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Publication Date

2024-09-01

DOI

10.1016/j.tranon.2024.102041

Peer reviewed

Translational Oncology 47 (2024) 102041

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon

Original Research

Krüppel-like factor 12 decreases progestin sensitivity in endometrial cancer by inhibiting the progesterone receptor signaling pathway

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ARTICLE INFO

Keywords:
KLF12
Endometrial Cancer
Progesterone Receptor
Progestin sensitivity

ABSTRACT

Objective: This study aimed to clarify the mechanism by which Krüppel-like factor 12 (KLF12) affects progesterone sensitivity in endometrial cancer (EC) through the progesterone receptor PGR signaling pathway.
Methods: The relationship of KLF12 with PGR in EC patients was examined by immunohistochemistry, and the expression of KLF12 and PGR in EC cell lines was detected by real-time PCR and western blotting. Cell proliferation assay, plate clone formation, cell apoptosis assay, and cell cycle analysis were conducted to determine the impact of KLF12 intervention on progesterone therapy. CUT&Tag analysis and the dual-luciferase reporter experiment were used to determine the underlying regulatory effect of KLF12 on the PGR DNA sequence. A subcutaneous xenograft nude mouse model was established to validate the *in vivo* effect of KLF12 on progesterone sensitivity via PGR expression modulation.
Results: KLF12 demonstrated decreased progesterone sensitivity and a negative correlation with PGR expression in EC tissues. Progesterone sensitivity was increased by KLF12 deficiency through PGR overexpression, a result that could be significantly reversed by PGR downregulation. PGR was identified as a target gene of KLF12, which could directly bind to the PGR promotor region and inhibit its expression.
Conclusion: This study is the first to investigate the effect of KLF12 expression on EC cell resistance to progesterone. Our results offer important mechanistic insight into the direct regulation of the PGR promoter region, demonstrating that KLF12 expression strongly suppressed the PGR signaling pathway and, as a result, reduced progesterone sensitivity in EC patients.

Introduction

Endometrial cancer (EC) ranks as the sixth most common type of cancer in women. It has been reported that there were 417,000 existing cases and 97,000 new deaths worldwide in 2020. In the United States, there were 66,200 new cases and 13,030 deaths reported in 2023 [1]. Notably, the incidence of EC has exceeded that of cervical cancer in developed countries, including Northern America and Eastern Europe [2]. In China, there has been a surge of up to 10.54 % in the annual incidence of EC over recent years, with a mortality rate reaching 2.53 %, as per the most recent data released by the National Cancer Center [3].

Krüppel-like factors (KLFs) are a group of DNA-binding transcriptional regulators that modulate cellular processes such as proliferation, apoptosis, and differentiation [4]. Previous studies have shown that KLFs have a crucial role in either inhibiting or promoting the growth of tumors. KLFs can impact cellular activities by controlling the signaling of steroid hormones [5,6]. KLF12, a member of the KLF family, was previously reported to inhibit the transcription of target genes through

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https://doi.org/10.1016/j.tranon.2024.102041
Received 2 November 2023; Received in revised form 11 May 2024; Accepted 20 June 2024

interaction with its amino-terminal PVDLS sequence and carboxyl-terminal binding protein [7]. Studies have confirmed the crucial role of KLF12 in the development of various forms of cancer. The pioneering research of Nakamura et al. revealed the significant role that KLF12 plays in propelling the growth of poorly differentiated gastric cancer, indicating that it could serve as a target for therapy [8,9].

Furthermore, KLF12 has been documented to impede target gene expression by binding to specific promoter regions, thereby serving as a potential diagnostic biomarker and treatment target in basal breast cancer [10]. The downregulation of KLF12 expression is thought to suppress the growth of breast cancer by regulating cell invasion, migration, and chemoresistance [11-13]. In EC, high expression of KLF12 is known to promote tumor cell proliferation, invasion, and migration [14].

Progesterone receptor (PGR), a member of the steroid hormone receptor family, regulates the expression of genes related to cell adhesion, invasion, apoptosis, and proliferation in the endometrium. It is a transcription factor and an essential tumor suppressor implicated in the regulation of genes associated with cell cycle, inflammation, and differentiation [15]. Progesterone inhibits the growth and spread of EC cells by attaching to PGR and enhances their specialization. The PGR consists of a DNA-binding domain (DBD) at its center and a ligand-binding domain (LBD) at its carboxyl terminus. It is produced through transcription from various promoter regions of the same gene, resulting in the formation of two isoforms: a 116 kDa progesterone receptor B (PRB) and an 82 kDa progesterone receptor A (PRA). The main difference between PRB and PRA is due to the presence of an extra 164 amino acid residues in the amino-terminal region of PRB [16]. *In vitro,* findings suggest PRB as the main isoform involved in mediating the tumor-suppressive effect of progesterone in the endometrium [17].

Research has discovered that KLFs have a role in the molecular processes of both uterine cells and tissues. However, there is currently no concrete data that establishes a direct connection between the known impacts of KLF12 and the PGR signaling pathway. To acquire a deeper understanding of the mechanism of progestin resistance and expand the appropriate use of progesterone in clinical practice, the present study investigated the regulatory relationship between KLF12 and the progesterone pathway. Additionally, this study aims to provide new insights for progesterone-based treatment in EC.

Materials and methods

Patient tissues and cell lines

Paraffin-embedded specimens were collected from 83 EC patients who underwent a hysterectomy in the Department of Gynaecology of Nanjing Drum Tower Hospital from September 2018 to September 2021, including stage I (n=58), stage II (n=20), stage III (n=23), and stage IV (n=5) samples. Matched adjacent normal tissues were obtained as control. Each patient signed informed consent before tissue specimen collection, and the institutional ethics committee of the Affiliated Nanjing Drum Tower Hospital of Nanjing University approved the study protocol. Human EC cell lines (MFE-296 cell line, Ishikawa cell line, HEC cell line, AN3CA cell line) were acquired from the Cell Research Center at Shanghai Institutes for Biological Sciences and cultured in DMEM (Bio-channel) supplemented with 10 % fetal bovine serum (Bio-channel), penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37°C with 5 % CO_2.

Immunohistochemistry (IHC)

IHC was performed on paraffin sections using antibodies against PGR (1:1000; Santa Cruz) and KLF12 (1:2000; Abcam). The immunostaining process was carried out according to the recommendations outlined in previous studies [18]. The sections were deparaffinized and rehydrated in graded ethanol solutions and subjected to antigen retrieval. The endogenous peroxidase activity was inhibited by treatment with specific blockers. Following 30 min of blocking, the sections were incubated with the primary antibodies overnight at 4 °C and then probed with an HRP-conjugated secondary antibody for 30 min. Later, the slides were incubated with DAB-substrate (Typing) and counterstained with hematoxylin. The sections were dehydrated using various graded ethanol solutions and left to dry at 25 °C. The sections were analyzed using semi-quantitative histologic scoring (H-score).

Lentivirus infection

Lentiviruses encoding GFP-KLF12 (KLF12) or GFP (as control) were purchased from Keygen. Cell transfections were performed using Lipofectamine 3000 reagent (Invitrogen) and HEK293T cells following the instructions provided by the manufacturer[19]. Ishikawa cells were transduced with the Lenti-KLF12 virus to induce KLF12 expression (sequence information: AGGTCGACTCTAGAGGATCCCGCCACCATGA ATATCCATATGAAGAG). Before transfection, Ishikawa cells seeded in six-well plates were cultured for 24 h until they reached 20-30 % confluency. The cells were treated with 10 μL lentiviruses and 40 μL HitransG for 2 days at 37 ℃. This was followed by discarding the supernatant, and stable transformants were isolated using puromycin (2 μg/mL) for 2 weeks. Ishikawa cells transfected with respective lentivirus are shown as Ish-ovklf12 or Ish-vector.

Plasmid transfection

The KLF12-shRNA (Tsingke Biotechnology, sequence information: CCGGGTGACCTTAGATAGCGTTAATCTCGAGATTAACGCTATCTAAGGTCAC TTTTTT) or PGR-shRNA (Tsingke Biotechnology, sequence information: CCGGGCTGCACAATTACCCAAGATACTCGAGTATCTTGGGTAATTGTGCAG CTTTTTT) plasmid was transfected into MFE296 cells to knockdown KLF12 or PGR expression, respectively. 293 T Cells in the logarithmic growth were seeded in a 6-well plate at a density of 6 $\times 10^5$. The cells were cultured in a 37°C incubator for 24 h and used for transfection when the cell density reached 70 %-80 %. The medium was replaced with serum-free medium for 2 h before transfection. The recombinant plasmids and helper plasmids PSPXA2 and PMD2G were mixed at a ratio of 4:3:1 to achieve a total DNA concentration of 2.5 μg. The mixture was then incubated at room temperature for 15 min. The mixture was then added to 293 T cell culture medium and cultured at 37 °C for 8 h. Then, the supernatant was discarded and resuspended with 2 mL of medium and incubated at 37 °C for 48 h and 72 h, respectively. The supernatant was collected and centrifuged at 2000 rpm for 10 min to precipitate. The day before transfection, Ishikawa or MFE296 cells seeded in six-well plates were cultured for 24 h until they reached 30 % confluency. The stable transfection procedure was performed using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. The definition of MFE296 cells transfected with different plasmids is shown as follows: MFE296-shKLF12, MFE296-shNC; MFE296-PRKD, MFE296-NC; 296shklf12-PRKD, 296shklf12-NC.

Real-time PCR

In this study, the product protocol was followed to extract total RNA from EC cells for comparing transcript levels of *KLF12* and *PGR*. Trizol reagent was used for the extraction process. The cells were lysed in 1 mL of Trizol, either in a regular 6-well plate or a 35 mm dish. The total RNA extraction was conducted according to the instruction manual. To perform a more detailed examination of transcript levels, we generated complementary DNA (cDNA) from the total RNA that was extracted. This was achieved using the HiScript II 1st Strand cDNA Synthesis Kit, which includes a gDNA wiper to eliminate any contamination from genomic DNA. RT-PCR was performed using primer sequences as follows:

GAPDH forward primer: 5′-ATCGTCCACCGCAAATGCTTCTA-3′
GAPDH reverse primer: 5'-AGCCATGCCAATCTCATCTTGTT-3′
KLF12 forward primer: 5'-CCTTTCCATAGCCAGAGCAG-3′
KLF12 reverse primer: 5'-TTGCATCCCTCAAAATCACA-3′
PGR forward primer: 5'-GGATTCAGAAGCCAGCCAGAG-3'
PGR reverse primer: 5'-CCACAGGTAAGGACACCATAATGA-3'
PRB forward primer: 5'-AGGTCTACCCGCCCTATCTC-3′
PRB reverse primer: 5'-AGTAGTTGTGCTGCCCTTCC-3'
PRA forward primer: 5'-AGGGCAATGGAAGGGCAG-3'
PRA reverse primer: 5'-TTCTAAGGCGACATGCTGGG-3'

Western blotting

Cells were lysed in a mixed buffer of RIPA, PMSF, and NaF (Vazyme) to extract proteins. Proteins were separated using SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Millipore). Appropriate primary and HRP-conjugated secondary antibodies were used for detection. To identify membrane proteins, a blocking procedure was carried out by incubating with 5 % non-fat milk for 2 h at room temperature. Subsequently, membranes were subjected to overnight incubation at 4°C with antibodies specific to GAPDH, PGR, and KLF12. Subsequently, the membranes were probed with secondary antibodies to detect the target proteins. The visualization of these specific proteins was accomplished using enhanced chemiluminescence (Bio-Rad, Hercules, California, USA).

Cell counting kit-8 (CCK-8) assay

The experiment started with 1×10^3 cells/well of 100 μL 10 % DMEM seeded in 96-well plates. Different amounts of MPA were applied to the cells after they had been incubated for the whole night. Subsequently, the cells were incubated at 37°C for 1-4 days, during which 10 μL CCK-8 (Vazyme) reagent was added at specific time points. Following a two-hour incubation period at 37°C, the absorbance at 450 nm was quantified following the protocol provided by the manufacturer.

Cell apoptosis assay

In brief, cells (1×10^5) were digested with trypsin and without EDTA and then centrifuged at 150 ×*g* at 4 °C for 5 min. The supernatant was discarded, and the cell pellet was washed twice with pre-cooled PBS. Following that, the cells were reconstituted in 100 μL of 1H binding buffer and subjected to staining with 5 μL of PI staining solution and 5 μL of Annexin V-FITC. The samples were gently mixed, incubated in the dark at room temperature (20-25°C) for 10 min, and gently treated with 400 μL 1× binding buffer. Flow cytometry analysis was performed within 1 h after staining.

Cell cycle detection

In brief, cells (1×10^6) were digested with trypsin and centrifuged at 2000 rpm at 4°C for 5 min. The obtained cell pellet was mixed with 500 μL of 70 % ethanol (pre-chilled) and left undisturbed for 2 h overnight. The fixed cells were maintained at 4 °C, and before staining, the fixative solution was washed off using PBS. The cells were centrifuged at 1,000 rpm for 3 min and treated with 500 μL PI/RNase A staining solution at room temperature in the dark for 30 min. After being filtered, the samples were introduced into the machine. At 488 nm, the excitation wavelength, red fluorescence, was observed.

Chromatin immunoprecipitation technology (CUT&Tag)

CUT&Tag was performed according to the published procedure [20] with modifications using the Hyperactive Universal CUT&Tag Assay Kit for Illumina (Vazyme). In brief, 6×10^6 Ishikawa cells in the logarithmic growth phase were collected and washed with 500 μL wash buffer and centrifuged at 2500 rpm for 5 min at room temperature. Cell pellets were resuspended in 100 μL wash buffer, followed by the addition of 10 μL activated ConA beads to the cell tubes. The tubes were then incubated at room temperature for 10 min. After removing the supernatant, the bead-bound cells were resuspended in 50 μL antibody buffer containing 5 μL rabbit anti-KLF12 antibody. Subsequently, the cells were incubated overnight at 4 °C, and the primary antibody was carefully discarded. A diluted anti-rabbit secondary antibody (1:100 in 50 μL Dig-wash buffer) was then added to the cells, which were incubated with rotation at room temperature for 1 hour. The cells were washed three times with 200 μL of Dig-wash buffer, followed by the addition of 2 μL of pA/G–Tnp and 98 μL of Dig-300 buffer. After a 1-hour incubation at room temperature, the samples were washed again with 200 μL of Dig-300 buffer three times. Subsequently, each sample was treated with 10 μL of 5 × TTBL mixed with 40 μL of Dig-300 buffer and incubated at 37 °C for 1 h. The interactions were stopped by adding 5 μL of Proteinase K, 100 μL of Buffer L/B, and 20 μL of DNA extraction beads, followed by a 10 min incubation at 55 °C. After discarding the supernatant, the beads were washed once with 200 μL of Buffer WA and twice with 200 μL of Buffer WB before being resuspended in 22 μL of nuclease-free water. For library amplification, 15 μL of purified DNA was combined with 25 μL of 2× CAM, along with 5 μL of uniquely barcoded i5 and i7 primers from the TruePrep Index Kit V2 for Illumina, resulting in a total volume of 50 μL. The sample was then subjected to a thermal cycler program consisting of 72 °C for 3 min, 95 °C for 3 min, 20 cycles of 98 °C for 10 s, 60 °C for 5 s, and 72 °C for 1 min, followed by holding at 4 °C. To purify the PCR products, 2× volumes of VAHTS DNA Clean Beads were added and incubated at room temperature for 5 min. The beads were washed twice with 200 μL of fresh 80 % ethanol and eluted in 22 μL of ddH2O. All CUT&Tag libraries were sequenced by Novogene using the Illumina NovaSeq 6000 platform in PE150 mode.

Dual-luciferase reporter assay

The sequence of the PGR promoter region found by CUT&Tag was inserted into the pGL3-basic luciferase vector. The primer sequences used to clone the PGR promoter from the genomic DNA of MFE296 cells were 5'-AAAGGTACCTCCAACGTGCCAATCAGG-3′ (forward) and 5'-AACCATCCCAATAATGCAC-3′ (reverse). Using the Fast Site-Directed Mutagenesis Kit (TianGen) following the manufacturer's instructions, site-directed mutagenesis was carried out to modify the KLF12-binding site found in the PGR promoter cloned in the pGL3-basic vector; wild-type (WT) luciferase reporter constructs served as templates. The primer sequence used for mutagenesis of the PGR promoter was 5'-GGCTGGAAAGGAAGGACGGGGGTGAGATATTACCTAATGG-3′ (mutated nucleotides underlined). Cells were seeded in a 24-well plate at a density of 6×10^4 cells per well before transfection. The cells were co-transfected with a mixture of luciferase reporter pGL3-basic vector containing PGR-WT or PGR-Mut sequences and corresponding control vectors for 48 h at 37°C, respectively. Cells were harvested, and the luciferase activities were measured using a dual-luciferase reporter assay system (Vazyme) according to the manufacturer's protocol.

Subcutaneous xenograft model

Female athymic BALB/c nude mice, aged 5 weeks, were procured from the Beijing Vital River Laboratory Animal Technology and raised in SPF breeding units. Subcutaneous injections of Ish-ovklf12 or Ish-vector cells (2×10^7 cells/0.10 mL PBS) were performed in both axillae, and transplanted tumors were observed after 3 weeks. Successful mouse models were assigned to each group (n=5) and received intraperitoneal injections of MPA (100 mg/kg) or PBS (50 mg/kg) every 2 days for a total of 9 times. Tumor growth and size were tracked by measuring the length and width with calipers, and tumor volumes were computed using the following formula: Volume = 1/2 × length (mm) × width^2 (mm). On day 42, the mice were euthanized by cervical dislocation.

Statistical analysis

The experiments were conducted thrice independently. A *t*-test for independent samples served the purpose of comparing two groups. ANOVA analysis was carried out to compare three or more groups. IHC data were analyzed using the Mann-Whitter test. P-values < 0.05 indicated statistical significance.

Results

Inverse correlation between KLF12 and PGR expression in EC

We performed IHC analysis on continuous tissue sections embedded in paraffin. As shown in Fig. 1, EC tissues showed significantly higher expression of KLF12 and significantly lower expression of PGR than normal endometrial tissues. Furthermore, a negative connection was found between the expression of PGR and KLF12. To induce KLF12 overexpression, we transfected the PCMV lentivirus into Ishikawa cells. Additionally, we produced MFE296-shKLF12 cells using plasmid transfection (Fig. 2A). Overexpression and knockdown experiments confirmed that KLF12 negatively regulated both the mRNA and protein expression of PGR (Fig. 2B-2I). Therefore, *PGR* has the potential to function as a target gene of KLF12. In summary, these results indicate a negative association between KLF12 and PGR expression.

KLF12 decreases EC progesterone sensitivity in vitro

From the correlation provided, we hypothesized that KLF12 had an impact on the responsiveness of EC to progesterone. We examined the biological role of KLF12 in the sensitivity of EC to progesterone. In a follow-up experiment, MFE296-shklf12 and Ish-ovklf12 cells and their corresponding controls were exposed to DMSO or increasing concentrations of MPA for 48 h. It is well established that decreased apoptosis and enhanced cell viability both contribute to hormone therapy's failure [21]. CCK-8 assay results consistently demonstrated the lower survival rate of MFE296-shklf12 cells than the control cells after 48 h treatment with different concentrations of MPA (Fig. 3A). In comparison with Ish-vector cells, Ish-ovklf12 cells showed almost tripled IC50 value after 48 h treatment with different MPA concentrations (Fig. 3B). Consequently, KLF12-deficient cells showed decreased viability and were more susceptible to low-dose progesterone. Following this, we adjusted the treatment dose for the shKLF12 group based on the IC50 value of MPA. The EC cells from the MFE296-shklf12 group showed a remarkable decline in survival, as observed by the CCK-8 and colony formation assays (Fig. 3C, D). Flow cytometry results revealed a substantial increase in the apoptosis of MFE296-shklf12 cells treated with MPA (Fig. 3E). EC cells from the 296-shklf12 group were arrested in G2/M and S phases (Fig. 3F). Ish-ovklf12 cells showed increased viability in the presence of the same concentration of MPA (Fig. 3G, H). Moreover, flow cytometry results demonstrated a higher proportion of apoptotic cells in the

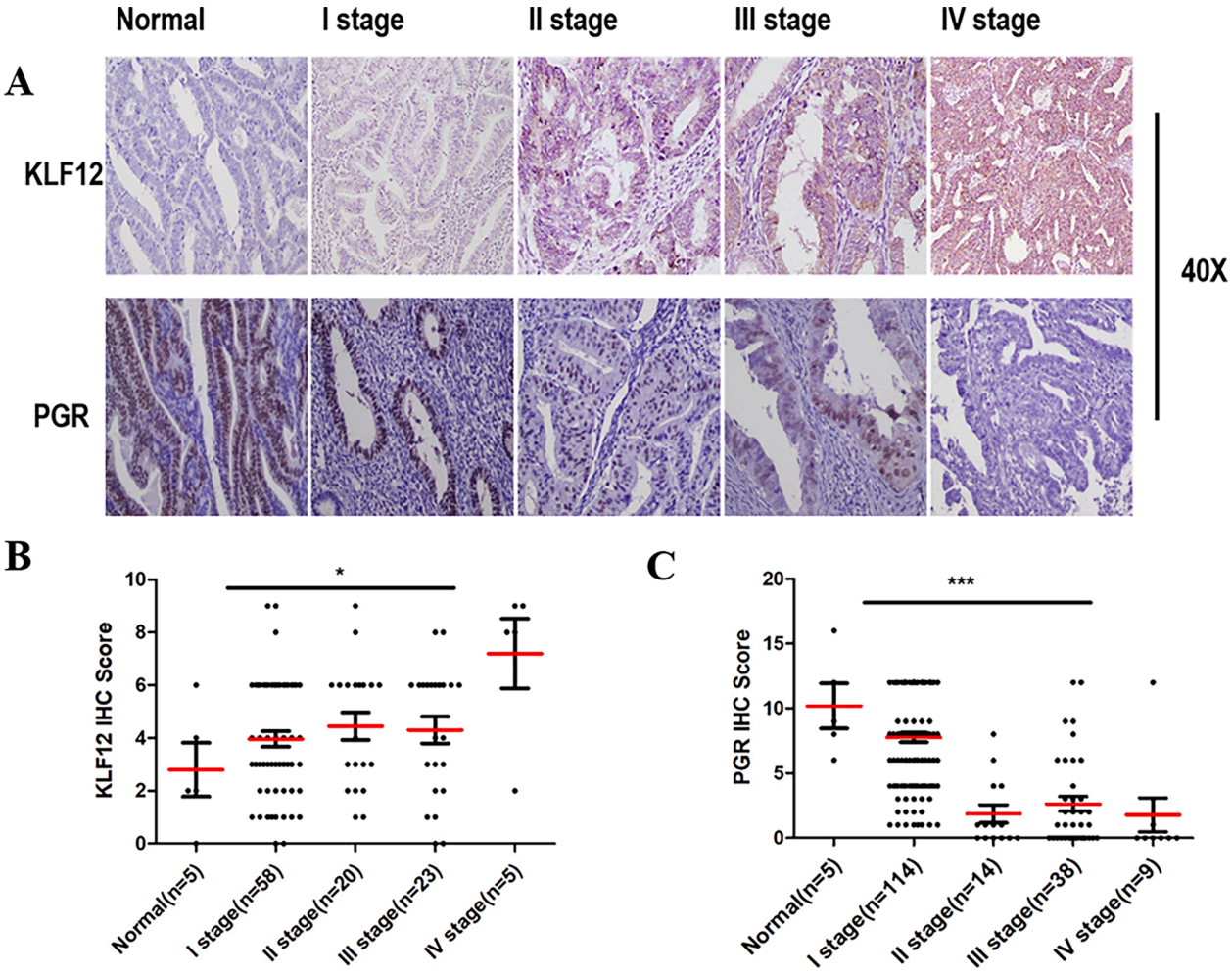

Fig. 1. KLF12 expression negatively correlated with PGR in EC tissues.
(A) IHC to detect the expression of KLF12 and PGR in normal endometrial and EC tissues. (B and C) Statistical charts of KLF12 and PGR expression in EC tissues at each stage. *P<0.05, **P<0.01, ***P<0.001.

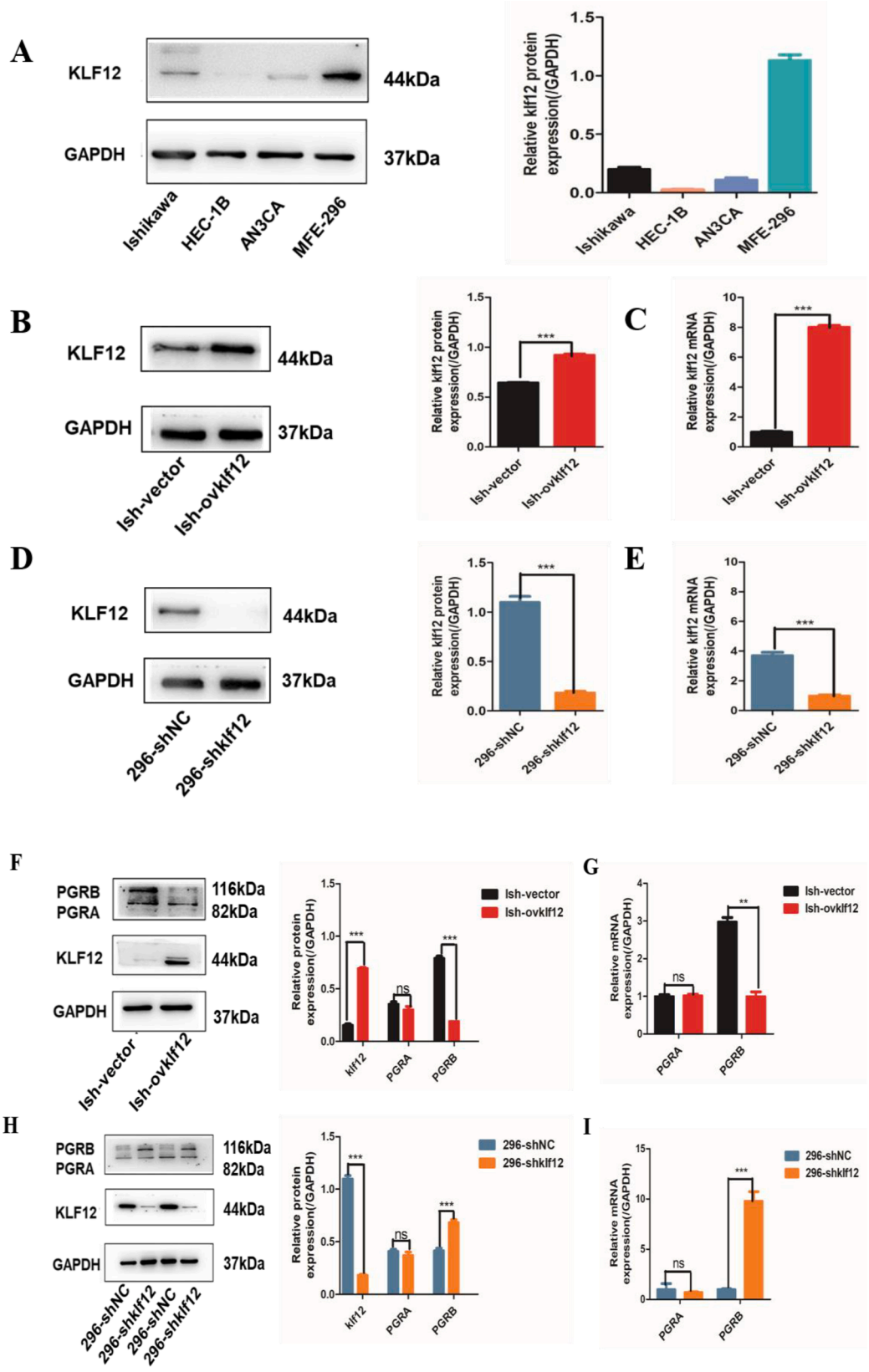

Fig. 2. KLF12 expression negatively correlated with PGR in EC cell lines.
(A) Relative expression of KLF12 protein in four EC cell lines (Ishikawa, HEC-1B, AN3CA, MFE-296). (B and C) Relative expression of KLF12 protein and mRNA after overexpression of KLF12 in Ishikawa cells. (D and E) Relative expression of KLF12 protein and mRNA after stable knockdown of KLF12 in MFE296 cells. (F and G) Western blotting and qPCR detected protein and mRNA expression, respectively, of PGR in Ishikawa cells overexpressing KLF12. (H and I) Western blotting and qPCR detected PGR protein and mRNA expression, respectively, after KLF12 knockdown in MFE296 cells. *P<0.05, **P<0.01, ***P<0.001, ns: not statistically significant.

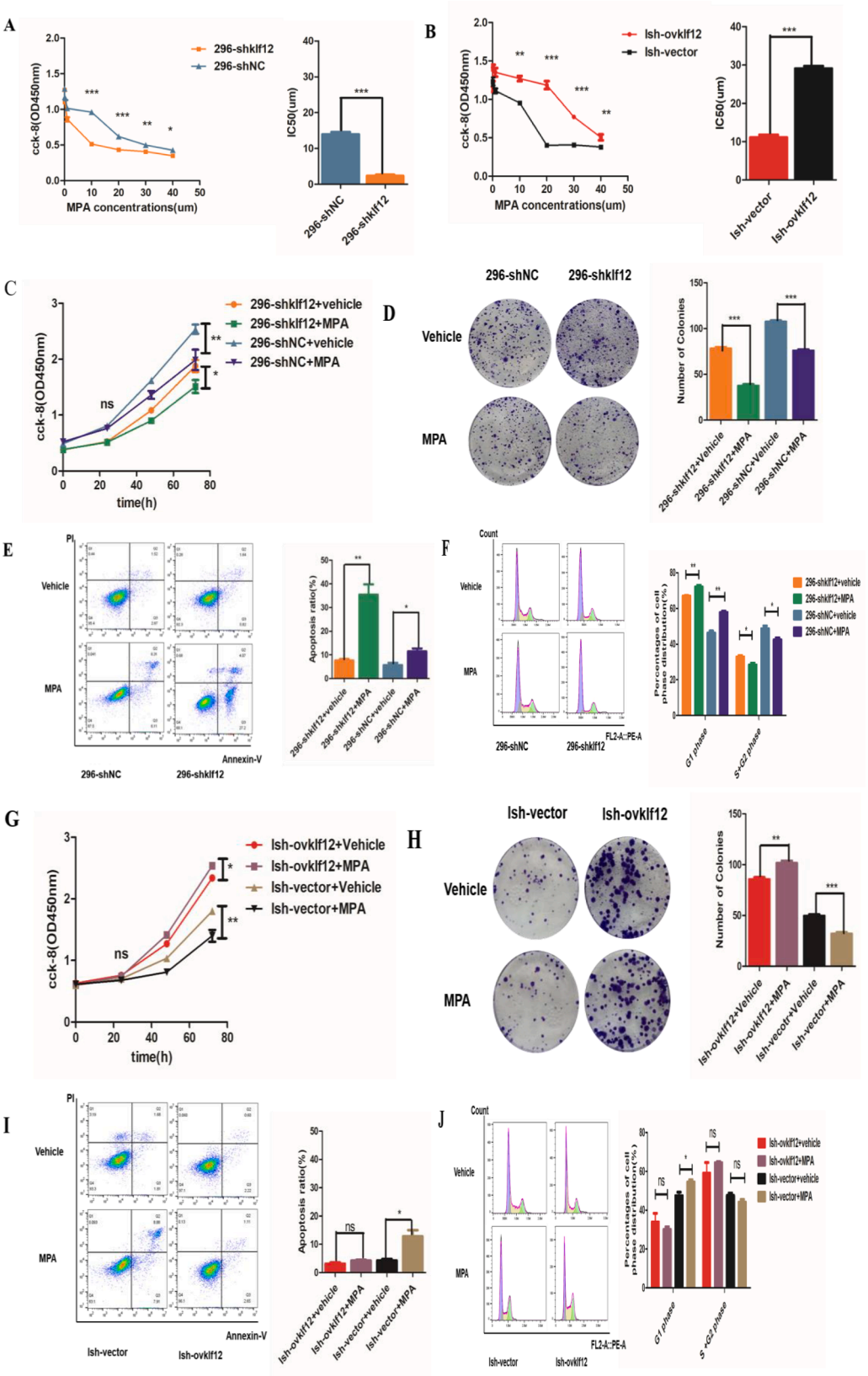

Fig. 3. The relationship of KLF12 with progesterone sensitivity.
(A and B) CCK-8 assay to detect drug resistance and calculate IC50. (C and G) Cell proliferation was measured by CCK-8 assay after treatment with 2 μm MPA. (E and H) Clone formation assay to determine the proliferative activity and clonal formation ability after treatment with 2 μm MPA. (E I) Cell apoptosis was detected by flow cytometry. (F and J) The proportion of cells in different cycle phases, as observed with flow cytometry. *P<0.05, **P<0.01, ***P<0.001, ns: Not statistically significant.

Ish-vector group than in the Ish-ovklf12 group after MPA treatment (Fig. 3I). The cells from the Ish-ovklf12 group were arrested in the G1 phase after MPA treatment (Fig. 3J). Collectively, these findings demonstrated that KLF12 knockdown had the opposite impact on EC cells and that KLF12 overexpression substantially aggravated progesterone insensitivity.

KLF12 regulates the promoter region of PGR

Ishikawa cells that overexpress KLF12 demonstrated increased proliferation when exposed to progesterone. Hence, we examined the interaction between KLF12 and PGR through the implementation of a CUT&Tag assay. The target genes expressed in Ish-ovklf12 cells were compared to those expressed in Ish-vector cells. The results revealed that the Ish-ovklf12 cells identified 12,991 peaks, while the control group identified 11,478 peaks. There were 4522 peaks shared by both groups, 8469 peaks unique to the Ish-ovklf12 cells, and 6956 peaks unique to the control group (Fig. 4A). The target genes with higher peaks in Ish-ovklf12 cells were selected. The distribution of peaks in different functional areas was calculated using the ChIP-seeker software. In the KLF12 overexpression group, 51.72 % of peaks were located in the Promoter region, while in the control group, 45.8 % were found in the same region (Fig. 4B, C). The target genes identified in Ish-ovklf12 cells were compared with those identified in control cells, and the target genes displayed higher peaks in the KLF12 overexpression group. The DNA that was obtained was examined using IGV visualization software.

As the peak value increased, the amount of DNA captured also increased. The maximum value in the PGR area of the test group was considerably higher than that in the control group (Fig. 4D). The recognition sequence of the transcription factor KLF12 in the promoter region of PGR was predicted through the JARSPER website, which is highly consistent with the primary binding sequence of the transcription factor KLF12 on the DNA of the target gene obtained in the Cut-tag experiment. Thus, KLF12 may directly bind to the promoter region of

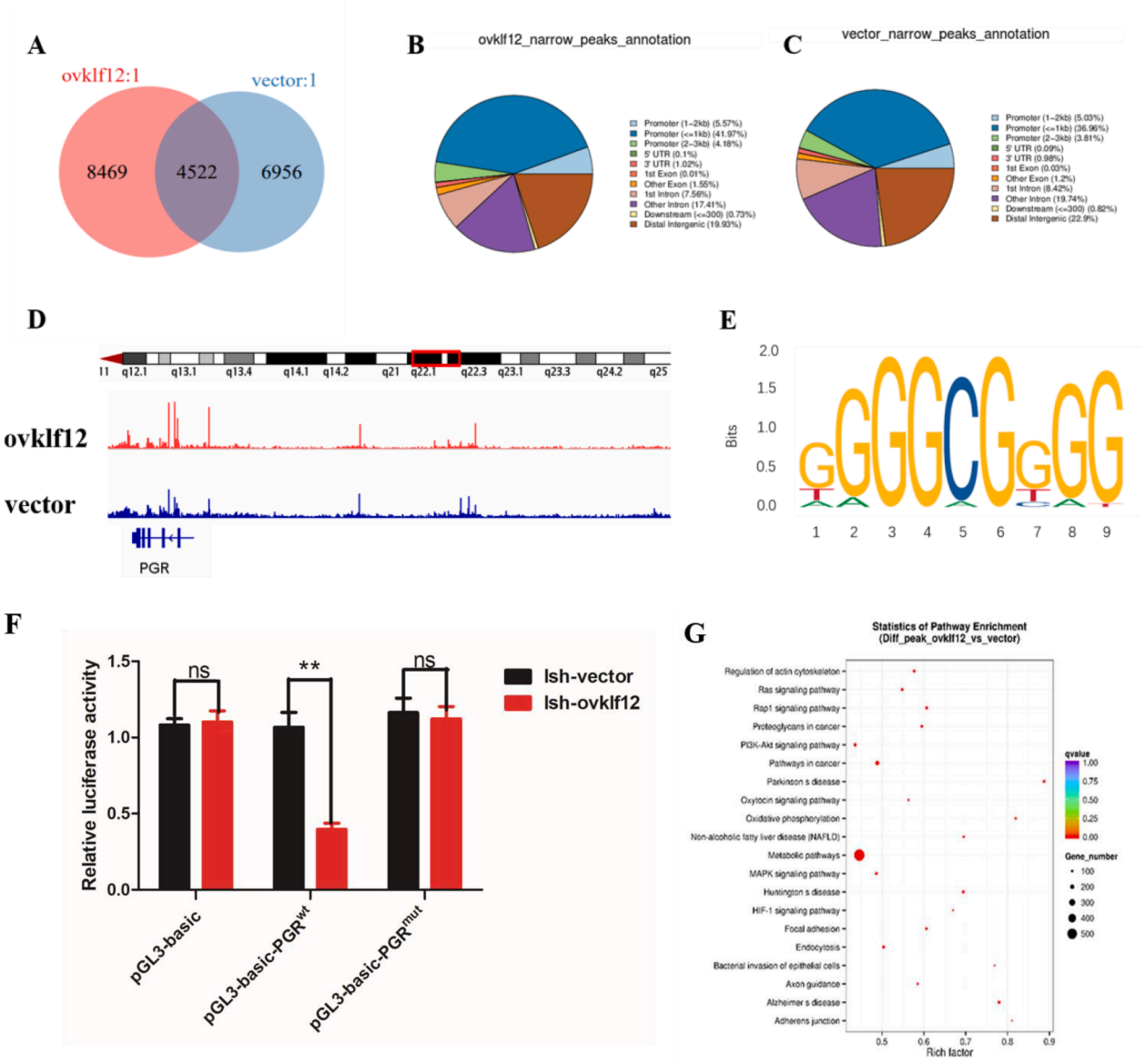

Fig. 4. KLF12 regulates the promoter region of PGR.
(A) Gene number captured by Ish-ovklf12 and Ish-vector. (B and C) DNA fragments bound to KLF12 captured by Ish-ovklf12 and Ish-vector. (D) IGV visualization software was used to analyze the amount of DNA captured; the higher the peak, the more the quantity of DNA captured. (E) The binding site of the PGR promoter region and KLF12. (F) Luciferase assays were performed after transfection with pGL3-basic-PGR^{mut} and pGL3-basic-PGR^{wt}. Relative luciferase activity was analyzed after 48 h treatment. (G) The main pathway for target gene enrichment that binds to KLF12. *P<0.05, **P<0.01, ***P<0.001.

PGR(Fig. 4E). To determine if the PGR promoter region is functional, we inserted the corresponding PGR promoter sequence to construct a PGL3-basic-PGR^{wt}-luciferase reporter. The mutations of select nucleotides in the PGR promoter made the reporter no longer responsive to KLF12 (Fig. 4F). Thus, we postulated that KLF12 impedes the interaction between PGR and progesterone response elements in the promoter region, leading to a decrease in the PGR-driven transcription of target genes. We analyzed the raw data obtained from CUT&Tag using KEGG enrichment analysis. This study revealed the top eight signaling pathways, one of which was the PI3K/AKT pathway (Fig. 4G).

Lack of KLF12 enhances progesterone sensitivity via PGR in EC cells

Based on the negative connection observed between KLF12 and PGR expression, we hypothesized that KLF12 contributes to progesterone resistance by inhibiting PGR expression. To achieve this objective, we introduced the PGR-shRNA plasmid into MFE296 cells (Fig. 5A, B) and observed a decrease in PGR expression when cells were co-transfected with shKLF12 and PGR-shRNA plasmids (Fig. 5C, D). As expected, the reduction in the cell viability caused by KLF12 deficiency was partially reversed after PGR inhibition. In addition, PGR downregulation diminished the effect of KLF12 deficiency on MPA-induced EC cell proliferation and apoptosis (Fig. 5E–J). The results obtained from the study of EC cells clearly emphasize the significance of suppressing PGR for the development of progesterone resistance mediated by KLF12. Previous research suggests that the PI3K/AKT pathway may have a crucial function in regulating KLF12 in EC. Through western blotting, we examined the expression of essential components in the AKT pathway and their phosphorylated versions. Our analysis revealed a significant decrease in the levels of P-AKT and Bcl-2 proteins in KLF12-deficient cells compared to the parental cells (**Fig. S1A, B**). P-AKT and Bcl-2 expression in KLF12-deficient cells can be successfully upregulated through the inhibition of PGR (**Fig. S1C, D**). Overall, KLF12 exerts a negative regulatory effect on PGR expression at the molecular level and stimulates the AKT signaling pathway.

KLF12 decreases EC progesterone sensitivity in vivo

We established a xenograft tumor model using Ish-ovklf12 and Ish-vector cells to investigate the role of KLF12 in the progesterone sensitivity of EC. All mice were subjected to MPA treatment, which exerted no inhibitory effect on KLF12 overexpression in the test group. However, MPA treatment significantly impeded tumor growth in the control group (Fig. 6A, B). IHC results revealed an apparent decrease in PGR expression after KLF12 overexpression (Fig. 6C). These findings provide evidence that KLF12 overexpression induces PGR reduction both *in vivo* and *in vitro* and correlates with diminished progesterone sensitivity.

Discussion

EC is one of the most prevalent malignancies of the reproductive system in women. The incidence and mortality of EC continue to rise with an increase in the prevalence of obesity and a decline in the rate of hysterectomy [22]. The KLF family is recognized for its crucial involvement in the regulation of the cancer-causing capabilities of steroid-responsive cells in the mammary and uterine endometrial cells [23]. As a transcription inhibitor, KLF12 inhibits gene expression by binding to the promoter region. KLF12 plays a critical role in the proliferation, invasion, and migration of EC cells. Gaining a deeper understanding of the interaction between KLFs and steroid hormone receptors would facilitate the identification of innovative treatment drugs designed explicitly for hormone-responsive cancers.

Primary progestin therapy is recommended for reproductive-aged women with well-differentiated early-stage disease when surgery is not feasible or in cases of recurrent or advanced EC [24]. Progestin therapy drugs, such as MPA, are frequently utilized. However, around 30 % of patients with early EC do not respond or only have a temporary response to progestin therapy. Additionally, 57 % of patients who initially respond to progestin therapy will experience a relapse [25,26]. Thus, primary or acquired progestin resistance has been a major clinical concern.

Progesterone therapy has generally demonstrated a higher response rate in EC patients with positive PGR testing than in those with negative PGR testing. PGR positive serves as a diagnostic for progesterone reactivity in addition to being a need for progesterone therapy [27]. Our results showed that KLF12 overexpression in EC cells can significantly downregulate the expression of PGR, while its expression silencing can induce opposite effects. Moreover, overexpression of KLF12 led to a decrease in the progesterone sensitivity of EC cells through PGR inhibition. The lack of KLF12 expression promoted the progesterone sensitivity of EC cells via upregulation of PGR expression, and this effect can be reversed mainly through PGR downregulation. In particular, our further analysis of the two PGR subtypes revealed that KLF12 had a significant impact on PRB expression but not PRA expression. While PRA inhibits steroid hormone receptors, including ESR, PRB is believed to be a more potent transcriptional activator. An increasing body of research indicates that PRB, not PRA, plays a crucial role in the pathophysiology of EC [28]. To better understand how EC responds to hormone therapy, future research should concentrate on examining the expression of these two distinct PGR isoforms during cancer and their link to clinicopathological characteristics. Our *in vivo* findings demonstrated significantly smaller tumor volumes in the Ish-vector group than in the Ish-ovklf12 group, suggesting that KLF12 can serve as a promising target to overcome progesterone resistance in EC.

Different tumors trigger the PI3K-AKT signaling pathway, which controls cell metastasis and metabolism. Even though PI3K-AKT is essential for mediating multidrug resistance, tumor chemoresistance may not always result from its activation alone [29,30]. AKT was found to be overactive in most ECs, and TCGA sequencing analysis revealed that over 90 % of endometrioid carcinomas exhibit genetic abnormalities in the PI3K/AKT pathway that contribute to increased AKT activity [31]. The PI3K-AKT signaling pathway was shown to promote progestin resistance in EC [32], which is in line with our KEGG analysis and experimental results. Furthermore, we discovered that the deletion or overexpression of KLF12 resulted in equivalent alterations in the level of P-AKT. Rescue tests showed that suppressing PGR in MFE296 and MFE296-shklf12 cells was able to reverse the observed phenotype. Therefore, it can be inferred that KLF12 causes progesterone resistance in endometrial cancer by controlling the PI3K-AKT pathway.

In our study, we performed tissue immunofluorescence colocalization and found KLF12 to be localized in the cytoplasm. PGR is mainly localized in the nucleus in the normal endometrial tissue, but its expression was primarily detected in the cytoplasm in EC (**Fig. S2**). This observation aligns with the results of IHC. As the EC stage increased, the expression of intranuclear PGR gradually declined. Current scientific theories state that the activation of the steroid receptor directly results in the binding of the complex produced by the steroid and its receptor to structures within the nucleus, which is a prerequisite for the target tissue's reaction to the steroid [33]. In approximately 60 % of samples obtained from neoplastic tissues, PGR was primarily located within the cytoplasm.

In many cases of malignant endometrial tissues, the transfer of PGR from the cytoplasm to the nucleus is inadequate, even under optimal *in vitro* conditions [34]. Hormones induce an unfavorable response from tumor tissues. Moreover, disorders of the receptor mechanism may be associated with decreased hormone receptor levels. To confirm these hypotheses, additional research is required.

In summary, the findings of this study indicate the ability of KLF12 to reduce the sensitivity of EC to progesterone through inhibition of PGR expression and phosphorylation of AKT and related proteins within the signaling pathway. These findings may help improve intervention efforts for predicting and reversing progesterone resistance in EC more

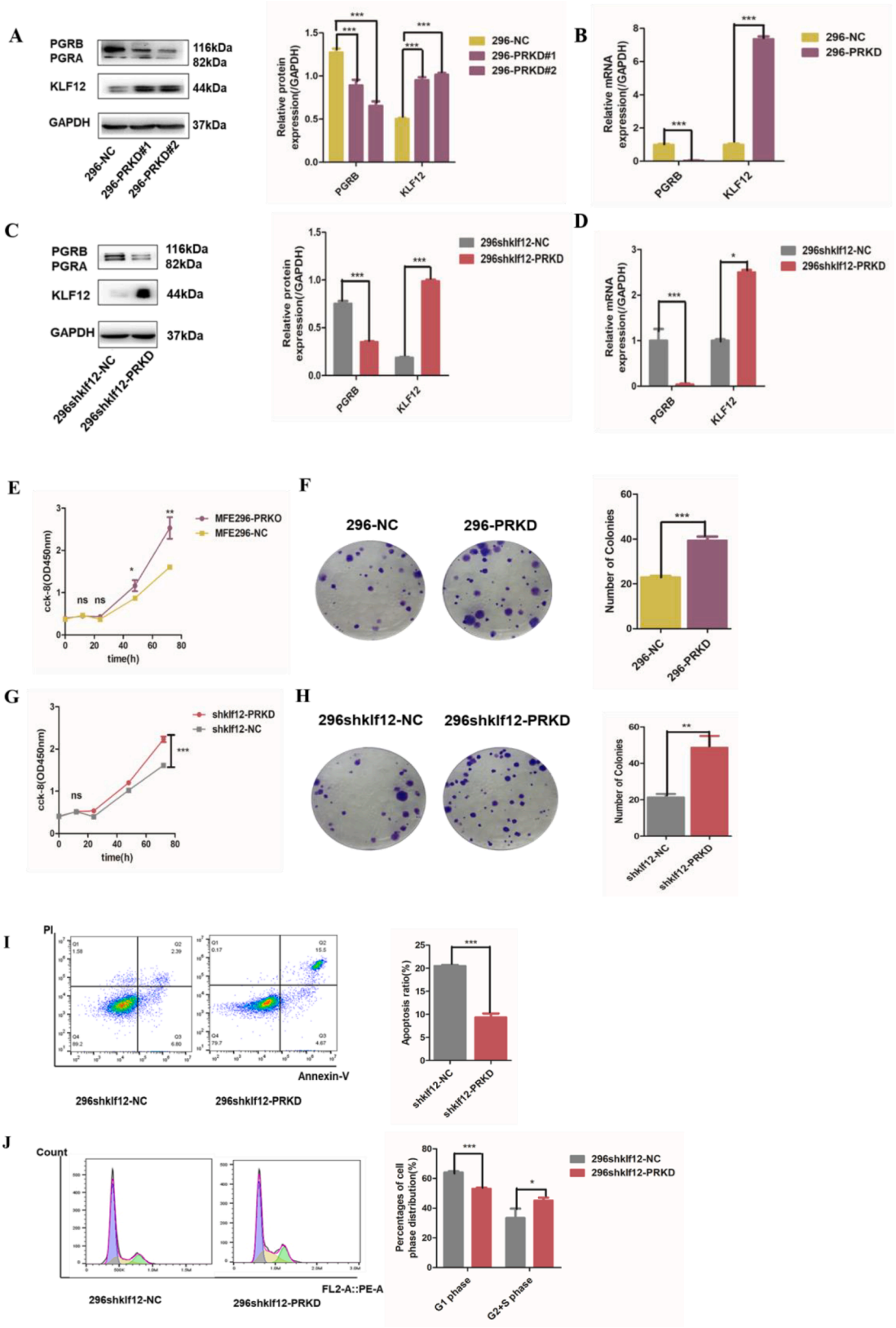

Fig. 5. KLF12 affects progesterone sensitivity via PGR.
(A and B) Relative expression of KLF12 and PGR proteins and mRNAs after PGR knockdown in MFE296 cells and (C and D) 296-shklf12 cells. (E and G) Cell proliferation was compared by CCK-8 and (F and H) clone formation experiments. (I) Cell apoptosis was detected by flow cytometry. (J) Proportion of cells in different cycle phases, as detected by flow cytometry. *P<0.05, **P<0.01, ***P<0.001.

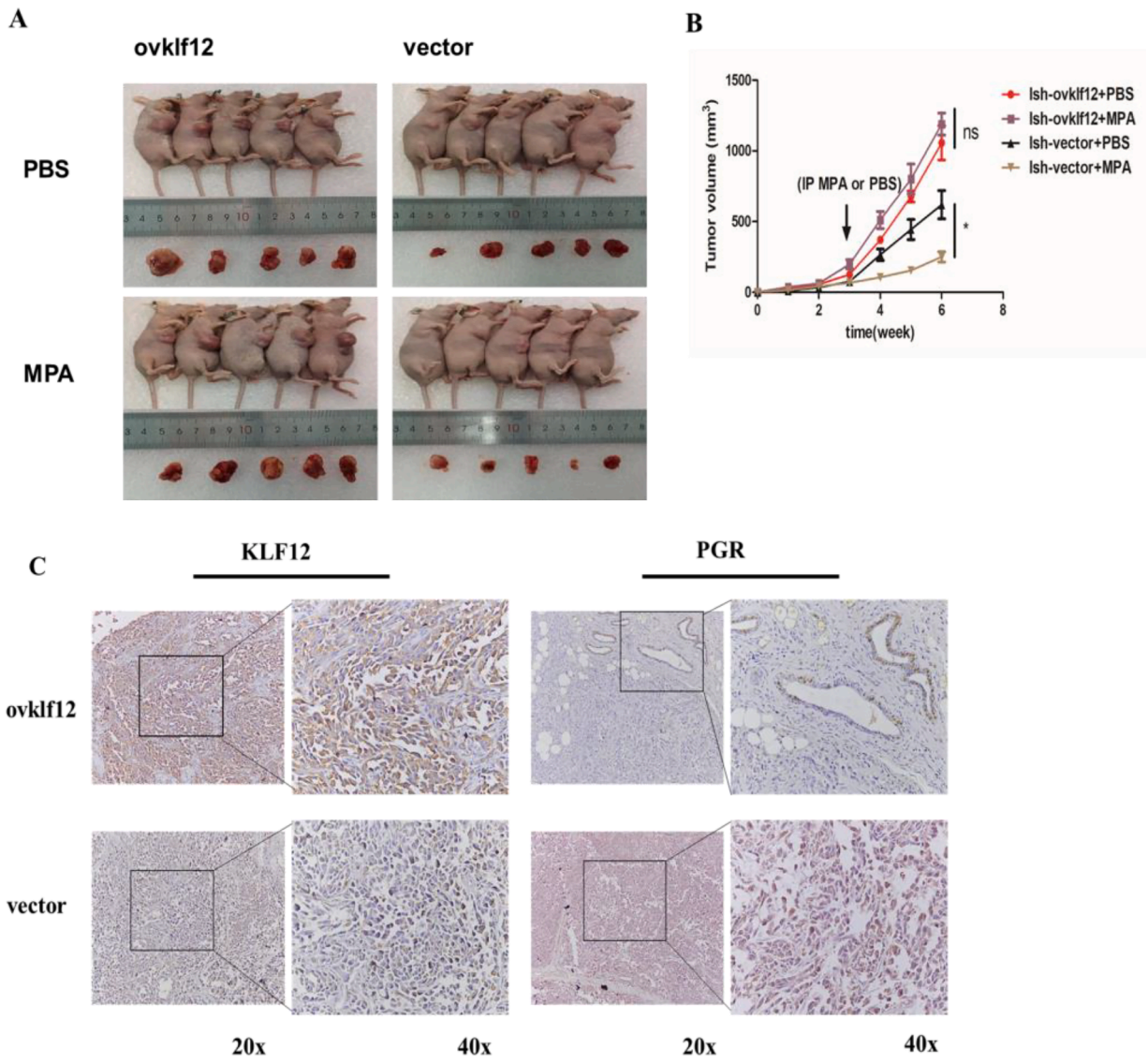

Fig. 6. KLF12 overexpression decreases EC progesterone sensitivity *in vivo*.
(A) Images of subcutaneous neoplasia at day 42. Ish-ovklf12 and Ish-vector cells were treated with MPA. (B) The subcutaneous tumor volume was measured at different time points, and the growth trend was noted. (C) Immunohistochemistry of mouse tumor tissues for KLF12 and PGR expression detection in Ish-ovklf12 + MPA and Ish-vector + MPA groups. Ip: intraperitoneal injection, *P<0.05, **P<0.01, ****P<0.001, ns: not statistically significant.

effectively. KLF12 may be a therapeutic target or a predictive biomarker for EC's progesterone insensitivity. Thus, targeting KLF12 could be a potential method for treating EC.

CRediT authorship contribution statement

Haimeng Shi: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **Jian Li:** Data curation, Formal analysis, Methodology, Validation, Writing – original draft. **Tong Yan:** Formal analysis, Supervision, Writing – review & editing. **Ling Zhou:** Formal analysis, Methodology, Software, Writing – original draft. **Yu Zhu:** Formal analysis, Methodology, Validation, Writing – original draft. **Feifei Guo:** Conceptualization, Investigation, Methodology, Supervision, Validation. **Sihui Yang:** Data curation, Software, Validation. **Xiangyi Kong:** Formal analysis, Methodology, Project administration. **Huaijun Zhou:** Formal analysis, Methodology, Software, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 82172819 and 81972434).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2024.102041.

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