

# Metformin attenuates ovarian cancer cell growth in an AMP-kinase dispensable manner

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## Abstract

Metformin, the most widely used drug for type 2 diabetes activates 5' adenosine monophosphate (AMP)-activated protein kinase (AMPK), which regulates cellular energy metabolism. Here, we report that ovarian cell lines VOSE, A2780, CP70, C200, OV202, OVCAR3, SKOV3ip, PE01 and PE04 predominantly express  $\alpha_1$ ,  $\beta_1$ ,  $\gamma_1$  and  $\gamma_2$  isoforms of AMPK subunits. Our studies show that metformin treatment (1) significantly inhibited proliferation of diverse chemo-responsive and -resistant ovarian cancer cell lines (A2780, CP70, C200, OV202, OVCAR3, SKOV3ip, PE01 and PE04), (2) caused cell cycle arrest accompanied by decreased cyclin D1 and increased p21 protein expression, (3) activated AMPK in various ovarian cancer cell lines as evident from increased phosphorylation of AMPK $\alpha$  and its downstream substrate; acetyl co-carboxylase (ACC) and enhanced  $\beta$ -oxidation of fatty acid and (4) attenuated mTOR-S6RP phosphorylation, inhibited protein translational and lipid biosynthetic pathways, thus implicating metformin as a growth inhibitor of ovarian cancer cells. We also show that metformin-mediated effect on AMPK is dependent on liver kinase B1 (LKB1) as it failed to activate AMPK-ACC pathway and cell cycle arrest in LKB1 null mouse embryo fibroblasts (mefs). This observation was further supported by using siRNA approach to down-regulate LKB1 in ovarian cancer cells. In contrast, metformin inhibited cell proliferation in both wild-type and AMPK $\alpha_{1/2}$  null mefs as well as in AMPK silenced ovarian cancer cells. Collectively, these results provide evidence on the role of metformin as an anti-proliferative therapeutic that can act through both AMPK-dependent as well as AMPK-independent pathways.

**Keywords:** metformin • ovarian cancer cells • LKB1 • AMPK • p21 • cyclin D1 • ACC • mTOR

## Introduction

Epithelial ovarian cancer is the fifth most lethal cancer and leading cause of death among women in United States. This high mortality rate (68%) reflects not only the fact that 75% of patients have extensive (>stage III) disease at diagnosis [1, 2], but also the limited efficacy of currently available therapies in recurrent disease. Following optimal cytoreductive surgery, ovarian cancer patients are commonly treated with carboplatin/cisplatin and paclitaxel, with only ~10–15% of >stage III patients remaining disease-free at 5 years. Potential novel therapeutic approaches that target one or more of the altered molecular pathways, involved in ovarian cancer pathology, may be beneficial to patients.

Metformin belongs to the biguanide class of drugs and is currently the single most prescribed oral anti-diabetic agent for type 2 diabetes. Beneficial cardiovascular effects of metformin intake have also been reported in diabetic patients, attributed to its positive action on lipid metabolism [3]. Anti-proliferative effects of metformin have been reported in cancers of the breast, glioma, prostate and colon [4–6], and more recently in ovarian and pancreatic cancer cells [7, 8]. Interestingly, metformin is commonly used in the treatment of polycystic ovary syndrome, a syndrome associated with increased risk of ovarian and endometrial cancers [9]. It exhibits anti-inflammatory properties, a potentially added attribute, because inflammatory mediators have been shown to play an important role in ovarian carcinogenesis. Collectively, these data support the role of metformin as an anti-cancer drug.

Research studies strongly suggest that the central mechanism of anti-proliferative action of metformin is *via* activation of liver kinase B1 (LKB1)-AMPK pathway, which has emerged as an attractive and widely studied target for cancer therapeutics. Mutations in LKB1 leads to Peutz–Jeghers syndrome, an autosomal

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dominant syndrome [10] characterized by benign gastrointestinal polyps and an increased risk of malignancy. LKB1 is implicated as a tumour suppressor gene in cancers of melanomas, non-small cell lung and pancreatic cancers. Most studies have attributed the anti-cancer effect of LKB1 due to activation of its downstream kinase, AMPK. AMPK is a heterotrimeric serine/threonine protein kinase that acts as an ultra-sensitive cellular energy sensor maintaining the energy balance within the cell [11]. AMPK is composed of a catalytic subunit ( $\alpha_1$  and  $\alpha_2$ ) and regulatory subunits ( $\beta_1$ ,  $\beta_2$ ,  $\gamma_1$ ,  $\gamma_2$  and  $\gamma_3$ ). It is activated in response to modulation of AMP/ATP ratio under stress conditions and also by certain cytokines and hormones like leptin and adiponectin [12]. Pharmacological compounds like aminoimidazole carboxamide ribonucleotide (AICAR), thiazolidinediones (TZDs), metformin and A-769662 also activate AMPK [13].

Recently, the role of AMPK in inhibiting proliferation has received attention in tumours of diverse origins [14]. AMPK is involved in cell cycle regulation, protein synthesis and modulates p53-p21 and TSC-2-mTOR nexus, respectively [15]. AMPK activation has been shown to also down-regulate PI3K-Akt pathway in cancer cells [16]. An alternate mechanism by which AMPK can exert anti-growth activity is through inhibition of lipid biosynthesis *via* inhibition of the acetyl co-carboxylase (ACC), a rate limiting enzyme of the fatty acid synthesis pathway [12]. Very little is known on the status of AMPK (basal enzymatic activity and the expression of its various subunits/isoforms) and use of metformin as a potential chemotherapeutic drug in ovarian cancer either *in vivo* or *in vitro*.

In the present study, we show that metformin inhibits ovarian cancer cell proliferation by modulating diverse pathways including inhibition of cell cycle, survival pathways, and/or protein and lipid biosynthetic pathways. Our studies also indicate that metformin can inhibit ovarian cancer cell growth in an AMPK dispensable manner.

## Material and methods

### Reagents and antibodies

Metformin, trypan Blue, MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, and propidium iodide were all from Sigma (St. Louis, MO, USA). C14-methionine, C14-palmitic acid and C14-acetate were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Antibodies to AMPK $\alpha_1$  was from Epitomics (Burlingame, CA, USA), AMPK $\alpha_2$ , AMPK $\beta_{1/2}$ , AMPK $\gamma_{123}$ , phospho-AMPK, phospho-ACC, phospho-Akt, phospho-mTOR, phospho-S6K, were from Cell Signaling (Danvers, MA, USA). AMPK $\alpha$  siRNA, LKB1 siRNA and respective non-target control were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA).

### Cell culture

Ovarian cancer cell lines, OVCAR3, OVCAR5 and CaOv3 were from ATCC (Manassas, VA, USA) and grown according to accompanied instructions.

OV202 is a low passage primary cell line generated at Mayo Clinic and grown in DMEM [17]. A2780, CP70, C200 cell lines were a kind gift of Dr. Tom Hamilton (Fox Chase Cancer Center, Philadelphia, PA, USA) and growth in RPMI medium was supplemented with insulin. PE01 and PE04 were a kind gift of Dr. Stephen Williams (Fox Chase Cancer Center) and grown in DMEM. SKOV3ip cell line was a gift from Dr. E. Vitetta (Cancer Immunology Center, Dallas, TX, USA) and grown in McCoy's medium. Immortalized ovarian surface epithelium (IOSE)-523 was from Dr. Nelly Auersperg (UBC, Canada). LKB1 $^{+/+}$  and LKB1 $^{-/-}$  mouse embryo fibroblasts (mefs) were a kind gift of Dr. Tomi Makela (University of Helsinki, Finland). AMPK $\alpha_{1/2}^{+/+}$  and AMPK $\alpha_{1/2}^{-/-}$  mefs were a kind gift from Dr. Keith R. Laderoute, (SRI International, Menlo Park, CA, USA).

### Proliferation assay

A total of  $2.5\text{--}5.0 \times 10^4$  cells were plated in 24-well plates in triplicates and treated with indicated concentrations of metformin every third day. Cell counts were done by trypan blue staining every day. Day 0 represents the day of treatment.

### Colony formation assay

A total of 2000–3000 cells were plated in triplicates in 6-well plates and treated with indicated concentrations of metformin. The cells were allowed to form colonies for up to 2–4 weeks (depending on the cell line) and media were replaced every third day. Colonies were stained with crystal violet or MTT and counted as indicated.

### Cell cycle analysis

Cellular DNA content was assessed by flow cytometry. Cells were cultured in 6-well plates and treated with indicated metformin concentrations. Attached cells were collected after trypsinization, washed and resuspended in 100  $\mu$ l of PBS and 5 ml of 70% ethanol was slowly added with continuous vortexing of the cells. The cells were fixed overnight at  $-20^\circ\text{C}$ . Next day, cells were spun, washed with PBS and suspended in 400  $\mu$ l of PBS with the addition of 10  $\mu$ g/ml RNaseA and 75  $\mu$ m propidium iodide. Cell cycle analysis was performed by flow cytometry (BD Biosciences, San Jose, CA, USA).

### Western blot

Immunoblot analysis with specific antibodies was performed as previously described [16].

### Protein synthesis

Equal number of ( $1 \times 10^6$ ) cells were plated in 100 mm dishes, treated with metformin for 24 hrs. During the last 4 hrs of incubation, cells were pulsed with  $^{14}\text{C}$ -methionine. Protein lysates were prepared as previously described [16]. A total of 150  $\mu$ g protein of each sample was precipitated with acetone overnight. The precipitated protein was pelleted and dissolved in  $1 \times$  Lamelli sample buffer. Incorporated radioactivity was determined using Beckman Coulter (Fullerton, CA, USA) counter.

## Fatty acid $\beta$ -oxidation

$\beta$ -oxidation of [ $1\text{-}^{14}\text{C}$ ] palmitic acid  $\beta$ - to acetate was measured in intact ovarian cancer cells suspended in HBSS as described before [18]. A2780, CP70, C200 and SKOV3ip cells were treated with indicated concentrations of metformin for 8 hrs. The reaction mixture of 6  $\mu\text{M}$  [ $1\text{-}^{14}\text{C}$ ] palmitic acid in Hank's buffered salt solution (HBSS) along with  $\alpha$ -cyclodextrin was added for 30 min. to intact cells. Reaction was stopped by 1 M KOH. After removal of denatured protein, supernatant was incubated at 60°C for 1 hr, neutralized with 6 M HCl and partitioned. The upper aqueous phase was used for measuring [ $1\text{-}^{14}\text{C}$ ] palmitic radioactivity.

## Lipid biosynthesis

The biosynthesis of non-polar and polar lipids was examined in A2780, CP70, C200 and SKOV3ip cells labelled with [ $1\text{-}^{14}\text{C}$ ] acetate. Cells were treated with metformin and pulsed with 1  $\mu\text{Ci}$  of [ $1\text{-}^{14}\text{C}$ ] acetate for final 2 hrs of incubation. After incubation, incorporation of labelled acetate in lipids was examined by extracting lipids using Folch method. Incorporation of labelled acetate into non-polar lipids was determined using high performance thin layer chromatography (HPTLC) followed by 3 days of exposure on X-ray film at  $-70^\circ\text{C}$ . Densitometric analyses are represented as bar graphs [19].

## Plasmid, transfections and luciferase assay

We obtained CyclinD1 luciferase construct from Dr. Janknecht (Mayo Clinic, Rochester, MN, USA) and p21 luciferase plasmid from Dr. S. Mujtaba (Mount Sinai School of Medicine, New York, NY, USA). Transfections were done using Lipofectamine-Plus reagent (Invitrogen, San Diego, CA, USA) in 24-well plates following the manufacturer's instructions. Cells were seeded at  $8 \times 10^4$  cells per well in 24-well plates (in triplicates) 1 day prior to transfection. A total of 0.15  $\mu\text{g}$  reporter luciferase constructs or empty vector as control were co-transfected with 0.01  $\mu\text{g}$  Renilla reporters. Luciferase activity was measured 24 hrs after transfection with Promega's Dual-Luciferase Reporter assay system (Promega, Madison, WI, USA) according to manufacturer's instructions. The relative light units are expressed after normalizing with Renilla luciferase to account for variability in transfection efficiency. For transfection with AMPK $\alpha$ , LKB1 and non-target siRNA, oligofectamine reagent (Invitrogen) was used. At 48 hrs after transfection protein lysates were prepared to examine the down-regulation of AMPK $\alpha$  protein expression and also plated for proliferation assay.

## Statistical analysis

Data are presented as mean  $\pm$  S.D. of at least three independent experiments. Differences between groups were analysed using two-tailed Student's *t*-test. The data were statistically analysed using the Student–Newman–Keuls test (GraphPad Software), with  $P < 0.05$  considered significant.

## Results

### Metformin inhibits proliferation of ovarian cancer cell lines

To examine the effect of metformin on ovarian cancer growth, various ovarian cancer cell lines (A2780, CP70, C200, OV202,

OVCAR3, SKOV3ip, PE01 and PE04) were treated with various concentrations of metformin (5–20 mM) and rate of cell proliferation was determined by trypan blue exclusion assay (0–7 days). Metformin treatment inhibited the growth of several ovarian cancer cell lines significantly in a time- and dose-dependent manner (Fig. 1) including cisplatin resistant CP 70 and C200 (Fig. 1B and C) and taxol resistant PE04 cell lines (Fig. 1H), but not to the same extent in the highly aggressive SKOV3ip1 cells (Fig. 1D). To determine if this inhibition was reflected in clonogenic survival, colony forming abilities of A2780, CP70, C200, SKOV3ip, PE01 and PE04 cells was estimated. Metformin treatment significantly attenuated clonogenic survival of ovarian cancer cell lines in a dose-dependent manner compared to untreated cells (Fig. 2). These results suggest that metformin treatment can result in a sustained inhibition of proliferation of various ovarian cancer cell lines, including chemoresistant cell lines (CP70, C200 and PE04) (Fig. 2B, C and F).

To check the effect of metformin on primary ovarian cells, we treated a SV40t/T immortalized ovarian surface epithelial cell line, IOSE-523 with metformin for 3 days. Metformin treatment (5–20 mM) did not have any significant effect on the growth of IOSE-523 cells, suggesting that its anti-proliferative effect is specific to ovarian cancer cells (Fig. S1A). HeLa cells treated with the same increasing doses of metformin (5–20 mM) for 3 days did not show any significant decrease in cell viability (Fig. S1B) indicating that the concentrations used in this study are not toxic to the cells.

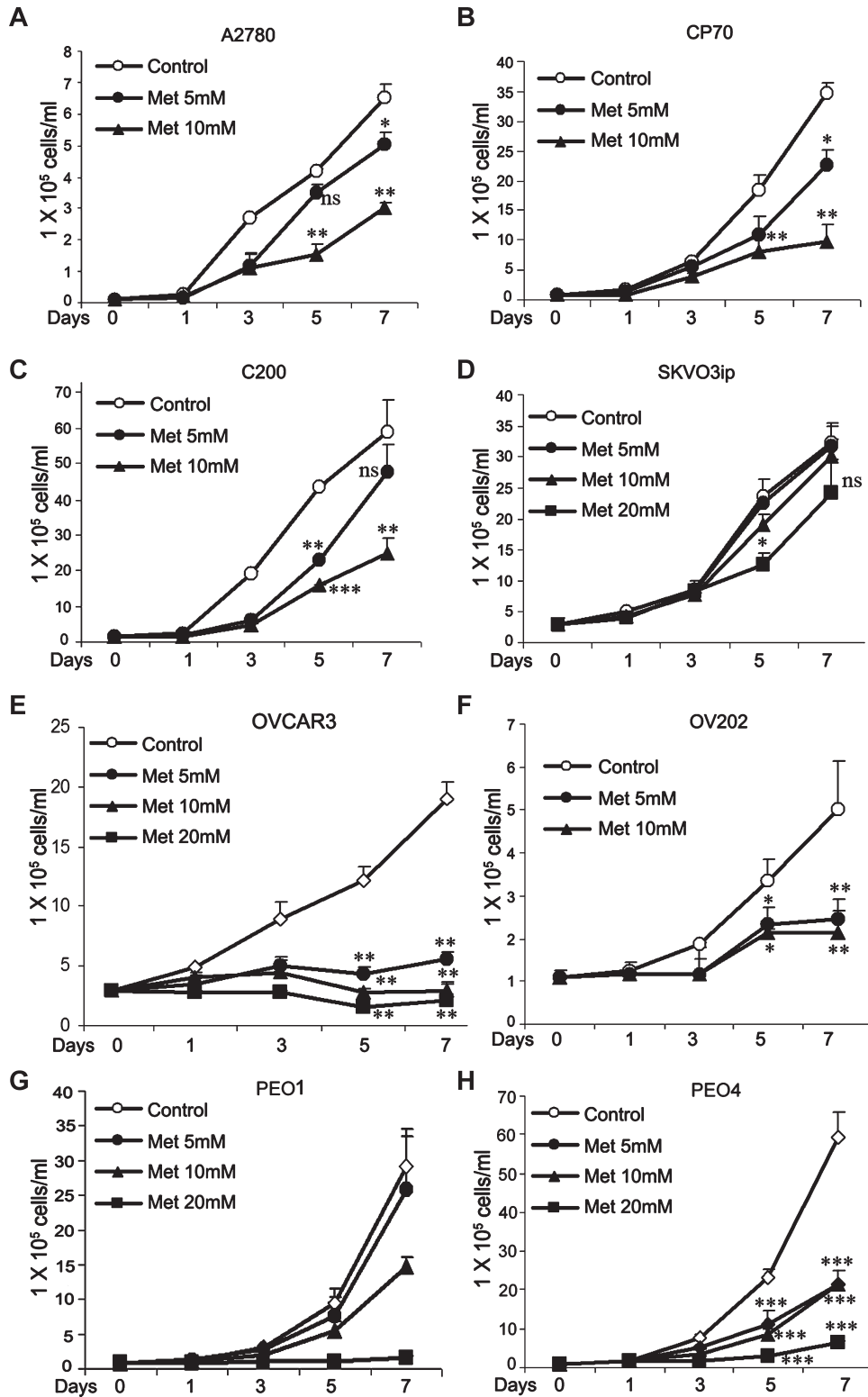
### Metformin causes cell cycle arrest in G1-phase

To determine if metformin-mediated inhibition of cell proliferation may reflect changes in cell cycle, we examined cell cycle distribution by flow cytometry. As depicted in Fig. 3A, metformin treatment led to accumulation of cells in G1-phase with corresponding decrease in the percentage of cells in the S-phase in all ovarian cancer cell lines tested compared to untreated cells. Consistent with this G1 arrest, immunoblot analysis revealed that metformin treatment resulted in reduced cyclin D1 levels with concomitant increase in the expression of p21 in A2780, CP70, C200 and SKOV3ip cells; however, there was no change in the expression of p27 (Fig. 3B). Densitometric representation of the data is shown in Fig. S2. Collectively, these results suggest that metformin inhibits cell cycle progression by modulating the expression of cell cycle proteins.

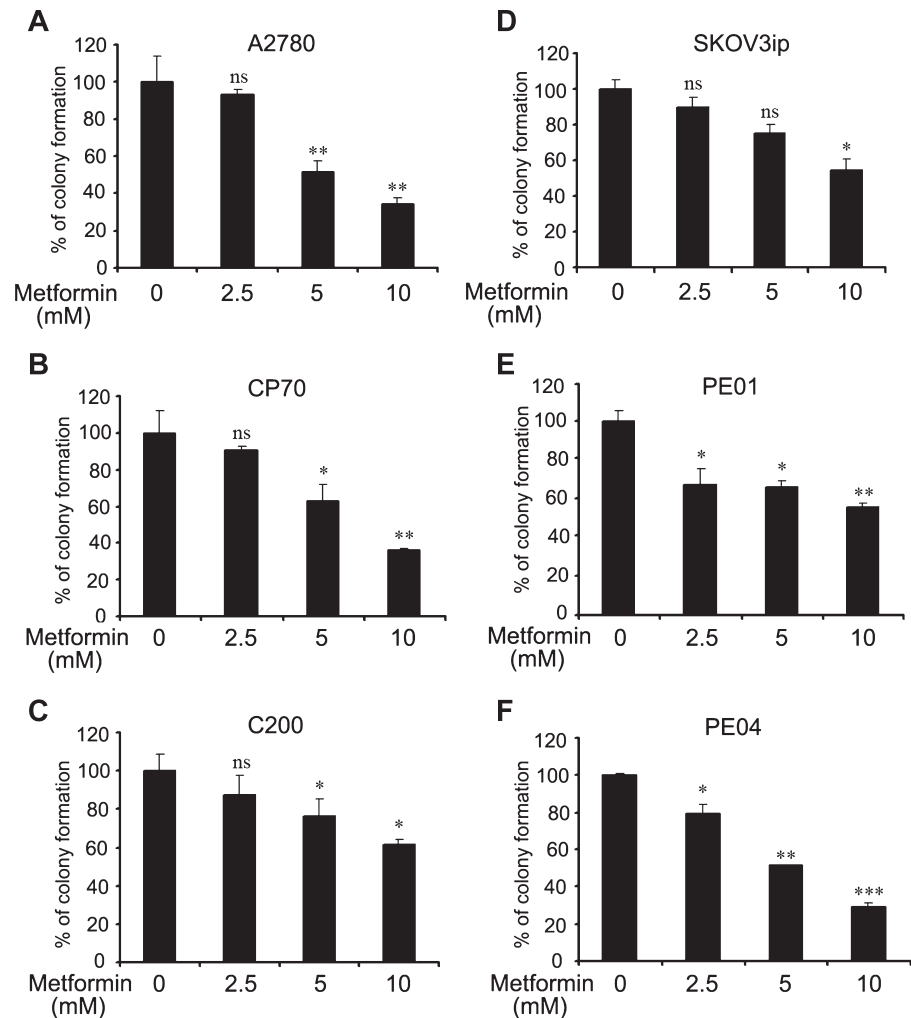
### Metformin activates AMPK in ovarian cancer cells

Immunoblot analysis of expression of AMPK subunits showed that AMPK $\alpha_1$  and  $\beta_1$  subunits were the predominant isoforms expressed in several ovarian cancer cell lines.  $\gamma_1$  and  $\gamma_2$  subunits were variably expressed, whereas  $\gamma_3$  was not detected (Fig. S3). With the exception of CaOV3, LKB1 was expressed in all tested cell lines, indicating that the LKB1-AMPK pathway is intact in most ovarian cancer cell lines.

Because metformin treatment results in the activation of AMPK, we determined its effect on AMPK activity in various ovarian



**Fig. 1** Metformin inhibits proliferation of ovarian cancer cell lines. Various ovarian cell lines (A2780, CP70, C200, OV202, SKOV3ip, OVCAR3, PEO1 and PEO4) were treated with metformin with indicated doses. Cells were counted from days 0 to 7, on alternate days by trypan blue staining. The data represent three separate experiments done in triplicates. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ , \* $P < 0.05$ ; NS: not significant, compared to untreated cells at respective time-point.



**Fig. 2** Metformin inhibits colony formation of ovarian cancer cells. 2000 cells/well (A2780, CP70, C200, SKOV3ip, PE01 and PE04) in 6-well plates were treated with indicated concentrations of metformin, every third day for 2–3 weeks until colonies were formed. The colonies were stained with MTT and counted. The data represent three separate experiments done in triplicates. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ , \* $P < 0.05$ ; NS: not significant compared to untreated cells.

cancer cell lines. Immunoblot analysis indicated that metformin-induced phosphorylation of AMPK $\alpha$  at Thr-172 and its downstream target ACC (Ser-79) in a dose-dependent manner (Fig. 4A). ACC is an important rate-controlling enzyme for the synthesis of malonyl-CoA, which is a critical precursor for biosynthesis of fatty acids and also a potent inhibitor of mitochondrial fatty acid oxidation [20]. Metformin treatment significantly enhanced the mitochondrial  $\beta$ -oxidation, further supporting the activation of AMPK and inhibition of ACC in A2780, CP70 and C200 cells (Fig. 4B).

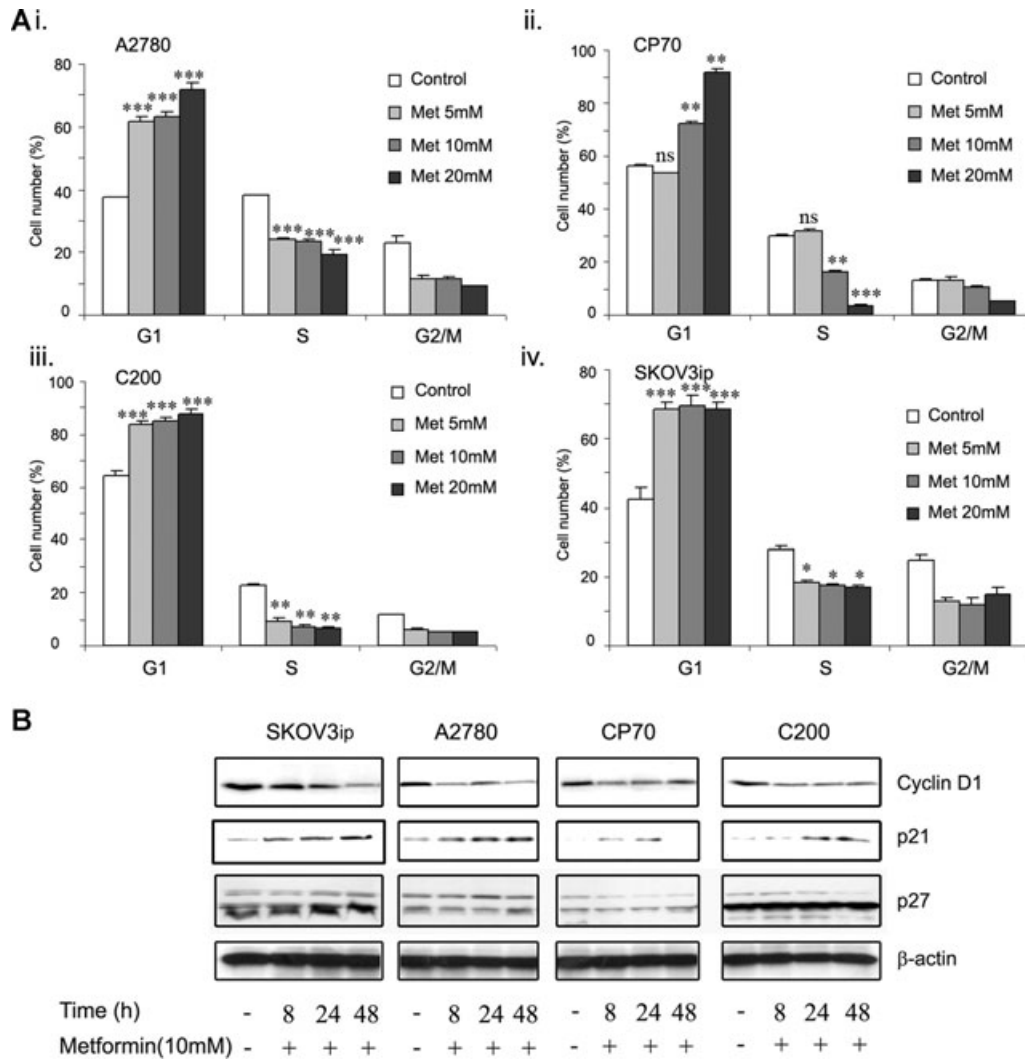
### Metformin inhibits m-TOR-S6RP-protein translation and lipid biosynthetic pathways

AMPK activation is associated with decreased activation of mTOR and S6K, a critical translational pathway for protein synthesis [21, 22]. Metformin treatment resulted in attenuated activation of mTOR and S6K as demonstrated by decreased phosphorylation of

mTOR and S6K in treated ovarian cancer cells compared to untreated cells (Fig. 5A). Metformin treatment also resulted in attenuation of Akt phosphorylation, which regulates mTOR *via* tuberous sclerosis complex-2 [23, 24], (Fig. 5A). These results indicate that metformin can not only inhibit mTOR but can also inhibit the pathway at an upstream level.

Because phosphorylation of mTOR and S6K is directly associated with increased protein synthesis, we examined protein biosynthesis using radioactive methionine as a tracer. As illustrated in Fig. 5B, metformin treatment inhibited the incorporation of [ $^{14}$ C]-methionine into proteins in a dose-dependent manner, further supporting the role of metformin-mediated inhibition of mTOR pathway in ovarian cancer cells. Collectively, these data indicate that metformin effectively inhibits the protein synthesis machinery in ovarian cancer cells.

AMPK also regulates lipid biosynthesis by phosphorylating and inhibiting activity of ACC and HMG CoA reductase, rate-limiting enzymes for fatty acid and cholesterol biosynthesis, respectively



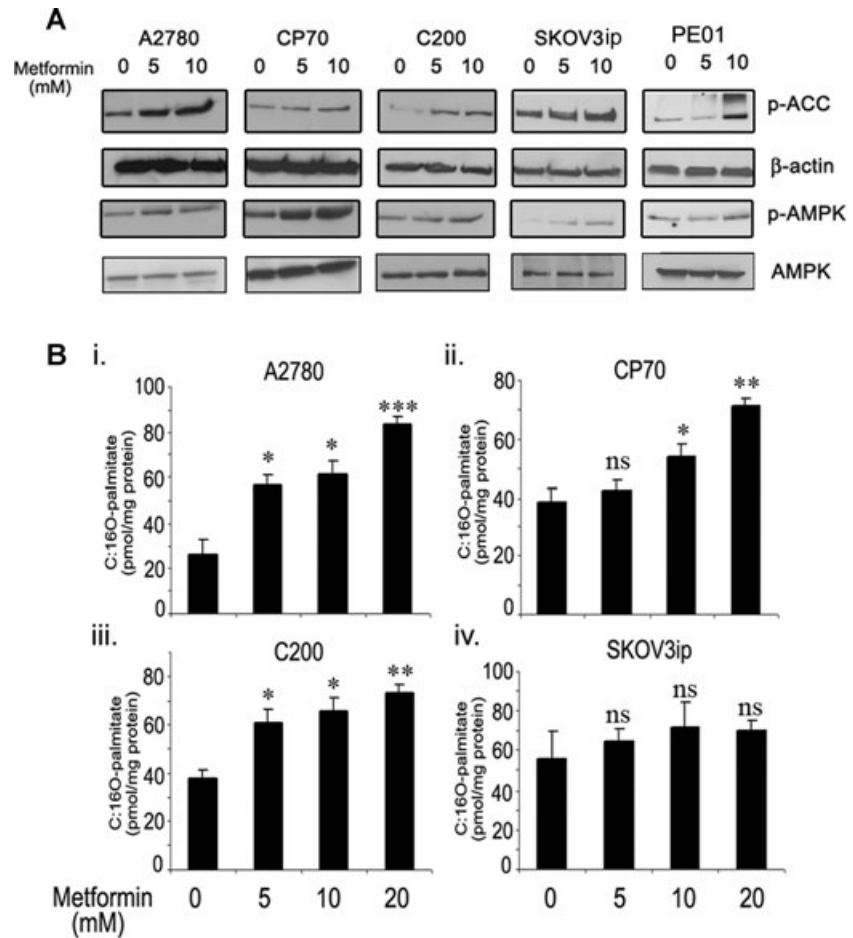
**Fig. 3** Metformin causes cell cycle arrest in G1-phase. (A) A2780, CP70, C200 and SKOV3ip cells were treated with metformin with indicated concentrations for 24 hrs. Cells were fixed overnight, stained with propidium iodide and flow-sorted. The data represent three separate experiments. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ , \* $P < 0.05$ ; NS: not significant compared to untreated cells. (B) Immunoblot analysis of ovarian cancer cells treated with metformin showing reduced cyclin D1 and up-regulated p21 levels. Blots are representation of two separate experiments.

[25, 26]. To examine the effect of metformin on lipid biosynthesis, various ovarian cancer cells were treated with metformin (5–20 mM) for 8 hrs followed by [ $^{14}$ C] acetate treatment for additional 4 hrs. Metformin treatment significantly inhibited the biosynthesis of both polar (phospholipids) and non-polar (cholesterol and triglycerides) lipids in ovarian cancer cell lines (Fig. 6). Collectively, these results suggest that metformin treatment leads to decreased protein translation and lipid biosynthesis, which may result in limited availability of essential cellular building blocks to the tumour cells, essential for cell growth/survival.

### LKB1 is required for activation of AMPK by metformin

The molecular mechanism of action of LKB1 is thought to involve in large part, as an activator of AMPK. To determine if LKB1 is

imperative in metformin-mediated cell cycle arrest, we employed LKB1 mefs. Metformin treatment resulted in robust activation of AMPK in LKB1 $^{+/+}$  mefs as demonstrated by phospho-AMPK and phospho-ACC levels, whereas metformin was unable to achieve AMPK activation in LKB1 $^{-/-}$  mefs (Fig. 7A). Metformin treatment resulted in S-phase arrest in LKB1 $^{+/+}$  mefs whereas the LKB1 $^{-/-}$  mefs showed no cell cycle arrest in any cell cycle phase (Fig. 7Bi and ii), suggesting metformin mediates its effect by activating LKB1. To check if a similar phenomenon was operative in ovarian cancer cells, we silenced LKB1 in A2780 cells using siRNA. Down-regulation of LKB1 (Fig. 7C) resulted in attenuation of phosphorylation of ACC compared to untransfected control (C) and non-target control (NT) siRNA transfected cells (Fig. 7D), suggesting that in the absence of LKB1, AMPK was not activated. Additionally, LKB1 siRNA down-regulated cells (Si) did not show any inhibition of proliferation compared to untransfected control (C) or non-target control (NT) siRNA transfected cells in response to metformin



**Fig. 4** Metformin treatment activates AMPK in ovarian cancer cell lines. **(A)** Ovarian cancer cells (A2780, CP70, C200, SKOV3ip and PE04) were treated with metformin with indicated concentrations for 12 hrs. Protein lysates were immuno-blotted against phospho-ACC and phospho-AMPK antibodies. Same blot was stripped and probed for β-actin to show equal protein loading. **(B)** A2780, CP70, C200 and SKOV3ip cells were treated with indicated concentrations of metformin for 8 hrs. Cells were pulsed with 6 μM [<sup>14</sup>C] palmitic acid. After partition of cells, the upper aqueous phase was taken for measurement of radioactivity. \*\*\**P* < 0.001; \*\**P* < 0.01, \**P* < 0.05; NS: not significant compared to untreated cells.

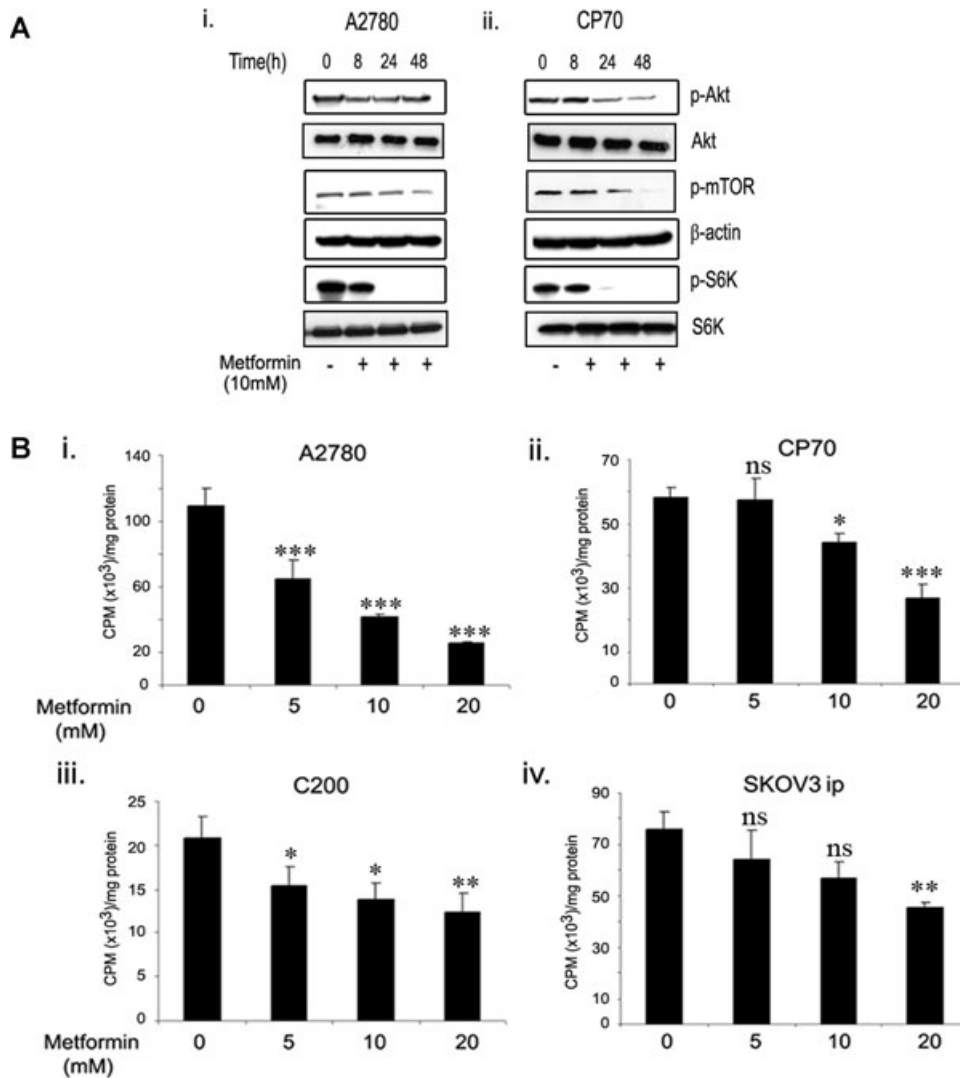
treatment (Fig. 7E). Together, these data indicate that metformin acts through LKB1 in order to activate AMPK, and in the absence of LKB1, is unable to mediate its anti-growth action.

### AMPK is dispensable for metformin-mediated anti-proliferative effect

To further support the conception that metformin-mediated effect is dependent on AMPK, we down-regulated its expression in A2780 cells using siRNA specific to AMPK $\alpha_1$  and examined its effect on ACC phosphorylation and cell proliferation. Immunoblot analysis shows efficient down-regulation of AMPK $\alpha_1$  and less phosphorylation of ACC in AMPK $\alpha_1$  siRNA transfected cells (Si) compared to non-target control siRNA transfected (NT) and untransfected (C) cells (Fig. 8A). However, despite lower expression of AMPK $\alpha_1$  in siRNA transfected A2780 cells, metformin treatment was able to inhibit cell growth (Fig 8B). This inhibition was significantly less (~20%) compared to AMPK expressing

parental or non-target control siRNA transfected control cells (~40–45%).

Because we could not achieve complete down-regulation of AMPK $\alpha$  expression in A2780 cells, we utilized AMPK $\alpha_{1/2}$  null ( $^{-/-}$ ) mefs as a tool to support our observation in A2780 cells. Metformin treatment at 5 and 10 mM induced AMPK activation in AMPK $\alpha_{1/2}^{+/+}$  mefs compared to AMPK $\alpha_{1/2}^{-/-}$  mefs, as observed by increased phosphorylation of ACC and AMPK (Fig. 8C). It also inhibited phosphorylation of S6RP in the AMPK $\alpha_{1/2}^{+/+}$  mefs compared to null, indicating that AMPK is essential for inhibition of protein synthesis by metformin. Interestingly, metformin treatment attenuated proliferation of both AMPK $\alpha_{1/2}^{+/+}$  and AMPK $\alpha_{1/2}^{-/-}$  mefs, although the AMPK $\alpha_{1/2}^{-/-}$  mefs seem to be modestly less sensitive to metformin's anti-proliferative effect (Fig. 8D). On the other hand, AMPK $\alpha_{1/2}^{-/-}$  mefs showed higher reporter (~2-fold) activity of CyclinD1 and less activity of p21 (~3-fold) compared to AMPK $\alpha_{1/2}^{+/+}$  mefs (Fig. 8E–F). Metformin treatment inhibited cyclin D1 and induced p21 luciferase reporter activity in both AMPK $\alpha_{1/2}^{+/+}$  and AMPK $\alpha_{1/2}^{-/-}$  mefs. However,



**Fig. 5** Metformin restricts mTOR-translation pathway in ovarian cancer cells. **(A)** A2780 and CP70 cells were treated with metformin (10 mM) at indicated time-points. Cells lysates were prepared and analysed for Akt (Ser-473), mTOR (Ser-1448) and S6-kinase phosphorylation by Western blot. The blots are representative of three independent experiments. **(B)** A2780, CP70, C200 and SKOV3ip cells were seeded and treated with metformin with indicated concentrations for 18 hrs followed by <sup>14</sup>C-methionine (20 μCi/mL) labelling for 4 hrs. <sup>14</sup>C radioactivity incorporated was assessed as described in material and methods. The data are representative of three separate experiments. \*\*\**P* < 0.001; \*\**P* < 0.01, \**P* < 0.05; NS: not significant compared to untreated cells.

the inhibition of cyclin D1 as well as induction of p21 by metformin was greater in AMPK $\alpha_{1/2}^{+/+}$  mefs compared to null mefs, indicating a role for AMPK in metformin's anti-proliferative effect, particularly pertaining to regulation of cell cycle proteins. These data suggest that although AMPK plays an elemental role in metformin's anti-growth action, it is not indispensable. These data lead us to conclude that metformin can still be effective as an anti-proliferative agent even in the absence of AMPK activation, possibly by modulating other oncogenic pathways either directly or through the action of LKB1.

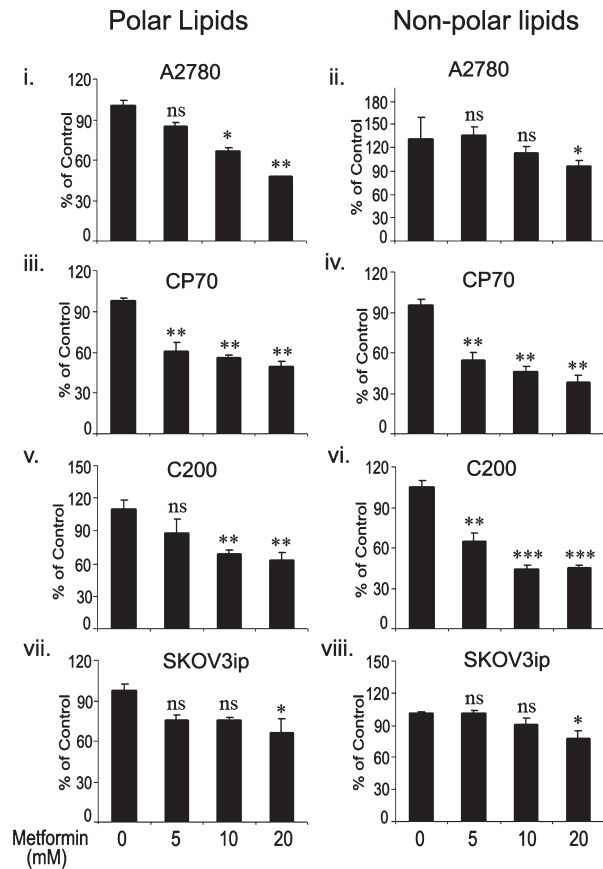
## Discussion

Ovarian cancer causes more deaths per year than any other cancer of the female reproductive system. One of the reasons

for this is the failure of current treatment regimens to control the spread of ovarian cancer. Therefore other novel approaches may improve the overall survival of patients with ovarian cancer.

Here, we investigated the therapeutic potential of metformin against ovarian cancer in a pre-clinical *in vitro* study. We document that metformin effectively inhibits proliferation of numerous ovarian cancer cell lines including cisplatin and taxol chemoresistant cell lines. Its treatment leads to G1 growth arrest with concomitant inhibition of cyclin D1 and induction of p21 expression. Metformin treatment resulted in the activation of AMPK which led to decreased protein and lipid biosynthesis. Our data also indicate that the anti-growth action of metformin is LKB1 dependent but, could be AMPK dependent or independent, implicating other currently unknown oncogenic pathways that could be modulated by metformin.





**Fig. 6** Metformin inhibits lipid biosynthesis in ovarian cancer cells. (A) A2780, CP70, C200 and SKOV3ip cells were treated with indicated concentrations of metformin. After 12 hrs treatment, cells were pulsed with 1  $\mu$ Ci of [ $^{14}$ C] acetate for an additional 4 hrs. Incorporation of labelled acetate into non-polar lipids was examined by HPTLC followed by 3 days exposure on X-ray film at  $-70^{\circ}\text{C}$ . Densitometric analysis was performed on the films and represented as bar graphs. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ , \* $P < 0.05$ ; NS: not significant, compared to untreated cells.

In recent years, various studies have shown that metformin has anti-tumour properties *in vitro* and *in vivo*. *In vitro* anti-proliferative effects of metformin have been reported in cancers of the breast, glioma, prostate and colon [4–6] and more recently in ovarian and pancreatic cancer cells [7, 8]. Various *in vivo* studies have shown that metformin effectively inhibits growth of colon cancer, mammary adenocarcinomas, pancreatic cancers, intestinal polyps and lung carcinoma cells [27].

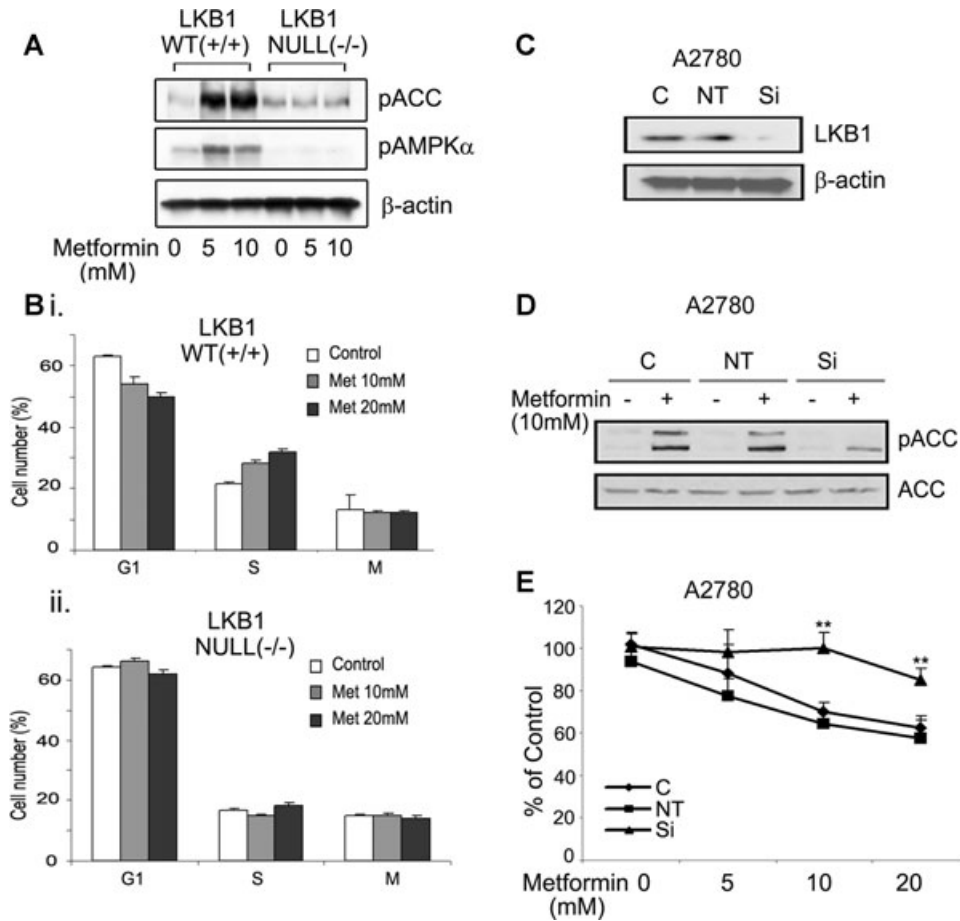
Our study also adds to these observations, where metformin treatment inhibits growth of ovarian cancer cell lines as shown by both MTT and colony formation assays. Metformin, however, did not inhibit the proliferation of immortalized ovarian surface epithelial cells, suggesting its specificity for ovarian cancer cells. In contrast to breast cancer cell lines [4, 28], where metformin treatment resulted in inhibiting Cyclin D1 with concomitant increase in cell

cycle inhibitors (p21, and p27), metformin treatment of ovarian cancer cells lines in this study resulted in no change in p27 expression, but induced G1-arrest accompanied by inhibition of cyclin D1 and increase in p21 expression.

AMPK activation by metformin treatment leads to attenuation of mTOR pathway resulting in the inhibition of protein translation and synthesis [29]. Our study also shows that metformin treatment results in the activation of AMPK and its downstream effector ACC in ovarian cell lines as seen in other cell types. Metformin treatment also resulted in the inhibition of mTOR and its downstream mediators, thereby, attenuating protein biosynthesis in ovarian cancer cells. Additionally, metformin also blocked Akt phosphorylation, indicating that it can quench the pathway upstream of mTOR. Whether this is a direct effect of metformin or is mediated through AMPK or LKB1 is not yet known. We have previously shown that another activator of AMPK, AICAR also inhibits Akt phosphorylation [16]. Recently it was shown that adiponectin-activated AMPK dephosphorylates Akt by increasing protein phosphatase 2A (PP2A) and reduces invasiveness in MDA-MB-231 breast cancer cells [30]. Additionally, energy depletion mediated activation of LKB1/AMPK has been reported to inhibit phosphatidylinositol 3-kinase/Akt signalling *via* IRS-1 phosphorylation to inhibit cell survival [31]. Based on these studies, it can be argued that metformin treatment may lead to attenuation of Akt activation by modulating LKB1/AMPK pathway.

Another major anabolic pathway modulated by AMPK activation is the lipid pathway. Activated AMPK phosphorylates ACC and inhibits its activity which results in inhibition of malonyl-CoA. Malonyl-CoA is not only a precursor of fatty acids but also a potent inhibitor of mitochondrial  $\beta$ -oxidation. In this study we show that metformin treatment not only induced  $\beta$ -oxidation of palmitic acid but also inhibited lipid synthesis, suggesting the activation of AMPK by metformin treatment in ovarian cancer cell lines. Reduction in these two vital biosynthetic pathways could lead to serious depletion of building blocks involving these macromolecules for the cancer cells, specifically when they have an increased demand due to their highly proliferative capacity.

There are contradicting reports on whether p53 status contributes to proliferation inhibition by metformin. Although Jones *et al.* [32] have shown a requirement of p53-phosphorylation for AMPK-induced cell cycle arrest, other reports from the same group [33] indicate that metformin selectively inhibits p53 negative tumour cell growth. In our study we show that metformin inhibited proliferation of ovarian cancer cell lines independent of p53 status. For example, metformin inhibited proliferation of OVCAR3 with p53 mutation [34] and A2780 with wild-type (WT) p53. However, in SV40T-immortalized ovarian surface epithelial cell line IOSE-523 with inactivated p53, the effect of metformin in inhibiting cell proliferation was time dependent. Although there was no significant decrease in the proliferation rate at 72 hrs, longer treatment of up to 7 days resulted in significant decrease in proliferation (data not shown). Testing a larger panel of cell lines with and without p53 could lead to a better understanding on the role of p53 in metformin-induced inhibition of proliferation.

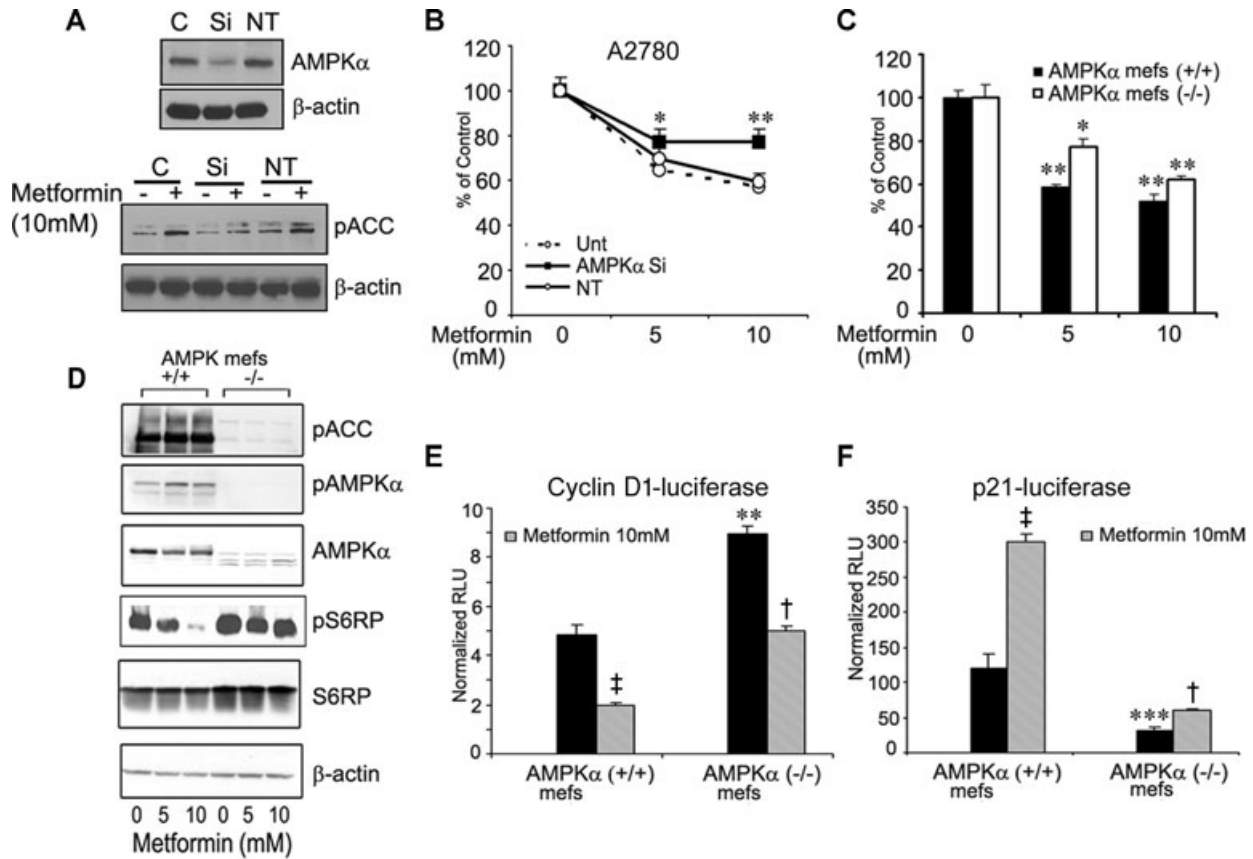


**Fig. 7** Metformin mediated its action *via* LKB1. **(A)** LKB1 null ( $LKB1^{-/-}$ ) and wild-type ( $LKB1^{+/+}$ ) mefs were treated with metformin (5–10 mM) and analysed by immunoblot for AMPK and ACC phosphorylation and  $\beta$  actin for equal protein loading. **(B)**  $LKB1^{-/-}$  and wild-type mefs were treated with metformin with indicated concentrations for 24 hrs. After overnight fixation and propidium iodide staining cells were flow-sorted. The data are representations of two separate experiments. **(C)** Immunoblot analysis revealed down-regulation of LKB1 in untransfected (C), siRNA (Si) and non-target siRNA control (NT) transfected A2780 cells at 48 hrs. **(D)** After 48 hrs transfection, similar sets were treated with metformin for 12 hrs and processed for immunoblot for p-ACC and total ACC. **(E)** A2780 untransfected (C), siRNA (Si) and non-target siRNA control (NT) transfected cells were treated with indicated concentrations of metformin for 48 hrs and assessed for proliferation by MTT.  $**P < 0.01$ , siRNA (Si) compared to C and NT.

It is interesting to note that although metformin treatment resulted in the inhibition of Cyclin D1 and activated p21 in SKOV3ip1 cells, it did not inhibit proliferation (Fig. 1D). Although we are at a loss to explain this observed effect in SKOV3ip1 cells, it is important to realize that these are extremely aggressive cells with constitutively active Akt [28] as well as overexpression of *c-erbB-2/v* oncogene [35] which could result in the activation of mTOR. Thus, the resistance to metformin-induced proliferation could be due to highly active oncogenic signalling in SKOV3ip1 cells. It is possible that these cells may require longer exposure with much higher concentrations of metformin (which may not be physiologically relevant) to overcome the resistance to proliferation inhibition. We also observed that although metformin did not inhibit proliferation of SKOV3ip1 cells with the concentrations tested, we saw inhibition of colony formation (Fig. 2D). Colony formation assays are the gold standard to determine the effect of drug response of cells over a longer time kinetics that is more reflective of therapeutic response.

Mechanism of action of metformin has been postulated to require LKB1. Using mefs from LKB1 knockout mice and silencing

LKB1 in ovarian cells, we show that metformin was neither able to induce AMPK activation in the absence of LKB1 nor induce cell cycle arrest or inhibit proliferation, indicating LKB1's important role in its anti-proliferative action. It is interesting to note that metformin hindered the proliferation of both AMPK null mefs and AMPK silenced ovarian cancer cells. These results seem to indicate an important role of LKB1 in metformin-mediated AMPK activation and its anti-proliferative effect in ovarian cancer cell lines. Conversely, metformin can still be effective in the absence of AMPK. Under this scenario, LKB1 probably plays a greater role than AMPK in metformin-mediated growth arrest. LKB1 not only regulates the activity of AMPK but 13 other AMPK-related kinases that also play a role in regulation of energy stress and cell polarity [36, 37]. We speculate that one or more of these downstream kinases may play a role in inducing cell cycle arrest in the absence of AMPK. Also, LKB1 by itself can cause G1 cell arrest [38, 39]. LKB1 mutant cells also have been shown to have increased expression of Cyclin D1 [39] and LKB1 has a direct role in transcriptional up-regulation of p21 *via* p53 in a promoter specific



**Fig. 8** Metformin can inhibit growth independent of AMPK. (A) Immunoblot analysis revealed the down-regulation of AMPK $\alpha_1$  in untransfected (C), siRNA (Si) and non-target siRNA control (NT) transfected A2780 cells at 48 hrs. Similar sets were treated with metformin (10 mM) for 12 hrs and processed for immunoblot for p-ACC and  $\beta$  actin (lower panel). (B) A2780 untransfected, siRNA and non-target siRNA transfected cells were treated with indicated concentrations of metformin for 48 hrs and assessed for cell number.  $**P < 0.01$ ,  $*P < 0.05$  compared to siRNA (Si) and non-target siRNA control (NT) transfected cells. (C) AMPK null (AMPK $^{-/-}$ ) and wild-type (AMPK $^{+/+}$ ) mouse embryo fibroblast (mefs) were treated with metformin (5–10 mM) and analysed by immunoblot for pAMPK and pACC, as well as for status of proteins of mTOR pathway. (D) AMPK $^{-/-}$  and wild-type mefs were treated with metformin (5–10 mM) for 72 hrs. MTT assay was performed to estimate cell viability. The data represent three separate experiments.  $**P < 0.01$ ,  $*P < 0.05$  compared to untreated AMPK null and wild-type mefs. (E, F) AMPK $^{-/-}$  and wild-type (AMPK $^{+/+}$ ) mefs were transfected with CyclinD1 and p21 reporter-luciferase constructs (0.2  $\mu$ g/24 well). The next day, the cells were treated with 10 mM metformin and 24 hrs later, luciferase activity was determined using dual luciferase assay system.  $***P < 0.001$ ,  $**P < 0.01$  AMPK $\alpha_{1/2}^{-/-}$  mefs compared to AMPK $\alpha^{+/+}$  mefs (black bars).  $\ddagger P < 0.001$ ,  $\dagger P < 0.01$  between metformin treated AMPK $\alpha^{-/-}$  mefs and AMPK $\alpha_{1/2}^{+/+}$  mefs.

manner [40, 41]. This suggests that LKB1, on activation, can cause cell cycle arrest independent of its activity on AMPK. Conversely, it may be that metformin as a drug has some direct effect in modulating cell cycle. In support of this, Isakovic *et al.*, showed metformin treatment caused G0/G1 cell cycle arrest resulting in apoptosis *via* mitochondrial depolarization and oxidative stress in glioma C6 cells while having no effect on primary astrocytes [5].

The mechanism by which metformin activates AMPK is not well understood. It is reported that metformin inhibits complex I of respiratory chain leading to an increase in the AMP levels which in turn leads to LKB1-AMPK activation [42]. Because most of the

mechanistic studies regarding metformin action are derived from studies in other tissues such as muscle, liver or fatty tissue, its effect on epithelial cells is not well known. The exact mechanism by which metformin confers its myriad effects needs to be studied in greater detail to get an insight into the role of AMPK-dependent and AMPK-independent pathways in cancer biology. More importantly, additional preclinical *in vivo* studies are needed to determine the effect of this drug in a therapeutic setting, other than diabetes.

Collectively our data strongly support the use of metformin as a therapeutic agent in ovarian cancer, alone or in combination with other standard chemotherapeutic drugs. Because the persistent growth of cancer cells is supported by their high metabolic

activity which is a result of higher activities of protein synthesis and lipogenic pathways [43], intervention by metformin in these basic cell survival pathways will certainly be detrimental to cancer cells. However, whether it is the modulation of cell cycle proteins or the inhibition of protein and lipid synthesis that is responsible for growth arrest or a combination of both requires additional investigation. Additionally, the success of metformin usage in treating patients with Polycystic ovarian syndrome, which can predispose women to ovarian and endometrial cancer, also highlights the beneficial effect metformin might have in other ovarian related disease. Recent reports by Cazzaniga *et al.*, that indicate a plan to initiate pre-surgical randomized clinical trial to evaluate the activity of metformin on tumour cell proliferation in breast cancer patients [44], opens the door for using metformin in other cancer types including ovarian cancer.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1 (A)** IOSE-523 cells were treated with indicated concentrations of metformin. Cell survival was assessed by MTT on day 3. NS: non-significant compared to untreated. **(B)** HeLa cells were treated with indicated concentrations of metformin. Cell survival was assessed by MTT on day 3. Data are representations of two individual experiments in triplicates. NS: non-significant compared to untreated.

**Fig. S2** Densitometric representation of immunoblot analysis of ovarian cancer cells treated with metformin showing reduced cyclin D1 and up-regulated p21 levels depicted in Fig. 3.

**Fig. S3** Expression of various AMPK subunits and their isoforms in ovarian cancer cell lines. Immunoblot analysis of various subunits of AMPK and their isoforms in several ovarian cancer cell lines including VOSE, CaOV3, OV167, OVCAR3, OVCAR5, OVCAR7, OVCAR10, SKOV3, OVCAR8, A2780, CP70 and C200 using specific antibodies. AMPK  $\alpha_2$  levels could be detected only when membrane was exposed more than 2 hrs, whereas other subunits were detected at exposure time of less than 2 min.  $\beta$ -actin was used to show equal protein loading. The data are representations of two separate experiments.

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