

## Original Article

# Overexpression of MEF2D contributes to oncogenic malignancy and chemotherapeutic resistance in ovarian carcinoma

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**Abstract:** The transcription factor MEF2 promotes survival in various cell types and a number of studies indicate that abnormal regulation of MEF2 is linked to oncogenicity in several carcinomas. We have found that MEF2D, a member of the MEF2 family, is upregulated in Ovarian Cancer (OC). Immunohistochemistry analysis of tumor sections of 402 OC patients revealed that MEF2D is significantly elevated at the protein level. We have also found that the expression level of MEF2D is associated with cisplatin-resistance and poor prognosis by a retrospective analysis. Furthermore, Downregulation of MEF2D by siRNA reduces proliferation and invasiveness of OC cells SKOV3 and OVCAR3, induces apoptosis in vitro, and abolishes OVCAR3 tumorigenicity in xenograft model. Mechanistic study via ChIP analysis identified two of MEF2D-targeted genes, HPSE and IKBKE, which are associated with tumor invasion and chemotherapy-resistance, in accord with MEF2D expression in OC. Remarkably, knock-down of MEF2D invariably lead to the downregulation of IKBKE and reversed cisplatin (DDP)-resistance in cisplatin-resistant cells SKOV3-DDP. Our results suggest that MEF2D promotes malignant biological behaviors and cisplatin-resistance in OC and establish MEF2D as a new therapeutic target in OC treatment.

**Keywords:** Ovarian cancer (OC), MEF2D, cisplatin-resistant, IKBKE, HPSE

## Introduction

Ovarian cancer (OC) is ranking the fifth among the most common cancer caused death in women, leading to the death of more than 150,000 patients per year worldwide [1, 2]. It is estimated that over 70% of OC patients are diagnosed at advanced stage of the disease, whereas the overall 5-year survival rate of epithelial OC remains only about 30% [3, 4]. The prognosis of OC is very poor on account of late diagnosis and limited options of effective treatment, this is mainly because of chemotherapy resistance post-surgery. The first-line or common chemotherapy after surgery is platinum, such as cisplatin or carboplatin combined with taxol [5-8]. Although 80 percent of patients are sensitive to first-line chemotherapy, over 80 percent of these patients develop resistance in the pro-

cess of chemotherapy [9, 10]. Thus, study of the molecular mechanisms underlying OC and the search for promising novel therapeutic strategies will be indispensable for improving the prognosis of patients with OC.

The myocyte enhancer factor 2 (MEF2) family of human transcription factors, consisting of four subtypes, MEF2-A, -B, -C and -D [11], play an important role in the transcription regulation of genes that control cell differentiation, proliferation, and apoptosis in a wide range of cell types [12-14]. Members of MEF2 family have been implicated in the development of a variety of cancers. MEF2B and MEF2C are highly mutated in diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) [11, 12, 15]. Chromosomal translocation in a rare form of acute lymphoblastic leukemia (ALL) results in fusion

between MEF2D and the RNA binding protein DAZAP1, and the fusion proteins MEF2D-DAZAP1 and DAZAP1-MEF2D appear to be oncogenic [13-15]. Retroviral insertion studies show that MEF2C and MEF2D are frequent targets of deregulation in mouse models of leukemia/lymphoma [16, 17], the viral integration events generally activate the expression of MEF2, which in turn cooperates with other oncogenic factors to drive tumor development [18, 19]. The expression of MEF2C is also activated in a subset of T-ALL cell lines and the overexpressed MEF2C promotes oncogenesis by inhibiting the expression of apoptosis factors such as NR4A1/NUR77 [20]. Overexpression of MEF2C is also associated with the self-renewal program in leukemia stem cells (LSC) induced by the MLL-AF9 fusion protein, a translocation product found in mixed lineage leukemia (MLL). Knockdown of MEF2C by short hairpin RNA inhibited tumor induction by LSC in vitro and in vivo [21]. These data suggest that deregulation of MEF2, either through mutation or aberrant expression, can lead to cancer development.

The present study focuses particularly on MEF2D. Since Prima and Hunger reported the first evidence of MEF2D as an oncogene in human leukemia malignancy [17], there have been several studies exploring the role of MEF2D in hepatocellular carcinoma; these studies reveal elevated expression of MEF2D in pancreatic cancer, nasopharyngeal carcinoma, non-small cell lung cancer and colorectal cancer [18-22]. Through the Oncomine database ([www.oncomine.org](http://www.oncomine.org)) we found that the DNA and mRNA copies of MEF2B and MEF2D were upregulated in OC compared with normal ovarian. Similar search in Cbioportal database ([www.cbioportal.org](http://www.cbioportal.org)) identified the highest genetic amplification of MEF2A and MEF2D in OC compared to other MEF2s. Furthermore, it has been reported that MEF2D expression was associated with OC progression-free time in public datasets [23]. However, the precise role of MEF2D in the initiation and progression of OC is still unclear.

We first evaluated the expression of MEF2D at protein levels in human OC samples and investigated correlations between MEF2D expression and clinicopathological parameters. We further showed that MEF2D knock-down could impair cell proliferation and migration, and

induce apoptosis, and abolish OVCAR3 tumorigenicity in xenograft model. Through ChIP analysis we have identified two MEF2D target genes, HPSE and IKBKE, which are associated with tumor invasion, metastasis, and cisplatin-resistance. Moreover, MEF2D knock-down resulted in IKBKE and HPSE downregulation and reversal of cisplatin (DDP) resistance in cisplatin-resistant cells SKOV3-DDP. Our findings suggest that MEF2D functions as a key oncogenic driver and also confers drug resistance in OC and could serve as a potential therapeutic target for treating this devastating disease.

### Material and methods

#### *Cell lines culture and establishment of DDP resistant cells*

Human ovarian cancer OVCAR3, SKOV3 cell lines and normal human ovarian epithelial cells IOSE were obtained from Sciencell (California, USA), and authenticated by short tandem repeat. All of the cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640; Gibco, USA), supplemented with 10% FBS (Gemini, France), 100 U/ml penicillin and 100 ug/ml streptomycin at 37°C in a 5% CO<sub>2</sub>, 95% air environment in humidified incubators. To establish DDP resistant OC cells, SKOV3 cells were treated with DDP (Shandong Qilu Pharmaceutical Factory, Shandong, China) in a step-wise manner from 0.2 to 2 ug/L over a period of 6 months. The DDP resistant cells were named as SKOV3/DDP.

#### *Immunohistochemistry and clinical samples*

Formalin-fixed paraffin-embedded human OC specimens from 105 patients (median age, 43 years) during 2010-2013 from Hunan Provincial Tumor Hospital and 297 patients (median age, 47 years) during 2010-2017 from The Second XiangYa Hospital of Central South University after obtaining written, informed consent from patients. Our experiment was approved by Medical Ethics of The Second XiangYa Hospital of Central South University. The average follow-up time of 3-year survival analysis was 31 months, and the longest was 48 months. The average follow-up time of 5-year survival analysis was 38 months, and the longest was 80 months. OC patients' pathological classification are according to the criteria of WHO Classification of Tumors of Female Reproductive Organs (WHO,

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2014). OC diagnosis was confirmed by histological examination. All patients were staged in accordance with FIGO standards (2009). OC patients were followed up by gynecologic reexamination after operation and chemotherapy. More than five years after treatment, the survival rate was evaluated by phone or with a questionnaire to check their health status. Then the tumor sections were treated with 0.01 mol/L sodium citrate buffer (pH 6.0) for antigen retrieval, followed by blocking with 3% hydrogen peroxide. The expression of MEF2D was assessed using standard immunohistochemical methods with primary antibody against MEF2D (Abcam, Cambridge, UK; 1:100 dilution), and secondary antibody. The intensity and extent of MEF2D staining signals were independently evaluated by two different pathologists. Staining intensity was graded as follows: 0, no staining; 1, mild staining; 2, moderate staining; and 3, intense staining. The staining area was scored as follows: 0, no staining of cells; and 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100% of cells stained. A summary of staining score (intensity and extension) index was used as the final staining score, graded as follows: 0-1, negative; 2-4, weakly positive; and 5-7, strongly positive, where both weakly positive and strongly positive samples were considered as positive for MEF2D expression.

### *RNA extraction and Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)*

Total RNAs were isolated from cells using a Trizol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was prepared using PrimeScript® RT reagent Kit (Takara, RRO47A, Dalian, China) following standard protocols. qPCR was performed using SYBR® Premix Ex Taq™ II (Takara, RR820A, Dalian, China) on the real time PCR instrument (Light Cycler 96, Roche) with the following two-stage program parameters: preincubated for 60s at 95°C and then 40 cycles of 5 sec 95°C and 30 s at 60°C. Results were shown as target mRNA levels relative to housekeeping gene GAPDH. The sequences of the primers used for this analysis are as follows:

MEF2D Forward, 5'-AGGGAATAACCAAAAAAC-TACAAA-3'; MEF2D Reward, 5'-GCTACATGAACACAAAAACAGAGACC-3'; IKBKE Forward, 5'-GAGAAGTTCGTCTCGGTCTATGG-3'; IKBKE Reward, 5'-TGCATGGTACAAGTCACTCC-3'; HPSE Forward, 5'-ACCAAACCTCAGGTACGCAG-3'; HP-

SE Reward, 5'-GTTCTGTCCGTCACCATTG-3'; DUSP6 Forward, 5'-ATGATAGATACGCTCAGACCG-3'; DUSP6 Reward, 5'-GATGTGCGACGACTCGTATAG-3'; KLF6 Forward, 5'-CTGCAGGAAAGTTTACACAAA-3'; KLF6 Reward, 5'-ACTCATC-ACTTCTTGCAAAACG-3'; KLF4 Forward, 5'-CAGCTTACCTATCCGATCC-3'; KLF4 Reward, 5'-GATGTACACCGGGTCCAATTC-3'; GAPDH Forward, 5'-CAGGAGGCATTGCTGATGAT-3'; GAPDH Reward, 5'-GAAGGCTGGGGCTCATTT-3'. All experiments were performed in triplicate.

### *Western blotting*

Treated cells or tissues were lysed on ice in RIPA buffer (Dingguo, China) containing 1 mmol/L fluoride (PMSF, Dingguo, China). Cell lysate or tissue samples containing 20 µg of protein were loaded into the wells of 8-12% sodium dodecyl sulfate-polyacrylamide gels electrophoresis carried out for 0.5 h at 80 V and then 1 h at 120 V, and proteins were transferred to PVDF membranes (Millipore, Bedford, MA). Membranes were then blocked in 5% skim milk for 1.5 h at room temperature, followed by incubation overnight at 4°C with specific primary antibodies (1:1000 dilution). After washing with TBST buffer three times (15 min each), membranes were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (1:10000 dilution; Proteintech, Chicago, IL) at room temperature for 1 h. Proteins were then detected using enhanced chemiluminescence reagents (NCM, China). The primary antibodies used in our study included anti-MEF2D, anti-cleaved caspase3 (Abcam, Cambridge, UK), anti-cyclinD1, anti-c-Myc, anti-caspase3 (Proteintech), anti-MMP9, anti-HPSE, anti-IBKKE (abclonal, Wuhan, China). GAPDH (Proteintech) was used as a loading control. Each experiment was performed in triplicate.

### *Small interfering RNA (siRNA) transfection*

Three different sequences targeting MEF2D were designed and provided by RIBOBIO (Guangzhou, China). siRNA transfection was performed according to the manufacturer's protocol. Briefly, cells were seeded into 6-well plates at a density of  $2 \times 10^4$  cells/well. After reaching 50%-60% confluence, cells were transfected with siRNA using riboFECT™ CP. After 24-48 h of incubation, knockdown efficiency was evaluated by qRT-PCR and Western Blot analysis.

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siRNAs with optimized knockdown efficiency were selected for subsequent experiments. The target sequences of the MEF2D siRNAs in MEF2D were as follows:

siMEF2D001GCAACAGCCTAAACAAGGT; siMEF2D002GTCTCCCAGTCTACTCATT; siMEF2D003-GCAACGCCGAGTTTACTCA.

### *Cell proliferation assays*

In the cell proliferation assay, cells were seeded in 96-well plates at the concentration of  $1 \times 10^4$ /well and transfected with MEF2D siRNA or negative control siRNA for 12, 24, 48 or 72 at 37°C with 5% CO<sub>2</sub>, after which 10 µl of the Cell Counting Kit-8 solution (CCK-8, Genview, China) was added to the medium. After 30 minutes incubation, the amount of orange formazan dye generated was determined by measuring the absorbance at 450 nm under microplate reader (Thermo Scientific, Carlsbad, CA, USA).

### *Cell invasion assays and cell scratch-wound assays*

Cell migration and invasion assays were performed using transwell inserts with 8-µm-pore filters (Corning, NY, USA). After transfection with MEF2D siRNA or negative control siRNA, cells were suspended in 200 µL of serum-free medium and reseeded in the upper chamber of Transwell inserts in 24-well plates. In invasion assays, the transwell membrane were precoated with Matrigel (Corning, NY, USA) and cultured for 16-24 h at 37°C. The bottom wells were filled with RPMI-1640 (Gibco, USA) supplemented with 20% FBS (Gemini, France). After culture for 24 or 48 h, the cells remaining on the surface of the upper well were removed using a cotton swab and the cells that crossed the filters were fixed in 10% formalin and stained with Crystal Violet Staining Solution (Beyotime, Shanghai, China). The cells were then photographed under an inverted microscope and counted at 100 × magnification. In cell scratch-wound assays, treated cells were seeded into 12-well plates and cultured until confluence. A wound was generated by scraping with a 100-µL pipette tip. The cells in the wounded monolayer were photographed at 100 × magnification and cell migration was assessed by measuring gap sizes in multiple fields at 0 h, 24 h and 36 h later.

### *Apoptosis assays*

To assess tumor cell apoptosis, we employed Annexin V-FITC/PI Apoptosis kit. Ovarian cancer cell lines OVCAR3 and SKOV3 were transfected by siMEF2D RNA or negative control siRNA. 2 days later cells were suspended in 200 µL binding buffer, and then 5 µl AnnexinV-FITC reagent and 5 µl PI Staining solution were added and further incubated for 10 min. Cell apoptosis rate was analyzed using flow cytometry (Millipore, USA).

### *Chromatin immunoprecipitation assay*

Chromatin immunoprecipitation assay (ChIP) was done following a procedure provided by a ChIP Assay Kit (Millipore). OVCAR3 and SKOV3 cells were cross-linked by formaldehyde (final concentration of 1%) for 10 min at 37°C, and then washed by cold PBS twice. The cells were lysed by 100 µl 1% SDS lysis buffer and sheared by sonication. Proteins and DNA were pulled down with MEF2D monoclonal antibody (61-0774, BD, San Jose, CA, USA). Anti-RNA Polymerase II was used as a positive control and normal mouse IgG as a negative control. Products of ChIP-PCR were separated on a 2% agarose gel. Immunoprecipitation of ChIP proteins were confirmed by Western blot analysis using anti-MEF2D.

The primers for ChIP-PCR were: HPSE1 (Sense primer) 5' TTCCTCGGCTCAAGCAATC 3'; (Anti-sense primer) 5' TGTACCACCAATAAGGCAACAA 3'; HPSE2 (Sense primer) 5' TTCCTCGGCTCAAGCAATC 3'; (Anti-sense primer) 5' TGTACCACCAATAAGGCAACAA 3'; IKBKE1 (Sense primer) 5' CTAGACCAACCTGCTCAATCCA 3'; (Anti-sense primer) 5' CCAAAGTGCCGGGATTACA 3'; IKBKE2 (Sense primer) 5' GTGGATCAGGAGGTTGGGAGA 3'; (Anti-sense primer) 5' CTGGGTCAAGTGATTCTTCTGC 3'.

### *In vivo xenograft study*

All animal experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Central South University, China. All animals were housed in a pathogen-free facility and maintained in a standard temperature- and light-controlled animal facility. Female SCID mice (4-6 weeks old) were

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purchased from Hunan SJA Laboratory Animal Co. Ltd in Changsha, China. All mice were injected subcutaneously with  $2 \times 10^6$  OVCAR3 cells suspended in 0.2 ml Matrigel. Mice were randomly selected for treatment with MEF2D siRNA or negative controls provided by RIBOBIO (Guangzhou, China), when tumor volume reached 200 to 500 mm<sup>3</sup>. For delivery of cholesterol conjugated RNA, 5 nmol RNA in 0.1 ml normal saline was locally injected into the tumor mass once every 3 days. Tumor size and the body weight of mice were measured 3 times each week. Mice were sacrificed after a moribund state was noted, as defined by evident, persistent shivering, extreme prostration, labored breathing, greater than 20% of body weight loss and/or a tumor greater than 2 cm with diabrosis. And then the MEF2D expression analysis of tumor mass was conducted by immunohistochemical evaluation.

### Statistical analysis

Data are presented as means  $\pm$  standard deviation (SD). Statistical analysis was performed using Graphpad software (version 6.0). Data were evaluated using ANOVA with LSD test for multiple comparisons and Student's *t* test between two groups. *P* < 0.05 was considered as statistical significant.

## Results

### Aberrant expression of MEF2D in OC

Based on public data deposited in Oncomine (www.oncomine.org), we found that MEF2D DNA copy number and mRNA expression is significantly upregulated in ovarian cancer tissues compared to normal ovarian tissues (**Figure 1A**). We assessed MEF2D protein level by western blot in two OC cell lines (OVCAR3, SKOV3) and normal Human Ovarian Epithelial Cells (IOSE), and the results showed that MEF2D was overexpressed in cancer cell lines compared with the normal IOSE (**Figure 1B**). To confirm the overexpression of MEF2D in ovarian carcinogenesis, Western blot was used to analyze 4 fresh ovarian normal tissue specimens and 12 fresh OC tissue specimens. MEF2D levels in OC tissues were greatly upregulated compared with normal ovarian tissues (**Figure 1C**). Encouraged by the consistent observations of MEF2D overexpression in OC in these studies, we conducted an extensive analysis of 402 OC

patient specimens at protein level by immunoblotting, while 10 normal ovarian samples were included as negative controls. Subsequently, all OC specimens were classified into groups with negative or positive MEF2D expression via quantitative analysis of protein staining. MEF2D protein was not detectable in normal ovarian samples while positive expression of MEF2D was observed in 237 of 402 (59.0%) OC tissues (**Figure 1D**). These results indicated that MEF2D expression was upregulated in both OC tissues and cells. Moreover, as described below, we have found that two of MEF2D target genes, IKBKE and HPSE, are highly correlated with the expression of MEF2D in OC. IKBKE and HPSE were found to be positively expressed in 15 of 20 (75%) and 13 of 20 (65%) of OC tissues respectively, while not detectable in normal ovarian samples (**Figure 1E**). Thus, IKBKE and HPSE were also upregulated in OC tissues compared with normal ovarian.

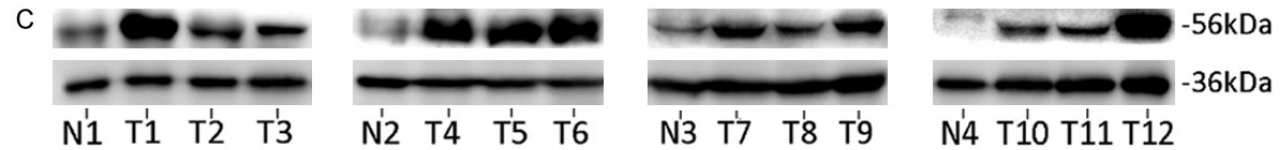
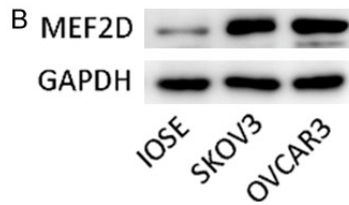
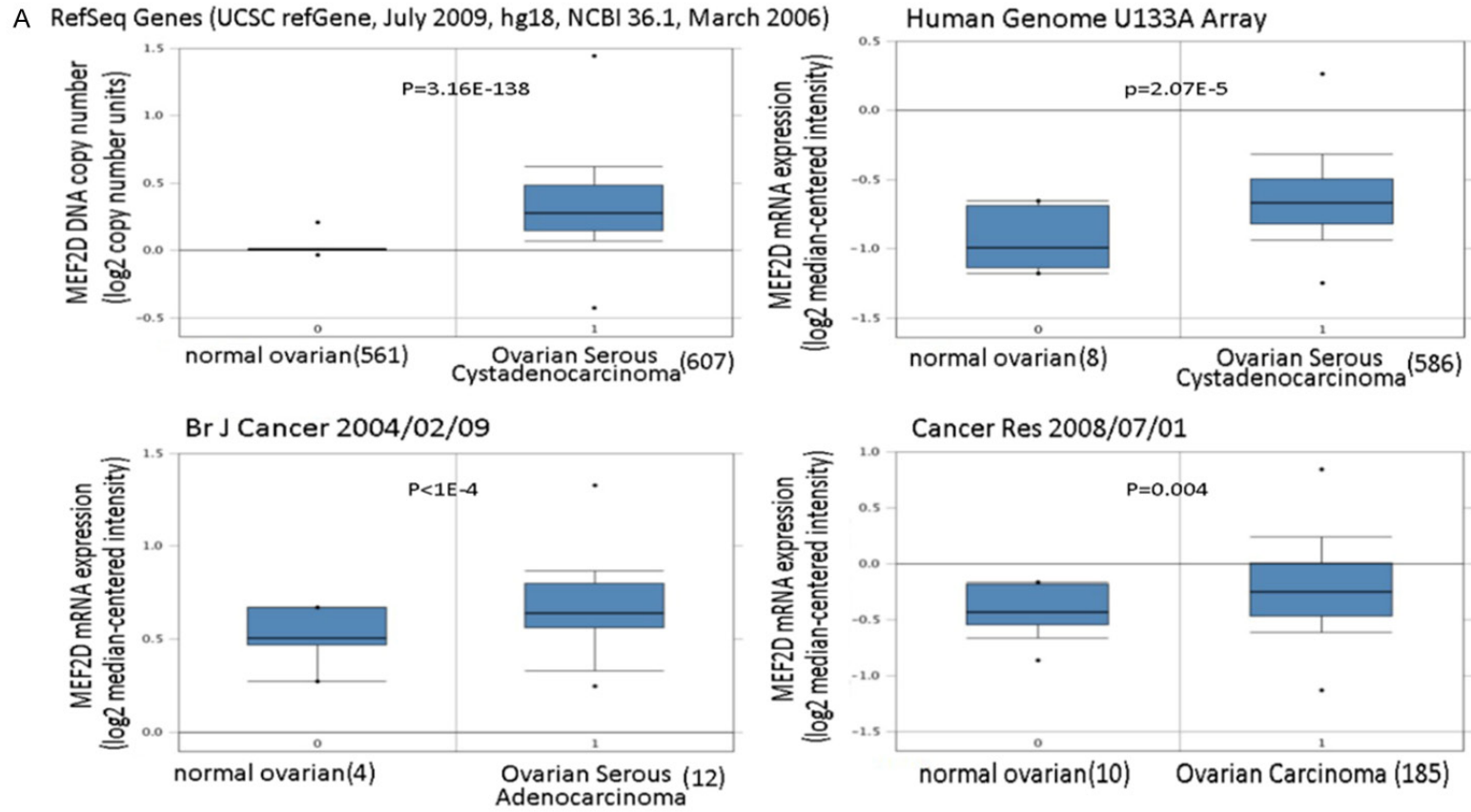
### MEF2D overexpression correlates with cisplatin-resistance and poor prognosis in OC patients

The clinical relationship between MEF2D expression and clinicopathological parameters in OC was further analyzed in a retrospective analysis to explore the importance of MEF2D expression through 402 OC samples mentioned above through IHC analysis. The representative images of OC samples with positive or negative MEF2D expression were shown in **Figure 2A**. 59.0% of OC samples showed positive MEF2D expression, whereas the percentages within chemotherapy resistance and sensitive subgroups were 80.1% and 48.7% respectively (*P* < 0.001). It also showed that high level of MEF2D was correlated with clinical stage (*P* = 0.046), pathological grade (*P* = 0.033) and histologic type (*P* = 0.028) (**Figure 2B**). Retrospective analysis also suggested that high level of MEF2D was correlated with 3-year survival rate (*P* = 0.0359) and 5-year survival rate (*P* = 0.008) (**Figure 2C, 2D**).

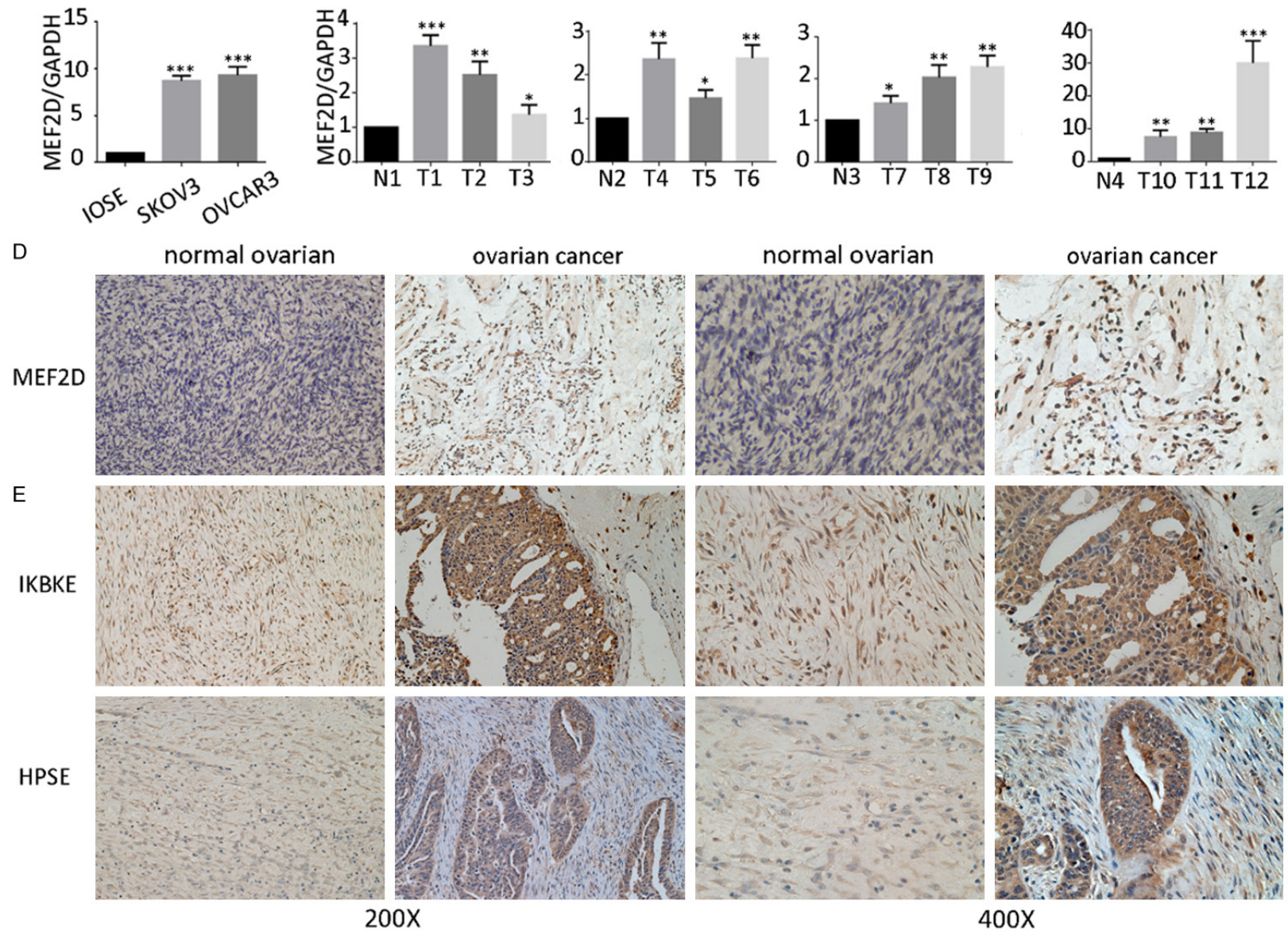
### Downregulation of MEF2D reduces proliferation and induces apoptosis in OC cells in vitro

To uncover the potential functions of MEF2D in OC, we designed 3 different siRNA products (siMEF2D-001, siMEF2D-002, and siMEF2D-003) and a scrambled siRNA as the negative control (siRNA-NC). Upon transfection into OV-

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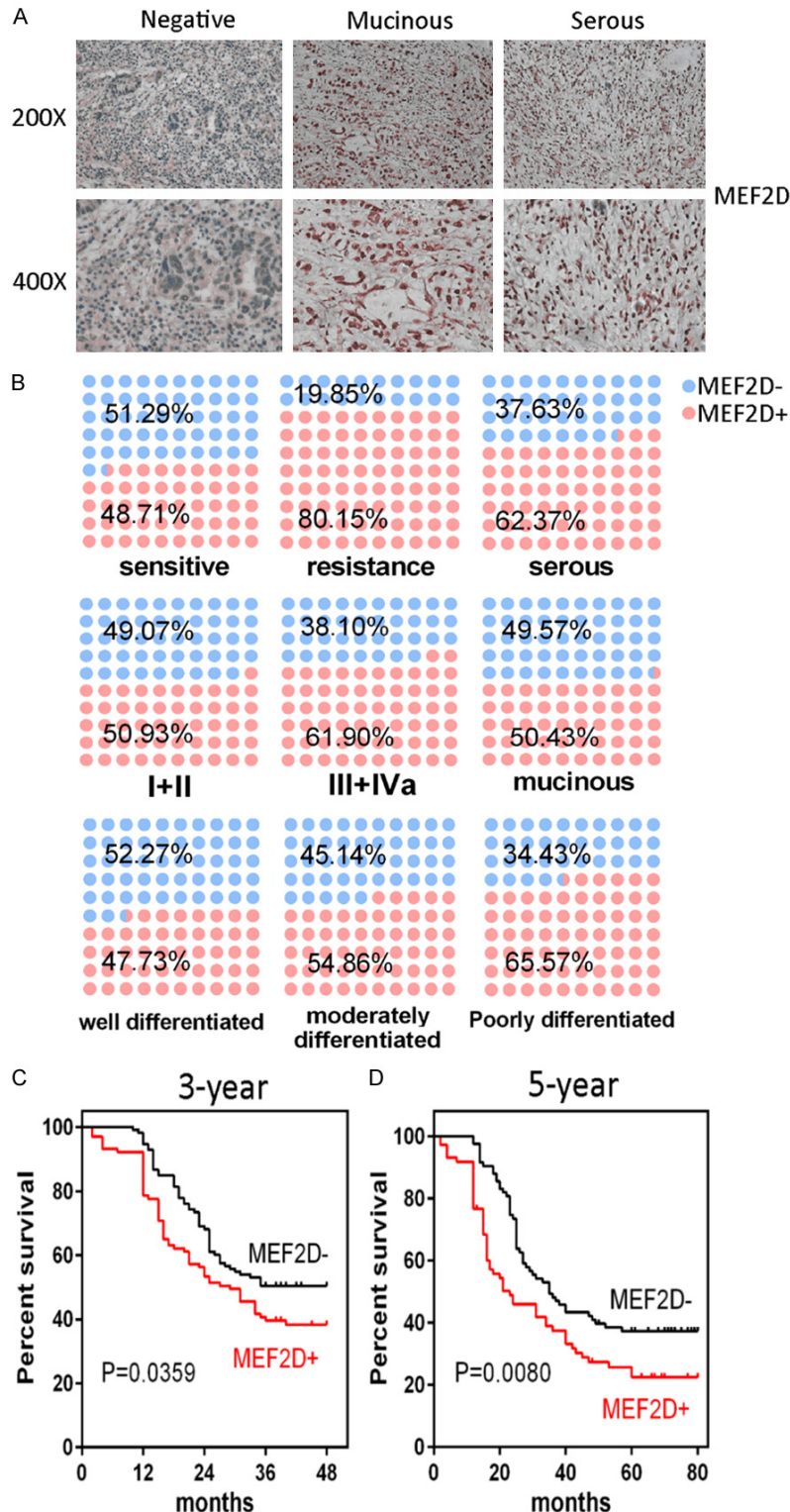
MEF2D promotes oncogenic malignancy and chemotherapeutic resistance in OC



**Figure 1.** Analyses of MEF2D expression in OC cell lines and patient samples. A. Data on MEF2D DNA copy number and mRNA expression in ovarian cancer and normal ovarian tissue from several study groups deposited in the OncoPrint database ([www.oncoPrint.org](http://www.oncoPrint.org)). B. The protein expression of MEF2D in normal human

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ovarian epithelial cells (IOSE) and human OC cells (OVCAR3, SKOV3) was determined by western blot assays. C. The protein expression of MEF2D in normal ovarian tissues (N1-N4) and human OC tissues (T1-T12) was determined by western blot assays. Error bars represent the s.d. of triplicate measurements. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . D. IHC analysis of MEF2D protein expression in normal ovarian tissues, and OC tissues Original magnifications:  $\times 200$  and  $\times 400$ . E. IHC analysis of IKBKE and HPSE protein expression in normal ovarian tissues, and OC tissues Original magnifications:  $\times 200$  and  $\times 400$ .



**Figure 2.** The relationship between MEF2D overexpression and cisplatin resistance & prognosis of OC patients. A. The representative microscopic photographs of negative and positive MEF2D expression in OC tissues by immunohistochemistry. B. Percentages of positive and negative MEF2D expression in different groups are shown in the charts. It showed that high level of MEF2D was correlated with chemotherapy resistance ( $P < 0.001$ ), clinical stage ( $P = 0.046$ ), pathological grade ( $P = 0.033$ ) and histologic type ( $P = 0.028$ ). C. 3-year Kaplan-Meier analysis of overall survival of patients during 2010-2015 with OC according to the expression level of MEF2D protein. D. 5-year Kaplan-Meier analysis of overall survival of patients during 2010-2013 with OC according to the expression level of MEF2D protein.

CAR3 and SKOV3 cells, western blot analysis indicated that both siMEF2D-001 and siMEF2D-002 could effectively knock down MEF2D expression in cell lines OVCAR3 and SKOV3. Thus, siMEF2D-001 and siMEF2D-002 were used in subsequent experiments. siMEF2D-001, siMEF2D-002 or siRNA-NC was transfected into SKOV3 and OVCAR3 cell lines, and cell growth assays were performed using CCK8 kits. The resulting growth curves demonstrated that knockdown of MEF2D in both OVCAR3 and SKOV3 cells significantly inhibited cell proliferation,



compared with the negative controls (**Figure 3A**). Moreover, the expression of proliferation-related protein C-myc and cyclin-D1 were evaluated by Western blot assays. Compared with the negative control group, knockdown of MEF2D in SKOV3 cells led to inhibition of the expression of C-myc and cyclin-D1 (**Figure 3C**). These results suggested that MEF2D are required in maintaining the proliferation of OC cells. To further investigate cell apoptosis in MEF2D knockdown OC cells, we assessed Annexin V-FITC/PI by flowcytometry. The rate of apoptosis was significantly higher in SKOV3 and OVCAR3 cells with siMEF2D than that in negative control (**Figure 3B**). Meanwhile, caspase3 and caspase8 expression in cells transfected with siMEF2D or negative control was assessed by western blot assays, and the result showed that apoptosis-related protein caspase3 and its cleaved form instead of caspase8 were much higher in cells transfected with siMEF2D001 and 002 than cells transfected by NC (**Figure 3C**). Taken together, these results suggest that MEF2D is involved in OC cell proliferation and survival by antagonizing intrinsic apoptosis pathways.

### *MEF2D promotes invasion and migration of OC cells in vitro*

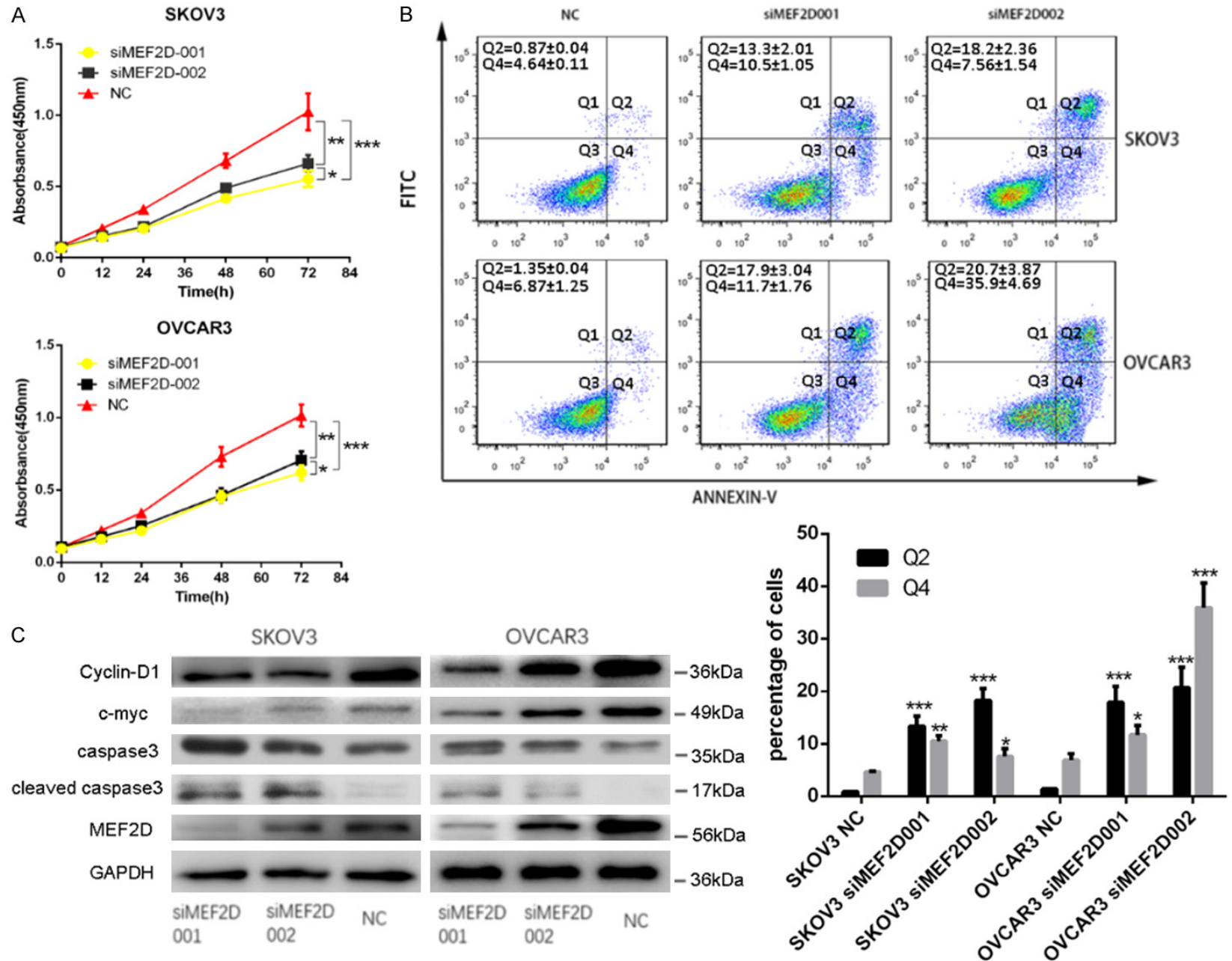
To further explore the functions of MEF2D in the tumorigenesis of OC, we next studied the impact of MEF2D on cell migration and invasion using cell scratch-wound healing assays and transwell cell migration and invasion assays. Scratch-wound healing assays revealed that silencing MEF2D in OVCAR3 and SKOV3 cells resulted in reduced wound healing ability, compared with negative control cells (**Figure 4A**). The results of transwell cell migration and invasion assays demonstrated that knockdown of MEF2D significantly reduced the number of cells on membrane filters compared with controls (**Figure 4B**). MMP9, which is a MEF2 targeted gene involved in cancer cell invasion and metastasis, was shown to be downregulated in OC cells transfected with siMEF2D, compared with negative control cells (**Figure 4C**). These results demonstrated that MEF2D plays a role in the invasion and metastasis of OC cells.

### *MEF2D induces the expression of HPSE and IKBKE in OC cells*

To further study the mechanism of MEF2D function in OC, we used GEO datasets (GDS-

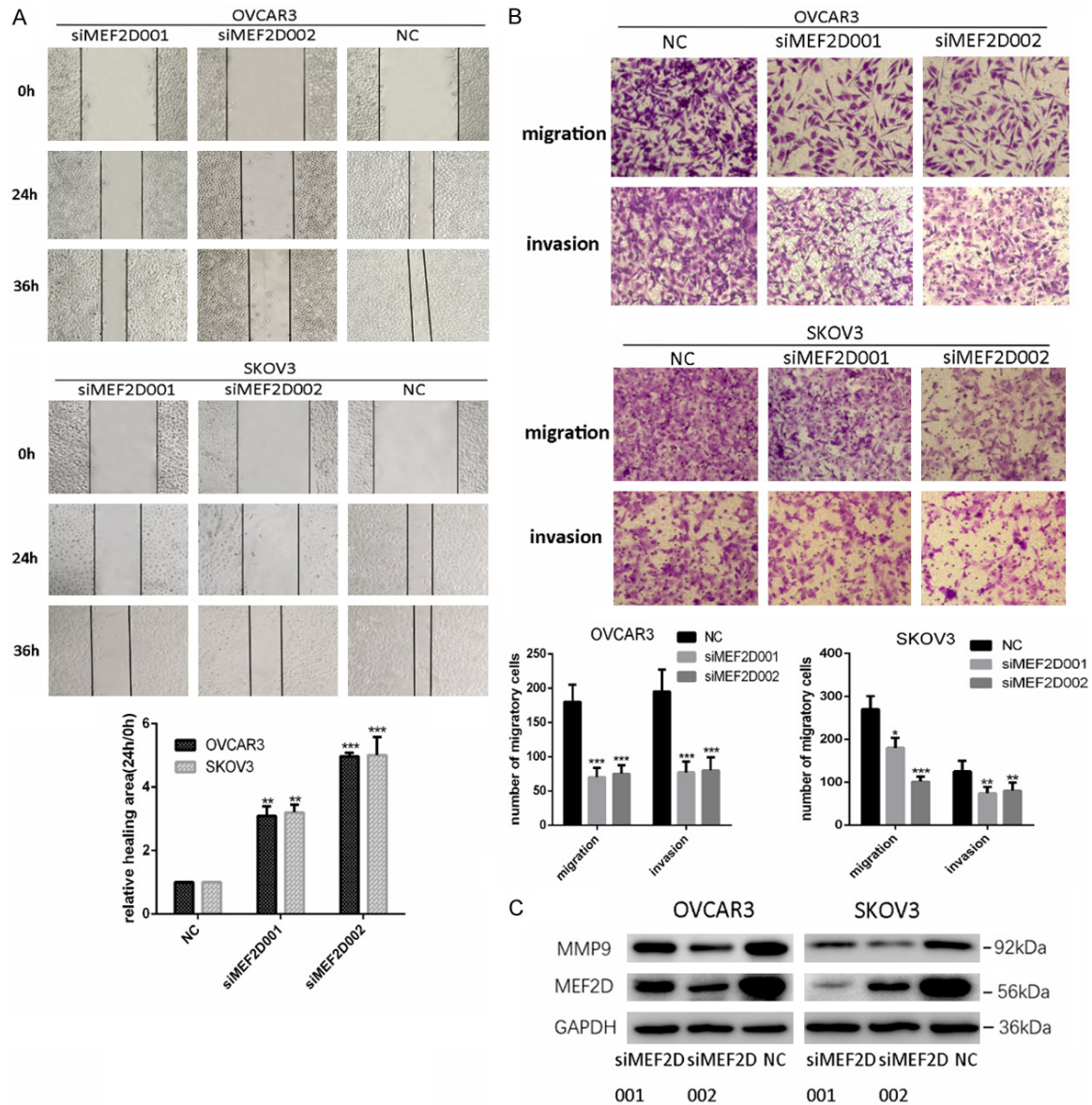
4950, GDS3952) to screen possible downstream genes of MEF2D through bioinformatics analysis tools. We identified several genes that were highly correlated with MEF2D and had also been reported as oncogenes in OC. We then investigated the binding scores of these candidate genes with MEF2D through prediction websites ([www.jasper.com](http://www.jasper.com)), which led us to choose HPSE, IKBKE, DUSP6, KLF4 and KLF6 for further investigation. To assess the role of MEF2D in these genes, we transfected SKOV3 and OVCAR3 cells with siMEF2D001 and siMEF2D002. The mRNA levels of HPSE, IKBKE, DUSP6, KLF4 and KLF6 assayed with qRT-PCR showed that silencing MEF2D expression significantly reduced the level of HPSE and IKBKE transcripts and elevated DUSP6 transcripts, while having no obvious effect on the mRNA expression of KLF4 or KLF6. (**Figure 5A**). Reducing MEF2D level also significantly downregulated the expression of HPSE and IKBKE as determined by Western blot assays (**Figure 5B**), but we haven't found the expression of DUSP6 upregulated after reducing MEF2D level. We then choose HPSE and IKBKE for further investigation. As a well-known oncogene, HPSE has been reported to be upregulated and play a critical role in proliferation and invasion in OC [24, 25], meanwhile IKBKE has also been reported to participate in tumor progression and cisplatin-resistance in OC [26]. Analysis of the HPSE and IKBKE gene promoter reveals the presence of a putative MEF2D binding site (5'-ACTAAAATAGA-3') shared by the two genes and MEF2D has high binding scores of these two genes through prediction websites ([www.jasper.com](http://www.jasper.com)) (**Figure 5C**). To test whether MEF2D binds to the promoters of HPSE and IKBKE, we carried out Chromatin immunoprecipitation (ChIP) assays. Anti-MEF2D antibody was used to immunoprecipitate the MEF2D-DNA in OVCAR3 and SKOV3 cells, while anti-RNA Polymerase II and IgG were used as positive and negative control. First, we confirmed that the anti-MEF2D antibody efficiently immunoprecipitated MEF2D protein (**Figure 5D**). PCR product of HPSE and IKBKE were both observed in the presence of MEF2D antibody and anti-RNA Polymerase II, but not in negative control IgG (**Figure 5D**). Our ChIP analysis showed that MEF2D bound specifically to a region within HPSE and IKBKE gene promoter respectively that contains the putative MEF2 site in SKOV3 and

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**Figure 3.** Effect of MEF2D knockdown on proliferation and apoptosis of OC cells in vitro. A. CCK-8 assays were performed to determine the effects of MEF2D knockdown on the proliferation of SKOV3 and OVCAR3 cells. Cell viability was determined at 0, 12, 24, 48 and 72 h. B. Flow cytometry assays were performed to determine the effects of MEF2D knockdown on the apoptosis of SKOV3 and OVCAR3 cells. C. Effects of MEF2D knockdown on proliferation-associated protein cyclin-D1 and c-myc and apoptosis-related protein caspase3 and cleaved caspase3 were analyzed by western blotting in SKOV3 and OVCAR3 cells. Error bars represent the s.d. of triplicate measurements. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

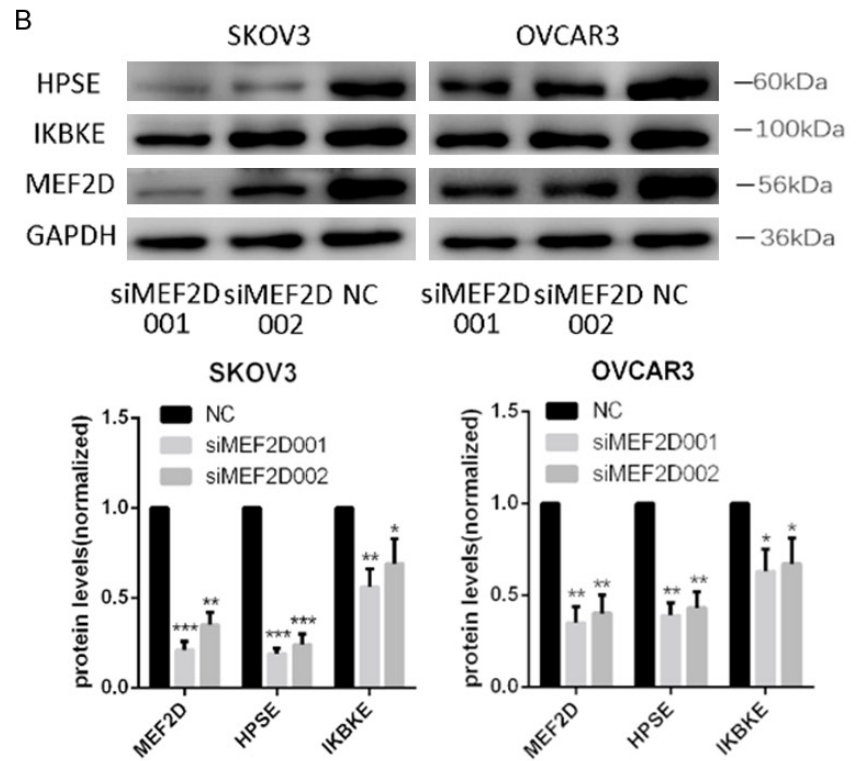
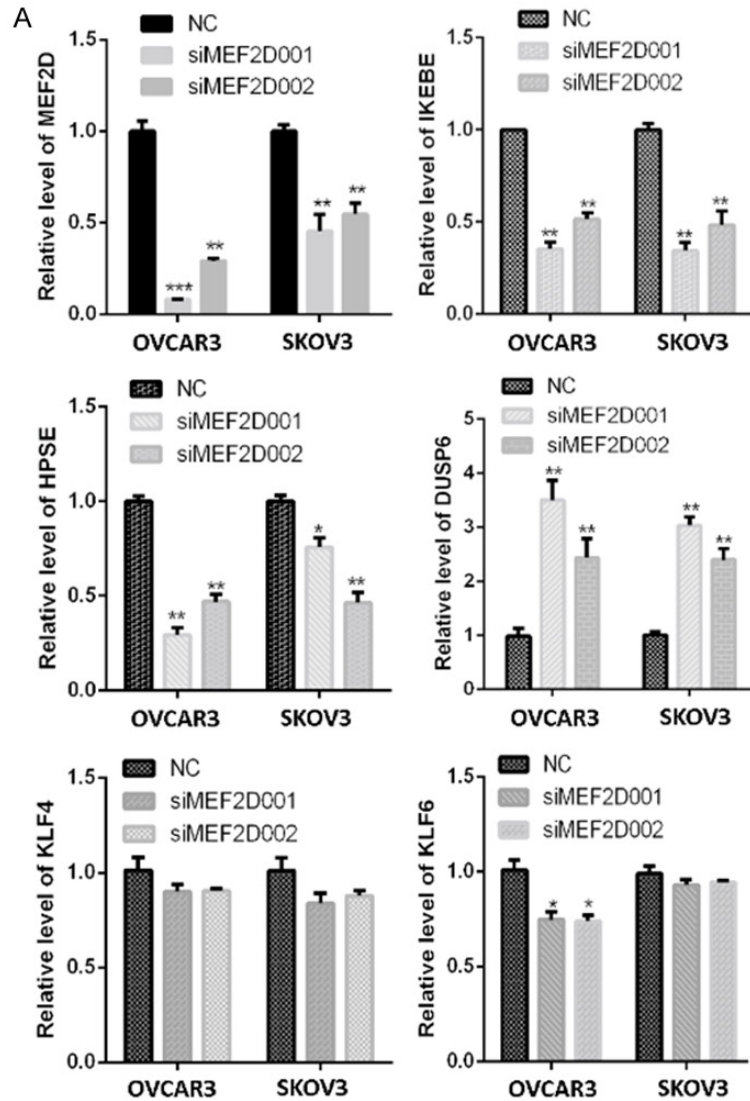


**Figure 4.** Effect of MEF2D knockdown on invasion and migration of OC cells in vitro. A. The migration abilities of SKOV3 and OVCAR3 were measured through testing the wound closure after MEF2D knockdown using wound healing assays. B. Transwell assays were used to detect the migration and invasion abilities after MEF2D knockdown in SKOV3 and OVCAR3 cells. Original magnifications,  $\times 200$  and  $\times 400$ . C. Effects of MEF2D knockdown on migration associated protein MMP9 were analyzed by western blotting in SKOV3 and OVCAR3 cells. Error bars represent the s.d. of triplicate measurements. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

OVCAR3 cells. These data suggest that MEF2D is likely an activator of IKBKE and HPSE gene

transcription in SKOV3 and OVCAR3 cancer cells.

MEF2D promotes oncogenic malignancy and chemotherapeutic resistance in OC



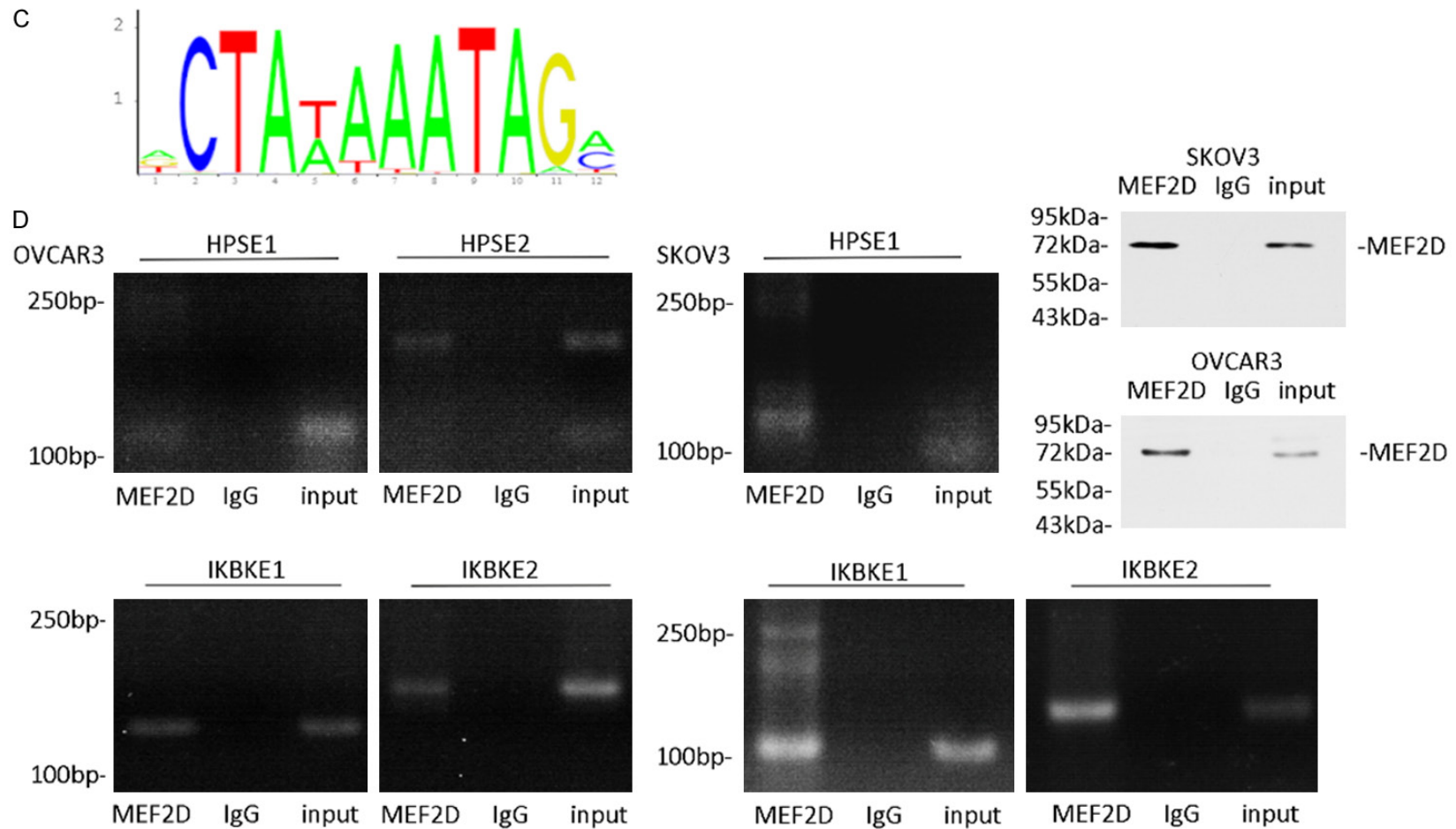
2 putative sites were predicted with these settings (80%) in sequence named **IKBE**

Model ID	Model name	Score	Relative score	Start	End	Strand	predicted site sequence
MA0773.1	MEF2D	12.617	0.917395744621662	608	619	1	actaaaaataca
MA0773.1	MEF2D	12.617	0.917395744621662	752	763	1	actaaaaataca

7 putative sites were predicted with these settings (80%) in sequence named **HPSE**

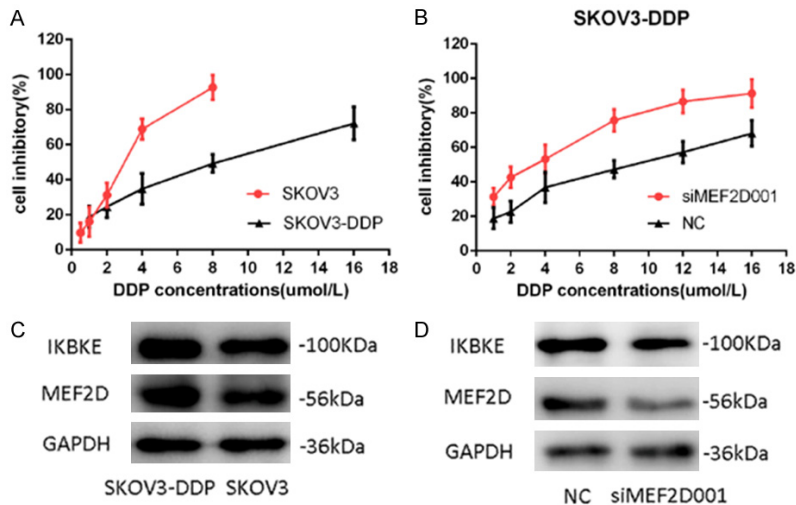
Model ID	Model name	Score	Relative score	Start	End	Strand	predicted site sequence
MA0773.1	MEF2D	12.617	0.917395744621662	61	72	1	actaaaaataca
MA0773.1	MEF2D	3.691	0.818310409898679	526	537	-1	aataataataat
MA0773.1	MEF2D	3.691	0.818310409898679	529	540	-1	aataataataat
MA0773.1	MEF2D	5.293	0.836093818617726	535	546	-1	tcttaaaaaataat
MA0773.1	MEF2D	4.591	0.828301088954323	697	708	-1	acaaaaaataca
MA0773.1	MEF2D	12.998	0.921625132088552	878	889	-1	aataaaaaataga
MA0773.1	MEF2D	4.536	0.827690547456479	1663	1674	1	gctaaatattgt

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**Figure 5.** ChIP analysis of MEF2D targeted genes in OC cells. A. Silencing MEF2D expression significantly reduced the level of HPSE and IKBKE transcript and upregulated DUSP6 transcript examined by qRT-PCR. Error bars represent the s.d. of triplicate measurements. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001. B. Reducing MEF2D level also significantly downregulated the expression of HPSE and IKBKE protein level determined by Western blot. Error bars represent the s.d. of triplicate measurements. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001. C. Analysis of the HPSE and IKBKE gene promoter reveals the presence of a putative MEF2D binding site (5'-ACTAAAATAGA-3') shared by the two genes and putative states of MEF2D binding on HPSE and IKBKE promoter were predicted through prediction websites ([www.jaspar.com](http://www.jaspar.com)). D. In ChIP assays the anti-MEF2D antibody actually immunoprecipitated MEF2D protein. PCR product of HPSE and IKBKE were both observed in the presence of MEF2D antibody and anti-RNA Polymerase II but not in negative control IgG.

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**Figure 6.** Effect of MEF2D knockdown on IKBKE expression and cisplatin resistance in OC cells. A. The sensitivity of SKOV3 parental and DDP-resistant cells (SKOV3/DDP) were evaluated by use of CCK-8 kits. B. siMEF2D can significantly increase the sensitivity of SKOV3/DDP cells to DDP treatment ( $P < 0.05$ ). C. SKOV3/DDP cells were transfected with siMEF2D001 or NC and the effect of MEF2D knockdown was identified by western blots. D. Knocked down MEF2D also reduced IKBKE expression in SKOV3/DDP cells.

*siMEF2D reduces the expression of IKBKE and reverses Cisplatin resistance in cisplatin-resistant OC cells*

To further determine the mechanism of MEF2D function in cisplatin-resistance in OC, we established cisplatin-resistant ovarian cells SKOV3/DDP. Firstly, the sensitivity of SKOV3 parental and DDP resistant cells was evaluated by use of CCK-8 kits. Our data showed that the established DDP resistant cells were much more resistant to DDP treatment as compared to their corresponding parental cells ( $P < 0.05$ ). The 48 h IC<sub>50</sub> values of DDP for SKOV3/DDP and SKOV3 were 10.251 and 3.081  $\mu\text{g/L}$ , respectively (**Figure 6A**). In order to investigate whether MEF2D was involved in the chemoresistance of OC cells, SKOV3/DDP cells were transfected with siMEF2D001 or NC (**Figure 6C**). Our results suggested that siMEF2D can significantly increase the sensitivity of SKOV3/DDP cells to DDP treatment (**Figure 6B**) ( $P < 0.05$ ). We further showed that knocked-down MEF2D also reduced IKBKE expression in SKOV3/DDP cells and reverse cisplatin-resistance (**Figure 6D**).

*Knocked-down MEF2D abolished tumorigenicity of ovarian carcinoma cells in vivo*

The role of MEF2D in tumor formation of OV-CAR3 cells was also investigated in vivo, using

a xenograft model in SCID mice. As shown in **Figure 7A**, tumors treated with MEF2D siRNA were significantly smaller than those treated with scrambled NC RNA. Tumor volume and weight were significantly inhibited in MEF2D-siRNA-injected tumors (**Figure 7A, 7B**), and immunohistochemical analysis showed that the MEF2D-siRNA group exhibited weaker expression of MEF2D (**Figure 7C**). These results suggest that MEF2D plays an important role in promoting OC tumorigenesis in vivo.

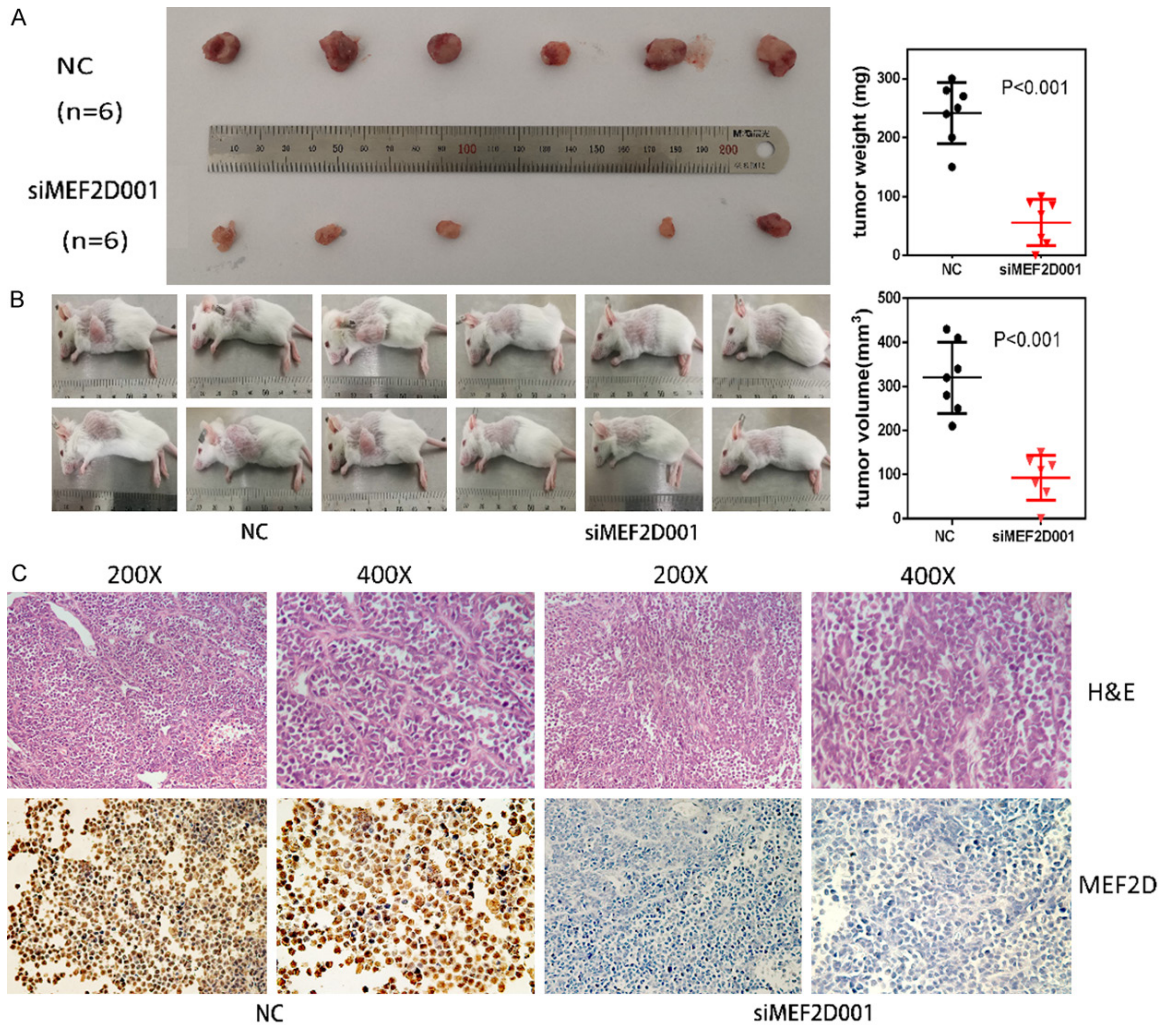
*Proposed model of MEF2D deregulation in ovarian carcinoma cells*

Our results suggest that deregulation of MEF2D plays a significant in ovarian carcinoma. In normal cells the activity of MEF2D is exquisitely regulated by the balancing act of co-repressors such as class IIa HDACs, Cabin 1/Cain, and co-activators such as CBP/p300, to ensure the proper regulation of downstream genes [27-29] (**Figure 8A**). This model of transcription regulation suggests that precise regulation of MEF2-dependent gene expression is critical to the normal function of cells, explaining why deregulation of MEF2 has been associated with a variety of oncogenic processes. In ovarian carcinoma cells (**Figure 8B**), the activity of MEF2D is upregulated by yet to be identified pathogenic mechanisms, leading to the abnormal expression of its downstream genes, including the upregulation of HPSE, IKBKE, MMP9 and possibly other genes. As secondary effects, HPSE could induce the expression of cyclin D1 and MMP9 which participate in cell proliferation and invasion in OC [25]. IKBKE could suppress the caspase3 and induce PARP, which participate in cisplatin-resistance in OC [26].

### Discussion

MEF2 regulates specific gene expression by recruiting transcription co-factors in a cell state and context dependent manner [30]. In most cells that express MEF2, at least a fraction of

## MEF2D promotes oncogenic malignancy and chemotherapeutic resistance in OC

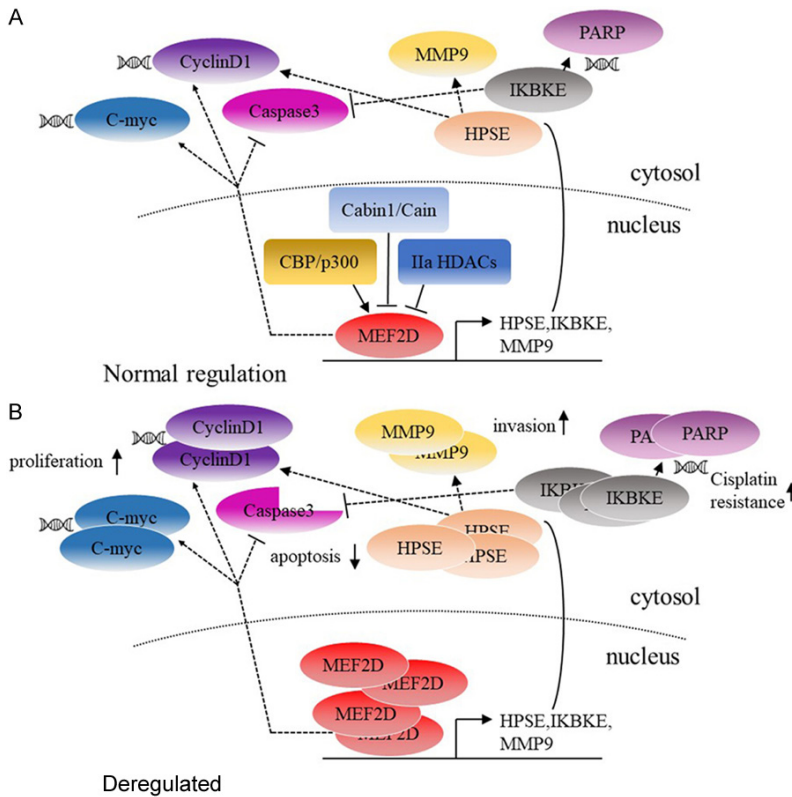


**Figure 7.** Effect of MEF2D knockdown on tumorigenicity of ovarian carcinoma cells in vivo. A, B. OVCAR3 cells were subcutaneously inoculated in SCID mice, which were randomly grouped to NC or siMEF2D001 (n = 6 for each group) and then injected with NC or siMEF2D001 every 3 days. 28 days later tumors were removed for analysis. Each tumor formed was volumed and weighted. The weight and volume of established tumors was measured and is shown in a scatter plot. C. Immunohistochemical analysis of MEF2D expression was performed on OVCAR3 tumor xenografts. The representative images are shown (with original magnification, of  $\times 200$  and  $\times 400$ ).

MEF2 is constitutively bound to DNA in the nucleus. In the resting state, MEF2 recruits co-repressors such as Cabin1/Cain and class IIa histone deacetylases (HDAC 4, 5, 7 and 9) to specific loci of the genome to inhibit the expression of target genes [31, 32]. Upon activation, the co-repressors dissociate from MEF2 through calcium-dependent mechanisms [33], the DNA-bound MEF2 then recruits co-activators such as CBP/p300 to activate transcription [27, 34, 35] (**Figure 8A**). Thus, the overall transcription state of MEF2-bound promoters depends on the availability of MEF2, its different co-factors and their binding interactions. Disruption of one or more these components

and steps could lead to diseases such as cancer. In a broad sense, MEF2 may not be simply classified as an oncogene or tumor suppressor gene because of its dual role in transcription activation and repression and its' ability to activate either pro- or anti-apoptotic programs in different contexts [27-29]. However, a number of studies have revealed that at least in some cancers MEF2, particularly MEF2D, acts as an oncogenic driver. For example, the ALL-associated MEF2D-DAZAP1 fusion protein is transcriptionally more active in luciferase assay than the wild type MEF2D [14]. This result is consistent with our finding that MEF2D play a key role in driving oncogenesis in OC.

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**Figure 8.** Signaling and transcription network of MEF2D and its deregulation in oncogenesis. A. In normal cells the activity of MEF2D is regulated by a number of co-factors, including co-repressors Cabin1/Cain, class IIa HDACs and co-activators CBP/p300, leading to the proper regulation of downstream genes. B. In diseased cells such as OC cells, the activity of MEF2D is deregulated leading to the abnormal expression of its downstream genes.

Our studies suggest that MEF2D serves as an oncogenic driver and also a key mediator of cisplatin-resistance in ovarian carcinoma. This conclusion is based on a number of independent and corroborating observations. First, elevated MEF2D expression was strongly associated with the chemotherapy-resistance and poor prognosis of OC in a retrospective analysis of 405 OC patients' specimens. Second, MEF2D knockdown resulted in attenuated tumorigenicity and inhibited the proliferation, migration and invasion of OC cells, meanwhile promoted the intrinsic apoptosis in vitro. Third, well-known oncogene, such as HPSE, or gene known to promote tumor growth and resistance to chemotherapy in OC, such as IKBKE, are targeted by MEF2D, and knockdown of MEF2D lead to the reduction of these genes and their activity in OC. Finally, MEF2D knockdown also lead to abolished tumorigenicity of ovarian carcinoma cells in vivo.

Several studies suggest that MEF2, through its interactions with cofactor Cabin1 or HDAC4, plays a critical role in regulating apoptosis programs in a variety of cells [27-29]. We speculate that MEF2D knockdown induced OC cell apoptosis may be related to these intrinsic apoptosis mechanisms regulated by MEF2 and its cofactor complexes. We found that MEF2D knockdown abolished tumorigenicity of ovarian carcinoma cells in vivo. We also found that MEF2D knockdown downregulated C-myc, Cyclin-D1 and MMP9 which are involved in cancer cell proliferation and invasion, meanwhile upregulated caspase3 which participated in cell apoptosis. These observations supported that MEF2D is involved in tumorigenesis and progression of OC (Figure 8B). An interesting question is whether mutation or aberrant expression of MEF2 cofactors, such as

Cabin1, class IIa HDACs and p300, are also involved in OC tumorigenesis and progression. Such question will be the subject of future studies.

Our studies also investigated potential target genes of MEF2D that may explain its role in OC. Both RT-qPCR and western blot assays demonstrated that HPSE and IKBKE were downregulated by MEF2D knockdown, while ChIP assays also suggested that MEF2D bound specifically to a region within the promoter of HPSE and IKBKE gene in SKOV3 and OVCAR3 cells, respectively. Previous studies showed that HPSE functioned as an oncogene in the proliferation and invasion in malignant glioma, hepatocellular carcinoma and OC through regulating the expression of cyclin-D1 and MMP9 [24, 25, 36-38]. IKBKE has also been reported to participate in progression and cisplatin-resistance in OC through regulating the expression of cas-



pase-3 and PARP, which was a well-known gene participating in cisplatin-resistance in many kinds of cancers [26], and chemosensitivity in non-small cell lung cancer induced by STAT3 and tobacco carcinogen [39]. Caspase-3 and PARP were also reported to be well-known gene participating in cisplatin-resistance in ovarian cancers [40]. Then we speculate that the relationship between these factors is constructed in this way (**Figure 8B**).

Taken together our studies suggest that MEF2D play a key role in the proliferation, migration and invasion of OC through HPSE, IKBKE, MMP9 and probably other downstream genes. The observations that siMEF2D reduced the expression of IKBKE a reversed cisplatin-resistance in OC cells also suggest that MEF2D and its downstream genes play a critical role in the chemotherapy-resistance of OC. These findings not only shed new insights into the oncogenic mechanisms of OC but also suggest potential new therapeutic approaches by targeting the MEF2D signaling/transcription pathway.

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### Disclosure of conflict of interest

None.

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