



Original Research

## Knockdown of MPP8 suppresses cell proliferation via regulation of HOXA5 in non-small cell lung cancer cells

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Received August 3, 2017; Accepted January 25, 2018; Published January 31, 2018

Doi: <http://dx.doi.org/10.14715/cmb/2018.64.1.6>

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**Abstract:** M-phase phosphoprotein 8 (MPP8) is reported to be closely implicated in cancer initiation and progression. In addition, the homeobox gene HOXA5 has been shown to play critical roles in hematopoiesis, embryogenesis, and tumorigenesis. Nevertheless, the functional relevance of MPP8 and its relation with HOXA5 in non-small cell lung cancer (NSCLC) is unknown. Therefore, the present study aimed to detect the expression profile of MPP8 in NSCLC and further explore its biological roles in lung cancer cells. Cell proliferation was measured by CCK-8 assay and EdU incorporation assay. Real-time PCR was applied to detect the mRNA expression of MPP8 and HOXA5. The protein levels of MPP8 and HOXA5 were evaluated by western blot. Our study found that the expression of MPP8 was significantly increased in the NSCLC tissue compared with the adjacent non-tumorous tissue. Compared with the human lung fibroblasts, the elevated gene expression of MPP8 was also detected in the human NSCLC cell lines including NCI-H23 and NCI-H1299. In addition, knockdown of MPP8 led to an obvious reduction in cell viability and DNA synthesis in NCI-H23 and NCI-H1299 cells. Furthermore, down-regulation of MPP8 resulted in elevated expression of HOXA5 in NSCLC cells both at the mRNA and protein levels. Moreover, depletion of HOXA5 abolished the anti-tumor function of MPP8 knockdown in NSCLC cells. The present study demonstrated that MPP8 was associated with NSCLC cell proliferation through regulation of HOXA5, suggesting that MPP8 may act as a novel therapeutic target for treatment of NSCLC.

**Key words:** Non-small cell lung cancer, MPP8, HOXA5, proliferation.

### Introduction

Lung cancer is the second most common cancer and the leading cause of cancer-related deaths in the world (1). Approximately 80% of lung cancers are classified as non-small cell lung carcinoma (NSCLC) with significant differences in the clinical and pathological manifestation of the small cell lung carcinoma (SCLC) (2). During the past several decades, various treatment modalities of lung cancer have been developed, including surgery, radiotherapy, chemotherapy and molecular targeted therapy (3, 4). However, most NSCLC patients received the curative treatment are still at a high risk of relapse. Therefore, it is urgent to broaden our understanding of the tumor molecular biology to develop novel therapeutic targets for lung cancer.

The M-phase phosphoprotein 8 (MPP8) was identified as a protein that is highly phosphorylated during M phase (5). It consists of two functional domains, a carboxy-terminal ankyrin domain and an amino-terminal chromodomain affinitive to trimethylated histone H3 lysine 9 (H3K9me3) residues (5). During the progression of cell cycle, MPP8 primarily localizes to chromatin and dissociates during interphase and early mitosis, respectively (6). Mechanically, the chromatin dissociation of MPP8 during metaphase to anaphase is regulated by cyclin B1-Cdk1 signals (7). Particularly, Kokura et al revealed that the expression of MPP8 was

increased in breast cancer cells, and knockdown of MPP8 led to an obvious reduction of cell migration and invasion, suggesting that MPP8 may serve as an oncogene in the cancer progression (8). Additionally, MPP8 has been demonstrated to be implicated in transcription regulation and cancer cell behaviors (9). The homeobox gene HOXA5 has been reported to be closely involved in several biological processes, including embryogenesis and hematopoiesis (5, 8). Moreover, deregulated expression of HOXA5 is implicated in the pathogenesis of several cancers, such as breast cancer, colorectal cancer and clear cell renal cell carcinoma (10-13). In human lung cancer, HOXA5 correlates with TNM stages, tumor size, and lymph node metastasis. Meanwhile, HOXA5 regulates cell proliferation, apoptosis, migration, and invasion in non-small cell lung cancer (NSCLC) cells (14-16). In the present study, we explored the expression and functional relevance of MPP8 in lung cancer as well as the underlying molecular mechanism.

### Materials and Methods

#### Clinical sample

Twenty-four patients pathologically diagnosed with NSCLC were recruited during May 2015 to December 2015. Lung cancer tissues and adjacent normal tissues were collected from these patients undergone routine surgery in the Department of Medical Oncology, Fujian

Cancer Hospital. No radiotherapy or chemotherapy had been conducted in these patients prior to the surgery. This study was approved by the Ethics Committee of the Puer People's Hospital, and informed consent was signed by all the participated patients.

### Cell culture

Human NSCLC cell lines, including NCI-H23 and NCI-H1299, and human lung fibroblasts WI-38 were purchased from the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All cells were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

### Lentiviral transduction

In brief, lentivirus carrying MPP8 shRNA sequence (GCAACAGATGCAATTCCTCAAGT) was generated by transfection of 80% confluent HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and then used to interference MPP8 expression.

### Cell viability assay

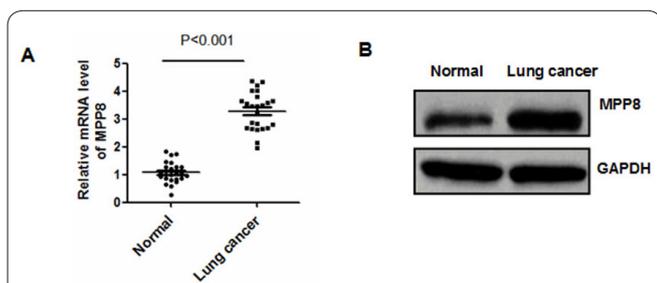
Cell viability was detected using the cell proliferation reagent WST-8 (Sigma, USA). After seeding cells into 96-well plates at  $1.0 \times 10^3$ /well, 10  $\mu$ L of CCK-8 was added to each well according to the manufacturer's protocol. Then, the cellular viability was measured using a spectrophotometer at 450 nm.

### EdU incorporation assay

Cells were seeded into the 96-well plates at a density of  $1.0 \times 10^3$ /well and were allowed to grow overnight in complete medium. Then, cells were incubated with 5-ethynyl-2'-deoxyuridine (EdU), and DNA synthesis was determined using an EdU cell proliferation detection Kit (RiboBio, China) according to the manufacturer's protocol.

### RNA extraction, cDNA synthesis, and real-time PCR

Total RNAs were isolated from cells with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA was reversely synthesized with 2  $\mu$ g of total RNA using a Takara RNA PCR kit according to the manufacturer's protocol (Takara, Japan). Real-time PCR was performed in a volume of 20  $\mu$ l using a SYBR Green Premix Ex Taq (Takara, Japan) on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). Primers for MPP8: F- 5' CATC-GACTCTGTGAGGGTCC-3', and R 5'-TGACTGTC-



**Figure 1.** Increased expression of MPP8 in NSCLC tissue. NSCLC tissues and the adjacent normal tissues were harvested and subject to real-time PCR (A) and western blot (B) for determination of MPP8 expression.

CAGAAAAACAGGA-3'; primers for HOXA5: F-5' TAACTGTGCATGTGCGTGGGA-3', and R 5'-GAGCCACTTCCAGAGTTCGT-3'. The relative mRNA expression of each gene was evaluated using the  $\Delta\Delta$ CT method.

### Western blot

Tumor cells were harvested with PBS, and lysed with ice-cold RIPA lysis buffer. The proteins were extracted and quantified using the BCA kit according to the manufacturer's protocol (Pierce, Rockford, IL, USA). After protein quantification, 10  $\mu$ g of proteins were subject to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF membrane. The membrane was blocked with 10% nonfat milk in PBS and then incubated with anti-MPP8 (dilution: 1:2000) and anti-HOXA5 antibodies (dilution: 1:2000) (Santa Cruz, CA, USA) overnight at 4 °C. Then, the membrane was washed in PBST for three times and incubated with the HRP-linked secondary antibodies (Santa Cruz, CA, USA). Protein bands were detected by (ECL) kit (Millipore, Fisher Scientific, USA) according to manufacturer's protocol. The relative protein levels were quantified to total GAPDH (Santa Cruz, CA, USA).

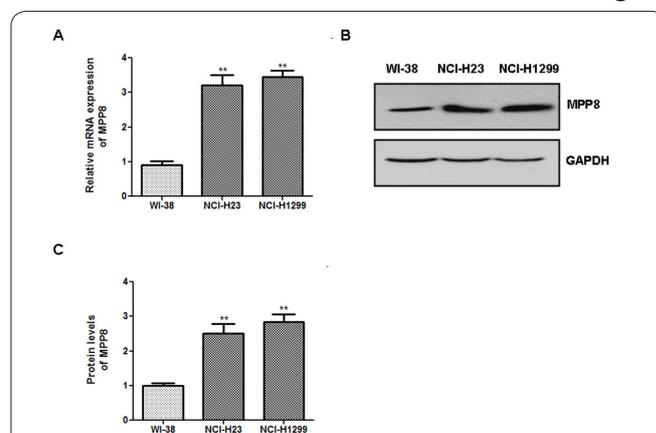
### Statistical analysis

Experimental were repeated at least three times, and the data were presented as mean  $\pm$  SD. Results were statistically evaluated with the ANOVA and Student's t-test using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Statistical significance is displayed as \* ( $P < 0.05$ ), or \*\* ( $P < 0.01$ ).

## Results

### Deregulated expression of MPP8 in lung cancer

In order to elucidate the role of MPP8 in lung cancer, we firstly detected the expression of MPP8 in the NSCLC tissue and adjacent non-tumorous tissue. Real-time PCR analysis and western blot showed that the mRNA and protein levels of MPP8 were elevated in the tumor sample compared with the normal tissue (Figure 1A and B). Additionally, we examined the expression of MPP8 in several NSCLC cell lines. As shown in Figure



**Figure 2.** Up-regulated expression of MPP8 in NSCLC cell lines. Human NSCLC cell lines (NCI-H23 and NCI-H1299) and the lung fibroblasts (WI-38) were subject to real-time PCR (A) and western blot (B). Protein expression was quantified with GAPDH served as internal loading control (C). \*\* $P < 0.01$ .

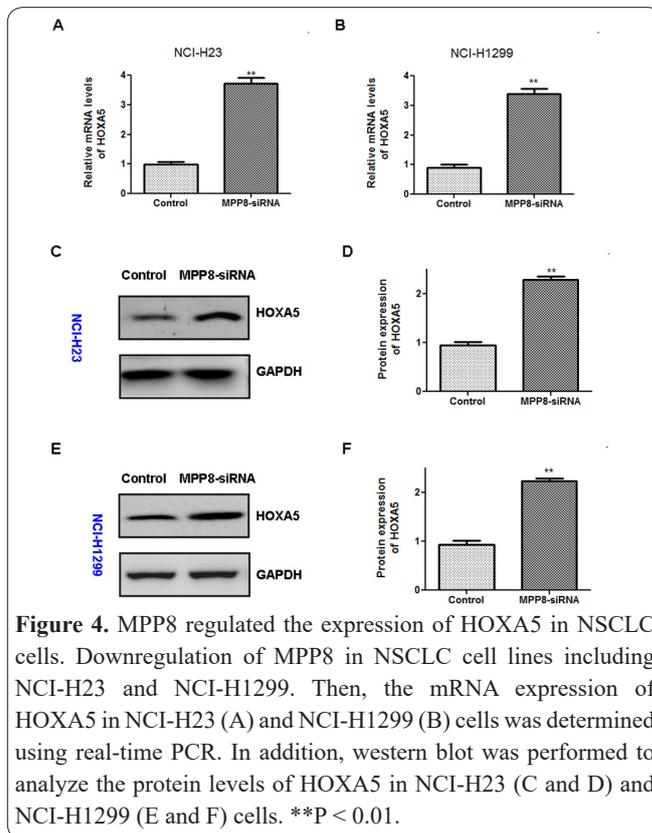
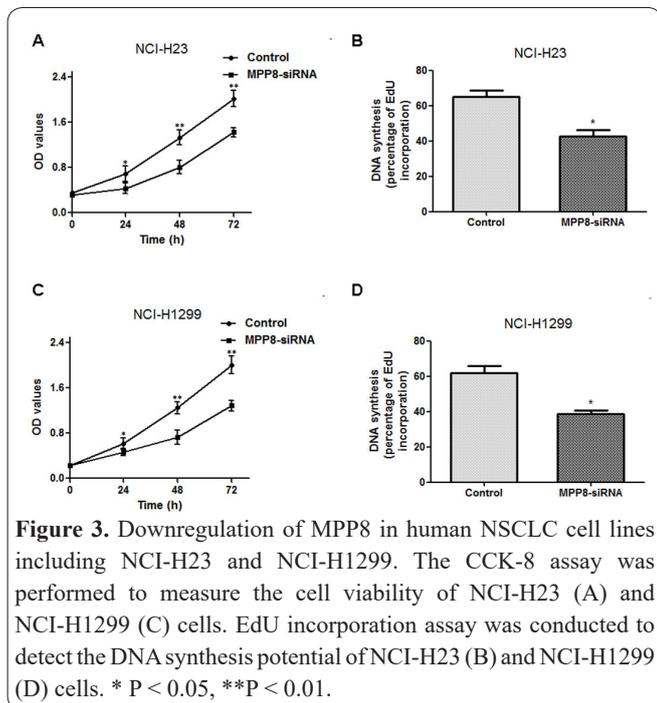
2A, these increased MPP8 transcripts were also observed in the human lung cancer cell lines (NCI-H23 and NCI-H1299) compared with the human lung fibroblasts WI-38. Consistently, we found that the protein expression pattern of MPP8 was elevated in the human lung cancer NCI-H23 and NCI-H1299 cells compared with WI-38 cells (Figure 2B and C). Collectively, these data suggested that MPP8 might be implicated in the NSCLC cell biological processes.

### MPP8 knockdown inhibited cell growth in human NSCLC cells

To further explore the functional role of MPP8, we transfected lung cancer NCI-H23 cells with lentivirus expressing MPP8 shRNA and successfully decreased the expression of MPP8 by more than 70%. After transfection, we performed a CCK-8 assay to evaluate the functional outcomes induced by down-regulation of MPP. Interestingly, we found that knockdown of MPP8 caused a significant reduction in cell viability (Figure 3A). Moreover, we conducted EdU incorporation assay to measure DNA synthesis ability in NSCLC cells after downregulation of MPP8. Results showed that MPP8 knockdown significantly inhibited the DNA synthesis in NCI-H23 cells (Figure 3B). Furthermore, we found that down-regulation of MPP8 also suppressed the cell growth (Figure 3C) and DNA synthesis (Figure 3D) of NCI-H1299 cells. Taken together, these data suggested that down-regulation of MPP8 could inhibit cell proliferation in NSCLC cells.

### Effects of MPP8 knockdown on HOXA5 expression in NSCLC cells

We further investigated the underline mechanism of MPP8 in the regulation of lung cancer development. Previous investigation has revealed that HOXA5 is involved in the genesis of several cancers, including lung cancer (14-16). In our study, we interfered the expression of MPP8 and found that the mRNA levels of HOXA5 were significantly increased in NCI-H23 (Figure 4A) and NCI-H1299 (Figure 4B) cells. Moreover,

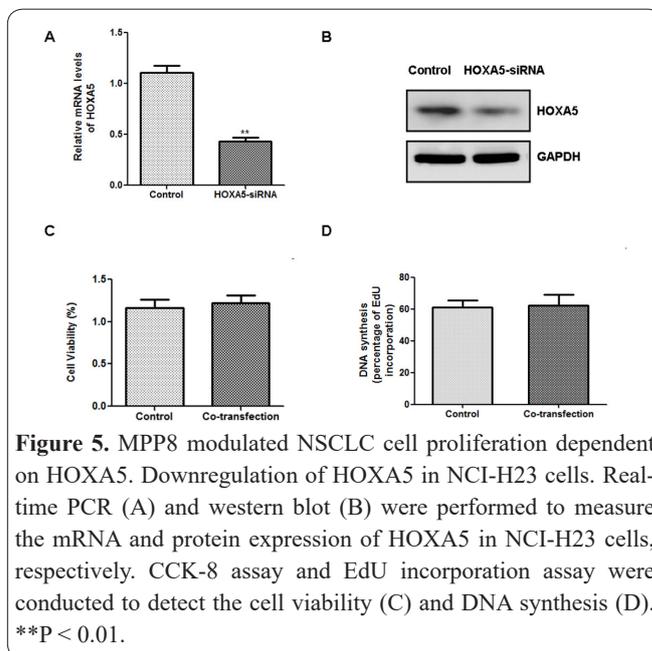


**Figure 4.** MPP8 regulated the expression of HOXA5 in NSCLC cells. Downregulation of MPP8 in NSCLC cell lines including NCI-H23 and NCI-H1299. Then, the mRNA expression of HOXA5 in NCI-H23 (A) and NCI-H1299 (B) cells was determined using real-time PCR. In addition, western blot was performed to analyze the protein levels of HOXA5 in NCI-H23 (C and D) and NCI-H1299 (E and F) cells. \*\* $P < 0.01$ .

western blot analysis showed that knockdown of MPP8 also enhanced the protein profile of HOXA5 in the human NSCLC cells lines including NCI-H23 (Figure 4C and D) and NCI-H1299 (Figure 4E and F). These results implied that there was a possible link between HOXA5 and MPP8 in human NSCLC cells.

### Effects of HOXA5 knockdown on the cell viability of NSCLC cells with depletion of MPP8

Given that HOXA5 levels were affected by depletion of MPP8, we thus silenced the expression of HOXA5 in NSCLC cells. As a result, real-time PCR showed that HOXA5 transcripts were efficiently decreased in lung cancer NCI-H23 cells (Figure 5A). Consistently, western blot analysis also revealed that the protein expression of HOXA5 was downregulated in tumor cells



**Figure 5.** MPP8 modulated NSCLC cell proliferation dependent on HOXA5. Downregulation of HOXA5 in NCI-H23 cells. Real-time PCR (A) and western blot (B) were performed to measure the mRNA and protein expression of HOXA5 in NCI-H23 cells, respectively. CCK-8 assay and EdU incorporation assay were conducted to detect the cell viability (C) and DNA synthesis (D). \*\* $P < 0.01$ .

(Figure 5B). As mentioned above, down-regulation of MPP8 could inhibit cell proliferation in NSCLC cells. We found that interfering with HOXA5 led no obvious differences in terms of cell viability (Figure 5C) and DNA synthesis (Figure 5D) of tumor cells downregulating MPP8. These data suggested that HOXA5 mediated the anti-tumor function of MPP8 in NSCLC cells.

## Discussion

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer death worldwide. The pathogenesis of lung cancer is believed to be composed of a series of events occurring at both the genetic and epigenetic levels (17, 18). Thus, the revelation of the molecular mechanism underlying lung cancer would greatly contribute to the development of novel therapy for such tumors. In the current study, we evaluated the expression pattern of MPP8 in the NSCLC tissue and the matched normal tissue. Furthermore, we explored the molecular mechanism of MPP8 in the regulation of cancer development.

It is well known that cancer initiation and progression are closely implicated in the uncontrolled cell growth. As a methyl-H3K9-binding protein, MPP8 has been shown to be expressed in the nucleus during the mitotic phase. Specifically, MPP8 was found to be phosphorylated in M-phase of cell cycle, suggesting that it could play a role in cell proliferation and cell cycle progression (5). Later investigations have demonstrated that depletion of MPP8 resulted in an obvious reduction in the cell growth and invasion in breast cancer cells (8). In a recent study, Liang *et al.* have found that lentivirus-mediated knockdown of MPP8 could suppress colon cancer cell proliferation and migration and induce tumor cell apoptosis (19). Mechanically, Kenji *et al.* demonstrated that MPP8 knockdown led to a reduction in the DNA methylation of E-cadherin CpG island, suggesting a mechanism by which MPP8 recognizes methyl-H3K9 marks and inhibit the expression of tumor suppressor; consequently, MPP8 plays a critical role in epithelial-to-mesenchymal transition (EMT) and metastasis (8). Based on these findings, we speculated that MPP8 may also be involved in the NSCLC progression. Our results showed that the transcripts profile of MPP8 was significantly increased in NSCLC tissues compared with the matched normal tissue. Moreover, the elevated MPP8 was also observed in the lung cancer NCI-H23 and NCI-H1299 cells compared with WI-38 cells. In addition, knockdown of MPP8 led to a significant reduction in cell viability and DNA synthesis in the lung cancer NCI-H23 and NCI-H1299 cells. Their findings suggested that down-regulation of MPP8 could be able to inhibit NSCLC cell proliferation.

Currently, four HOX gene clusters have been discovered in mammals and they act as critical regulators during embryonic development (20, 21). Through targeting a battery of genes, HOX genes play important role in a variety of cellular biology including cell growth, differentiation, invasion and metastasis (22-24). HOXA5 has been shown to be involved in the regulation of lung development. Mandeville *et al.* found that knockout of HOXA5 in mice led to impaired lung maturation and function (25). Moreover, deregulated

expression of HOXA5 has been shown to be involved in carcinogenesis, such as NSCLC, breast cancer, colorectal cancer and clear cell renal cell carcinoma (10-13). In the present study, knockdown of MPP8 led to increased gene expression of HOXA5 in NCI-H23 and NCI-H1299 cells. In addition, to evaluate the role of HOXA5 in the context of MPP8-knockdown cells, we depleted HOXA5 expression in lung cancer cells. Compared with the control group, we didn't observe significant differences in cell proliferation in NSCLC cells after interfering with MPP8 and HOXA5. These results suggested that HOXA5 was associated with the anti-tumor function of MPP8 in NSCLC cells.

In conclusion, our present study demonstrated that MPP8 participated in the proliferation of NSCLC cells through regulation of HOXA5. These novel findings implied that knockdown of MPP8 may be developed into an effective therapeutic approach for NSCLC treatment.

## Conflict of interest

Authors declared no conflict of interest.

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