

Targeting FoxM1 inhibits proliferation, invasion and migration of nasopharyngeal carcinoma through the epithelial-to-mesenchymal transition pathway

CHAO YU, LILI CHEN, LIN YIE, LEI WEI, TAOYU WEN,
YANAN LIU and HONGYAN CHEN

Department of Otolaryngology Head and Neck Surgery, The First Affiliated Hospital,
Chongqing Medical University, Chongqing 400016, P.R. China

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Abstract. High expression levels of the forkhead box M1 (FoxM1) transcription factor are associated with metastasis and poor prognosis of malignancies. However, little is known concerning its function in nasopharyngeal carcinoma (NPC). The present study aimed to investigate the impact of FoxM1 inhibition on the migration and invasion of NPC cells and the potential mechanisms. The effects of FoxM1 inhibitor treatment and FoxM1 silencing on the proliferation, migration and invasion of NPC CNE-1 and CNE-2 cells were examined by CCK-8, Transwell migration/invasion and colony formation assays. The effects of stable FoxM1 silencing on the growth and lung metastasis of implanted NPC were evaluated. The relative levels of FoxM1, zinc finger E-box binding homeobox 2 (ZEB2), Snail2 and E-cadherin in the different groups of NPC cells and tumors were determined by quantitative real-time PCR, western blotting and immunohistochemical assays. Treatment with thioestrepton, FoxM1 inhibitor, significantly reduced the survival of NPC cells. Treatment with thioestrepton and/or knockdown of FoxM1 inhibited the anchorage-independent proliferation, migration and invasion of NPC cells. Inhibition of FoxM1 also increased the relative levels of E-cadherin, but reduced ZEB2 and Snail2 expression in NPC cells. Stable FoxM1 silencing inhibited the growth and lung metastasis of implanted NPC *in vivo*, which was associated with increased levels of E-cadherin, but decreased ZEB2 and Snail2 expression in the NPC tumors.

In conclusion, our data clearly indicate that knockdown of FoxM1 inhibited the growth and metastasis of human NPC by modulating epithelial-to-mesenchymal transition (EMT), and FoxM1 may be a potential target for the intervention of NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is a tumor arising from the epithelial cells in the nasopharynx. Although NPC is rare in Western countries, its incidence remains high in Southern China and Southeast Asia (1). Although the etiology of NPC remains unclear, many factors such as Epstein-Barr virus infection, environmental factors and genetic alterations are associated with the development of NPC (2-5). More importantly, patients with early-stage NPC are difficult to diagnose and without intervention, NPC can easily metastasize into local lymph nodes, leading to poor prognosis. Currently, patients with NPC can be treated with chemoradiotherapy and sequential radiotherapy and chemotherapy (1). However, the efficacy of these therapeutic strategies is limited by their undesirable side effects, local recurrence and distant metastasis (6). Therefore, understanding the molecular mechanisms underlying the metastasis of NPC may reveal new targets for the design of new therapies for patients with NPC (7).

Forkhead box M1 (FoxM1) is a member of the forkhead family of transcription factors and is ubiquitously expressed in proliferating and regenerating mammalian cells (8,9). FoxM1 is a key regulator of both G1-S and G2-M phases of the cell cycle and mitotic spindle integrity (10). High levels of FoxM1 expression are associated with the development of various cancers, including hepatocellular, prostate, lung, glioma, cervical and gastric cancers (11-15). Furthermore, FoxM1 overexpression can promote epithelial-to-mesenchymal transition (EMT) and enhance the invasion and migration of several types of cancers (16-19). Hence, FoxM1 may be a potential therapeutic target for the development of anticancer treatments (20-22). However, little is known concerning the role of FoxM1 expression in the proliferation, migration and invasion of NPC cells.

In the present study, we examined the impact of treatment with FoxM1 inhibitor or FoxM1 silencing on the survival,

Correspondence to: Dr Hongyan Chen, Department of Otolaryngology Head and Neck Surgery, The First Affiliated Hospital, Chongqing Medical University, 1 Yixueyuan Road, Yuzhong, Chongqing 400016, P.R. China
E-mail: chenhongyan20145@163.com

Abbreviations: NPC, nasopharyngeal carcinoma; FoxM1, forkhead box M1; EMT, epithelial-to-mesenchymal transition; Thios, thioestrepton; siCtr, siControl

Key words: nasopharyngeal carcinoma, invasion, migration, EMT signaling, thioestrepton

anchorage-independent proliferation, migration and invasion of human NPC cells *in vitro* and *in vivo*. Furthermore, we determined the potential effects of treatment with a FoxM1 inhibitor or FoxM1 silencing on the EMT process in NPC cells. Our findings indicate that FoxM1 positively regulates the proliferation, migration and invasion of NPC by enhancing the EMT process.

Materials and methods

Cell lines and reagents. Human nasopharyngeal cancer cell lines, CNE-1 and CNE-2, purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) were maintained in RPMI-1640 medium supplemented with 10% newborn calf serum (NBCS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The 293T cell line was obtained from Invitrogen (Carlsbad, CA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin.

Thiostrepton is a cycling polypeptide antibiotic with selective inhibitory activity against FoxM1 (23) and was purchased from Tocris Cookson (Ellisville, MO, USA). Antibodies against FoxM1, E-cadherin, zinc finger E-box binding homeobox 2 (ZEB2), zinc finger protein Snail2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Growth inhibition assay. The impact of inhibition of FoxM1 on the proliferation of NPC cells was determined by a growth inhibition assay using the Cell Counting Kit-8 (CCK-8; Beyotime, China), according to the manufacturer's instructions. Briefly, CNE-1 and CNE-2 cells at a density of 3,000 cells/well were cultured in 96-well plates overnight and were treated in pentaplicate with thiostrepton at various concentrations (0, 1, 2, 4, 8 and 16 µmol/l) for 48 or 72 h. During the last 2 h of culture, the cells were exposed to a water-soluble tetrazolium salt of WST-8 (10 µl/well), and the levels of the resulting WST-8 formazan in individual wells were measured at 450 nm using a microplate reader (Biotek, Winooski, VT, USA).

RNA isolation, reverse transcription and qRT-PCR. Total RNA was extracted from individual groups of NPC cell lines using TRIzol (Takara, Shiga, Japan), and the resulting RNA (5 µg/sample) was reversely transcribed into cDNA using the First Strand cDNA kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The relative levels of target gene mRNA transcripts to control GAPDH in individual samples were determined using the specific primers and SYBR-Green system according to the manufacturer's instructions (Takara). The sequences of primers were: sense, 5'-GCT TGC CAG AGT CCT TTT TGC-3' and antisense, 5'-CCA CCT GAG TTC TCG TCA ATG C-3' for human FoxM1 (123 bp); sense, 5'-CGG GAA TGC AGT TGA GGA TC-3' and antisense, 5'-AGG ATG GTG TAAGCG ATG GC-3' for human E-cadherin (154 bp); sense, 5'-GAT GCC GCG CTC CTTCCT GG-3' and antisense, 5'-GGG GGA CTC ACT CGC CCC AA-3' for human Snail2 (168 bp); sense, 5'-TGC CAC CAG TCC AGA CCA GTA T-3' and antisense, 5'-GCT

CCA GTT GGG TAG GTG TAG G-3' for human ZEB2 (173 bp); sense, 5'-GAA GGT CGG AGT CAA CGG ATT T-3' and antisense, 5'-CGC TCC TGG AAG ATG GTG A-3' for human GAPDH (119 bp). The PCR amplification was performed in triplicate at 94°C for 3 min and subjected to 35 cycles of 94°C for 40 sec and 61°C for 45 sec, followed by one cycle of extension at 72°C for 10 min. The relative levels of gene mRNA transcripts to control GAPDH were calculated by normalizing to the control and using the 2^{-ΔΔCt} method.

Lentivirus construction and infection. Individual lentivirus plasmid vectors for expressing small interfering RNA (siRNA) specific for FoxM1 or control siRNA were constructed. After sequencing, the generated plasmids, together with two packaging plasmids (pVSVG and 48.91; Cambridge, MA, USA) were co-transfected into 293T cells by calcium phosphate transfection, respectively. Another type of lentivirus was established for expressing firefly luciferase. Forty-eight hours after transfection, the media of the cultured 293T cells were collected and after centrifugation, the titers of the generated individual types of lentiviruses were measured by flow cytometry. CNE-2 cells (5×10⁵/well) were infected with the FoxM1-siRNA or control siRNA expressing lentivirus combined with the lentivirus for expressing firefly luciferase at an MOI of 50 to generate firefly luciferase and FoxM1 stable silenced CNE-2 cells.

Gene silencing using siRNA. FoxM1 siRNA and scrambled control siRNA were purchased from Invitrogen. CNE-1 and CNE-2 cells were transfected with either FoxM1-specific or control siRNA by Lipofectamine 2000 reagent (Invitrogen) as described previously (24).

In vitro cell migration and invasion assays. CNE-1 and CNE-2 cells at a density of 8×10⁴ cells/well were cultured in the presence or absence of 8 µmol/l thiostrepton in the top wells of 24-well Transwell plates (Corning Inc., Corning, NY, USA) that had been coated with fibronectin. After the cells were cultured for 24 h, the cells on the bottom surface of the top well were stained with Giemsa and examined under a microscope in a blinded manner. In addition, CNE-1 and CNE-2 cells were transfected with FoxM1-specific or control siRNA for 48 h and these cells were harvested for assessment of their migration in the presence or absence of 8 µmol/l thiostrepton.

To determine the impact of FoxM1 inhibition on NPC invasion, CNE-1 and CNE-2 cells at a density of 8×10⁴ cells/well were cultured in the presence or absence of 8 µmol/l thiostrepton in the top wells that had been pre-coated with 24 mg/ml Matrigel (R&D Systems, Minneapolis, MN, USA). Moreover, the FoxM1-specific or control siRNA-transfected CNE-1 and CNE-2 cells were tested for their invasion in the presence or absence of 8 µmol/l thiostrepton.

Colony formation assay. The impact of FoxM1 inhibition on anchorage-independent proliferation of NPC cells was assessed. CNE-1 and CNE-2 cells at 2×10³ cells/well were mixed with 1 ml of culture medium containing 0.3% (w/v) agar, 20% FBS and layered over a basal layer of 1 ml of culture medium containing 0.6% (w/v) agar and 20% FBS in 6-well plates in the presence or absence of 8 µmol/l of thiostrepton.

The cells were exposed to fresh medium containing the drug weekly and cultured for 2-3 weeks. The numbers of individual colonies containing >50 cells were counted under a microscope in a blinded manner. The colony formation efficiency of individual groups of cells was calculated as: (the numbers of colonies/numbers of cells inoculated) x 100%.

In addition, the generated FoxM1 shRNA stably expressing CNE-2 and control CNE-2 cells (2×10^3 cells/well) were also tested for their anchorage-independent proliferation and the formed colonies were examined under a fluorescence microscope.

Western blotting. CNE-1 and CNE-2 cells were treated with, or without, 8 μ mol/l of thioestrepton for 48 h. Similarly, the CNE-1 and CNE-2 cells were transfected with control or FoxM1-specific siRNA for 24 h and treated with, or without, thioestrepton for 48 h. The cells were harvested and lysed using a nuclear protein extraction kit (Takara). After quantification of the protein concentrations, the cell lysates (30 μ g/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Hybond-enhanced chemiluminescence nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). After being blocked with 5% fat-free dry milk, the membranes were probed with polyclonal antibodies against FoxM1 (C-20), E-cadherin, Slug, ZEB-2 and monoclonal anti-GAPDH antibody (all from Santa Cruz Biotechnology). After being washed with TBST, the bound antibodies were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized using enhanced chemiluminescence (ECL; GE Healthcare). The relative levels of target proteins to the control GAPDH expression were determined by densitometric scanning and calculated using ImageJ software.

Animal experiments. Male BALB/C nude mice at 4-5 weeks of age were purchased from the Shanghai Institute of Material Medicine, Chinese Academy of Science (Shanghai, China) and housed in a specific pathogen-free (SPF) animal facility in our campus. The experimental protocol was established in accordance with institutional guidelines, and was approved by the Animal Use and Care Committee of Chongqing Medical University.

The control CNE-2 cells stably expressing firefly luciferase and CNE-2 cells stably expressing the FoxM1-specific shRNA were harvested, washed with PBS, adjusted to 2×10^6 /ml in PBS. To induce tumors *in vivo*, individual mice were injected subcutaneously with 2×10^6 control CNE-2 or FoxM1-specific shRNA stably expressing CNE-2 cells into the flank (n=10 per group). Some of the mice were monitored for the growth of implanted NPC cells weekly up to 30 days post inoculation. The volume of the formed tumor in individual mice was calculated according to the following formula: tumor volume = (length x width²)/2. At the end of this experiment, the mice were anesthetized and sacrificed. Their subcutaneous tumors were dissected out and the tumor volumes were measured. In addition, the remaining mice were monitored for lung metastasis up to 60 days post inoculation. Their lungs were dissected and examined for the numbers of metastatic tumors in the lungs. Briefly, the lung tissues were imaged, fixed with 10% formalin and paraffin-embedded. The lung tissue sections

(4 μ m) were stained with hematoxylin and eosin (H&E) and every 100th section was examined under a light microscope.

Immunohistochemistry. The dissected subcutaneous tumors were fixed in 10% formalin and paraffin-embedded. The tumor tissue sections (4- μ m) were deparaffinized, rehydrated, and treated with 3% H₂O₂ in methanol, followed by antigen retrieval with citrate buffer (pH 6.0) in a high pressure steamer. After being blocked with 5% fat-free dry milk in TBST, the sections were incubated with polyclonal antibodies against FoxM1, E-cadherin, ZEB2 and Snail2, respectively. The bound antibodies were detected with HRP-conjugated secondary antibodies and visualized with DBA staining, followed by microimaging under a microscope.

Statistical analysis. Data are expressed as representative images or the means \pm SD. The difference among groups was determined by the Student's t-test or repeated one-way ANOVA using SPSS 17.0 software. A P-value of <0.05 was considered to indicate statistical significance.

Results

Targeting of FoxM1 expression reduces the viability of NPC cells. FoxM1 is crucial for the proliferation and differentiation of tumor cells and its expression is associated with the development of different types of cancers (14). Thioestrepton is a specific inhibitor of FoxM1 (25) and can inhibit proteasomal properties (26). To explore the role of FoxM1 in regulating NPC cell proliferation, we first tested the impact of FoxM1 inhibition on the viability of NPC cells *in vitro*. Human NPC CNE-1 and CNE-2 cells were treated with the indicated concentrations of thioestrepton for 48 and 72 h, and their cell viability was determined using the Cell Counting Kit-8. As shown in Fig. 1A, treatment with different doses of thioestrepton for 48 h reduced the relative viability of the CNE-1 cells in a dose-dependent manner, and treatment with thioestrepton for 72 h further reduced the relative viability of the CNE-1 cells. A similar pattern of thioestrepton toxicity against CNE-2 cells was observed. At a concentration of 8 μ M, thioestrepton reduced the relative viability of both cell lines by ~58 and 54%, respectively. Hence, thioestrepton reduced the relative viability of NPC cells in a dose- and time-dependent manner.

Next, we examined the effect of thioestrepton on anchorage-independent proliferation of NPC cells by colony formation assays. We found that treatment with 8 μ M thioestrepton significantly inhibited the numbers of formed CNE-1 and CNE-2 colonies *in vitro* (P<0.05 for both cells, Fig. 1B). To further investigate the effect of FoxM1 inhibition on anchorage-independent proliferation of NPC cells, we generated FoxM1-specific shRNA expressing or control lentiviruses and infected CNE-2 cells to establish FoxM1-specific shRNA stably expressing CNE-2 cell lines. Subsequently, we tested the impact of FoxM1 silencing on anchorage-independent proliferation of CNE-2 cells *in vitro*. We found that stable knockdown of FoxM1 expression significantly reduced the numbers of formed CNE-2 colonies *in vitro* (P<0.05, Fig. 1C). These three lines of evidence indicated that targeting of FoxM1 inhibited the proliferation of NPC cells and reduced the relative viability of NPC cells *in vitro*.

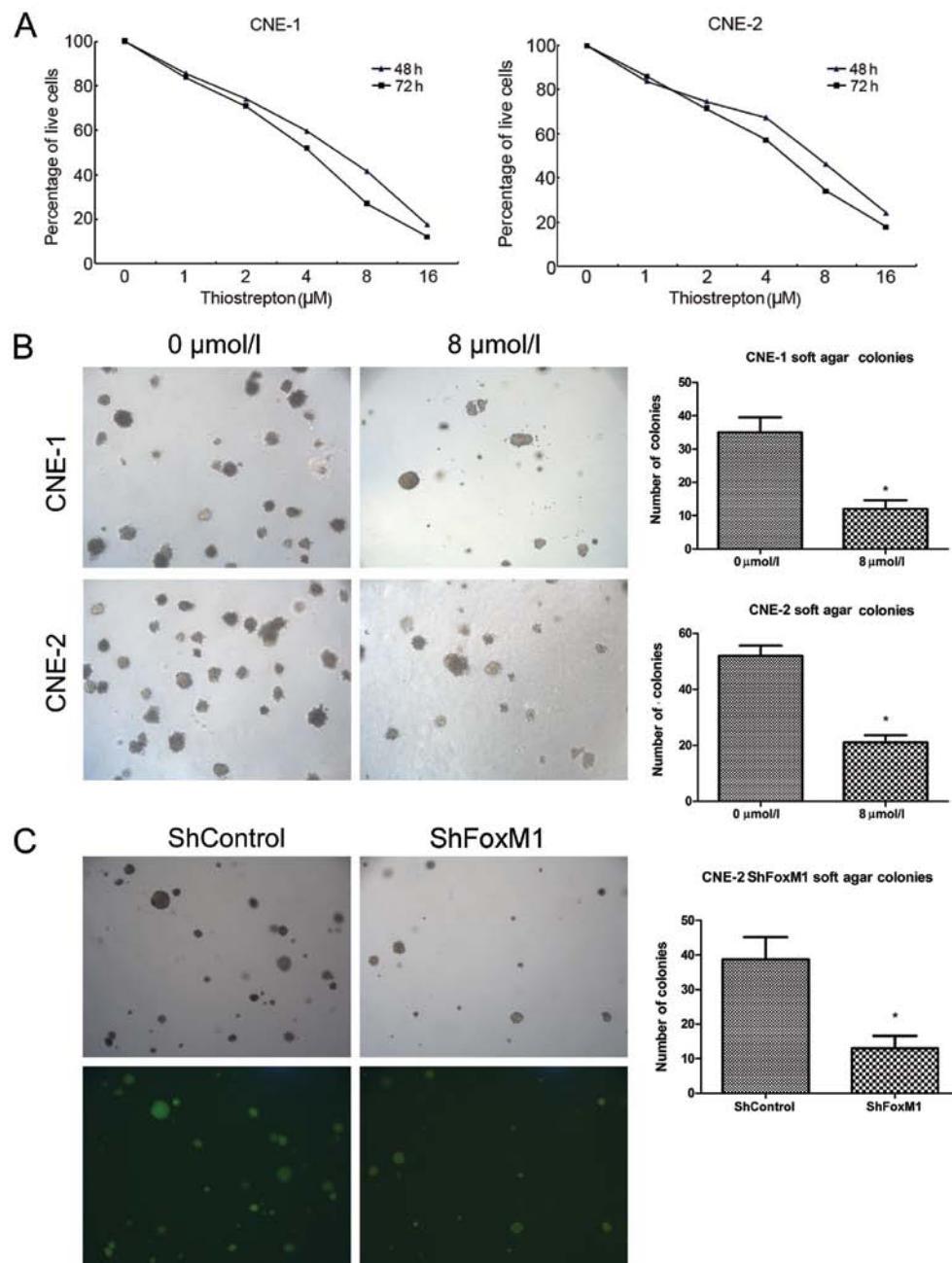


Figure 1. Thiostrepton has a potent cytotoxic effect on NPC cells *in vitro*. (A) CNE-1 and CNE-2 cells were treated with thiostrepton (0, 1, 2, 4, 8 and 16 μ M) for 48 or 72 h, and the survival of cells was determined using the CCK-8 assay. Data are the means (%) related to the controls (100%) from three separate experiments. (B) Thiostrepton inhibited the colony formation of NPC cells. CNE-1 and CNE-2 cells were treated with, or without, 8 μ M thiostrepton and cultured in soft-agar to assess the colony formation. (C) Knockdown of FoxM1 inhibited the colony formation of the CNE-2 cells *in vitro*. The FoxM1-specific shRNA stably expressing shFoxM1 and control shControl CNE-2 cell lines were established, and the colony formation was determined by soft-agar cultures. Data are representative images or expressed as the means \pm SD of individual groups of cells from three separate experiments. * $P < 0.05$ vs. the controls. NPC, nasopharyngeal carcinoma; FoxM1, forkhead box M1.

Targeting of FoxM1 inhibits the migration and invasion of NPC cells in vitro. FoxM1 can modulate MMP production and regulate cell migration and invasion (10,17). Accordingly, we tested the effect of thiostrepton on the migration of NPC cells *in vitro* by a Transwell migration assay. We found that treatment with 8 μ M thiostrepton significantly reduced the numbers of migrated CNE-1 and CNE-2 cells ($P < 0.05$ for both cell lines, Fig. 2A). Similarly, in comparison with that of the unmanipulated cells, transfection of CNE-1 and CNE-2 cells with control siRNA did not change the numbers of migrated NPC cells while transfection with FoxM1-specific siRNA significantly

reduced the numbers of migrated CNE-1 and CNE-2 cells ($P < 0.05$ for both cell lines). More importantly, the numbers of migrated CNE-1 and CNE-2 cells that had been transfected with control siRNA and treated with 8 μ M thiostrepton were similar to that of the CNE-1 and CNE-2 treated with 8 μ M thiostrepton (data not shown) while treatment with the same dose of thiostrepton further reduced the numbers of migrated CNE-1 and CNE-2 cells that had been transfected with FoxM1-specific siRNA. These data clearly indicated that treatment with thiostrepton and FoxM1 silencing synergistically inhibited the migration of CNE-1 and CNE-2 cells *in vitro*. A similar pattern of FoxM1

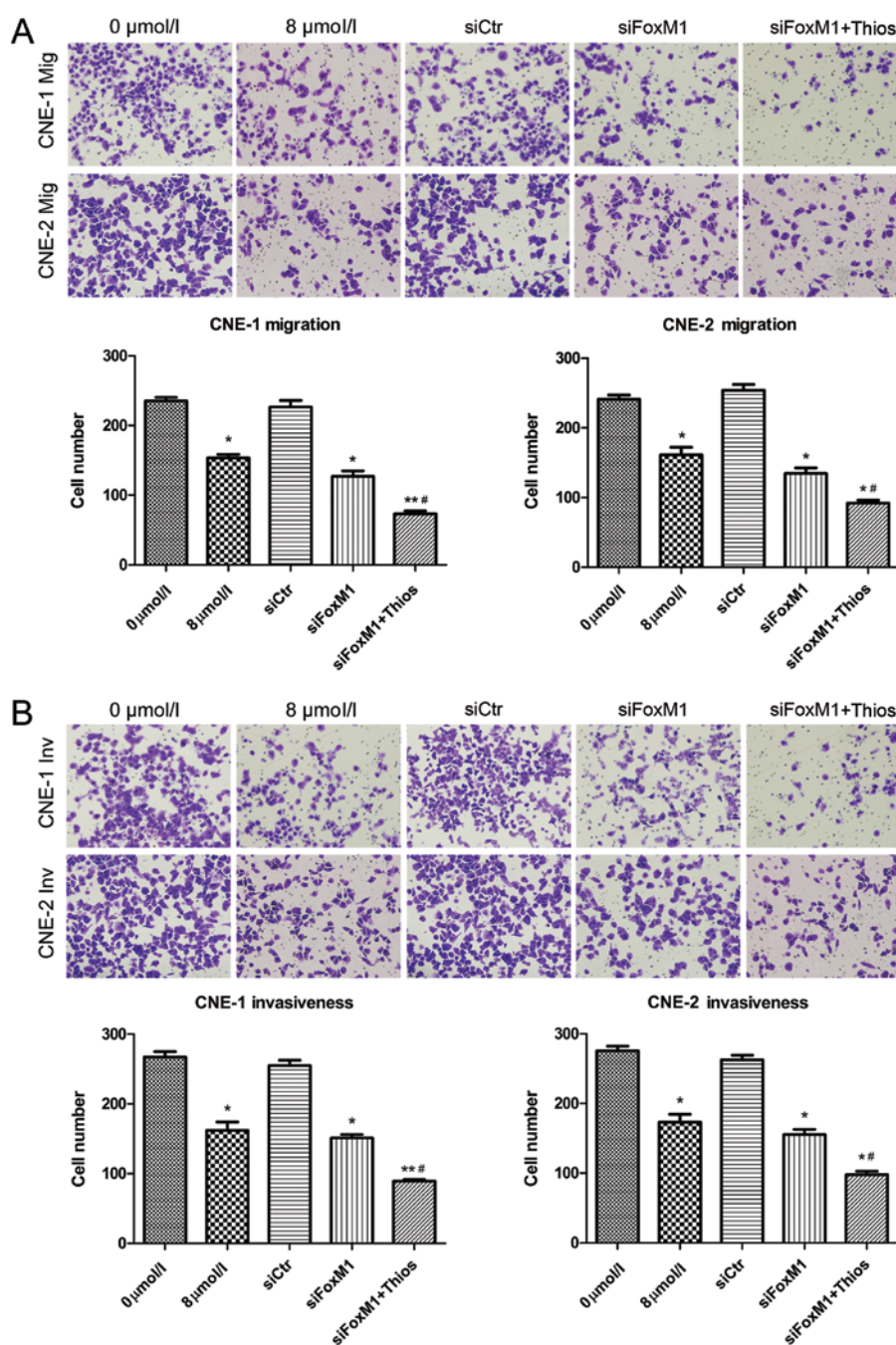


Figure 2. Thiostrepton and FoxM1-silencing synergistically inhibit the migration and invasion of NPC cells *in vitro*. CNE-1 and CNE-2 cells were treated with, or without, 8 μM thiostrepton and/or transfected with control siRNA or FoxM1-specific siRNA, and the migration and invasion of these cells were determined using Transwell migration or invasion assays. (A) Thiostrepton and FoxM1-silencing synergistically inhibited the migration of NPC cells. (B) Thiostrepton and FoxM1-silencing synergistically inhibited the invasion of NPC cells. Data are representative images or expressed as the means \pm SD of the migrated or invaded cells of each group from three separate experiments. * $P < 0.05$, ** $P < 0.01$ vs. the controls; # $P < 0.05$ vs. the siFoxM1 cells or the cells treated with 8 μM thiostrepton alone. NPC, nasopharyngeal carcinoma; FoxM1, forkhead box M1; siRNA, small interfering RNA; Thios, thiostrepton.

inhibition on the invasiveness of both CNE-1 and CNE-2 cells was observed (Fig. 2B). These data clearly demonstrated that targeting of FoxM1 inhibited the migration and invasion of the NPC cells *in vitro*.

Targeting of FoxM1 inhibits the EMT process in NPC cells. The EMT process is crucial for the migration and invasion of cancer cells and during the EMT process, downregulation of E-cadherin and upregulation of ZEB2 and Slug expression occur in cancer cells. To understand the potential mechanisms

underlying the regulatory effect of FoxM1 inhibition on the migration and invasion of NPC cells, we tested the impact of FoxM1 inhibition on the expression of E-cadherin, ZEB2 and Slug in the CNE-1 and CNE-2 cells by quantitative RT-PCR and western blot assays. As shown in Fig. 3A, in comparison with that in the unmanipulated control NPC cells, treatment with thiostrepton significantly reduced the relative levels of FoxM1, ZEB2 and Slug mRNA transcripts in the CNE-1 and CNE-2 cells ($P < 0.05$). In contrast, treatment with thiostrepton significantly upregulated the relative levels of E-cadherin

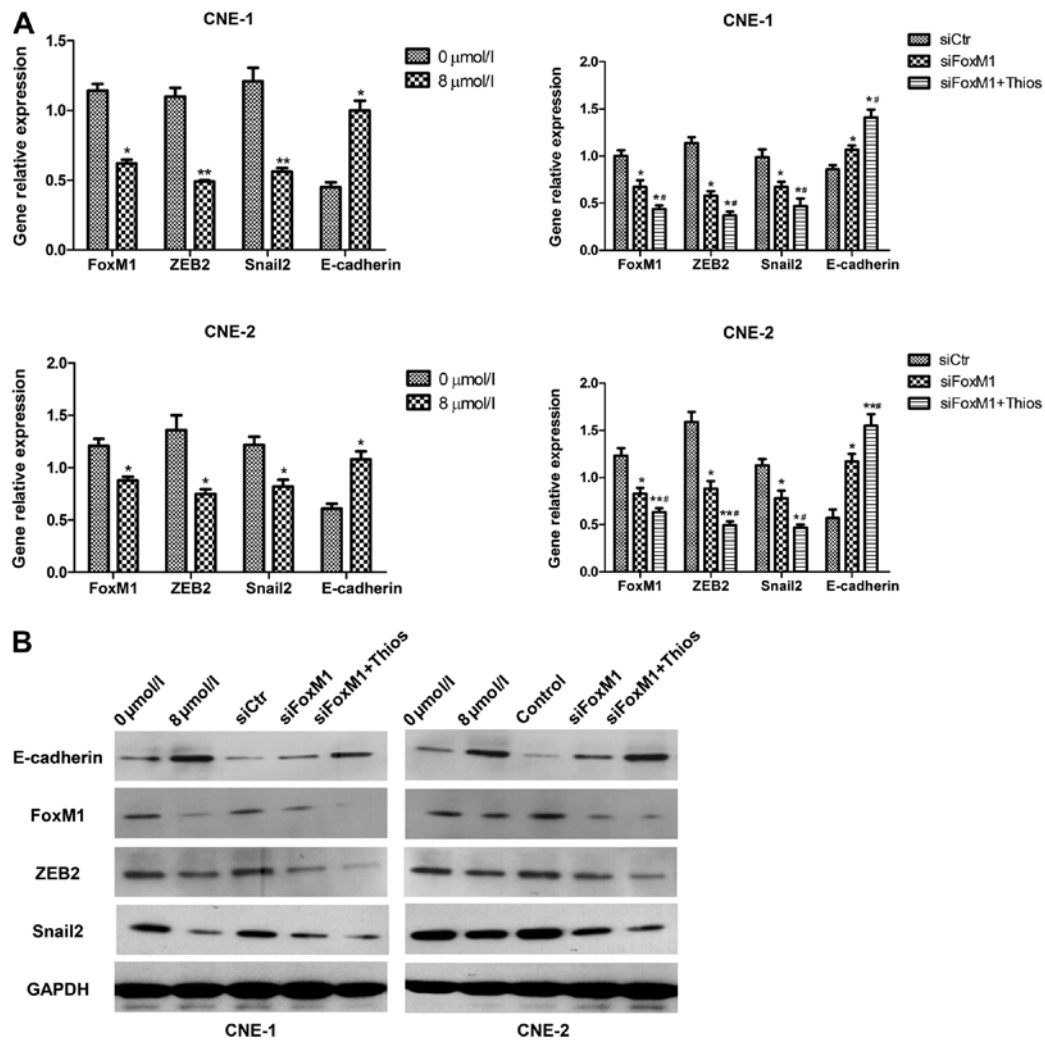


Figure 3. Thiostrepton and FoxM1-silencing synergistically modulate the relative expression levels of EMT-related regulators in NPC cells. CNE-1 and CNE-2 cells were treated with, or without, 8 μM thiostrepton and/or transfected with control siRNA or FoxM1-specific siRNA and the relative levels of the indicated gene mRNA transcripts to control GAPDH were determined by quantitative RT-PCR and western blot assays. (A) Quantitative RT-PCR analysis. (B) Western blot analysis. Data are representative images or expressed as the means \pm SD of the relative levels of gene mRNA transcripts from three separate experiments. * $P < 0.05$, ** $P < 0.01$ vs. the controls; # $P < 0.05$ vs. the siFoxM1 cells or the cells treated with 8 μM thiostrepton alone. NPC, nasopharyngeal carcinoma; FoxM1, forkhead box M1; siRNA, small interfering RNA; Thios, thiostrepton.

mRNA transcripts in both NPC cell lines. Similarly, transfection with FoxM1-specific siRNA significantly reduced the relative levels of FoxM1 mRNA transcripts, as compared to that in the control siRNA-transfected NPC cells, demonstrating effective knockdown of FoxM1 expression in both NPC cell lines. Furthermore, knockdown of FoxM1 expression significantly reduced the relative levels of ZEB2 and Slug mRNA transcripts in both NPC cell lines, but significantly upregulated the relative levels of E-cadherin mRNA transcripts in both cell lines. In addition, treatment with thiostrepton further significantly reduced the relative levels of FoxM1, ZEB2 and Slug mRNA transcripts, but elevated the relative levels of E-cadherin mRNA transcripts in the FoxM1-silencing CNE-1 and CNE-2 cells.

Western blot analyses revealed that the targeting of FoxM1 by treatment with thiostrepton or transfection of FoxM1-specific siRNA not only significantly reduced the relative levels of FoxM1, ZEB2 and Slug expression, but also significantly enhanced the relative levels of E-cadherin expression in CNE-1 and CNE-2 cells ($P < 0.05$, Fig. 3B). Targeting of

FoxM1 by both treatment with thiostrepton and siRNA-based FoxM1 silencing synergistically inhibited the FoxM1, ZEB2 and Slug expression, but enhanced the E-cadherin expression in both NPC cell lines *in vitro*. Collectively, these data suggest that targeting of FoxM1 expression significantly inhibits the EMT process, which may inhibit the migration and invasion of NPC cells.

Targeting of FoxM1 expression significantly inhibits the growth and migration of implanted NPC in vivo. To determine the effect of FoxM1 silencing on the growth and migration of NPC tumors *in vivo*, we implanted the FoxM1 shRNA stably expressing or control CNE-2 cells into nude mice. We found that while the tumors induced by control CNE-2 cells rapidly grew with time, the tumors induced by the FoxM1 shRNA stably expressing CNE-2 cells grew slowly. As a result, the volume of the tumors derived from the FoxM1 shRNA-expressing CNE-2 cells was significantly less than that of the tumors derived from the control CNE-2 cells ($P < 0.05$, Fig. 4A). Further analyses indicated that the mean

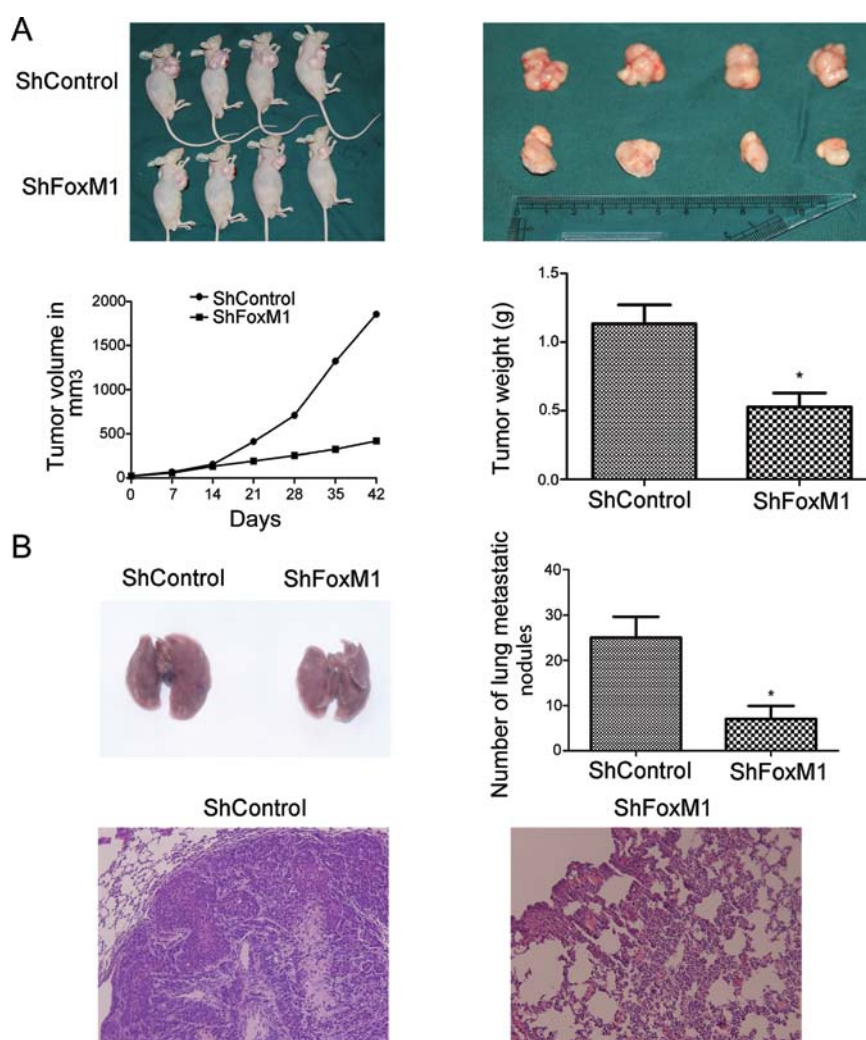


Figure 4. Stable knockdown of FoxM1 expression inhibits the growth and metastasis of implanted NPC *in vivo*. Nude mice were inoculated with ShControl or ShFoxM1 CNE-2 cells, and the growth of implanted NPC cells as well as the metastatic lung tumors were assessed by measuring the volumes and weights of the tumors and the numbers of lung metastatic nodules. Lung images are also shown. (A) FoxM1-silencing inhibited the growth of implanted NPC tumors. (B) FoxM1-silencing inhibited the lung metastasis of implanted NPC. Data are representative images expressed as the means or means \pm SD of each group of mice (n=10 per group). * P <0.05 vs. the controls. NPC, nasopharyngeal carcinoma; FoxM1, forkhead box M1.

weight of the tumors derived from the FoxM1 shRNA stably expressing CNE-2 cells was significantly less than that of the tumors derived from the control CNE-2 cells (P <0.05, Fig. 4A). More importantly, the number of metastatic nodules in the lungs of the FoxM1 shRNA-expressing tumors were significantly less than that in the control tumors (P <0.05, Fig. 4B). Immunohistochemical analyses revealed that the intensity of anti-FoxM1, anti-ZEB2 and anti-Slug staining in the tumors of the FoxM1 shRNA stably expressing tumors was obviously less than that in the control tumors (Fig. 5). In contrast, the intensity of anti-E-cadherin staining in the tumors of the FoxM1-shRNA stably expressing tumors was markedly stronger than that in the control tumors. Collectively, targeting of FoxM1 expression significantly inhibited the growth and lung metastasis of implanted NPC cells *in vivo*, associated with inhibition of the EMT process in NPC cells.

Discussion

FoxM1 overexpression has been found in a variety of aggressive human carcinomas (11,20,27-29) and is associated

with the early steps of metastasis of pancreatic and prostate cancers (17,30,31). In the present study, we examined the role of FoxM1 in the proliferation, migration and invasion of human NPC cells. We found that inhibition or knockdown of FoxM1 significantly reduced the survival of NPC cells and inhibited the anchorage-independent proliferation, migration and invasion of NPC cells. Furthermore, FoxM1 silencing inhibited the growth and lung metastasis of implanted NPC tumors in mice. Inhibition of FoxM1 or knockdown of FoxM1 expression increased the relative levels of E-cadherin, but reduced ZEB2 and Snail2 expression in the NPC cells and tumors. More importantly, treatment of FoxM1-silenced CNE-2 cells with the FoxM1 inhibitor synergistically enhanced the inhibitory effects. These novel data clearly indicate that knockdown of FoxM1 inhibits the growth and metastasis of human NPC.

Cancer metastasis involves a series of complex steps, including decreased adhesion, increased motility, cell attachment, matrix dissolution and migration (18). During tumor progression, cancer cells undergo dramatic changes in cytoskeletal organization to adopt an invasive phenotype and eventually metastasize to other organs (16). The EMT process

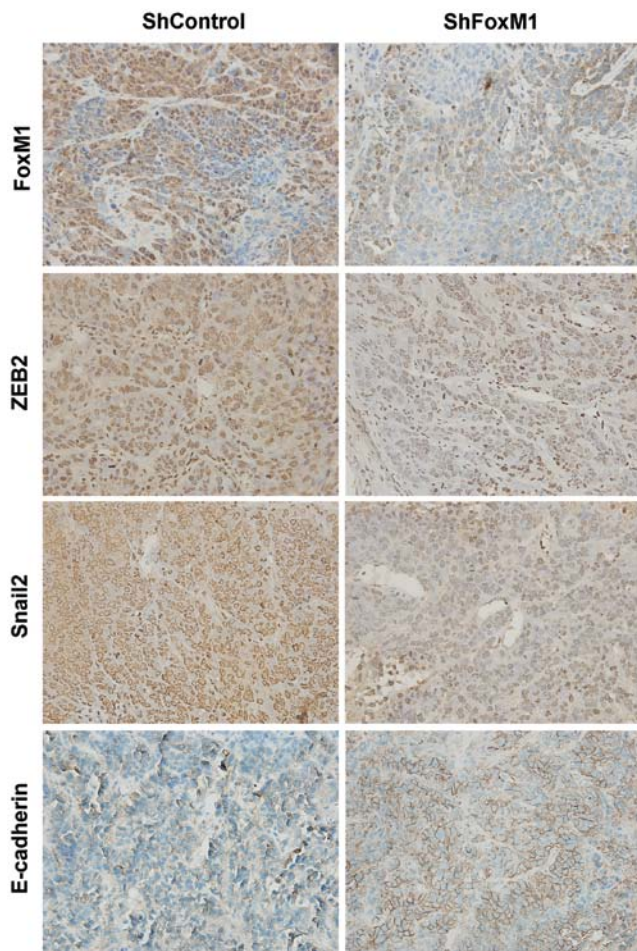


Figure 5. Immunohistochemical analysis of the expression of EMT-related regulators in NPC tumors. The dissected tumors from each group of mice were analyzed for the expression of FoxM1, ZEB2, Snail2 and E-cadherin by immunohistochemistry. Data are representative images of gene expression in the tumor sections from each group of mice (n=6 per group). NPC, nasopharyngeal carcinoma; FoxM1, forkhead box M1; EMT, epithelial-to-mesenchymal transition; ZEB2, zinc finger E-box binding homeobox 2.

has been thought to be crucial for the migration and metastasis of cancer (32,33). During the EMT process, cancer cells usually lose E-cadherin expression and gain ZEB2, Snail2, vimentin and N-cadherin expression (34-38). In the present study, we found that treatment with the FoxM1 inhibitor or knockdown of FoxM1 expression significantly increased the relative levels of E-cadherin expression, but decreased the ZEB2 and Snail2 expression in NPC cells and related NPC tumors. Previous studies have shown that FoxM1 can stimulate the expression of MMP-2, MMP-9 and vascular endothelial growth factor (VEGF) in pancreatic cancers and promotes the metastasis of prostate cancer (17,30,31). More importantly, we found that inhibition or knockdown of FoxM1 expression inhibited the migration of NPC cells *in vitro* and the lung metastasis of implanted NPC *in vivo*. Our findings extended these findings and support the notion that FoxM1 positively regulates the EMT process in cancer cells. Our findings also provide a new explanation why high levels of FoxM1 expression are associated with a poor prognosis of NPC (21). Hence, FoxM1 may be a potential target for the intervention of NPC.

Previous studies have shown that FoxM1 is a regulator of cell cycling and is important for the maintenance of genomic

stability and chromosomal integrity (8-10). Furthermore, FoxM1 has been shown to stimulate Snail2 expression in pancreatic cancer cells (17). We found that knockdown of FoxM1 expression not only reduced the relative levels of Snail2 and ZEB2 expression, but also upregulated E-cadherin expression in NPC cells. These data suggest that FoxM1 may directly or indirectly stimulate ZEB2 expression in NPC cells. Although FoxM1 has been recognized as a tumorigenesis-promoting transcription factor, FoxM1c transactivates the expression of mouse and human E-cadherin, a tumor suppressor (38). Indeed, endothelial cell-specific FoxM1 knockout promoted urethane-induced lung tumors in mice. It is possible that FoxM1 has dual functions in regulating tumorigenesis. We found that knockdown of FoxM1 inhibited the EMT process, proliferation and migration of NPC cells, suggesting that FoxM1 may act as a tumorigenesis- and metastasis-promoting transcription factor in NPC.

In summary, our data indicated that treatment with a FoxM1 inhibitor or knockdown of FoxM1 expression reduced the survival of NPC cells and inhibited the anchorage-independent proliferation, migration and invasion of NPC cells. Knockdown of FoxM1 inhibited the tumor growth and lung metastasis of NPC cells in mice. The inhibition of FoxM1 downregulated ZEB2 and Snail2 expression, but upregulated E-cadherin expression in the NPC cells and related tumors, indicating that knockdown of FoxM1 inhibited the EMT process in NPC. Collectively, our data suggest that FoxM1 may be a positive regulator of NPC metastasis and a potential target for prognosis and therapy. Therefore, our findings may provide new insights into molecular regulation by FoxM1 of the metastasis of NPC and may aid in the design of new therapies for intervention of NPC in the clinic.

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