Int J Clin Exp Pathol 2017;10(12):12003-12009
www.ijcep.com /ISSN:1936-2625/IJCEP0065492

Original Article

Repression of M-phase phosphoprotein 8 inhibits melanoma growth and metastasis *in vitro* and *in vivo*

Bo Yuan[1], Lei Lin[2], Zhen-Yi Ying[1], Meng-Xia Ying[1], Qiong-Yan Zhou[1], Lei Shi[1]

[1]*Department of Dermatology, The Affiliated Hospital of Medical School, Ningbo University, Ningbo 315020, China;* [2]*Department of Aesthetic Medicine, Ningbo College of Health Sciences, Ningbo, China*

Received September 13, 2017; Accepted October 30, 2017; Epub December 1, 2017; Published December 15, 2017

Abstract: Metastatic melanoma accounts for the majority of skin cancer deaths due to its aggressiveness and high resistance to current therapies. M-phase phosphoprotein 8 (MPP8) has been shown to bind to methylated H3K9 and promote tumor cell motility and invasion. The current study aimed to investigate the role of MPP8 in melanoma growth and metastasis. Our results showed that MMP8 was up-regulated in the metastatic melanoma specimens. Knockdown of MPP8 inhibited melanoma cell growth both *in vitro* and *in vivo*. Furthermore, down-regulation of MPP8 induced S-phase cell cycle arrest as well as altered expression of cell cycle-related proteins in melanoma cells. In addition, repression of MPP8 inhibited the migration and invasion of melanoma cells both *in vitro* and *in vivo*. Taken together, these data suggest that MPP8 knockdown could inhibit the growth and metastasis of melanoma cells and provide novel therapeutic target for melanoma treatment.

Keywords: Melanoma, MPP8, growth, metastasis

Introduction

Melanoma is one of the most malignant types of skin cancers with rapid progression and poor prognosis. It has been estimated that the incidence of melanoma has been increasing steadily worldwide in the past 20 years [1, 2]. In the early stages, malignant melanoma can be successfully cured by surgical resection. However, it is extremely difficult to treat the metastatic melanoma which is resistance to currently available therapies. Therefore, revelation of the molecular mechanism underlying the melanoma metastasis is critical for developing novel therapeutic means of skin cancers [3].

Metastasis is a complex series of steps in which cancer cells leave the original site and migrate to other sites to form new tumors [4]. Epithelial-mesenchymal transition (EMT) refers to the transition from an epithelial to a mesenchymal cell phenotype characterized by altered gene expression and cell morphology. Accumulating evidence has suggested that EMT is closely associated with the infiltration transfer of tumor cells in breast cancer, lung cancer, hepatocellular carcinoma and melanoma [5, 6].

The M-phase phosphoprotein 8 (MPP8), firstly identified as a methyl-H3K9-binding protein, displays increased expression levels in various human tumor cells [7]. Functional analysis reveals that MPP8 plays an important role in the tumor cell invasion and migration [7]. Mechanically, MPP8 targets the E-cadherin gene promoter and mediates the expression of this critical regulator of EMT via its methyl-H3K9 binding ability [8, 9]. In the current study, we for the first time investigated the role of MPP8 in the melanoma metastasis using *in vitro* and *in vivo* models.

Materials and methods

Human tissue specimens

Human melanoma tissues were obtained from the Department of Dermatology at Affiliated Hospital of Ningbo University School of Medicine And Ningbo Clinical Pathology Diagnosis

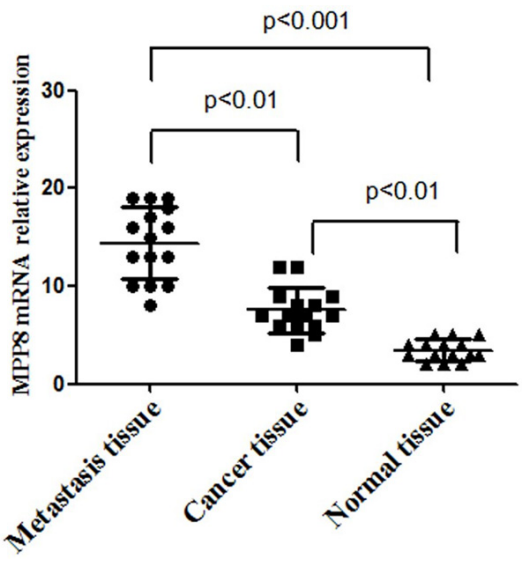

Figure 1. Up-regulation of MPP8 in the melanoma specimens. qRT-PCR was performed to measure the mRNA expression of MPP8 in metastatic melanoma and melanoma in situ. **P<0.01.

Center. All human materials were used in accordance with the policies of the institutional review board at Affiliated Hospital of Ningbo University School of Medicine.

Cell culture

A375 melanoma cells were purchased from the Chinese academy of sciences (Shanghai, China) and cultured in DMEM with 10% FBS. All maintenance media contained 100,000 U/L penicillin and 100 mg/L streptomycin.

MTT assay

Cells were adjusted into a density of 5×10^4 per ml. Cells were seeded 100 μl/well into 96-well plates for MTT detection. The plates were then incubated at 37°C for 48 hours. 20 ml of MTT stock solution (5 mg/ml) was added to each well and incubated for 4 hours. Absorbance of the reaction was measured with a spectrophotometer at 490 nm.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen, America) according to the manufacturer's instructions. cDNA was synthesized with 2 μg of total RNA. The PCR reaction was performed in a volume of 20 μl and done for triplicates. MPP8 primers are: 5'AAAGCAAGGTCTCCCCACAAG'3, and 5'GGTCTGTGCT AGATCAAAAGGCA3'. Relative gene expression was evaluated using the ΔΔCT method.

Colony formation

Cells were seeded in 6-wells plates and cultured in DMEM for different time points. After that, cells were washed, fixed with methanol, and stained with a 5% Giemsa solution. Colonies were photographed (100 × magnification) on 5 different fields and the relative colony number was calculated.

Flow cytometry

Cells were seeded in 60 mm plates and cultured in DMEM media. Then, cells were collected, washed in PBS, and fixed in ice-cold 70% ethanol for 24 h. At last, cells were incubated with PI and subject to flow cytometry. At least three independent experiments were repeated.

Transwell migration and wound healing migration assays

Cell motility was measured by transwell migration and wound healing migration assays. In brief, cells were harvested, resuspended in serum-free medium, and then transferred to the transwell chambers (BD Biosciences, San Jose, CA). The chambers were then incubated for 3 h in culture medium with 10% FBS in the bottom chambers before analysis. The cells were stained with 0.05% crystal violet and counted under a microscope and the cell number was calculated. For wound healing assay, the monolayer culture was scrape-wounded with a sterile micropipette tip in order to create a gap. Then, cell migration was photographed into the wounded region following the scraping.

Western blot

Cells were harvested, washed with cold PBS, and lysed in the lysis buffer. Proteins were subject to SDS-PAGE and then transferred to PVDF membrane. The membrane was blocked with 5% BSA in TBST buffer and incubated with the primary antibodies followed by appropriate horse radish peroxidase-conjugated secondary antibodies. At last, the protein expression was measured using ECL detection with beta-actin served as controls.

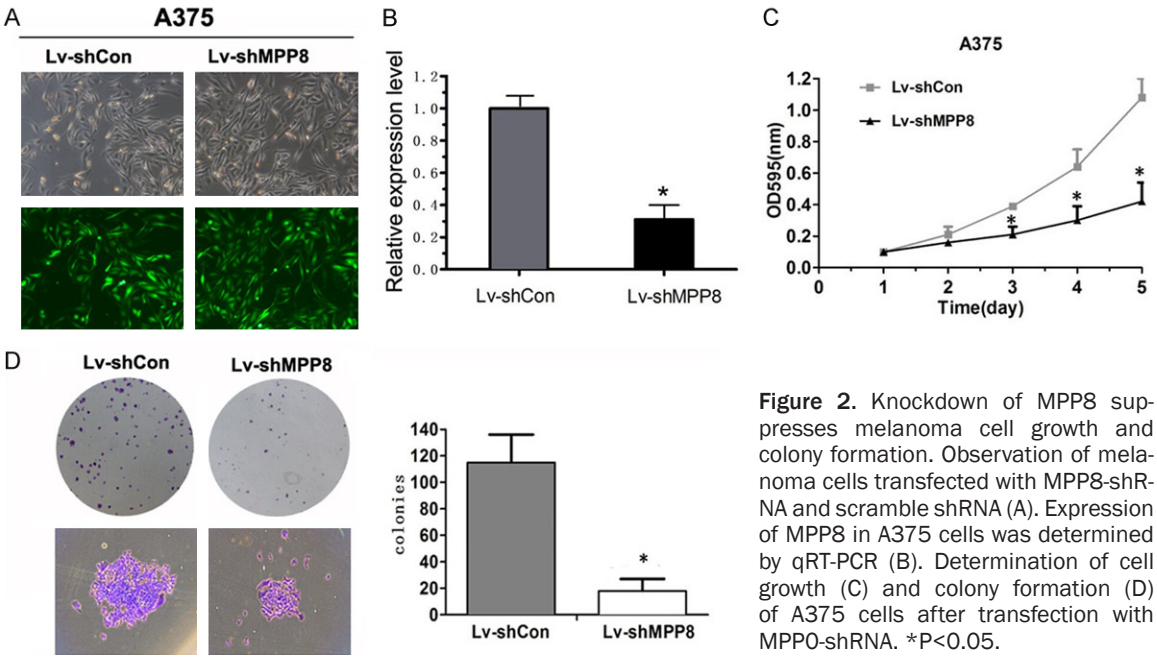

Figure 2. Knockdown of MPP8 suppresses melanoma cell growth and colony formation. Observation of melanoma cells transfected with MPP8-shRNA and scramble shRNA (A). Expression of MPP8 in A375 cells was determined by qRT-PCR (B). Determination of cell growth (C) and colony formation (D) of A375 cells after transfection with MPP0-shRNA. *P<0.05.

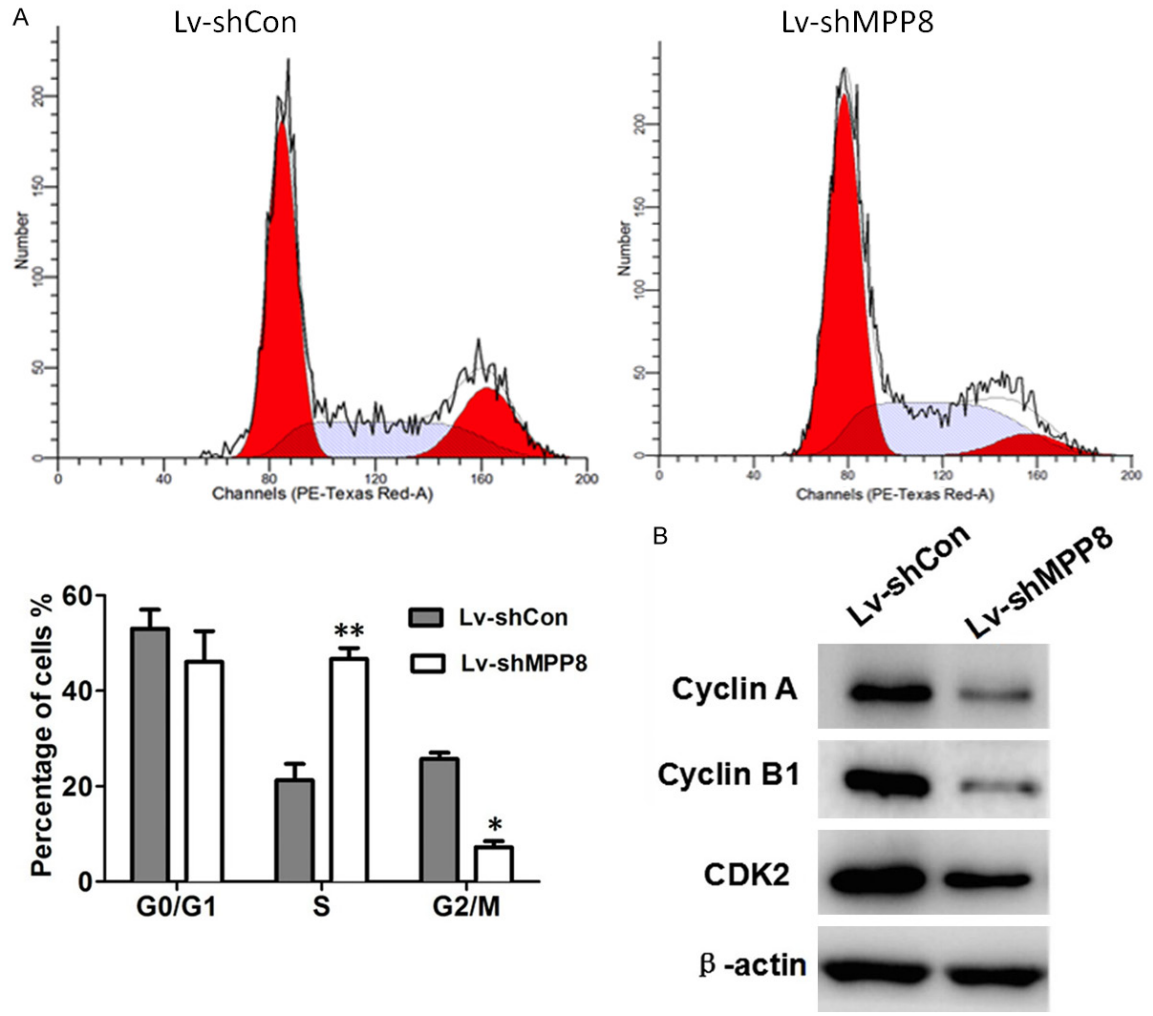

Figure 3. MPP8 knockdown induces cell cycle arrest in melanoma cells. Cell cycle distribution of A375 cells was measured by flow cytometry (A). Western blot was performed to detect the expression of cell cycle-related proteins (B).

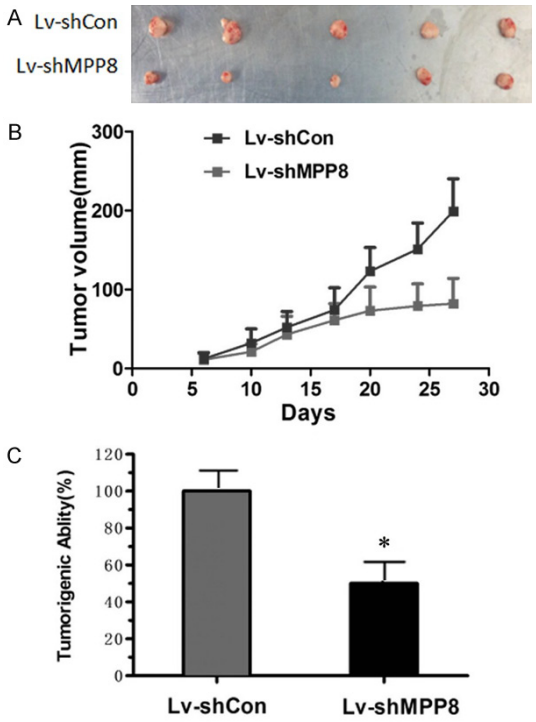

Figure 4. Down-regulation of MPP8 inhibits melanoma cell growth *in vivo*. Nude mice were given subcutaneous inoculation in the flank with 5×10^6 cells with or without shMPP8 to establish a melanoma xenograft model (A). The volume (B) and tumorigenic ability (C) of A375 cells transfected with or without MPP8-shRNA were determined.

Xenograft model

The animal experiment was approved by the institutional animal ethics committee. Female nude Balb/c mice, aged between six-eight weeks, were bred in the animal house, kept in pathogen specific free facility with free-accessible food and water. For tumor formation assay, 5×10^6 cells were injected subcutaneously in the flanks of mice (five mice in each group). The tumor volume was measured using the formula: volume = 1/2 × length × $(\text{width})^2$.

Statistical analysis

Each experiment was repeated at least three times, and the data were presented as mean ± SD followed by analysis using SPSS 17.0. For comparison of the two groups, the Student's t-test was used. For comparisons among groups, one-way ANOVA was applied. $P<0.05$ was considered as statistically significant.

Results

MPP8 is up-regulated in the melanoma specimens

Firstly, we examined the expression of MPP8 in tumor samples derived from twenty patients with melanoma. As shown in **Figure 1**, the mRNA level of MMP8 exhibited an obvious increase in metastatic tumors compared with the melanoma in situ ($P<0.01$). These data indicate that MPP8 may function as an oncogene in melanoma.

Knockdown of MPP8 suppresses melanoma cell growth and colony formation

In order to validate the oncogenic role of MPP8, we silenced its expression in a melanoma cell line A375 using lentivirus-mediated transfection of MPP8-shRNA. Then, the stable cell line with MPP8 knockdown was selected using puromycin and observed under fluorescence microscope (**Figure 2A**). Then, qRT-PCR revealed that MPP8 was down-regulated by more than 70% in shMPP8-transfected A375 cells (**Figure 2B**). Consequently, down-regulation of MPP8 significantly inhibited the proliferation (**Figure 2C**) and colony formation (**Figure 2D**) of A375 cells.

Knockdown of MPP8 induces cell cycle arrest in melanoma cells

Next we determined the role of MPP8 knockdown on cell cycle distribution in melanoma cells. A375 cells transfected with MPP8-shRNA or scramble shRNA were subjected to flow cytometry analysis. We found that down-regulation of MPP8 significantly increased cell number in S-phase without an increase of that in G0/G1-phase and decreased cell number in G2/M-phase (**Figure 3A**). On the molecular level, down-regulation of MPP8 reduced the expression levels of several cell cycle-related proteins including Cylin A, Cyclin B1 and CDK2 (**Figure 3B**). These data suggest that MPP8 knockdown induces cell cycle arrest in S-phase in melanoma cells.

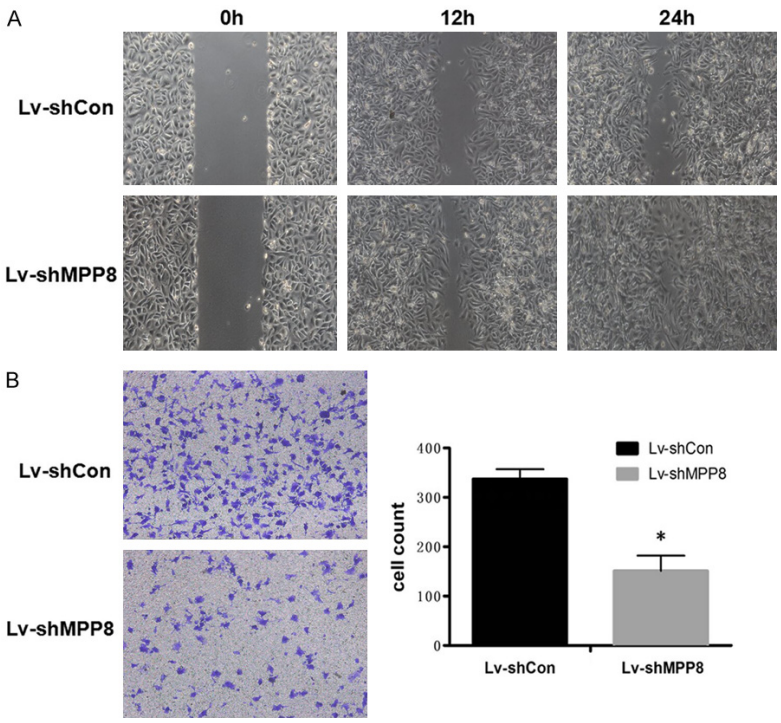

Figure 5. Knockdown of MPP8 suppresses melanoma cell migration and invasion in vitro. Melanoma cells transfected with or without MPP8-shRNA were subjected to wound healing and transwell invasion assay for measurement of migration (A) and invasion (B) abilities, respectively. *P<0.05.

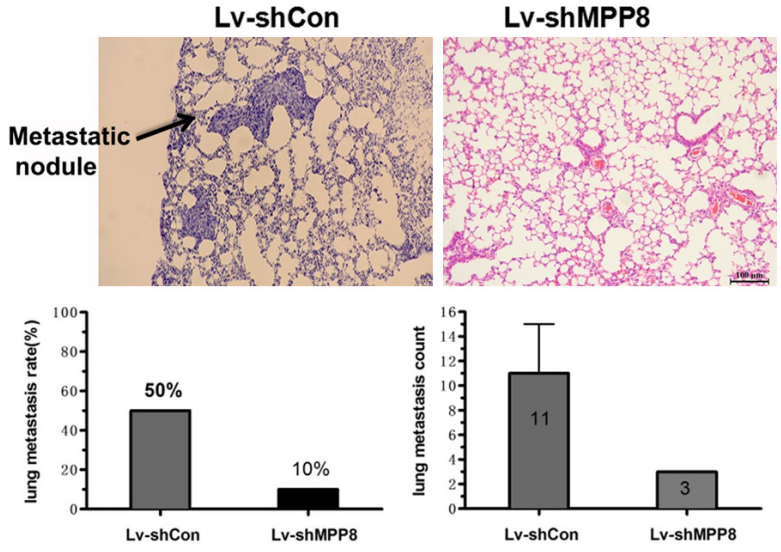

Figure 6. Down-regulation of MPP8 inhibits the lung metastasis of melanoma cells. Nude mice were injected with melanoma cells with or without stably silenced MPP8 and the lung metastatic potential of A375 cells was determined.

Knockdown of MPP8 inhibits melanoma cell growth in vivo

Furthermore, we tested the inhibitive effects of MPP8 silencing on melanoma growth in a xenograft model. Nude mice were given subcutaneous inoculation in the flank with 5×10^6 cells with stable down-regulation of MPP8 (**Figure 4A**). When the tumors reached a palpable size, their volumes were measured every three days for four weeks. We found that down-regulation of MPP8 obviously decreased the tumorigenic ability of melanoma cells (**Figure 4B**) and tumor volume (**Figure 4C**). Taken together, these results demonstrate that knockdown of MPP8 could suppress melanoma cell growth *in vivo*.

Knockdown of MPP8 inhibits the migration and invasion of melanoma cells both in vitro and in vivo

To verify that the knockdown of MPP8 was capable of affecting cell motility and invasion, we did wound healing assay and transwell invasion assay with melanoma cells. We found that down-regulation of MPP8 greatly inhibited A357 melanoma cell migration (**Figure 5A**). Additionally, transwell assay showed that the number of migrating cells per high-power field significantly decreased in melanoma cells stably transfected with MPP8-shRNA (151.4±25.6) compared with the control group (332.0±22.1) (**Figure 5B**).

Furthermore, we validated the inhibitive role of MPP8 knockdown on cell mobility in an in a xenograft model. Consistently, *in vivo* test also demonstrated that down-regulation of MPP8 dramatically decreased the lung metastasis rate (10% vs. 50%) and count (3 vs. 11) (**Figure 6**). Taken together, these data suggest that MPP8 knockdown is capable of inhibiting melanoma cell mobility *in vitro* and *in vivo*.

Discussions

Metastatic melanoma accounts for the majority of skin cancer-related deaths due to its highly aggressiveness and acquired drug resistance [10, 11]. Therefore, it is urgent to develop new therapeutic approaches for successful treatment of this devastating disease. In the current study, we evaluated the role of MPP8 in the melanoma and found that inhibition of MPP8 could serve as a novel therapeutic modality for melanoma.

MPP8 was originally identified as a novel M phase phosphoprotein by expression cloning [12]. It is composed of two functional domains, a carboxy-terminal ankyrin domain and an amino-terminal chromodomain with an affinity for trimethylated histone H3 lysine 9 (H3K9me3) residues [7, 13]. Thus, MPP8 plays a critical role in heterochromatin organization through regulating the interplay between DNA methylation and histone H3 methylation [14]. Given that carcinogenesis results from not only the genetic alterations but also the epigenetic modifications, we consider that MPP8 might be involved in the pathogenesis of melanoma. In our study, we examined the transcriptional expression of MPP8 and found that MPP8 was up-regulated in the tumor specimen from melanoma patients, implicating MPP8 as a potential oncogene in melanoma.

The pathophysiolgical function of MPP8 in cancers is poorly understood, although current evidences support its role in tumor cells invasion and motility through epigenetic regulation of E-cadherin [7, 15]. We further investigated its role in cell growth, colony formation and cell cycle using stably MPP8 knockdown melanoma cells. We found that down-regulation of MPP8 could inhibit melanoma cell growth *in vitro* and *in vivo*, and induce S-phase cell cycle arrest.

Metastasis is a complex series of events in which cancer cells leave the original tumor site and migrate to other parts of the body through the bloodstream, the lymphatic system, or by direct extension [16-18]. Consequently, tumor cells are able to populate and flourish in new tissue habitats and cause organ dysfunction and even death [19-21]. In our study, wound healing and transwell invasion assay showed that MPP8 knockdown slowed the metastatic potential of melanoma cells. In addition, shRNA-mediated knockdown of MPP8 reduced the lung metastasis in a xenograft model. Taken together, these data suggest that MPP8 down-regulation suppresses melanoma metastasis both *in vitro* and *in vivo*.

In conclusion, our study for the first time demonstrates that down-regulation of MPP8 could inhibit melanoma growth, invasion and metastasis both *in vitro* and *in vivo*. These evidences suggest that MPP8 may be a novel therapeutic target for treatment of malignant melanoma.

Acknowledgements

This work was supported by the Ningbo Municipal Natural Science Funds (NO. 2014A-610224).

Disclosure of conflict of interest

None.

Address correspondence to: Lei Shi, Department of Dermatology, The Affiliated Hospital of Medical School, Ningbo University, Ningbo 315020, China. Tel: 86-574-87035307; E-mail: lindalei@163.com

References

[1] Shain AH, Yeh I, Kovalyshyn I, Sriharan A, Talevich E, Gagnon A, Dummer R, North J, Pincus L, Ruben B, Rickaby W, D'Arrigo C, Robson A, Bastian BC. The genetic evolution of melanoma from precursor lesions. N Engl J Med 2015; 373: 1926-36.

[2] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015; 65: 5-29.

[3] Orgaz JL, Sanz-Moreno V. Emerging molecular targets in melanoma invasion and metastasis. Pigment Cell Melanoma Res 2013; 26: 39-57.

[4] Hutchinson L. Skin cancer: novel resistance mechanism revealed. Nat Rev Clin Oncol 2012; 9: 5.

[5] McCaffrey LM, Macara IG. Epithelial organization, cell polarity and tumorigenesis. Trends Cell Biol 2011; 21: 727-35.

[6] Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002; 2: 442-54.

[7] Kokura K, Sun L, Bedford MT, Fang J. Methyl-H3K9-binding protein MPP8 mediates E-cadherin gene silencing and promotes tumour cell motility and invasion. EMBO J 2010; 29: 3673-87.

[8] Chang Y, Horton JR, Bedford MT, Zhang X, Cheng X. Structural insights for MPP8 chromo-

domain interaction with histone H3 lysine 9: potential effect of phosphorylation on methyllysine binding. J Mol Biol 2011; 408: 807-14.
[9] Li J, Li Z, Ruan J, Xu C, Tong Y, Pan PW, Tempel W, Crombet L, Min J, Zang J. Structural basis for specific binding of human MPP8 chromodomain to histone H3 methylated at lysine 9. PLoS One 2011; 6: e25104.
[10] Gibney GT, Kudchadkar RR, DeConti RC, Thebeau MS, Czupryn MP, Tetteh L, Eysmans C, Richards A, Schell MJ, Fisher KJ, Horak CE, Inzunza HD, Yu B, Martinez AJ, Younos I, Weber JS. Safety, correlative markers, and clinical results of adjuvant nivolumab in combination with vaccine in resected high-risk metastatic melanoma. Clin Cancer Res 2015; 21: 712-20.
[11] Livingstone E, Swann S, Lilla C, Schadendorf D, Roesch A. Combining BRAF(V) (600E) inhibition with modulators of the mitochondrial bioenergy metabolism to overcome drug resistance in metastatic melanoma. Exp Dermatol 2015; 24: 709-10.
[12] Matsumoto-Taniura N, Pirollet F, Monroe R, Gerace L, Westendorf JM. Identification of novel M phase phosphoproteins by expression cloning. Mol Biol Cell 1996; 7: 1455-69.
[13] Bua DJ, Kuo AJ, Cheung P, Liu CL, Migliori V, Espejo A, Casadio F, Bassi C, Amati B, Bedford MT, Guccione E, Gozani O. Epigenome microarray platform for proteome-wide dissection of chromatin-signaling networks. PLoS One 2009; 4: e6789.
[14] Murata K, Sato S, Haruta M, Goshima T, Chiba Y, Takahashi S, Sharif J, Koseki H, Nakanishi M, Shimada M. Physical interaction between MPP8 and PRC1 complex and its implication for regulation of spermatogenesis. Biochem Biophys Res Commun 2015; 458: 470-5.
[15] Sun L, Kokura K, Izumi V, Koomen JM, Seto E, Chen J, Fang J. MPP8 and SIRT1 crosstalk in E-cadherin gene silencing and epithelial-mesenchymal transition. EMBO Rep 2015; 16: 689-99.
[16] Bishop JM. Molecular themes in oncogenesis. Cell 1991; 64: 235-48.
[17] Chiba M, Aldaz CM, Conti CJ, Klein-Szanto AJ. Metastatic potential of mouse skin carcinomas produced by different protocols of chemical carcinogenesis. Invasion Metastasis 1991; 11: 288-96.
[18] Branco-Price C, Evans CE, Johnson RS. Endothelial hypoxic metabolism in carcinogenesis and dissemination: HIF-A isoforms are a NO metastatic phenomenon. Oncotarget 2013; 4: 2567-76.
[19] Parmiani G. Melanoma cancer stem cells: markers and functions. Cancers (Basel) 2016; 8.
[20] Huang FJ, Steeg PS, Price JE, Chiu WT, Chou PC, Xie K, Sawaya R, Huang S. Molecular basis for the critical role of suppressor of cytokine signaling-1 in melanoma brain metastasis. Cancer Res 2008; 68: 9634-42.
[21] Chiang AC, Massague J. Molecular basis of metastasis. N Engl J Med 2008; 359: 2814-23.