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Metformin inhibits growth of eutopic stromal cells from adenomyotic endometrium via AMPK activation and subsequent inhibition of AKT phosphorylation: a possible role in the treatment of adenomyosis

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Abstract

Adenomyosis is a finding that is associated with dysmenorrhea and heavy menstrual bleeding, associated with PI3K/AKT signaling overactivity. To investigate the effect of metformin on the growth of eutopic endometrial stromal cells (ESCs) from patients with adenomyosis and to explore the involvement of AMP-activated protein kinase (AMPK) and PI3K/AKT pathways. Primary cultures of human ESCs were derived from normal endometrium (normal endometrial stromal cells (N-ESCs)) and adenomyotic eutopic endometrium (adenomyotic endometrial stroma cells (A-ESCs)). Expression of AMPK was determined using immunocytochemistry and western blot analysis. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assays were used to determine the effects of metformin and compound C on ESCs and also to detect growth and proliferation of ESCs. AMPK and PI3K/AKT signaling was determined by western blotting. A-ECSs exhibited greater AMPK expression than N-ESCs. Metformin inhibited proliferation of ESCs in a concentration-dependent manner. The IC_{50} was 2.45 mmol/l for A-ESCs and 7.87 mmol/l for N-ESCs. Metformin increased AMPK activation levels (p-AMPK/AMPK) by 2.0±0.3-fold in A-ESCs, 2.3-fold in A-ESCs from the secretory phase, and 1.6-fold in the proliferation phase. The average reduction ratio of 17β-estradiol on A-ESCs was 2.1±0.8-fold in proliferative phase and 2.5±0.5-fold in secretory phase relative to the equivalent groups not treated with 17β-estradiol. The inhibitory effects of metformin on AKT activation (p-AKT/AKT) were more pronounced in A-ESCs from the secretory phase (3.2-fold inhibition vs control) than in those from the proliferation phase (2.3-fold inhibition vs control). Compound C, a selective AMPK inhibitor, abolished the effects of metformin on cell growth and PI3K/AKT signaling. Metformin inhibits cell growth via AMPK activation and subsequent inhibition of PI3K/AKT signaling in A-ESCs, particularly during the secretory phase, suggesting a greater effect of metformin on A-ESCs from secretory phase.
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Introduction

Women with dysmenorrhea, chronic pelvic pain, and heavy menstrual bleeding present a diagnostic and therapeutic challenge. Adenomyosis is a commonly recognized cause of the above-mentioned symptoms. Adenomyosis is characterized by the presence of ectopic endometrial tissue in the myometrium, with adjacent smooth muscle hyperplasia (Devlieger *et al*. 2003, Harvey & Warwick 2010). Adenomyosis is usually found in multiparous, pre-menopausal women older than 30 years, particularly those with previous cesarean section or other uterine surgery (Takeuchi & Matsuzaki 2011).

Adenomyosis is usually diagnosed by histological evaluation of a hysterectomy specimen showing invasion of endometrial tissue into the myometrium (Brown *et al*. 1991). Ultrasound or magnetic resonance imaging (MRI) may aid in the nonsurgical diagnosis of adenomyosis, with MRI having 78–88% sensitivity and 67–93% specificity (Ascher *et al*. 1994, Reinhold *et al*. 1996, Atri *et al*. 2000, Dueholm *et al*. 2001, Bazot *et al*. 2001).

Adenomyosis is non-neoplastic and hence is not associated with significant mortality. It does, however, cause substantial morbidity and reduced quality of life for the many individuals it affects. Patients typically present with menorrhagia, dysmenorrhea, metrorrhagia, and dyspareunia, which may be a cause of reduced fertility (Devlieger *et al*. 2003, Takeuchi & Matsuzaki 2011). Surgical hysterectomy remains the only definitive treatment (Brown *et al*. 1991, Devlieger *et al*. 2003). Surgery is usually physiologically and psychologically traumatic for patients of reproductive age, highlighting the need to develop novel and effective treatments.

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There is substantial evidence to indicate that adenomyosis and endometriosis are influenced and promoted by the effects of estrogen (Cakmak *et al.* 2009). Indeed, the estrogen agonist tamoxifen has been successfully used to generate mouse models of adenomyosis (Li *et al.* 2011). Leyendecker *et al.* (2009) have proposed a tissue injury and repair theory of adenomyosis, in which microtrauma at the endometrial–myometrial junction causes local hyperestrogenism and enhanced peristaltic activity within the zone of the endomyometrial junction. Estrogen is known to act through the MAPK and PI3K/AKT/mTOR pathways, raising the possibility that targeting the PI3K pathway may provide a novel approach for the treatment of these conditions (Cakmak *et al.* 2009, Leyendecker *et al.* 2009, Makker *et al.* 2012). In support of this possibility, other workers have reported that overactivation of PI3K signaling occurs in eutopic and ectopic endometria (Zhang *et al.* 2010).

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase complex, comprising a catalytic α-subunit and regulatory β- and γ-subunits (Hardie 2011). AMPK signaling regulates energy metabolism and is activated in response to cellular stresses that deplete cellular energy levels and increase the AMP:ATP ratio (Carling 2004). Once activated, AMPK restores cellular energy levels by stimulating catabolic pathways, such as glucose uptake, glycolysis, and fatty acid oxidation, and by halting ATP-consuming processes, such as fatty acid, cholesterol, and protein synthesis (Lim *et al.* 2009). AMPK signaling is also linked to the PI3K pathway (Tao *et al.* 2010), which is an important regulator of cell survival (Chen *et al.* 2010). AMPK signaling has, therefore, been investigated as a potential therapeutic target for the treatment of diseases with aberrantly activated PI3K signaling, such as carcinomas (van Veelen *et al.* 2011, Carling *et al.* 2012, Martelli *et al.* 2012). To date, however, to our knowledge no studies have investigated whether AMPK may play a role in adenomyosis.

Metformin is a biguanide drug that is widely used as a first-line pharmacological treatment in patients with type 2 diabetes (Cantrell *et al.* 2010). Metformin exerts its effects through the activation of AMPK in muscle, adipose tissue, and liver (Towler & Hardie 2007). However, little is known about its effects on endometrial tissues, and whether these effects may include actions via PI3K/AKT signaling that would be beneficial for the treatment of adenomyosis.

In this *in vitro* study, we explored the role of AMPK signaling in the adenomyotic eutopic endometrium and investigated the effects of activation and inhibition of AMPK signaling on the proliferation of eutopic stromal cells. We also assessed the role of PI3K/AKT signaling and investigated the possible value and mechanism of metformin in the treatment of adenomyosis.

Materials and methods

Reagents and antibodies

Collagenase IA, trypsin, metformin, and 17β-estradiol (E_2) were obtained from Sigma Corporation and compound C from Merck Serono. Penicillin and DMEM:nutrient mixture F-12 (DMEM/F12) (1:1) were purchased from HyClone Corporation (Beijing, China). Charcoal-stripped fetal bovine serum (FBS), in which hormones and certain growth factors had been excluded, was obtained from Gibco (Invitrogen). Rabbit primary antibodies against human AMPK, p-AMPK (Thr172), AKT, p-AKT (Ser473), and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA). Mouse antibodies against human vimentin and cytokeratin were obtained from Cell Signaling Technology. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies and diaminobenzidine (DAB) staining kits were purchased from Jingmei Biotech Co. Ltd. (Shanxi, China). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), RIPA buffer, and the Mammalian Cell Extraction Kit were purchased from BioVision (Milpitas, CA, USA). The ECL Plus Western Blotting Detection System was obtained from Amersham Biosciences and the Bradford protein assay kit from Bio-Rad.

Compound C was dissolved in DMSO at a stock concentration of 10 mmol/l and stored at 4 °C. A final compound C concentration of 40 μmol/l was used in cell cultures, and the final concentration of DMSO was $<0.1\%$. The same volume/concentration of DMSO was used in control experiments. Metformin was dissolved in double-distilled water at a stock concentration of 10 mmol/l and stored at 4 °C. E_2 was dissolved in absolute ethyl alcohol at a stock concentration of 10 mmol/l and stored at 4 °C. A final E_2 concentration of 10 nM was used in cell cultures, such that the final concentration of absolute ethyl alcohol was $<0.1\%$. The same concentration/volume of DMSO were used in control experiments.

Subjects and specimens

The experiments were performed using endometrial biopsies of eutopic endometrium taken from 34 patients with adenomyosis, 42.1 ± 2.4 years of age. Fifteen tissue samples were taken during the proliferative phase (42.7 ± 1.9 years) and 19 during the secretory phase (41.3 ± 2.4 years). The diagnosis was confirmed by pathological examination, which excluded the presence of other gynecological diseases. Endometrial biopsies taken from 19 women of reproductive age (mean: 43.7 ± 3.2 years) without endometriosis undergoing bilateral tubal ligation were used as a control group. None of the participants had received hormonal therapy during the 6 months prior to surgery.

The phase of the menstrual cycle (proliferative phase or secretory phase) was determined on the basis of the time of the last menstruation, E_2 and progesterone levels of participants before surgery, findings during surgery, and the histological pattern of the endometrium (Noyes *et al.* 1975).

All participants provided written informed consent prior to biopsy; the use of human tissues was approved by the Institutional Review Board of Shandong Provincial Hospital Affiliated to Shandong University (China).

Purification of endometrial stromal cells

The isolation and culture of endometrial cells were based on methods described in previous studies (Sugawara *et al.* 1997, Yang *et al.* 2007), with slight modifications. Briefly, specimens obtained during surgery were placed immediately in ice-cold sterile PBS and transported to the laboratory. Tissues were washed two times with sterile PBS, then homogenized, and incubated with 0.25% collagenase type IA, in a shaking water bath for 1 h at 37 °C. The collagenase activity was terminated by the addition of three volumes of pre-warmed PBS.

The cell suspension was sequentially filtered through a 154 μm monofilament nylon mesh and then through a 38.5 μm monofilament nylon mesh. The resulting cell suspension was collected and centrifuged at 110 ***g*** for 10 min to obtain stromal cells. The pellet was re-suspended in DMEM/F12 (1:1) medium containing 10% FBS and incubated in cell culture dishes for 2 h at 37 °C in 95% air and 5% CO_2. The medium was then replaced with fresh medium; non-attached cells were discarded and the attached stromal cells were cultured further.

The culture medium was changed every 2–3 days. Normal endometrial stromal cells (N-ESCs) and adenomyotic eutopic ESCs (A-ESCs) were isolated and cultured in DMEM/F12 (1:1) medium, containing 10% charcoal-stripped FBS, penicillin (100 U/ml), and streptomycin (100 U/ml) at 37 °C in a humidified environment with 5% CO_2 in air. The cultured endometrial cells were characterized by immunocytochemical staining for vimentin and cytokeratin (Bruse *et al.* 2005); meanwhile, the purity of ESCs was tested.

Immunocytochemistry

The cultured ESCs on coverslips were washed twice with PBS and fixed in 4% paraformaldehyde (pH 7.0) for more than 24 h. The cells were then washed in PBS, blocked with 10% normal goat serum for 30 min, and incubated overnight with rabbit anti-human-AMPK primary antibody (diluted 1:500 in PBS) and with mouse antibodies against human vimentin and cytokeratin at 4 °C. HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were used as secondary antibodies. HRP activity was detected using DAB tetrahydrochloride. Slides were counterstained with hematoxylin before mounting. Slides incubated with PBS instead of primary antibody were used as negative controls, and immunostaining with rabbit anti-human-β-actin antibody (at the same dilution) was used as a negative control for nuclear staining.

The immunocytochemical score was evaluated on the basis of the percentage of positively stained ESCs (0, no staining; 1, 1–10% positively staining cells; 2, 11–50% positively staining cells; 3, 51–80% positively staining cells; and 4, 81–100% positively staining cells). The immunoreactivity was shown as a staining intensity score. Staining intensity was scored as follows: 0, negative; 1, weak staining; 2, moderate staining; and 3, strong staining.

For each sample, two slides were assessed independently by two independent observers. Evaluation of slides for immunostaining was undertaken in a blinded fashion, without any knowledge of the clinical or pathological data.

Cell proliferation assays

The effect of metformin on cell proliferation was examined by the MTT assay. ESCs were plated in 96-well plates at a concentration of 1×10^3 cells/well. After attachment, cells were treated with different doses of metformin/compound C for 0 min, 15 min, 1 h, and 24 h. MTT assays were performed as described previously (Towler & Hardie 2007). In brief, MTT (5 mg/ml) was added to the 96-well plates at a volume of 10 μl/well, and the plates were incubated for 4 h. The MTT reaction was terminated by removal of the culture medium containing MTT, and 100 μl DMSO per well were added and incubated at RT on a shaker for 10 min to ensure that the crystals had dissolved sufficiently. Absorbance values were measured at 595 nm. Cell proliferation (percentage of control) was calculated as follows: absorbance (experimental group)/absorbance (control group). Cell proliferation inhibition (percentage of control) was calculated as follows: 100%−cell proliferation (percentage of control). Each experiment was performed in duplicate and repeated six times to assess result consistency.

Western blotting

ESCs were washed twice with ice-cold PBS. Cell lysates were prepared by incubation in RIPA buffer (1% NP40, 0.5 sodium deoxycholate, and 0.1% SDS) for 30 min on ice followed by centrifugation at 120 000 r.p.m. (approximately 15 000 ***g***) for 20 min. The protein concentration of the extracts was determined using a Bradford protein assay kit. Equal amounts of protein (30 μg) were separated by electrophoresis on a 10% SDS–PAGE and transferred onto a PVDF membrane (Hybond-P PVDF membrane; Amersham). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 for 1 h and incubated overnight with a 1:1000 diluted primary antibody at 4 °C. The membrane was then washed and incubated for 1 h with a peroxidase-conjugated secondary antibody (diluted 1:10 000).

Antibody binding was detected using an ECL detection system (GE Healthcare, Princeton, NJ, USA). Western blotting densitometric analysis was performed using an AlphaImager 2200 gel documentation system with image analysis software. The band densities for AMPK and p-AMPK as well as Akt and p-Akt were normalized to that of β-actin. Each experiment was repeated in triplicate.

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 Software (SPSS, Inc.). Data were expressed as the means ± S.E.M.S. Comparisons between groups were performed using Student's *t*-test or one-way ANOVA followed by a Tukey's *post-hoc* test (for comparisons between treated and control ESCs and between groups in the cell proliferation experiments). For the analyses of experiments with three or four replicates, we used a non-parametric method. The data were shown as the median ± range. Values of $P<0.05$ were considered statistically significant.

Results

Verification for the purity of ESCs

Immunocytochemical staining for vimentin and cytokeratin was performed to test whether the purified cells were ESCs. As shown in Fig. 1, the purified cells were positive for vimentin and negative for cytokeratin, confirming that these cells were indeed ESCs. The purity of the ESCs was 98% after the first and second passages.

AMPK expression in ESCs

Immunocytochemical and western blotting analyses of AMPK expression were performed to investigate AMPK expression in ESCs, as shown in Fig. 2A. Immunostaining of AMPK was observed in the cytoplasm of both N-ESCs and A-ESCs. A-ESCs exhibited greater AMPK expression than N-ESCs ($P<0.05$; Table 1). Eight samples were employed, ESCs isolated from four samples from control group (mean age: 40.5±2.3 years) and four from adenomyosis group (mean age: 40.8±2.3 years). Figure 2B showed that AMPK expression levels (AMPK/β-actin) in A-ESC and N-ESC groups were 83.4±13.8 and 58.9±9.8% respectively (i.e. 1.4-fold more).

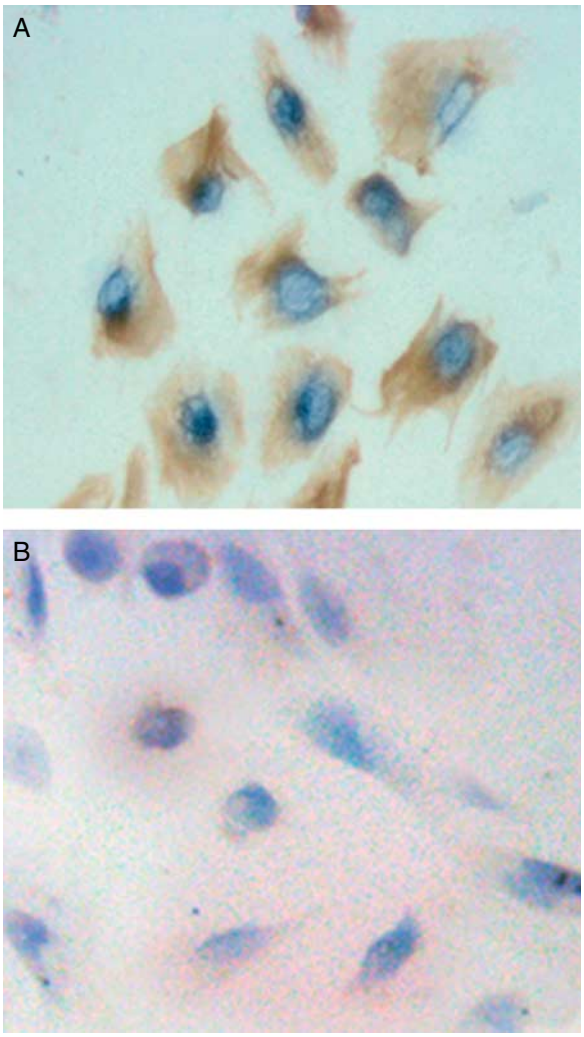

Figure 1 Immunocytochemical staining for vimentin and cytokeratin expression. (A) The cytoplasm of cells was positive for vimentin. The nuclei were stained with hematoxylin. Magnification, ×400. (B) The cytoplasm of cells was immunonegative for cytokeratin. The nuclei were stained with hematoxylin. Magnification, ×400.

Compound C inhibited AMPK activation in ESCs

ESCs isolated from six endometria samples, three from control group (mean age: 42.5±1.8 years) and three from adenomyosis group (mean age: 40.8±2.1 years), were used in these experiments. Compound C, a selective inhibitor of AMPK, was found to significantly inhibit the phosphorylation of AMPK in a time-dependent manner, in both N-ESCs and A-ESCs (Fig. 3). After 1 h of exposure, the average inhibition was 2.4±0.5-fold in A-ESCs and 2.9±0.9-fold in N-ESCs. In both A-ESCs and N-ESCs, there was no difference in the inhibition effect between 1 and 24 h groups ($P>0.5$). On the basis of these findings, ESCs pretreated for 1 h with 40 μM compound C were used in the follow-up experiments.

Metformin inhibits the proliferation of ESCs via AMPK activation

The same batch cells as was used for the experiments for which the results are illustrated in Fig. 3 were used for MTT assay. The effect of metformin on the proliferation of N-ESCs and A-ESCs were shown in Fig. 4A. Exposure to metformin for 24 h resulted in marked, concentration-dependent inhibition of growth of both A-ESCs and N-ESCs. The mean IC_{50} value for A-ESCs was 2.45 and 7.87 mmol/l for N-ESCs (i.e. threefold less).

A-ESCs isolated from four women (mean age: 40.8 ±2.3 years) with metformin were used in the western blot analysis. In the absence of compound C, metformin was associated with a significant 2.0±0.3-fold increase in AMPK activation (p-AMPK/AMPK) compared with controls (Fig. 4B, $^*P<0.001$), whereas in the presence of compound C, this effect was diminished. The relative activated AMPK levels in A-ESCs in control, metformin-, compound C-, and compound C+metformin-treated groups were 43.9±1.6, 83.2±6.1, 23.2±5.2, and 25.1±4.1% respectively (Fig. 4B).

Metformin shows pronounced effects on activation of AMPK signaling in A-ESCs from secretory phase than in cells from proliferative phase

As shown in Fig. 5A, samples of endometrium were taken from six women, 43.2±1.5 years of age, with a diagnosis of adenomyosis, three in secretory phase and three in proliferative phase. Metformin inhibited the growth of A-ESCs from proliferative

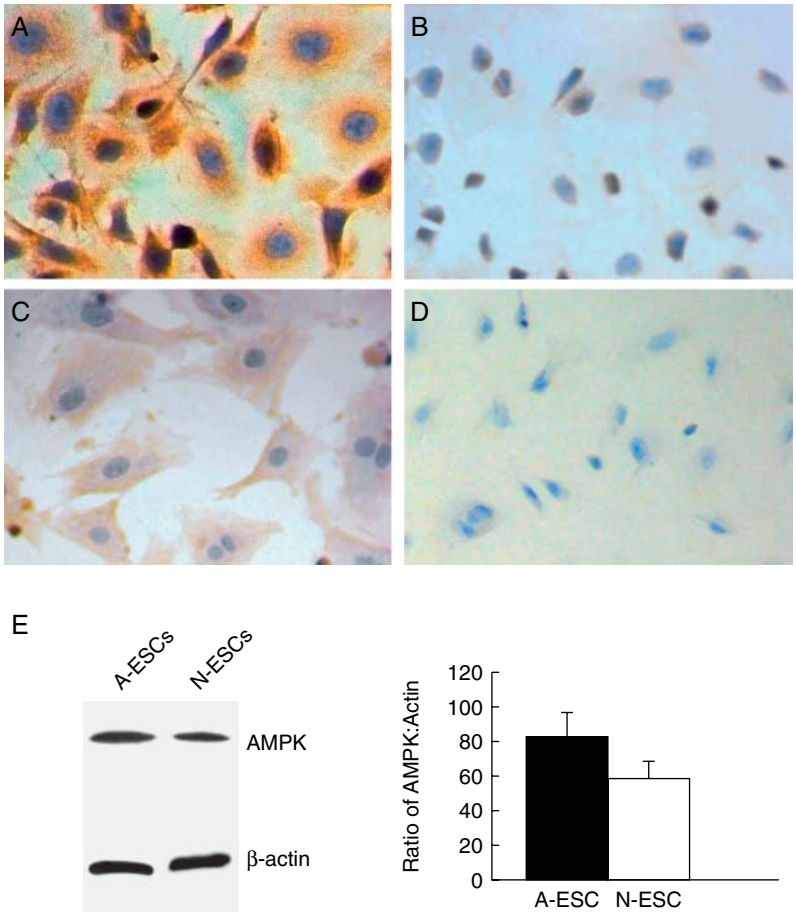

Figure 2 (A) Immunocytochemistry staining for AMPK expression. (a) A-ESCs with AMPK antibody, (b) negative controls for A-ESCs using PBS instead of AMPK as primary antibody, (c) N-ESCs with AMPK antibody, and (d) negative controls for N-ESCs using PBS instead of AMPK as primary antibody. Immunostaining of AMPK was observed in the cytoplasm of both A-ESCs and N-ESCs. The nuclei were stained with hematoxylin. Magnification, ×400. (B) AMPK expression levels (AMPK/β-actin) in A-ESCs ($n=4$) and N-ESCs ($n=4$). Protein expression was normalized to β-actin. AMPK expression levels in A-ESC and N-ESC groups were 83.4±13.8 and 58.9±9.8% respectively (i.e. 1.4-fold more). Data are shown as the median±range.

phase by 47.8±1.2% and cells from secretory phase by 58.0±0.9% relative to controls. Metformin-induced inhibition of cell growth was abolished by pretreatment with compound C (40 μM for 1 h).

E_2 significantly inhibited AMPK activation in both A-ESCs from proliferative phase and cells from secretory phase (Fig. 5B). The same batch cells with E_2 were used in western blot analysis; the result showed that the average reduction ratio was 2.1±0.8-fold in proliferative phase and 2.5±0.5-fold in secretory phase.

In order to test our hypothesis, we employed 18 more samples from patients with adenomyosis (mean age: 42.1±1.8 years), nine in secretory phase and nine in proliferative phase. The effects of metformin on activation of AMPK signaling differed between the A-ESCs from the proliferative phase and from the secretory phase. As shown in Fig. 5C, in the absence of compound C, the effects of metformin on activation of AMPK signaling (p-AMPK/AMPK) were obvious (Fig. 5C, $*P<0.001$). The relative activated AMPK levels in cells during the secretory phase and proliferative phase were 86.8±4.8 (2.3-fold increase vs control) and 60.2±2.5 (1.6-fold increase vs control) respectively. Pretreatment with compound C (40 μM for 1 h) diminished this effect.

AMPK activation inhibits the PI3K/AKT pathway in A-ESCs

The data presented earlier suggested that metformin inhibited proliferation of A-ESCs through effects on the AMPK pathway. As previous reports revealed, AMPK is upstream of the PI3K/AKT pathway (Hanna *et al.* 2012). The researchers wanted to know whether AMPK is upstream of the PI3K/AKT pathway in A-ESCs. We used the same-generation of cells that were used for the experiments that yielded the results illustrated in Fig. 5C from 16 women (eight in secretory phase and eight in proliferative phase) in western blot analysis. We investigated the effects of metformin (with or without pretreatment compound C 40 μM for 1 h) on AKT phosphorylation. As illustrated in Fig. 6A and B, the inhibitory effects of metformin on AKT activation

Table 1 Histological score (HSCORE) values of total AMPK expression in A-ESCs and N-ESCs.

	No. of slides	**HSCORE** (mean±S.D.)
A-ESCs	30	3.4±0.1
N-ESCs	17	2.2±0.2

$P<0.05$, A-ESCs vs N-ESCs.

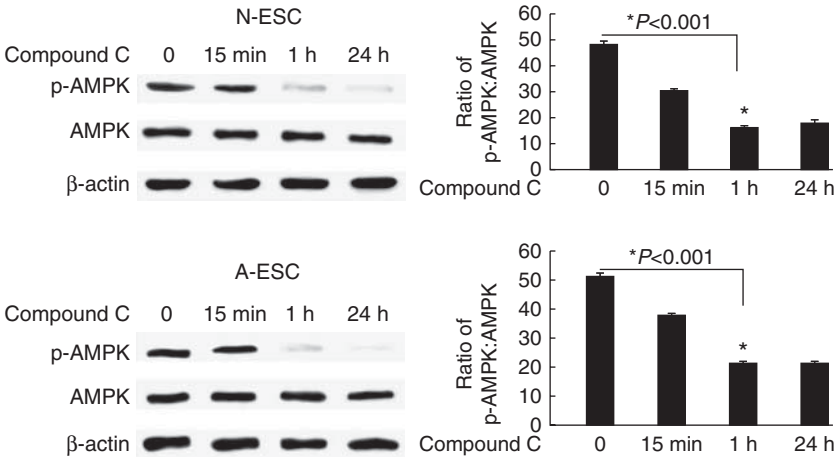

Figure 3 Compound C inhibited AMPK activation in ESCs. Compound C was found to significantly inhibit the phosphorylation of AMPK in a time-dependent manner, in both N-ESCs ($n=3$) and A-ESCs ($n=3$). In the A-ESC group, the ratio of p-AMPK:AMPK was 21.15±1.2% at 1 h and 21.53±0.1% at 24 h. In the N-ESC group, the ratio was 16.06±0.7 and 18.06±1.4% respectively. After 1 h of exposure, the average inhibition was 2.4±0.5-fold in A-ESCs and 2.9±0.9-fold in N-ESCs. In both A-ESCs and N-ESCs, there was no difference regarding the inhibitory effect between 1 and 24 h groups ($P>0.5$). The concentration of CC is 40 μmol/l, *$P<0.001$ vs control group. Data are shown as the median±range.

(p-AKT/AKT) were more pronounced in A-ESCs from the secretory phase (3.2-fold inhibition vs control) than in those from the proliferation phase (2.3-fold inhibition vs control). The inhibitory effect of AKT phosphorylation was abolished by compound C pretreatment (Fig. 6A and B, $P<0.001$). The results showed that PI3K/AKT pathway can be inhibited by upregulating AMPK phosphorylation with metformin.

Discussion

Adenomyosis has been described as an 'elusive disease' or 'enigma' (Thomas & Clark 1989), and there is a genuine need for new chemotherapeutic strategies to treat it. Adenomyosis results from the growth of ectopic endometrium, a process that requires coordination of cellular energy status (Osteen *et al.* 2002). In a previous study, researchers showed that activation of the energy-sensing AMPK pathway can inhibit cell proliferation in lung cancer cells (Su *et al.* 2010). In this study, AMPK expression level is higher in A-ECSs than in N-ESCs. On the basis of these discoveries, we considered it worthwhile to investigate AMPK as a possible target for therapeutic intervention in adenomyosis.

AMPK expression has been previously studied in other tissues, such as hippocampal neurons and fibroblasts (Amato *et al.* 2011), but, to our knowledge, no report about AMPK expression in ESCs has been published. In this study, we used immunostaining and western blot analysis to evaluate the expression levels of AMPK in N-ESCs and A-ESCs. Our results indicated that AMPK expression was higher in the cytoplasm of A-ESCs than in that of N-ESCs. This indicates that if we find drugs with an effect on the AMPK signaling pathway this might lead to breakthroughs in the treatment of adenomyosis.

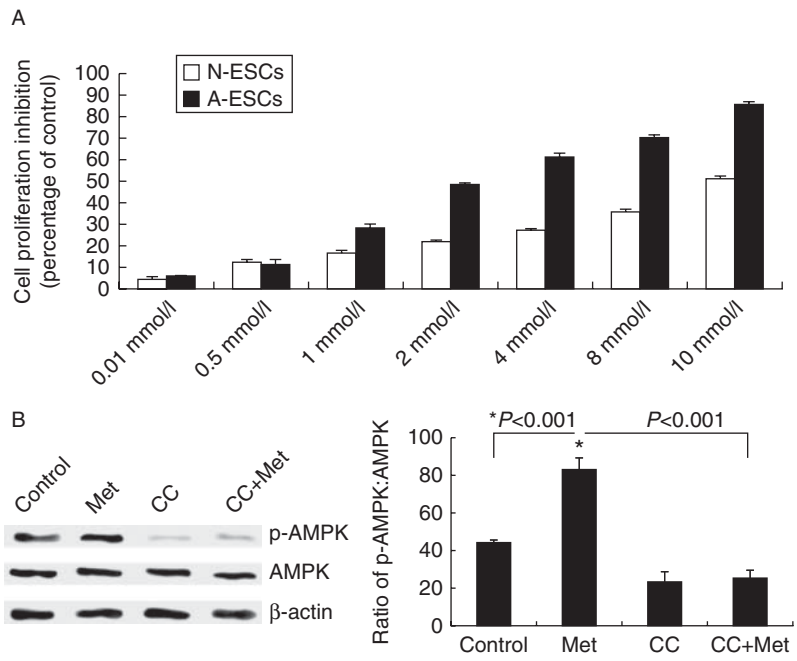

Figure 4 (A) Met inhibited proliferation of ESCs. In both N-ESCs ($n=3$) and A-ESCs ($n=3$), and Met (0.01–10 mmol/l) inhibited cell proliferation in a concentration-dependent manner. Met was found to be more effective in A-ESCs (IC_{50} 2.45 mmol/l) than in N-ESCs (IC_{50} 7.87 mmol/l) (i.e. threefold less). (B) Met increased AMPK activation in A-ESCs. A-ESCs were incubated for 1 h with or without 40 μmol/l of CC prior to stimulation with 2 mmol/l Met for 24 h. Protein expression was normalized to β-actin. In the absence of CC, Met was associated with a significant 2.0±0.3-fold increase in AMPK activation (p-AMPK/AMPK) compared with controls (*$P<0.001$), whereas in the presence of CC, this effect was diminished. The relative activated AMPK levels in A-ESCs in control, Met-, CC-, and CC+Met-treated groups were 43.9±1.6%, 83.2±6.1%, 23.2±5.2%, and 25.1±4.1% respectively, $n=4$. Met, metformin; CC, compound C. Data are shown as the median±range.

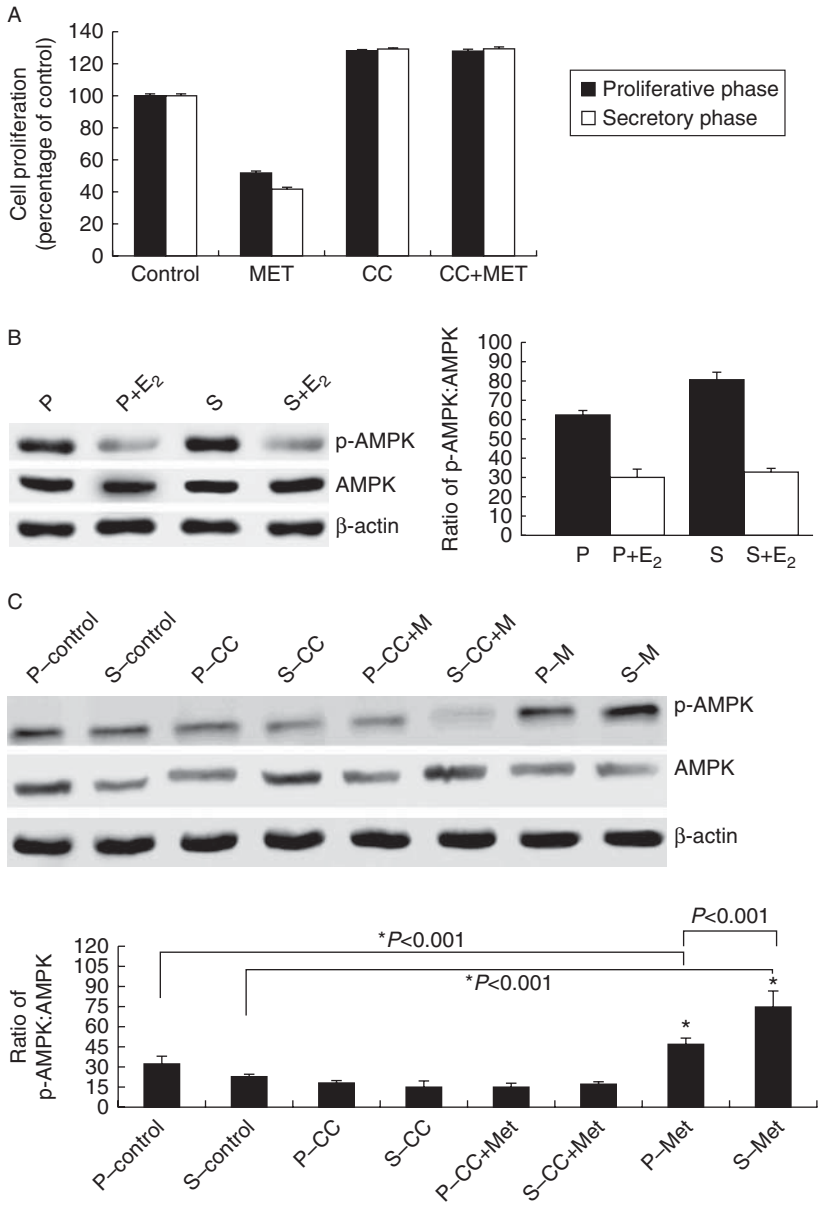

Figure 5 (A) Met inhibition of the growth of A-ESCs alters with the menstrual cycle. A-ESCs were incubated for 1 h with or without 40 μmol/l of CC prior to stimulation with 2 mmol/l Met for 24 h. A-ESC growth was assessed by MTT assay. Met inhibited growth of A-ESCs from the proliferative phase ($n=3$) by 52.2±1.2% and of cells from the secretory phase ($n=3$) by 42.0±0.9% relative to controls. (B) E_2 significantly inhibited AMPK activation. A-ESCs from the proliferative phase ($n=3$) and from the secretory phase ($n=3$) were incubated with or without 10 nM E_2 for 24 h. Protein expression was normalized to β-actin. The average reduction ratio was 2.1±0.8-fold in the proliferative phase and 2.5±0.5-fold in the secretory phase. (C) The effect of Met on activation of AMPK signaling was more pronounced in A-ESCs from the secretory phase. A-ESCs were incubated for 1 h with or without 40 μM CC prior to stimulation with 2 mmol/l Met for 24 h. Protein expression was normalized to β-actin. S, secretory phase ($n=9$); P, proliferative phase ($n=9$). *$P<0.001$ vs control group; $P<0.001$ secretory phase+Met vs proliferative phase+Met. Met, metformin; CC, compound C. Data are expressed as mean±S.E.M.

Metformin, a biguanide drug widely used for the treatment of type 2 diabetes (Radziuk *et al.* 2003), is considered as an insulin sensitizer (Polyzos *et al.* 2010). Metformin also has beneficial effects on endometrial abnormalities, such as hyperplasia and cancerous lesions (Palomba *et al.* 2009). Expression of the insulin receptor gene is regulated during the menstrual cycle, enabling insulin to inhibit stromal cell decidualization (Lockwood *et al.* 2007). Recent evidence suggested that metformin inhibits hepatic gluconeogenesis by inhibiting mitochondria complex I activity (Owen *et al.* 2000) and activating its downstream target, AMPK, which can regulate multiple signaling pathways controlling cell proliferation.

We found that metformin was associated with a significant increase in AMPK activation. The effects of metformin on activation of AMPK signaling and inhibition of A-ESC proliferation were more pronounced during the secretory phase, suggesting that application of metformin to patients with adenomyosis during secretory phase would be more effective.

Metformin has been the agent most frequently studied in patients with polycystic ovarian syndrome (Cheang & Nestler 2004). It has been suggested that metformin may reduce the risk of first-trimester miscarriage (Glueck *et al.* 2001). Meta-analysis supports the hypothesis that metformin is beneficial in improving clinical pregnancy and ovulation rates (Tang *et al.* 2010). In our study, the inhibitory effect by metformin on endometrial cells was dose dependent. The concentration of metformin required for inhibition of cell proliferation (2 mM) was much higher than the doses used clinically

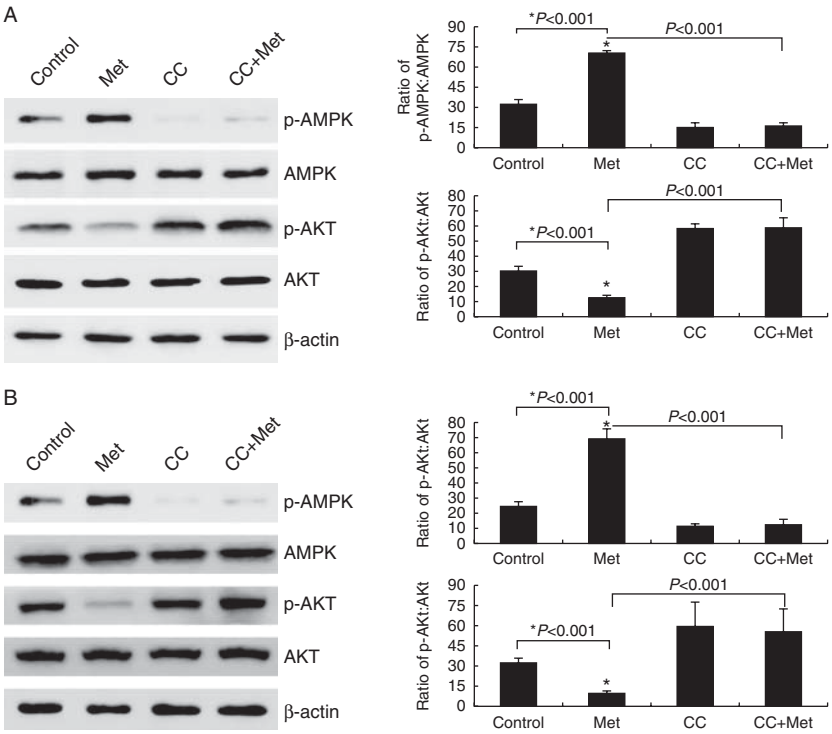

Figure 6 AMPK activation inhibited AKT signaling in A-ESCs. (A) A-ESCs from proliferative phase, $*P<0.001$ vs control group ($n=8$). (B) A-ESCs from secretory phase, $*P<0.001$ vs control group ($n=8$). A-ESCs were incubated for 1 h with or without 40 μmol/l CC prior to stimulation with 2 mmol/l Met for 24 h. Protein expression was normalized to β-actin. The inhibitory effects of Met on p-AKT/AKT were 3.2-fold inhibition in the secretory phase vs control and 2.3-fold inhibition in the proliferative phase. The inhibitory effect of AKT phosphorylation was abolished by CC pretreatment ($P<0.001$). Met, metformin; CC, compound C. Data are expressed as the mean±S.E.M.

(i.e. 0.465–2.5 mg/l; Al-Jebawi *et al.* 1998). The very large doses of metformin used in our *in vitro* cultures cannot be replicated clinically. Further research is needed using our data to search for other compounds that increase AMPK activation more effectively for treatment of adenomyosis.

Interestingly, adenomyosis has been reported to be associated with enhanced expression of nerve growth factor β (Li *et al.* 2011), tyrosine kinase receptor B (Huang *et al.* 2011), and PAK1 (Kim *et al.* 2010), all of which are thought to signal through PI3K. PI3K/AKT signaling plays an important role in cell survival. It is aberrantly activated in adenomyosis (Zhang *et al.* 2010). Previous studies showed that AMPK acts upstream of the PI3K/AKT pathway in hippocampal neurons and fibroblasts (Amato *et al.* 2011). Here, we found that metformin can increase AMPK phosphorylation and decrease AKT phosphorylation in A-ESCs. An AMPK inhibitor can inhibit AMPK phosphorylation and increase AKT phosphorylation in A-ESCs. These findings indicate a functional interaction between the AMPK and PI3K/AKT pathways in A-ESCs, with AMPK activation leading to inhibition of AKT signaling. AKT phosphorylation was negatively correlated with AMPK activation in A-ESCs. So in A-ESCs, AMPK also acts upstream of the PI3K/AKT pathway. Also, metformin acts as a novel AKT inhibitor and can be used to inhibit the proliferation of human A-ESCs.

Consistent with our findings, metformin has been reported to be as effective as letrozole (an aromatase inhibitor that reduces estrogen levels) in inducing regression of endometriotic tissue in a rat model of endometriosis (Oner *et al.* 2010). It has also been shown to repress tumor growth in xenograft tumor models of breast, prostate, and colon cancers (Belda-Iniesta *et al.* 2011). Recent epidemiological evidence suggests that metformin can lower the risk of cancer and reduce cancer incidence and cancer-related deaths among diabetic patients (Decensi *et al.* 2010). Furthermore, a retrospective cohort study of patients with early-stage endometrial cancer showed that patients receiving metformin and adjuvant chemotherapy had a higher response rate than those receiving adjuvant chemotherapy alone (Jalving *et al.* 2010). These findings have prompted further research into the potential role of metformin in cancer treatment and prevention (Ben Sahra *et al.* 2010).

In conclusion, our study showed that metformin can inhibit the proliferation of A-ESCs through activation of AMPK and inhibition of the PI3K/AKT signaling pathway. This study indicates that metformin may be an option for treatment of adenomyosis and needs further investigation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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