Brusatol Sensitizes Endometrial Hyperplasia and Cancer to Progestin by Suppressing Nrf2-Tet1-AKR1C1-Mediated Progestin Metabolism

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Research

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Abstract

Background

Progestin resistance is the main obstacle for the conservative therapy to maintain fertility in women with endometrial hyperplasia and cancer. Up to 30% of endometrial cancers fail to respond to progestin, a rate that has not significantly changed due to the lack of a detailed understanding of progestin resistance.

Methods

Cell Counting Kit-8 (CCK-8) assays were used to detect the synergistic effects of brusatol in combination with progestin. Using commercial kits, the conversion of progestin to 20α-dihydroxyprogesterone following btusatol treatment or AKR1C1 silence was investigated. The correlation betwwen AKR1C1 expression profile and prgestin response was further analyzed in paired endometrial hyperplasia and cancer samples from the same individuals before and after progestin therapy. The effects of brusatol-mediated reversal of progestin resistance was explored in both mouse xenograft and human organoid models. DNA dot blot, HMeDIP, and dural-luciferase reporter assays were performed to uncover the mechanism through which brusatol inhibits AKR1C1 and sensitizes endometrial hyperplasia and cancer to progestin.

Results

Brusatol sensitizes endometrial cancer cell to progestin by downregulating the expression of Nrf2 and its target AKR1C1. Increased AKR1C1 facilitated production of 20-α-dihydroxyprogeserone and was associated with declined progesterone. Suppression of AKR1C1 by brusatol resulted in decreased progesterone catabolism and maintained potent progesterone to inhibit endometrial cancer growth. Aberrant overexpression of AKR1C1 was found in paired endometrial hyperplasia and cancer samples from the same individuals with progestin resistance, whereas attenuated or loss of AKR1C1 was observed in post-treatment samples with well progestin response as compared with paired pre-treatment tissues. Tet1 was identified as a novel Nrf2 target gene. It in turn upregulated AKR1C1 expression by increasing hydroxymethylation levels in its promoter regions.

Conclusions

We found that Nrf2-Tet1-AKR1C1 axis plays an essential role in progestin resistance, and brusatol sensitizes endometrial hyperplasia and cancer to progestin by suppressing Nrf2-Tet1-AKR1C1-mediated progestin metabolism. Our findings suggest that AKR1C1 expression pattern may serve as an important biomarker of progestin resistance in endometrial hyperplasia and cancer .

Background

Epidemiologic studies have revealed that atypical hyperplasia or endometrial intraepithelial neoplasia and well-differentiated cancer tend to occur in younger women [1, 2]. These women, especially those of

child-bearing age, have a strong desire to maintain their fertility. For these individuals, conservative management with progestin is the optimal choice. However, approximately 30% of patients develop progestin resistance, and there are no current effective therapeutic strategies to overcome this obstacle.

In past decades, several mechanisms have been proposed to explain progestin resistance, such as deficiency of progestin receptor (PR) and downregulation of ERα expression, aberrant survivin expression as well as increased TGF-EGFR signaling [3–8]. 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 drug resistance [9, 10]. However, as a transcript factor, how it involves in progestin resistance is poorly understood.

AKR1C1 is well characterized as a target gene of Nrf2 [11, 12]. Increased AKR1C1 expression has been described in a previous study and might be associated with the pathological progression of endometrial cancer [13, 14]. AKR1C1 functions as 20α-hydroxysteroid dehydrogenase and inactives progesterone by forming 20α-dihydroxyprogesterone, a metabolite with weak progestin function [15, 16]. In this reaction, AKR1C1 exhibits a high catalytic efficiency with a K_M of 5.7 μM and a K_{cat} 0.93/min [17]. Although AKR1C1 has been reported to diminish the protective effect of progesterone by inactivating progesterone in diseased endometrium[13], whether AKR1C1 is associated with progestin resistance in endometrial hyperplasia and cancer is not clear. Base on these findings, it is interesting to identify whether AKR1C1-mediated conversion of therapeutic potent progestin to less active 20α-dihydroxyprogesterone is responsible for Nrf2-driven progestin resistance.

In our previous study, we demonstrated that Tet1-dependent DNA hydroxymethylation contributes to elevated Nrf2 expression in endometrial cancer [18]. Meanwhile, the parallel increases in levels of Tet1 and Nrf2 have been observed in progressive endometrial lesions. Tet1 is an important components of the ten-eleven translocation 5-methylcytosine dioxygenase family, which is responsible for the conversion of 5-mC to 5-hmC [19]. Aberrant expression of Tet1 is associated with the development of multiple types of cancer [20, 21]. Therefore, the role of Tet1 in Nrf2-driven progestin resistance need to be further clarified.

Brusatol was first discovered by Ren D. 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 expression via ubiquitination-dependent degradation [22]. Many studies showed that the inhibitory activity of brusatol is not restricted to Nrf2 ; it can also rapidly and potently decrease the expression of sevral other proteins including HIF-1α, p38, STAT3 and SQSTM1 [23–25], which implies that brusatol is a global protein synthesis inhibitor [26–28]. Despite ubiquitination-dependent degradation is an important manner for brusatol to suppress protein expression, increasing evidence illustrates 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.

Here, we present data that Nrf2-Tet1-AKR1C1 axis plays an essential role in progestin resistance. AKR1C1 was identified as a key scavenger of progestin and a mediator of Nrf2-Tet1 driven progestin resistance. Aberrant expression of AKR1C1 was observed in endometrial hyperplasia and cancer patient samples with poor progestin response, which suggests that AKR1C1 is a specific marker to identify progestin resistance. Moreover, 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. Ishikawa cells are derived from a well-differentiated endometrioid adenocarcinoma, so they were used to illuminate some of the molecular mechanisms underlying progestin resistance as an in vitro model of hyperplasia. HEK-293 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in DMEM:F12 medium (1:1, GIBCO) containing 10% fetal bovine serum (FBS; 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% CO2.

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

To investigate the roles of Nrf2 and AKR1C1 in progestin resistance, stable cell lines with Nrf2/AKR1C1 overexpression or AKR1C1 depletion were established using a retrovirus system as described previously [9, 10]. Transient transfection of the indicated plasmids or siRNA was performed with Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA). The cells were treated with progestin or brusatol either alone or in combination for 48 hours, and proliferation was measured with a CCK8 assay.

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 harvested after various treatments and lysed with RIPA buffer to extract total protein. After the protein concentration was quantified, 50 μg of protein per lane was loaded onto an SDS-polyacrylamide gel, electrophoresed and transferred to PVDF membranes, which were incubated overnight with primary antibodies against Nrf2 and Tet1 (Sigma Aldrich); α-tubulin (Abcam); and AKR1C1, AKR1B10, GCLM, NQO1, GAPDH and HO-1 (Santa Cruz Biotechnology). After the membranes were washed and treated 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

A dot blot assay was carried out as previously described. After conducting the indicated treatments, 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 10% skim milk for 1 hour before it was incubated with 5hmC primary antibody (1:500 dilution, Active Motif) overnight at 4°C. After the membrane was washed and treated with HRP-conjugated secondary antibody, it was subjected to ECL and scanned to visualized bound antibodies. Methylene blue (MB) 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 sampled with 100 ng DNA was analyzed by one-way analysis of variance (ANOVA). Endometrial cancer cells were transfected with pPB-Tet1 plasmid and subjected to brusatol treatment, after which total DNA was extracted and sheared via ultrasonication. 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.

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 [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 were treated with various drugs were harvested and lysed to determine the 20α-dihydroxyprogesterone concentration with analysis kits (XQ-EN15767, Xinquan Company, Shanghai, China).

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. The patients' clinical information is listed in Table 2. The pathological diagnosis of endometrial hyperplasia or well-differentiated carcinoma 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: no response 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; partial response, no residual hyperplasia/endometrial intraepithelial neoplasia (EIN) 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 complete response, attenuated endometrial glands with decidualized stroma. The IHC assay was performed as previously described [6, 9, 18].

Human endometrial organoids culture

Human endometrial organoids culture was carried out as previous report [29]. Briefly, fresh hyperplasia endometrial biopsies or endometrial cancer tissues were collected, followed by enzymatically digested and centrifugated. The pellets were resuspended in diluted ice-cold Matrigel medium mix. Fifty-microlitre drops of Matrigel–cell suspension were plated into 24-well plate and overlaid with organoid Expansion Medium (ExM) . The medium was changed every 2–3 d. Cultures were passaged by manual pipetting every 7–10 d. After various treatments with drugs, the organoids were collected and fixed with 4% paraformaldehyde on ice, followed by resuspending and embedding the organiods with 3% low melting point agarose for H&E and IHC analysis.

In vivo xenograft mouse model

Nude mice were subcutaneously injected with 1×10^6 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 among multiple groups were made with one-way ANOVA followed by Dunnett'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 by downregulating the expression of Nrf2 and its downstream genes

To detect whether brusatol enhances progestin sensitivity, we first determined the effect of brusatol on cell proliferation in endometrial hyperplasia and cancer cells. As shown in Figure 1a, brusatol dramatically suppressed cellular growth in a dose-dependent manner, which paralleled the Nrf2 and its relative gene expression profiles. Meanwhile, brusatol significantly suppressed the expression of Nrf2 and the relative genes in a time-dependent manner (Fig. 1b). The combined effect on cellular growth in Figure 1c has confirmed that compared with brusatol or MPA alone, brusatol combined with MPA markedly suppressed the growth of endometrial cancer cells. To further define that Nrf2 signaling plays an essential role in progestin resistance, Ishikawa-Nrf2 cells were established by overexpression of Nrf2 and monitored its response to progestin. As expected, the lack of response to progestin has been observed

compared with Ishikawa-vector cells (Fig. 1d). The corresponding Nrf2 signaling proteins and relative Tet1 has been determined by western blot in both cell lines, as shown in Figure 1e, MPA obviously 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 Tet1 and AKR1C1 (Fig. 1e).

Brusatol sensitizes endometrial cancer cell to progestin by impairing Nrf2-AKR1C1 mediated progestin metabolism

To assess the mechanism through which brusatol reversed progestin resistance, the association between Nrf2 and AKR1C1 has been defined firstly. As shown in Supplementary Figure 1, several AREs has been found in AKR1C1 promoter region, which implies that it is a Nrf2 target gene as previously reported [11, 12]. Indeed, its expression profile paralleled with other Nrf2 target genes in the presence of tBHQ, an Nrf2 inducer (Fig. 2a). 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. 2b), which suggests AKR1C1 mediated Nrf2-driven progestin resistance. Next, we tried to figure out whether progestin metabolism alteration by AKR1C1 plays an essential role in the failure responsing to progestin. We detected the conversion of progestin to 20α-dihydroxyprogesterone. Overexpression of AKR1C1 resulted in an increase of 20α-dihydroxyprogesterone and reduced levels of progesterone in cell lysates in a dose-dependent manner (Fig. 2c, d). Thus, we speculate that reduced progestin metabolism activity and retained progestin levels due to silencing of AKR1C1 may contribute to increased sensitivity to progestin as we observed above (Fig. 2b). Therefore, targeting AKR1C1 and progestin metabolism is a novel therapy strategy. As shown in Figure 2e, brusatol, as a specifical inhibitor of Nrf2, also inhibitted the expression AKR1C1 in both Ishikawa and Ishikawa-Nrf2 cells. Consistent with this result, the increased 20α-dihydroxyprogesterone and reduced progestin by overexpression of AKR1C1 were effectively blocked by brusatol treatment (Fig. 2f, g). 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 the clinical informations were listed in Table 2. As shown in Figure 2h and Supplementary Figure 2A-C, atypical complex hyperplasia (ACH) endometrial tissues showed strong AKR1C1 staining, whereas atrophic glands that successfully responded to progestin therapy showed loss of AKR1C1 expression. In cases with partial response, we observed normal glands with negative AKR1C1 staining and around 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 cases with a partial response in which the patients underwent two therapy cycles with progestin; however, the atrophic glands with negative AKR1C1 staining was clearly observed after the second progestin therapy cycle with complete response

to progestin (Fig.S2D). 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 that showed positive AKR1C1 expression (Fig.S2E); 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 3.

In vivo effects of brusatol sensitizes precancerous endometrial lesions and endometrial cancer to progestin

Our above results have shown that brusatol suppressed progestin metabolism through the Nrf2-AKR1C1 signaling axis and sensitizes endometrial cancer to progestin. We therefore investigated the effects of brusatol on tumor growth in vivo when the mices were injected with Ishikawa and Ishikawa-Nrf2 cells. As shown in Figure 3a and b, the growth of tumors in nude mice was significantly suppressed regardless injection with Ishikawa or Ishikawa-Nrf2 cells when it exposed to both brusatol and MPA, while treatment with MPA or brusatol alone only slightly inhibited tumor growth (Fig. 3b). We further used a human endometrial organoids model to re-evaluate this suppression effect. As shown in Figure 3c, combined treatment with brusatol and MPA dramatically reduced the number of organoids derived from a type I endometrial cancer patient. Immunohistochemistry analyses showed that partial of endometrial cancer or hyperplasia glands were reversed as normal glands by combined treatment with brusatol and MPA (Fig. 3d). Meanwhile, a declined expression of AKR1C1 was also observed in the brusatol plus MPA group in the organoid model (Fig. 3e). In sum, our data suggest that brusatol can sensitize precancerous endometrial lesions and endometrial cancer cells to progestin via the Nrf2-AKR1C1 signaling axis.

Suppression hydroxymethylation of AKR1C1 through declined Tet1 contributes to brusatol-enhanced progestin sensitivity

Methylcytosine dioxygenase Tet1 is involves in the epigenetic gene regulation. It catalyzes the conversion of 5-mC to 5-hmC, leading to hydroxymethylation and methylation changes in the promter region. We have demonstrated Tet1-mediated hydroxymethylaiton involves in regulation of Nrf2 transciptional activity. Therefore, we speculated Tet1 may play a role in Nrf2-driven progestin resistance. As shown in Figure 4a, elevated Nrf2 enhanced Tet1 and AKR1C1 expression, and this upregulation can be blocked by brusatol in a dose-dependent manner. We subsequently found that overexpression of Tet1 induced AKR1C1 expression, while the upregulation was inhibited by brusatol treatment (Fig. 4b). In addition, dot blot assay indicated brusatol potently decreased the level of total DNA hydroxymethylation in Ishikawa cells (Fig. 4c). We next measured the enrichment of 5-hmC in the genetic regions of AKR1C1 using hydroxymethylated DNA immunoprecipitation (hMeDIP). Results showed Tet1 enhanced hydroxymethylation in AKR1C1 promoter region, while it can be ameliorated by brusatol (Fig. 4d). To investigate the effect of these regulatory mechanisms on Nrf2-mediated resistance to progestin and brusatol-enhanced progestin sensitivity, a 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. 4e). These data suggest that Nrf2-Tet1-AKR1C1

signal pathway plays a critical role in progestin resisance and is a critical target for brusatol to reverse the resistance.

Nrf2 promotes Tet1 expression by binding AREs in the Tet1 promoter region

In our previous study, similar expression profiles between Nrf2 and Tet1 have been detected by IHC in consecutive sections of endometrials tissue samples from hyperplasia, to EAH, progressed to endometrial carcinoma[18]. We thereby further evaluate the possible regulation relationship between both proteins. The parallel expression patterns between Tet1 and Nrf2 in response to brusatol implies that Nrf2 may play an essential role in regulating Tet1 expression (Fig. 1a and b, Fig. 4a). To further confirm that Tet1 is also a target gene of Nrf2, ectopic Nrf2 was transfected into Ishikawa and ECC1 cells, and the expression pattern of Tet1 and other Nrf2 targeting genes was estimated. As shown in Figure 5a, 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. 5b). 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. 5c). Meanwhile, it was found that Tet1 also bears four AREs in the promoter region within 5000 bp from the transcription start site (Figure S3). To identify which ARE is necessary for Nrf2 to regulate Tet1, four truncations targeting the indicated AREs were constructed (Fig. 5d). The dual luciferase reporter assay revealed that each truncation could respond to Nrf2 as long as it contains the ARE1 sequence (Fig. 5e). Furtherly, it was found that Nrf2 transfection could enhance the luciferase activity in wild ARE1 report plasmid, whereas has little effect on mutant ARE1 report plasmid, which implys that ARE1 plays an essential role in Nrf2-driven Tet1 overexpression.

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 conservative treatment. Previously, we demonstrated that brusatol, a specific inhibitor of Nrf2, could sensitize endometrial cancer cells to progestin; however, the detailed molecular mechanism are still not completely understood. Here, we found that Nrf2/Tet1 signaling enhanced progestin metabolism via AKR1C1 with a epigenetic mechanism, which resulted in progestin resistance(Fig. 6). This also provided a possible critical target for brusatol to reverse progestin resistance.

Since progestin resistance abrogates the therapy effect of progestin, numerous studies focus on the mechanism how it happens. Reduced PR expression is thought to be one of the critical mechanisms of progestin resistance due to desensitization to progestin [4]. Aberrant expressions of survivin and GloI were illustrated involvement in progestin resistance [3, 8, 30, 31]. In addition, disordered signaling pathways were

also linked to progestin resistance including abnormally activated PI3K/AKT, Fas/FasL and Nrf2 signaling [7, 9, 32–35]. In current study, we found a novel mechanism mediated by Nrf2 which is different from previous reports. An enhanced metabolism of progestin mediated by Nrf2-Tet1-AKR1C1 axis is associated with the lack of response to progestin. The increase expressions of Tet1, Nrf2 and AKR1C1 resulted in more therapeutic progestin to convert to less potent metabolite 20α-dihydroxyprogesterone, an inactive form of progestin, and might finally lead to the failure of progestin therapy. This kind of drug-resistant mechanism is different with the well-known functions of Nrf2, such as increased oxidative stress, enhanced drug efflux or reduced drug uptake [36–38]. In last decades, majority of studies pay more attention to the ligand and ignore the change of progestin itself [39–43]. Thus, our current study highlights the variation of progestin metabolism and demonstrated it is also a key target for successful progestin therapy. Previously, we found that brusatol could reverse progestin resistance in endometrial precancer and cancer cells through inhibition of Nrf2 expression [9], but the underlying mechanism is not completely understood. Here, the decline expressions of Nrf2, Tet1 and AKR1C1 by brusatol may attenuate the catabolism of progestin, which in turn result in enhanced suppression on cellular growth in the presence of MPA.

Functional analysis revealed that AKR1C1 inactivates progestin by forming 20α-dihydroxyprogesterone, which prompted us to investigate the metabolism of progestin in precancerous endometrial tissues and endometrial cancer. To our knowledge, the critical oncogenic mechanism driving the formation of type I endometrial cancer from simple, complex or atypical hyperplasia is persistent estrogen stimulation without an opposing effect from progestin. The main function of progestin is to regulate the differentiation of endometrial epithelial cells and limit cell proliferation [44, 45]. Dysregulation of progestin metabolism may contribute to the formation of endometrial lesions and even lead to the loss of the therapeutic effect of exogenous progestin. In the current study, AKR1C1 overexpression enhanced progestin catabolism and formed less active 20α-dihydroxyprogesterone in endometrial cancer cells. 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-dihydroxyprogesterone, which have lower affinities towards the progesterone receptors, the double side effects further contribute to this disease [16]. Similarly, this might be one of the another mechanisms by which Nrf2-mediated progestin resistance.

Since AKR1C1 plays an essential role in progestin resistance, targeting this gene is a possible therapeutic strategy for reversing progestin resistance. We found that brusatol significantly suppressed AKR1C1 expression and blocked AKR1C1-mediated progestin metabolism. In addition, brusatol combined with MPA not only potently reduced the number but also inhibited AKR1C1 expression in these human endometrial cancer organoids. Thus, blocking progestin metabolism may be the main molecular mechanism by which brusatol resensitizes endometrial cancer cells to progestin by Nrf2-Tet1-AKR1C1 signaling. Detect the local metabolism of progestin in endometrial lesion tissue is difficult, however, the expression profile of AKR1C1 may represent the metabolic activity of progestin, to a certain extent. IHC assay discovered that AKR1C1 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 clearly indicate that AKR1C1 is a good marker for identifying patients with a poor response to progestin. Interesting, it exhitted an inverse expression pattern of AKR1C1 between stromal and glandular epithelial cell. 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 [46–51]. Therefore, we consider that the possible explanation behind this phenomenon is 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. So, an increase AKR1C1 expression in stromal cells could guarantee a low level of progestin by enhancing progestin catabolism. By contrast, in the 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.

Nrf2, a target molecule of brusatol, contributes to drug resistance in a broad spectrum of cancer cell types via the “dark side” effect. Brusatol is functional as a specific inhibitor of Nrf2 and enhances Nrf2 degradation via an ubiquitination-dependent pathway [22]. But how it facilitates suppression of endometrial cancer cell proliferation via Nrf2 has not been clarified. Tet1 has been involved in chemoresistance in endometrial cancer with a parallel Nrf2 expression pattern [18]. Mechanistic studies also revealed that overexpression of Nrf2 elevated Tet1 expression. Here, we found that the Tet1 promoter region contains four AREs, and the first ARE was identified as essential for Nrf2-mediated regulation of Tet1 expression. Thus, Tet1 may serve as a novel Nrf2 target gene. This is consistent with our previous findings that overexpression of Nrf2 elevated Tet1 expression [18]. Moreover, knocking down Tet1 enhanced MPA-induced proliferation inhibition in ishikawa-Nrf2 cells. This suggests that Tet1 is required for Nrf2 induced progestin resistance. AKR1C1 has been identified as another Nrf2 target gene in previous studies [11, 12], and we found that it contains six AREs within its promoter region. Whether AKR1C1 involves in Nrf2-Tet1 mediated progestin resistance has not been revealed in previous study. In current study, it was found AKR1C1 has been upregulated by Tet1 via hydroxymethylation mechasnism. However, this kind of epigenetic modification can be erased by brusatol. It implies that the lack of hydroxymethylation due to downregulation of Tet1 contributes to brusatol-enhanced progestin sensibility in endometrial cells.

Conclusions

In this study, we found that Nrf2/Tet1/AKR1C1-mediated dysfunction of progestin metabolism is a possible molecular mechanism of progestin resistance. Downregulating Nrf2, Tet1 and AKR1C1 expression and attenuating progestin metabolism via brusatol treatment may prove to be useful to

overcome progestin therapy failure. Our findings provide a novel insight into progestin resistance and confirm that AKR1C1 may be a useful biomarker for predicting progestin resistance.

Abbreviations

Tet1: tet methylcytosine dioxygenase 1; Nrf2: nuclear factor erythroid 2-related factor 2; AKR1C1: aldo-keto reductase family 1 member C1; PR: progestin receptor; MPA: medroxyprogesterone acetate; HMeDIP: hydroxymethylated DNA immunoprecipitation; CCK-8: Cell Counting Kit-8; ACH: atypical complex hyperplasia.

Declarations

Ethics approval and consent to participate

Human endometrial hyperplasia and cancer tissue samples were obtained from Shanghai General Hospital Affiliated to Shanghai Jiaotong University and Shanghai First Maternity and Infant Hospital Affiliated to Tongji University School of Medicine. Patients informed consent was obtained. All animal studies were approved by the Animal Ethics Committee of Shanghai General Hospital experimental protocols.

Consent for publication

Not applicable.

Availability of data and material

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

This study was conceived by Z.B., S.W., and J.L.; Z.B.and M.H. designed the study; M.H., D.S., J.Y.,Y.F.,Z.Q.,B.H.,and Q.Z. performed experiments; X.C.,Y.W.,H.Z.,Y.W.,Y.J.F,W.Z., and H.L.provided reagents and conceptual advice; Y.J.F. and H.L.provided reagents;Z.B.,J.L.and M.H. wrote the paper with comments from all authors.

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Tables

Table 1. Primers for amplification of the squence on AKR1C1 promoter region in the HMeDIP assay

Primer No.	Primer sequence
Primer 1	Forward 5'- GATTTCTTGTTTCCTTGTATGCC -3'
	Reverse 5'- CAACAACAACAAATAGTCGG -3'
Primer 2	Forward 5'- GGCAGGTTCTCAGTCAAGGC -3'
	Reverse 5'- AGCAATTCAAAAGCCATTGG -3'
Primer 3	Forward 5'- CACTGCAAGTTGTGACTACT -3'
	Reverse 5'- TGGTGAATAATCCTCGCATG -3'
Primers 4	Forward 5'- CCGCTAGAGGTTTCTGTATT -3'
	Reverse 5'- GTGGTCATGATACACTCATT-3'

Table 2. Endometrial cancer/precancer patients treated with progestins

Case no.	Age (years)	Progestin dose	Treatment duration (months)	Diagnosis	Findings after progestin treatment
CR					
1	30	MPA 160 mg	3	ACH	Progestin effects
2	33	MPA 160 mg	2	CH	CH, SM
		Diane-35, LNG-IUS	3	CH	Progestin effects
3	34	MPA 160 mg	4	ACH	Progestin effects
4	28	MPA 160 mg	3	ACH	ACH, SM
		MPA 160 mg	3	ACH	Focal CH, SM, Progestin effects
		Drospirenone 3 mg	3	ACH	Progestin effects
5	49	MPA 160 mg	3	ACH	Progestin effects
6	29	MPA 160 mg	3	ACH	Progestin effects
7	37	MPA 160 mg	7	ACH	Focal ACH, SM, Progestin effects
		MPA 160 mg	3	ACH	Progestin effects
8	38	Progesterone 100 mg	3	ACH	ACH
		MPA 80 mg	6	ACH	Progestin effects
9	33	Drospirenone 3 mg	4	ACH	Focal ACH, SM, Progestin effects
		MA 160 mg	3	ACH	Progestin effects
10	26	MA 160 mg	3	ACH	Progestin effects
11	42	MPA 80 mg	3	SH, focal ACH	Progestin effects
12	32	MPA 160 mg+Progesterone 200 mg	3	CH	Progestin effects
13	24	Norethindrone 5 mg	3	CH	Focal CH, SM, Progestin effects
			3	CH	Progestin effects
14	29	MPA 160 mg	3	ACH	ACH, SM
		MA 160 mg	3	ACH	Progestin effects
15	28	MPA 160 mg	3	ACH	ACH, SM
		MPA 160 mg	3	ACH	Progestin effects
16	28	MPA 80 mg	6	ACH	Progestin effects

PR		MPA 80 mg		SH, focal CH	
1	25	MA 160 mg	3		Focal crowded glands with an irregular shape, SM, Progestin effects
2	25		3	ACH	
3	30	MPA 160 mg	3	CH	Focal crowded glands, SM, proliferative endometrium
4	30	MPA 160 mg	4	ACH	Focal disordered proliferative glands, Progestin effects
5	40	MPA 160 mg	1	ACH	
6	42	MA 160 mg	2	ACH	Dilated and irregular glands are present, Progestin effects
7	33	MA 80 mg	3	ACH	Focal crowded glands with an irregular shape, SM, Progestin effects
8	39	MPA 160 mg	40 days	CH, focal atypia	
NR		MPA 80 mg		ACH	Dilated and irregular glands are present
1	30	Metformin 160 mg	2		Focal crowded glands, SM, Progestin effects
2	35		3	ACH	
		MPA 160 mg	3	ACH	Focal crowded glands, SM, Progestin effects
3	39	MPA 160 mg	2	ACH	
4	33	MPA 160 mg	3	ACH	ACH
		MPA 160 mg		ACH	ACH
5	30	MPA 40 mg+Metformin 500 mg	3	ACH	ACH
6	28	MPA 160 mg	3	ACH	ACH, Proliferative endometrium
7	26	MA 160 mg	3	ACH	Focal ACH, SM, Progestin effects
8	35	MA160 mg	3	ACH	ACH, SM
		MA160 mg	3	ACH	Focal ACH, SM, Progestin effects
9	25	MA160 mg	3	ACH	Progestin effects
10	46	MPA 160 mg	80 days	CH	Focal ACH, SM, Progestin effects
		MA160 mg		CH	ACH
		MA80 mg			Focal ACH, SM, Progestin effects
					Focal CH, SM, Progestin effects
					Focal CH, SM, Progestin effects

Table 3. Comparisons of AKR1C1 expression among responders, partial responders and nonresponders to progestin therapy

	Before progestin treatment Mean (range)	After progestin treatment Mean (range)	P-values
CR			
Epithelial cell index	144 (40-300)	38 (0-100)	<0.05
Stromal cell index	10 (0-30)	174 (90-240)	<0.001
PR			
Epithelial cell index	83 (5-160)	120 (70-200)	0.7000
Stromal cell index	10 (0-20)	30 (10-60)	0.5000
NR			
Epithelial cell index	150 (100-200)	232 (90-300)	0.1429
Stromal cell index	4 (0-15)	10 (10-70)	<0.05

Student t-test.

Figures

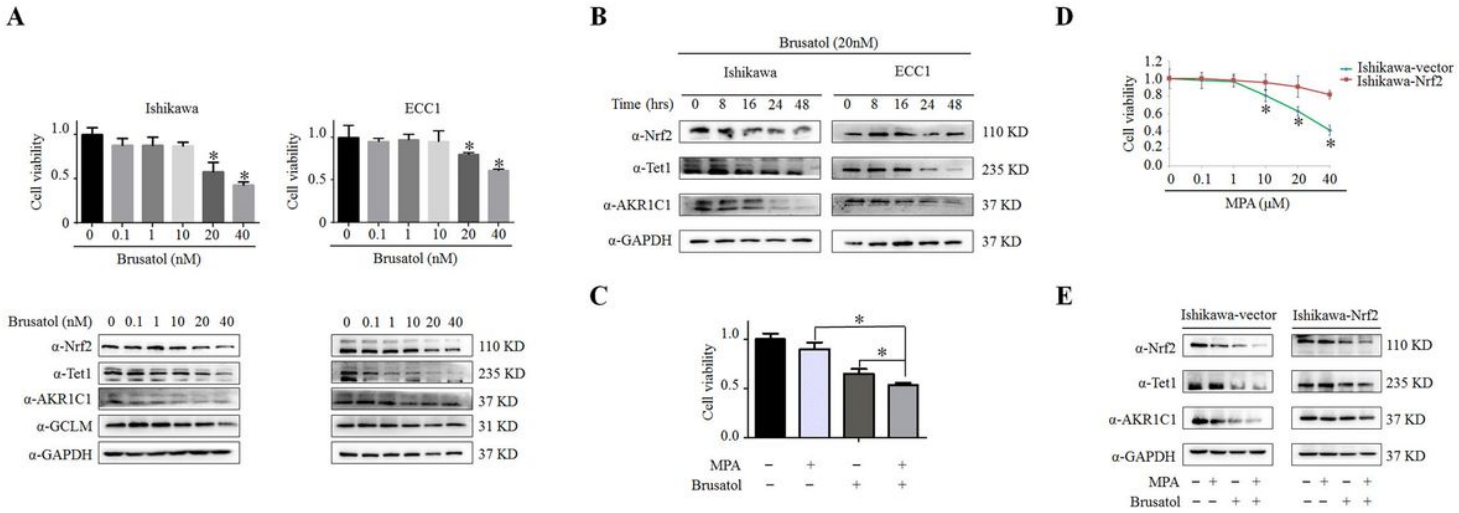

Figure 1

Brusatol sensitizes endometrial cancer cell to progestin by downregulating the expression of Nrf2 and its downstream genes. A, Ishikawa and ECC1 cells were treated with the indicated doses of brusatol for 48 hours, and cell viability was estimated by the CCK8 assay (upper pannel). The expression of Nrf2 was detected by western blot (lower pannel). B, Both cell lines were treated with 20 nM brusatol for the

indicated time and the expression levels of Nrf2 and its downstream proteins were analyzed by immunoblotting. C. Endometrial cancer cells were treated with the MPA (20 μM) or brusatol (20 nM) alone, or combined MPA and brusatol for indicated time, and the proliferative activity was determined by the CCK8 assay. D, A stable cell line with high levels of Nrf2 was established, and its response to progestin was evaluated. *p<0.05. E, The expression patterns of Nrf2, Tet1 and AKR1C1 were detected by immunoblotting when the parental or Nrf2 stably transfected Ishikawa cells were exposed to progestin or brusatol either alone or in combination.

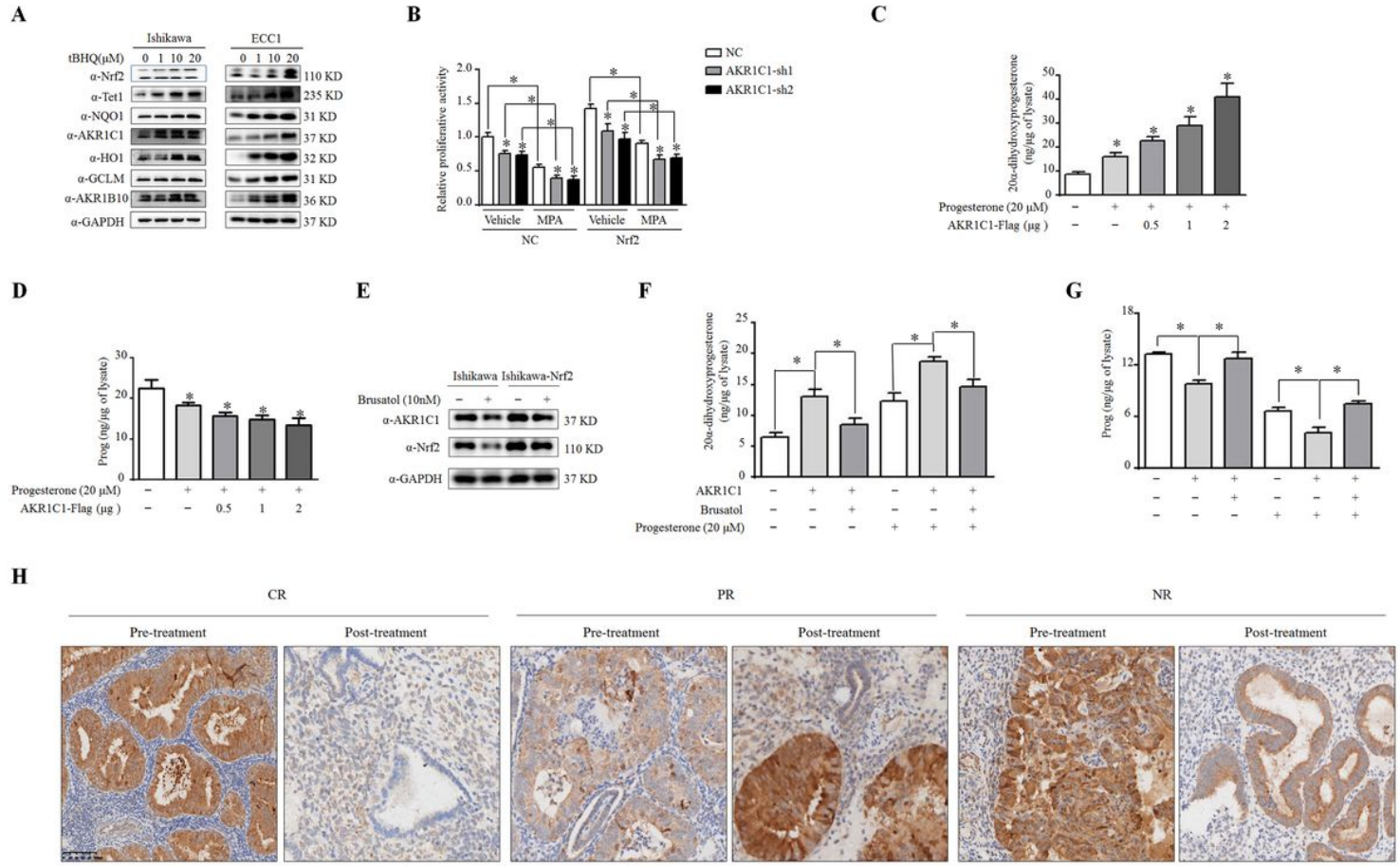

Figure 2

Brusatol sensitizes endometrial cancer cell to progestin by enhancing progestin metabolism via Nrf2-AKR1C1 signal pathway. A, tBHQ, an Nrf2 inducer, increased the expression of AKR1C1 and Nrf2-related proteins. B, AKR1C1 was silenced in control and of Nrf2 overexpresed Ishikawa cells, and a CCK8 assay was performed in the presence of MPA (20 μM). Overexpression of AKR1C1 increased 20α-dihydroxyprogesterone levels (C) and reduced progesterone levels (D). E, Brusatol inhibited Nrf2 and AKR1C1 in both Ishikawa and Ishikawa-Nrf2 cells. Meanwhile, Ishikawa cells were transfected with AKR1C1 plasmid before they were treated with brusatol in the presence or absence of progesterone. The 20α-dihydroxyprogesterone (F) and progesterone levels (G) were detected. *p<0.05. H, Aberrant expression of AKR1C1 in endometrial tissues before and after progestin treatment. IHC assay was used to determine AKR1C1 expression in the patients with different responses to progestin as follows: CR, complete response ; PR, partial response; NR, no response. Original magnification in this panel is 200×.

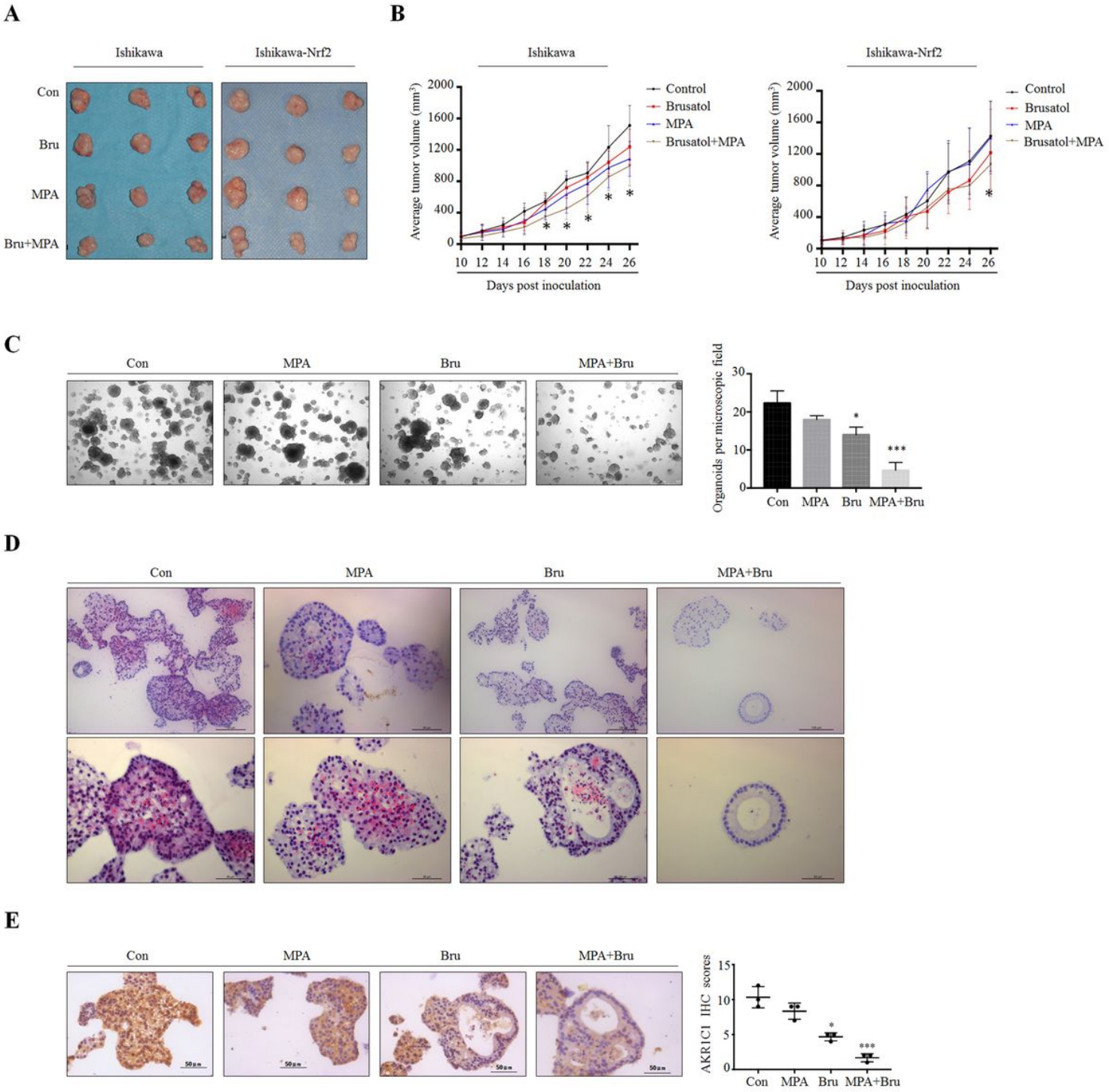

Figure 3

In vivo effects of brusatol-mediated reversal of progestin resistance. A, Representative picture of the xenograft tumor subjected to the indicated treatments. B, The volume of the xenograft tumors were estimated. *p<0.05. C. Numbers of organoids after indicated treatments. *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.

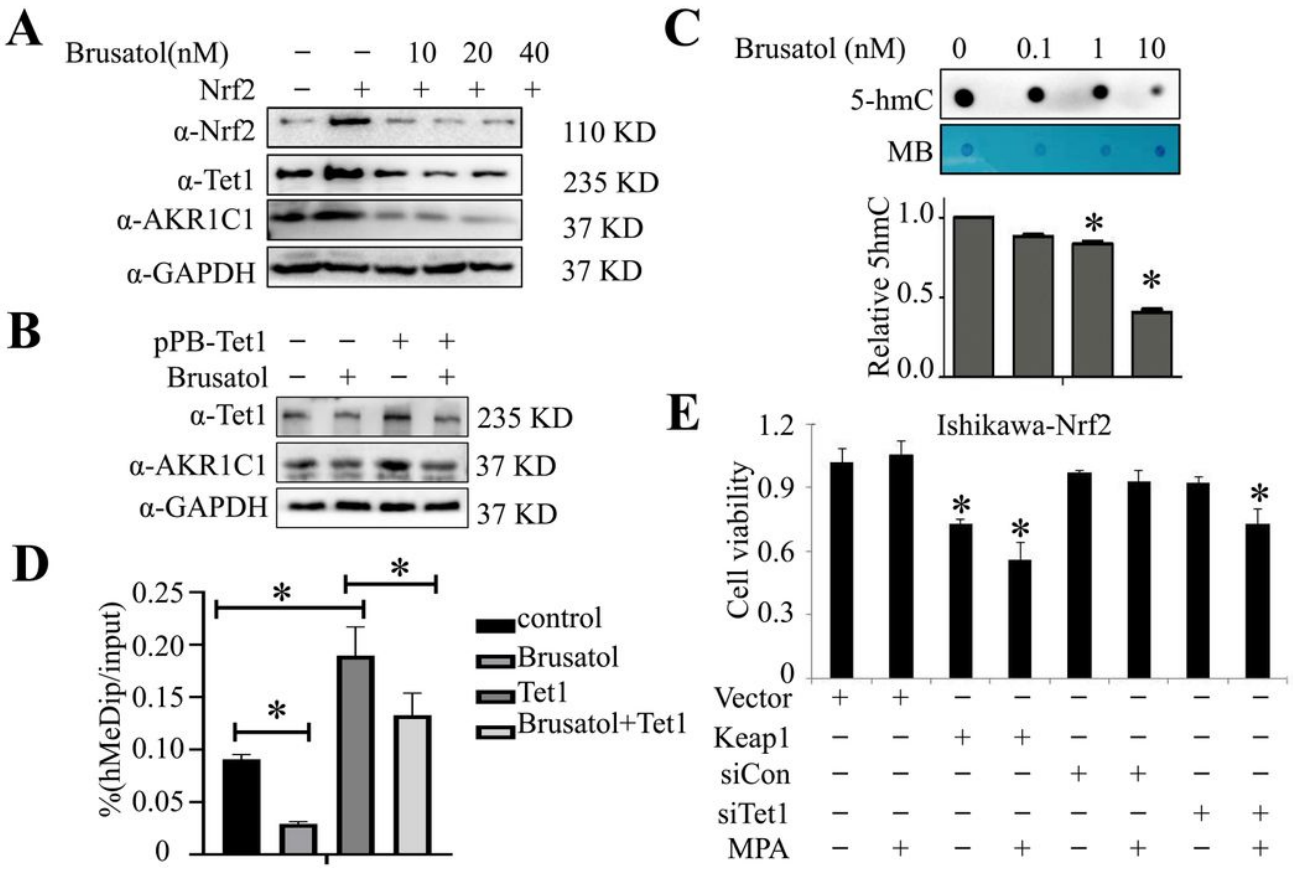

Figure 4

Suppression hydroxymethylation of AKR1C1 through declined Tet1 contributes to brusatol-enhanced progestin sensitivity. A, Brusatol mitigated the effect of Nrf2 by decreasing Tet1 and AKR1C1 expression. B, Ishikawa cells were transfected with Tet1 overexpression plasmid. Cells were then treated with or without 20 nM brusatol and AKR1C1 level was detected by immmunobloting. C, Brusatol suppressed genomic hydroxymethylation status. Ishikawa cells were treated with the indicated dose of brusatol for 48 hours, and 5hmC levels in total DNA were detected by dot blot assay, *p<0.05. D, Brusatol abrogated hydroxymethylation in AKR1C1 promoter region. Cells that underwent the indicated treatments were subjected to the hMeDIP assay. *p<0.05. E, Keap1 overexpression or Tet1 knockdown affected the growth of Ishikawa-Nrf2 cells, *p<0.05.

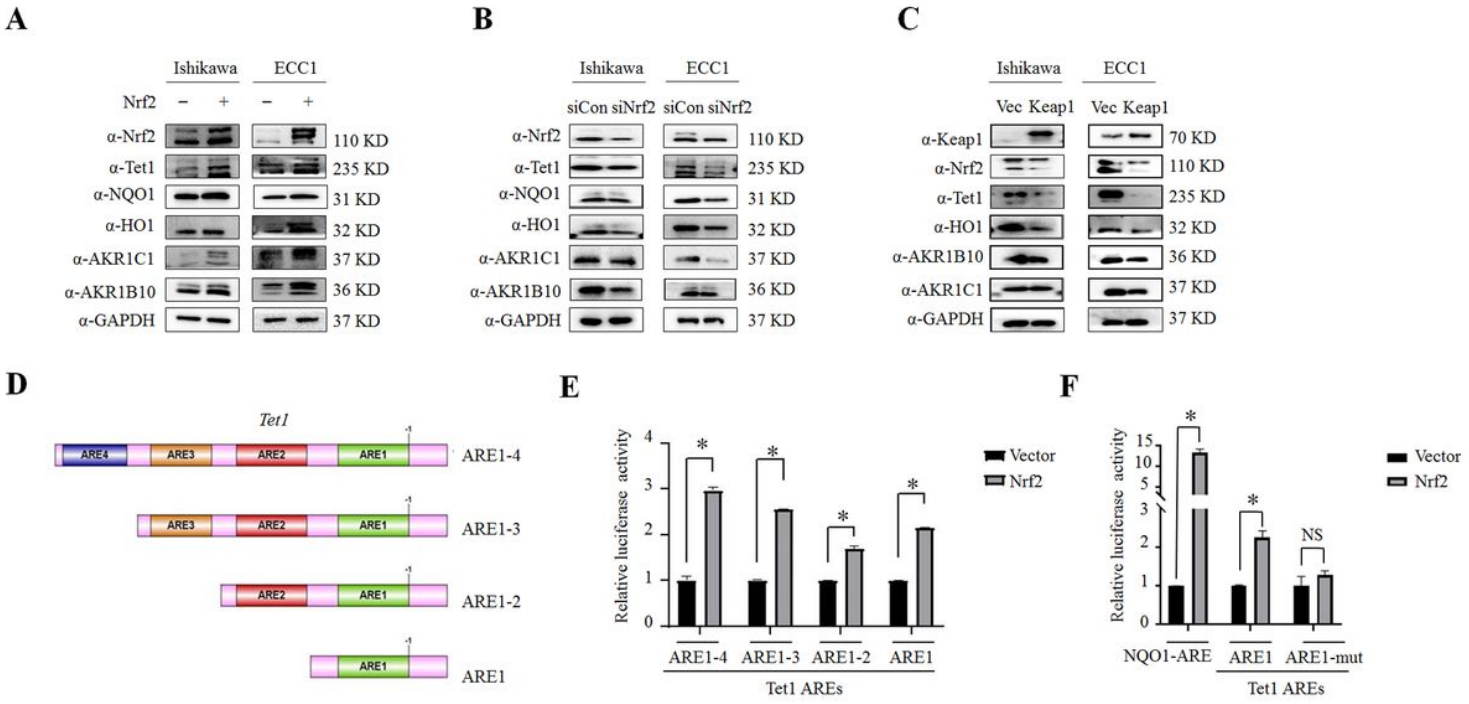

Figure 5

Nrf2 promotes Tet1 expression by binding AREs in the Tet1 promoter region. Transient ectopic expression of Nrf2 (A) elevated the levels of Tet1 and other target proteins while depletion of Nrf2 by siRNA (B) or transfection of a Keap1-overepxressing plasmid (C) suppressed the expression of the target proteins and Tet1. D, Schematic diagrams illustrating the construction of Tet1-ARE luciferase reporter plasmids with the indicated AREs. E, Luciferase activity in endometrial cancer cells transfected with different Tet1-ARE constructs after Nrf2 overexpression was assessed by the dual luciferase reporter assay. *p<0.05 compared with the vector control. F,The effect of Nrf2 on wild or mutant Tet1 ARE's luciferase activity. *p<0.05, transfected NQO1 ARE serve as a positive control.

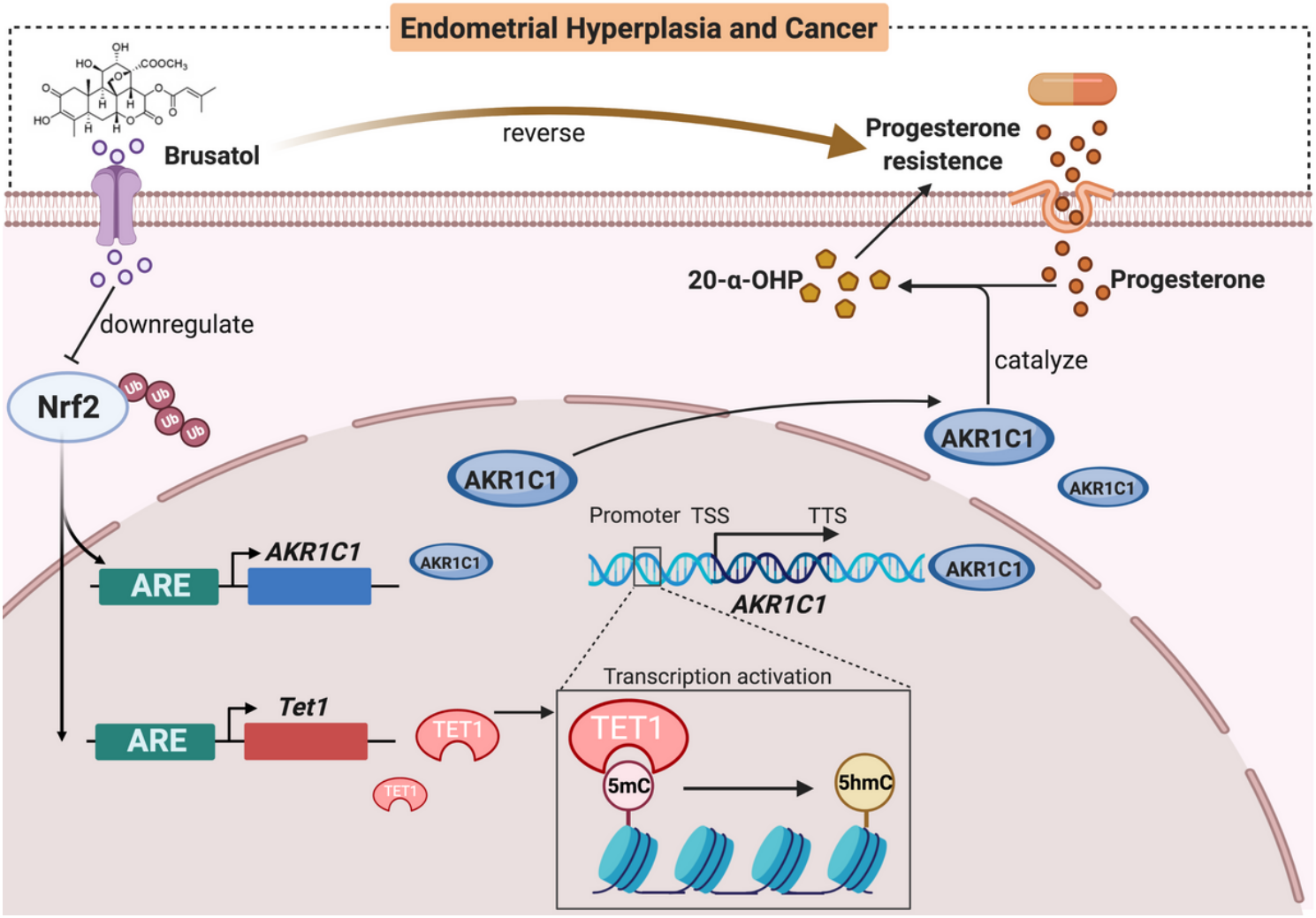

Figure 6

A proposed model illustrating that brusatol suppresses progestin metabolism and sensitizes precancerous/endometrial cancers to progestin. Nrf2 binds to the AREs in the Tet1 promoter region and enhances Tet1 expression, which in turn facilitating AKR1C1 expression via hydroxymethylation modification. Furthermore, high levels of Nrf2 enhance progestin metabolism by upregulating AKR1C1, which contributes to progestin resistance due to loss of drug function in progestin treatment. This kind of progestin resistance can be reversed by brusatol.

Supplementary Files

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