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ORIGINAL ARTICLE

Cyclophilin A promotes non-small cell lung cancer metastasis via p38 MAPK

Yinan Guo[1], Mei Jiang[1], Xiaoting Zhao[1], Meng Gu[1], Ziyu Wang[1], Shaofa Xu[2] & Wentao Yue[1]

1 Department of Cellular and Molecular Biology, Beijing Chest Hospital, Capital Medical University/Beijing Tuberculosis and Thoracic Tumor Research Institute, Beijing, China

2 Department of Thoracic Surgery, Beijing Chest Hospital, Capital Medical University/Beijing Tuberculosis and Thoracic Tumor Research Institute, Beijing, China

Keywords

Cyclophilin A (CypA); metastasis; non-small cell lung cancer (NSCLC); p38 MAPK.

Correspondence

Shaofa Xu, Department of Thoracic Surgery, Beijing Chest Hospital, Capital Medical University/Beijing Tuberculosis and Thoracic Tumor Research Institute, 9#, Beiguan Street, Tongzhou, Beijing 101149, China.
Tel: +86 10 8950 9181
Fax: +86 10 8050 7349
Email: shaofa_xu@hotmail.com

Wentao Yue, Department of Cellular and Molecular Biology, Beijing Chest Hospital, Capital Medical University/Beijing Tuberculosis and Thoracic Tumor Research Institution, 9#, Beiguan Street, Tongzhou, Beijing 101149, China.
Tel: +86 10 8950 9372
Fax: +86 10 8050 7349
Email: yuewt@ccmu.edu.cn

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Abstract

Background: Cyclophilin A (CypA) is associated with metastasis in diverse cancers; however, its role in lung cancer metastasis and the underlying mechanisms remain poorly understood. Our study investigated the effect of CypA on non-small cell lung cancer (NSCLC) metastasis in vitro and in vivo to determine its mechanisms.

Methods: In this study, A549 and H1299 cell lines with downregulated and overexpressed CypA, respectively, were constructed by lentivirus transfection of NSCLC cells. in vitro experiments, including wound healing and transwell assays and Western blotting, showed that CypA promoted cancer cell migration and epithelial-mesenchymal transition in NSCLC. Lung metastasis mouse models were used for the first time to confirm that CypA promoted NSCLC metastasis in vivo. The p38 inhibitor SB203580 was used to show that p38 MAPK is involved in CypA-mediated NSCLC metastasis.

Results: Wound healing and transwell assays showed that the migration of both A549 and H1299 cells decreased in the CypA downregulated group and increased in the CypA overexpressed group. CypA also positively promoted the expression of epithelial-mesenchymal transition-relevant proteins. Results of mouse models confirmed that the tumor metastasis rate was much higher in the CypA overexpressed than in the CypA downregulated group. In addition, SB203580 inhibited NSCLC cell migration significantly in the CypA overexpressed group, while the difference in the CypA downregulated group was not significant.

Conclusions: In conclusion, this study demonstrated that CypA promotes NSCLC cancer metastasis via p38 MAPK.

Introduction

Lung cancer is the leading cause of death worldwide, with very high mortality and morbidity. Non-small cell lung cancer (NSCLC) is the main type and comprises almost 85% of all lung cancers.[1] NSCLC is often diagnosed at advanced stage, making treatment difficult. Cancer metastasis is one of the main reasons for this problem; thus, assessing the mechanisms of cancer cell metastasis attracts increasing attention.

Cyclophilin A (CypA) is a member of the cyclophilin (Cyp) family, intracellular binding proteins for the potent immunosuppressive drug cyclosporine A (CsA), with peptidyl-prolyl cis-transisomerase (PPIase) activity.[2] CypA is widely expressed in normal cells and is overexpressed in various cancers, including hepatocellular,[3] non-small cell

lung,[4–6] pancreatic,[7] breast,[8,9] colorectal,[10] and squamous cell cancers,[11,12] and melanoma.[13] Many researchers have assessed CypA function in cancer. It has been suggested that CypA plays an important role in the whole pathological process of cancer development. Choi *et al.* found that CypA overexpression promotes cancer cell proliferation and blocks hypoxia-induced apoptosis.[14] Howard *et al.* demonstrated that CypA overexpression stimulates cancer cell growth in NSCLC, with cancer cell growth inhibited by CypA knockdown.[4,5] Boulos *et al.* found that at high CypA levels, the protein interacts with the proline-containing peptide in the transmembrane domain of CD147, thereby stimulating human pancreatic cancer cell proliferation.[15] Accumulating evidence indicates that CypA is associated with metastasis in diverse cancers. A previous study showed that CypA promotes human hepatocellular carcinoma cell metastasis via MMP3 and MMP9 regulation.[16] A recent study found that CypA expression is relevant to lymph node metastasis in esophageal squamous cell carcinoma.[17] However, the role and mechanisms of CypA remain poorly understood.

Our previous studies have found that metastasis is reduced after downregulating CypA expression.[18] Expression levels of some epithelial-mesenchymal transition (EMT)-related proteins are also changed simultaneously, as well as phosphorylated-p38. This suggests that p38 MAPK is involved in the regulation of metastasis by CypA. The purpose of our present study was to determine the effect of CypA on NSCLC metastasis in vitro and in vivo, and to investigate if CypA promotes NSCLC metastasis via p38 MAPK.

Methods

Cell culture

Human lung adenocarcinoma cell lines A549 and H1299 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Gibco, Los Angeles, CA, USA), at 37°C in a humidified chamber containing 5% CO_2. Both cell lines express CypA, as confirmed by Western blot.

Lentivirus infection of cells

CypA knockdown cells (CypA-KD) and control cells (NC) were generated with the A549 and H1299 cell lines, as previously described[18] CypA overexpressing cells (CypA-KD-OE) and control cells (CypA-KD-Vector) were obtained based on CypA-KD cell lines. Target cells were seeded into 96-well culture plates at 5000/well. The human CypA gene coding sequence was ligated into the GV208-GFP vector (GeneChem Co. Ltd, Shanghai, China), with the empty vector used as a control. Twenty-four hours after infection, green fluorescent protein expression was detected by fluorescence microscopy (Nikon, Tokyo, Japan) to determine infection efficiency. Cells were cultured for an additional two weeks prior to harvest, when CypA expression was assessed by Western blot. For animal studies, cells were transfected with a GV208-Luciferase-Vector, and stable cell lines were selected by treatment with puromycin (Sigma-Aldrich, St Louis, MO, USA); puromycin-resistant clones were obtained after four weeks. Luminescence was observed 15 minutes after a reaction with D-Luciferin.

Wound healing assay

Cells were seeded in six-well plates and incubated overnight to obtain confluent monolayers for wounding. Wounds were generated with a sterile pipette tip and observed every two hours along the scratch. For inhibition studies, SB203580 was dissolved in dimethyl sulfoxide (DMSO) and added to the cell culture medium at a final concentration of 10 μM. SB203580 was added immediately after wounding.

Transwell migration assay

Cell migration assay was carried out in a 24-well Transwell unit with 8 μm pore polycarbonate membrane. After overnight starvation, the cells were resuspended and plated in the upper compartment with serum-free medium with or without 10 μM SB203580. The lower compartment was filled with medium containing 10% FBS as a chemoattractant. After 24 hours, the cells in the upper compartment were removed completely by gentle swabbing. The cells that had migrated to the lower surface of the membrane were subject to crystal violet staining and counted in five high power fields (200× magnification). Triplicate samples were tested, and data are presented as mean ± standard error of the mean.

Western blot

Protein levels were measured with a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's instructions. Equal amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto NC membranes (Millipore, Billerica, MA, USA). The membranes were probed with target antibodies in 5% (w/v) bovine serum albumin in tris-buffered saline plus tween 20 (50 mM Tris–HCl, 138 mM NaCl, and 0.1% Tween-20, pH7.6). Secondary antibodies conjugated to horseradish peroxidase were diluted 1:2500 before use. Protein signals were detected with the SuperSignal West Femto

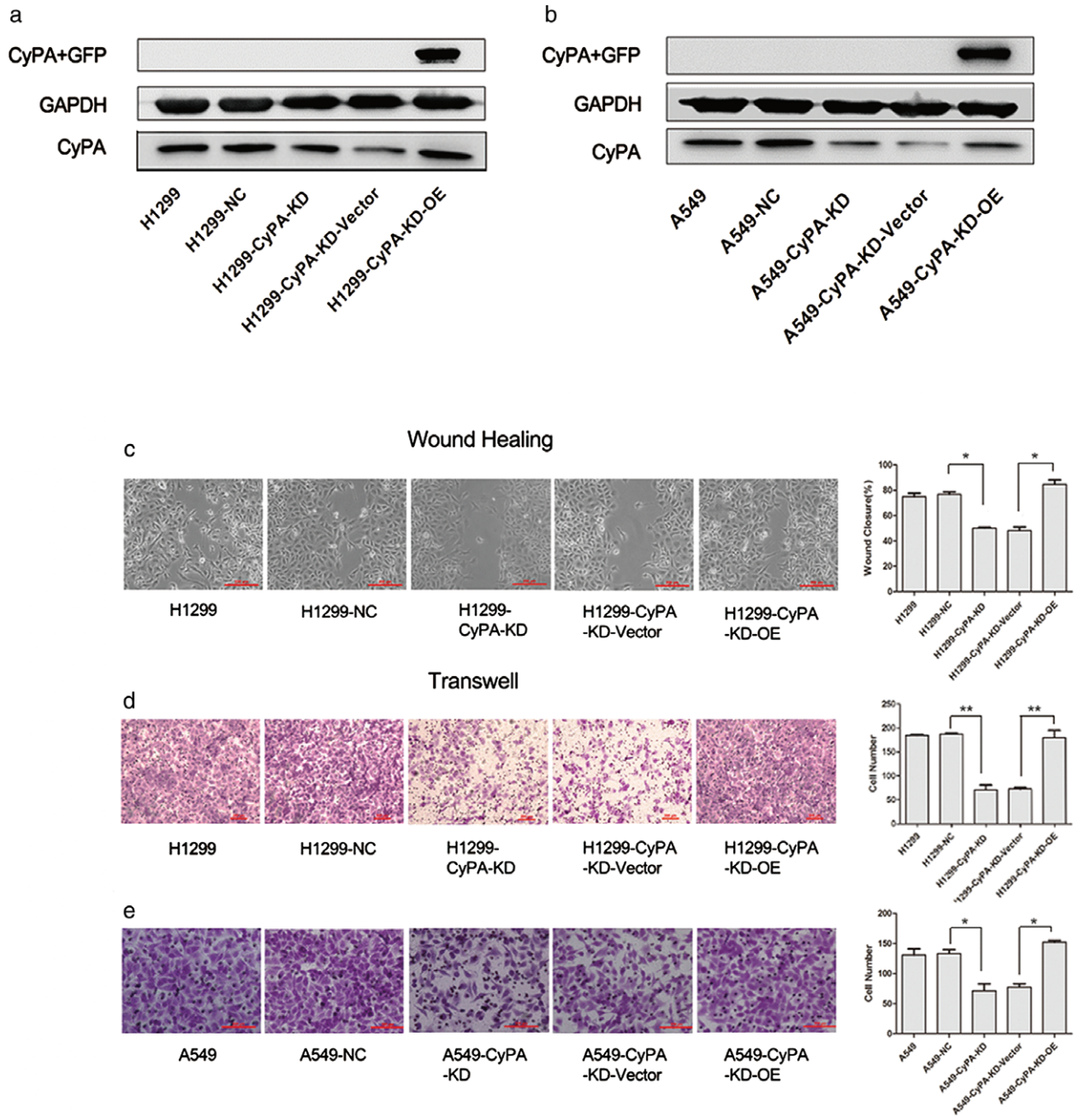

Figure 1 CypA expression and effect on non-small cell lung cancer cell migration. (**a,b**) CypA knockdown (KD) and overexpressing (OE) cells and respective controls were successfully constructed in H1299 and A549 cell lines. The CypA-GFP fusion protein is about 37 KD. (**c**). Wound healing assay was performed with H1299 CypA KD and OE cell lines. Cells were cultured in serum-free medium after being scratched with a pipette tip and imaged after 24 hours. Areas of wound closure (%) at 24 hours following wound generation were calculated. (**d,e**) Transwell migration assay was performed with H1299/A549 CypA downregulated and overexpressed cell lines. Cells on the lower side of the insert filters were fixed, stained, and counted under a microscope after 24 hours. Scale bars, 200 μM. Data are expressed as mean ± standard error of mean triplicate values from three separate experiments. $^{**}P < 0.05$ (*t*-test). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Chemiluminescent Detection System (Pierce Chemical Co.). Primary antibodies included rabbit anti-p38, anti-pp38, anti-β-catenin, anti-vimentin, and anti-snail antibodies (Abcam, Cambridge, UK).

Animal studies

Animal experiments were approved by the Ethics Committee of Beijing Chest Hospital, Capital Medical University, and complied with the Beijing Laboratory Animal Welfare and

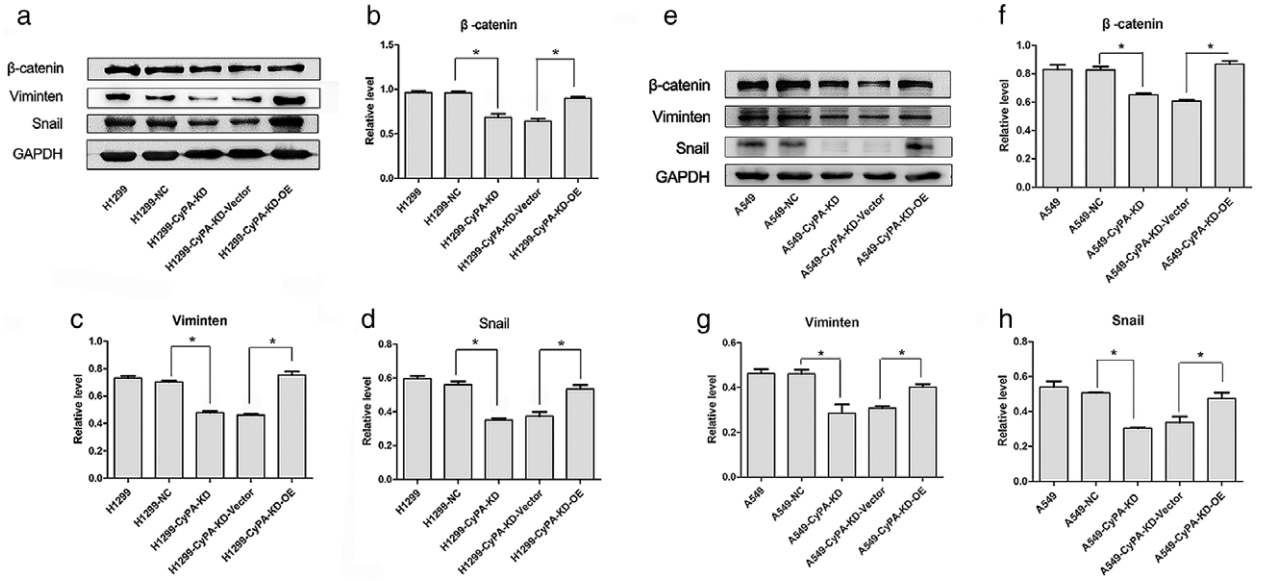

Figure 2 Effects of CypA on epithelial-mesenchymal transition-relevant proteins. Expression levels of β-catenin, vimentin, and Snail were detected by Western blot in (**a**) H1299 CypA knockdown (KD) and overexpressing (OE) cell lines and (**e**) A549 CypA KD and OE cell lines. Relative expression levels of β-catenin, vimentin, and Snail in (**b**–**d**) in H1299 and (**f**–**h**) in A549. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal loading control. Data are representative of three independent experiments (mean ± standard error of the mean). $*P < 0.05$, $**P < 0.01$ (*t*-test).

Ethical Guidelines of the Beijing Administration Committee of Laboratory Animals. NOD/SCID mice (male, 6–8 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), and divided into three groups of five. A549 CypA-KD, A549 CypA-KD-OE, and A549 cells (two million) were injected via the tail vein. Photographs were acquired on an IVIS Lumina XRMS Series III Multi-Species Optical and X-Ray Imaging System (PerkinElmer Inc., Waltham, MA, USA) after 14 weeks. D-Luciferin salt (200 μL*15 mg/mL) (Sigma-Aldrich) was injected intraperitoneally 15 minutes before imaging.

Statistical analysis

Data are presented as mean ± standard error of the mean from at least three independent experiments. One-way analysis of variance and Student's *t*-tests were used for comparisons. SPSS version 20.0 (IBM Corp., Armonk, NY, USA) was used for all analyses, with $P < 0.05$ considered statistically significant.

Results

CypA levels correlate with non-small cell lung cancer (NSCLC) cell migration in vitro

CypA KD and OE cell lines were constructed successfully in both A549 and H1299 cell lines. CypA protein levels were assessed by Western blot (Fig 1a,b). Wound healing and transwell migration assays were used to determine the effects of CypA on cell migration. The wound healing assay showed that CypA downregulation resulted in significantly decreased migration of NSCLC cells ($P < 0.05$); when CypA was overexpressed, the NSCLC cell migration was recovered accordingly in the H1299 cell line (Fig 1c). The Transwell migration assay in H1299 cells (Fig 1d) yielded similar findings of the effects of CypA on NSCLC cell migration. Similar results were also obtained in the A549 cell line (Fig 1e). These results suggested that CypA positively regulated NSCLC cell migration.

CypA promotes epithelial-mesenchymal transition in NCSLC cells

To assess the effect of CypA on EMT, we next quantified the protein expression levels of vimentin, Snail and β-catenin by Western blot in both A549 (Fig 2a-d) and H1299 (Fig 2e-h) cell lines. Interestingly, the protein expression levels of β-catenin (Fig 2b,f), vimentin (Fig 2c,g) and Snail (Fig 2d,h) were significantly decreased after CypA downregulation. In addition, CypA overexpression resulted in enhanced expression of β-catenin, vimentin and Snail. These results suggest that CypA promoted EMT in NCSLC cells; EMT is

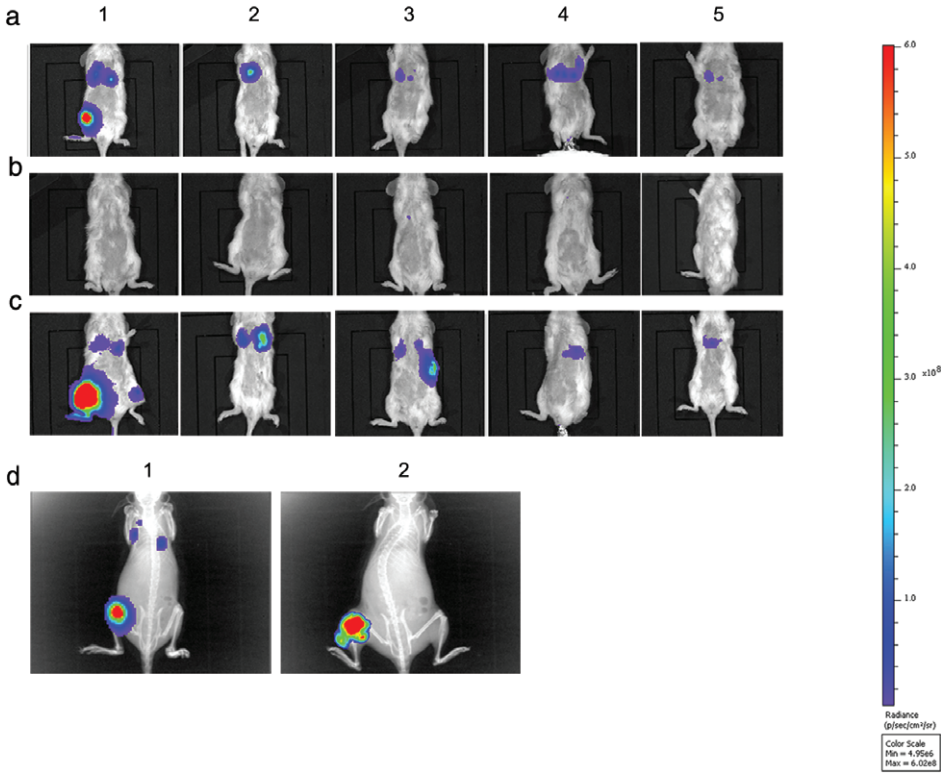

Figure 3 CypA affects non-small cell lung cancer metastasis in vivo. Cells were injected into NOD/SCID mice (200 μL; 10 million/mL/per mouse). Photographs were acquired after 14 weeks. (**a**) A549, (**b**) A549 CypA-knockdown (KD), and (**c**) A549-CypA-KD-overexpressing (OE) groups. (**d**) X-ray showing overt bone metastasis. D-1, X-ray image of A-1; D-2, X-ray image of C-1.

thus involved in CypA-mediated metastasis of NSCLC cells.

CypA facilitates NSCLC metastasis in vivo

To assess the effects of CypA on NSCLC cells in vivo, a lung metastasis mouse model of NSCLC was generated. A total of 15 NOD/SCID male mice were randomly divided into three groups of five. A549 CypA-KD and A549 CypA-KD-OE cells were injected via the tail vein, with A549 cells used as a control. IVIS Lumina XRMS Series III was used to observe tumor formation after 14 weeks. Consistent with the in vitro data, the metastasis rate of the CypA-KD group was significantly lower than that of the CypA-OE group (Fig 3). Lung metastasis was observed in 100% (5/5) mice in both A549 CypA-KD-OE and A549 groups, while the A549 CypA-KD group had 0% (0/5). In addition, osseous metastasis was found in 20% (1/5) of mice in the A549 CypA-KD-OE and A549 groups. These results demonstrated that CypA promoted NSCLC metastasis in vivo.

p38 phosphorylation is positively affected by CypA

Our previous study demonstrated that p38 phosphorylation is decreased with CypA downregulation.[18] To confirm the above results, we monitored the phosphorylation levels of p38 not only in CypA downregulated cell lines, but also in CypA-OE cells by Western blot. P38 phosphorylation levels were significantly decreased in the H1299 CypA-KD group ($P < 0.05$) compared to the control groups (Fig 4a,b). CypA was upregulated in the H1299 CypA-OE group (Fig 4a,b) resulting in increased p38 phosphorylation levels ($P < 0.05$). Similar results were obtained for the A549 CypA KD and OE groups (Fig 4c,d). These findings suggested that CypA positively regulated p38 phosphorylation in NSCLC cells.

p38 is required for CypA-induced metastasis in NSCLC

To determine whether p38 is involved in NSCLC metastasis, the p38 inhibitor SB203580 was used to inhibit p38

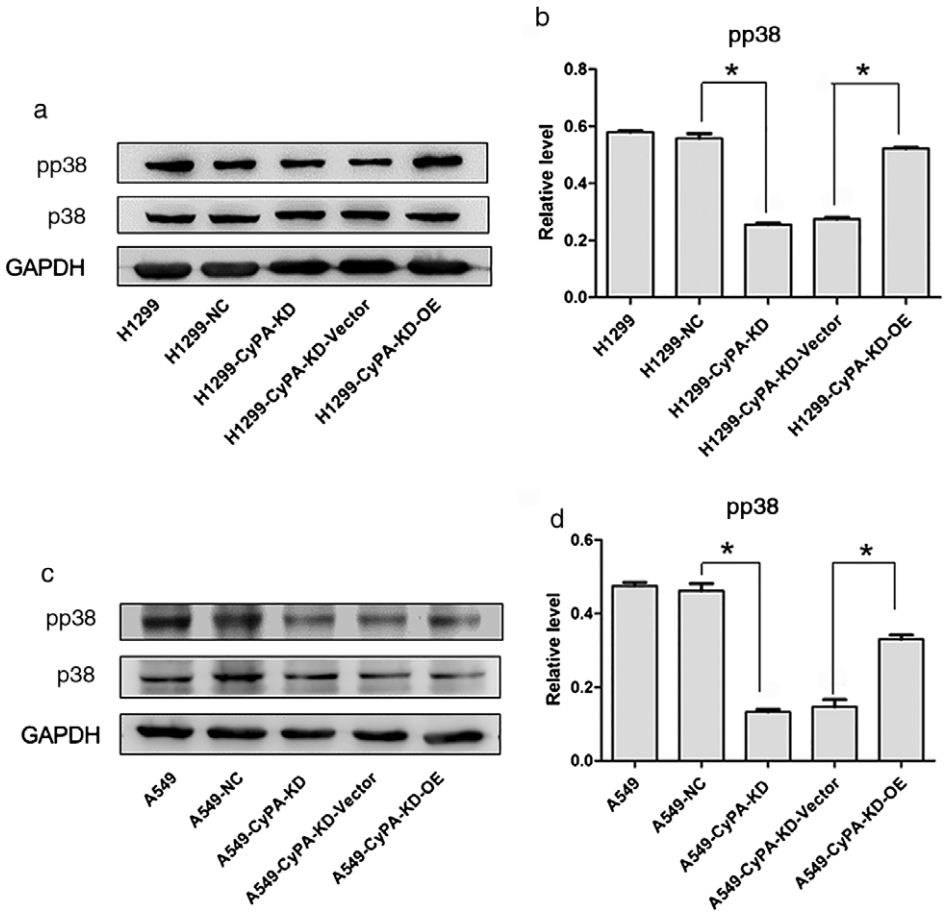

Figure 4 CypA levels affect p38 phosphorylation. p38 and phospho-p38 (pp38) levels in the (**a**) H1299 and (**c**) A549 groups. (**b,d**) Relative levels of pp38, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal loading control. In all graphs, data are representative of three independent triplicate experiments (mean ± standard error of the mean). *$P < 0.05$ (*t*-test).

phosphorylation. Cells were incubated with SB203580, and control cells treated with DMSO. Wound healing assay showed that SB203580 significantly inhibited NSCLC cell migration in the H1299 CypA-KD-OE group ($P < 0.05$), while no significant difference was found between H1299 CypA-KD and control cells (Fig 5a). In addition, transwell migration assay was used to confirm the results of wound healing assay. The number of migrated cells was remarkably reduced by SB203580 in the H1299 CypA-KD-OE group ($P < 0.05$), while in the H1299 CypA-KD group SB203580 had no obvious effect (Fig 5b). Western blot was used to assess the effects of the p38 inhibitor on EMT-relevant proteins in CypA KD and OE cell lines (Fig 5c), respectively. Protein expression levels of β-catenin, vimentin and Snail were significantly inhibited by SB203580 in CypA-OE cells ($P < 0.05$) (Fig 5d–h). Meanwhile, there was no significant difference in CypA-KD cells between the experimental and control groups. These findings suggested that p38 is required for CypA-mediated EMT and metastasis in NSCLC.

Discussion

This study demonstrated that CypA promotes NSCLC metastasis via p38 MAPK and EMT, both in vitro and in vivo.

In vitro, we first constructed CypA KD and OE cells with human lung adenocarcinoma cancer cell lines H1299 and A549. We found that CypA overexpression significantly promoted NSCLC cell migration by wound healing and transwell migration assays in the H1299 and A549 cell lines. Meanwhile, Western blot showed that CypA positively regulated β-catenin, vimentin, and Snail protein expression levels. These results suggest that CypA plays an important role in EMT and metastasis of NSCLC. Similar results have been reported for other cancer types. A previous study of breast cancer cells demonstrated that prolactin binds CypA for cancer progression and tumor metastasis.[8] Another study reported that CypA is one of the malignant transformation-related proteins in esophageal squamous cell carcinoma.[12] Al-Ghoul *et al.* also demonstrated that CypA is expressed at higher levels in metastatic melanoma compared to primary melanoma and normal fibroblasts.[19]

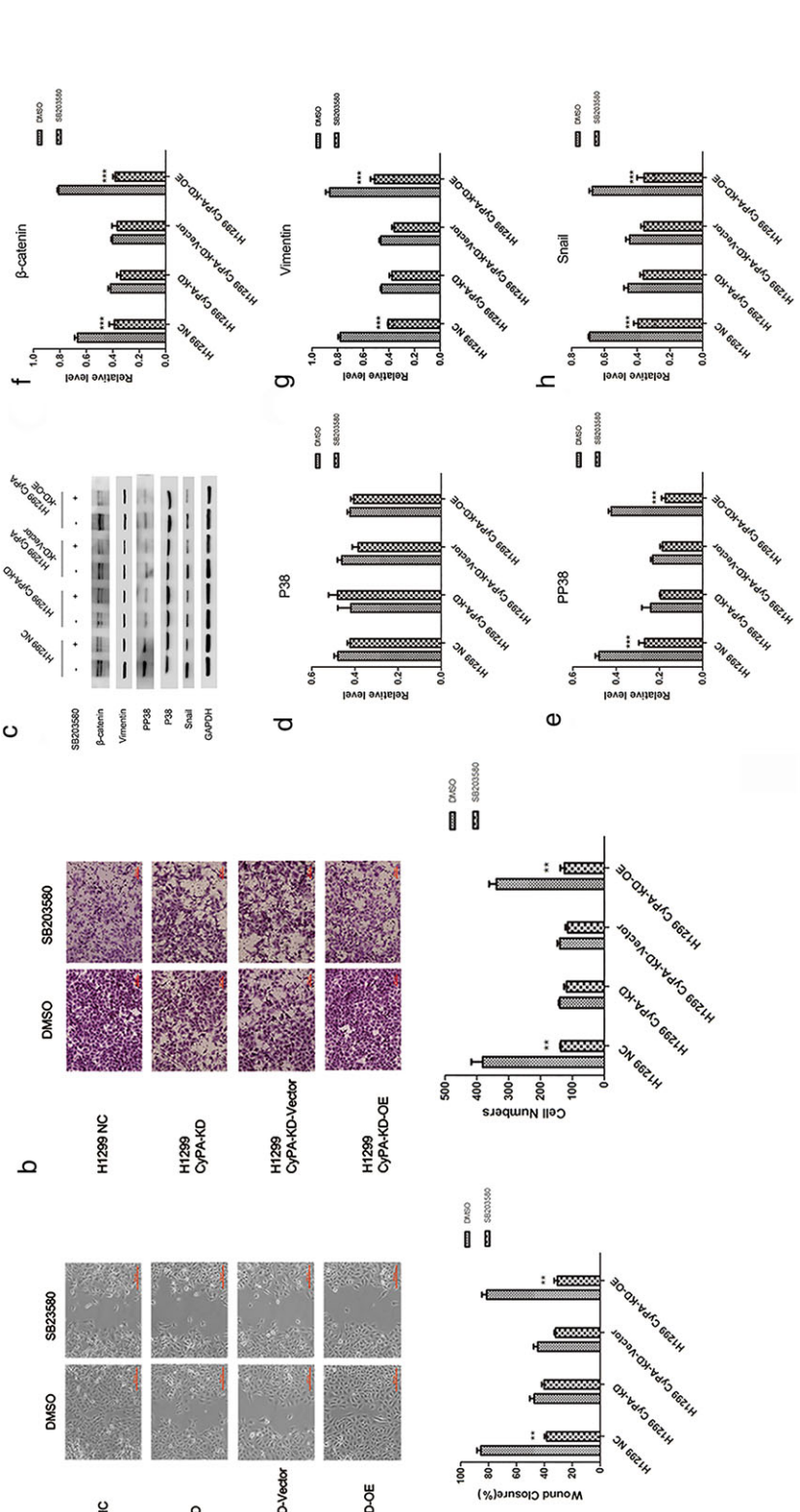

Figure 5 SB203580 inhibits CypA-mediated migration of non-small cell lung cancer cells and CypA-mediated epithelial-mesenchymal transition induction. (a) Wound healing assay was performed with H1299 CypA knockdown (KD) and overexpressing (OE) cell lines. Cells were cultured in serum-free medium with 10 μM SB203580 or dimethyl sulfoxide (DMSO) after being scratched with a pipette tip and imaged after 24 hours. (**b**) Transwell migration assay was performed with H1299 CypA KD and OE cell lines. Cells were plated in the upper compartment with serum-free medium containing 10 μM SB203580 and DMSO in the experimental and control groups, respectively. Cells on the lower side of the insert filters were fixed, stained, and counted under a microscope after 24 hours. (**c**) Western blot was used to detect protein expression levels of p38, phospho-p38 (pp38), β-catenin, vimentin, and Snail. (**d–h**) Relative levels of p38, pp38, β-catenin, vimentin and Snail. Scale bars, 200 μm. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal loading control. Data are presented as mean ± standard error of the mean from three independent triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ by one way analysis of variance compared to control cells in the same group.

These findings suggest that increased levels of CypA may promote cancer cell metastasis.

To confirm the effect of CypA on NSCLC metastasis in vivo, mouse models were generated. A549, A549 CypA-KD, and A549 CypA-KD-OE cells were injected into NOD/SCID mice for 14 weeks. Lung metastasis occurred in all mice in the A549 and A549 CypA-KD-OE groups, but not in the A549 CypA-KD group. These findings indicated that CypA remarkably promotes NSCLC metastasis in vivo; however, the mechanism by which CypA regulates metastasis of NSCLC or other cancers is not clearly understood.

In our previous study, p38 was considered a probable target by which CypA affects NSCLC metastasis.[18] In this study, cells were treated with SB203580, the most widely used p38 inhibitor; control cells were treated with DMSO. Interestingly, cell migration was inhibited by SB203580 in A549 CypA-KD-OE and H1299 CypA-KD-OE cells. In addition, the expression levels of several EMT-relevant proteins were also decreased in CypA-OE cells after treatment with SB203580. Meanwhile, there was no significant difference in CypA-KD cells compared to control cells. These findings suggest that CypA effects metastasis positively through p38 MAPK rather than in other pathways in NSCLC. P38, first detected by Brewster and Han in different experiments, activates p38 MAPK to affect the invasive and metastatic capabilities of cancer cells by regulating specific signaling molecules.[20,21] Hsieh *et al.* found inhibited cell migration and invasion in SK-Hep-1 cells treated with SB203580.[22] P38 MAPK is known to be required for the invasion and metastasis of cancer cells. Several MMPs are regulated by p38 in bladder, breast, liver, skin keratinocyte, and prostate cell lines derived from human tumors.[23–27] Consistent with our findings, other studies have demonstrated that CypA induces p38 activation.[28,29] However, Kim *et al.* reported that p38 phosphorylation is inhibited by CypA overexpression in the HEK293 cell line.[30] This indicates a different relationship between CypA and p38 MAP kinase in distinct cells. Our results suggested that the β-catenin/Wnt signaling pathway may be correlated with CypA-mediated metastasis in NSCLC, which may be another focal point to investigate the mechanism of tumor metastasis in the future.

Overall, we provided evidence that CypA promotes EMT and metastasis in NSCLC via p38 MAPK in vitro and in vivo. Therefore, targeting CypA may constitute a potential early diagnostic or therapeutic approach in NSCLC.

Acknowledgments

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Disclosure

No authors report any conflict of interest.

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