INTRODUCTION

Epidemiologic studies have revealed that atypical hyperplasia and well-differentiated cancer tend to occur in younger women. These women, especially those of child-bearing age who wish to become pregnant, have a strong desire to maintain their fertility. For these individuals, conservative management with progestin is the optimal choice. However, ~30% of the patients develop progestin resistance.

In the past decades, several mechanisms have been proposed to explain progestin resistance, such as deficiency of progesterin receptor (PR), downregulation of ERα expression, aberrant survivin expression as well as increased TGF-EGFR signaling. Recently, downregulation of NRF2 was stated to potentially improve the response of patients with endometrial cancer to progestin therapy or chemotherapy, while high levels of NRF2 contribute to therapy resistance, which may represent another molecular mechanism involved in progestin resistance.

AKR1C1 is well characterized as a target gene of NRF2. In our previous study, TET1 has been found to play a role in metformin-enhanced progestin sensitivity. We have also demonstrated that TET1-dependent DNA hydroxymethylation contributes to elevated NRF2 expression in endometrial cancer. Therefore, the role of TET1 in NRF2-driven progestin resistance needs to be further clarified.

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Brusatol was first discovered by Ren et al. and identified as an inhibitor of the NRF2 pathway. It sensitizes multiple types of cancer cells to anti-cancer drugs by downregulating NRF2 via ubiquitination-dependent degradation\textsuperscript{22}. Many studies showed that brusatol can potently decrease the expression of other proteins including HIF-1α, p38, STAT3 and SQSTM1\textsuperscript{23–25}, which implies that brusatol is a global protein synthesis inhibitor\textsuperscript{26–28}. Despite ubiquitination-dependent degradation is an important manner for brusatol to suppress protein expression, increasing evidences illustrated brusatol can also regulate its targets at the transcriptional level. However, little is currently known about how brusatol transcriptionally mediates its targets and whether brusatol is involved in NRF2-driven progestin resistance.

In our reports, AKR1C1 was identified as a key scavenger of progesterin and a mediator of NRF2-TET1 driven progestin resistance. Ablent expression of AKR1C1 was observed in endometrial cancer patient samples with poor progestin response, which suggests that AKR1C1 is a specific marker to identify progestin resistance. Moreover, progestin resistance due to the NRF2-TET1-AKR1C1 signaling axis can be reversed by brusatol in precancerous and cancerous endometrial cells.

**MATERIALS AND METHODS**

**Cell lines and cell culture**

Human endometrial cancer cell lines (Ishikawa and ECC1) were maintained in our laboratory. HEK-293 cells were purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM: F12 medium (1:1, Gibco) containing 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA). 100 U/ml penicillin G and 100 µg/ml streptomycin (Life Technologies, Inc., Rockville, MD) and placed in a 37°C incubator with a humidified atmosphere containing 5% CO\textsubscript{2}.

**Establishment of stable cell lines, transient transfection, small interfering RNA transfection and progestin treatment**

Stable cell lines with NRF2/AKR1C1 overexpression or AKR1C1 depletion were established using a retrovirus system as described previously\textsuperscript{11,10}. Transient transfection of the indicated plasmids or siRNA was performed with Lipofectamine\textsuperscript{TM} 3000 (Invitrogen, Carlsbad, CA, USA).

**Drug treatment and cell proliferation assay**

Endometrial cancer cells were treated with MPA, brusatol, or tBHQ for the indicated times. Cell proliferation was measured with a CCK8 assay.

**Immunoblot analysis**

Cells were lysed with RIPA buffer to extract total protein. In total, 50 µg of protein per lane was loaded onto an SDS-polyacrylamide gel, electrophoresed and transferred to PVDF membranes, which were incubated with the indicated secondary antibodies, detection of the protein bands was carried out using a chemiluminescence detection system (ECL detection kit; Pierce, Rockford, IL). Each experiment was performed at least three times.

**Dot blot and hMeDIP assay**

We extracted total DNA and performed a gradient dilution of the samples. The dilutions of total DNA were dropped on nitrocellulose membranes, which were baked at 80°C for 10 min. The membrane was blocked with 5% skim milk for 1 h before it was incubated with ShmC primary antibody (1:500 dilution, Active Motif) overnight at 4°C. After HRP-conjugated secondary antibody incubation, the membrane was subjected to ECL and scanned to visualize bound antibodies. Methylene blue staining served as a loading control. Quantification of the dot blots from three independent assays was calculated with Gel-Pro analyzer software (Media Cybernetics). The gray intensity of dots subjected to ECL bands was quantified. The DNA fragments were incubated with 5hmC antibody (Active Motif) and pulled down to amplify the AKR1C1 gene promoter via real-time PCR. The primers used are listed in Table 1.

**Table 1. Primers for amplification of the sequence on AKR1C1 promoter region in the hMeDIP assay.**

| Primer No. | Primer sequence |
|------------|-----------------|
| Primer 1   | Forward 5′- GATTTCCTTGTTCCCTGTATGCC-3′ |
|            | Reverse 5′- CAACACAAACAAATGTCGG-3′ |
| Primer 2   | Forward 5′- GGCGAGTTCTCTAAGCAGGC-3′ |
|            | Reverse 5′- AGCAATTTAAGAGCTTGG-3′ |
| Primer 3   | Forward 5′- CACTGCAAAGTGTGACT-3′ |
|            | Reverse 5′- TGTTGATAATCTCCGATG-3′ |
| Primer 4   | Forward 5′- CGGCTAGAGGTCTGTATTA-3′ |
|            | Reverse 5′- GGTGCAATGACACTCATT-3′ |

**ARE constructs and dual luciferase reporter assay**

The wild type or mutant TET1 AREs were amplified and cloned into pGL4.27 plasmids (Promega) as previously described\textsuperscript{18}. Ishikawa cells were cotransfected with pGL4.27-TET1-ARE plasmids, pRL-SV40-Renilla plasmid (Promega) and NRF2 plasmid, and the relative luciferase activity was determined by a Dual Luciferase Assay Kit (Promega).

**20α-dihydroxyprogesterone concentration detection**

Endometrial cancer cells treated with indicated drugs were harvested and lysed to determine the 20α-dihydroxyprogesterone concentration with the analysis kits (XQ-EN15767, Xinquan, Company, Shanghai, China). The detailed procedure has been finished as the manual described. And high performance liquid chromatography (HPLC) also was used to quantified the 20α-dihydroxyprogesterone after treatment the endometrial cancer cell with progestagen alone or combine with brusatol and progesterone. In brief, the specific steps are to add 80% methanol to the standard 20α-dihydroxyprogesterone stock buffer and prepare solutions with final gradient concentrations of 100, 50, 20, 10, 5 and 2 µg/ml, respectively. Then set the instrument parameters, according to the set chromatographic and mass spectrometry conditions, add the prepared standard solution or samples into the injection bottle, and the peak at Rt (retention time) = 2.72 (min) was identified to be 20α hydroxyprogesterone. For data analysis, we use MultiQuant software for integration, get the standard curve, and use the standard curve for content calculation. Through calculation, the equation \( y = 277.45x - 30.741 \) \((R^2 = 0.9996)\) is obtained. Calculate the 20α dihydroxyprogesterone concentration based on the area under the curve in the figure according to the formula.

**Selection of matched cases, tissue processing and immunohistochemical (IHC) analysis**

Thirty-four pairs of endometrial samples before and after progestin treatment were assessed in this study. Sixteen patients showed complete response (CR) to progestogen therapy, eight patients showed partial response (PR), and ten patients showed no response (NR). The pathological diagnosis of endometrial hyperplasia was reviewed and confirmed by gynecological pathologists (YJ and WZ) on the basis of the WHO classification. Specifically, the different pathological statuses based on progestin response were defined as follows: NR or residual disease, any architectural abnormalities such as clusters of crowded glands, papillary structures, and complex types of glands with or without cytologic atypia either alone or in combination; PR, no residual hyperplasia but an incomplete response or abnormal glands or any residual architectural abnormalities that do not reach the level of residual disease or nonresponsive disease; and CR, attenuated endometrial glands with decidualized stroma. The IHC assay was performed as previously described\textsuperscript{15,16}. The “Index in epithelial/stromal cell” is defined as the H-score of AKR1C1 reactivity graded on a score ranging from 0 to 100 based on the product of staining intensity (0–3) and percentage (0–100) of the cells stained. Score of 0 represented negative expression. A staining intensity score of 1–3 represented weak, moderate and strong staining, respectively. All IHC slides were reviewed independently by two investigators.

**Human endometrial organoid culture**

According to the previous research\textsuperscript{17}, human endometrial organoid expansion medium (ExM) was as follows: DMEM/F12-glutamine medium.
(Gibco, USA) with B27 supplement minus vitamin A (Thermo, USA), N2 supplement (Thermo, USA), Primocin 100 µg/ml (Invivogen, France), N-Acetyl-L-cysteine 1.25 mM (Sigma, USA), A83-01 500 nM (MEC, USA), nicotinamide 10 nM (Sigma, USA), rh-EGF 50 ng/ml, rh-Noggin 100 ng/ml, rh-HGF 50 ng/ml, rh-Rspondin-1 500 ng/ml and rh-FGF-10 100 ng/ml (Peprotech, USA). Briefly, fresh endometrial cancer tissues were collected, followed by digested with Collagenase IV (Gibco, USA) and centrifugated. The cell pellets were resuspended in ice-cold Matrigel (Corning, USA) and ExM mix. In total, 50 µl drop of Matrigel–cell suspension were plated into the center of wells of 24-well plate. The plate was placed in the incubator for 20 min for solidification and overlaid with organoid ExM. The medium was changed every 2–3 days. Cultures were passaged by manual pipetting every 7–10 days. Endometrial organoids were replanted on 24-well plate and treated with DMSO, MPA 20 µmol/l, Brusatol 10 nmol/l and both for 3 days. Then the samples were photographed under microscope and collected and fixed with 4% paraformaldehyde on ice, followed by resuspending, centrifugating and embedding the organoids with 3% low melting point agarose. The agarose cube with organoids was dehydrated and paraaffin embedded for further HE and IHC staining.

In vivo xenograft mouse model
Nude mice were subcutaneously injected with 1 × 10⁶ endometrial cancer cells. After the mice were treated with MPA and brusatol either alone or in combination for 30 days, they were sacrificed. The tumors were harvested for IHC analysis as previously described.²²

Statistical analysis
SPSS 19.0 was used for data analysis. Comparisons of proliferation, the dot blot assay, the dual luciferase reporter assay and the western blot results after various treatments between two groups were made with Student’s t test. p < 0.05 was considered a significant difference when compared with the control group.

RESULTS
Brusatol sensitizes endometrial cancer cell to progestin in vitro
We first determined the effect of brusatol on cell proliferation in endometrial cancer cells. As shown in Fig. 1A, brusatol suppressed cellular growth in a dose-dependent manner. In addition, brusatol significantly suppressed the expression of NRF2 and the relative genes in a dose and time-dependent manner (Fig. 1A lower panel, B). NRF2 is reported to be implicated in chemoresistance. We therefore tried to detect whether brusatol, an NRF2 inhibitor, could enhance progestin sensitivity. Compared with brusatol or MPA alone, brusatol combined with MPA markedly suppressed the growth of endometrial cancer cells (Fig. 1C). Next, Ishikawa-NRF2 cells were established by overexpression of NRF2 and we found cells overexpressing NRF2 were resistant to progestin (MPA) therapy. Importantly, brusatol sensitized Ishikawa-NRF2 cells to MPA (Fig. 1D). The corresponding NRF2 signaling proteins and relative TET1 have been determined by western blot in Ishikawa and Ishikawa-NRF2 cells. MPA suppressed NRF2 expression in Ishikawa cells whereas showed little effect on Ishikawa-NRF2 cells. However, brusatol combined with MPA almost eliminated NRF2 expression in both Ishikawa and Ishikawa-NRF2 cells. Similar changes have also been observed in the protein levels of AKR1C1 and TET1 (Fig. 1E).

Brusatol impairs NRF2-AKR1C1-mediated progestin metabolism
To assess the mechanism which brusatol reversed progestin resistance, the association between NRF2 and AKR1C1 has been defined firstly. As shown in Supplementary Fig. 1, several AREs have been found in AKR1C1 promoter region, which implies that it is a NRF2 target gene as previously reported.¹¹,¹² Moreover, knockdown of AKR1C1 by selective small interfering RNAs notably facilitated the suppression on cellular growth in the presence of MPA with or without NRF2 transfection (Fig. 2A), which suggested AKR1C1-mediated NRF2-driven progestin resistance. Next, we tried to figure out whether progestin metabolism alteration by AKR1C1 plays an essential role in the progestin treatment failure. We detected the conversion of progestin to 20α-dihydroxyprogesterone (20α-OH). Overexpression of AKR1C1 resulted in an increase of 20α-dihydroxyprogesterone and reduced levels of progesterone in cell lysates in a dose-dependent manner (Fig. 2B). Moreover, the HPLC analysis indicated brusatol impaired the reversion of progesterone to 20α-OH. We found that the level of 20α-dihydroxyprogesterone significantly decreased after adding brusatol (Fig. 2C). Consistent with this result, the increased 20α-OH and reduced progesterin by overexpression of AKR1C1 were effectively blocked by brusatol treatment (Fig. 2D). Thus, we speculate that AKR1C1-mediated progesterin metabolism may contribute to sensitivity to progestin therapy as we observed above.

Hydroxymethylation suppression of AKR1C1 promoter region through declined TET1 contributes to brusatol-induced progestin sensitivity
We next tried to explore how brusatol regulated AKR1C1. Our data indicated brusatol reduced the level of AKR1C1 in endometrial cancer cells. Despite ubiquitination-dependent degradation is an important manner for brusatol to suppress the target protein expression, increasing evidences illustrate brusatol can also regulate its targets at the transcriptional level. Methylcytosine dioxygenase TET1 is involved in the epigenetic gene regulation. It catalyzes the conversion of 5-mC to 5-hmC, leading to hydroxymethylation and methylation changes in the promoter region. In our previous study, similar expression profiles between NRF2 and TET1 have been detected by IHC in consecutive sections of endometrial tissue samples from hyperplasia, to EAH, progressed to endometrial carcinoma.²⁸ Considering brusatol is an NRF2 inhibitor, we thereby further evaluated the possible regulation relationship between NRF2 and TET1. NRF2 was transfected into Ishikawa and ECC1 cells, and the expression pattern of TET1 and other NRF2 targeting genes was estimated. As shown in Fig. 3A, NRF2 overexpression resulted in a significant increase in the expression of TET1 protein as well as other target proteins, including NQO1, HO1, AKR1C1, and AKR1B10, whereas knocking down NRF2 led to decreased expression of these proteins (Fig. 3A, right panel). Similarly, we transfected endometrial cancer cells with a plasmid overexpressing Keap1, an E3 ligase responsible for the degradation of NRF2, and we then found it reduced the expression of NRF2 and its downstream target molecules, including TET1 (Fig. 3B). Meanwhile, it was found that TET1 also bears four AREs in the promoter region within 5000 bp from the transcription start site (Fig. S2). To identify which ARE is necessary for NRF2 to regulate TET1, four truncations targeting the indicated AREs were constructed (Fig. 3C). The dual luciferase reporter assay revealed that each truncation could respond to NRF2 as long as it contains the ARE1 (Fig. 3D, left). Subsequently, it was found that NRF2 transfection could enhance the luciferase activity in wild type ARE1 reporter plasmid, whereas had little effect on mutant ARE1 (Fig. 3D, right), which implies that ARE1 plays an essential role in NRF2-driven TET1 overexpression.

We have demonstrated brusatol inhibited TET1 expression (Fig. 1B, E) and we speculated brusatol might regulate TET1 at the transcriptional level. We found elevated NRF2 enhanced the expression of TET1 induced by AKR1C1 expression, and this upregulation can be blocked by brusatol in a dose-dependent manner (Fig. 3E). We subsequently found that overexpression of TET1 induced AKR1C1 expression, while the upregulation was inhibited by brusatol treatment (Fig. 3F). In addition, a dot blot assay indicated brusatol potently decreased the level of total DNA hydroxymethylation in Ishikawa cells (Fig. 3G). We next measured the enrichment of 5-hmC in the genetic regions of AKR1C1 using hMeDIP. Results showed TET1 enhanced hydroxymethylation in AKR1C1 promoter region, while
it can be impaired by brusatol (Fig. 3H). To investigate the effect of these regulatory mechanisms on NRF2-mediated progestin resistance and brusatol-induced progestin sensitivity, a cell counting kit-8 (CCK8) assay was performed when Keap1 was overexpressed or TET1 was silenced in Ishikawa-NRF2 cells. Interestingly, either Keap1 overexpression or TET1 knockdown could sensitize endometrial cancer cells to MPA (Fig. 3I). These data suggest that NRF2-TET1-AKR1C1 signal pathway plays a critical role in progestin resistance and brusatol transcriptionally regulates AKR1C1 to reverse the resistance.

**Brusatol sensitizes endometrial cancer to progestin ex vivo and in vivo**

Our above results have shown that brusatol suppressed progestin metabolism through the AKR1C1 inhibition and sensitized endometrial cancer to progestin. We therefore investigated the effects of brusatol on tumor growth in vivo when the mice were subcutaneously injected with Ishikawa and Ishikawa-NRF2 cells. As shown in Fig. 4A, B, tumor growth in nude mice received Ishikawa cell injection was significantly suppressed when it exposed to combined brusatol and MPA, while treatment with MPA or brusatol alone only slightly inhibited tumor growth. While in mice injected with Ishikawa-NRF2 cells, MPA alone showed little effect on tumor growth; however, brusatol in combination with MPA markedly suppressed tumor growth (Fig. 4B). We further used a human endometrial organoid model to re-evaluate this suppression effect. As shown in Fig. 4C, combined treatment with brusatol and MPA dramatically reduced the number of organoids derived from a type I endometrial cancer patient. H&E staining analyses showed that partial of endometrial cancer glands were reversed as normal glands by combined treatment with brusatol and MPA (Fig. 4D). Meanwhile, a declined expression of AKR1C1 was also observed in the brusatol plus MPA group in the organoid model (Fig. 4E). In summary, our data suggested that brusatol can
sensitize endometrial cancer to progestin via downregulating AKR1C1.

Validation of AKR1C1 as a prognostic marker of progestin resistance

We next tried to validate the significance of AKR1C1 in predicting progestin resistance. It is difficult to determine the local metabolism of progestin in human endometrial lesion tissues, but monitoring the progestin metabolism activity indirectly by detecting AKR1C1 expression profile with IHC assay is reasonable. Thirty-four pairs of endometrial tissues collected before and after progestin treatment were evaluated, and 16 patients showed CR, 8 patients showed PR, and 10 patients showed NR. As shown in Fig. 5A, atypical complex hyperplasia endometrial tissues showed

![Graph](image1)

**Fig. 2** Brusatol sensitizes endometrial cancer cell to progestin by enhancing progestin metabolism. A AKR1C1 was silenced in control and Nrf2 overexpressed Ishikawa cells, and a CCK8 assay was performed in the presence of MPA (20 μM). B Overexpression of AKR1C1 increased 20α-dihydroxyprogesterone levels and reduced progesterone levels. p < 0.05 compared with the blank control. C HPLC analysis indicated brusatol blocked progesterone catabolism. The conversion of progesterone to 20α-dihydroxyprogesterone after brusatol treatment was quantified by calculating the area under the curve (right panel). p < 0.05. D Ishikawa cells were transfected with AKR1C1 plasmid before they were treated with brusatol in the presence or absence of progesterone. The 20α-dihydroxyprogesterone (left) and progesterone levels (right) were detected. p < 0.05.
strong AKR1C1 staining, whereas atrophic glands that successfully responded to progestin therapy showed loss of AKR1C1 expression. In cases with PR, we observed normal glands with negative AKR1C1 staining and surrounding hyperplasia glands with strong AKR1C1 expression prior to progestin treatment. After progestin treatment, atrophic glands with negative AKR1C1 staining and the remaining hyperplasia glands with strong staining were observed on the same slide. Moreover, positive AKR1C1 staining was exhibited in tissues pre- and post-progestin treatment from the same patient with poor progestin response, which suggests that AKR1C1 is a potential marker for identifying progestin resistance. The same staining pattern was observed in a case with a PR in which the patient underwent two therapy cycles with progestin; however, the atrophic glands with negative AKR1C1 staining was clearly observed after the second progestin therapy cycle with CR to progestin (Fig. 5B). Interestingly, inverse expression profiles of
AKR1C1 in stromal cells and gland cells have been observed, especially in stromal cells with decidual changes around the atrophic endometrial glands, AKR1C1 was positively expressed (Fig. 5C). These data imply that stromal cells play a role in gland epithelial cell proliferation. The expression patterns of AKR1C1 among patients with different responses to progestin are summarized in Table 2.

DISCUSSION

Women of reproductive age with atypical hyperplasia, endometrial intraepithelial neoplasia, or well-differentiated endometrial cancer have a strong desire to preserve their fertility. Currently, the optimal therapeutic strategy for these women is conservative treatment with a high dose of progestin. However, a low response or resistance to progestin is the main obstacle for successful treatment. Here, we found that brusatol reversed NFR2-driven progestin resistance and suppressed progestin catabolism via AKR1C1 with an epigenetic mechanism (Fig. 6).

Since progestin resistance abrogates the therapy effect of progestin, numerous studies have focused on how it happens. Reduced PR expression is thought to be one of the critical mechanisms of progestin resistance due to desensitization to progestin5. Aberrant expressions of survivin and GloI were illustrated involvement in progestin resistance6,8,10,11. In addition, disordered signaling pathways were also linked to progestin resistance including abnormally activated PI3K/AKT, Fas/FasL and NFR2 signaling7,9,32-35. In current study, we found a novel mechanism which is different from previous reports. An enhanced metabolism of progestin mediated by NFR2-TET1-AKR1C1 axis is associated with the lack of response to progestin. The increase expression of TET1, NFR2 and AKR1C1 resulted in converting more therapeutic progestin to less potent metabolite 20α-dihydroxyprogesterone, and might finally lead to the failure of progestin therapy. This kind of drug-resistant mechanism is different with the well-known functions of NFR2, such as increased oxidative stress, enhanced drug efflux or reduced drug uptake16-18. In last decades, a majority of studies paid more attention to the ligand and ignored the change of progestin itself39-41. Thus, our current study highlights the variation of progestin metabolism and demonstrated it is also a key target for successful progestin therapy.

Brusatol, as a quassinoid natural product, is widely acknowledged as a potent NFR2 pathway inhibitor showing greater than 50% inhibition of luciferase activity at nanomole concentration, while its general effect on translation appears at higher concentration42. Consistently, we found brusatol could significantly inhibit endometrial cancer cell proliferation at nanomole concentration alone or with MPA. Considering NFR2 as an essential role in progestin resistance and the known inhibition of NFR2 by brusatol in progestin resistance6, we further investigated the underlying detailed mechanism of brusatol regulating progestin metabolism mediated by NFR2. Here, the decline expressions of NFR2, TET1 and AKR1C1 by brusatol may attenuate the catabolism of progestin, which in turn results in enhanced suppression on endometrial cellular growth in the presence of MPA.

Functional analysis revealed that AKR1C1 inactivates progesterin by forming 20α-dihydroxyprogesterone, which prompted us to investigate the metabolism of progesterin in precancerous endometrial tissues and endometrial cancer. The main function of progesterin is to regulate the differentiation of endometrial epithelial cells and to limit cell proliferation45,46. Dysregulation of progesterin metabolism may contribute to the formation of endometrial lesion and even lead to the loss of the therapeutic effect of exogenous progesterin. Previous study demonstrated that increased expression of AKR1C1 and AKR1C3 in endometriosis not only decreased expression of progesterone receptor B but also induced production of less active metabolite, 20α-dihydroxyprogesterone47. Similarly, this might be one of another mechanism by which NFR2-mediated progestin resistance.

NFR2, a target molecule of brusatol, contributes to drug resistance in a broad spectrum of cancer cell types via the “dark side” effect. Brusatol is a specific inhibitor of NFR2 and enhances NFR2 degradation via a ubiquitination-dependent pathway48. But how it suppresses endometrial cancer cell proliferation via NFR2 has not been clarified. TET1 has been involved in chemoresistance in endometrial cancer48. Here, we found that the TET1 promoter region contains four AREs, and the first ARE was identified as essential for NFR2-mediated regulation of TET1 expression. Thus, TET1 may serve as a novel NFR2 target gene. This is consistent with our previous findings that overexpression of NFR2 elevated TET1 expression18. Moreover, knocking down TET1 enhanced MPA-induced proliferation inhibition in Ishikawa-NRF2 cells. This suggests that TET1 is required for NFR2 induced progesterin resistance. AKR1C1 has been identified as another NFR2 target gene in previous studies11,12. In this study, we found TET1 is transcriptionally regulated by NFR2 and AKR1C1 is upregulated by NFR2-TET1 via an increase of hydroxymethylation level in the promoter region. However, this kind of epigenetic modification can be erased by brusatol. Briefly, the two different mechanisms of NFR2 regulating AKR1C1 are as follows: (1) direct transcriptional regulation by NFR2 through ARE element located at the AKR1C1 promoter; (2) indirect NFR2-AKR1C1 interaction mediated by hydroxymethylation by TET1. We speculate the above two mechanisms contribute equally to brusatol attenuating endometrial cancer resistance to progesterin by inhibiting NFR2, which remains to be verified in future studies.

We found that brusatol significantly suppressed AKR1C1 expression and blocked AKR1C1-mediated progestin metabolism. In addition, brusatol combined with MPA potently reduced AKR1C1 expression in the human endometrial cancer organoids. Thus, blocking progesterin metabolism may be the molecular mechanism by which brusatol sensitizes endometrial cancer cells to progestin. Detection the local metabolism of progesterin in endometrial lesion tissue is difficult; however, the expression profile of AKR1C1 may represent the metabolic activity of progesterin, to a certain extent. IHC assay indicated that AKR1C1
was strongly expressed in paired tissues from the same individual with progestin resistance regardless pre- or post-progestin treatment. Conversely, in the cases with good response to progestin, high level of AKR1C1 expression disappeared in atrophic glands underwent progestin administration compared with that of pre-treatment. These data indicated that AKR1C1 is a good marker for identifying patients with a poor response to progestin. Interestingly, an inverse expression pattern of AKR1C1 between stromal and glandular epithelial cell has been observed. AKR1C1 is overexpressed in the stromal cells compared with around atrophic glands in patients with a good response to progestin, whereas loss of AKR1C1 is observed in stromal cells compared with the hyperplasia or cancer glands in the patients with progestin resistance. Previous studies demonstrated that endometrial stromal cells contribute to endometrial regeneration, repair and inhibit endometrial epithelial cell growth by secreting growth factors or hormones.\textsuperscript{47–52} Therefore, we consider that it is no longer necessary to maintain a high level of progestin to limit glandular epithelial cell excessive growth in the atrophic glands, if it shows a well response to progestin treatment. An increased AKR1C1 expression in stromal cells could guarantee a low level of progestin by enhancing progestin catabolism. By contrast, in the

**Fig. 4**  **Ex vivo and in vivo effects of brusatol-mediated reversal of progestin resistance.**  
**A** Representative pictures of the xenograft tumor subjected to the indicated treatments. **B** The final volume of the xenograft tumors was estimated at the time of sacrifice. *p < 0.05. **C** Numbers of organoids after indicated treatments were shown. *p < 0.05, ***p < 0.001 when compared with the control. These organoids were collected for H&E staining (D) and IHC assay against AKR1C1 (E). Bru represents brusatol. *p < 0.05, ***p < 0.001 when compared with the control.
progestin-resistant cases, the lack of progestin catabolism due to loss AKR1C1 in these stromal cells resulted in a high level of progestin to suppress hyperplasia glandular epithelial cellular growth with a compensatory manner.

However, there are some limitations in this work that could be addressed in the future studies. Firstly, brusatol, as a general protein inhibitor, might improve progestin resistance through other non-NRF2 dependent pathways besides our findings. Meanwhile, there may be some other NRF2 specific inhibitors such as Halofugionne, or unknown compounds potentially promoting progestin effect by NRF2, to be further investigated.

In conclusion, our findings elaborate the mechanisms of brusatol sensitizing endometrial cancer to progestin by suppressing progestin metabolism mediated by NRF2-TET1-AKR1C1 pathway, providing a novel insight into progestin resistance. And we confirm that AKR1C1 may be a useful biomarker for progestin-resistant cases.

### Table 2. Comparisons of AKR1C1 expression among responders, partial responders and non-responders to progestin therapy.

|                     | Before progestin treatment | After progestin treatment | p values |
|---------------------|----------------------------|---------------------------|----------|
| Complete response (CR) | Mean (range)               | Mean (range)              | <0.05    |
| Index in epithelial cell | 144 (40–300)              | 38 (0–100)                |          |
| Index in stromal cell | 10 (0–30)                  | 174 (90–240)              | <0.001   |
| Partial response (PR) |                           |                           |          |
| Index in epithelial cell | 83 (5–160)                | 120 (70–200)              | 0.7000   |
| Index in stromal cell | 10 (0–20)                  | 30 (10–60)                | 0.5000   |
| No response (NR)     |                            |                           |          |
| Index in epithelial cell | 150 (100–200)            | 232 (90–300)              | 0.1429   |
| Index in stromal cell | 4 (0–15)                  | 10 (10–70)                | <0.05    |

Student’s t test.
predicting progestin resistance in the treatment of endometrial atypical hyperplasia and endometrial cancer.

DATA AVAILABILITY
The data used and analyzed during the current study are available from the corresponding author on reasonable request.

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