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MED28 and forkhead box M1 (FOXM1) mediate matrix metalloproteinase 2 (MMP2)-dependent cellular migration in human nonsmall cell lung cancer (NSCLC) cells

Nien-Tsu Hsieh[1] | Chun-Yin Huang[1] | Chien-Cheng Li[2] | I-Ching Wang[2,3] | Ming-Fen Lee[1,4]

[1]Department of Nutrition, China Medical University, Taichung, Taiwan, R.O.C

[2]Institute of Biotechnology, National Tsing Hua University, Hsinchu, Taiwan, R.O.C

[3]Department of Life Sciences, National Tsing Hua University, Hsinchu, Taiwan, R.O.C

[4]Department of Nutrition and Health Sciences, Chang Jung Christian University, Tainan, Taiwan, R.O.C

Correspondence
Ming-Fen Lee, Department of Nutrition, China Medical University, 91 Hsueh-Shih Road, 40402 Taichung, Taiwan, R.O.C.
Email: leemf@mail.cmu.edu.tw; summerblueair@yahoo.com

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Abstract

Non-small-cell lung cancer (NSCLC) accounts for the majority of the lung cancer cases that have become a leading cause of cancer deaths worldwide. Overexpression of transcription factor forkhead box M1 (FOXM1) is involved in the inauspicious development of several types of cancer, including lung tumor aggressiveness. Our laboratory has previously found that MED28, a Mediator subunit for transcriptional activation, modulates cell growth, epithelial-mesenchymal transition, migration, and invasion in human breast cancer cells. The objective of the current study is to investigate the potential role of MED28 and FOXM1 in NSCLC. In addition to A549 and PC9 cells, we also used a doxycycline-inducible system to generate FOXM1-overexpressed A549-DN cells, and we explored the connection of MED28 with FOXM1 and their effect on migration. Herein, we report that the increased expression levels of both MED28 and FOXM1 elevated the expression of matrix metalloproteinase 2 (MMP2), a metastasis marker, which enhanced cell migration and matrigel invasion of NSCLC cells. Furthermore, MED28 interacted with FOXM1, and both exhibited a mutual effect on the expression and subcellular localization. Moreover, MED28 small interfering RNA-mediated MMP2 gene suppression could be attenuated by inducible expression of a constitutively active form of FOXM1, which consequently restored the migration and invasion ability of NSCLC cells. Our data indicate that MED28 interacts with FOXM1, and each affects the expression and localization of the other, and, more importantly, both regulate MMP2-dependent migration and invasion in human lung cancer cells.

KEYWORDS
forkhead box M1 (FOXM1), invasion, lung cancer, MED28, migration, matrix metalloproteinase 2 (MMP2)

1 | INTRODUCTION

Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, including collagenases, gelatinases, stromelysins, matrilysins, and membrane type proteases, facilitate the destruction and remodeling of extracellular matrix in normal physiology and pathological conditions (Yan & Boyd, 2007). One critical consequence of MMP malfunction is its association with carcinogenesis. Dysregulation of MMPs may contribute to cancer development, including tumor growth, angiogenesis, epithelial-mesenchymal transition, and metastasis (Vincenti & Brinckerhoff, 2007). Therefore, MMPs have been proposed as novel biomarkers and potential therapeutic targets in cancer (Roy, Yang, & Moses, 2009).

Abbreviations: DOX, doxycycline; FBS, fetal bovine serum; FOX, forkhead box; MMP, matrix metalloproteinase; NSCLC, non-small-cell lung cancer; siRNA, small interfering RNA.

Forkhead box (FOX) M1 (FOXM1), a member of the FOX transcription factor family, plays a critical role in cell proliferation as a key regulator of both G_1-S and G_2-M stages through the cell cycle in normal cells (Wang et al., 2005; Wierstra, 2013). The human *FOXM1* gene encodes at least three isoforms of FOXM1 proteins through alternative splicing, where FOXM1b and FOXM1c are transcriptional activators to promote the expression of target genes, yet FOXM1a is considered transcriptionally inactive (Ma et al., 2005; Yao, Sha, Lu, & Wong, 1997). FOXM1 is highly expressed during embryogenesis but confined to actively replicating cells in adult tissues. Overexpression of FOXM1 has been reported in various tumor cell lines and multiple types of cancer in human, including lung cancer, breast cancer, and colorectal cancer (Kalin, Ustiyan, & Kalinichenko, 2011). A growing body of evidence indicates the role of FOXM1 in tumorigenesis and cancer progression, including angiogenesis, migration, and invasion (Laoukili, Stahl, & Medema, 2007; Raychaudhuri & Park, 2011).

The mammalian Mediator complex, a multiprotein coactivator, assists in the transcriptional activation of the protein-coding genes (Sato et al., 2004). In addition to the role in transcribing RNA polymerase II genes, recent literature has indicated that several Mediator subunits are associated with human diseases, including various types of malignancy (Spaeth, Kim, & Boyer, 2011). Although the underlying mechanisms are not fully understood in each case, overexpression or mutation of the genes encoding for some of the subunits have been identified in several types of cancer (Schiano et al., 2014). For example, the expression of Mediator subunit MED15 with its clinical implications in head and neck squamous cell carcinoma has been identified (Shaikhibrahim et al., 2015). Recently, we have reported that MED28, another Mediator subunit, is involved in cell growth in human breast cancer cells and colorectal cancer cells (Huang et al., 2015; Lee, Hsieh, Huang, & Li, 2016). Therefore, mammalian Mediator subunits display novel cellular roles in addition to their function in transcriptional activation.

Non-small-cell lung cancer (NSCLC) accounts for the majority of lung cancer cases. The unfavorable prognosis of the malignancy has become a leading cause of cancer deaths worldwide because this disease is usually unnoticeable until the patient exhibits clinical manifestations associated with metastasis at the late stage. Xu et al. (2013) reported that FOXM1 overexpression in tumor tissues promotes metastasis, which is significantly associated with the poor prognosis of NSCLC. Furthermore, overexpression of both MED28 and FOXM1 has also been reported in lung cancer samples in the Oncomine database (Supporting Information Figure S1); however, whether there is any correlation between MED28 and FOXM1 in lung cancer is unclear at present. Therefore, this study was aimed to investigate the potential connection of MED28, a Mediator subunit, and FOXM1, a transcription factor, in MMP2-dependent migration and invasion in human NSCLC cells.

2 | MATERIALS AND METHODS

2.1 | Chemicals, reagents, and antibodies

All chemicals and reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO) unless indicated otherwise. Antibodies for MED28 and FLAG-tag was obtained from GeneTex, Inc. (Irvine, CA) and Proteintech Group, Inc. (Rosemont, IL), respectively. Antibodies for FOXM1, GAPDH, β-actin, and α-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX), and MMP2 antibodies were purchased from Abcam (Cambridge, MA). Specific small interfering RNA (siRNA) pools for MED28 were from Sigma-Aldrich Corporation; MMP2 and FOXM1 siRNA pools were from Santa Cruz Biotechnology, Inc. Sequences of the siRNA for control, MED28, FOXM1, and MMP2 were listed in Supporting Information Table S1. The MMP2-luciferase reporter plasmid containing the full-length of the human *MMP2* promoter was kindly provided by Dr. Douglas D. Boyd (MD Anderson Cancer Center, Houston, TX) (Bian & Sun, 1997). The RSV-β-galactosidase plasmid was a gift from Dr. Amy Yee (Tufts University, Boston, MA), and the pINDUCER20 (ORF-UN) was a gift from Dr. Stephen Elledge (# 44012; Addgene plasmid; Meerbrey et al., 2011). The pInducer-GFP-FOXM1 was constructed by cloning an N-terminal deletion FOXM1b into pINDUCER20 (Wang et al., 2010). Overexpression plasmids for human complementary DNA (cDNA) clones of FOXM1b and Myc-DDK-tagged-MED28 (FLAG-MED28) were from OriGene Technologies, Inc. (Rockville, MD).

2.2 | Cell culture

All cell culture reagents and materials were obtained from Thermo Fisher Scientific (Waltham, MA) unless indicated otherwise. Human embryonic kidney 293 (293T) cells were obtained from Dr. Tzong-Der Way at China Medical University (Taichung, Taiwan). A549 and PC9 human NSCLC cell lines were from the American Type Culture Collection (Manassas, VA). A549-DN cells were established by infecting pInducer-GFP-FOXM1 lentivirus in A549 cells. Upon the addition of doxycycline (DOX), these cells can express an N-terminal deletion FOXM1 product with stronger transcriptional activity than that of the endogenous counterpart. All cells were maintained at 37°C in a 5% CO_2 incubator, and they were cultured in Roswell Park Memorial Institute 1640 medium except 293T cells, which were cultured in Dulbecco's Modified Eagle Medium. All media contained 10% fetal bovine serum (FBS), 100,000 units/L penicillin, and 100 mg/L streptomycin. For RNA interference and overexpression experiments, cells were transfected using Lipofectamine® RNAiMAX transfection reagent and Lipofectamine® 3000 transfection reagent, respectively.

2.3 | RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted by Total RNA Mini Kit (NovelGene, Taipei, Taiwan), and the cDNA was synthesized by iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using iQ™ SYBR® Green Supermix (Bio-Rad) and detected by CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The following primers were used: MED28 (forward, 5′-TTCGA ACCGGTGTTGATCAG-3′; reverse, 5′-GCCAATGCCTCAGCTTTGTC-3′); FOXM1 (forward, 5′-GTGTTTAAGCAGCAGCAG-3′; reverse, 5′-GTACC AGGTATGAGCTGAC-3′); GAPDH (forward, 5′-CGACCACTTTGTCA

AGCTCA-3′; reverse, 5-AGGGGAGATTCAGTGTGGTG-3′). The protocol was as follows: cDNA synthesis (1 cycle of 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min), DNA denaturation (1 cycle of 95°C for 3 min), PCR amplification (35–40 cycles of 95°C for 10 s and 60°C for 30 s), and melt curve analysis (60–95°C, increment 0.5°C, 5 s/step). Data were analyzed by CFX Manager™ 3.0 Software (Bio-Rad).

2.4 | Cell lysates and western blot analysis

Cells were lysed in ice-cold Nonidet P-40 Tris-based (NP-40/Tris) lysis buffer containing 1% NP-40, 150 mM NaCl, and 50 mM Tris (pH 8.0), centrifuged at 16,000*g* for 15 min, and the supernatant was saved. Protein concentration was measured by the Bradford assay (Bio-Rad). Fifty micrograms of total cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes from EMD Millipore Corporation (Billerica, MA). The membranes were incubated in blocking solution containing 5% nonfat dry milk, 20 mM Tris-HCl (pH 7.6) and 0.1% Tween 20 for 1 hr at room temperature. After washing three times, the membranes were incubated with specific antibodies overnight at 4°C. The next day, the membranes were probed with appropriate secondary antibodies, conjugated with horseradish peroxidase, from Jackson Immuno Research Inc. (West Grove, PA). The signal was detected by enhanced chemiluminescence solution from Biokit Biotechnology Inc. (Miaoli, Taiwan) and the images were analyzed by ChemiDoc™ XRS+ system from Bio-Rad.

2.5 | Coimmunoprecipitation assay

After cotransfected with FLAG-MED28 and FOXM1b overexpression plasmids for 48 hr, 293T cells were lysed in 1% NP-40/Tris lysis buffer. SureBeads protein A or G (Bio-Rad) were mixed with anti-FLAG, anti-FOXM1, or normal rabbit IgG at room temperature for 10 min. After unbound antibodies were removed, the mixture containing beads-antibodies was incubated with cell lysates at room temperature for 1 hr with gentle rotation. After three washes, the immunoprecipitated products were extracted for 10 min at 70°C from a dilution of 3× elution buffer containing 187.5 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 0.03% bromophenol blue, and 300 mM dithiothreitol.

2.6 | Reporter assay

Cells were seeded at a density of 5×10^4 cells/well on 24-well plates. After plating, cells were cotransfected with the MMP2-luciferase reporter plasmid and the RSV-β-galactosidase plasmid. For RNA interference experiments, cells were also transfected with nontargeting control, MED28-specific, or FOXM1-specific siRNA for 48 hr; for overexpression experiments, cells were cotransfected with pcDNA control, FOXM1, or MED28 expression plasmid for 24 hr. Cell lysates were collected in lysis solution, and the activities of luciferase and β-galactosidase were measued by Dual-Light® System (Applied Biosystems, Bedford, MA) and detected by Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT).

2.7 | Wound-healing assay

A549 or A549-DN cells were transfected with MED28 siRNA, MMP2 siRNA, or nontarget siRNA one day before subjected to wound-healing assay. The next day, cells (1×10^4 cells/well) were plated on Culture-Insert 2 well in μ-Dish (ibidi GmbH, Martinsried, Germany) and cultured for 24 hr. After removing the Culture-Insert, the Well was filled with an appropriate medium in the presence or absence of 1 μg/mL of DOX and allowed for wound-healing. Representative phase-contrast images were taken under 100× magnification at the indicated time points.

2.8 | Matrigel invasion assay

Matrigel inserts for 24-well chambers were purchased from Corning Incorporated (Oneonta, NY) and used as described in the manufacturer's guidelines for use. The upper chamber contained 5×10^4 cells in serum-free medium, and the lower compartment contained culture medium with 10% FBS in a 37°C, 5% CO_2 incubator. After 48 hr, the noninvading cells from upper chamber were removed using cotton swabs, and the cells on the lower surface were fixed with 90% methanol, stained with Giemsa, and counted. The migrated cells were counted in five, randomly selected microscopic fields (200× or 400× magnification). Error bars represent the variation of the cell number between the selected fields.

2.9 | Immunofluorescence analysis

Cells were seeded at a density of 5×10^3 cells/well on 8 well Chamber (ibidi). Cells were transfected with MED28 siRNA or nontarget siRNA for 72 hr or treated with DOX for 24 hr, and then fixed with 4% paraformaldehyde for 10 min followed by permeabilization with 0.25% Triton X-100 for 5 min. Next, at room temperature, cells were blocked with BlockPRO™ Blocking Buffer (Visual Protein, Taipei, Taiwan) for 1 hr, incubated with specific primary antibodies for 2 hr, and stained with secondary antibodies for 1 hr in the dark. The chamber slide was then mounted with mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific), and cells were visualized under Leica TCS SP2 Spectral Confocal & Multiphoton System (Leica Microsystems, Wetzlar, Germany). Representative images were taken under 630× magnification.

2.10 | Statistical analysis

Data are expressed as mean ± *SD* from at least triplicate experiments. Statistical significance was analyzed using Student's *t* test, and the results were considered significantly different at $P < 0.05$.

3 | RESULTS

3.1 | MED28 regulated the expression of MMP2 and acted upstream of MMP2

Our laboratory has previously discovered that MED28 regulates cellular migration in human breast cancer cells (Huang, Chou, Hsieh,

Chen, & Lee, 2012; Lee, Pan, Chiou, Cheng, & Huang, 2011); therefore, we raised the question if MED28 also regulates cellular migration in human lung cancer cells. First, we examined if the suppression of MED28 was involved in migration and invasion of lung cancer cells. After we suppressed the expression of MED28 by MED28-specific siRNA (siMED28) and confirmed MED28 knockdown by RT-PCR and western blotting analyses in A549 human lung cancer cells (Figure 1a), we investigated the effect of siMED28 on cellular migration. As shown by wound-healing assay, the migratory potential of A549 cells was reduced upon MED28 knockdown (Figure 1a). Suppression of MED28 not only inhibited the expression of MMP2 (Figure 1b) but also Matrigel invasive ability in both A549 and PC9 human lung cancer cells (Figure 1c). Next, we ectopically overexpressed MED28 and found correspondingly increased expression of MMP2 (Figure 1d). Exogenous MED28 expression was also accompanied by enhanced invasion in A549 cells (Figure 1e).

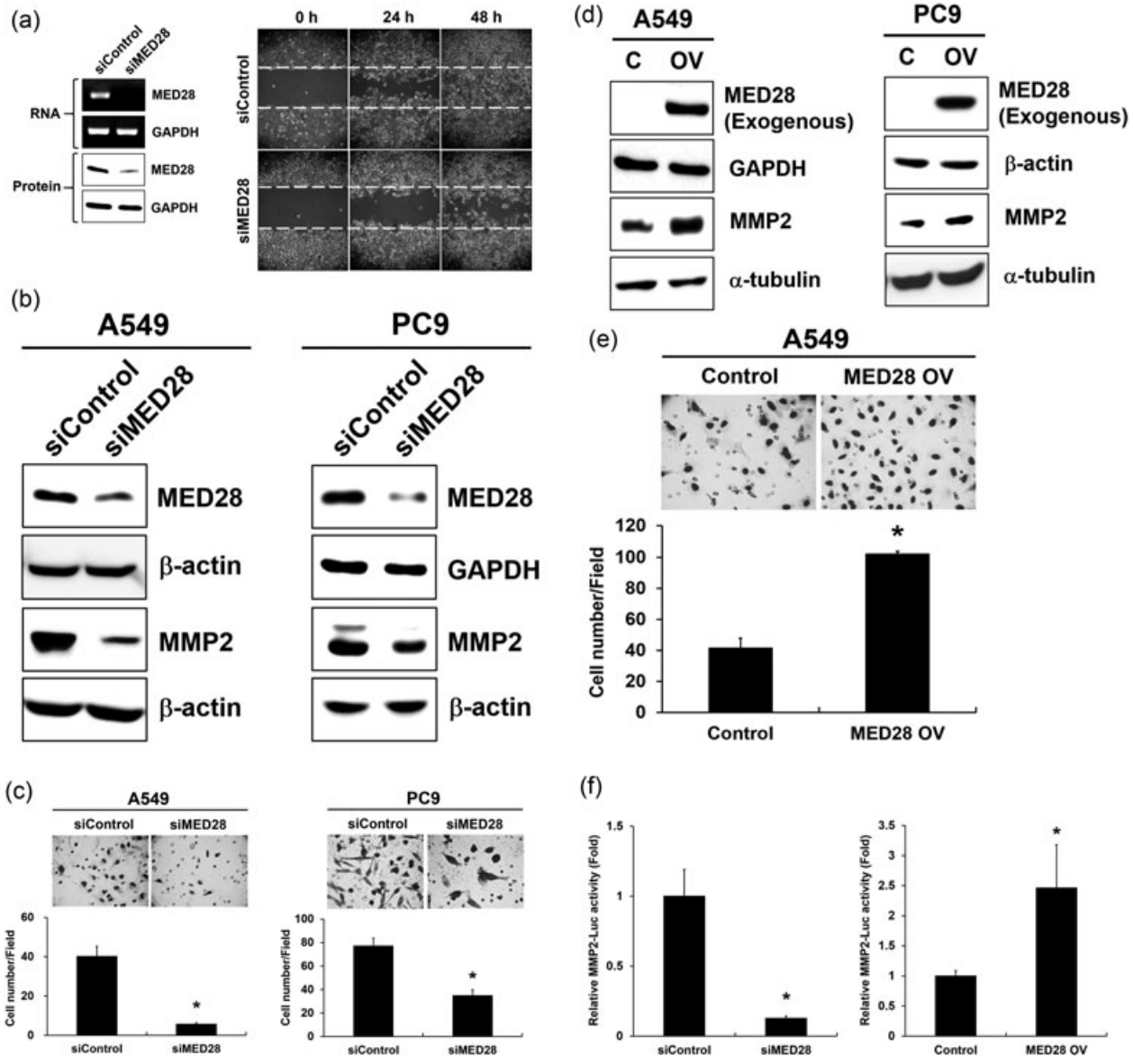
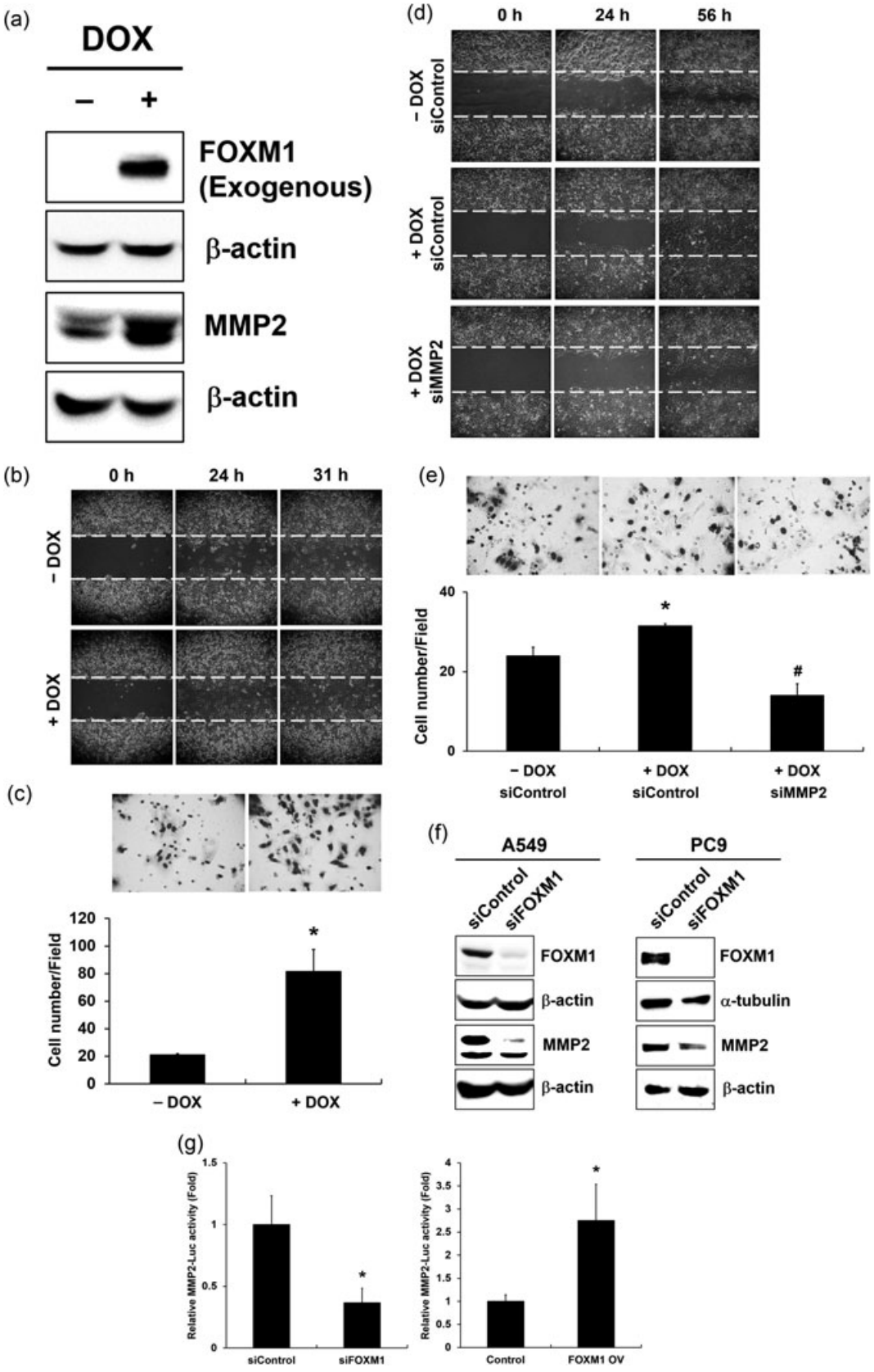


FIGURE 1 MED28 modulated the MMP2 expression, cell migration, and invasion in human non-small-cell lung cancer (NSCLC) cells. (a) After confirmation of MED28 suppression, A549 cells were subjected to wound-healing using Culture-Insert 2 well in μ-Dish (ibidi GmbH), and representative phase-contrast images were taken (100×). (b–e) After transfected with MED28 siRNA (siMED28) or FLAG-MED28 cDNA (OV) with respective control, control siRNA (siControl), or empty vector (C), for 24 hr, cells were subjected to western blotting (b,d). For invasion assay (c,e), cells were seeded with serum-free medium in the upper chamber and allowed to migrate for 48 hr to the lower compartment containing complete medium. The invading cells were photographed and counted in five, randomly selected fields (200× for A549 and 400× for PC9). (f) After transfected with siMED28 or MED28 OV for 48 hr, A549 cells were subjected to reporter assay by cotransfecting with an MMP2-luciferase (MMP2-Luc) reporter gene and an RSV-β-gal plasmid for 24 hr. Relative MMP2-Luc activity was the ratio of MMP2-Luc activity after normalization with β-galactosidase activity with respect to control. All data are expressed as mean ± *SD*, $n = 3$; $^*p < 0.05$ as compared with respective control. cDNA: complementary DNA; MMP2: matrix metalloproteinase; 2 OV: overexpression; *SD:* standard deviation; siRNA: small interfering RNA


(a)
DOX
–
+
FOXM1 (Exogenous)
β-actin
MMP2
β-actin
(b)
0 h
24 h
31 h
– DOX
+ DOX
(c)
Cell number/Field
120
100
80
60
40
20
0
*
– DOX
+ DOX
(d)
0 h
24 h
56 h
– DOX siControl
+ DOX siControl
+ DOX siMMP2
(e)
Cell number/Field
40
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– DOX siControl
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(f)
A549
PC9
siControl
siFOXM1
FOXM1
β-actin
MMP2
β-actin
siControl
siFOXM1
FOXM1
α-tubulin
MMP2
β-actin
(g)
Relative MMP2-Luc activity (Fold)
1.5
1
0.5
0
*
siControl
siFOXM1
Relative MMP2-Luc activity (Fold)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
FOXM1 OV

We then tested whether MED28 regulated MMP2 at the transcriptional level as identified before in breast cancer (Huang et al., 2012). As shown by the reporter gene assay in Supporting Information Figure S2, MED28 overexpression led to the upregulation of the *MMP2* promoter activity in 293T cells. Moreover, the transcriptional activity of the MMP2 reporter gene also corresponded to the expression levels of MED28 in A549 cells (Figure 1f). These data indicated that, as in human breast cancer cells, MED28 regulated migration and invasion where MMP2 appeared to be a downstream effector in human lung cancer NSCLC cells.

3.2 | FOXM1 regulated the expression of MMP2 and acted upstream of MMP2

FOXM1 was reported to regulate the expression of MMP2 and MMP2-mediated invasion in glioma cells (Dai et al., 2007) and osteosarcoma cells (Wang et al., 2008). We overexpressed FOXM1 by employing a DOX-inducible system in A549 cells to generate A549-DN cells, and we used these cells to explore the effect of FOXM1 on the expression of MMP2 and migration. The expression of MMP2 was enhanced in the presence of DOX in A549-DN cells with a corresponding enhancement in cellular migration and invasion (Figure 2a–c). However, the enhancement in migration and invasion by DOX induction was attenuated upon the addition of MMP2 siRNA in A549-DN cells (Figure 2d,e), indicating that MMP2 was a downstream target of FOXM1, which was responsible for the migration and invasion. When we knocked down endogenous FOXM1 in A549 or PC9 cells, the expression of MMP2 was also reduced (Figure 2f). FOXM1 overexpression led to upregulation of MMP2 as shown by the reporter gene assay in 293T cells (Supporting Information Figure S3). In addition, as in the case of MED28, the activity of the MMP2 reporter gene also corresponded to the expression level of FOXM1 in A549 cells (Figure 2g). These data indicated that FOXM1 indeed regulated the expression of MMP2, as well as migration and invasion in human NSCLC cells.

3.3 | MED28 and FOXM1 interacted with each other and affected the expression and subcellular localization of the other, and both regulated MMP2-dependent migration and invasion in human lung cancer cells

Overexpression of both MED28 and FOXM1 has been reported in lung cancer samples in the Oncomine database (Supporting Information Figure S1), whereas no evidence as yet indicates any correlation between MED28 and FOXM1. We then raised the question if the regulation of MMP2 by MED28 and FOXM1 identified in the current study is a dependent or independent event. If the first scenario turns out to be the case, then what is the control hierarchy between these two proteins? To address this issue, we first reconfirmed that both FOXM1 and MED28 acted upstream of MMP2 as shown by the corresponding expression and transcriptional activity of MMP2 upon knockdown or overexpression of MED28 and FOXM1 individually in A549 cells (Figure 3a). FOXM1, a transcription factor, and MED28, a Mediator subunit, are both involved in the transcriptional activation of multiple genes, and in the current case, controlling the expression of MMP2. This similarity prompted us to test if both proteins may also interact with each other. When we coexpressed both FLAG-MED28 and FOXM1, followed by coimmunoprecipitation in 293T cells, we found that FLAG-MED28 coimmunoprecipitated with FOXM1 and vice versa (Figure 3b), suggesting that MED28 and FOXM1 existed together in a complex. To explore any possible control hierarchy with respect to MMP2 between MED28 and FOXM1, beyond protein–protein interaction, we further determined their mutual effect. First, we suppressed the expression of MED28 or FOXM1 by MED28-specific or FOXM1-specific siRNA in A549 cells, and we determined the messenger RNA (mRNA) expression levels of both genes. As shown in Figure 3c, suppression of either gene also inhibited the mRNA expression of the other. In both A549 and PC9 cells, the expression of the FOXM1 protein was reduced upon MED28 knockdown (Figure 3d), and the expression of the MED28 protein was also reduced upon FOXM1 knockdown (Figure 3e). Next, we determined the overexpression effect of one gene on the other. When we overexpressed MED28, we observed an increase in the expression of FOXM1, and vice versa (Figure 3f,g). Because FOXM1 and MED28 are transcription factor and Mediator subunit, respectively, both proteins are likely to present in the nucleus. Therefore, we next tested if the localization of either protein could be altered by the expression status of the other. As shown in Figure 3h, in the control cells, the localization of FOXM1 was more concentrated in the nuclei with a clearer nuclear boundary; in contrast, upon MED28 knockdown (siMED28), the expression level of FOXM1 appeared lower and the subcellular localization of FOXM1 appeared more diffuse towards the periphery of the cell. When we overexpressed FOXM1 by DOX induction in A549-DN cells, the localization of MED28 appeared to be more prominent in the nucleus than that of untreated cells (Figure 3i). Next, we explored the potential effect between the interaction of FOXM1 and MED28 on MMP2, cellular migration, and invasion. The enhancement of MMP2 expression through the DOX (FOXM1-inducible) system was attenuated upon MED28 knockdown in A549-DN cells (Figure 3j), suggesting that MED28 was involved in the FOXM1-regulated expression of MMP2. Moreover, the inhibitory effect

FIGURE 2 FOXM1 modulated MMP2 expression, cellular migration, and invasion in human NSCLC cells. (a–c) A549-DN cells were treated with or without doxycycline (DOX; 1 μg/mL) for 24 hr followed by Western blotting (a), wound-healing assay (b), or Matrigel invasion assay (c). (d,e) A549-DN cells were transfected with MMP2 siRNA (siMMP2) in the presence or absence of DOX and then subjected to wound-healing assay (d) or Matrigel invasion assay (e). Representative images were 100× magnification for the wound-healing assay and 200× magnification for the invasion assay. (f) Cells were transfected with control siRNA (siControl) or FOXM1 siRNA (siFOXM1) and then subjected to western blotting. (g) After transfected with siFOXM1 or FOXM1b cDNA (OV) for 48 hr, A549 cells were subjected to reporter assay. All data are expressed as mean ± *SD*, $n = 3$; $^{*}p < 0.05$ as compared with –DOX/siControl or respective control; $^{\#}p < 0.05$ as compared with +DOX/siControl. cDNA: complementary DNA; MMP2: matrix metalloproteinase; OV: overexpression; *SD*: standard deviation; siRNA: small interfering RNA

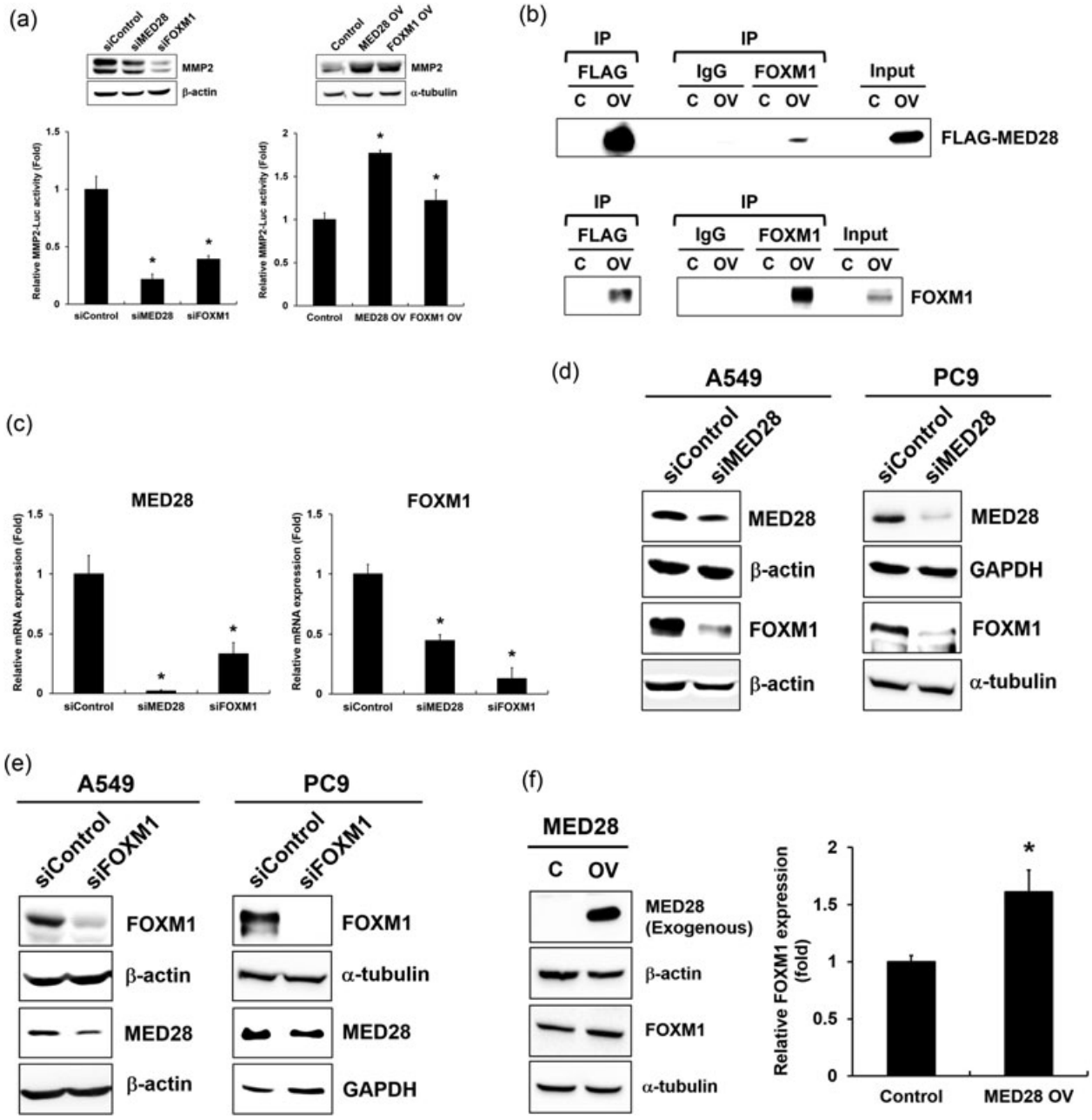
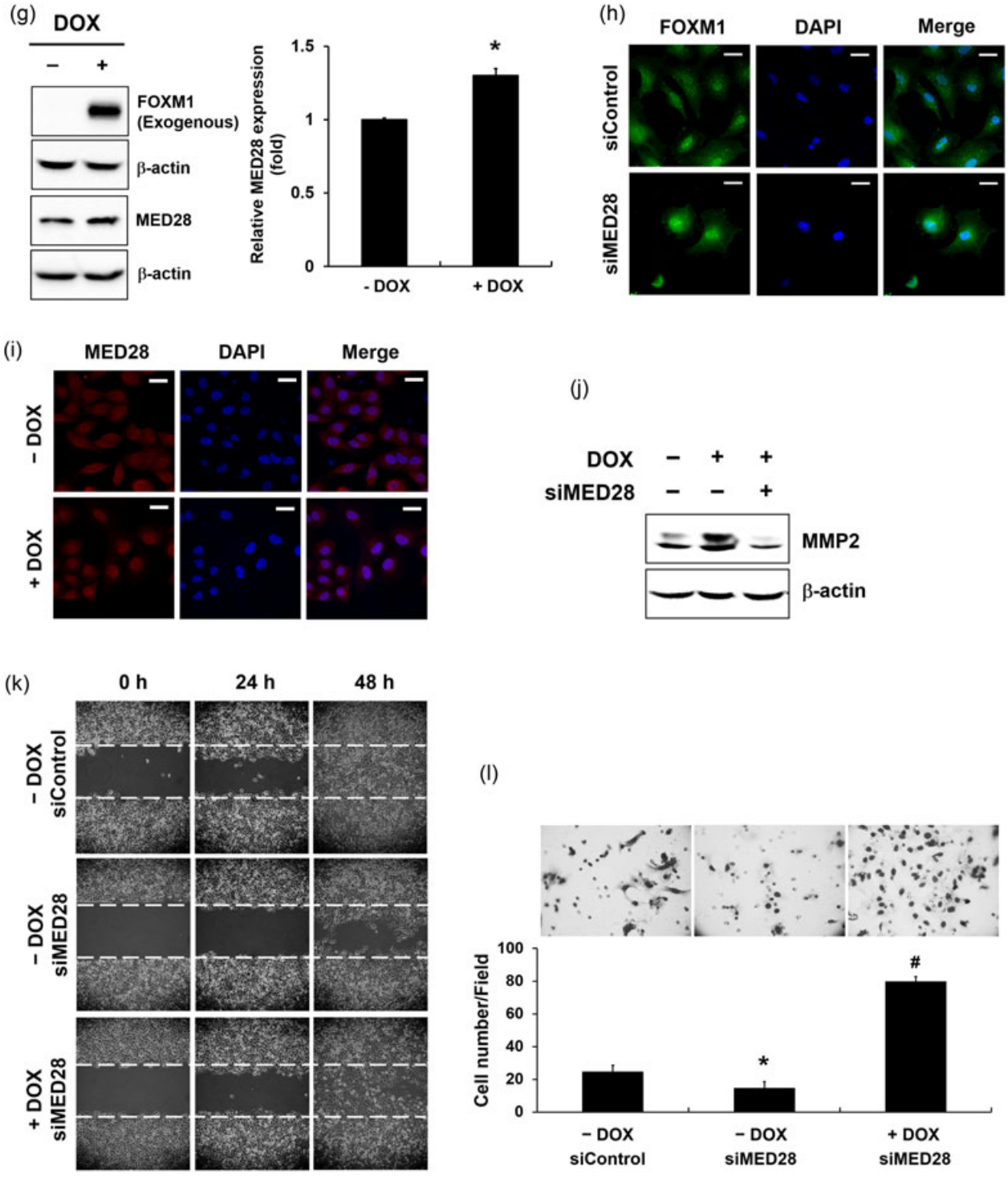


FIGURE 3 MED28 and FOXM1 interacted with each other, affected the expression and subcellular localization of the other, and both regulated the expression of MMP2 and controlled cell migration. (a) After transfected with specific siRNA for MED28 (siMED28) or FOXM1 (siFOXM1) or expression vector (OV) for MED28 or FOXM1b for 24 hr, A549 cells were subjected to western blotting or MMP2-luciferase reporter gene assay. (b) 293T cells were cotransfected with the FLAG-MED28 and FOXM1 expression vectors for 48 hr and immunoprecipitated (IP) with the indicated antibodies, followed by western blotting. C: Untransfected control; OV: overexpression. (c) A549 cells were subjected to siMED28 or siFOXM1, followed by quantitative real-time PCR assay. (d,e) Cells were transfected with siMED28 (**d**) or siFOXM1 (e), and then subjected to western blotting. (f) A549 cells were transfected with FLAG-MED28 expression vector (OV) for 48 hr and then subjected to western blotting. (g) A549-DN cells were treated with doxycycline (DOX; 1 μg/mL) for 24 hr and then subjected to western blotting. (h,i) A549 cells were transfected with siMED28 for 72 hr (h), A549-DN cells were treated with vehicle (−) or DOX (+) for 24 hr (i), and subjected to immunofluorescence analysis. (Scale bar = 25 μm). (j-l) A549-DN cells were transfected with siMED28 in the presence or absence of DOX and then subjected to Western blot analysis (j), wound-healing assay (k), or Matrigel invasion assay (l). Representative images were 100× magnification for the wound-healing assay and 200× magnification for the invasion assay. All data are expressed as means ± *SD*, $n = 3$; $^{*}p < 0.05$ as compared with −DOX/siControl or Control; $^{\#}p < 0.05$ as compared with −DOX/siMED28. MMP2: matrix metalloproteinase; *SD*: standard deviation; siRNA: small interfering RNA



of siMED28-mediated suppression of cell migration and Matrigel invasion could be rescued by inducible expression of FOXM1 (Figure 3k,l). Taken together, our data indicate that MED28 interacts with FOXM1, and each affects the expression and localization of the other; more important, both regulate MMP2-dependent migration and invasion in human lung cancer cells (Figure 4).

4 | DISCUSSION

Among the MMP family members, MMP2 and MMP9, gelatinase A and B, respectively, often overexpressed in solid tumors, including breast, colorectal, and lung, are considered as cancer biomarkers (Bauvois, 2012). These proteins are involved in the progression of

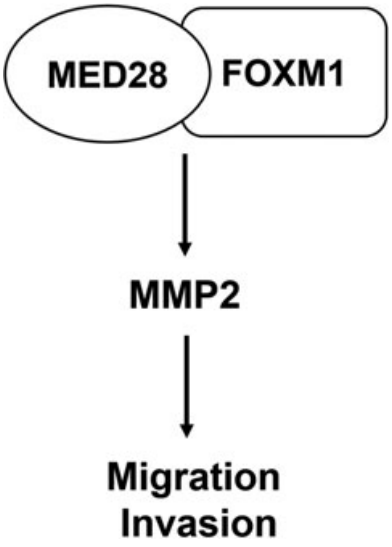


FIGURE 4 Model of MED28 and FOXM1 in cellular migration and invasion in human non-small-cell lung cancer (NSCLC) cells. MED28 and FOXM1 interact and both regulate the expression of MMP2, which ultimately lead to cellular migration and invasion in human NSCLC cells. MMP2: matrix metalloproteinase

cancer, including epithelial-mesenchymal transition, migration, and invasion. The putative FOXM1/MMP/migration and invasion axis has been reported in oral cavity squamous cell carcinoma (Chen et al., 2009) and papillary thyroid carcinoma (Ahmed et al., 2012), among other malignancy. FOXM1 directly regulates the expression of MMP2, but indirectly regulates MMP9 through JNK1-dependent mechanism to promote migration and invasion in U2OS osteosarcoma cells (Wang et al., 2008). In addition, FOXM1 upregulates the expression of MMP2 and promotes invasion in glioma progression (Dai et al., 2007). In this regard, our current work identified the role of FOXM1 in regulating MMP2 in lung cancer. Employing a DOX-inducible system to examine the effect of FOXM1 on MMP2, we found that FOXM1 sits upstream of MMP2 and controls MMP2-dependent migration, which is confirmed by the data that MMP2 knockdown attenuated the enhancement effect on migration and invasion upon DOX induction (Figure 2d,e).

Increasing literature addressed the individual roles of Mediator subunits in the progression of cancer (Schiano et al., 2014). In bladder cancer, MED19 has been reported to mediate cell proliferation and migration (Yuan et al., 2017). Kuuselo, Savinainen, Sandstrom, Autio, & Kallioniemi (2011) described that the suppression of MED29 decreases migration and invasion in pancreatic cancer cells. In addition, MED30 regulates the proliferation, migration, and invasion of gastric cancer cells (Lee, Han, Baek, Kim, & Oh, 2015). Recently, our laboratory has reported that MED28, a Mediator subunit, is involved in epithelial-mesenchymal transition and cell migration in human breast cancer cells (Huang et al., 2012; Huang et al., 2017). MED28 regulates the expression of MMP2 and cellular migration in human breast cancer cells (Huang et al., 2012). Suppression of MED28 blocked cellular migration and invasion accompanied by reduced expression of MMP2, whereas overexpression of MED28 upregulated the MMP2 expression and enhanced cellular migration (Huang et al., 2012). We also have reported that MED28 enhanced epidermal growth factor-induced migration by upregulating the expression of MMP9 in MDA-MB-231 human breast cancer cells (Lee et al., 2011). It appears that MED28 directly mediates MMP2 activation and subsequent cell migration, and, with additional stimuli, MED28 induces the expression of MMP9 in human breast cancer cells. Similarly, in human lung cancer cells, we also found that the upregulation of MMP9 by MED28 was mostly only prominent in the presence of growth factors or cytokines (data not shown). The current study reported a role of MED28 in cellular migration in lung cancer cells where MED28 upregulates MMP2 and promotes subsequent cell migration. Together with the previous findings in our laboratory, MED28 indeed plays an important role in cancer development, at least in the case of breast cancer and lung cancer.

Both MED28 and FOXM1 can modulate cell growth (Huang et al., 2015; Lee et al., 2016; Wang et al., 2005; Wierstra, 2013), and this role may affect the cell numbers after knockdown or overexpression. Therefore, the effect of MED28 and/or FOXM1 on migration and invasion may not be completely independent of their role in cell growth. However, for the invasion assay, we maintained the cells with serum-free medium after serum starvation overnight to allow for synchronization and minimize the confounding effect of proliferation (Figure 1c,e; Figure 2c,e; Figure 3l). For the migration data, the effect of MED28 or FOXM1 was already distinguishable at 24 hr (Figure 1a; Figure 2b,d; Figure 3k). In addition, both MED28 and FOXM1 regulated the expression of MMP2, which, in turn, was involved in migration and invasion (Figures 1, 2, and 3). Together, although we cannot rule out any interfering effect of growth-promoting mode of MED28 and FOXM1 on migration and invasion, our current study indeed indicated that MED28 and FOXM1 regulated MMP2-dependent migration and invasion.

Unlike transcription factors, Mediator subunits such as MED28 do not bind to a consensus DNA-binding site, rather, they mediate transcription through protein–protein interaction with transcription factors and/or other coactivators (Sato et al., 2004). In contrast, FOXM1 usually acts as a transcription factor to promote tumor

development by regulating a whole spectrum of genes involved in tumor cell growth, angiogenesis, and migration among other carcinogenesis events. FOXM1-binding sites have been identified in the promoter of *MMP2* (Dai et al., 2007), yet how exactly that MED28 activates the MMP2 expression is unclear. One would ask how FOXM1 and MED28 interact to regulate the transcriptional activation of their downstream target gene such as MMP2 in the current case? It appears that MED28 and FOXM1 recruit each other to stay in the nucleus (Figure 3h,i), and perhaps by doing so they promote the expression of their target gene. One conceivable scenario is that the interaction of MED28 with FOXM1 could facilitate the activation of the FOXM1 expression. Previous studies indicate that FOXM1 regulation through a positive autoregulatory loop where FOXM1 activates its own mRNA and protein expression (Halasi & Gartel, 2009). By interacting with FOXM1, MED28 could help FOXM1 to stay in the nucleus and maintain its expression. Another possible situation is that the interaction of MED28 with FOXM1 could recruit other transcription factor and/or coactivator for transcriptional activation. Zhang et al. (2011) reported that, in glioma cells, FOXM1 interacts with β-catenin, promotes the nuclear localization of β-catenin, and enhances its transcriptional activity, thereby controlling the downstream target genes of Wnt/β-catenin signaling. Therefore, FOXM1 can be a critical regulator of Wnt/β-catenin signaling in tumors, such as glioma, rather than the main transcription factor that directly promotes tumorigenesis. Kim, Xu, Hecht, & Boyer (2006) reported a direct interaction between β-catenin and MED12, a Mediator subunit, by coimmunoprecipitation assays using LiCl-treated nuclear extracts from HeLa and BG-1 cells, as well as by glutathione S-transferase pulldown assays; furthermore, this interaction is required for the transcriptional activation event of Wnt/β-catenin signaling. Interestingly, our laboratory has recently reported that, in human colorectal cancer cells, suppression of MED28 reduces the nuclear β-catenin expression with correspondingly lower expression of c-Myc and cyclin D1, two Wnt/β-catenin target genes (Lee et al., 2016). Although MED28 appears involved in Wnt/β-catenin signaling, whether MED28, as a Mediator subunit, also interacts with β-catenin as MED12 is unclear at present. However, the interaction of MED28 and FOXM1 could bring MED28 to β-catenin in close vicinity, and together FOXM1 and MED28 could regulate Wnt/β-catenin signaling.

Interestingly, in searching for potential transcription factor-binding sites in the DNA regulatory region of MED28, we came across the ChIP-seq datasets from the ENCODE Transcription Factor Targets dataset and identified putative FOXM1-binding sites (Rouillard et al., 2016). Although the prediction was not confirmed yet, this information could indeed provide a possible rationale that FOXM1 enhanced the expression of MED28 (Figure 3g,i). The findings in NSCLC cells that MED28 knockdown attenuated the FOXM1-upregulated MMP2 expression (Figure 3j) and inducible expression of FOXM1 relieved the inhibitory effect of siMED28-mediated suppression on cell migration and Matrigel invasion (Figure 3k,l) further support the putative synergistic interaction between MED28 and FOXM1 and reiterate the significance of their role in NSCLC.

In this study, we identified a connection between MED28 and FOXM1 and their link in human lung cancer cells such that MED28 and FOXM1 may interact with each other and sit upstream of MMP2, thereby controlling the activation of MMP2 and modulating MMP2-dependent migration in human lung cancer cells. Overexpression of both MED28 and FOXM1 enhanced the MMP2 expression and migration, and suppression of either MED28 or FOXM1 inhibited the MMP2 expression and migration. FOXM1 appeared to bind to MED28, and they both assisted each other staying in the nucleus. The link with FOXM1, a pro-oncogenic transcription factor and as a regulator of MMP2, further support the critical roles of MED28, a multifaceted protein, in cancer. In addition to NSCLC, our group has previously identified a role of MED28 in the malignancy of breast cancer as indicated by its effect on migration and invasion, as well as cell morphology of human breast cancer cells (Huang et al., 2012; Huang et al., 2017). Moreover, we have also reported that MED28 is involved in cell growth in both human breast cancer cells and colorectal cancer cells (Huang et al., 2015; Lee et al., 2016). Our findings further emphasize the significance of MED28 and imply the prospective translational application of MED28 in tumorigenesis and cancer development.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ORCID

Chun-Yin Huang http://orcid.org/0000-0001-7699-5727
Ming-Fen Lee http://orcid.org/0000-0003-2557-6527

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