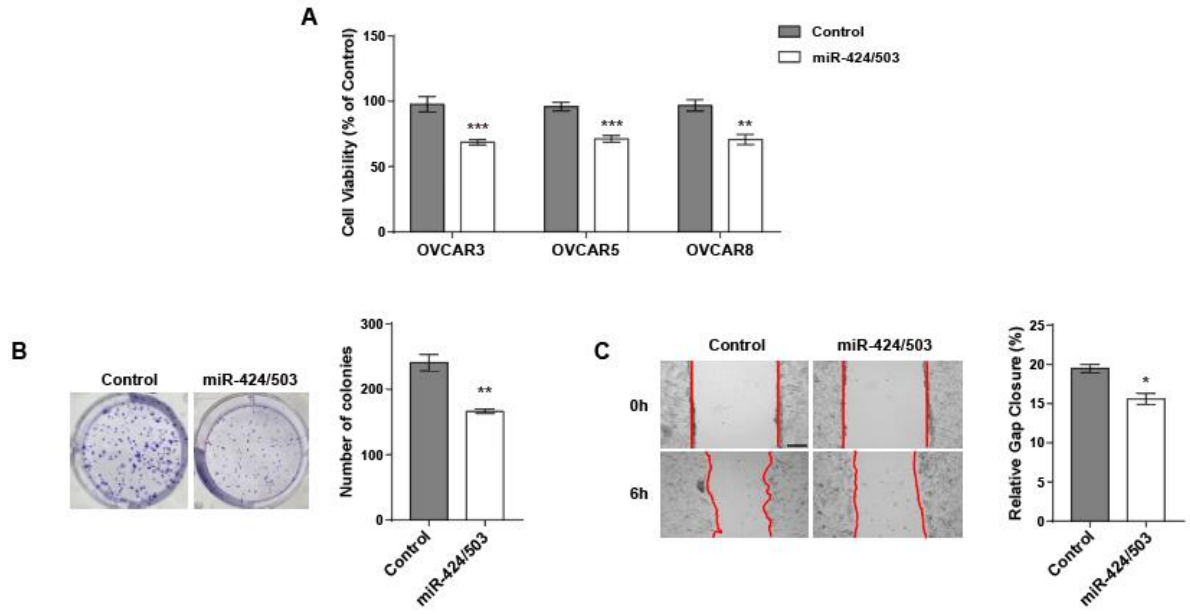

Figure S5. Cell viability, migration and colony assay after the overexpression of miR-424 and miR-503 in adherent OVCAR3, OVCAR5 and OVCAR8 cells.

(A) Cell viability after the overexpression of miR-424 and miR-503 in adherent OVCAR3, OVCAR5 and OVCAR8 cells. (B) Colony formation was determined using crystal violet staining 14 days after the miR-424 and miR-503 overexpression in adherent OVCAR8 cells. (C) Migration assay after miR-424/503 overexpression in adherent OVCAR8 cells. Scale bar: 200 μm. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by Student's *t*-test.

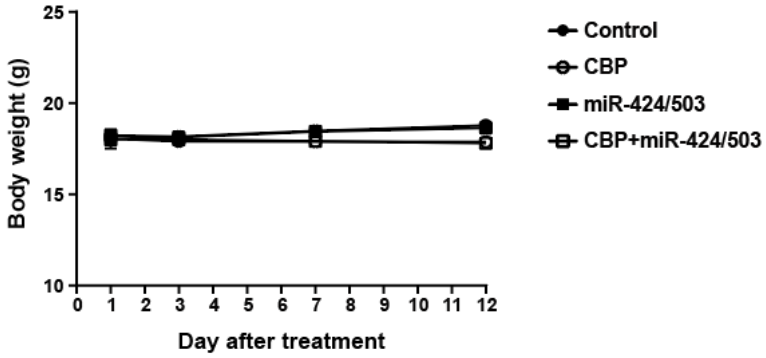

Figure S6. The effect of CBP and miR-424/503 on the body weight of SKOV3 xenograft mice.

No significant changes were observed in body weights among the different groups.

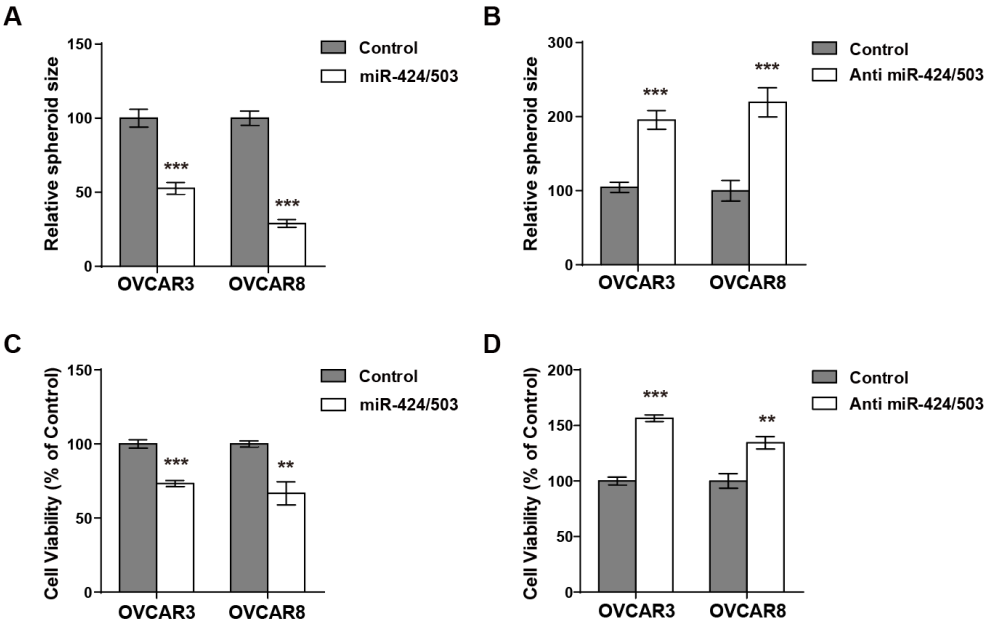

Figure S7. Cancer stem cell-like properties following the overexpression or inhibition of miR-424/503 in OVCAR3 and OVCAR8 cells.

The sizes of the spheroids after the overexpression (A) or inhibition (B) of miR-424/503 in OVCAR3 and OVCAR8 cells. Cell viability after the overexpression (C) or inhibition (D) of miR-424/503 in OVCAR3 and OVCAR8 spheroids. $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test.

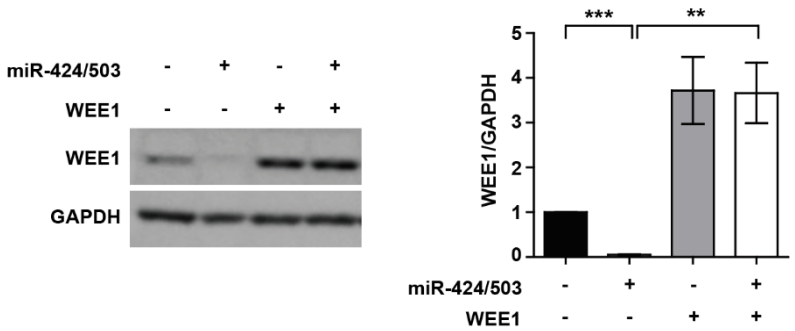

Figure S8. WEE1 protein expression levels after concurrent miR-424/503 and WEE1 overexpression. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to controls by unpaired two-tailed Student's *t-test* or one-way ANOVA with Bonferroni's multiple comparison test. Error bars, standard error of the mean.

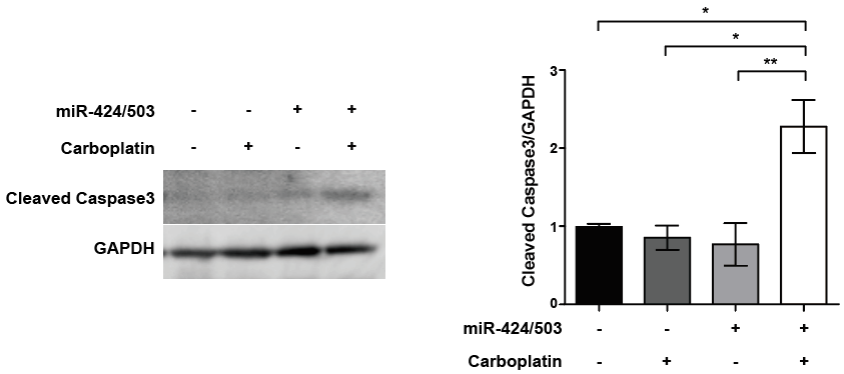

Figure S9. Increased caspase-3 cleavage in response to miR-424/503 overexpression upon carboplatin treatment of SKOV3 spheroids.

*P < 0.05, **P < 0.01 by one-way ANOVA, with Bonferroni *post-hoc* test.

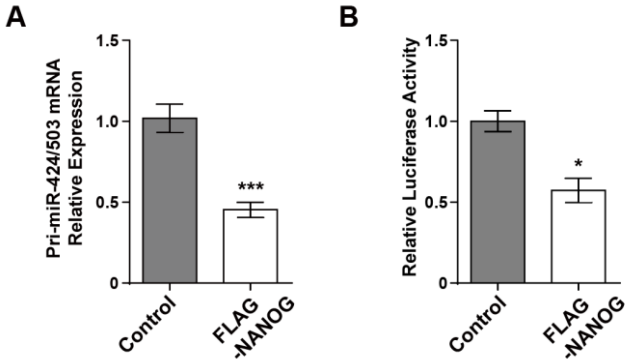

Figure S10. NANOG regulates miR-424/503 expression.

(A) Pri-forms of miR-424 and miR-503 expression in response to NANOG overexpression. (B) Relative luciferase activity of SKOV3 cells co-transfected with miR-424/503 promoter luciferase construct and NANOG. *P < 0.05, ***P < 0.001 by Student's t-test.

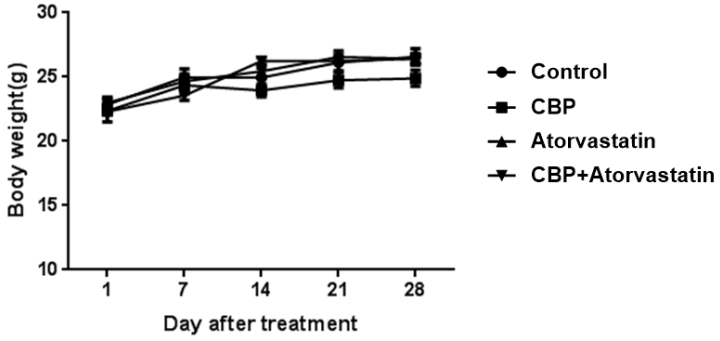

Figure S11. The effect of CBP and atorvastatin on the body weight of SKOV3 xenograft mice.

No significant changes were observed in body weights among the different groups.

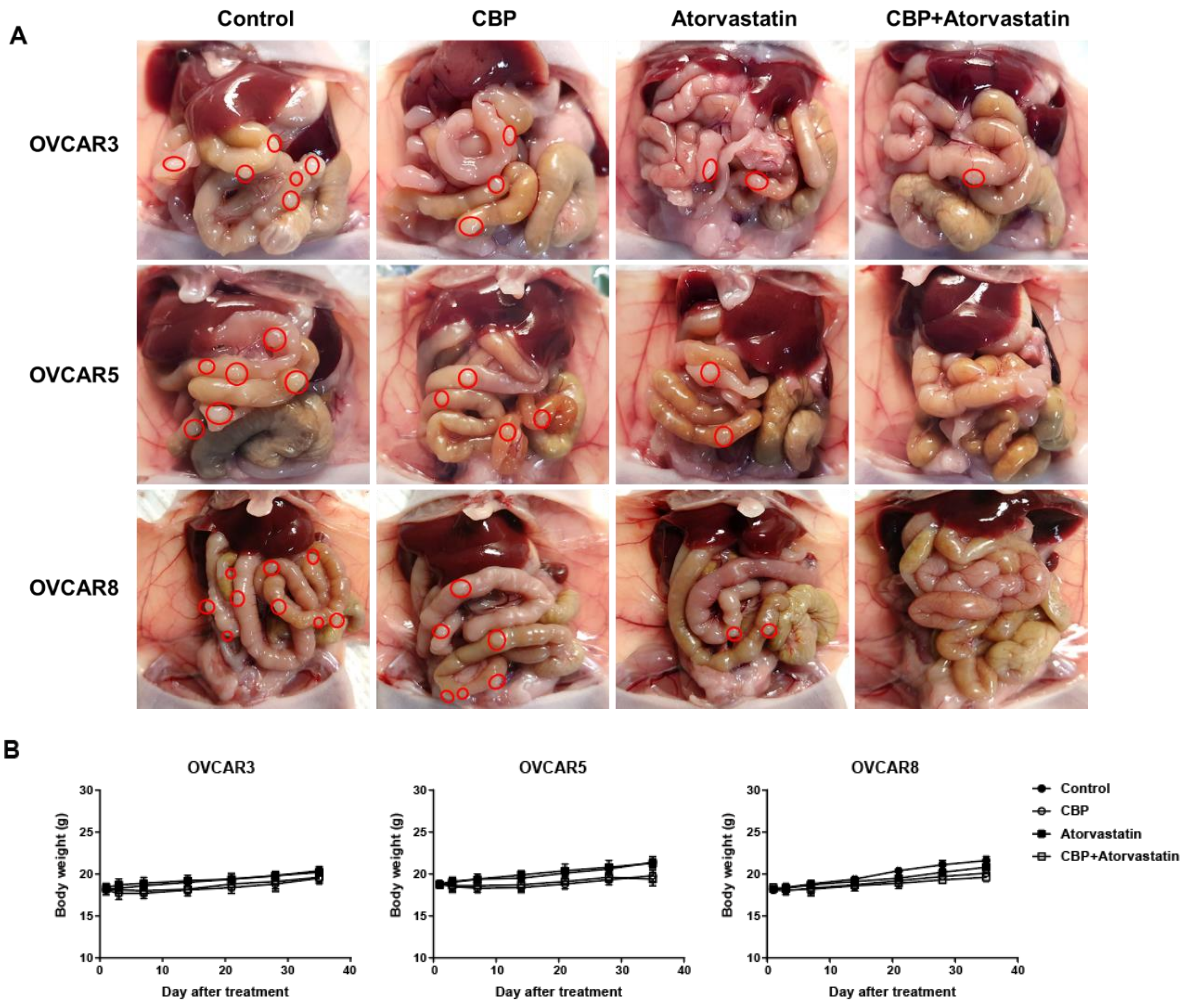

Figure S12. Combined treatment with carboplatin and atorvastatin inhibited ovarian cancer in the OVCAR3, OVCAR5 and OVCAR8 xenograft mouse model.

(A) Representative images of the peritoneal cavities of mice showing reduction in peritoneal tumors in the CBP, atorvastatin, and atorvastatin/CBP groups compared to that in the control group. (B) The effect of CBP and atorvastatin on the body weight of OVCAR3, OVCAR5 and OVCAR8 xenograft mice. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by one-way analysis of variance (ANOVA), with Bonferroni *post-hoc* test.

References

1. Kim, J, Kang, Y, Kojima, Y, Lighthouse, JK, Hu, X, Aldred, MA, McLean, DL, Park, H, Comhair, SA, Greif, DM, *et al.* (2013). An endothelial apelin-FGF link mediated by miR-424 and miR-503 is disrupted in pulmonary arterial hypertension. *Nat Med* **19**: 74-82.
2. Lee, A, Papangeli, I, Park, Y, Jeong, HN, Choi, J, Kang, H, Jo, HN, Kim, J, and Chun, HJ (2017). A PPARgamma-dependent miR-424/503-CD40 axis regulates inflammation mediated angiogenesis. *Sci Rep* **7**: 2528.
3. Lanczky, A, and Gyorffy, B (2021). Web-Based Survival Analysis Tool Tailored for Medical Research (KMplot): Development and Implementation. *J Med Internet Res* **23**: e27633.