Methylation-associated silencing of miR-638 promotes endometrial carcinoma progression by targeting MEF2C

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Abstract. Promoter methylation-associated silencing of cancer-associated microRNAs (miRNAs) is a common epigenetic mechanism during tumorigenesis in various types of human cancer. However, this has not been comprehensively examined in endometrial carcinoma (EC). In the present study, an miRNA microarray consisting of 1,347 common human miRNAs was used to select potential tumor suppressive miRNAs that were hyper-methylated in EC. This led to the identification of miR-638, miR-210 and miR-3665. The methylation status of miR-638 was examined by bisulfite sequencing polymerase chain reaction and miR-638 expression was measured by TaqMan miRNA assays. EC cell lines transfected with vectors overexpressing miR-638, its target gene myocyte enhancer factor 2C (MEF2C) or both, were constructed. Dual-luciferase reporter assays, a xenograft mouse model and rescue experiments were designed to study miR-638 and its target gene MEF2C. The results indicated that the promoter region of miR-638 was highly methylated and the expression of miR-638 was significantly downregulated in cancerous tissues from 42 patients with EC who underwent surgical resection. Additionally, a low expression of miR-638 was significantly associated with advanced Federation of Gynecology and Obstetrics stage and was demonstrated to indicate shorter disease-free survival. Functional studies indicated that the overexpression of miR-638 in EC cell lines inhibited *in vitro* tumor progression and *in vivo* tumorigenicity. MEF2C was verified as a direct target of miR-638 and was demonstrated to mediate the tumor-suppressive function of miR-638 in EC.

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Introduction

The epigenetic regulation of cancer-associated microRNAs (miRNAs) was reported to be indispensable during tumor initiation and cancer progression (1,2). In terms of tumor-suppressive miRNAs, aberrant methylation in the CpG islands of their promoters, was demonstrated to be one of the most common epigenetic mechanisms that modulated their expression and biological function (2). A number of miRNAs, including miR-132, miR-148a, miR-107, miR-181 and miR-34, have been identified to be epigenetically silenced by the hypermethylation of CpG islands in/near the promoter region (3-5). However, miRNA methylation status in endometrial carcinoma (EC) has not been comprehensively investigated.

miR-638, a newly characterized cancer-associated miRNA, is differentially expressed in various types of human cancer and participates in a number of biological processes during tumorigenesis (6-9). However, the data concerning its exact biological roles in different cancers are conflicting and poorly understood. miR-638 is upregulated and acts as a tumor promoting miRNA in prostate cancer (7), esophageal carcinoma (9), breast cancer (9) and melanoma (10). Nevertheless, miR-638 was demonstrated to be downregulated and to function as a tumor suppressive miRNA in gastric (8,11), cervical (12) and breast cancer (13), and hepatocellular carcinoma (14). The methylation status, expression level and biological roles of miR-638 have not been investigated in EC.

The present study identified potential anti-tumor miRNAs that were significantly downregulated by promoter methylation using a microarray, among which miR-638 was selected to be further investigated. The methylation status, expression level, biological function, and molecular targets of miR-638 were examined. The aim of the present study was to comprehensively identify potential promoter methylation-silenced cancer-associated miRNAs in EC and to specifically study the regulatory mechanisms of miR-638 in EC in depth.

Materials and methods

Cell culture and demethylation treatment. The human EC KLE and Ishikawa (ISH) cell lines, which have already been authenticated, were purchased from the American Type Culture Collection, which had already been authenticated. Both cell lines were maintained in DMEM medium. For DNA demethylation treatment, EC cell lines were treated with 3 μM 5-aza-2-deoxycytidine for 72 h at 37˚C.

Clinical samples. A total of 68 consecutive paired EC tissues with cancerous and non-cancerous tissues were obtained from patients who underwent primary resection without neoadjuvant therapy at the Fudan University Shanghai Cancer Center (FUSCC) between January 2014 and April 2014 and who were pathologically diagnosed with endometrioid adenocarcinoma or serous carcinoma of the endometrium. All specimens were frozen in liquid nitrogen immediately following surgical removal and were then stored at -80˚C. Subsequently, tissue samples from 42 patients were randomly selected for further research. Informed consent was obtained from the patients and the study was approved by the Committee for the Ethical Review of Research at FUSCC. The characteristics of the patients are summarized in Table SI.

RNA isolation, miRNA microarray analysis and semi-quantitative polymerase chain reaction (PCR). Total RNA was extracted from freshly-frozen tissue samples and cell lines using TRIzol® reagent (Thermo Fisher Scientific, Inc.). miRNAs were isolated using an mirVana™ miRNA isolation kit (Thermo Fisher Scientific, Inc.). For the microarray analysis, purified total RNAs from EC cells lines treated with 5-aza-2-deoxycytidine or saline, and then hybridized with a microchip using an miRNA Complete Labeling and Hyb kit (Agilent Technologies, Inc.). The signals were then detected using the Agilent Microarray Scanner and the images were analyzed using the Agilent Feature Extraction Software version 10.5.1.1 (Agilent Technologies, Inc.). Significantly upregulated miRNAs following 5-aza-2-deoxycytidine treatment were identified. TaqMan miRNA assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used to quantify the relative expression of miR-638. Semi-quantitative PCR with SYBR green I was used to compare the relative expression of specific mRNAs using a SYBR® Premix Ex Taq™ kit (Takara Biotechnology Co., Ltd.). The thermocycling conditions for miR-638 were as follows: An initial step at 95˚C for 15 sec followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. Amplified fragments were then detected on 4% agarose gel electrophoresis containing ethidium bromide using a ChemiDoc XRS system and Quantity One 1.0 software (Bio-Rad Laboratories, Inc.). The primers used are listed in Table SII.

DNA methylation analysis. Genomic DNA was extracted from patient tissues and cell lines using the QIAamp DNA formalin-fixed paraffin-embedded Tissue kit (Qiagen, Inc.). Sodium bisulfite modification of DNA was performed using the EpiTect Bisulfite kit (Qiagen, Inc.). PCR products were recovered using Qiagen gel DNA kits (Qiagen, Inc.) and then sequenced at GeneTech (Shanghai) Co., Ltd. The primer sequences are listed in Table SII.

Vector construction and cell transfection. miR-638 mimic (5'-AGGGATCGCGGGCGGGTGGCGGCCT-3'), miR-638 mimic negative control (5'-AGGTACGAAACGCTAAGAAT-3'), short hairpin RNA targeting MEF2C (shMEF2C; 5'-GGACAAGGAATGGGAGGATAT-3') and shMEF2C negative control (5'-UCUCCGAUGCAGGCUCAAC-3') were synthesized by Shanghai GenePharma Co., Ltd. For transfection, 100 ng miR-638 mimic, miR-638 mimic negative control, shMEF2C or shMEF2C negative control was transfected using Lipofectamine™ 2000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). In addition, a specific vector containing the entire coding sequence of MEF2C but without its 3'-untranslated region (UTR), as well as its negative control, were designed by Shanghai GenePharma Co., Ltd. The lentiviral packing kit used to transfect the various vectors were purchased from the Open Biosystems, Inc. and transfection was performed at a final concentration of 50 nM. 293T cells were purchased from the Cell Bank of Chinese Academy of Sciences. Generally, EC cell lines with $5x10^4$ cells were infected with $1x10^8$ lentivirus-transducing units. Following transfection for 24 h, the cells were cultured with 1 μg/ml puromycin for 2 weeks, and then stably transduced EC cell lines were selected.

Cell viability, migration and invasion assays and apoptosis analysis. Cell viability was investigated using Cell Counting Kit-8 and colony formation assays. Cell migration and invasion assays were performed using Transwell cell migration assay kits and BioCoat™ Matrigel® Invasion Chambers (Corning Incorporated), respectively. For the migration assay, $5x10^4$ cells were seeded into the upper chamber and incubated for 24 h at 37˚C in 5% CO_2. For the invasion assay, $1x10^5$ cells were plated on chambers pre-coated with 1.6 mg Matrigel (Corning, Inc.) and incubated for 36 h at 37˚C in 5% CO_2. Subsequently, cells were fixed, stained with 0.5% crystal violet solution at room temperature for 10 min, photographed and counted under a light microscope (magnification, x200).

Cell apoptosis was analyzed with the Annexin V-fluorescein isothiocyanate/propidium iodide Apoptosis Detection kit. Briefly, $1x10^5$ cells were re-suspended in 500 μl binding buffer and stained with 5 μl Annexin V and 5 μl PI in the dark at room temperature for 20 min. The samples were then analyzed using a FACScan flow cytometer and the CellQuest™ Pro 1.0 software (BD Biosciences), according to the manufacturer's instructions.

Western blot analysis. Cells were harvested and lysed in ice-cold radioimmunoprecipitation assay lysis buffer. Protein concentrations were determined by the BCA Protein assay reagent and measured using a NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific, Inc.). Subsequently, 10 μg protein/lane was separated by 10% SDS-PAGE gels and then transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk for 2 h at room temperature and then incubated with primary antibodies against MEF2C (cat. no. 5030; Cell Signaling Technology, Inc.), matrix metalloproteinase (MMP)2 (cat. no. 40994;

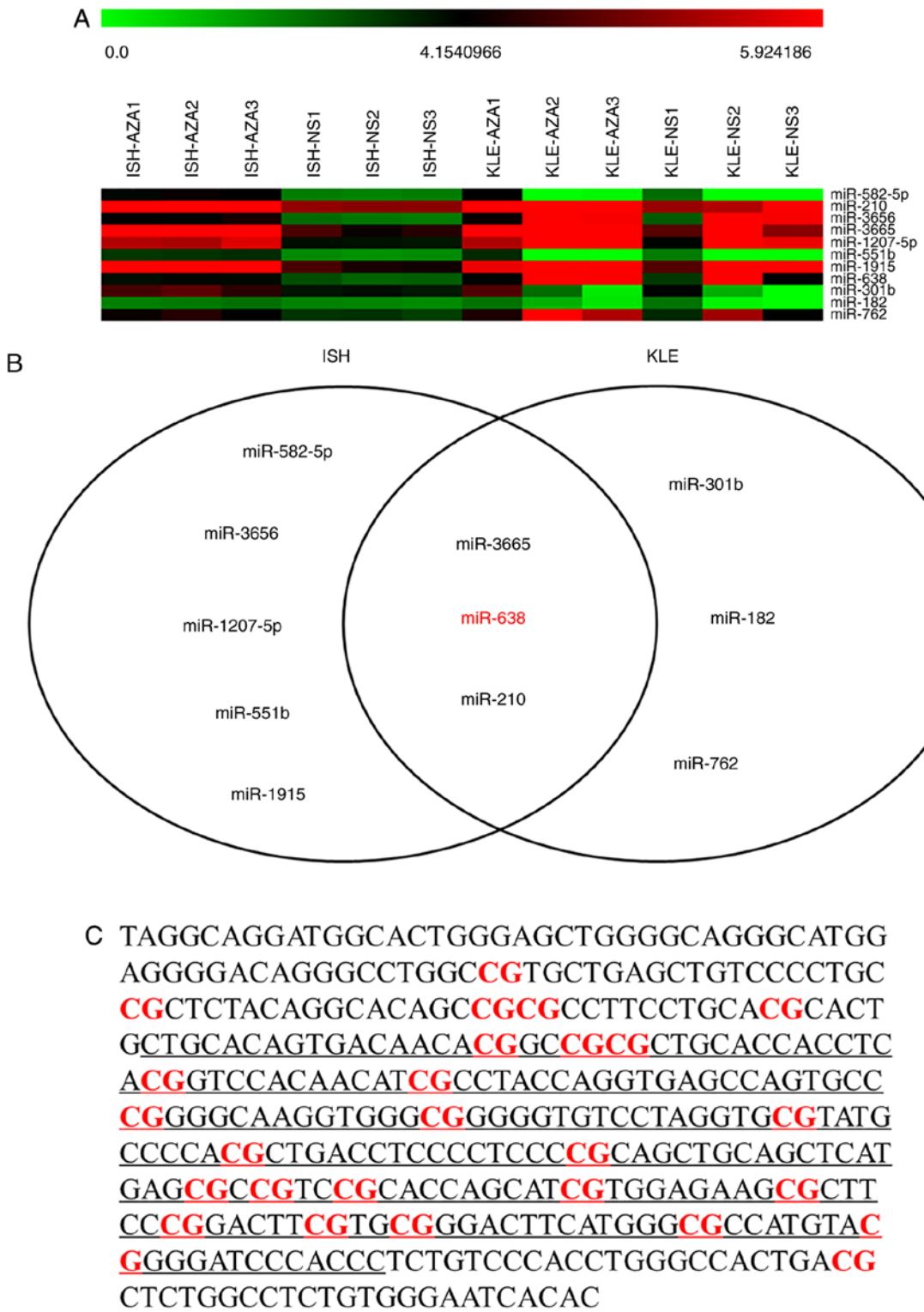

Figure 1. miR-638 gene is hyper-methylated in EC. The EC ISH and KLE cell lines were treated with 3 μM 5-aza-2'-deoxycitidine for 72 h. (A) An miRNA microarray was used to measure the relative expression of 1,347 common human miRNAs. A total of 11 miRNAs were identified as significantly upregulated after 5-aza-2'-deoxycitidine treatment ($P<0.05$ with fold-change >2). (B) Venn diagram of shared miRNA between the ISH and KLE cell lines (C) DNA sequence located ~5kb upstream of miR-638. The underlined sequence delineates the promoter region. CG dinucleotides exhibiting differential methylation are marked red.

Cell Signaling Technology, Inc.), MMP9 (cat. no. 13667; Cell Signaling Technology, Inc.), vascular endothelial growth factor (VEGF; cat. no. ab69479; Abcam), cyclin D1 (cat. no. 2922; Cell Signaling Technology, Inc.), cyclin-dependent kinase (CDK)2 (cat. no. 2546; Cell Signaling Technology, Inc.), CDK4 (cat. no. 12790; Cell Signaling Technology, Inc.) and tubulin (cat. no. 5568; Cell Signaling Technology, Inc.) overnight at 4˚C. All aforementioned antibodies were used at 1:1,000. Then, the membranes were incubated with the corresponding secondary antibodies overnight at 4˚C. The secondary antibodies used

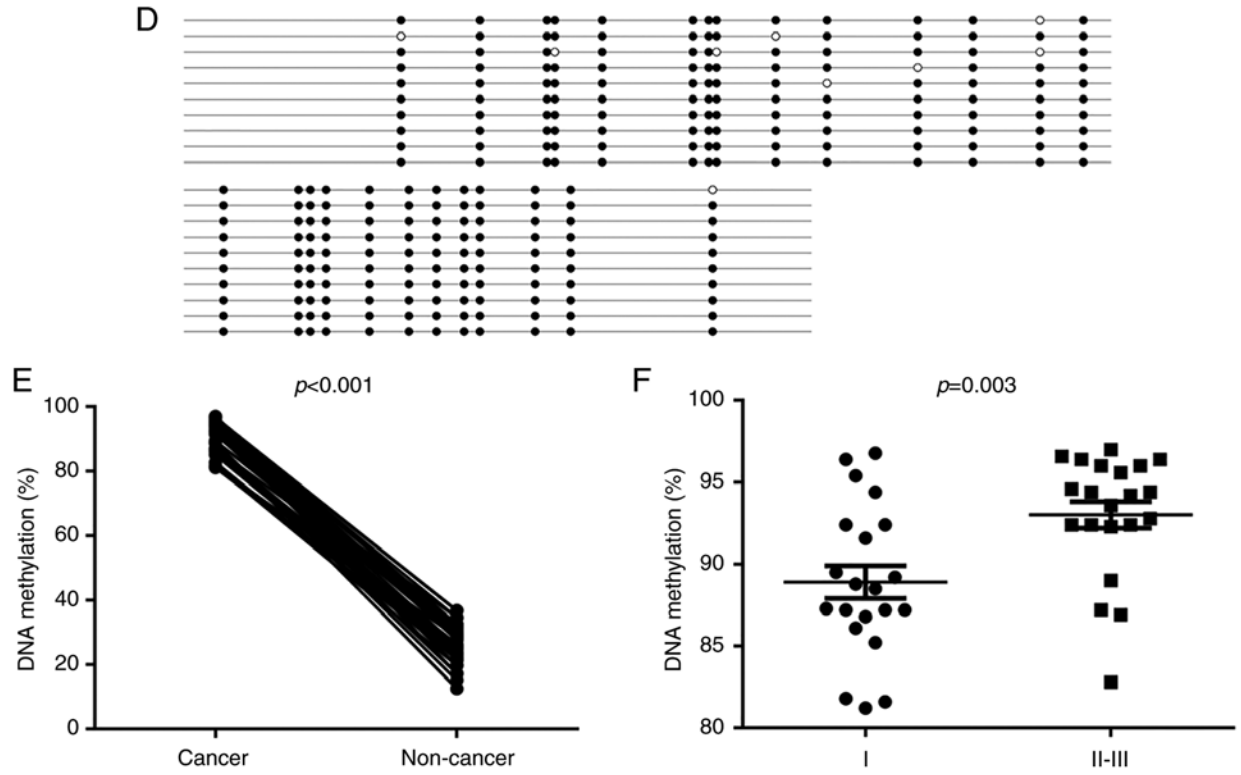

Figure 1. Continued. miR-638 gene is hyper-methylated in EC. The EC ISH and KLE cell lines were treated with 3 μM 5-aza-2'-deoxycitidine for 72 h. (D) Bisulfite sequencing analysis to determine the DNA methylation status of the promoter of miR-638 gene, showing the result from an individual patient. The black dots represent methylated CpG sites and white dots represent un-methylated CpG sites. (E) The DNA methylation level of miR-638 in 42 paired EC tissues and adjacent non-cancer tissues were assessed by pyrosequencing and TaqMan quantitative polymerase chain reaction, respectively. (F) Associations between the DNA methylation level of miR-638 with Federation of Gynecology and Obstetrics stage. The statistical significance of differences between different groups was calculated using the Student's t-test. miR, microRNA; EC, endometrial carcinoma; ISH-AZA, ISH cell line following 5-aza-2'-deoxycitidine treatment; KLE-AZA, KLE cell line following 5-aza-2'-deoxycitidine treatment; ISH-NS, ISH negative control; KLE-NS, negative control.

in the present study included: Horseradish peroxidase-conjugated secondary antibodies against rabbit (cat. no. SA00001-2; 1:10,000) and mouse (cat. no. SA00001-1; 1:10,000), purchased from Proteintech Group, Inc. Finally, protein bands were visualized using a chemiluminescence detection system (Bio-Rad Laboratories, Inc.). Protein expression was quantified using Image-Pro® Plus software (version 6.0; Media Cybernetics, Inc.).

In vivo tumorigenicity. A total of 100 4-week-old female nude mice were purchased from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences and placed in laminar flow cabinets under specific pathogen-free conditions for 1 week. Then, 5-week-old mice were randomly grouped (6 mice/treatment group) and $1x10^6$ cells were injected subcutaneously into each mouse. The tumor size was recorded weekly. A total of 50 days following cancer cell injection, the mice were euthanized and the tumor weight was determined. All of the mouse experiments were performed under the guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals. The study protocol was approved by the Committee on the Use of Live Animals in Teaching and Research of Fudan University.

Target prediction and luciferase assay. DIANA-microT-CDS (http://diana.imis.athena-innovation.gr/DianaTools/index.php), TargetScan (http://www.targetscan.org/) (15) and miRNA (www.miRNA.org) were used for the prediction of miR-638 target genes. Luciferase constructs were generated by ligating oligonucleotides containing the wild-type or a 4 bp mutation in the putative target site of the MEF2C 3'-UTR into the Psi-CHECK2 vector (Promega Corporation) downstream of a luciferase gene. The wild-type/mutant type vectors were co-transfected with miR-638 mimics/negative control into 293T cells (Cell Bank of Chinese Academy of Sciences), using Lipofectamine (Thermo Fisher Scientific, Inc.). After 48 h, the cell lines were harvested and the luciferase activity of each group was detected using the dual-luciferase reporter assay system (Promega Corporation), with *Renilla* luciferase selected as the internal control.

Statistical analysis. All results are presented as the mean ± standard deviation. All statistical analyses were performed using GraphPad Prism software 7.0 (GraphPad Software, Inc.) and SPSS v.22.0 (IBM Corp.). For comparisons, one-way analysis of variance, *t*-test, and Pearson chi-square test were performed as indicated. Tukey's post hoc test was used for multiple comparisons test. Disease free survival (DFS), defined as the time interval between surgery to disease recurrence or mortality due to any cause, was estimated using the Kaplan-Meier method and differences among subgroups were investigated by the log-rank test. The correlations between relative miR-638 expression and DNA methylation level, as well as between relative miR-638 expression and

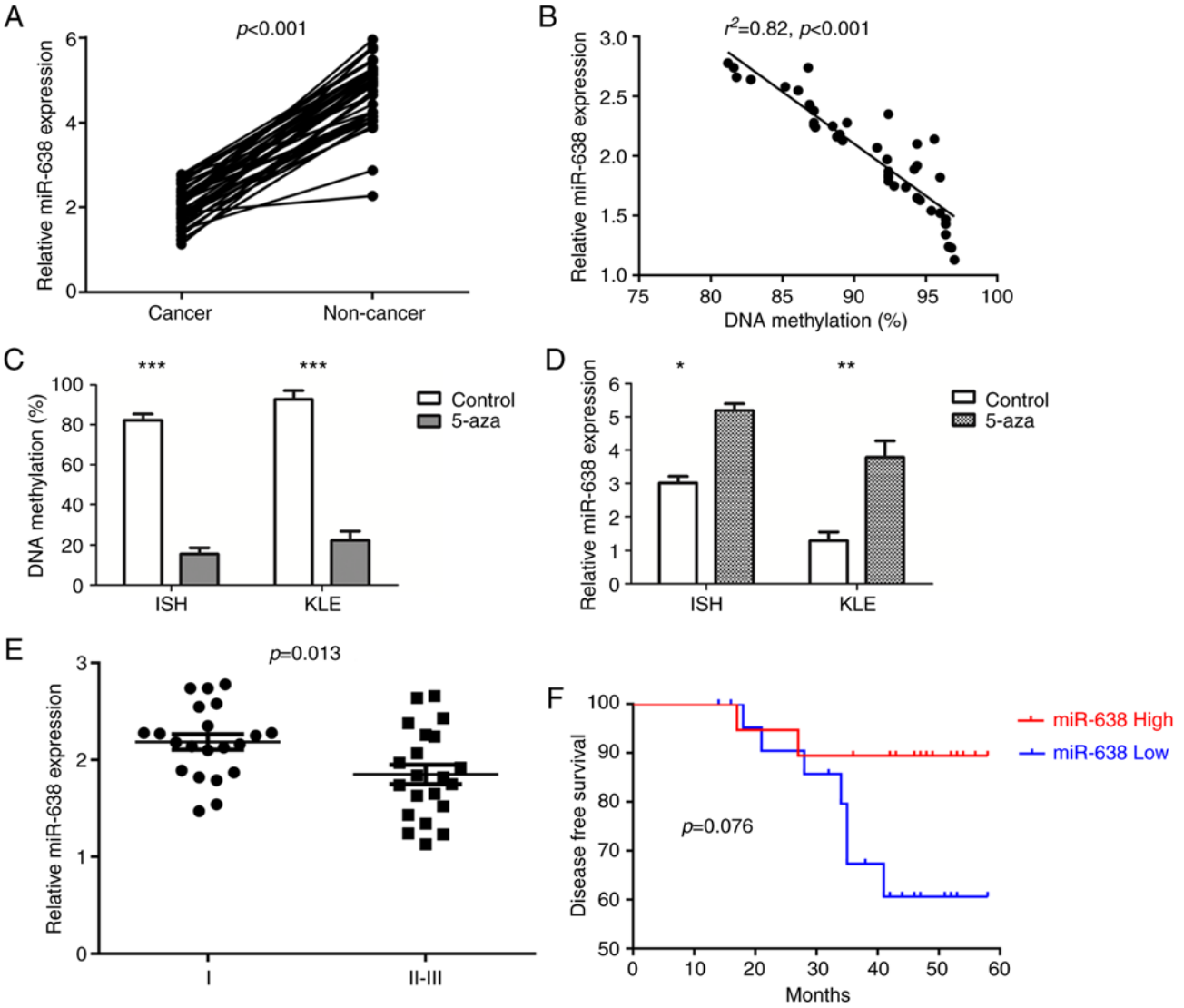

Figure 2. miR-638 expression is downregulated in EC. (A) Relative expression of miR-638 in 42 paired endometrial carcinoma tissues and adjacent non-cancer tissues, was assessed by TaqMan quantitative polymerase chain reaction. U6 served as an internal control. (B) The correlation between relative miR-638 expression and the DNA methylation level was evaluated using Spearman's correlation analysis. (C and D) Endometrial carcinoma cell lines were treated with 3 μM 5-aza for 72 h. Then, the (C) DNA methylation level and (D) relative miR-638 expression were examined. The results are presented as the mean ± standard deviation of values obtained from 3 independent experiments. (E) Associations between relative miR-638 expression with Federation of Gynecology and Obstetrics stage. The statistical significance of differences between different groups was calculated using the Student's t-test. (F) Kaplan-Meier survival curves comparing patients with an increased expression level of miR-638 and those with a decreased expression level of miR-638. The median expression level of miR-638 was selected as the cut-off point. *P<0.05, **P<0.01 and ***P<0.001. miR, microRNA; EC, endometrial carcinoma; 5-aza, 5-aza-2'-deoxycitidine.

relative MEF2C mRNA expression, were determined using the Spearman correlation coefficient. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-638 gene is hyper-methylated in EC. To identify potential cancer-associated miRNAs whose promoters were highly methylated in EC, the EC ISH and KLE cell lines were treated with demethylation agents for 72 h, and the expression levels of 1,347 common human miRNAs were measured using microarray analysis (Fig. 1A). With the cut-off criteria of P<0.05 and fold change >2, miR-638, miR-3665 and miR-210 were revealed to be significantly upregulated in both cell lines (Fig. 1B). miR-638 was selected for further study as it was a newly identified cancer-associated miRNA, and data concerning its specific biological roles in different types of human cancer are conflicting (9). A total of 2 CpG-rich regions at/near the promoter of miR-638 gene were identified using CpG Island Searcher (15). The first CpG island overlapped with the open reading frame sequence of the miR-638 gene and the second CpG island was located about 5kb upstream of the miR-638 gene (Fig. 1C). The methylation statuses of consecutive CpG sites in the second CpG island were highly concordant (Fig. 1D) and the mean value was used as representative of the methylation level of the miR-638 gene. A significantly higher degree of DNA methylation was identified in the cancerous tissues compared with the non-cancerous tissues among clinical samples from 42 patients with EC (Fig. 1E). The associations between the DNA methylation level of miR-638 in cancerous tissues with various clinicopathological parameters, including age, primary tumor size, Federation of

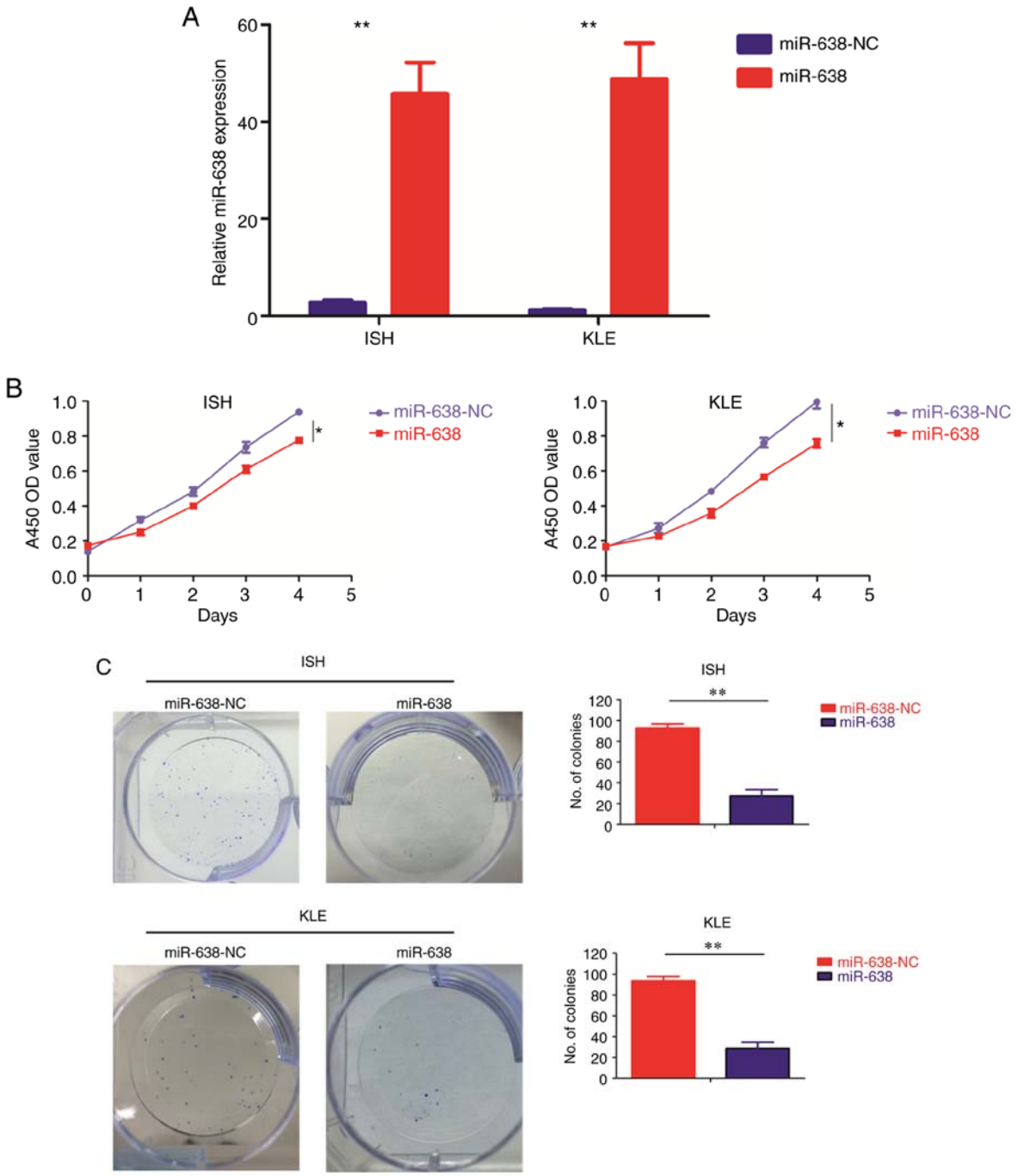

Figure 3. miR-638 functions as a tumor-suppressive miRNA in endometrial carcinoma. The 2 endometrial carcinoma ISH and KLE cell lines were stably transfected with an miR-638 lentiviral vector (miR-638) or its negative control (miR-638-NC), respectively. (A) Relative miR-638 expression was examined by TaqMan quantitative polymerase chain reaction. (B and C) Cell proliferation was examined using (B) Cell Counting Kit-8 and (C) colony formation assays.

Gynecology and Obstetrics (FIGO) stage (2014 version) (16), tumor differentiation and tumor histology were also analyzed. A significant positive correlation between miR-638 promoter methylation level and FIGO stage was identified (Fig. 1F). Of note, no statistical significance was identified when comparing the methylation (P=0.679) or the relative expression (P=0.523) levels of miR-638 in the cancerous tissue from the 36 patients with endometrial adenocarcinoma with that from the 6 patients with serous carcinoma.

miR-638 expression is downregulated in EC. To examine the effect of miR-638 promoter region methylation on miR-638 expression, the relative miR-638 expression levels were measured in 42 paired EC samples. A significant downregulation of miR-638 expression in cancerous tissues was detected (Fig. 2A). Furthermore, a significant inverse correlation between miR-638 DNA methylation and miR-638 expression was demonstrated (Fig. 2B). Additionally, DNA methylation and miR-638 expression levels were investigated in ISH and KLE cell lines treated with 5-aza-2'-deoxycitidine. Both cell lines exhibited hypermethylation of the miR-638 promoter region prior to treatment and the DNA methylation levels decreased significantly (Fig. 2C), whereas miR-638 expression was increased significantly following treatment (Fig. 2D).

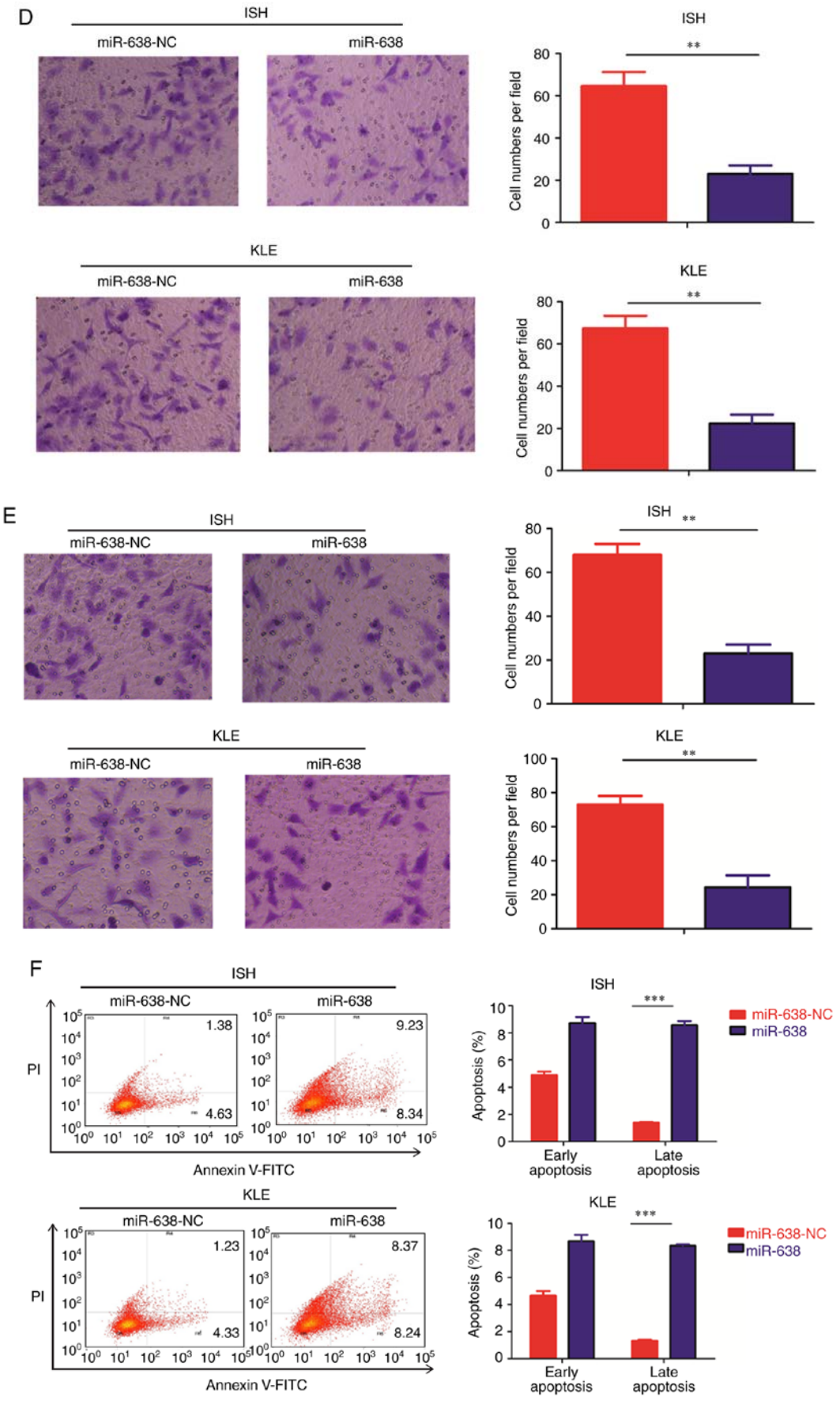

Figure 3. Continued. miR-638 functions as a tumor-suppressive miRNA in endometrial carcinoma. The 2 endometrial carcinoma ISH and KLE cell lines were stably transfected with an miR-638 lentiviral vector (miR-638) or its negative control (miR-638-NC), respectively. (D and E) Cell migration and invasion were investigated by (D) migration and (E) invasion assays, respectively. The number of cells that invaded through the membrane was counted in 10 fields using a x20 objective lens. Original magnification, x200. (F) At 48 h post-transfection, apoptosis was measured by the flow cytometric analysis of cells stained with Annexin V-FITC and PI.

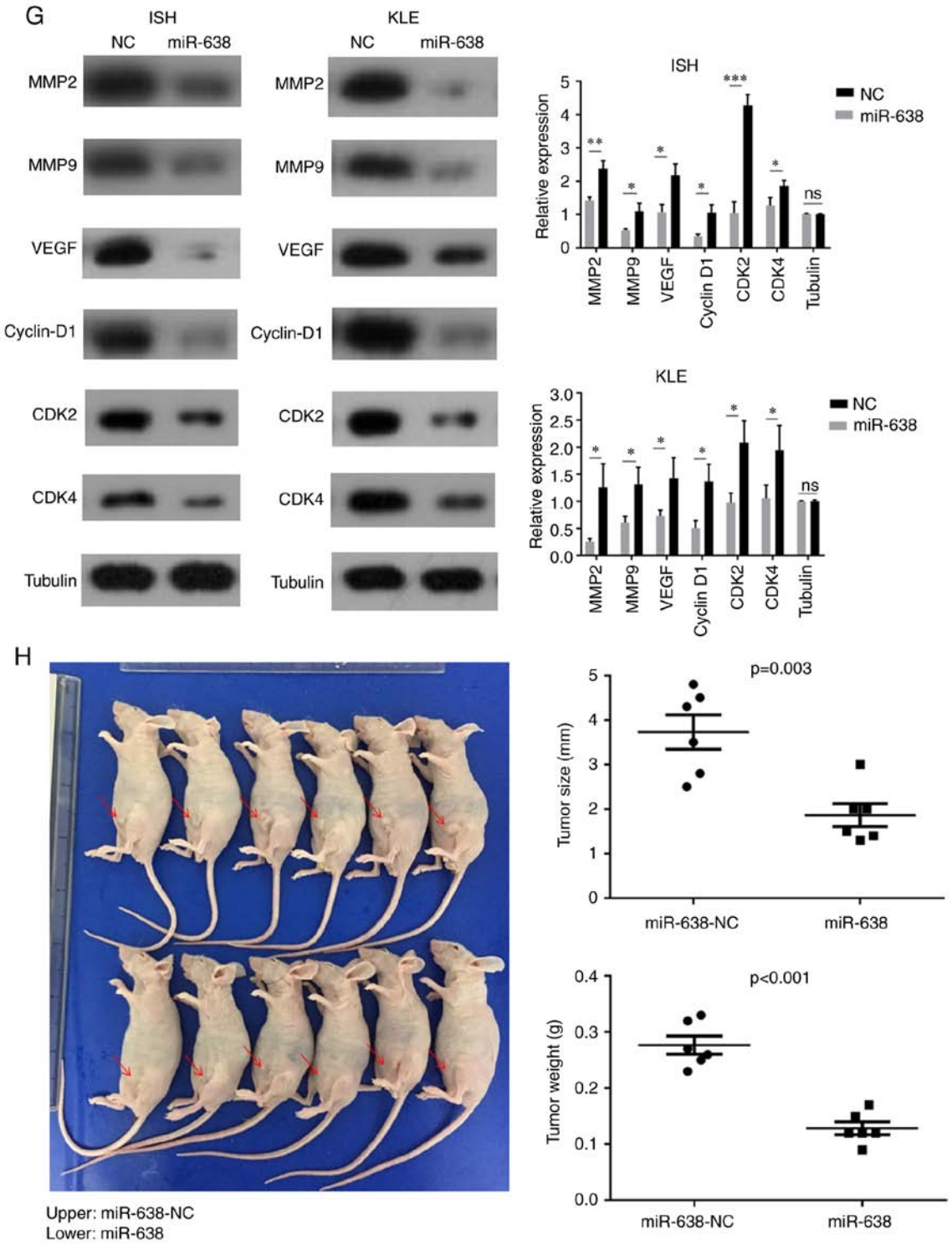

Figure 3. Continued. miR-638 functions as a tumor-suppressive miRNA in endometrial carcinoma. The 2 endometrial carcinoma ISH and KLE cell lines were stably transfected with an miR-638 lentiviral vector (miR-638) or its negative control (miR-638-NC), respectively. (G) Proteins that are frequently altered in various types of human cancer were subjected to western blot analysis using the indicated antibodies. (H) *In vivo* tumorigenicity was investigated in a nude mouse xenograft model. Tumor size and weight were measured on day 50 after cancer cell injection. All results are presented as the mean ± standard deviation of values obtained from 3 independent experiments. Statistical significance was calculated using the Student's t-test. *P<0.05, **P<0.01 and ***P<0.001. miR, microRNA; FITC, fluorescein isothiocyanate; PI, propidium iodide; NC, negative control.

When examining the association between miR-638 expression with the aforementioned common clinical variables, a significant negative correlation between miR-638 expression and FIGO stage was identified (Fig. 2E). Furthermore, with a median follow-up of 48.0 months (range, 14-58), 9 patients exhibited disease recurrence, and those with increased expression levels of miR-638 exhibited a prolonged DFS (Fig. 2F).

miR-638 functions as a tumor suppressive miRNA in EC. EC cell lines transfected with miR-638 mimics (miR-638) or its corresponding negative control (miR-638-NC), were

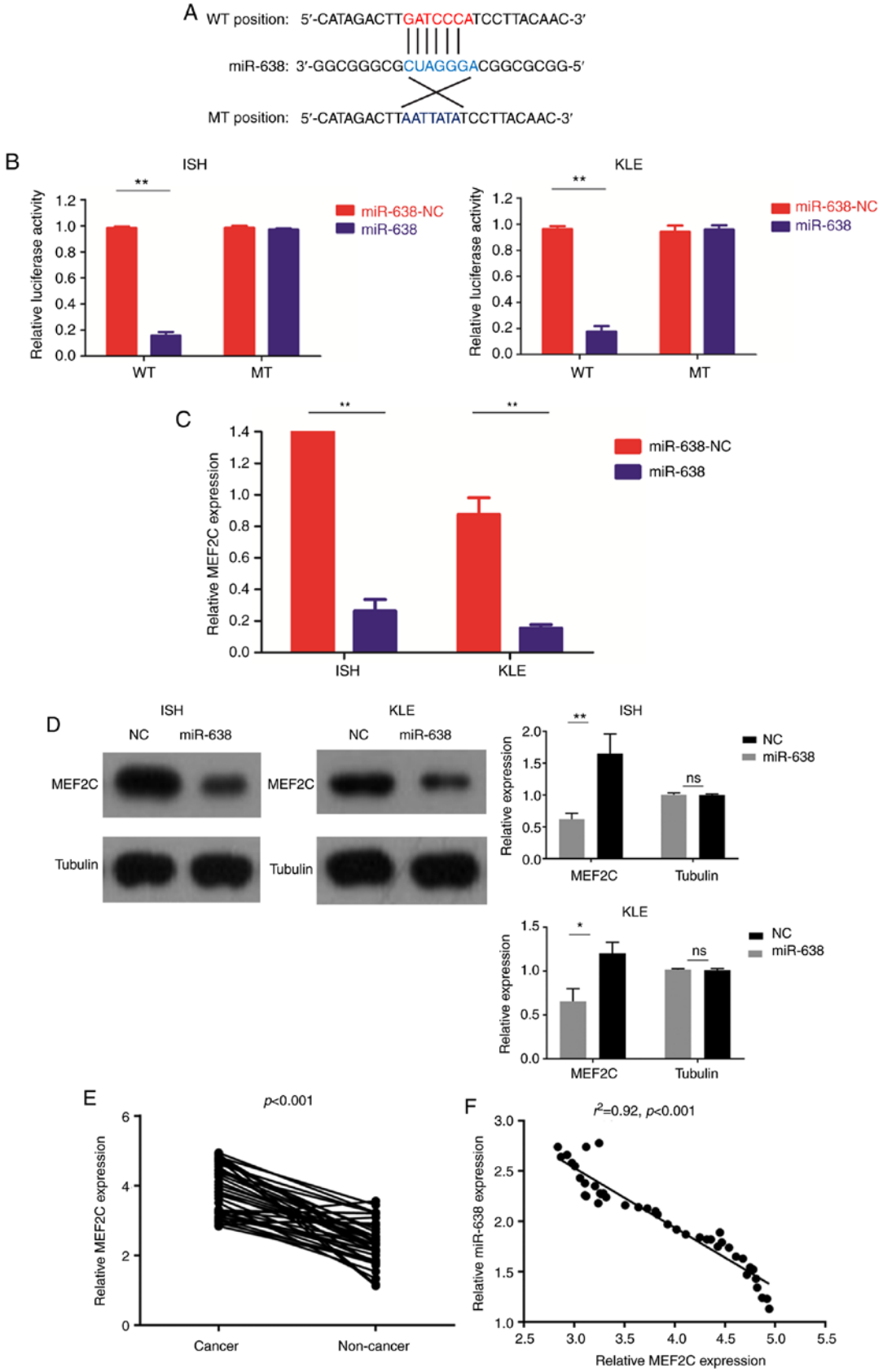

Figure 4. miR-638 downregulates MEF2C expression by directly targeting its 3'-UTR. (A) Human MEF2C 3'-UTR fragment containing the WT or MT miR-638-binding sequence. (B) 3'-UTR luciferase reporter assays in 293T cells. 293T cells were co-transfected with miR-638 or its negative control vector and a luciferase reporter construct containing the wild-type or mutant MEF2C 3'-UTR. For each experiment, the data were normalized to the luciferase activity detected in cells transfected with the control vector. (C and D) Relative MEF2C mRNA expression and protein expression in ISH and KLE cell lines that were stably transfected with miR-638 lentiviral vector (miR-638) or its negative control (miR-638-NC). (E) Relative expression of MEF2C mRNA in 42 paired endometrial carcinoma tissues and adjacent non-cancer tissues were assessed by quantitative polymerase chain reaction. GAPDH served as an internal control. (F) The correlation between relative miR-638 expression and relative MEF2C mRNA expression was evaluated using Spearman's correlation analysis. *P<0.05 and **P<0.01. miR, microRNA; WT, wild-type; MT, mutant; UTR, untranslated region; NC, negative control; ns, not significant.

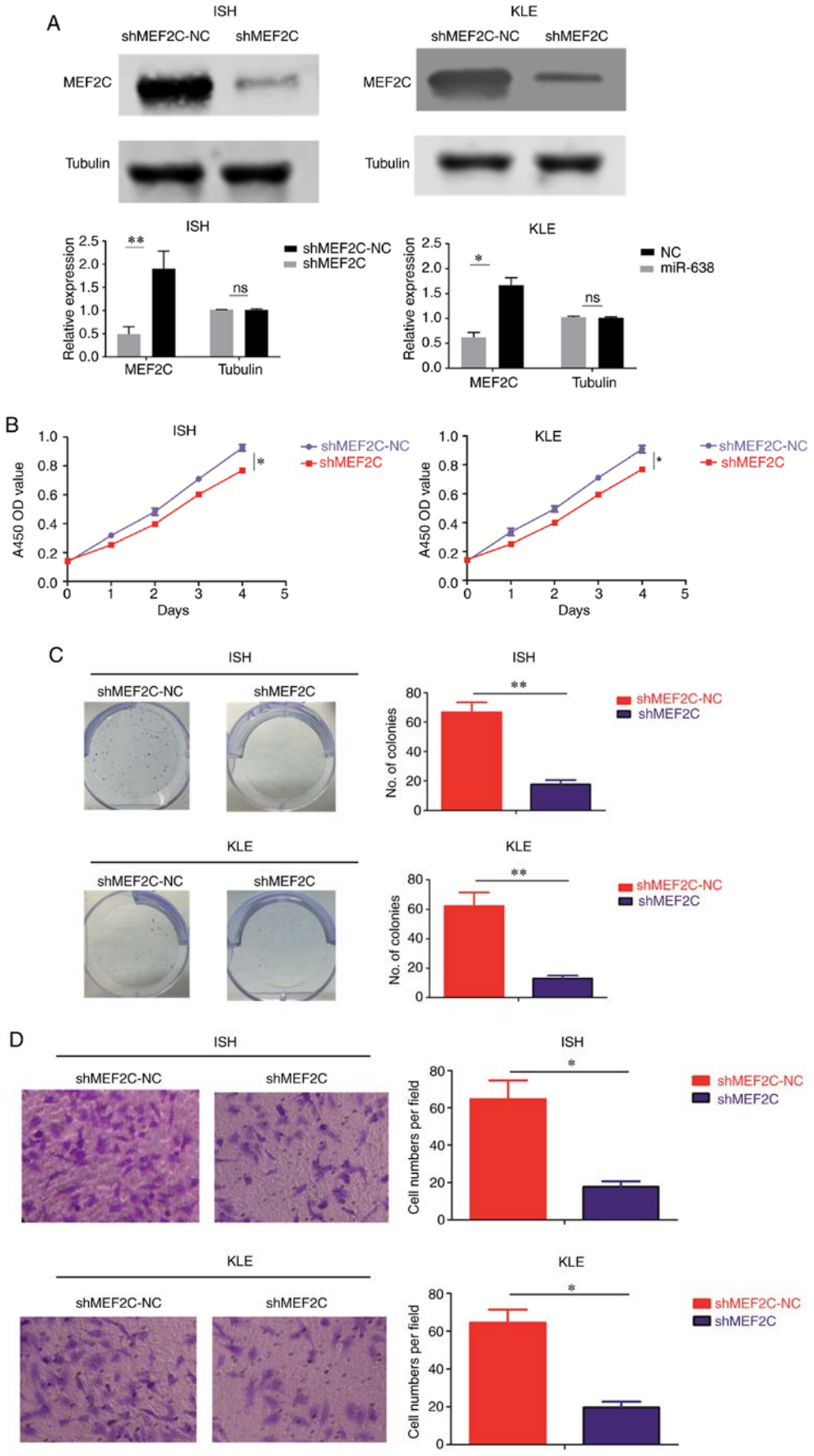

Figure 5. MEF2C functions as a onco-protein in EC. The 2 EC ISH and KLE cell lines were stably transfected with shMEF2C lentiviral vector (shMEF2C) or its negative control (shMEF2C-NC), respectively. (A) MEF2C expression was examined by western blot analysis using the indicated antibodies. (B and C) Cell proliferation was examined by (B) Cell Counting Kit-8 and (C) colony formation assays. (D) Cell migration and invasion were investigated by migration and invasion assays, respectively. The number of cells that invaded through the membrane was counted in 10 fields using a x20 objective lens. Original magnification, x200.

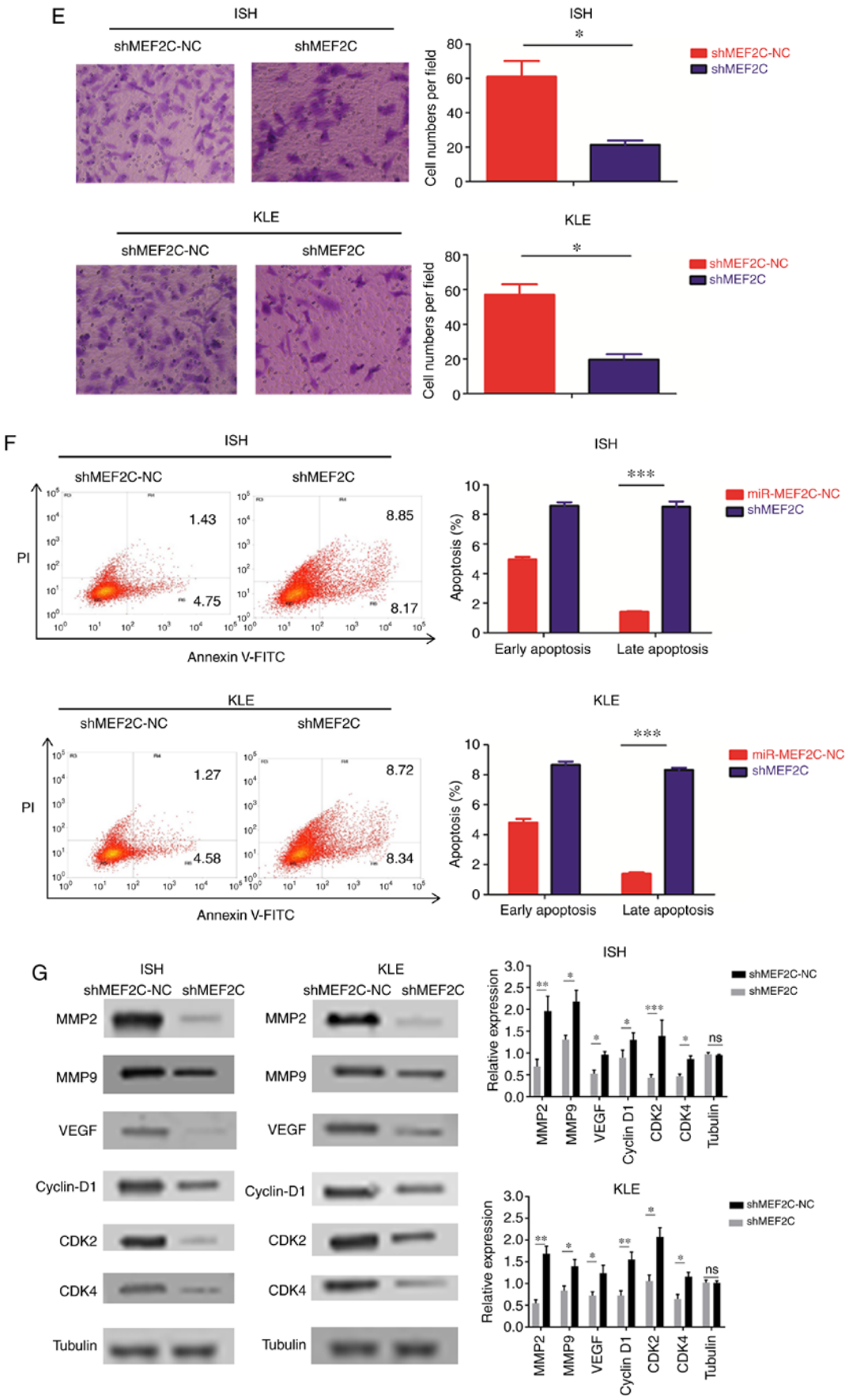

Figure 5. Continued. MEF2C functions as a onco-protein in EC. (E) Cell migration and invasion were investigated by migration and invasion assays, respectively. The number of cells that invaded through the membrane was counted in 10 fields using a x20 objective lens. Original magnification, x200. (F) At 48 h post-transfection, apoptosis was measured by the flow cytometric analysis of cells stained with Annexin V-FITC and PI. Statistical significance was calculated using the Student's t-test. (G) MMP2, MMP9, VEGF, cyclin D1, CDK2 and CDK4 expression levels were examined using western blot analysis with the indicated antibodies. Tubulin expression was selected as a control.

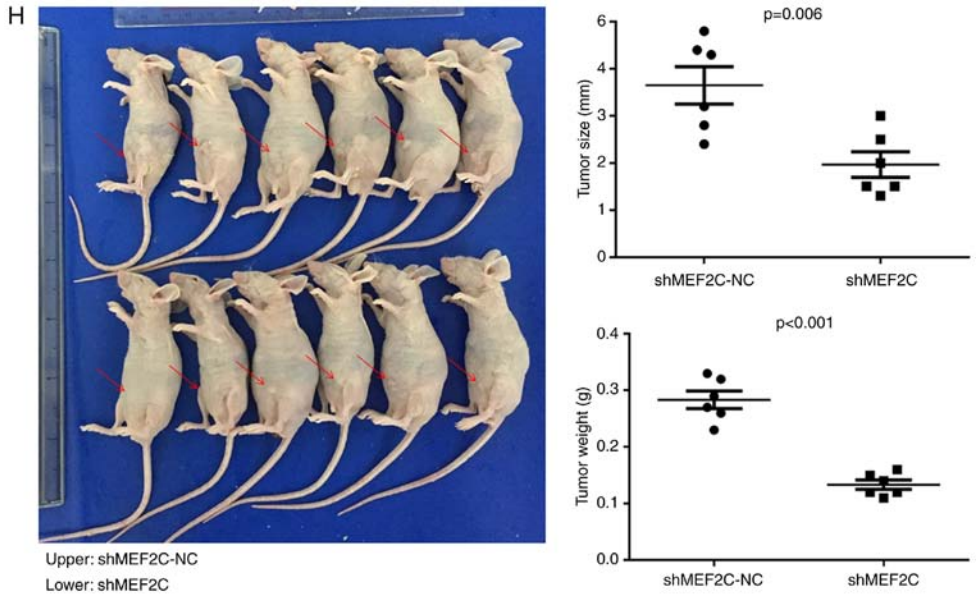

Figure 5. Continued. MEF2C functions as a onco-protein in EC. (H) *In vivo* tumorigenicity was investigated in a nude mouse xenograft model. Tumor size and weight were measured on day 50 after cancer cell injection. All results are presented as the mean ± standard deviation of values obtained from 3 independent experiments. *P<0.05, **P<0.01 and ***P<0.001. MEF2C, myocyte enhancer factor 2C; EC, endometrial carcinoma; sh, short hairpin; miR, microRNA; FITC, fluorescein isothiocyanate; PI, propidium iodide; NC, negative control; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; CDK, cyclin-dependent kinase.

constructed (Fig. 3A). Overexpression of miR-638 repressed cell proliferation (Fig. 3B and C), migration (Fig. 3D) and invasion (Fig. 3E), and promoted cell apoptosis (Fig. 3F). Furthermore, miR-638 overexpression led to the downregulation of MMP2, MMP9, VEGF, cyclin D1, CDK2 and CDK4 (Fig. 3G).

EC cell lines stably transfected with miR-638 mimics or its negative control were subcutaneously implanted into nude mice. It was identified that the overexpression of miR-638 significantly decreased tumor growth compared with controls (Fig. 3H).

miR-638 directly targets MEF2C. MEF2C was identified to be one of the targets of miR-638 using several commonly used microRNA database. To validate this result, a luciferase reporter assay was employed. The entire wild-type 3'-UTR of MEF2C or the mutant 3'-UTR with a 4 bp mutation in the seed region was cloned downstream of the luciferase gene open reading frame (Fig. 4A). Luciferase activity was significantly decreased upon transfection of miR-638 mimics in the wild type reporter. Conversely, luciferase activity of mutant reporter was unaffected following transfection with miR-638 mimics or the control vector (Fig. 4B). In addition, the MEF2C mRNA (Fig. 4C) and protein (Fig. 4D) expression levels were substantially decreased following miR-638 overexpression in both EC cell lines. Furthermore, in the 42 paired clinical samples, MEF2C mRNA levels were significantly increased in the cancerous tissues (Fig. 4E) and a significant inverse correlation between MEF2C mRNA level and miR-638 expression was demonstrated (Fig. 4F). Of note, no statistical significance was revealed when comparing the MEF2C mRNA levels in the cancerous tissue from the 36 patients with endometrial adenocarcinoma with that from the 6 patients with serous carcinoma (P=0.472).

MEF2C mediates the tumor suppressive effect of miR-638 in EC. EC cell lines were transfected with lentivirus encoding shMEF2C, which mediated the stable knockdown of endogenous MEF2C. siRNA-mediated knockdown of MEF2C inhibited cell proliferation, migration and invasion, but promoted cell apoptosis. It also downregulated the expression levels of MMP2, MMP9, VEGF, cyclin D1, CDK2 and CDK4 and repressed *in vivo* tumor growth in mouse models (Fig. 5).

Next, a rescue experiment was performed to examine the biological and molecular associations between MEF2C and miR-638 in EC. A specific lentiviral vector (MEF2C analog) that encoded the entire coding sequence of MEF2C but lacked its 3'-UTR was constructed, in order to ectopically express MEF2C without affecting the expression of miR-638 and its numerous other targets. EC cell lines stably transfected with miR-683 mimics and/or MEF2C analog were selected, and MEF2C expression levels in these cells is presented in Fig. S1. Overexpressing miR-638 led to significant downregulation of MEF2C, while co-transfection of miR-638 mimics and the MEF2C analog (miR-638 + MEF2C) rescued the expression of MEF2C. Using the 3 specifically designed EC cell lines (miR-638-NC, miR-638 and miR-638 + MEF2C), it was demonstrated that the ectopic expression of MEF2C significantly abrogated the tumor-suppressive effect induced by miR-638 (Fig. 6).

Discussion

Epigenetic modifications of cancer-associated miRNAs have been demonstrated to have important roles during tumorigenesis (1,4). In the present study, miR-638 was verified to be downregulated by promoter methylation in EC. The promoter region of the miR-638 gene was hyper-methylated in samples from patients with EC and EC cell lines, which is consistent

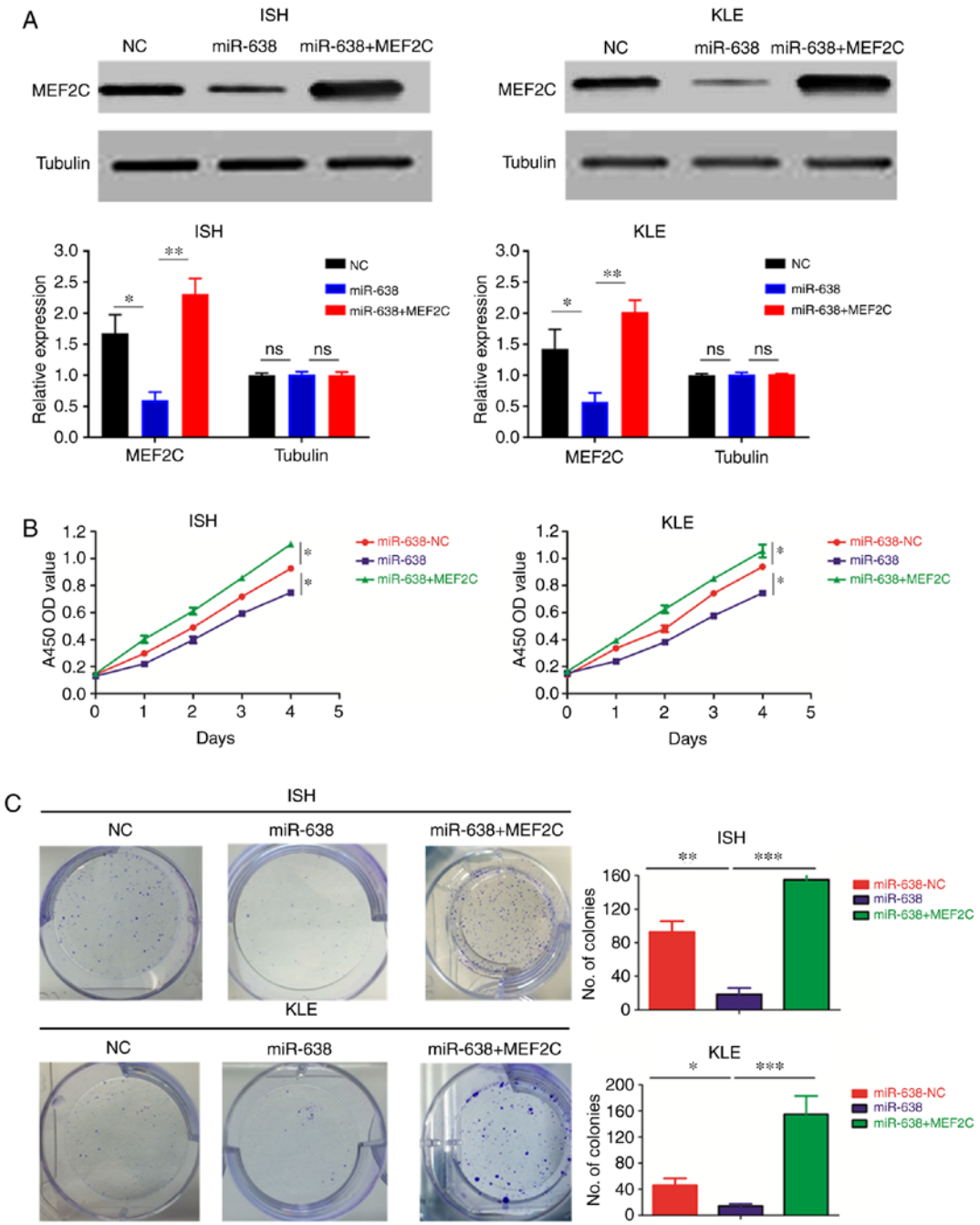

Figure 6. MEF2C mediates the tumor-suppressive function of miR-638. A total of 3 cell lines were used in these experiments: EC cell lines transfected with negative control (miR-638-NC), miR-638 mimics (miR-638) and miR-638 mimics and a MEF2C-expressing vector that encoded the entire coding sequence of MEF2C but lacked the 3'-UTR (miR-638 + MEF2C). (A) MEF2C expression was examined by western blot analysis using the indicated antibodies. (B and C) Cell proliferation was examined by (B) Cell Counting Kit-8 and (C) colony formation assays.

with a previous study of miR-638 in colorectal carcinoma (17). In addition, the promoter methylation level of miR-638 and miR-638 expression were significantly associated with FIGO stage. Furthermore, an increased expression level of miR-638 was observed to be associated with improved DFS. Notably, all of the patients in the present study were grouped into FIGO stages I and II-III, and the majority of patients were diagnosed FIGO stage I clinically, which was similar to previous studies (18,19). Although a trend towards an increased level of methylation and decreased expression of miR-638 gene were observed among patients with FIGO stage III disease when compared with that of patients with FIGO stage II disease, no statistical significance was identified, most likely due to the small sample size. In order to further investigate the prognostic significance of methylation status and expression level of miR-638 among patients with FIGO stage II and stage III separately, future studies with larger sample size are required.

As is known, there are two main clinic-pathological subtypes of EC; the estrogen-associated type I EC, primarily endometrioid adenocarcinoma, and the non-estrogen-associated type II EC, including uterine papillary serous carcinoma, clear cell carcinoma and others. In the present study, down-regulation of miR-638 by its promoter region methylation and upregulation of MEF2C were observed in both endometrioid

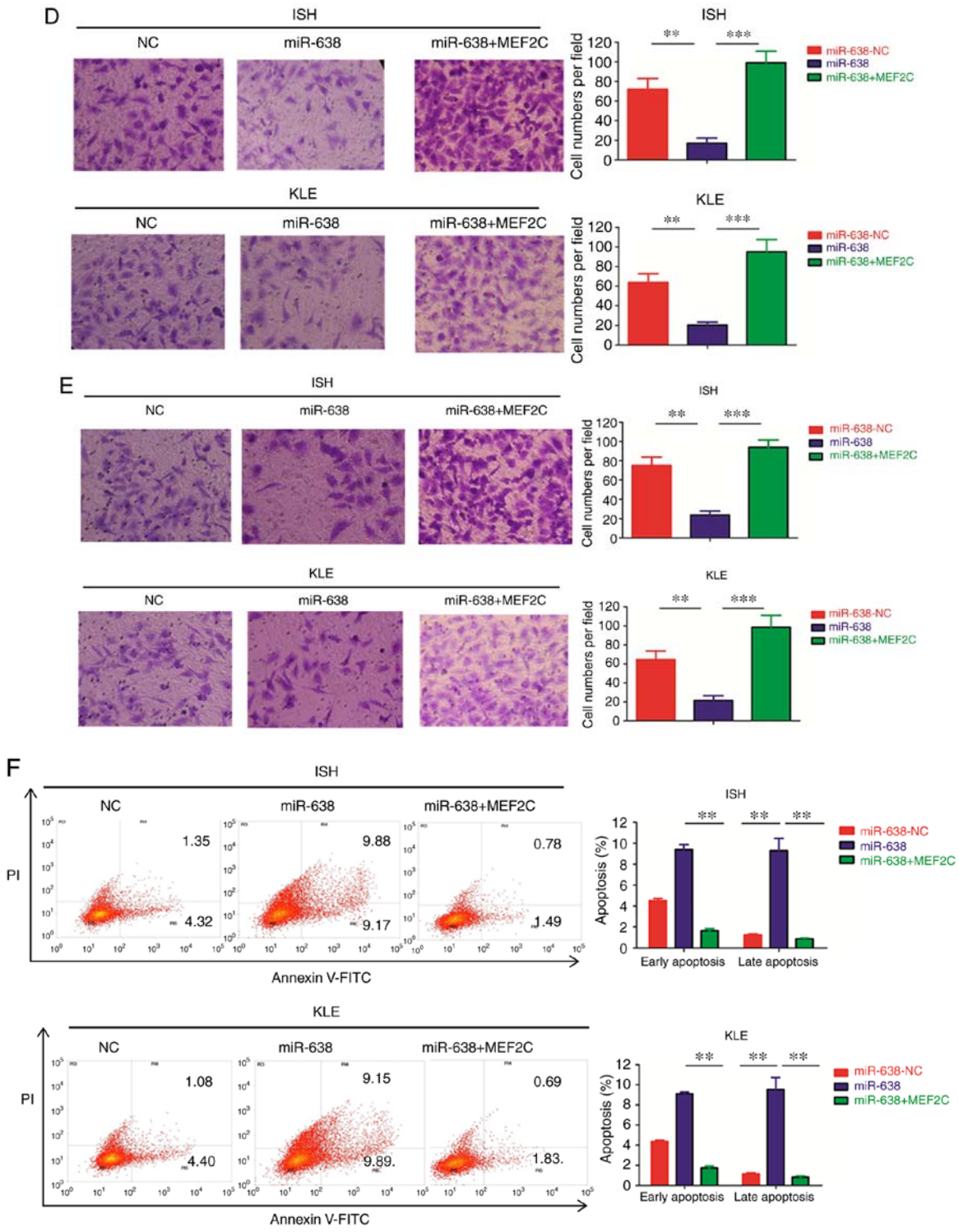

Figure 6. Continued. MEF2C mediates the tumor-suppressive function of miR-638. (D and E) Cell migration and invasion were investigated using (D) migration and (E) invasion assays, respectively. The number of cells that invaded through the membrane was counted in 10 fields using a x20 objective lens. Original magnification, x200. (F) At 48 h post-transfection, apoptosis was measured by the flow cytometric analysis of cells stained with Annexin V-FITC and PI.

adenocarcinoma and serous carcinoma, despite considerable differences in genetic backgrounds, molecular aberrations and drug sensitivities being reported between these 2 pathological subtypes of EC (20-22). Estrogen-associated type I EC frequently exhibits mutations in *PTEN*, catenin beta 1, PIK3 catalytic subunit alpha, AT-rich interaction domain (ARID)1A, *KRAS* and SWItch/Sucrose non-fermentable chromatin remodeling complex genes such as ARID5B. Conversely, as one of the most common type of non-estrogen-associated endometrial carcinoma, serous carcinoma of the endometrium normally has extensive copy number alterations, and often shares genomic features with ovarian serous and basal-like breast carcinomas (20,21,23). Nevertheless, there are certain similarities between these two different histologic subtypes. A pooled analysis of individual-level data from 10 cohort and 14 case-control studies identified that patients with these 2

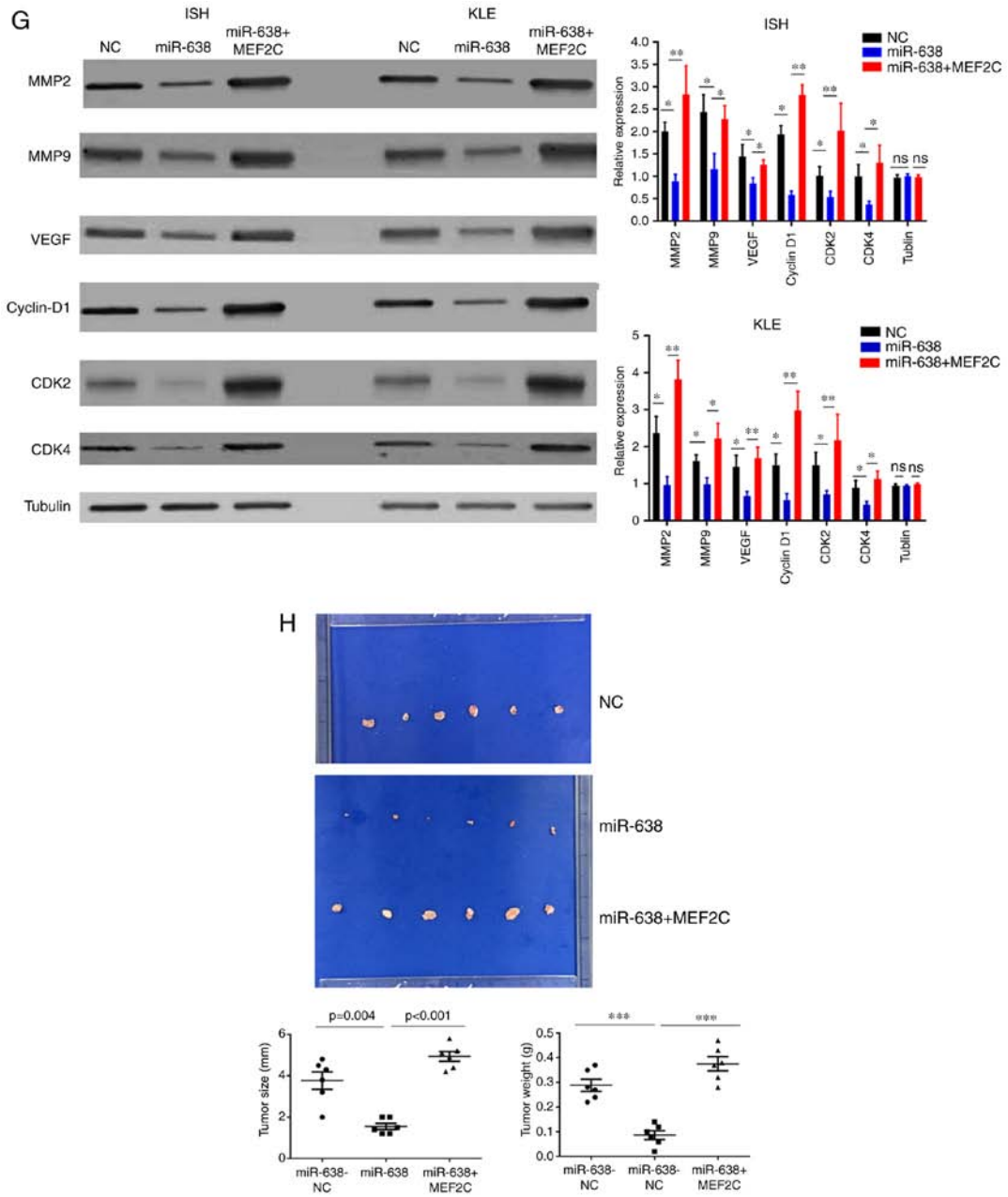

Figure 6. Continued. MEF2C mediates the tumor-suppressive function of miR-638. (G) MMP2, MMP9, VEGF, cyclin-D1, CDK2 and CDK4 expression levels were measured using western blot analysis with the indicated antibodies. Tubulin expression was selected as the control. (H) *In vivo* tumorigenicity was investigated in a nude mouse xenograft model. Tumor size and weight were measured on day 50 after cancer cell injection. All results are presented as the mean ± standard deviation of values obtained from 3 independent experiments. Statistical significance was calculated using the Tukey's post hoc test. *P<0.05, **P<0.01 and ***P<0.001. MEF2C, myocyte enhancer factor 2C; sh, short hairpin; miR, microRNA; FITC, fluorescein isothiocyanate; PI, propidium iodide; NC, negative control; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; CDK, cyclin-dependent kinase.

endometrial cancer types shared a number of common etiologic factors, including parity, oral contraceptive use, cigarette smoking, age at menarche and presence of diabetes (24). Furthermore, shared molecular regulatory mechanisms among these 2 pathological subtypes of EC have been previously reported, such as solute carrier family 1 member 5 regulating glutamine uptake and cell growth in EC (25). In the present study, the differences in methylation status and expression levels of miR-638, as well as expression levels of MEF2C, were compared among cancerous tissues and non-cancerous tissues in clinical samples from patients with endometrioid adenocarcinoma and serous carcinoma. Additionally, no statistical significance was identified when comparing the methylation or expression levels of miR-638 gene in the cancerous tissues from the patients with endometrioid adenocarcinoma with that from the patients with serous carcinoma. Furthermore, 2 different cell lines were selected for analysis in the present study. ISH was isolated from a patient with estrogen receptor-positive, well-differentiated endometrioid adenocarcinoma, and KLE was isolated from a patient with less differentiated and highly aggressive, type II endometrium carcinoma, and the results were confirmed in both cell lines. Taken together, silencing miR-638 through promoter methylation may be a shared epigenetic regulatory mechanism among

these 2 different histologic subtypes of EC, which warrants future validation.

Additionally, miR-3665 and miR-210 were also identified as potential miRNAs that may be epigenetically regulated by promoter hypermethylation. miR-3665 is associated with influenza virus (26) and tuberculosis infection (27), but its associations with human cancer has rarely been reported. However, the downregulation of miR-210 expression by the hypermethylation of its promoter region has been reported (28,29).

The biological roles of miR-638 appear to be cancer type-dependent (6-9) and it functions as a tumor suppressive miRNA in EC. Liu *et al* (30) reported that miR-638, along with 3 other miRNAs, predicted survival of patients with nasopharyngeal carcinoma. miR-638 was upregulated in esophageal squamous cell carcinoma and breast cancer cells, and it promoted starvation- and rapamycin-induced autophagy by targeting DACT3/Wnt/β-catenin signaling. Ma *et al* (6) revealed that miR-638 was downregulated in colorectal cancer and served as a tumor suppressive miRNA by targeting SRY-box transcription factor 2. Similarly, Zhang *et al* (8) demonstrated that miR-638 mediated its tumor suppressive function by targeting phospholipase D1 in gastric cancer. All these data suggest that miR-638 is associated with various processes during tumorigenesis, but mediates its versatile effects by targeting distinct downstream genes in different types of human cancer. To the best of our knowledge, the present study is the first comprehensive investigation of the status and biological role of miR-638 in EC. The results demonstrated that miR-638 was downregulated by promoter hypermethylation and functioned as a powerful tumor suppressive miRNA by targeting MEF2C in EC.

MEF2C, a myogenic differentiation transcription factor, was recently characterized as a potent onco-protein in various types of human cancer, in particular leukemia (31,32). MEF2C was activated by NK2 homeobox 5 or BCR-ABL (33,34), participated in the regulation of homing and invasiveness (35) and was associated with chemotherapy resistance (36) or patient prognosis in various types of leukemias (37). Furthermore, several studies have investigated its expression and biological function in solid tumors. Zhang *et al* (38) identified that Yin Yang-1 suppressed the invasion and metastasis of pancreatic ductal adenocarcinoma by downregulating MMP10 via a mucin 4, cell surface associated/Erb-B2 receptor tyrosine kinase 2/p38 mitogen-activated protein kinase (MAPK)/MEF2C-dependent mechanism. Bai *et al* (39) demonstrated that MEF2C mediated VEGF-induced vasculogenic mimicry, angiogenesis and migration/invasion, with involvement of the p38 MAPK and protein kinase C signaling pathways, and that the nuclear translocation of MEF2C inhibited cancer proliferation via blockade of Wnt/β-catenin signaling in hepatocellular carcinoma. Ignatius *et al* (40) revealed that the Notch receptor 1/snail family transcriptional repressor 1/MEF2C pathway regulated growth and self-renewal of tumor-propagating cells in embryonal rhabdomyosarcoma. The present study demonstrated that the downregulation of MEF2C inhibited cell proliferation, migration, invasion and promoted cell apoptosis in EC, partly through the upregulation of MMP2, MMP9, VEGF, cyclin D1, CDK2 and CDK4. It is important to note that the expression of MEF2C was not successfully significantly upregulated by transfecting a vector overexpressing MEF2C. We hypothesized that there may be certain feedback regulatory systems that affect the expression of MEF2C and it may be difficult to further upregulate the expression of MEF2C in a state of sustained high expression in EC cells. VEGF, MMP2 and MMP9 are well-recognized angiogenic factors in EC (41), whereas cyclin D1, CDK2 and CDK4 are common regulators of the cell cycle and apoptosis (42). Therefore, we hypothesized that MEF2C promotes EC progression partly through the regulation of angiogenesis, cell cycle and apoptosis, which warrant further research. Additionally, CDK4/6 inhibitors such as palbociclib and abemaciclib, were demonstrated to exhibit preliminary anti-tumor potency in EC cell lines and animal models, and particularly in patients with genomic aberrations that activate cyclin D1 (43,44). Further research focusing on the detailed molecular networks of miR-638/MEF2C/cyclin D1 signaling may lead to the improved identification of patients who could benefit the most from CDK4/6 inhibitors.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HW and YR designed the study. JN and SL conducted the experiments and wrote the manuscript. BS and WT performed the experiments. JN, SL and YR analyzed the data. All authors read and approved the final version of the manuscript.

Ethics committee approval and patient consent

The study was approved by the Committee for the Ethical Review of Research at Fudan University Shanghai Cancer Center, and written informed consent was obtained from the patients. The protocol for the animal study was approved by the Committee on the Use of Live Animals in Teaching and Research, Fudan University, Shanghai.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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