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Metformin Inhibit Lung Cancer Cell Growth and Invasion *in Vitro* as Well as Tumor Formation *in Vivo* Partially by Activating PP2A

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: The aim of this study was to investigate whether PP2A activation is involved in the anti-cancer activity of metformin.

Material/Methods: A549 and H1651 human lung cancer cells were constructed with stable α4 overexpression (O/E α4) or knockdown of PP2A catalytic subunit A/B(sh-PP2Ac). Influences of okadaic acid (OA) treatment, O/E α4 or sh-PP2Ac on metformin treated cells were investigated by cell viability, proliferation, apoptosis, and Transwell invasion assay *in vitro*. Protein expression levels of Bax, Bcl-2, Myc, and Akt as well as serine phosphorylation level of Bax, Myc, and Akt were examined by western blot. For *in vivo* assays, wild type (WT) or modified A549 cells were subcutaneously injected in nude mice, and metformin treatment on these xenografted tumors were assayed by tumor formation assay and western blot detecting cell proliferation marker PCNA (proliferating cell nuclear antigen) as well as protein expression level and serine phosphorylation level of Akt and Myc.

Results: Metformin treatment significantly reduced A549 or H1651 cell growth and invasive capacity *in vitro* as well as Ser184 phosphorylation of Bax, Ser62 phosphorylation of Myc, and Ser473 phosphorylation of Akt, all of which could be partially attenuated by OA treatment, O/E α4 or sh-PP2Ac. Metformin treatment also significantly reduced tumor formation in vivo as well as protein expression of PCNA, Akt, Myc, and serine phosphorylation of the latter 2, which can be partially blocked by O/E α4 or sh-PP2Ac.

Conclusions: Metformin reduced lung cancer cell growth and invasion *in vitro* as well as tumor formation *in vivo* partially by activating PP2A.

MeSH Keywords: Lung Neoplasms • Metformin • Mycolic Acids • Phosphorylation

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Background

Lung cancer is currently the second most often diagnosed cancer type and leading cause of cancer-related death over the globe [1]. Aside from surgery, therapy against lung cancer has evolved from chemotherapy to targeted therapy and immunotherapy, greatly improved prognosis and survival of patients diagnosed with early-to-mid stage lung cancer, but survival of those with late stage lung cancer remains poor [1,2]. Recent findings have revealed the novel anti-cancer activity of a conventional hypoglycemic drug for anti-diabetic purpose, metformin, against lung cancer and many other cancer types [3,4]. In practice, metformin is found to attenuate lung cancer development either alone or synergistically with chemo/targeted therapy, significantly reducing lung cancer risk and mortality [5–7]. However, the molecular mechanisms of these effects of metformin remains poorly understood. It is generally believed that the anti-cancer activity of metformin is mediated by AMPK activation in either LKB1-dependent or independent manner, although other molecular mechanisms aside from AMPK activation are also implied to mediate the anti-cancer effect of metformin [8–11]. Data from research emphasizing the involvement of AMPK activation in metformin's anti-lung cancer effect also showed that AMPK knockout or sufficient knockdown did not completely relieve the inhibitory effect of metformin on lung cancer development [8,10].

We deeply reviewed current literatures regarding the molecular action of metformin and found that Kickstein et al. reported on the direct activation of PP2A by metformin treatment: metformin disrupts the interaction between the catalytic subunit of PP2A and α4 and MID1, the 2 of which function as inhibitors of PP2A catalytic activity, and this mechanism was later confirmed by Hettich et al. [12,13]. PP2A is a well-defined tumor suppressor protein in lung cancer as well as in other cancer types [14]. In lung cancer, PP2A has been found to suppress the oncogenic activity of Akt and Myc by catalyzing their serine de-phosphorylation, as summarized by Walter et al. [15]. Xin et al. reported that dephosphorylation of Bax catalyzed by PP2A increases its pro-apoptotic activity, while Shouse et al. demonstrated that tumor suppressive role of PP2A is partially p53 dependent [16]. Notably, Chen et al. reported that α4 functioning as endogenous PP2A inhibitor is frequently upregulated in breast, liver, and lung cancers, and artificial α4 overexpression granted non-transformed cells with tumor forming ability in nude mice [17]. Disruption of MID1-α4/PP2A complex by metformin treatment was also reported by Demir et al. in prostate cancer cells *in vitro*, in which they found that MID1 or α4 knockdown mimicked the anti-cancer effect of metformin in an AMPK-independent manner [18]. Sacco et al. demonstrated that metformin treatment increased the protein expression of PP2A catalytic and regulatory subunit as well as the holoenzyme assembly in breast cancer cells *in vitro* [19]. Metformin was proposed to attenuate Alzheimer or Parkinson disease-like neuropathy by reducing the phosphorylation of tau protein or α-synuclein, respectively, in a PP2A dependent manner [20–23], but recent research claimed that metformin reduced endometrial cancer development by inhibiting PP2A [24]. These previous findings led us to the hypothesis that PP2A activation is involved in the anti-cancer activity of metformin, which was tested using A549 non and H1651 human -small cell lung cancer (NSCNC) cells in the present research.

Material and Methods

Cell culture, transfection, and treatment

A549 and H1651 human non-small cell lung cancer (NSCLC) cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were preserved in liquid nitrogen after shipment and were used on passage 2 to 5. A549 and H1651 cells were cultured in RPMI-1640 medium (STEMCELL Technologies, Vancouver, Canada) supplemented with 10% FBS (STEMCELL Technologies) and 100 U/mL of penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) in a cell incubator with 37°C, 5% CO_2 atmosphere. α4 overexpression in A549 or H1651 cells was achieve by lentiviral transfection, and A549 or H1651 cell line with stable knockdown of A/B catalytic subunit of PP2A was constructed by lentiviral transfection of targeting shRNA. Lentiviral vectors described above were constructed by Genecopoeia (Rockville, MD, USA) and were used following manufacturer's instructions. Metformin hydrochloride (Tocris Bioscience, Bristol, UK) was pre-diluted in complete culture medium as 10× stock and preserved under –8°C before use. OA (Tocris Bioscience) was pre-diluted in DMSO as 100x stock and preserved under –20°C before use.

Cell viability, proliferation, apoptosis, and Transwell invasion assay

A549 and H1651 cell viability was assayed using CCK-8 cell counting kit (Dojindo, Kumamoto, Japan) following manufacturer's instructions. Then, 1.5×10^4 cells of each experimental group were equally seeded on 96-well plates and were treated as indicated for 48 hours before cell viability assay. Cell proliferation was assayed using Click-iT™ EdU microplate assay kit (Thermo Fisher Scientific) following manufacturer's instructions. Cells were treated as indicated for 48 hours before assay. Apoptosis assay was performed using TiterTACS *in situ* detection kit (R&D Systems, Minneapolis, MN, USA) following manufacturer's instructions. Cells were treated as indicated for 48 hours before assay. Transwell assay was performed using Matrigel-coated Transwell inserts (with 8.0 mm pore membrane, Corning Incorporated, Corning, NY, USA). Briefly, equal amounts of cells of each group were seeded in the insert chamber with

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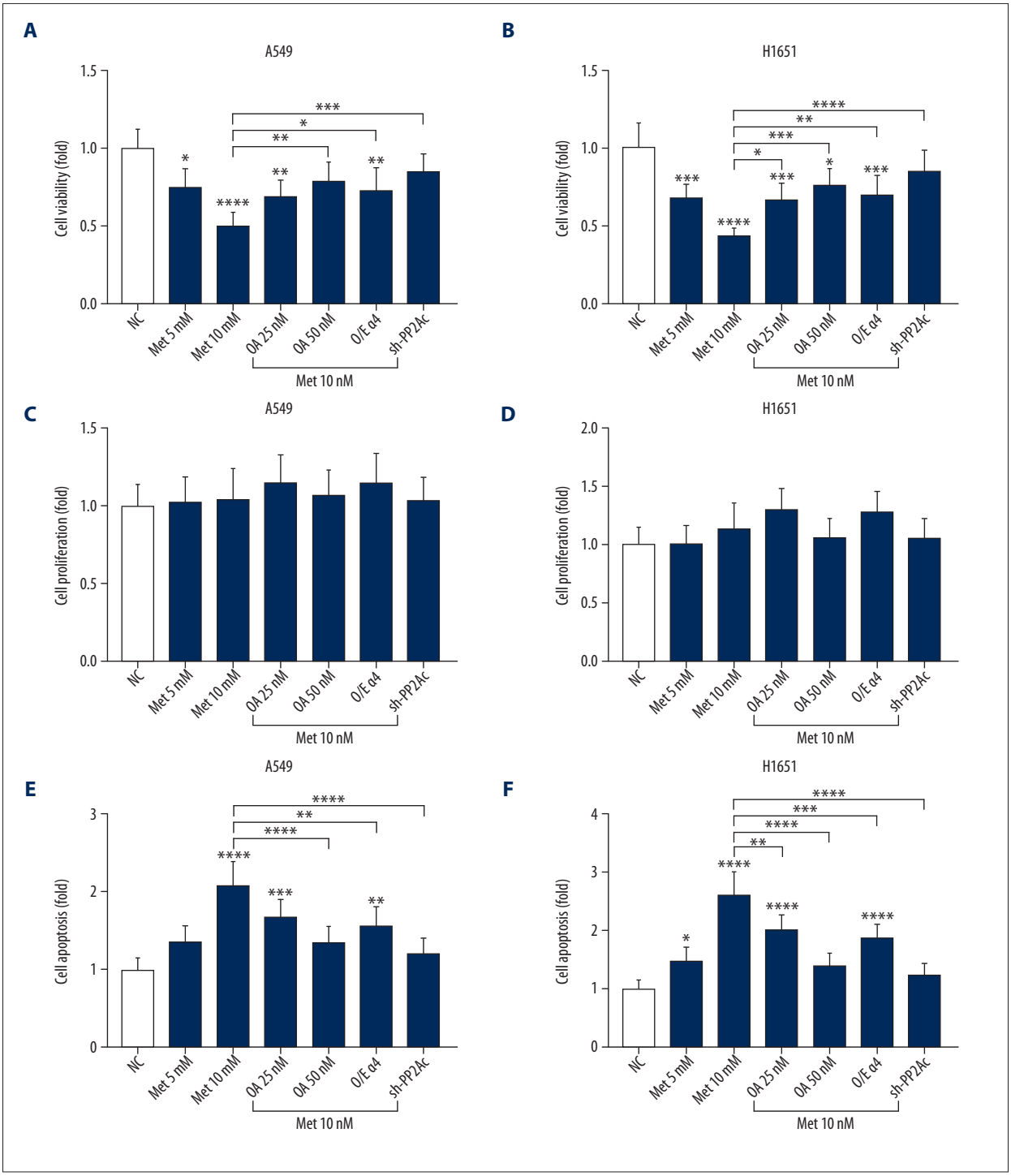
serum-free culture medium and inserted in complete culture medium with 10% FBS. After incubation for 24 hours, cells migrated to the bottom of the chamber were stained with crystal violet and counted under microscope.

Western blot

Western blot was performed using homemade, reducing polyacrylamide (Bio-Rad, Hercules, CA, USA) gel. After being separated by electrophoresis and transferred onto nitrocellulose membrane (Bio-Rad), proteins of interest were blotted with primary and HRP-conjugated secondary antibodies, which



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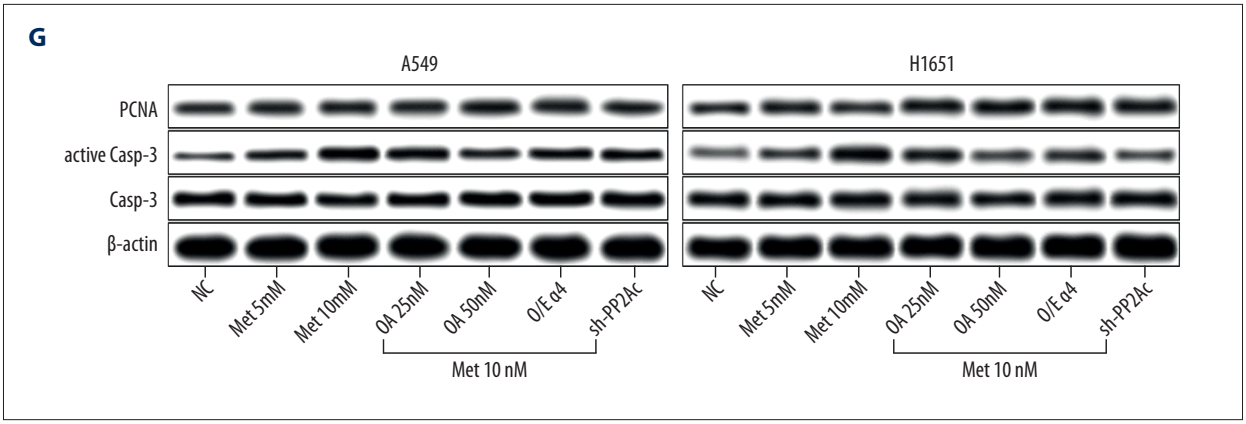

Figure 1. Metformin treatment inhibited A549 and H1651 cell proliferation and survival *in vitro* partially in a PP2A catalytic activity-dependent manner. (**A, B**) Metformin dose-dependently reduced A549 and H1651 cell viability after treatment for 48 hours, which was partially attenuated by okadaic acid treatment (OA), α4 overexpression (O/E α4) or knockdown of PP2A catalytic subunit 1 and B (sh-PP2Ac). (**C, D**) Treatment as in A and B for 48 hours showed no significant influence on cell proliferation. (**E, F**) Metformin dose-dependently increased A549 and H1651 cell apoptosis in *in vitro* cell culture, which was partially rescued by OA treatment, O/E α4 or sh-PP2Ac. (**G**) Western blot detecting the protein expression level of PCNA, caspase-3 and active caspase-3 in cells after treatment in **C–F**. Data in **A–F** was normalized to the mean value of NC group in the same panel. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$.

were then detected by incubation with fluorescent ECL substrate (BosterBio, Pleasanton, CA, USA) and x-ray film (MBL International, Woburn, MA, USA). Protein expression was semi-quantified by comparing the gray scale of band of each protein visualized on x-ray film to that of the housekeeping protein β-actin processed under same conditions. Gray scale analysis was performed using ImageJ software. Primary antibodies used for western blot are as follows: PCNA (orb214367, rabbit polyclonal, Biorbyt, San Francisco, CA, USA), caspase-3 (orb153764, rabbit polyclonal, Biorbyt), active caspase-3 (ab2324, rabbit polyclonal, Abcam, Cambridge, MA, USA), Bax (NBP1-28566, mouse monoclonal, Novus Biologicals, Littleton, CO, USA); phospho-Bax (Ser184, PA5-39778, rabbit polyclonal, Thermo Fisher Scientific); Bcl-2 (658702, mouse monoclonal, BioLegend); c-Myc (sc-40, mouse monoclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA); phospho-c-Myc (Ser62, 13748, rabbit monoclonal, Cell Signaling Technology, Danvers, MA, USA); Akt (NBP2-44110, mouse monoclonal, Novus Biologicals); phosphor-Akt (Ser473, NB100-56749, mouse monoclonal, Novus Biologicals); β-actin (ab20272, mouse monoclonal, Abcam). HRP-conjugated secondary antibodies used for western blot are as follows: Rabbit Anti-Mouse IgG H&L (ab6728, Abcam); Goat Anti-Rabbit IgG H&L (ab6721, Abcam)

Tumor formation assay

Xenograft model was constructed by injecting A549 cells with different modifications subcutaneously on Nu/J nude mice (Jackson Laboratory, Bar Harbor, ME, USA) held under sterile condition with free access to standard mouse chow (Harlan, Indianapolis, IN, USA) and sterile water before experiment. After 4 to 5 days when xenografted tumors were detectable, mice were fed with sterile water dissolved with 5 mg/mL metformin until euthanized. Xenografted tumors were then dissected and weighted before homogenization with a tissue grinder. Proteins of interest in tumor tissue homogenates were detected by western blot as described. Animal care and use in the present research was approved by the ethical review committee of the First Affiliated Hospital of Zhejiang University.

Statistical analysis

Statistical analysis was performed using SPSS Software (ver. 17). Each data represents the results of 6 independent replicate experiments and was presented as mean ± SDS unless otherwise indicated. All data were presented as fold induction when applicable. One-way analysis of variance (ANOVA) with Tukey's post hoc test was used for significance test. A P value <0.05 was considered significant.

Results

Anti-cancer effect of metformin is partially depended on the catalytic activity of PP2A

To examine our hypothesis that activation of PP2A is involved in the anti-cancer activity of metformin treatment, we employed OA for inhibiting the catalytic activity of PP2A in A549 and H1651 human NSCLC cells [12,25]. We also constructed α4 overexpression (O/E α4) or double knockdown of PP2A catalytic subunit A and B (sh-PP2Ac) to reduce PP2A activity in the

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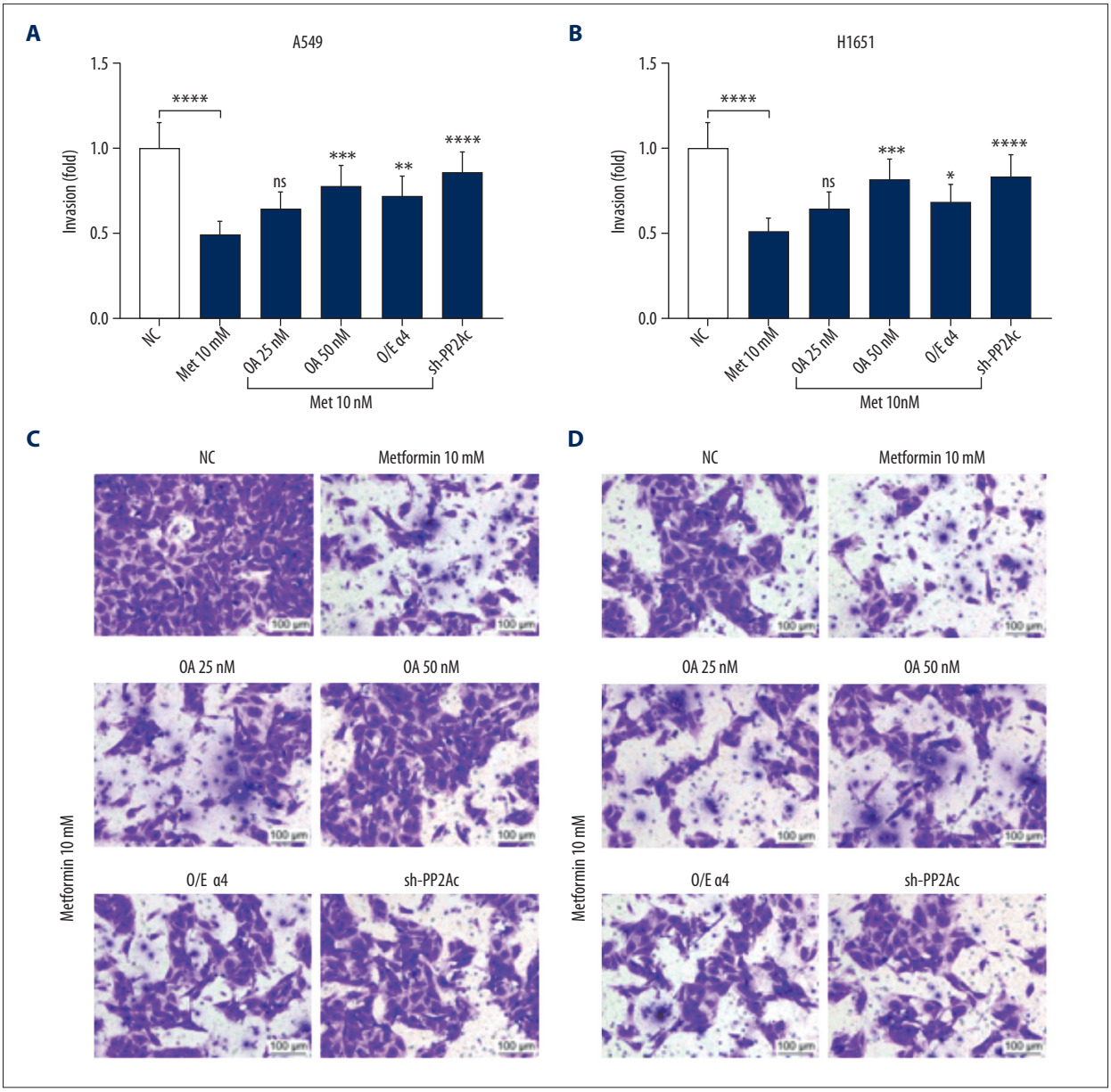

Figure 2. Metformin treatment inhibited A549 and H1651 cell invasion capacity *in vitro* partially in a PP2A catalytic activity-dependent manner. (**A–D**) Metformin dose-dependently reduced Transwell invasion of A549 and H1651 cells *in vitro,* which was partially attenuated by okadaic acid treatment (OA), α4 overexpression (O/E α4) or knockdown of PP2A catalytic subunit **A and B** (sh-PP2Ac). **C and D** are representative results of Transwell assay in **A and B**, respectively. Data in **A and B** was normalized to the mean value of NC group in the same panel. ns – non-significant; * *P*<0.05; ** *P*<0.01; *** *P*<0.001; **** *P*<0.0001.

2 cell lines [17]. Significant α4 overexpression and sufficient inhibition of PP2A catalytic subunit A and B expression were verified by RT-qPCR and western blot (data not shown). Cell functional assay results showed that treatment with metformin significantly reduced A549 and H1651 cell viability while increasing apoptosis *in vitro* in a dose dependent manner, leaving the cell proliferation status intact, and these effects could be partially reversed by combined treatment with OA, α4 overexpression or PP2Ac knockdown (Figure 1A–1F). Cell proliferation and apoptosis in A549 and H1651 cells after treatment in Figure 1C–1F were further verified by western blot detecting the protein expression level of cell proliferation and apoptosis marker genes PCNA and caspase-3 (Figure 1G). Transwell invasion assay using Matrigel-coated Transwell chamber demonstrate that treatment with metformin at 10 mM significantly reduced invasive capacity of A549 or H1651 cells by nearly 50% *in vitro*, which could be partially reversed by OA co-treatment at 50 nM, α4 overexpression or PP2Ac knockdown (Figure 2).

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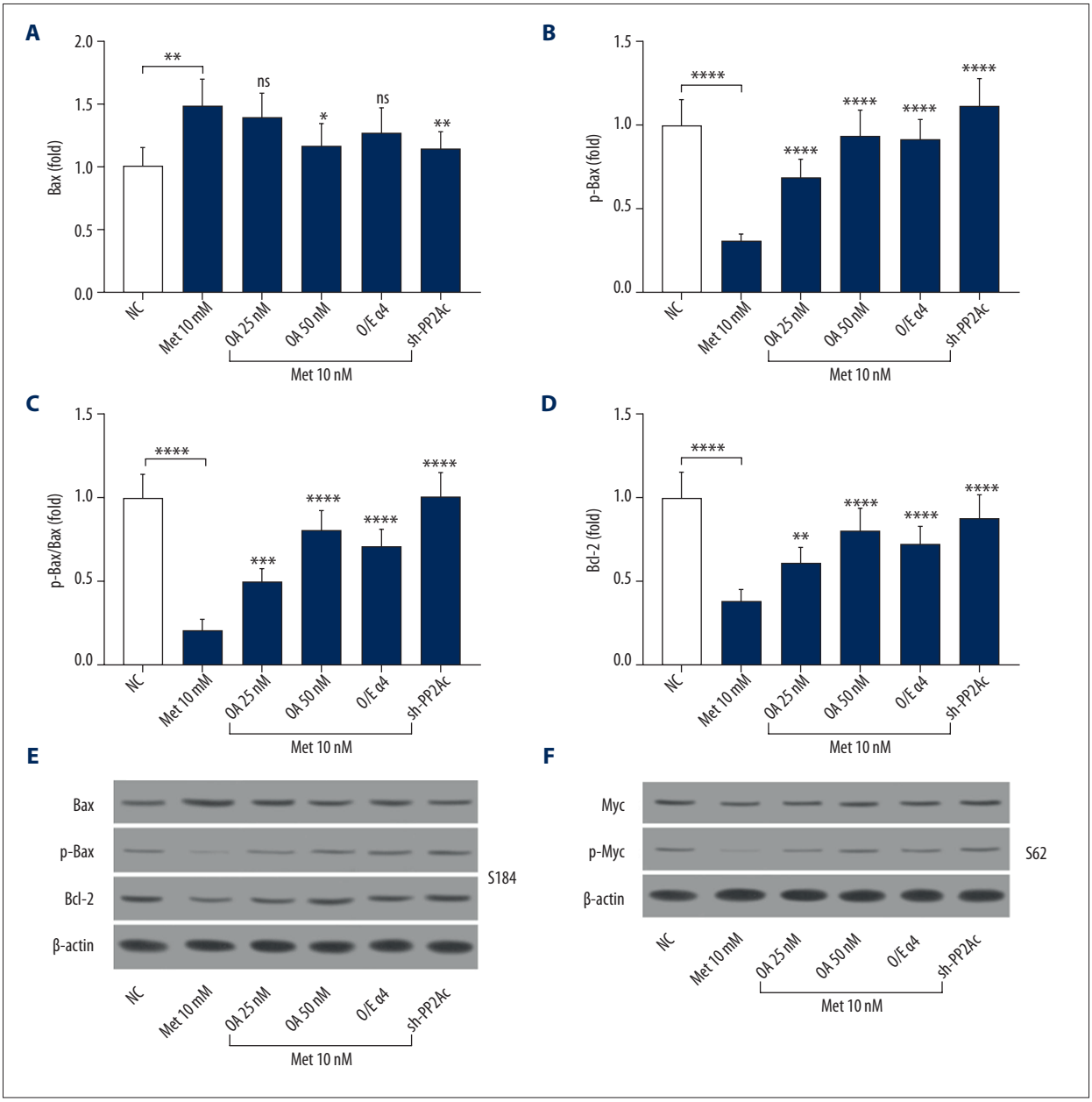
Among these results, knockdown of PP2A catalytic domain A and B showed the most significant interference on the inhibitory effect of metformin treatment on cell viability, survival and invasion *in vitro*. These data suggested that the anti-cancer effect of metformin is partially depended on the catalytic activity of PP2A.

Metformin treatment increased PP2A catalytic activity

PP2A has been demonstrated to suppress lung cancer cell growth and tumor development by decreasing the signal transduction of key oncogenic signaling pathways, including PI3K-Akt and Myc, as well as by increasing the pro-apoptotic activity of Bax via its serine phosphatase activity [15,25]. We therefore investigated whether metformin treatment would influence the serine phosphorylation level of Bax, Myc, and Akt on PP2A sensitive site in A549 cells (Figure 3). Western blot assays revealed that metformin treatment significantly increased Bax protein expression level and decreased its Ser184 phosphorylation level, resulting in a dramatic decrease in p-Bax/Bax ratio (Figure 3A–3C, 3E); metformin treatment also significantly decreased Bcl-2 protein expression level in A549 cells, possibly due to the increase in cell apoptosis (Figure 3D, 3E). Metformin treatment significantly decreased Ser62 phosphorylation of Myc protein and S473 phosphorylation of Akt protein that are known to be the target site of PP2A [15] (Figure 3F–3L). Notably,



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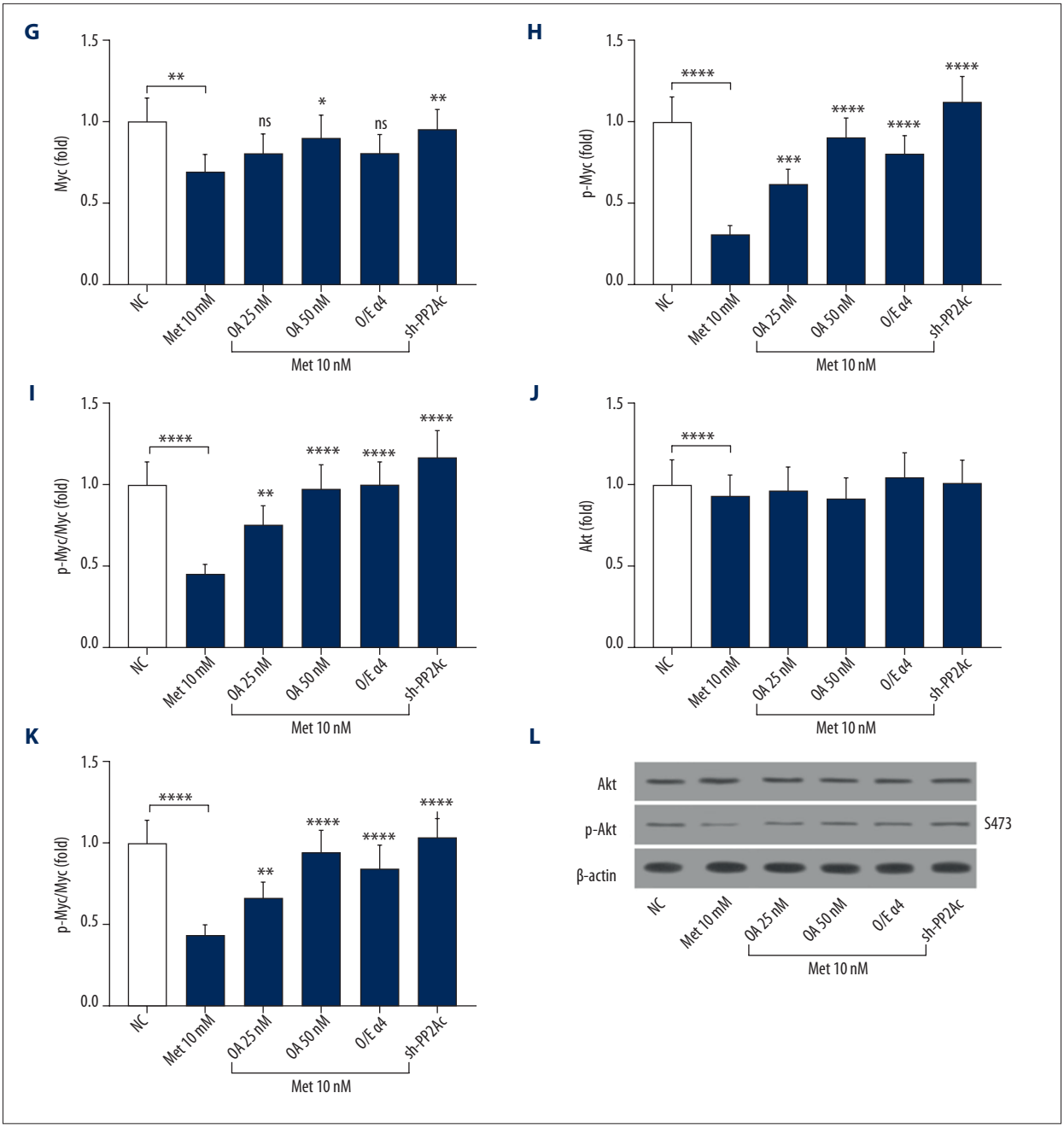

Figure 3. Western blot detecting Bax, Bcl-2, Myc, and Akt protein expression as well as serine phosphorylation of Bax, Myc, and Akt at indicated sites in A549 cells. (**A–D**) Bax and Bcl-2 protein expression as well as serine phosphorylation of Bax in A549 cells. (**G–K**) Myc and Akt protein expression as well as serine phosphorylation of Myc and Akt in A549 cells. (**E, F, L**) Representative western blot results. Data in other panels was normalized to the mean value of NC group in the same panel. ns, non-significant; * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$.

metformin treatment showed no significant influence on Akt protein expression level but significantly reduced that of Myc protein, probably due to the ubiquitination-mediated degradation of Myc protein after loss of Ser62 phosphorylation catalyzed by PP2A [26] (Figure 3G, 3J). Our western blot assay further revealed that these influences of metformin treatment on protein expression and serine phosphorylation of Bax, Bcl-2, Myc, and Akt proteins can be significantly reversed by OA co-treatment in a dose dependent manner, α4 overexpression or PP2Ac knockdown (Figure 3). These data clearly demonstrated that metformin treatment could decrease the serine phosphorylation of PP2A substrate proteins, which could be partially

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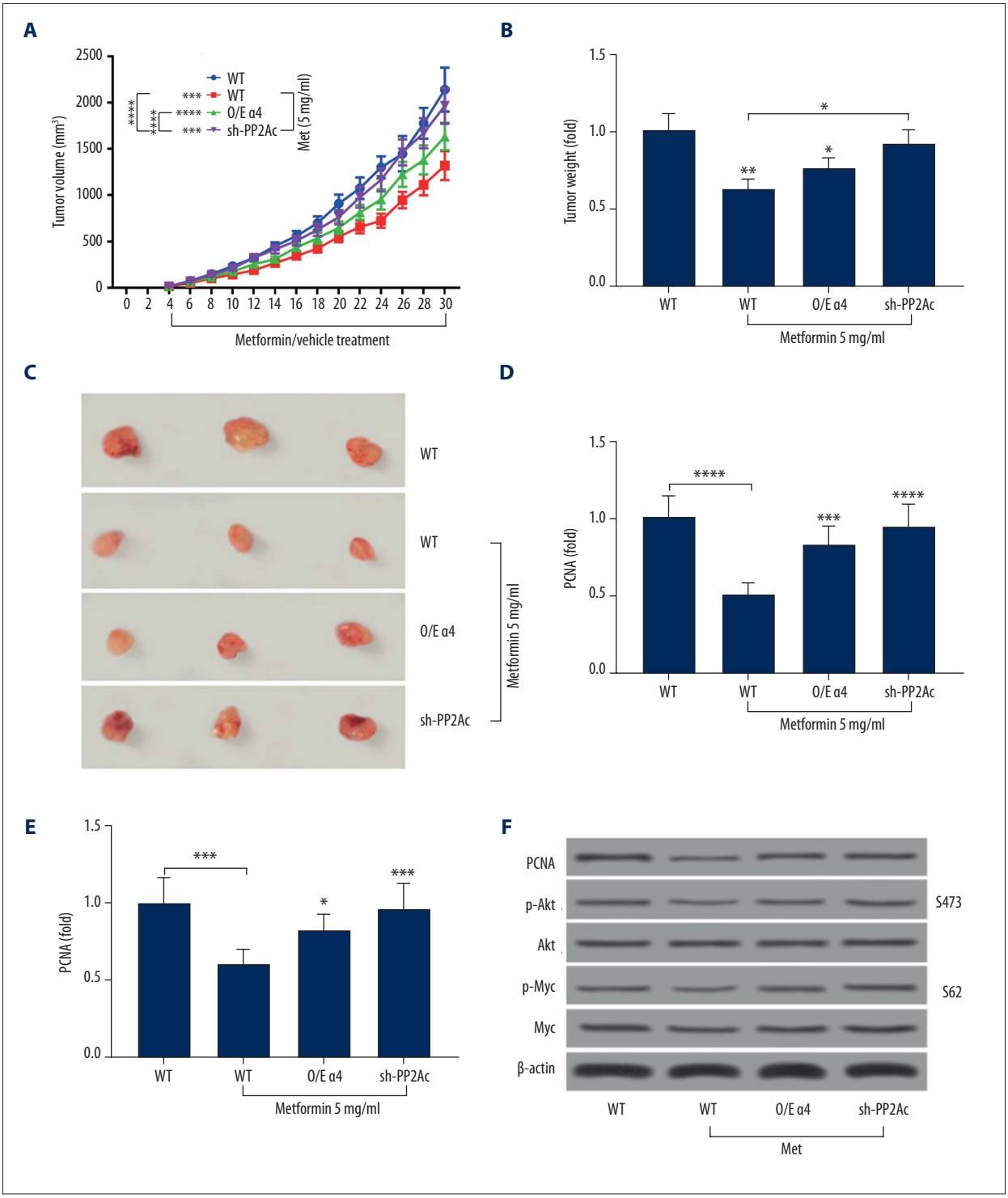
reversed by inhibition of PP2A catalytic activity through various means, suggesting that metformin could activate PP2A in lung cancer cells *in vitro*.

Metformin treatment reduced tumor growth *in vivo*

We next investigated whether the anti-cancer effect of metformin *in vivo* is also mediated at least in part by PP2A. We constructed xenograft mouse model using A549 cells that were wild type (WT), α4 overexpression (O/E α4) or PP2Ac knockdown



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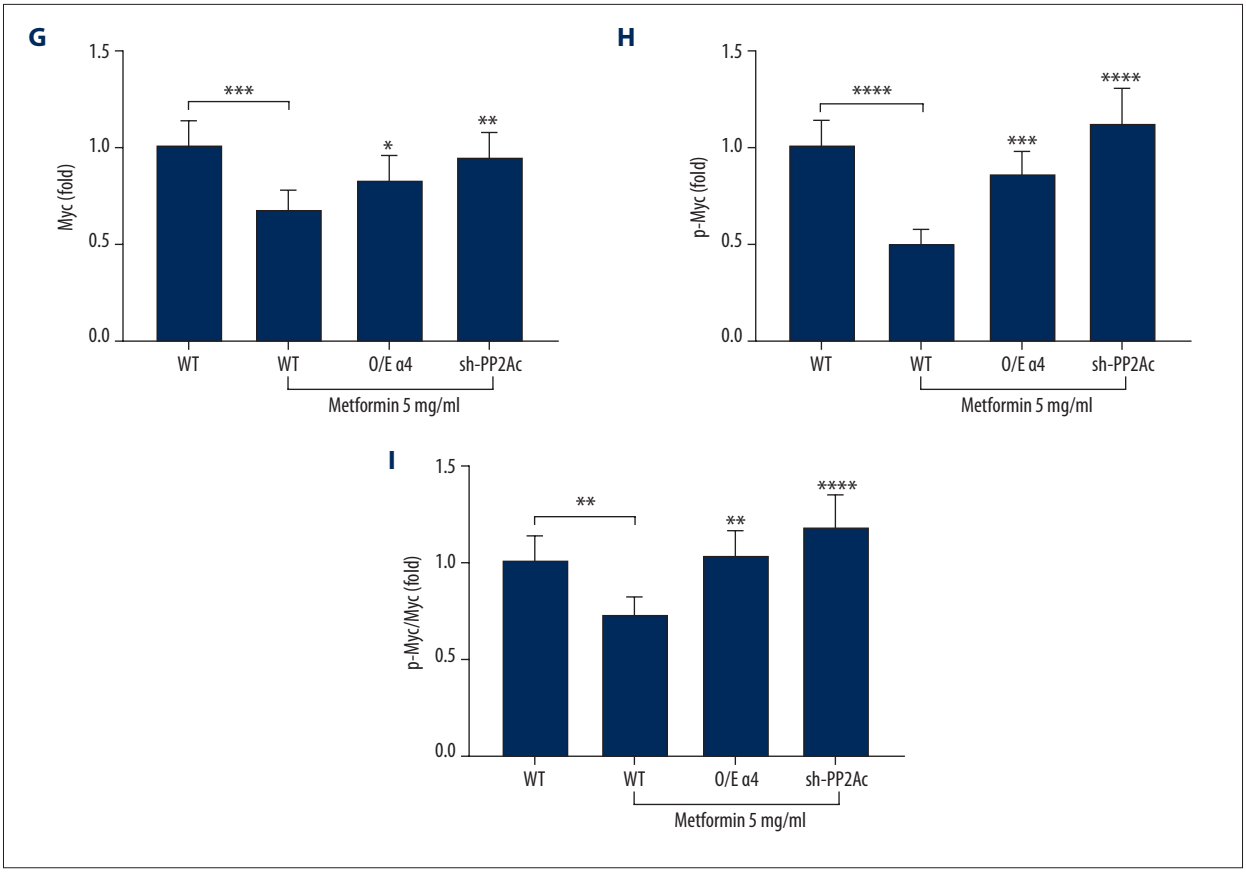

Figure 4. Metformin treatment inhibited tumor formation capacity of A549 in xenograft model partially in a PP2A catalytic activity-dependent manner. (**A**) Tumor growth curve of each group. (**B, C**) Tumor mass at endpoint of the tumor formation assay. (**D–I**), western blot detecting indicated protein expression level or serine phosphorylation level in xenografted tumor tissue. (**F**) Representative western blot result. Data in **B** presented as mean ±SD represents 3 independent replicate experiments. Data in **B, D, E, G–I** was normalized to the mean value of WT group in the same panel. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$. WT, wild type.

(sh-PP2Ac). Metformin oral treatment significantly attenuated the tumor volume increase in nude mice burdened with WT A549 xenografted tumor, while xenografted tumors constructed with O/E α4 or sh-PP2Ac A549 cells showed resistance to metformin treatment, marked by the accelerated tumor volume growth comparing to WT (Figure 4A–4C). Western blots examining tumor tissue cells at the end of the tumor formation assay showed that cell proliferation in tumor tissue was significantly reduced by metformin treatment, which was partially reversed by α4 overexpression or PP2Ac knockdown; similar to *in vitro* experiment results in Figure 3, western blots examining xenografted tumor tissues showed that phosphorylation of S473 on Akt protein and Ser62 on Myc protein were significantly reduced by metformin and partially rescued by α4 overexpression or PP2Ac knockdown (Figure 4D–4I). These data suggested that the *in vivo* anti-cancer effect of metformin is also partially dependent on PP2A catalytic activity, possibly by reducing the signal transduction of Myc protein and Akt protein.

Discussions

The anti-cancer activity of metformin treatment in various types of cancers has been acknowledged in recent years. The molecular mechanisms of these effect, however, remains to be further elucidated. A better understanding of the molecular mechanism of action of metformin might aid the development of novel therapeutic strategy for cancer management. Recognition of AMPK activation by metformin involved in its anti-cancer effect dawned in 2007 when Buzzai et al. reported the selective inhibition of metformin treatment on p53 deficient liver cancer cells but not wild type counterparts [27]. In lung cancer cells, metformin-induced AMPK activation has been shown to inhibit lung cancer cell growth *in vitro* and *in vivo* either alone or in synergy with other anti-cancer therapy [5,10,28]. However, after carefully examining the data presented by current reports, we believe that the anti-cancer effect of metformin on lung cancer cannot be explained solely by AMPK activation.

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Analogously, Lin et al. reported that the anti-cancer and cisplatin-sensitizing effect of metformin treatment in NSCLC cells were partially mediated by an AMPK-independent STAT3 dephosphorylation mechanism [11].

During our literature research, we noticed that metformin treatment could directly interfere with the association of PP2A catalytic subunit, α4, and MID1, thus liberating the catalytic activity of PP2A [12,13]. PP2A has long been confirmed as a tumor suppressor in lung, breast, and ovarian cancers, although PP2A inhibition was also suggested to induce mitotic catastrophe and cell death in senescent cancer cells [14,29,30]. Our present study aimed to test whether PP2A activation was involved in the anti-cancer effect of metformin treatment on lung cancer. Our data showed that decreasing the PP2A catalytic activity either by OA treatment, α4 overexpression or stable knockdown of PP2A, catalytic subunit A and B significantly attenuated the inhibition of NSCLC cell growth or invasive capacity *in vitro* by metformin treatment, and PP2Ac knockdown most significantly obstructed metformin's effect. Tumor formation assay also confirmed that α4 overexpression or PP2Ac knockdown desensitized A549 cells to metformin treatment-induced inhibition of tumor growth *in vivo*. Western blot assay results further demonstrated that metformin treatment lowered Ser473 phosphorylation of Akt that would result in its inactivation, Ser62 phosphorylation of Myc that would lead to its degradation, as well as Ser184 phosphorylation of Bax known to increasing its pro-apoptotic activation, and these effects were significantly weakened by OA treatment, α4 overexpression, or PP2Ac knockdown [15,25]. Collectively, our results suggested that PP2A activation by metformin is involved in its anti-cancer effect on lung cancer. Re-activation of PP2A by metformin or other targeted approach might aid lung cancer treatment.

Interestingly, our *in vitro* experimental data suggested that metformin treatment reduced lung cancer cell growth *in vitro* primarily by inducing apoptosis, considering that the cell viability assay we performed evaluated the number of living cells from the same amount of initial seeding cells; However, by western blot assay detecting cell proliferation marker PCNA and apoptosis marker caspase-3 activation, we found that cell proliferation in xenografted tumor was reduced by metformin treatment, and caspase-3 activation level was not significantly altered (data not shown). We inferred from these data that metformin might exhibit additional effect that reduced cancer cell proliferation *in vivo*, which warranted further investigation. Various research employed cell viability assay to evaluate the cell proliferation-inhibiting effect of metformin on lung cancer cells [31,32], but this technique is questionable, because cell viability assays reflects the change in viable cell number, which could be the overall effect of cell proliferation plus cell apoptosis. It should also be noted that sufficient knockdown of PP2A catalytic subunits or inhibitor treatment did not completely block the anti-cancer effect of metformin treatment in other experimental conditions. Although activation of PP2A by metformin has been suggested as AMPK-independent, possibility remains that activation of AMPK and PP2A might synergize to inhibit tumor development. Besides AMPK or PP2A activation, it has also been suggested that metformin inhibits lung cancer development through other mechanisms. For instance, Jin et al. demonstrated that metformin treatment reduced the mRNA and protein expression of E2F8, a presumed oncogenic transcription factor in lung cancer that functions by promoting cell cycle progression [33]; Yu et al. demonstrated that metformin treatment upregulated Sirtuin 1 to reduce acetylation of NF-E2-related factor 2, thus reducing its transcriptional activity and sensitizing lung cancer cells to epigallocatechin-3-gallate induced apoptosis [34]. Whether these phenomena are direct effects of metformin or indirect through AMPK or PP2A activation needs to be further elucidated. Previous research also pointed to the synergy effect of metformin and chemo- or targeted therapy, especially EGFR tyrosine kinase inhibitor [35,36]; whether PP2A is involved in these synergic effects should also be further investigated.

Conclusions

In the present study, our data clearly demonstrated that metformin reduced lung cancer cell growth and invasion *in vitro* as well as tumor development *in vivo* partially by activating PP2A, which inhibited the activation of tumor promoting proteins such as Bax, Myc, and Akt by catalyzing their dephosphorylation.

Conflict of interest

None.

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