Original Article
Mechanism of progestin resistance in endometrial precancer/cancer through Nrf2-survivin pathway

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Abstract: Progestin is commonly used for young patients suffering from endometrial hyperplasia or cancer. However, there is approximately 30% failure rate with unclear mechanism. We investigated if Nrf2-survivin pathway contributes the progestin resistance (PR) in this setting. Current study detected Nrf2 and survivin protein expression in post progestin treated endometrial tissue samples by using immunohistochemistry. Transfection of Nrf2 and survivin into endometrial cancer cells in vitro was done to determine the roles of Nrf2 and survivin in progestin resistance. Silence of survivin was then performed to explore if Nrf2-driven progestin resistance is mediated by survivin. Medorxyprogesterone acetate (MPA) and metformin were applied to examine the cellular proliferations under the controlled conditions. Overexpression of survivin and Nrf2 were found in progestin-resistant endometrial samples as well as in those areas with only partial responses after MPA treatment. In contrast, all responded endometrial tissue with complete decidualization showed negative expression of these two biomarkers. Exogenous overexpression of Nrf2 and survivin resulted in progestin resistance. In addition, reduction of survivin in endometrial cancer cells overcame the Nrf2 overexpression induced progestin resistance. Furthermore, Nrf2 and survivin expressions were effectively suppressed after withdrawal of MPA. Interestingly, metformin increased the progestin sensitivity by down regulation of Nrf2 and survivin. The findings suggest that dysregulation of Nrf2-survivin may represent part of the molecular mechanisms of progestin resistance in endometrial cancer. Detecting survivin and Nrf2 may predict progestin resistance, while targeting Nrf2 and survivin may represent a promising prevention and treatment strategy for endometrial cancer.

Keywords: Endometrial cancer, endometrial precancer, endometrial hyperplasia, progestin resistance, survivin, Nrf2

Introduction

Type I endometrial cancer is encountered most frequently in the peri- and postmenopausal women, when the hormone level is out of balance. It can also occur, however, in young women and even teenagers, in whom anovulatory cycles are common. Endometrial precancers, referred as atypical hyperplasia or endometrial intraepithelial neoplasia, and well differentiated endometrial endometrioid cancers tend to occur in younger women. Surgical management with hysterectomy may not be an ideal approach for those patients when they either have a desire to maintain their fertility or not suitable for surgery. When this happens, pro-gestin treatment as a conservative management is commonly applied. However, approximately 30% of such patients fail to respond to progestin therapy [1-3]. The molecular mechanism of progestin resistance is currently unclear.

To better understand the mechanism of progestin resistance, significant research efforts including ours have been made in the last 2 decades. Downregulation of progestin receptor (PR) resulting from continuous progestin administration leads to desensitization to progestin, which was thought to be one of the reasons of progestin resistance [4, 5]. The other molecules including those in the EGF/EGFR and insulin sig-

naling pathways may also contribute to progestin resistance [4, 5]. In our earlier studies, significant decrease of survivin expression in human tissues with endometrial hyperplasia was seen in progestin responders, whereas, no significant level changes of survivin expression were found in those non-responders [1]. It was additionally noted that increased ratio of Fas/FasL expression is consistent with a better response to progestin treatment, while dysregulation of Fas/FasL expression contributes to progestin resistance [6]. Despite all these efforts and progresses received in the past, however, the detail molecular mechanism is still unclear. Increasing evidence from recent studies showed that the transcription factor NF-E2-related factor 2 (Nrf2) plays a critical role in cancer recurrence through increased tolerance to adjuvant chemo- and/or radiation therapies. The mechanisms of Nrf2 mediated drug resistance involve multiple genes and details of the molecular pathways of such drug resistance have been summarized elsewhere [7-13]. It has recently been reported that Nrf2 involves in endometrial cancer progestin resistance [3, 14], however, how does it contribute to progestin resistance remains to be clarified.

Evidence accumulated over the past 10 years suggests that survivin, a small inhibitor of apoptosis (IAP) protein has multiple functions as an essential regulator of cell division, a modulator of apoptotic and non-apoptotic cell death, and a promoter of angiogenesis. The survivin gene has been mapped to the chromosomal region 17q25, and encodes a 1.9-kb transcript, which contains 4 exons resulting in a 142-amino acid (16.5 kDa) protein. In the anti-apoptotic network, survivin interacts with other adaptors or cofactors, provides a heightened cell survival threshold and hinges on multiple signaling pathways. In contrast, survivin is highly expressed in most human tumors, including lung, breast, and prostate cancer. In human endometrium, survivin is expressed in normal and proliferative endometrium and is overexpressed in hyperplastic and malignant endometrium. Overexpression of survivin in neoplastic endometrium suggests that survivin may play an important role in the process of estrogen dependent endometrial carcinogenesis. Meanwhile, progesting resistance may also be related to the survivin overexpression since it blocks cellular apoptotic pathway [15-17]. With

these understandings, we hypothesize that Nrf2-driven progestin resistance is probably mediated through survivin regulation to make the cells of endometrial cancer or hyperplasia not to respond progestin treatment properly.

In this study, we examined the role of Nrf2 and survivn in the process of progestin resistance with the following approaches: 1) to test the level of Nrf2 and survivin proteins expression in post progestin-treated endometrial samples; 2) to examine the relationship between Nrf2 and survivin in terms of progestin resistance through a molecular manipulation process; and 3) to test if the progestin resistance could be reversed by addition of metformin, and the effects of the agent on Nrf2 and survivin expression.

Material and methods

Patients and tissue samples

A total of 35 patients were enrolled in the study. These included 26 atypical hyperplasia, 3 atypical hyperplasia bordering endometrial cancer, and 6 well differentiated endometrioid carcinoma. Pathological diagnosis of endometrial hyperplasia or well-differentiated carcinoma was reviewed and confirmed by a gynecologic pathologist (WZ) according to WHO classification [18]. Patient age ranged from 23 to 38 with an average age of 31.6 years. Among the 35 patients, 15 were known to have polycystic ovarian syndrome, 3 had Lynch syndrome, and 17 had unknown etiology. Regarding the body weight, we summarized the BMI for the studied patients. There were 20 patients their BMI more than 35, 8 between 25 and 35, and 7 were 25 or less but within normal range. All patients received at least 6 months of Medorxyprogesterone acetate (MPA) treatment and followed in the clinic regularly. Endometrial curetting samples were received at 6 months (n = 14), 9 months (n = 10), and between 10 and 14 months (n = 11). Patients were considered to have complete regression of hyperplasia (responders) if post progestin treated samples showed decidualized stroma with attenuated endometrial glands in more than 95% of the samples and without any evidence of residual hyperplasia or cancer; If more than 50% of the entire sample contained residual hyperplasia similar or worse than the findings prior to the progestin treatment, the patient was consid-

ered to have persistent disease (non-responder); If residual hyperplastic glands comprising 50% or less, it was classified as partial response. The criteria for the responders vs non-responders were referenced based on previous publications [1, 3, 6].

All the tissue samples were obtained from the Department of Pathology, University of Arizona and the study was approved by the Institutional Review Board.

Immunohistochemical staining

IHC analyses of Survivin and Nrf2 protein levels were performed as previously described. Briefly, specimens were de-paraffinized in xylene and rehydrated in a graded series of ethanol, subsequently, endogenous peroxidase activity was blocked by a 10-minute treatment with 3.0% hydrogen peroxide. Following, sections were subjected to antigen retrieval by boiling in citrate buffer (pH 6.0) and incubated for 30 min with 0.01% Trixon, then incubated for 20 min with 5% bovine serum albumin. The 5 um sections were incubated overnight either with rabbit anti-survivin antibody (diluted to 1:100; Sigma, St. Louis, MO, USA) or with anti-Nrf2 antibody (diluted to 1:100; Abcam, Cambridge, UK), followed by a 50-minute incubation with biotinylated secondary antibody (Dako, Carpinteria, CA, USA). Omitted primary antibodies served as negative controls. Expression of Nrf2 or Survivin protein were assessed using a semi-quantitative method: the slides were evaluated for the percentage of positively stained cells (0-4) and the intensity of the staining (0-3). Index of Nrf2 or Survivin expression was calculated as percentage × intensity of the staining. Therefore, score 0 present negative, 1-4 as weak positive, 5-8 as positive, and 9-12 as strong positive. All IHC slides were reviewed independently by two investigators.

Cell lines and cell culture

The RL95-2 cell line, a hormone-responsive Type I endometrial cancer cell line purchased from American Type Culture Collection (ATCC). The cells with positive estrogen and progesterone receptor expression were maintained in Dulbecco's modified Eagle's medium (DMEM) F-12 1:1 medium (GIBCO) with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA), 100 U/ml penicillin, sodium pyruvate and L-glutamine in a humidified atmosphere of 5% CO_2 at 37°C.

Transient transfection of plasmids and hormone administration

To investigate the roles of Nrf2 and survivin in progestin resistance, exogenous transfection of Nrf2 and survivin were performed. Briefly, after serum starvation for 24 hours, RL95-2 cells were transfected with pCI-Nrf2 and pcDNA-survivin plasmid using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, respectively. After transfection for 16 hours, Nrf2-transfected or survivin-transfected cells and their corresponding control cells were treated with different dose of MPA for another 48 hours. Metformin (1 mM) was used for the progestin resistant reserve tests. Cell proliferation was determined by the MTT assay. All experiments represented results of triplicate test.

Small interfering RNA transfection and hormone stimulation

RL95-2 derived stable cell lines, with incorporation of Nrf2 or an empty vector, were established using lentivirus system as described previously. Stable RL95-2 cells were continuously cultured in medium containing 1.5 µg/ml puromycin (sigma). The acute knockdown of survivin in above stable cell lines was performed as previously described. Briefly, cells were seeded in 0.1 ml of growth medium in 96-well plate without antibiotics. After 24 hours, transfection of survivin siRNA was done according to the manufacturer's instructions with Hi-perfect transfection reagent (Qiagen, Boston, Massachusetts). After knockdown for 16 hours, the cells were treated with different dose of MPA for another 48 hours. The cell viability was then determined by MTT assay.

Western blot analysis

Western blots were carried out as described elsewhere [19]. Briefly, cells treated with various drugs and hormones were lysed, and proteins (60 µg) were separated by SDS-PAGE. After transferring to polyvinylidene fluoride (PVDF) membranes, proteins of interest were determined using specific antibodies.

MTT assay

The MTT assay was performed to determine cell viability. Briefly, RL95-2 cells were plated in a 96-well plate (2,000 cells per well) and incu-

Table 1. Survivin and Nrf2 expression in progestin treated endometrial samples

Marker scores	Responders (n = 18)	Partial Responders (n = 6)	Non-responders (n = 11)	p Values
Survivin	0.52 ± 0.03	6.15 ± 0.82	8.52 ± 1.25	< 0.001
Nrf2	0	4.21 ± 0.62	5.12 ± 0.48	< 0.001

bated for 24 hours. The culture medium was then changed to serum-free DMEM F-12 1:1 medium for 24 hours. After various hormone and metformin treatments, MTT solution (20 μL of 5 mg/mL MTT in PBS) was added to the treated cells. After 4 hours of incubation at 37°C, the culture medium was removed, and 150 μL of dimethylsulfoxide was added to dissolve the formazan. Finally, absorbance at 490 nm was measured with a GENios multifunction reader (Tecan, Zurich, Switzerland).

Statistical analysis

The statistical significance of the differences in the IHC staining in endometrial tissues was calculated using the Chi-squared test. The differences in various protein levels and cell proliferation between groups were analyzed using the Student's t-test. A two-sided test with $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SAS Release 8.02 (SAS Institute Inc., Cary, NC, USA) or SPSS 11.0 (SPSS Inc., Chicago, IL).

Results

Patients responding to progestin treatment

All H&E slides from studied patients were reviewed under light microscopy. Based on the defined criteria, we have identified overall responses to progestin treatment as follows. There were 18 progestin responders (successfully treated), 6 partial responders, and 11 non-responders (failures to response to progestin treatment).

Nrf2 and survivin expression in post progestin-treated endometrial samples

Immunohistochemical stain showed that survivin and Nrf2 expression were positively correlated to the status of progestin resistance. In the responder group, survivin and Nrf2 were basically negative in all 18 endometrial samples of patients who received progestin treatment. In contrast, both survivin and Nrf2 were

highly expressed in the samples of partial responders and non-responders ($P < 0.001$). The detailed results are summarized in **Table 1**. Interesting to note, responded areas of partial responding cases showed negative staining of both markers, too. Survivin and Nrf2 expression were mainly cytoplasmic in endometrial epithelial cells. No statistical differences of the marker stains were observed between cases of atypical hyperplasia versus endometrioid carcinoma (data not shown). Representative pictures of survivin and Nrf2 expression are presented in **Figure 1**.

Nrf2 and survivin enhanced the progestin resistance

To determine the role of survivin and Nrf2 in endometrial cancer progestin resistance, we overexpressed Nrf2 and survivin by transfecting each plasmid. As shown in **Figure 2A**, increased level of Nrf2 expression resulted in progestin resistance. Similarly, transfection of survivin led to reduced susceptibility to progestin treatment (**Figure 2B**). These findings suggest that survivin plays an important role in endometrial cancer progestin resistance.

Survivin expression controlled by Nrf2 in the process of progestin resistance

To examine if survivin overexpression is driven by Nrf2 in the scenario or progestin resistance, we established Nrf2-overexpressed RL95-2 stable cell line (RL95-2-Nrf2). siSurvivin and its negative control (siCon) were transfected in to RL95-2-Nrf2 cells, respectively. Compared with the cells transfected with siCon, the cells with siSurvivin showed a significant suppression of survivin expression (**Figure 3A**), which subsequently enhanced the sensitivity to progestin treatment (**Figure 3B**).

After the plasmids with different Nrf2 doses constructed, we examined if the level of survivin expression could be regulated accordingly. As shown in **Figure 4A**, a dose response relationship between survivin expression and the dose of Nrf2 plasmid was observed. This relationship was further confirmed by using different doses of tBHQ to replace Nrf2 plasmids (**Figure 4B**). The results suggest that Nrf2 directly regulates survivin expression.
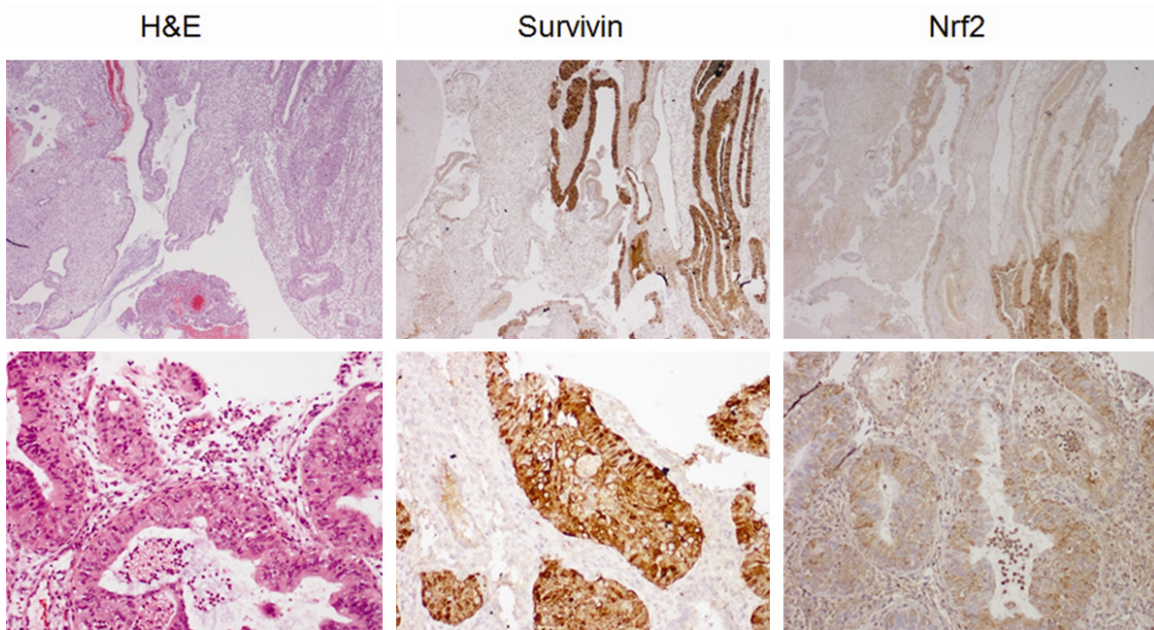
Figure 1. Expression of surviving and Nrf2 in endometrial precancer/cancer tissues after progestin treatment. An example of partial response to progestin treatment is shown on the upper panel, while non-response of a carcinoma case is on the lower panel. Staining of survivin and Nrf2 were performed in the deeper sections of the same tissue block. Within the partial response case, area at the left side represented endometrium responded to progestin treatment, while right side was partial responded area (H&E, upper left panel). As illustrated, survivin and Nrf2 were stained only in the partially responded area (upper panel) and all non-responding cancerous area (lower panel).
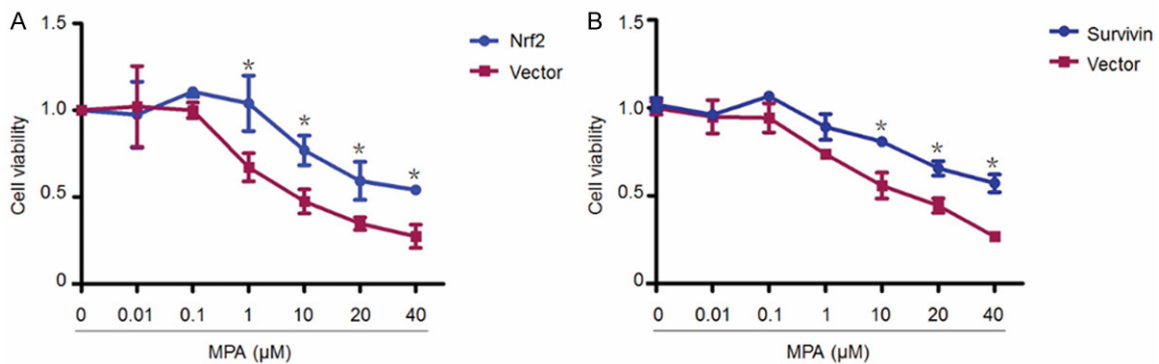

Figure 2. Effect of Nrf2 and survivin on the susceptibility to progestin treatment. Nrf2 plasmid (A) and survivin plasmid (B) were transiently transfected into endometrial cancer RL-95-2 cells, following treated with indicated dose of MPA for 48 hours. MTT assay was used to determine the relative proliferative ability of the cells. The cancer cells survived better after transfection of either Nrf2 or survivin.

Nrf2, survivin and PR expressions after MPA withdrawal

As hormone withdrawal usually causes 'breakdown' of the endometrium, we assessed whether the removal of MPA may change Nrf2, survivin and PR expression level in the RL95-2 cell line. MPA removal experiments showed that the level of Nrf2 and survivin expression became apparent at 48 hours and was most pro-

nounced at 72 hours (**Figure 5**). In contrast, the PR expression level increased in the time course of MPA withdraw experiments.

Progestin resistance overcome by metformin through down-regulation of survivin and Nrf2

It has been shown that metformin is able to reverse progestin resistance for patients with atypical endometrial hyperplasia [12, 20]. The
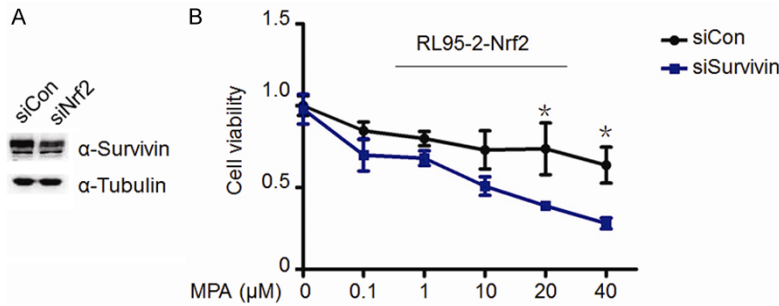
Figure 3. Survivin mediated Nrf2-driven progestin resistance in endometrial cancer cells. Survivin siRNA was transfected into stably overexpressed Nrf2 RL-95-2 cells. Western blot was used to detect the efficiency of the gene silence with siCon as a negative control (A). MTT assay was used to examine the proliferative activity after treatment with indicated doses of MPA (B). After the level of survivin was knocked down, the cancer cells survived significantly less (P < 0.01).
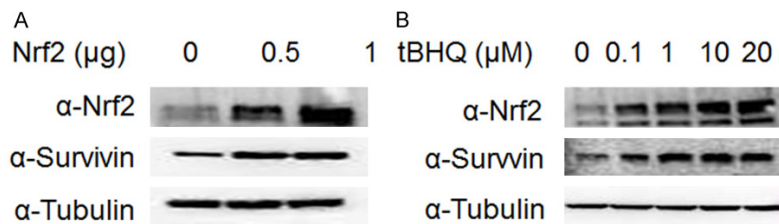

Figure 4. Nrf2 upregulated survivin expression in endometrial cancer cells. Different doses of Nrf2 plasmids were transfected into RL-95-2 cells for 24 hours and the effect of Nrf2 on survivin expression was determined by Western blot (A). Different doses of tBHQ were used to treat endometrial cancer cells in a similar condition as a control (B). The cells were harvested after 24 hours in a culture system to investigate the effect of Nrf2 inducer on Nrf2 and survivin expression. The level of survivin expression positively correlated with Nrf2 levels.
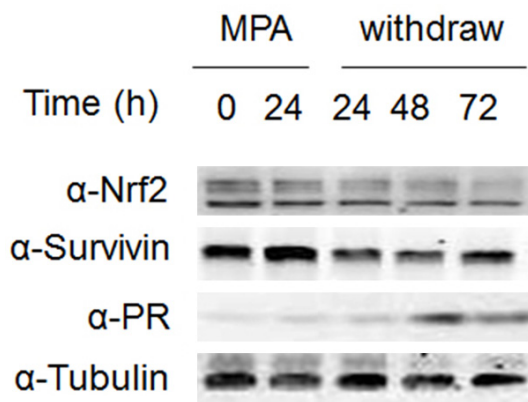

Figure 5. Effect of MPA withdrawal on Nrf2, survivin and progestin receptor expressions. Western blot was used to determine the expressions of Nrf2, survivin and progestin receptor in RL95-2 cells after MPA withdrawal. Both Nrf2 and survivin proteins were significantly down-regulated in the time course of MPA withdrawal, whereas the level of progestin receptor (PR) increased with the time.

cell viability assay revealed that treatment with 10 μM MPA alone has no significant inhibition effect on progestin-resistant RL-2-Nrf2 cells compared with its control, whereas treatment with metformin (1 mM) plus MPA remarkably reduced the cell viability of both control and progestin-resistant cells (P < 0.05) compared with treatment with MPA or metformin individually. This may attribute to the decrease of Nrf2 and survivin protein levels by metformin as shown in **Figure 6**.

Discussion

Endometrial carcinoma is one of the leading causes of death in women. More than 80% of endometrial cancers are adenocarcinoma (type I), and approximately 14% occurs in younger women who have wished to preserve fertility [21, 22]. For these patients, hysterectomy with bilateral oophorectomy is not the first choice. Therefore, in clinical practice, MPA, a synthetic progestin, is commonly used for those patients who suffer from estrogen driven endometrial neoplastic diseases and meanwhile who desire future fertility. Although MPA treatment has an encouraging 70% response rate, approximately 30% of patients are resistant to progestin therapy, thus it presents a major obstacle for the treatment. Meanwhile, researches to overcome progestin resistance become a heated topic in the field. It is widely accepted that loss of PR impairs the therapeutic effect of progestin administration, low dose and long term MPA-induced progestin-resistant endometrial cancer cells presented a declined PR expression pattern, which could be one mechanism of progestin resistance in endometrial precancer/cancer [23-27]. We previously reported that high level of survivin was present in patients who fail to respond to progestin therapy, whereas decrease of survivin expres-
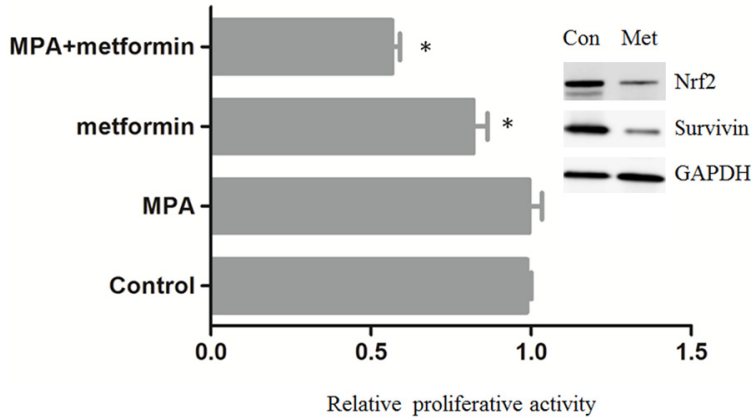
Figure 6. Metformin reversed progestin resistance by inhibition of Nrf2 and survivin expression. Western blot was used to determine the effect of metformin on Nrf2 and survivin expression. Treatment with MPA plus metformin (1 mM) enhanced the sensitivity of RL95-2-Nrf2 cells to MPA administration. *$P < 0.05$ by student's *t*-test, when compared with the control group.

sion can be seen in progestin responders [1]. Recently, Nrf2, a transcript factor, has been demonstrated to contribute to endometrial cancer progestin resistance [3, 14]. However, it is not clear if Nrf2-drived progestin resistance is mediated by survivin.

Survivin plays an essential role in the drug resistant phenotype of multiple human cancers [28-30]. Enforced high survivin expression could effectively suppress apoptosis induced by chemotherapeutic agents and increase drug resistance in various cancers [28-30]. As previously demonstrated, survivin is overexpressed in neoplastic endometrial tissues including atypical endometrial hyperplasia and well differentiated endometrial cancer [1]. Based on these understandings, we hypothesize that the elevated survivin and Nrf2 in endometrial cancer may be responsible to the emergence of progestin resistance. To further investigate how Nrf2 and survivin contribute to endometrial precancer or cancer progestin resistance, we performed the mechanism related experiments in vitro. Transfection of Nrf2 significantly increased resistance to progestin treatment. Conversely, knockdown of Nrf2 in these stable cells sensitized them to progestin treatment. In terms of survivin regulation, the results paralleled those of Nrf2 (**Figure 2**). Exogenous overexpression of survivin also resulted in an enhanced progestin resistance. However, while maintaining a high level of Nrf2 in Nrf2 stably-

expressed endometrial cancer cells, reducing survivin level alone overcame the Nrf2-associated progestin resistance (**Figure 3**). These findings indicate that Nrf2-survivin pathway may not only be responsible to the progestin resistance, but also shows survivin being one of the key downstream regulators. Recently, we have demonstrated that progestin resistance may also be mediated by Nrf2-AKR1C1 pathway [31]. Both Nrf2 mediated survivin and AKR1C1 pathways are probably linked by the down regulation of progesterone receptor B by decreasing progestin-dependent PR activation [32]. This is consistent with what we observed in our current study that the level of Nrf2-survivin was decreased, while progesterone receptor was elevated in endometrial cancer cells after MPA withdrawal. This is also supported by our previous observations [1, 6]. It seems that the decreased level of progestin potentiated by either survivin or AKR1C1 may limit its interactions with progesterone receptors and probably contribute to the progestin resistance.

Metformin (dimethylbiguanide) is a biguanide drug that has been widely used as a first-line pharmacologic treatment of type 2 diabetes. It has recently been demonstrated to possess anti-proliferative properties that can be exploited for the prevention and treatment of a variety of cancers including endometrial cancers [33-35]. The mechanism of metformin action is complex, all processes ultimately lead to enhanced tissue insulin sensitivity and reduction of blood glucose and insulin levels. However, recent studies have suggested that metformin has direct anticancer effects, part of which may be related to the induction of apoptosis pathway [36]. Current study showed that reduction of Nrf2-survivin is benefit to endometrial cancer cells responding to progestin. In our previous study we have shown that metformin is able to reverse progestin resistance in the endometrial cancer cell lines by decreasing the level of Nrf2 expression [31]. These findings are consistent with the clinical and experimen-

tal studies previously [20, 37]. Our finding of metformin reduced the level of survivin expression in endometrial cancer further supports that therapeutic effect of metformin may also involve Nrf2-survivin pathway. It may be worth a clinical trial to apply metformin simultaneously or prior to progestin treatment for those endometrial precancer or cancer patients in near future.

In conclusion, our findings show that survivin expression in endometrial samples is mainly controlled by Nrf2. The Nrf2-survivin pathway plays an important role in progestin resistance for patients with endometrial precancers/cancers. Detecting survivin and Nrf2 may predict progestin resistance, while targeting Nrf2 and survivin may represent a promising prevention and treatment strategy for endometrial cancer.

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Disclosure of conflict of interest

None.

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