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Effects of Metformin on Cellular Proliferation and Steroid Hormone Receptors in Patient-Derived, Low-Grade Endometrial Cancer Cell Lines

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Abstract

Endometrial cancer (EC) is the most common gynecologic malignancy and is the result of disruption of the balance between estrogen-stimulated growth and progesterone-induced growth modulation. Metformin has been shown to inhibit EC proliferation; however, its role in early-stage EC and its effects on steroid hormone receptors have not been adequately explored. Our aim was to examine the effects of metformin on cellular proliferation in patient-derived, low-grade EC cell lines and to determine whether it directly modulates steroid hormone receptor expression. Two novel EC cell lines were produced (EM2 and 3) from endometrial tumor tissue obtained from women undergoing surgery. Cellular proliferation was determined by the 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay, and in both cell lines, metformin decreased cell proliferation in a dose-dependent (10-200 μmol/L) manner and induced apoptosis as measured by cleaved PARP. Furthermore, metformin abrogated the effects of E2 on cell proliferation. Using quantitative real-time polymerase chain reaction and Western immunoblotting, metformin significantly decreased estrogen receptor (ER) α messenger RNA abundance but did not consistently affect the expression of progesterone receptor. Estrogen receptor α protein levels significantly decreased across all metformin doses tested, which resulted in a significant decrease in the expression of the ER targets genes Keratin-19 and Wnt-1 inducible signaling pathway 2. In addition, metformin increased phosphorylation of AMPK in a dose-dependent manner (10-200 μmol/L) indicating an effect on mammalian target of rapamycin (mTOR) signaling. Our data suggest that metformin therapy represents a potential fertility-sparing option for women with early-stage EC, given its capacity to inhibit EC cell proliferation, ERα expression, and the mTOR cell proliferation pathway.

Keywords

metformin, endometrial cancer, steroid hormones

Introduction

Endometrial cancer (EC) is the most common gynecologic malignancy, and approximately 55 000 new cases are diagnosed every year in the United States.[1,2] The majority of women diagnosed with EC are postmenopausal, with approximately 5% of the patients being diagnosed <40 years old. However, the number of reproductive-age women with the diagnosis of EC is likely to increase, as women continue to delay child bearing. Another risk factor, obesity, is also increasing in the United States. Other risk factors include genetic predisposition, polycystic ovarian syndrome, and anovulatory cycles. These conditions cause a hyperestrogenic state with absence of progesterone, which likely predisposes a woman to the development of estrogen-dependent endometrioid carcinoma.[3] There are 2 histology categories for endometrial carcinoma: estrogen-dependent endometrioid carcinoma (type I), which tends to be low grade at an early stage, and estrogen-independent nonendometrioid carcinoma (type II), which often is advanced stage and is serous, clear cell, or high-grade cancer.[4] Typically, the recommended treatment for stage I EC remains total hysterectomy and bilateral salpingo-oophorectomy with or without

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lymphadenectomy.[4,5] However, in women who desire fertility, alternative treatments must be considered.

The endometrium is a cyclic component of the uterus. Its growth and function are controlled by a balance between estrogen-stimulated stromal and epithelial cell proliferation and inhibition of this process by progesterone. A potential for hyperplasia and carcinoma development occurs when this balance is disrupted. Endometrial carcinoma is known to be related to overexposure to estrogen without progesterone-induced maturation. Progestin therapy, typically with medroxyprogesterone acetate (MPA), has been used to reverse endometrial hyperplasia and used as a nonsurgical treatment for low-grade EC.[6-8] However, response to this therapy is not complete in all patients and may not be definitive, as some patients experience recurrence.[9,10] The variable and incomplete response may be due to downregulation of progesterone receptor (PR) expression in endometrial cells in response to progestin therapy.[11,12] This paradigm has led to further investigation into hormonal regimens that target the estrogen receptor (ER) and PR, with the aim of preventing the decrease in PR expression and thus maintaining responsiveness to progestin therapy to reduce estrogenic effects on endometrial cell proliferation.

Estrogen and progesterone are steroid hormones and exert their effects through intranuclear receptors (ERα, PR-A, and PR-B). Progesterone receptor is encoded by a single gene, but it has 2 major isoforms: PR-A and PR-B that are identical except that the PR-B protein contains an additional 164 amino acids at the amino terminus, with this structural difference resulting in different functional characteristics for each isoform.[13] Progesterone receptor-A induces cell senescence and opposes the actions of estrogen by inhibiting the function of ERα, whereas PR-B is a more potent progesterone-induced transcriptional activator and induces the secretory phenotype in the endometrium.[14-17]

Expression of PR isoform is important in the maintenance of endometrial homeostasis, and its disruption may contribute to a malignant phenotype.[18] Expression of PR has been shown to decrease during EC progression, and PR status correlates with grade, histology, adnexal spread, age, and recurrence rate in stage I EC. As expected, when comparing PR-rich to PR-poor tumors, the response rate to progesterone is less with PR-poor tumors.[7] This further highlights the need to identify new approaches to maintain PR expression and sensitize tumors to PR-based therapy.

Metformin is a biguanide that induces muscular uptake of glucose from the blood and is first-line therapy for type 2 diabetes mellitus (DM).[19] Large population-based cohort and case–control studies have shown that patients with type II DM who are taking metformin have reduced carcinogenic events and a reduced cancer mortality rate.[20,21] These studies led to extensive preclinical models in breast, lung, liver, kidney, lymphoma, prostate, ovarian, and ECs that demonstrate metformin modulates multiple molecular pathways directly and indirectly that result in a decrease in growth and proliferation of tumor cells.[22] Given that hyperinsulinemia is also a risk factor for EC, metformin may have a particularly important role in preventing and treating EC.[23] This was further confirmed by a recent meta-analysis that showed metformin use was associated with a decreased risk of EC as well as a favorable survival outcome in patients with EC taking metformin.[24]

Recently, Cantrell et al studied the effects of metformin in ECC-1 and Ishikawa EC cell lines and found that metformin inhibits proliferation of EC cells through inhibition of the phosphatidylinositol 3-kinase-AKT/mammalian target of rapamycin (mTOR) pathway by inducing phosphorylation of AMPK in a concentration-dependent manner.[25] Similarly, Xie et al found that metformin promotes PR expression and enhances the inhibitory effect of MPA on proliferation in both Ishikawa and HEC-1B cell lines.[26] The concern with these studies is the use of commercial cell lines, which are often overpassaged and may no longer have key gene functions and morphology consistent with the original low-grade EC cells, and both studies fail to determine the effect of metformin on ERα expression.[27] The Ishikawa cell lines have been shown to have genotype changes over time due to multiple cell passage level, which may result in contamination or genetic drift.[28] Therefore, the aim of our study is to establish cell lines from patients with the diagnosis of stage Ia1 EC and assess the effects of metformin on PR-A, PR-B, and ERα expression and cellular proliferation. Our hypothesis is that metformin will decrease cellular proliferation by decreasing ERα and increasing PR expression.

Materials and Methods

Generation of Patient-Derived Cell Lines

Women with a preoperative diagnosis of low-grade EC were consented to participate in the study. This study was approved by the institutional review board (#2015-0133), and the tumor collection protocol was followed. All 3 women enrolled underwent a total hysterectomy, and after the hysterectomy was performed, the uterus was bivalved and a section of the endometrial tumor was obtained intraoperatively. The final pathology from the patient was reviewed and confirmed to be stage IA grade 1 endometrial carcinoma. The tumor specimens were immediately processed for cell dispersion and primary culture.

Tumor tissue was placed in Dulbecco's modified Eagle's medium (DMEM) and dissected into 2- to 4-mm pieces, and cells were dispersed using an ultrasonic dissociator (MACS Dissociator, Miltenyi Biotec, Germany). Cells were then passed through a 100-μmol/L and a 40-μmol/L strainer. Between each strainer, the cells were pelleted and resuspended in 5 mL phosphate-buffered saline. After the final centrifuge, the pellet was suspended in DMEM containing 10% (v/v) fetal bovine serum (FBS) and 1% penicillin, streptomycin, and actinomycin.

The cell line was grown, and the cells were split at 80% to 90% confluence. To ensure accurate development of an EC cell line, cellular markers were used and multiple passages (>10) were performed. Two of the 3 cell lines (EM2 and EM3)

survived multiple passages and expressed markers consistent with epithelial cancer cells.

The cell lines were maintained in DMEM-1× containing 10% FBS and 1% penicillin and streptomycin in an incubator with 5% CO_2 at 37°C. The cells were routinely passaged every 3 to 5 days.

Cell Proliferation Assays

The 2 EC cell lines were plated and grown in a 12-well plate at a concentration of 5×10^5 cells/well for 24 hours. The cells were then treated with varying doses and combinations of metformin hydrochloride, MPA, progesterone (P4), and estradiol (E2; all from Sigma Aldrich, St Louis, Missouri) for 24 and 72 hours. 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium (MTT) of 100 μL was added to each well and was then incubated at 37°C for 2 hours. The MTT reaction was terminated, and the formazan crystals were dissolved with the additional of 300 μL N-propranolol. The plates were mixed to ensure complete solubilization, and the absorbance was read at 600 nmol/L. Given the yellow tetrazolium MTT is reduced by metabolically active cells, the treatment groups with more active cellular proliferation resulted in increased intracellular purple formazan and a resulting higher absorbance rate. The effect of metformin, MPA, P4, and estradiol was calculated as percentage of cell viability compared to a nontreated group of cells grown in the same 12-well plate. Every experiment was performed in triplicate and repeated 3 times.

Polymerase Chain Reaction

The EC cells were plated at 5×10^5 cells/well in 6-well plates and treated for varying time lengths (30 minutes, 2 hours, 4 hours, 12 hours, 24 hours, 48 hours, and 72 hours) with different doses (10, 100, and 200 μmol/L) of metformin. The cells were then harvested as a cell pellet. The NucleoSpin RNA protocol was followed (Macherey-Nagel, Bethlehem, Pennsylvania). RA1 buffer and β-mercaptoethanol were added to the cell pellet and vortexed vigorously. The viscosity was reduced, and the lysate was then cleared by filtration through a NucleoSpin Filter (MACHEREY-NAGEL GmbH & Co. KG, reference #740955). Ethanol (70%) was added to the homogenized lysate and was then loaded to a NucleoSpin RNA Column (MACHEREY-NAGEL GmbH & Co. KG, reference #740955). The column was then treated with a membrane desalting buffer to make the rDNAse digest more effective. rDNase and reaction buffer for rDNase were placed directly on the column silica membrane and incubated for 45 minutes. The membrane was then washed 3 times, and finally, the RNA was eluted in RNAase-free water. The RNA was then reverse transcribed with a super reagent consisting of primers, RT buffer, deoxynucleotide triphosphates (dNTPs), Dithiothreitol (DTT), and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, California). Real-time polymerase chain reaction (PCR) was then performed in the presence of SYBR green (Applied Biosystems, Foster City, California) in an ABI PRISM 7500 sequence detector (Applied Biosystems). The cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and then 60°C for 1 minute. The cycle at which the fluorescence reached a present threshold (cycle threshold [C_T]) was determined. Abundance of the gene of interest messenger RNA (mRNA) relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was calculated using the ΔC_T method [relative mRNA abundance $= 2^{-(\text{CT gene of interest} - \text{CT GAPDH})}$]. Given there is no specific primer for PRA and PR total includes both PRA and PRB, PRA was calculated as a subtraction of C_T PRB from C_T PR total. The primers purchased from Invitrogen had the following sequences: PRB primer with the sequence AAGGGGAGTCCAGTCGTCATG, the PR total primer sequence was CATTGGGCGTTCCAAATGA, and the ER sequence was TGAAAGGTGGGATACGAAAAGAC. All of the PR levels obtained were normalized to the C_T of GAPDH. The relative levels of mRNA were then compared. All experiments were performed in triplicate, and the real-time PCR was repeated 3 times.

Western Immunoblotting

Initially, the EC cell lines were evaluated via Western blot analysis to ensure they were consistent with an epithelial cancer cell. The following antibodies were evaluated and detected: vimentin (cat #5741 S; Cell signaling, Danvers, MA), Pax-8 (cat #10336-1-AP; Proteintech group, Rosemont IL), cytokeratin-8 (cat #ab53280; Abcam, San Francisco, CA), PR (H190, cat #sc-7208; Santa Cruz Biotechnology, Dallas, TX), and ER (cat #sc-8002; Santa Cruz Biotechnology, Dallas, TX).

Both EC cell lines were then plated at 5×10^5 cells/well in 6-well plates and treated with different doses of metformin (10, 100, and 200 μmol/L). The cells were harvested as a cell pellet, and cell lysates were prepared in radioimmunoprecipitation buffer. Protein quantification was performed for each sample, and an equal amount of protein was loaded into the gel electrophoresis apparatus and run at 145 V for 1 hour. The gel was then transferred onto a nitrocellulose membrane using the iBlot Transfer system (ThermoFisher Scientific, Waltham, MA). The membrane was blocked with 5% milk for 1 hour at room temperature. The primary antibody was added at the appropriate dilution and amount and was incubated overnight at 4°C. The following day the membrane was washed, and the secondary antibody was added at the correct dilution and rocked on a shaker for 1 hour at room temperature. Using either Lumilight or Lumilight+ (Roche Kit) depending on the antibody, the membrane was then developed using an enhanced chemiluminescence detection system. After developing, the membrane was washed and reprobed for GAPDH (Santa Cruz cat #sc47724, 1:1000) to ensure equal lane loading. The protein bands were then quantified by densitometry with the Image J program (JAVA, NIH, Bethesda, MD). To investigate the role of metformin on steroid hormone receptors, ER and PR antibodies (1:250) were used. Similarly, to assess the effect of metformin on the mTOR pathway and apoptosis, *P*-AMPK (cat #2535 S, 1:250; Cell signaling, Danvers, MA), AMPK (cat #2603 S, 1:500; Cell

Table 1. Patient Characteristics for Generation of the Cell Lines.

	Patient 1	Patient 2	Patient 3
Age, years	74	69	65
BMI, kg/m^2	31.5	43.4	28.8
Pathology	Endometrial carcinoma; Endometrioid type grade 1, stage IA	Endometrial carcinoma; Endometrioid type grade 1, stage IA	Endometrial carcinoma; Endometrioid type grade 1, stage IA
Histology (200×)			

Abbreviation: BMI, body mass index.

signaling, Danvers, MA), p-S6 (cat #4858 S, 1:500; Cell signaling, Danvers, MA), S6 (cat #2217 S, 1:1000; Cell signaling, Danvers, MA), 4EBP1 (cat #9644 S, 1:1000; Cell signaling), poly (ADP-ribose) polymerase (PARP) (cat #9532 S, 1:500; Cell signaling, Danvers, MA), and cleaved PARP (cat #5625 S, 1:500; Cell signaling, Danvers, MA) antibodies were used. All experiments were performed in triplicate.

Statistical Analysis

All data are presented as mean $\pm$ standard error. The Shapiro-Wilk test was used as a normality test with a cutoff of 0.05. A 2-tailed Student t test was used to perform a preselected 2 group comparison and a 1-way analysis of variance followed by either a Tukey or a Dunnett multiple comparisons test was used when assessing 3 or more groups, and a value of $P < .05$ was considered significant.

Results

Metformin Decreases Cell Viability and Increases Cellular Apoptosis in Patient-Derived, Early-Stage EC Cell Lines

The enrolled patients had the final diagnosis of stage IA grade 1 endometrial carcinoma, endometrioid type. The patient characteristics are listed in Table 1. The second and third cell lines (EM2 and EM3) were passaged over 25 times with continued growth, and marker expression was stable. Therefore, EM2 and EM3 were used for further analysis. To ensure the cells were consistent with epithelial EC cells, several cellular markers were evaluated, including vimentin, ER and PR, PAX-8, and cytokeratin 8 (Figure 1A). Consistent with an EC and other commercially available cell lines, all markers were detected in both cell lines. Using these primary cells, we first explored the effects of metformin on cell survival using cellular viability assays. We found that metformin significantly decreased cell viability in a dose-dependent manner (Figure 1B) and increased apoptosis based on cleaved PARP levels (Figure 1C). Given metformin had been previously shown to inhibit the mTOR pathway through the activation of AMPK, we also explored the effects on metformin on the mTOR pathway to assess whether this is the mechanism driving the decrease in cell viability. We found that metformin increased phosphorylation of AMPK in both cell lines, suggesting that its effects on cell viability may be due to increased pAMPK and concomitant decrease in mTOR (Figure 4).

Metformin's Effects on Cellular Survival is Through the Regulation of ERα Expression

To mimic the estrogen-predominant environment typically found in women with EC as well as to ascertain the mechanism by which metformin is decreasing cell survival, we examined whether metformin abrogates the proliferative activity of estradiol. As expected, we found that estradiol alone increased cell viability (Figure 2A); however, in the presence of metformin, the proliferative effects of estradiol were completely blunted (Figure 2A). Consistent with our previous results, metformin alone decreased cell viability, and this was accompanied by a significant decrease in ERα protein and mRNA abundance in a dose-dependent manner ($P < .05$; Figure 2B-C). Furthermore, metformin also decreased expression of the estradiol/ERα responsive genes keratin 19 and Wnt-1 inducible signaling pathway 2; (WISP-2; Figure 3A).

We next examined the effects of metformin on PR expression, given previously reported data and the hypothesis that increased PR resulted in a decrease in PR desensitization. In both cell lines, the abundance of PRB was significantly lower

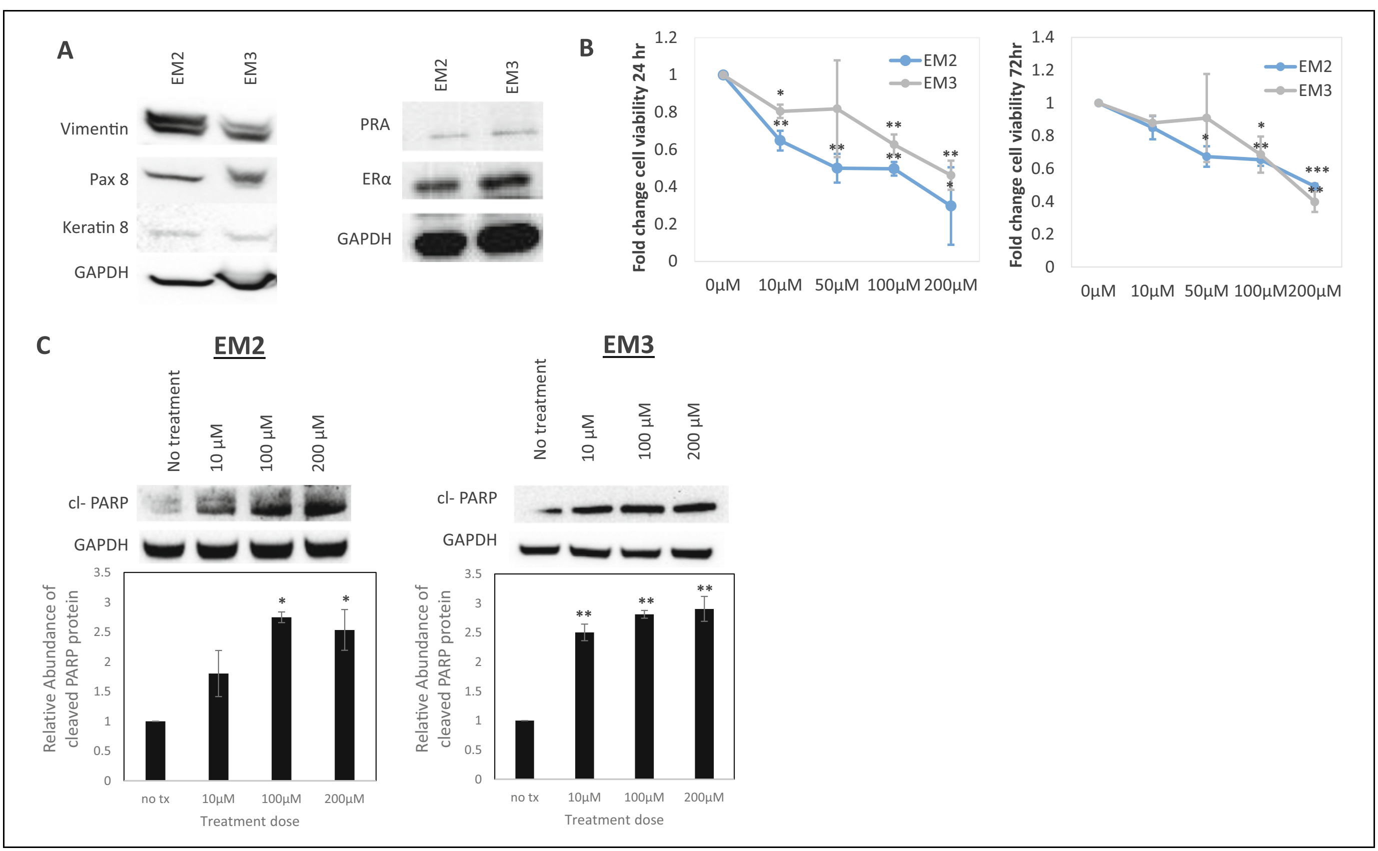

Figure 1. A, Characterization of the 2 endometrial cancer cell lines. Both cell lines (EM2 and EM3) are consistent with epithelial endometrial cancer cells as they have the following cellular markers: vimentin, estrogen and progesterone receptors, PAX-8, and keratin 8. B, Metformin at increasing doses results in a decrease in cell viability based on 24- and 72-hour 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium (MTT) results. C, Increasing doses of metformin resulted in elevated cleaved PARP protein levels, consistent with apoptosis (*$P < .05$, **$P < .01$, ***$P < .001$).

than progesterone receptor total (PRT) and therefore by inference PRA, as PRA is calculated based on PRT and PRB levels. Due to this lower baseline expression, PRB protein was not detectable by immunoblot assay. The EM2 cell line did not have any statistically significant changes in PRT, PRB, or PRA mRNA or protein levels with increasing doses of metformin (Figure 3B and C). However, with the EM3 cell line PRT, PRB, and PRA mRNA expression increased with a metformin dose of 100 and 200 μmol/L. Similarly, the PRA protein levels increased with a metformin dose of 100 and 200 μmol/L (Figure 3B and C). This suggests that the effect of metformin on the progesterone steroid hormone receptor varies in different ECs, as it had a profound effect on PR expression and protein level in one cell line but had no effect in the other.

Finally, to ascertain the importance progesterone on these primary cells, we assessed the effects of progesterone therapy on cell viability. Progesterone in the 2 cell lines did not affect viability and had no effect on metformin-induced inhibition of cell viability (Figure 3D). When treated with P4, MPA, metformin, or the combination, ERα abundance decreased, with the most significant decrease noted when both cell lines were treated with the combination of MPA and metformin (Figure 3E).

Discussion

This study is the first to use patient-derived cell lines to identify the function of metformin on the steroid hormone receptors and cell viability. In an era of targeted therapies, patient-derived cell lines provide a unique opportunity to study the cancer process without the potential for changes in phenotype of established cell lines. This study confirmed a decrease in ERα expression with the treatment of metformin as well as a possible increase in PR in some cell lines, which is consistent with prior studies.[25,26] In addition, the decrease in cellular proliferation may be through AMPK activation and inhibition of the mTOR pathway.[26] There is known cross talk between these signaling pathways, including the mTOR and the steroid hormone receptor pathways, which may be altered in EC. Type I EC is a hormonally dependent disease in which the balance between estrogen and progesterone is disrupted, and the excess estrogen stimulation leads to increased cell proliferation.

As expected, any condition in which there is excess estrogen such as obesity, diabetes mellitus, insulin resistance, and polycystic ovary syndrome (PCOS) may have a higher predisposition to endometrial malignancy. In the presence of hyperinsulinemia, metformin is an effective treatment for anovulation in PCOS.[23]

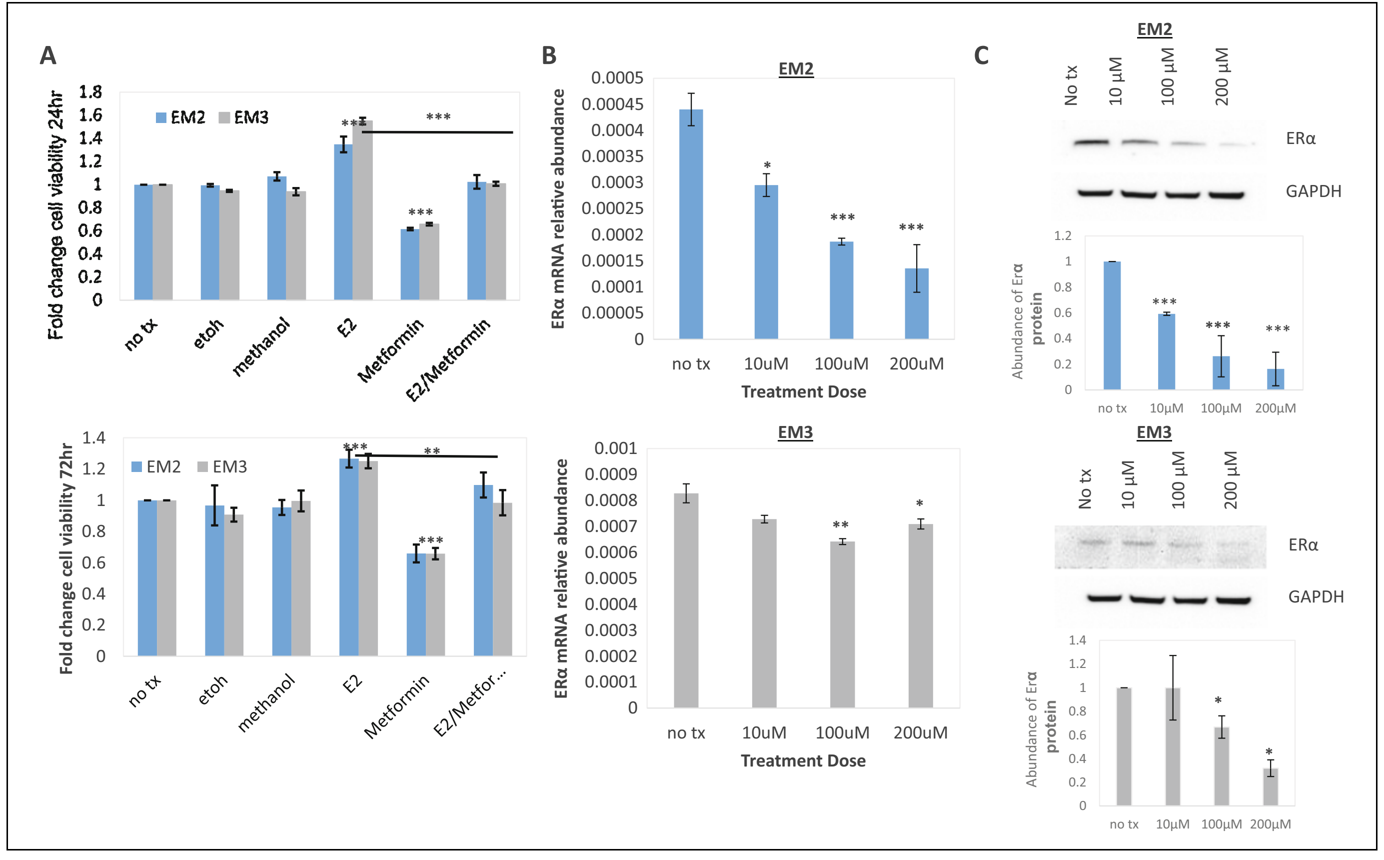

Figure 2. A, Estrogen results in increased cell viability while metformin decreases cell viability. Neither media in which the medications are dissolved results in a significant increase or decrease in cell viability. B and C, Estrogen steroid receptor messenger RNA (mRNA) and protein levels are decreased with 72 hours of 100 and 200 µmol/L metformin treatment in both EM2 and EM3 cell lines (*$P < .05$, **$P < .01$, ***$P < .001$).

This is likely due to the relationship between insulin resistance and sex hormone levels. Insulin resistance and hyperinsulinemia reduce the production of sex hormone-binding globulin by the liver, resulting in an increase in the circulating levels of estradiol.[29] The estradiol level may be further elevated due to peripheral aromatization of androgens to estrogens in the adipose tissue. This elevation in estrogen may lead to the development of sex-hormone-dependent cancers such as EC.[30]

Prior studies focused on the effects of metformin on activated protein kinase (AMPK) phosphorylation and its effect on the mTOR pathway and cellular proliferation.[31,32] Activation of AMPK leads to a decrease in energy consuming processes, such as cellular proliferation, and an increase in processes that generate energy and thus lower the serum glucose and insulin levels.[33] In our cellular model, an expected increase in AMPK phosphorylation was noted in a dose-dependent manner, which may contribute to the decrease in cellular proliferation demonstrated by the MTT assay. However, in EC, the effect of metformin may be more reliant on the interplay between insulin resistance and sex hormones.

There is a known interaction between estrogen, progesterone, and their receptors, as transcription of the PR gene is induced by estrogen and inhibited by progestins.[34,35] Interestingly, this estrogen receptor effect preferentially induces PRA and not PRB, which was the predominant PR increased in our cellular model. Levels of PRB in our cellular model were extremely low and likely had no effect. Decreased expression of PRA has been shown in a mouse model to be responsible for progesterone resistance in EC cells.[36] One of the pitfalls of progestin therapy for the treatment of EC is this concern for progestin-induced progesterone resistance. If this is due to a decrease in PRA, metformin may be beneficial, as it increased PRA in 1 cell line in the current model. Given this was not demonstrated in both cell lines, this effect may not be universally present in all ECs. Interestingly, EM2 demonstrated a greater decrease in ER with metformin treatment than EM3, but EM3 was the only cell line with an increase in PRA. There was no difference in cell proliferation or cleaved PARP levels suggesting that it may be a combination of the increase in PR and decrease in ER that affects cell proliferation and may vary between ECs. Progestins are also typically antagonistic to estrogen-stimulated cell proliferation in the endometrium, and prior studies show PRA directly inhibits ER function in a dominant-negative manner.[16,37] In our cell lines, metformin decreased ER and increased PRA, suggesting metformin may have a synergistic effect on ER and PR to decrease the response to estrogen and potentially reduce the amount of progestin therapy resistance due to PR downregulation with progestin therapy.

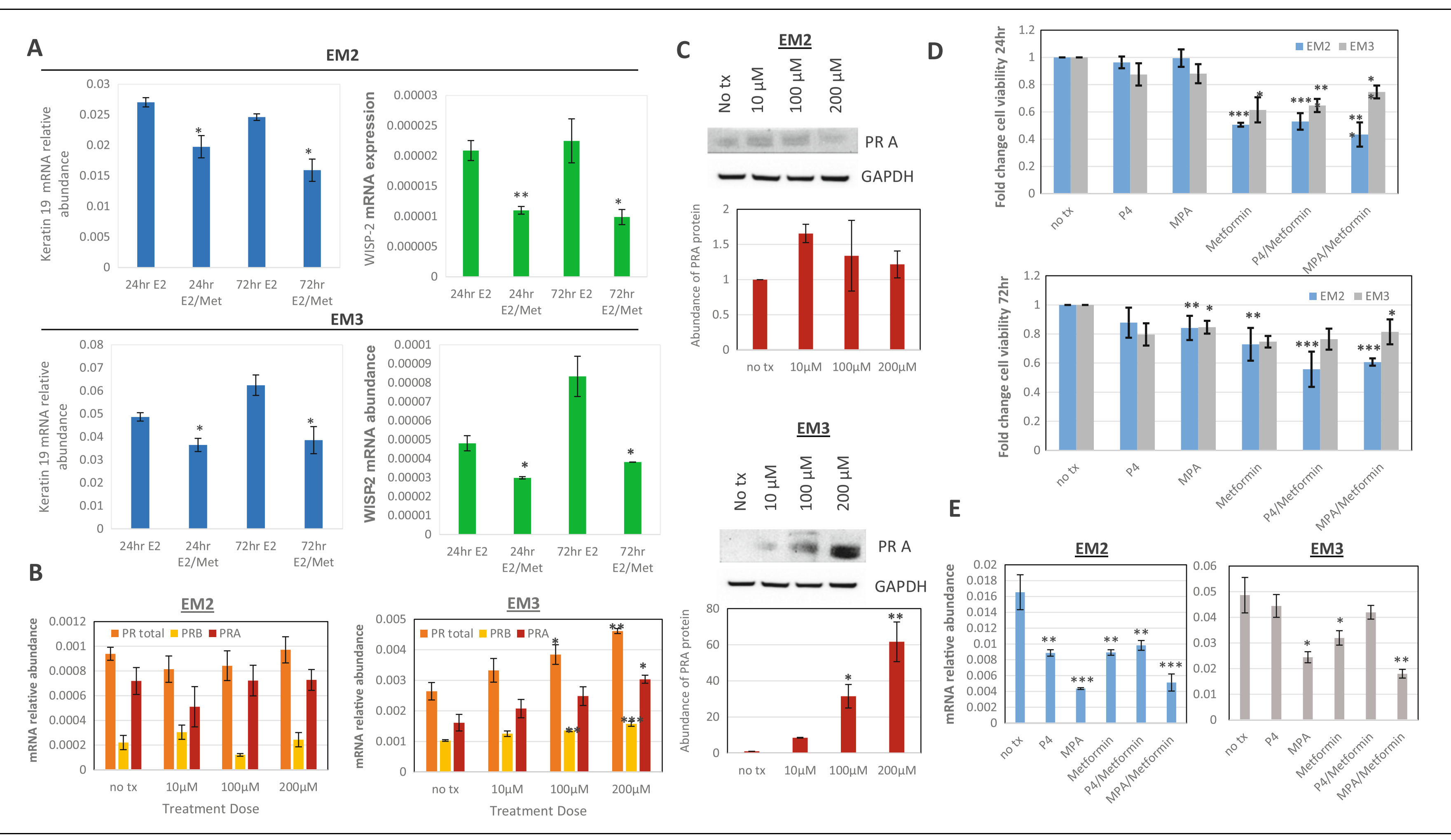

Figure 3. A, Estrogen receptor target genes, Keratin 10, and Wnt-1 inducible signaling pathway 2 (WISP-2), are downregulated with 24 and 72 hours of 100 μmol/L metformin treatment in both EM2 and EM3 cell lines. B and C, Increasing doses of metformin treatment result in an increase in PRT, progesterone receptor (PR) B, and PRA messenger RNA (mRNA) and protein levels in EM3 but has no effect on the PR expression in EM2 cell line. D, Progesterone therapy (P4 or medroxyprogesterone acetate) alone did not result in a significant decrease in cell viability as metformin therapy. There did not appear to be a synergistic effect with P4 or medroxyprogesterone acetate (MPA)on antiproliferation. E, Progestin therapy, metformin, and the combination result in a decrease in estrogen receptor mRNA expression, with the combination of MPA with metformin having the greatest downregulation effect (*$P < .05$, **$P < .01$, ***$P < .001$).

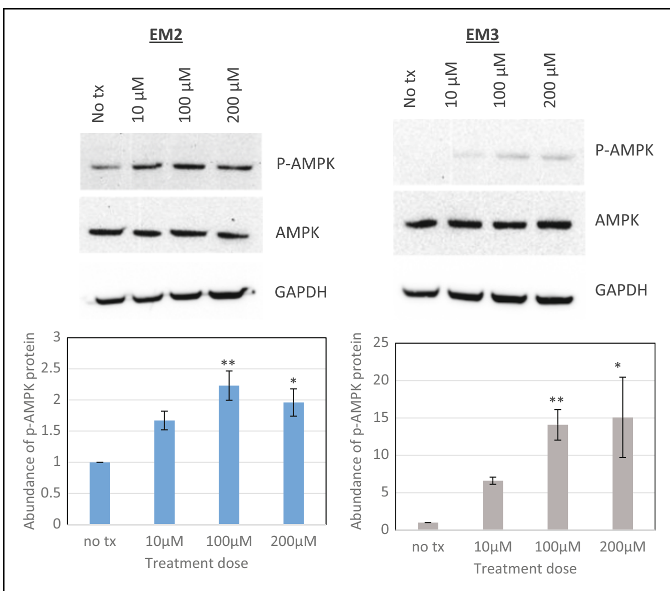

Figure 4. Treatment with metformin results in increased phosphorylation of AMPK in a dose-dependent manner but has a more pronounced effect in the EM3 cell line (*$P < .05$, **$P < .01$, ***$P < .001$).

The WISP-2 gene is a known estrogen responsive gene that has been extensively studied in breast cancer cells, which is another cancer dependent on estrogen cell growth and proliferation.[38] The gene is located downstream from Wnt-1 signaling, which has a known role in carcinogenesis. Keratin 19 is also a known estrogen responsive gene that has also been studied in breast cancer cell lines. Keratin 19 is one of the major proteins of the nuclear matrix-intermediate filament network that forms a continuous cell skeletal network and is thought to play a critical role in cell shape and function.[39] In both cell lines, the expression of WISP-2 and Keratin-19 increased with estrogen therapy and was decreased with the addition of estrogen and metformin, suggesting metformin not only decreases ER mRNA and protein expression but also decreases the downstream effects of estrogen.

Expression of ER and PR are associated with low-grade and early-stage tumors.[40] Unlike most endometrial cells grown in vitro (Hec50, KLE), both EM2 and EM3 express endogenous ER.[14] This suggests that both cell lines are responsive to estrogen-stimulated proliferation. Similar to studies performed in ER-positive breast cancer cell lines (MCF7), downregulation of ERα protein levels provides a therapeutically advantageous condition in which estradiol-stimulated proliferation is halted.[41] Therefore, in this cellular model, it is proposed that cellular proliferation is decreased for 2 reasons: downregulation of estrogen receptors and thus decreased response to estradiol-induced proliferation and activation of AMPK that decreases mTOR activation and decreased cellular proliferation.

Metformin is a commonly prescribed medication for the treatment of diabetes mellitus type 2. The mechanism of action is primarily to inhibit hepatic glucose production and increase glucose uptake in peripheral tissues, such as skeletal muscle. Metformin is a relatively inexpensive medication with minor side effects, including gastrointestinal side effects, vitamin B12 deficiency, and lactic acidosis. Lactic acidosis is the most serious side effect but only occurs in 3 of 100 000 patients with years of use and is lower if it is not used in patients older than 80 years or with hepatic, renal, or cardiac compromise.[42] Metformin is also safe during pregnancy.[43] Therefore, patients with low-grade EC who are planning to defer definitive surgical management would be able to continue metformin therapy until achieving pregnancy.

Clinical and laboratory studies using metformin to treat endometrial hyperplasia have been described. Treatment with metformin in addition to either progestin therapy or oral contraceptives has been shown to reverse atypical endometrial hyperplasia in some patients.[44,45] Interestingly, Markowska et al studied endometrial samples of women with EC and found that there is not a significant difference in ER and PR expression between diabetic and nondiabetic women with EC, but treatment with metformin decreased endometrial ER expression in diabetic women.[46] A similar antiproliferative mechanism was proposed in breast cancer, where a link between metformin and ER reduction was noted.[47] A preoperative window study treated women with EC with metformin for up to 4 weeks prior to surgical staging, and the effects on the mTOR pathway and steroid hormones was evaluated by immunohistochemistry. Metformin resulted in a decrease in proliferation and decreased expression of *P*-AMPK and ER but had no effect on PR expression.[48] The animal models had similar results. In both studies, metformin was found to have antiproliferative effects on the endometrium in a mouse model with endometrial hyperplasia.[49,50] Ideally, the different effects of metformin on steroid hormone receptors and the mTOR pathway should be investigated in a mouse model.

This study provides further evidence as to the mechanism through which metformin may affect cellular proliferation in EC. Future studies in a mouse model and humans need to be completed prior to recommending metformin for use in EC treatment. Ideally, a randomized controlled trial comparing conversion response of low-grade EC to normal endometrium with progesterone therapy alone versus progesterone therapy in addition to metformin needs to be performed to fully determine the benefit of metformin in this patient population. The study participants could be followed after conversion to normal endometrium and maintained on metformin to determine whether there is a decrease in time to regression back to EC. This study provides promising data for use of metformin in EC treatment, given its effects in our patient derived cellular model as well as its low cost and minimal potential side effects.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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