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Original Articles

A20-mediated deubiquitination of ERα in the microenvironment of CD163$^+$ macrophages sensitizes endometrial cancer cells to estrogen

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ABSTRACT

Continuous estrogen signaling is thought to be the main mechanism causing endometrial cancer (EC). Studies have demonstrated that CD163$^+$ macrophages could promote the development of estrogen-dependent EC, but the mechanisms involved remain unclear. We found that CD163$^+$ macrophages were the dominant macrophages in atypical endometrial hyperplasia and cancer, and their infiltration was positively associated with ERα expression. CD163$^+$ macrophages mainly increased ERα protein levels but with little upregulatory effect on *ESR1* (ERα coding gene) transcripts. The ubiquitin-editing enzyme A20, screened from the endometrial microarray obtained from mice receiving a high-fat diet and sustained estrogen-intervened, was highly expressed in endometrial lesions rich with CD163$^+$ macrophages, and positively correlated with ERα expression. Similarly, A20 and ERα were both upregulated by CD163$^+$ macrophages via cytokines such as IL1α, IL17A and TNFα. Mechanistically, A20 overexpression in EC cells prolonged ERα protein half-life without affecting *ESR1* transcripts. A20 increased functional ERα protein levels and enhanced estrogen-driven EC cell proliferation through preventing ERα protein degradation by its deubiquitinase activity. Our study revealed that A20-mediated deubiquitination of ERα might be an important mechanism by which CD163$^+$ macrophages sensitize EC cells to estrogen.

1. Introduction

It is generally accepted that persistent estrogen stimulation without progesterone protection contributes to abnormal endometrial proliferation and even endometrial endometrioid carcinoma (EEC) [1]. Patients with endometrial hyperplasia and EEC are likely to be complicated with metabolic syndromes, which put the body into a mild inflammatory state [2,3]. This chronic inflammation is a well-known carcinogenic factor even for hormone-related cancers [4]. Studies have reported that certain inflammatory cytokines (e.g. IL6) in the microenvironment of hormone-related tumors can upregulate hormone biosynthesis or hormone receptor expression to activate the hormone signaling and to accelerate tumor cell proliferation [5–9]. However, the role of chronic inflammation in hormone-related carcinogenesis including endometrial cancer (EC) is far from being understood. Therefore, exploring how chronic inflammation increases estrogen sensitivity in the endometrium might help in understanding the relationship between chronic inflammation and estrogen-dependent EC, and in the

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development of more effective preventive and treatment measures.

Macrophages are sentinel immune cells that infiltrate tumors abundantly during the early stages of tumor development [10]. As an important component of chronic inflammation, infiltrating macrophages can promote cell proliferation and metastasis in hormone-related tumors such as breast, prostate, and endometrial cancers, by activating hormone signaling [7,11–13]. We previously demonstrated that $CD163^+$ macrophages could promote estrogen-driven EC cell proliferation via IL17A-mediated epigenetic modulation of the *ESR1* (ERα coding gene) promoter [6]. Notably, IL17A showed significant upregulation of ERα protein levels in EC cells compared with upregulation of its mRNA levels. This suggested that post-transcriptional or post-translational effects might be involved in the mechanism of ERα protein upregulation in the microenvironment of EC with $CD163^+$ macrophages.

It has been reported that ERα protein level is not linearly related with its mRNA level in breast cancer [14,15]. An important mechanism found is the post-translational modification that regulates ERα protein turnover [16]. As a protein post-translational modification, ubiquitination modulation plays an important role in ERα protein stability and transactivation of its target genes such as *TFF1* and *CTSD*, which contains estrogen response element (ERE) at their promoter regions [17]. The binding of estrogen and ERα promotes ERα phosphorylation which can recruit co-factors to bind ERE and regulate ERα target genes. Meanwhile, activated ERα can be recognized by ubiquitin ligases that prime the 26S proteasome for ERα degradation [16,18,19]. In breast cancer, Pin1 stabilizes ERα protein by blocking its phosphorylation-dependent ubiquitination and degradation to increase breast cancer cell proliferation [20]. In contrary, Speckle-type POZ protein (SPOP) is an important E3 ligase enzyme which can destabilize the ERα protein, whose mutation in EC leads to defectiveness in regulating ERα ubiquitination and degradation and results in sustained estrogen signaling [21]. These suggested that aberrant ERα ubiquitination modulation leads to estrogen signaling dysregulation, which increases the risk of estrogen-related cancers.

A20, encoded by *TNFAIP3* in chromosome 6, is an important ubiquitin-editing enzyme. A20 contains an N-terminal OTU-type deubiquitinase (DUB) domain and seven zinc-finger domains at the C-terminus, which maintains protein stability though its deubiquitinase and promotes protein degradation through its E3 ubiquitin ligase, respectively [22]. Whether infiltrating $CD163^+$ macrophages regulate ERα protein through deubiquitination modification by A20 in EC has not been clarified.

In this study, we found that A20-mediated deubiquitination of ERα in a tumor microenvironment rich with $CD163^+$ macrophages played an important role in estrogen-driven endometrial carcinogenesis. Our study revealed that A20 may act as an oncogene for pathological ERα activation. In this way antagonizing A20 might be a potential therapeutic strategy to prevent estrogen-dependent EC.

2. Materials and methods

2.1. Ethics statement

This study complied with the tenets of the Helsinki Declaration and the National Guidelines for Animal Use in Research (China). This study was approved by the Medical Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University (Shanghai, People's Republic of China, hereafter referred to as 'Ob&Gyn Hospital').

2.2. Endometrial sample collection

Endometrial tissues in normal proliferative phase (PP), or with hyperplasia, atypia hyperplasia (EAH), and EEC (including endometrioid adenocarcinoma grade 1, grade 2, and graded 3) were obtained from patients who underwent hysterectomy at Ob&Gyn Hospital from January 2016 to November 2017. None of the patients received hormone therapy within 3 months before surgery. Normal endometrium was obtained from patients receiving hysterectomy because of leiomyoma, cervical high-grade squamous intraepithelial lesion (HSIL) or cervical cancer. Pathological diagnosis of endometrial samples was independently confirmed by at least two pathologists.

Twenty-two fresh endometrial tissues were collected for flow cytometric analysis and patient information was listed in Supplementary Table S1. A further 29 cases were collected for IHC staining and patient information was listed in Supplementary Table S2.

2.3. Fresh endometrium preparation and flow cytometric analysis

Fresh endometrium was prepared as previously described [23] and seen in Supplementary materials and methods. Cells from fresh endometrium were labeled with fluorescent-tagged antibodies and then detected by flow cytometry (Beckman). Anti-Hu CD45-BV510, anti-Hu IFNγ-Alexa 647, anti-Hu TGFβ-BV421, anti-mIgG2b Kpa-BV510, anti-mIgG1 Kpa-Alexa 647, and anti-mIgG1 Kpa-BV421 antibodies were purchased from BD Pharmingen, while anti-Hu CD14-PerCP/Cy5.5, anti-Hu CD86-PE/Cy7, anti-Hu CD163-PE, anti-mIgG1 κ Isotype-PerCP/Cy5.5, anti-mIgG1 κ Isotype-PE/Cy7, and anti-mIgG1 κ Isotype-PE antibodies were purchased from Biolegend.

2.4. Immunohistochemical (IHC) staining

IHC staining was performed as previously described [24]. Primary antibodies used in IHC staining included CD163 (Abcam, ab182422), A20 (Cell Signaling Technology, D13H3), and ERα (Cell Signaling Technology, D8H8). Semi-quantitative optical analysis was performed as previously described (6).

2.5. Establishment of mouse model and microarray analysis

Female C57BL/6 mice received bilateral oophorectomy at 6–8 weeks and then were separated randomly into two groups. The mice in the control group were fed with normal chow diet while the other group received a high-fat diet (Research Diets, D12492) and subcutaneously implantation of an estrogen-releasing pellet (Innovative Research of America, NE-121, 0.36 mg/pellet). After 12 weeks of intervention, the endometrial samples were collected and total RNAs were extracted for microarray analysis using the Affymetrix GeneChip Mouse Transcriptome Array 1.0 by GMINIX (Shanghai, China). Microarray data was uploaded to the Gene Expression Omnibus repository, with the GEO accession number GSE102103 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102103).

2.6. Cell lines

EC cell lines RL95-2, Ishikawa, and SPEC2 were kindly provided by Dr. Yu Yinhua (MD Anderson Cancer Center, Houston, TX) and HEC-1A was kindly provided by Dr. Wei Lihui (Peking People Hospital, Beijing, China). EC cell line KLE, human acute monocytic leukemia cell line THP-1, and human embryonic kidney (HEK) 293T cells were purchased from ATCC (USA). RL95-2, Ishikawa, SPEC2, and HEC-1A cells were cultured in DMEM/F12 medium, and KLE and HEK-293T in DMEM medium, while THP1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS).

2.7. Expression constructs

The A20 plasmids were purchased from Addgene (http://www.addgene.org/) and subcloned into pCMV-Myc and pCMV-Flag expression vectors. A20 N-terminal DUB domain, C-terminal E3 ligase (ZnF1-7) domain and their corresponding mutant structures as described were also subcloned into pCMV-Myc and pCMV-Flag expression vectors [25].

ERα was subcloned into pCMV-Flag, pCMV-Myc and pCMV-Flag-HA (FH) expression vectors.

2.8. Gene transfection and real-time PCR

Plasmids and siRNAs were transfected using Lipofectamine 3000 and RNAiMAX (Invitrogen) according to the manufacturer's protocol. siRNA sequences were listed in Supplementary Table S3. The mRNA levels were measured by real-time PCR (RT-PCR) using SYBR Premix Ex Taq (Takara, China). Primers were listed in Supplementary Table S4.

2.9. Drug intervention

The following drugs were used for cell interventions as indicated: phorbol-12-myristate-13-acetate (PMA) (Sigma Aldrich, P8139), IL4 (Sigma Aldrich, SRP3093), IL13 (Sigma Aldrich, SRP3274), recombinant human IL1α (Novoprotein, C070), recombinant human IL6 (Novoprotein, C027), recombinant human IL10 (Novoprotein, C027), recombinant human MCP2 (Novoprotein, C063), recombinant human GM-CSF (Novoprotein, C003), recombinant human IL17A (Sigma Aldrich, SRP3080), IL17A receptor antagonist (R&D systems, MAB177), pyrrolidine dithiocarbamate (PDTC) (Sigma Aldrich, P8765), MG132 (Selleckchem Houston, USA, S2619), 17β-estrogen (Sigma Aldrich, E2758), cyclohexamide (CHX) (Enzo, ALX-380269), and vehicles included ethanol (EtOH), dimethyl sulfoxide (DMSO), and PBS. Prior to E2 intervention, cells were cultured in phenol red-free medium supplemented with 10% charcoal-stripped FBS.

2.10. ESR1 dual-luciferase reporter assays

Cells were transfected with pGL3-*ESR1* promoter, Firefly plasmid, and pGL4.74 [hRluc/TK] Renilla plasmid, which acted as the internal control. The pGL3-*ESR1* promoter plasmids contain 4000 bp upstream of the transcription initiation site. The cells were lysed to measure the luciferase activity by VARIOSKAN FLASH (Thermo Scientific) using a dual luciferase reporter kit (Promega, E1910).

2.11. Cytokine arrays

The supernatants of THP-1, $CD163^+$ Mø, HEC-1A cells and $CD163^+$ Mø and HEC-1A co-culture were collected for cytokine arrays (RayBio Human Inflammation Antibody Array G3, Guangzhou, Shanghai). Forty-two cytokines and their fluorescent values were described in detail in Supplementary Table S5.

2.12. Immunofluorescent staining

Cell immunofluorescent staining was performed as previously described [24]. Primary antibodies against A20 (Santa Cruz Biotechnology, sc-166692) and ERα (Sigma Aldrich, SAB4500812) were incubated with cells overnight at 4 °C followed by incubation with secondary fluorescent-tagged antibody (anti-mouse, Alexa Fluor® 488 and 555, Life Technology) for 1 h. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Beyotime, China) for 5 min. Fluorescence was detected using a fluorescence microscope (Nikon).

2.13. Co-immunoprecipitation (Co-IP) and western blotting (WB)

Exogenous and endogenous co-IP procedures were performed as described previously [21]. Primary antibodies were as follows: ERα (Sigma Aldrich, SAB4500812), A20 (Abcam, ab92324), p65 (Abcam, 32536), p-p65 (Abcam, 76302), Myc-tag (MBL, M047-7), Flag-tag (MBL, M185-7), HA-tag (MBL, M180-7), GAPDH (Cell Signaling Technology, 14C10), and β-actin (Beyotime, AA128).

2.14. In vitro ubiquitination assays

In vitro ubiquitination assays were performed as previously described [26]. HEK-293T cells were lysed with 0.5% NP40 buffer and then incubated with anti-Myc affinity gel (Selleck, B23401) or anti-FLAG antibody-conjugated M2 agarose beads (Sigma) overnight at 4 °C. Ubiquitination levels of ERα were analyzed by western blotting.

2.15. Cell proliferation and apoptosis

5-Ethnyl-2'-deoxyuridine (Edu) staining, CCK8 (DOJINDO, Japan) and cell apoptosis (DOJINDO, Japan) were performedas described [21,27,28].

2.16. Statistics

All experiments were repeated at least three times in this study. SPSS 19.0 (IBM SPSS Software) was used for statistical analyses. Student's t-test, one-way or two-way ANOVA, and Spearman's correlation analysis were used for further analyses. P-values of 0.05 were considered statistically significant. Error bars indicate standard deviation (SD) in the graphs.

3. Results

3.1. $CD163^+$ $TGFβ^+$ macrophages are the dominant macrophages in endometrial lesions

Studies have shown that infiltrating macrophages are positively related to the risk of EC [11,29]. We first investigated whether $CD163^+$ macrophages are the dominant macrophages in endometrial lesions. Tissue-infiltrating lymphocytes ($CD45^+$) and monocytes ($CD14^+$) in fresh endometrial tissue from patients (PP, EAH, and EEC) were labeled and analyzed by flow cytometry (Fig. 1A). CD14 is mainly expressed on the surface of macrophages [30]. We found that the $CD14^+/CD45^+$ lymphocyte ratio was significantly increased in EAH and EEC compared with normal endometrium (Fig. 1B), indicating increased infiltrating macrophages in EAH and EEC lesions. As macrophages have pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes, we further used CD molecules and cytokines to label M1 (CD86, INFγ) and M2 (CD163, TGFβ) macrophages in $CD14^+$ monocytes [31–33]. The ratio of $CD163^+TGFβ^+$ to $CD86^+INFγ^+$ macrophages was elevated in both EAH and EEC lesions (Fig. 1C), demonstrating that $CD163^+TGFβ^+$ macrophages (M2 macrophages) were the dominant macrophages in EAH and EEC.

Because macrophages play important roles in the innate immune system, we used a mouse model to determine whether an innate immune response existed in EC-prone environments. Ovariectomized female mice were fed a high-fat diet for three months and subcutaneously implanted with a 17β-estrogen pellet to mimic the in vivo environment of EC patients. Endometrial samples from the mice were then harvested for microarray analysis. Further Gene Ontology analysis proved that innate immune responses in the endometrium were substantially induced by estrogen exposure and high-fat intake (Fig. 1D). Therefore, we speculated that $CD163^+TGFβ^+$ macrophages (hereafter called $CD163^+$ macrophages) might be the dominant macrophages in endometrial lesions and are involved in estrogen-driven endometrial carcinogenesis.

3.2. A20 and ERα are positively correlated with the amount of $CD163^+$ macrophages in endometrial lesions

As previously reported, $CD163^+$ macrophages promoted EC cell proliferation through upregulation of ERα expression [6]. $CD163^+$ macrophages were successfully induced (Supplementary Fig. S1), and the culture medium (CM) was extracted and then added to EC cells to verify the role of $CD163^+$ macrophages in regulating ERα expression.

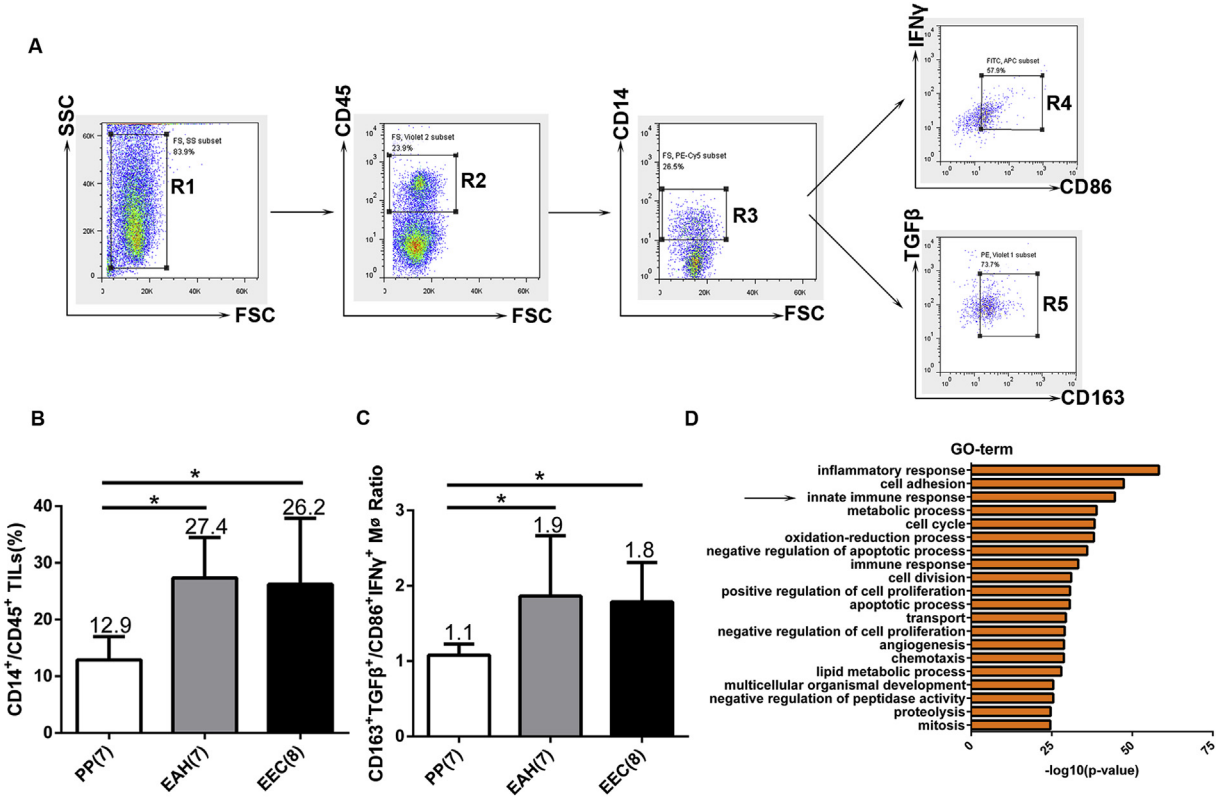

Fig. 1. CD163$^+$TGFβ$^+$ macrophages are the dominant macrophages in endometrial cancer lesions. A. Flow diagram of flow cytometry analysis. Cells of R1 (FSC vs. SSC) represent endometrial stromal cells including total leukocytes. Gate R2 is involved in gate R1; cells of gate R2 represent CD45$^+$ cells. Gate R3 is involved in gate R2; R3 cells represent CD14$^+$CD45$^+$ cells. R4 and R5 are involved in gate R3; R4 cells represent CD86$^+$INFγ$^+$ macrophages and R5 cells represent CD163$^+$TGFβ$^+$ macrophages. **B.** CD14$^+$/CD45$^+$ lymphocyte ratio in fresh endometrium from patients with PP, EAH, and EEC was analyzed by flow cytometry. The results were shown as a percentage in total CD45$^+$ cells. **C.** CD163$^+$TGFβ$^+$/CD86$^+$INFγ$^+$ macrophage ratio in fresh endometrium from patients with PP, EAH, and EEC was analyzed by flow cytometry. **D.** GO analysis of estrogen- and high-fat diet-induced differential expression genes (DEGs) in mouse endometrial samples. The top twenty functions induced by estrogen and high-fat diet are presented. PP, proliferative phase endometrium; EAH, endometrium atypical hyperplasia; EEC,endometrial endometrioid carcinoma. Values indicate mean ± S.D., n (PP) = 7, n (EAH) = 7, n (EEC) = 8, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

CD163$^+$ macrophage CM upregulated ERα expression in EC cells, while slightly activating *ESR1* mRNA transcription without affecting its promoter activity (Fig. 2A and B), suggesting that CD163$^+$ macrophages might upregulate ERα protein post-transcriptionally or post-translationally.

We next performed microarray analysis of mouse endometrium under estrogen and high-fat diet intervention and found a gene set associated with ubiquitin enzymes was dysregulated (Fig. 2C). CD163$^+$ macrophage CM intervention further confirmed the same change in ubiquitin enzymes in HEC-1A cells (Supplementary Fig. S2). Among the ubiquitin-editing enzymes identified, A20 was markedly elevated in the CD163$^+$ macrophage CM intervention test (Fig. 2D). As ubiquitination modification is an important post-translational mechanism for ERα protein modification [16], we thereby evaluated the potential relationship among A20, ERα expression, and the number of infiltrating CD163$^+$ macrophages in human endometrial tissues (Fig. 2E). IHC staining showed small numbers of CD163$^+$ macrophages in the stroma of normal proliferating endometrium. The number of CD163$^+$ macrophages increased gradually when endometrial lesions developed from hyperplasia to EC (Fig. 2F). Since the stroma gradually decreased in the lesion tissues from G1 to G3, the amount of infiltrating CD163$^+$ macrophages also consistently decreased in the same proportion in IHC staining. Consistent with CD163$^+$ macrophage infiltration, A20 and ERα were both highly expressed and positively correlated in hyperplasia, EAH, and EEC G1 (Fig. 2G), but decreased gradually when the lesions progressed from G1 to G3. We further analyzed A20 protein levels in ERα-positive or ERα-weak positive EC cell lines (Ishikawa, ECC1, RL95-2, and HEC-1A) and ERα-negative EC cell lines (KLE and SPEC2) (Fig. 2H). ERα and A20 protein expression changed by a similar degree in EC cell lines. These findings suggested that A20 and ERα protein were positively correlated in the microenvironment of CD163$^+$ macrophages.

3.3. A20 and ERα are upregulated by CD163$^+$ macrophages via cytokines

To explore the potential mechanisms involved in CD163$^+$ macrophage-mediated upregulation of ERα protein, CD163$^+$ macrophages and HEC-1A co-culture medium were collected to assess cytokine changes by cytokine array (Fig. 3A). Cytokines such as GM-CSF, IL1α, IL6, IL10, IL17A, MCP2, and TNFα, increased remarkably in the supernatant of co-culture systems compared with that from CD163$^+$ macrophages or HEC-1A alone (Fig. 3B). We then asked whether CD163$^+$ macrophages upregulated A20 and ERα in a paracrine manner through these cytokines. Interestingly, IL-1α, IL17A, and TNFα increased A20 and ERα expression in EC cells (Fig. 3C). We next selected IL17A for further investigation. IL17A increased A20 and ERα expression in a dose- and time-dependent manner, and neutralization of IL17A with its antagonistic antibody blocked IL17A-induced A20 and ERα expression (Supplementary Fig. S3).

As IL1α, IL17A, and TNFα regulate the NF-κB pathway, NF-κB inhibitor PDTC was used to test whether this pathway is involved in the upregulation of A20 and ERα expression. Data showed that PDTC blocked cytokine-induced expression of A20 and ERα (Fig. 3D). IL1α and TNFα upregulated A20 transcripts without affecting ERα transcripts, while IL17A upregulated ERα transcripts very slightly (Fig. 3E). These findings suggested that IL1α and IL17A might upregulate A20

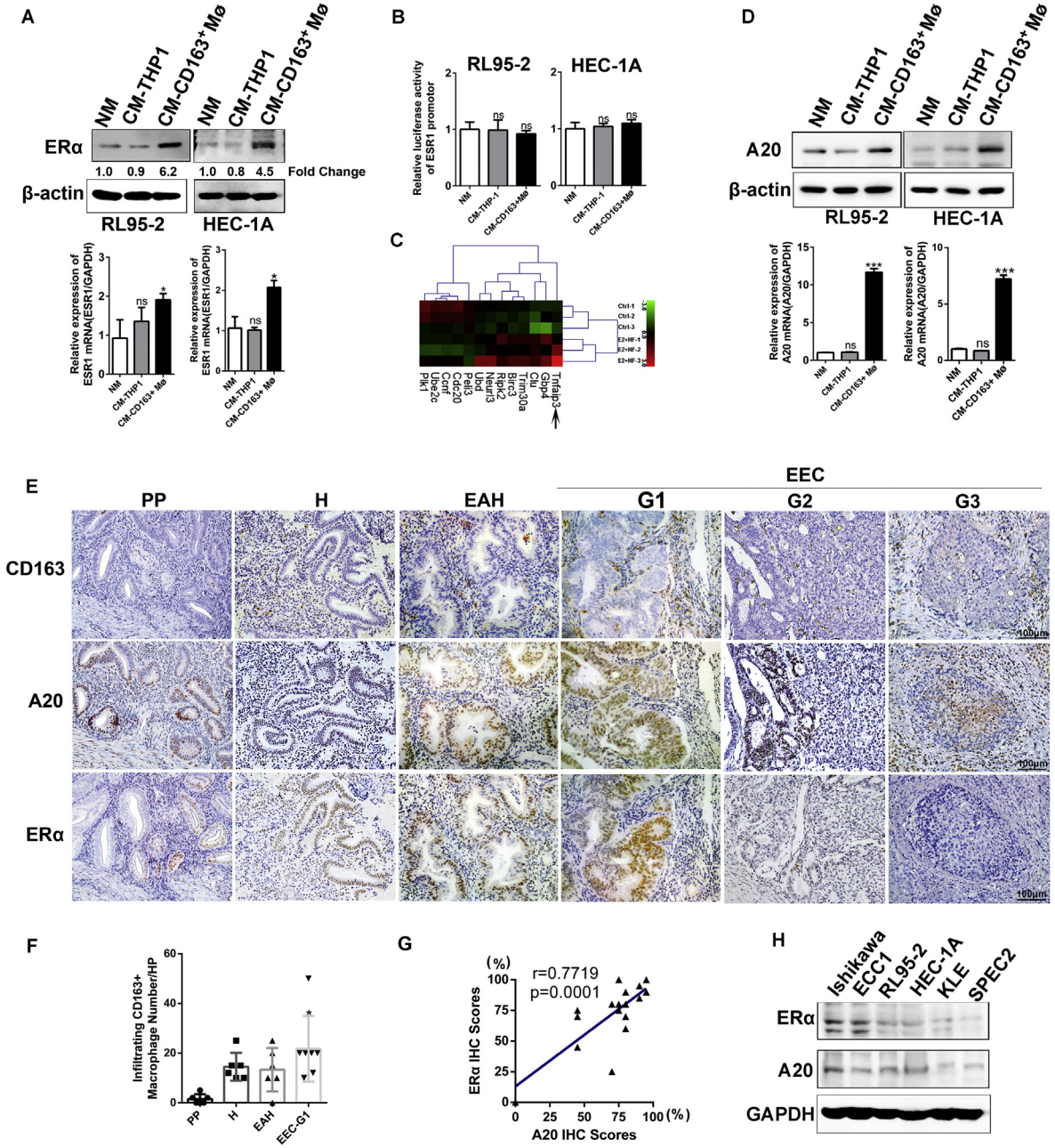

Fig. 2. A20 and ERα are positively correlated with the amount of CD163$^+$ macrophages in endometrial lesions. A. CD163$^+$ macrophage culture medium (CM) upregulated ERα protein expression but slightly upregulated *ESR1* mRNA levels in EC cells. Cells were cultured in normal medium (NM), THP1 CM, and CD163$^+$ macrophage CM for 16 h and 36 h with the most significant effect for RT-PCR and western blotting. The fold change of ERα relative to control group was calculated from densitometry. **B.** CD163$^+$ macrophage CM did not have significant transactivation effect on the *ESR1* promotor in EC cells. Cells were transfected with pGL3-*ESR1* promoter plasmids and pGL4.74 [hRluc/TK] (internal control) for 18 h and then cultured in normal medium (NM), THP1 CM, and CD163$^+$ macrophage CM for 18 h. **C.** Ubiquitin enzymes in mouse endometrium regulated by prolonged estrogen and high-fat diet intervention. The samples of endometrium were collected for microarray analysis. The mRNA levels of *TNFAIP3* were increased in the intervention group (black arrow). **D.** CD163$^+$ macrophage CM upregulated mRNA and protein levels of A20 in EC cells. Cells were cultured in NM, THP1 CM, and CD163$^+$ macrophage CM for 16 h and 36 h for RT-PCR and western blotting analysis. **E.** IHC staining for CD163, A20, and ERα expression in endometrial hyperplasic lesions. Original magnification 200 ×. Scale bars, 100 μm. **F.** The number of CD163$^+$ macrophages increased gradually from normal endometrium in the proliferative phase to EC. **G.** The staining intensity of A20 and ERα is scored by semi-quantitative optical analysis. Correlation of A20 and ERα IHC scores was analyzed by Spearman's correlation. **H.** ERα and A20 protein expression changed to a similar degree in EC cell lines. Baseline A20 and ERα protein expression in different EC cell lines was analyzed by western blotting. PP, proliferative phase; H, hyperplasia; EAH, endometrial atypical hyperplasia; G1, endometrioid adenocarcinoma grade 1; G2, endometrioid adenocarcinoma grade 2; G3, endometrioid adenocarcinoma grade 3. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

and ERα through NF-κB signaling. Since these cytokines mainly upregulated ERα protein with little effect on *ESR1* transcripts in EC cells, we speculated that A20-mediated post-translational modulation of ERα might be an important mechanism for increasing ERα abundance in the infiltrating macrophage-rich microenvironment of EC.

3.4. A20 increases ERα protein by preventing protein degradation

To test our hypothesis that A20 upregulated ERα protein in EC cells post-translationally, we compared ERα protein levels and *ESR1* mRNA levels through plasmid transfection of A20 in EC cells. Overexpression

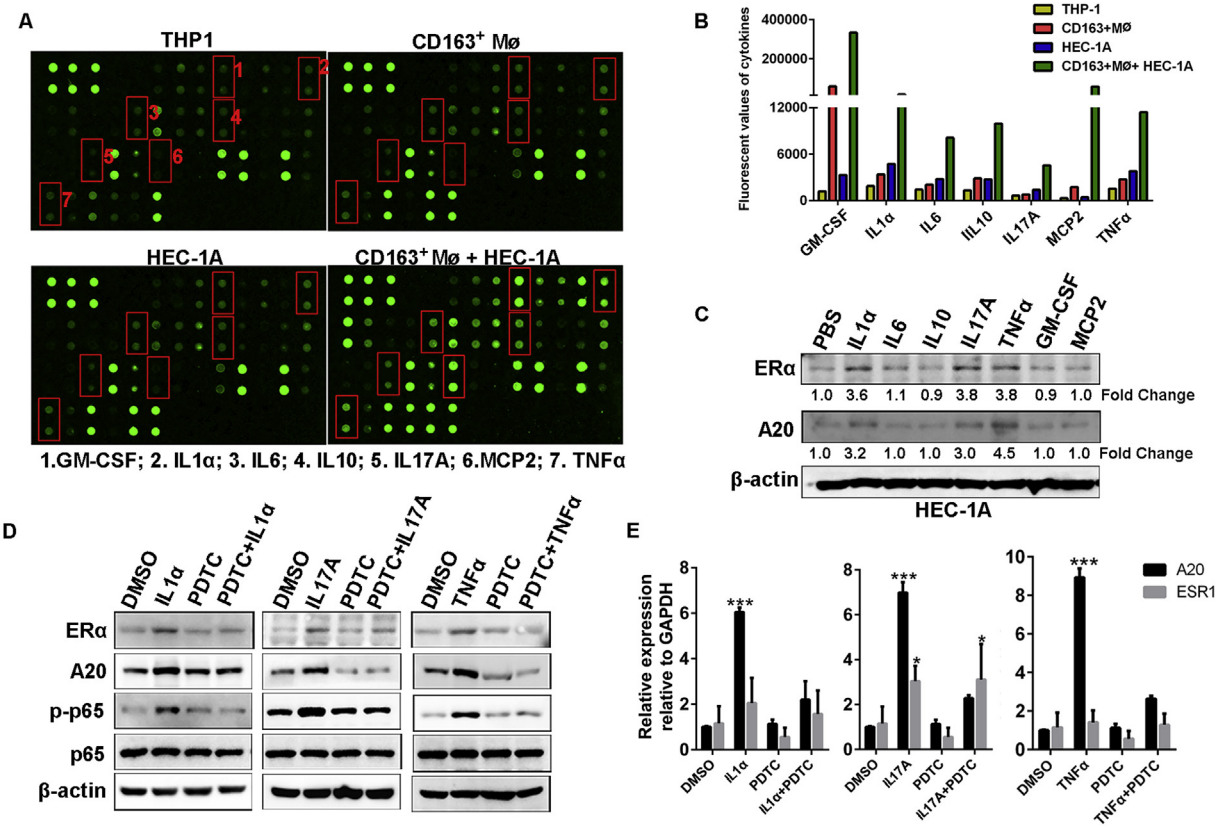

Fig. 3. A20 and ERα are upregulated by CD163$^+$ macrophages via cytokines. A. Co-culture of CD163$^+$ macrophages and HEC-1A cells significantly upregulated the production of cytokines. CD163$^+$ macrophages and HEC-1A were co-cultured for 24 h. Culture medium from this co-culture system as well as those culturing THP1 cells, CD163$^+$ macrophages, and HEC-1A cells alone were separately extracted. Cytokines in the culture medium were detected by cytokine array. Red boxes indicate GM-CSF, IL1α, IL6, IL10, IL17A, MCP2, and TNFα, as marked in the key. **B.** Fluorescence values of seven cytokines. **C.** Dysregulated cytokines at a dose of 50 ng/ml upregulated A20 and ERα protein expression, especially IL1α, IL17A, and TNFα. HEC-1A cells were treated with these cytokines for 36 h and then harvested for western blotting. The fold change of ERα and A20 relative to control group was calculated from densitometry. **D.** IL1α, IL17A, and TNFα upregulated A20 and ERα expression through NF-κB signaling. HEC-1A cells were pretreated with NF-κB inhibitor 20 μM pyrrolidine dithiocarbamate (PDTC) for 4 h and then were treated with these cytokines for 36 h. **E.** IL1α, and TNFα upregulated A20 transcripts without affecting ERα transcripts, while IL17A upregulated ERα transcripts very slightly. HEC-1A cells were treated with these cytokines for 16 h and then harvested for RT-PCR. $^*P < 0.05$, $^{***}P < 0.001$.

of A20 upregulated ERα protein levels but not the corresponding *ESR1* mRNA levels (Fig. 4A and B). Luciferase reporter assays in HEK-293T cells confirmed that there was no activating effect of A20 on the *ESR1* promoter (Fig. 4C). Furthermore, silencing of A20 by A20-targeted siRNAs led to a decrease in ERα protein and *CSTD* mRNA (ERα target gene) without affecting *ESR1* mRNA (Fig. 4D and E). These results all suggested that A20 might regulate ERα post-translationally.

As A20 is an important ubiquitin-editing protein that regulates protein degradation or stability, we supposed that A20 might upregulate ERα protein through prevention of ERα protein degradation. Overexpression of A20 prolonged the half-life of endogenous ERα protein and downregulation of A20 expression by using siRNAs towards A20 shortened the half-life of endogenous ERα protein in the presence of protein synthesis inhibitor CHX (Fig. 4F and G). Taken together, these findings suggest that A20 increased ERα protein stability by protecting ERα protein from degradation.

3.5. A20 interacts with ERα

We next investigated whether A20 stabilized ERα protein through protein interactions. Exogenous and endogenous co-IP experiments were performed to assess the binding of ERα and A20. Reciprocal co-IP assays confirmed that A20 interacted with ERα (Fig. 5A and B). Furthermore, endogenous A20 and ERα were co-localized in the nuclei of EC cells (Fig. 5C). These suggested that A20 might form a complex with ERα protein in cell nuclei to stabilize ERα protein.

3.6. A20 stabilizes ERα protein through deubiquitination of ERα protein

As A20 was found to interact with ERα and stabilize its protein structure, experiments were conducted to determine which domain of A20 was involved. A20 N-terminal DUB domain, C-terminal E3 ligase ZnF1-7 domain, and their corresponding mutant structures were established (Fig. 6A). Co-IP assays showed that ERα could bind with the N-terminal and C-terminal of A20 and their mutant structures (Supplementary Fig. S4), but ectopically expressed A20 N-terminal domain stabilized ERα protein instead of A20 C-terminal domain (Fig. 6B). As the DUB activity of A20 is attributed to its N-terminal OTU domain, we deleted the OTU domain from A20 to further confirm our hypothesis. Deletion of the A20 OTU domain had no stabilization effect on co-transfected ERα protein in HEK-293T cells (Fig. 6C). Similarly, enzymatic dead mutant (C103A) A20 also had no stabilization effect on co-transfected ERα protein (Fig. 6D). HEC-1A cells transfected with different domains of A20 showed that only the A20 DUB domain stabilized ERα protein (Fig. 6E), which suggested that the A20 OTU domain was required for ERα stabilization.

To determine whether A20 regulates ERα polyubiquitination, HA-Ub and Myc-ERα were coexpressed in HEK-293T cells with different doses of wild-type (WT) A20, and then in vitro ubiquitination assays were conducted to detect ERα ubiquitination levels. We observed a dose-dependent reduction in the ubiquitination levels of ERα with increasing transfection of A20 (Fig. 6F). To prove whether A20 DUB was involved in the decrease of ERα ubiquitination levels, HA-Ub and Myc-

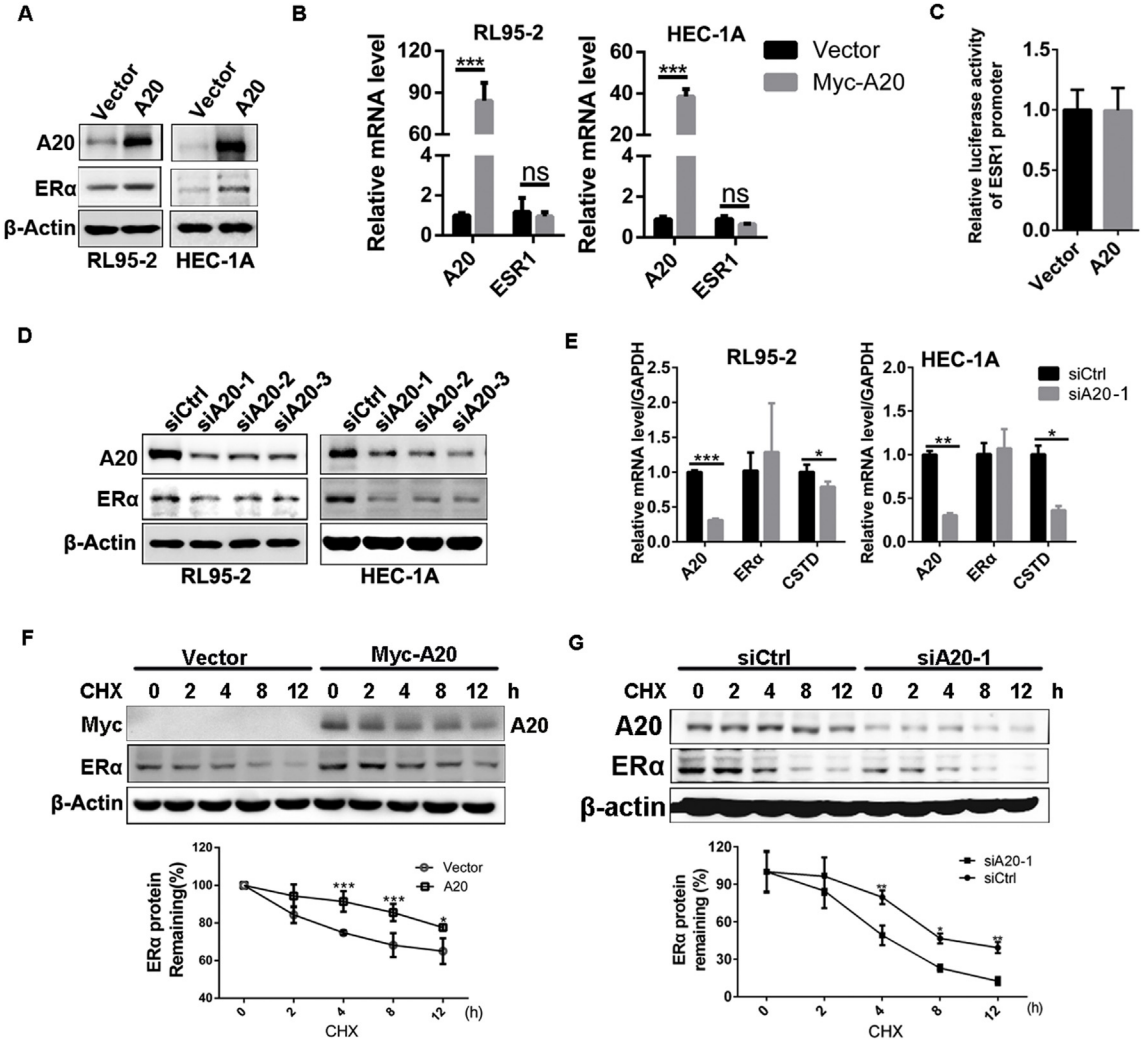

Fig. 4. A20 increases ERα protein by preventing protein degradation. A. Ectopically expressed A20 upregulated endogenous ERα protein levels in EC cells. EC cells were transfected with A20-expressing plasmids or empty vector and western blotting was used for evaluation of A20 and ERα protein levels in EC cells. **B.** A20 overexpression had no effect on *ESR1* mRNA levels in EC cells. EC cells were transfected with A20-expressing plasmids or empty vectors and RT-PCR was used to analyze *ESR1* mRNA levels. **C.** Ectopically expressed A20 did not activate *ESR1* transcription. HEK-293T cells were transfected with pGL3-*ESR1* promoter plasmids and pGL4.74 [hRluc/TK] plasmids for 18 h, and then transfected with A20-expressing plasmids and empty vectors for 18 h. Dual-luciferase reporter assays were conducted for detection of *ESR1* promotor activity. **D.** Silencing of A20 downregulated endogenous ERα protein levels in EC cells. EC cells were transfected with three independent siRNAs targeting A20 at different sites or siCtrl for 36 h. A20 and ERα were detected by western blotting. **E.** Silencing of A20 by siA20-1 resulted in decreased *CSTD* (ERα target gene) but not *ESR1* mRNA levels. EC cells were transfected with siA20-1 or siCtrl for 16 h. RT-PCR was used to analyze *ESR1* and *CSTD* mRNA levels. **F-G.** overexpression or downregulation of A20 level prolonged or shortened ERα protein half-life in HEC-1A cells respectively. HEC-1A cells were transfected with A20-expressing plasmids or empty vector, or siRNAs towards A20 or siCtrl for 24 h respectively. Cells were then treated with 50 μg/ml protein synthesis inhibitor cyclohexamide (CHX) and harvested at indicated time points for evaluation of ERα protein levels by western blotting. The intensity of ERα was normalized to the intensity of β-actin at each time point and then expressed as the percentages of the original value without treatment (time point, 0 h). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

ERα were co-expressed in HEK-293T cells with different domains of A20 and enzymatic dead mutant (C103A). The results showed that only the normal DUB domain of A20 could reduce ERα polyubiquitination (Fig. 6G). As SPOP is an important E3 ubiquitin ligase of ERα for proteasomal degradation [21], we also detected protein stability and ubiquitination level of ERα respectively in the presence of SPOP and/or A20. The data shown demonstrated that A20 and SPOP played opposite roles in regulating ERα stability and ubiquitination modification (Supplementary Figs. S5A–B). All these findings support the concept that A20 stabilizes ERα protein through preventing protein degradation via deubiquitination of ERα.

3.7. Overexpression of A20 increases sensitivity of EC cells to estrogen via regulation of ERα

To investigate whether EC cells with A20 overexpression possess more functional ERα protein, we evaluated the enhancement effect of A20 on estrogen sensitivity in EC cells. Cell proliferation and apoptosis assays showed that A20 WT increased EC cell proliferation and decreased cell apoptosis in the presence of E2, but A20 enzymatic dead mutant C103A (mutant type, MT) has no such effects (Fig. 7A–C). And this is also true for the effect of A20 on ERα protein level (Fig. 7D). The results of xenograft model in unterine horns of ovariectomized female mice was also consistent with that of in-vitro study (Supplementary Figs. S6A–B). Furthermore, A20 stabilized ERα in a dose-dependent manner in HEK-293T cells, and ERα protein abundance was more pronounced after estrogen treatment (Fig. 7E). To sum up, A20 can

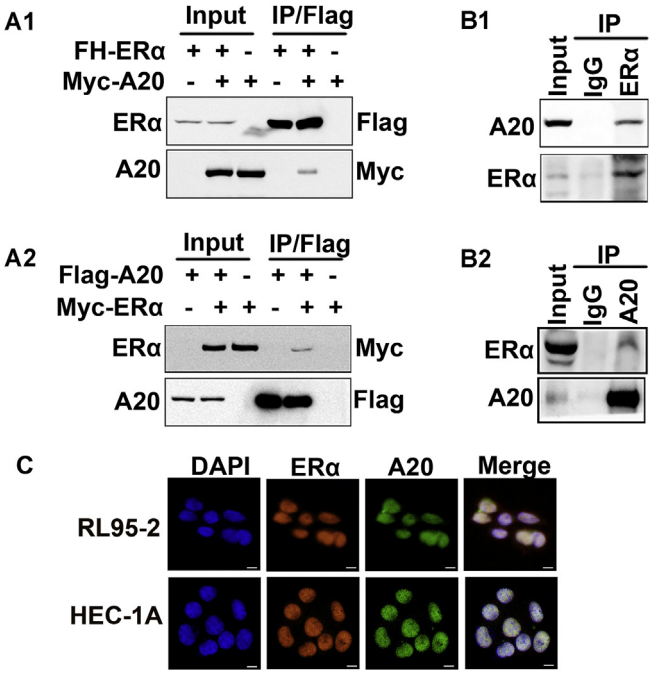

Fig. 5. A20 interacts with ERα. A. Ectopically expressed A20 and ERα showed a mutual interaction. HEK-293T cells were co-transfected with Myc-A20 and Flag-HA (FH)-ERα constructs (A1) or Flag-A20 and Myc-ERα (A2) for 30 h. After being treated with 20 μM MG132 for 8 h, cell lysates were prepared for co-IP with anti-FLAG beads and western blotting analysis. **B**. Endogenous A20 and ERα proteins interacted with each other in HEC-1A cells. After being treated with 20 μM MG132 for 8 h, cell lysates were prepared for co-IP with anti-ERα (B1) or anti-A20 (B2) antibody and western blotting analysis. **C.** ERα and A20 co-localized in EC cell nuclei. EC cells were immunostained with anti-ERα (red) and anti-A20 (green) antibodies and visualized by confocal microscopy. DAPI (blue) was used to indicate cell nuclei. Scale bars.

stabilize ERα protein and maintain estrogen signaling by inhibiting ERα ubiquitination degradation through interactions with ERα protein. This sustained estrogen signaling renders patients more susceptible to estrogen-dependent EC.

4. Discussion

In this study, we demonstrated that CD163$^+$ macrophage-associated chronic inflammation could stabilize ERα protein through A20-mediated deubiquitination modifications to sensitize EC cells to estrogen. Therefore, our study revealed that post-translational regulation was also an important mechanism involved in infiltrating macrophage-induced upregulation of estrogen sensitivity in endometrium.

Macrophage infiltration is one of the main characteristics of chronic inflammation that is closely related to endometrial carcinogenesis [10,12]. In this study, we found that CD163$^+$ macrophage infiltration is the early event of endometrial tumorigenesis. Although endometrial atypical hyperplasia is distinct from endometrial cancer, it is a precancerous stage which develops gradually into EEC G1 [34,35]. The findings that CD163$^+$ macrophages were abundantly infiltrated in EAH and EEC G1 suggested that CD163$^+$ macrophages might be involved in the progress from EAH to EEC. In this study, continuous section slides of endometrial lesions were used to stain A20, ERα and CD163 by immunohistochemistry, which indirectly proved that A20 and ERα co-localized in endometrial gland cells in CD163$^+$ macrophage-rich mesenchyme around endometrial glands. The expression of both A20 and ERα increased in EEC G1 compared with normal endometrium. However, when the lesions progressed from EEC G1 to G3, ERα presented with more pronounced decline than A20. These results suggest that when cells gained cancerous property under the stimulation of estrogen, estrogen is no longer the primary engine for cancer progression [36]. Therefore, we see low or even loss expression of ERα but moderate expression of A20 in EEC G3. In other words, EC no longer relies on ERα signaling or A20-mediated ERα protein stability when EEC G1 progressed to G3.

CD163$^+$ macrophage-associated chronic inflammation can provide a carcinogenic microenvironment [37,38]. In EC, the aromatase activity of stromal cells is upregulated by IL6, suggesting increased 17β-estrogen bio-synthesis [5]. Moreover, EC patients with insulin resistance showed increased GPER expression in EC tissues compared with those without insulin resistance [8,9,39]. Consistent with these reports, our findings also suggested that upregulation of ERα protein by CD163$^+$ macrophage-associated chronic inflammation is an important mechanism for estrogen-driven endometrial carcinogenesis.

The mechanisms by which CD163$^+$ macrophages increased ERα protein abundance are intriguing. In the present study, we demonstrated that A20 could positively regulate ERα protein stability through its N-terminal deubiquitinase activity. In conjunction with our recent report [6], we surmised that an inflammatory microenvironment with CD163$^+$ macrophages could upregulate ERα expression by both upregulating ERα expression through TET1-mediated epigenetic modulation of *ESR1* and stabilizing ERα protein through A20-mediated deubiquitination modification. Furthermore, the synergistic effects of these two mechanisms would be helpful for cascade amplification of ERα signaling, which ultimately promote the development of EC.

Reports showed that ERα ubiquitination modulation by E3 ligases or deubiquitinases significantly changed ERα expression and its function. There are three possible situations regarding ERα protein stability and transcriptional activity for ERα target genes: (a) increased ERα stability and transcriptional activity for ERα target genes (e.g. by RNF31, Pin1, MUC1) [20,40,41]; (b) increased ERα stability but decreased transcriptional activity for ERα target genes (e.g. by OTUB1) [42]; and (c) decreased ERα stability but increased transcriptional activity for ERα target genes (e.g. by E6PA, SPOP, SUG1, CUEDC2) [16,21,43,44]. These reports support a model in which transcriptional activity for ERα target genes can be related to ERα protein turnover to influence estrogen signaling. In this study, we found that A20-mediated deubiquitination of ERα protein could positively regulate ERα protein abundance and enhance estrogen signaling.

It has been reported that A20 expression is lower in ERα-positive breast cancer samples and cell lines [45], and A20 protein is overexpressed in human basal-breast cancer cell [46]. Contrary to breast cancer, our data demonstrated positive correlation between A20 and ERα both in endometrial tissues and EC cell lines. We supposed this difference might be due to the different nature of endometrial and breast cancer. Increased A20 expression can exhibit pro- or anti-apoptotic activity that depends on the cell type or context [47,48]. Our findings that A20 up-regulated proliferation in both ERα highly expressed (RL95-2) and moderately expressed (HEC-1A) EC cells lines suggested that A20 could increases estrogen sensitivity in ERα expressing EC cells.

There are some limitations in this study. For example, while we demonstrated that CD163$^+$ macrophages increased A20-mediated ERα abundance in EC cells through secreting cytokines such as IL1α, IL17A, and TNFα, it is not well understood as to whether macrophages affect EC cells through direct contact. Future investigations are warranted to solve these questions.

In summary, our data provides insights into the impact of A20 on ERα protein abundance in EC rich with CD163$^+$ macrophages. Insulin resistance is one of the important clinical manifestations of endometrial lesions [49,50], but the mechanisms involved are not clear [51]. In this study, we showed that CD163$^+$ macrophages-A20 mediated increased estrogen sensitivity might be one of the key mechanisms through which insulin resistance promotes carcinogenesis of endometrial cancer and negatively affect conservative treatment using progestin in endometrial cancer. Our study provides rational to target insulin resistance or A20

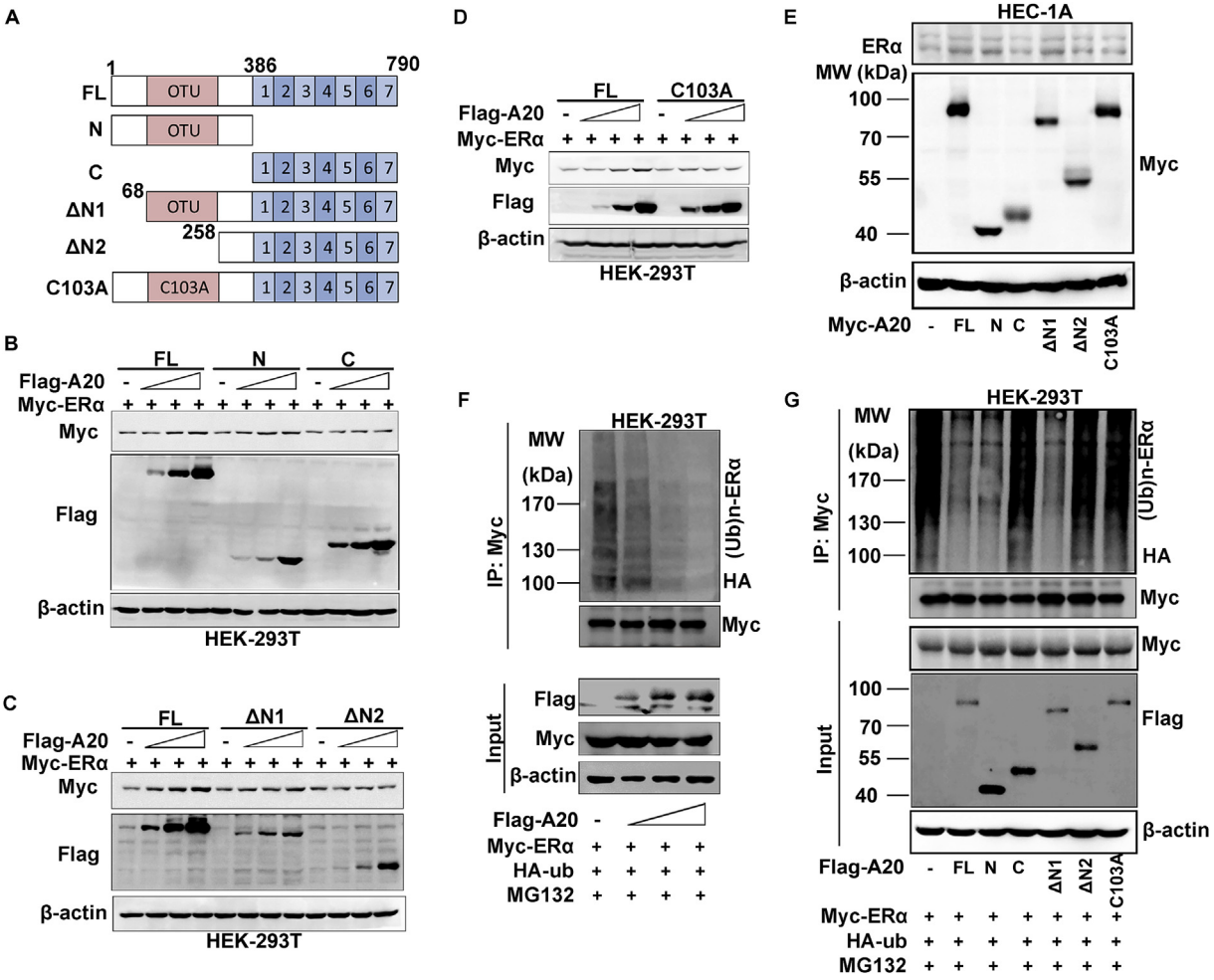

Fig. 6. A20 stabilizes ERα protein through deubiquitination of ERα protein. A. A schematic representation of the A20 deletion mutants. FL, full long; N, N terminus; C, C terminus, including seven zinc finger motifs; OTU, ovarian tumor domain; C103A, OTU domain was replaced with non-functional C103A mutant. **B.** The A20 N terminus upregulated ERα protein levels in a dose-dependent manner while the A20C terminus did not have this function. HEK-293T cells were transfected with 0.5 μg Myc-ERα and Flag-A20 FL/N/C plasmids at doses of 0, 0.25, 0.5, or 1.0 μg for 36 h before harvesting for western blotting. **C.** A20 and its ΔN1 structure upregulated ERα protein levels in a dose-dependent manner while A20 ΔN2 structure did not have this function. HEK-293T cells were transfected with 0.5 μg Myc-ERα and Flag-A20 FL/ΔN1/ΔN2 plasmids at doses of 0, 0.25, 0.5, or 1.0 μg for 36 h before harvesting for western blotting. **D**. A20C103A mutant could not upregulate ERα protein levels. HEK-293T cells were transfected with 0.5 μg Myc-ERα and Flag-A20 FL/C103A plasmids at doses of 0, 0.25, 0.5, or 1.0 μg for 36 h before harvesting for western blotting. **E.** A20 OTU domain upregulated endogenous ERα expression levels in EC cells. Cells were transfected with the indicated plasmids for 36 h, and then endogenous ERα expression levels were evaluated by western blotting. **F.** A20 attenuated ERα polyubiquitination in a dose-dependent manner. HEK-293T cells were transfected with Flag-A20, Myc-ERα, and HA-ub for 30 h and then treated with 20 μM MG132 for 8 h. Myc proteins collected by immunoprecipitation were analyzed by western blotting for detection of ubiquitination levels. **G**. A20 OTU domain attenuated ERα polyubiquitination. HEK-293T cells were transfected with the indicated plasmids for 24 h followed by treatment with MG132 for 8 h. Myc proteins collected by immunoprecipitation were analyzed by western blotting for detection of ubiquitination levels.

for preventing endometrial cancer and improve progestin-based conservative treatment in early stage endometrial cancer.

Authors' contributions

The conception and design of the study: Xiaojun Chen and Congjian Xu.

The acquisition of data: Qiaoying Lv, Liying Xie, Yali Cheng, Weiwei Shan, Chengcheng Ning, Bingying Xie, Bingyi Yang, Xuezhen Luo, Qizhi He, Qin Zhu.

Analysis and interpretation of data: Qiaoying Lv, Yali Cheng, Yingli Zhang, Zhenbo Zhang, Chenji Wang.

Drafting the article: Qiaoying Lv, Yue Shi and Liying Xie.

Revising it critically for important intellectual content: Xiaojun Chen, Zhenbo Zhang, Chenji Wang.

Final approval of the version to be submitted: Xiaojun Chen and Congjian Xu.

Declarations of interest

None.

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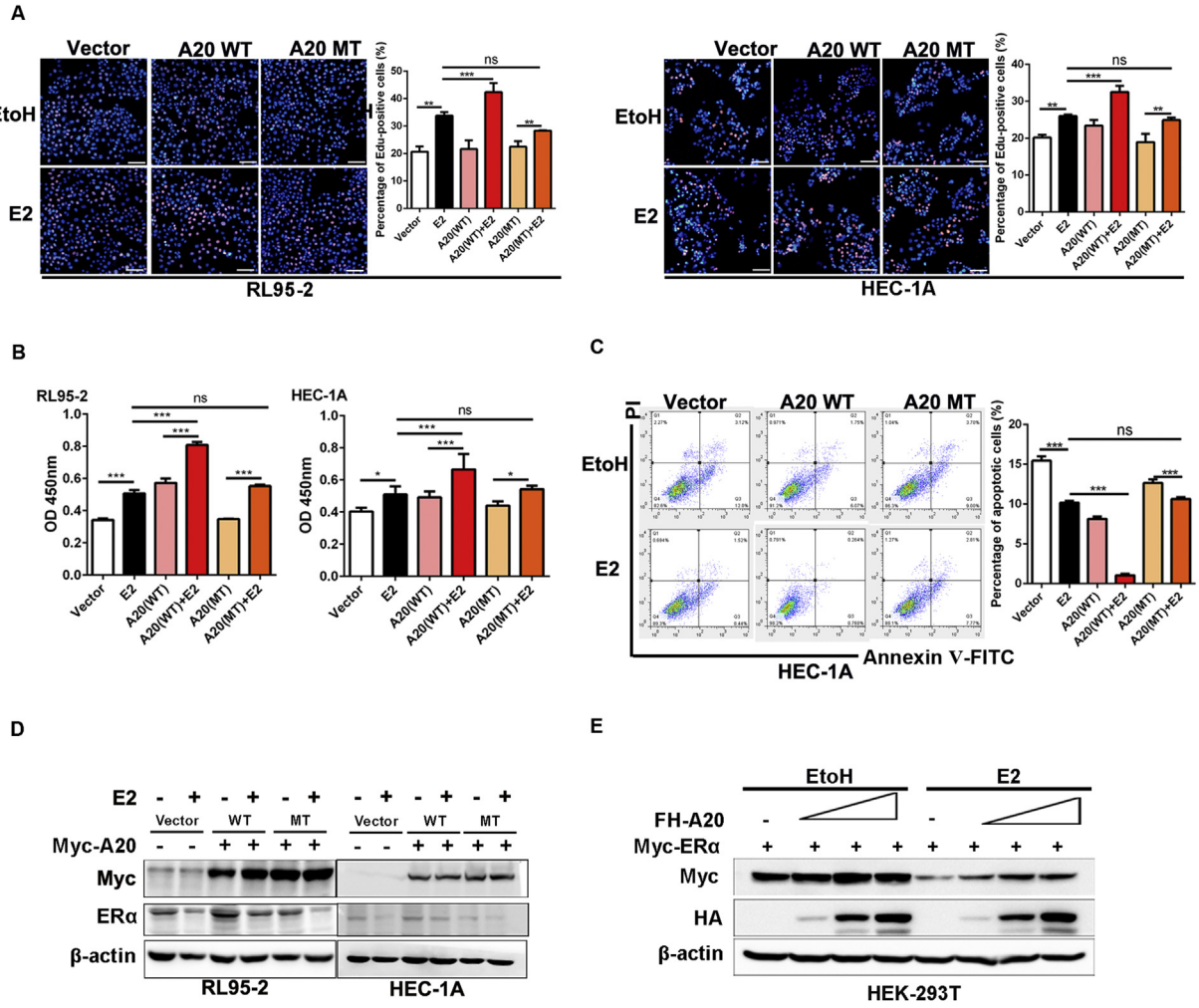

Fig. 7. Overexpression of A20 increases sensitivity of EC cells to estrogen via regulation of ERα. A-B. A20 WT promoted EC cell proliferation in the presence of E2, but A20 MT had no such effect. EC cells were treated with 100 nM E2 for 48 h after transfection with A20 plasmids or empty vector for 8 h. **A.** EdU staining was performed to evaluate cell mitosis. Cells stained with EdU (red fluorescence) were in the S phase of mitosis, and all cells were stained with Hoechst (blue fluorescence) to detect nuclei. Representative images (left column) and percentage of Edu-positive EC cells (right column) were presented. WT, wild type; MT, mutant type, C103A; Scale bars, 100 μm. **B.** CCK8 assay was performed to evaluate cell proliferation. **C.** A20 WT inhibited EC cell apoptosis in the presence of E2, but A20 MT had no such effect. HEC-1A cells were initially transfected with A20-expressing plasmids or empty vectors for 8 h and then treated with E2 for 48 h. Flow cytometry was performed to evaluate the percentage of apoptotic cells in HEC-1A cells. **D.** A20 upregulated ERα expression in the absence or presence of E2, but A20 MT had no such effect. EC cells were treated with 100 nM E2 for 48 h after transfection with A20-expressing plasmids or empty vectors for 8 h. Cells were harvested for western blotting analysis. **E.** A20 dose-dependently stabilized ERα protein in the presence of E2. HEK-293T cells were transfected with A20-expressing plasmids or empty vectors for 24 h and then were treated with E2 or ethanol vehicle (EtOH) for 24 h $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.10.019.

References

[1] R. Kaaks, A. Lukanova, M.S. Kurzer, Obesity, endogenous hormones, and endometrial cancer risk: a synthetic review, Cancer Epidemiol. Biomark. Prev. 11 (2002) 1531–1543 http://www.ncbi.nlm.nih.gov/pubmed/12496040.

[2] V. Rosato, A. Zucchetto, C. Bosetti, L. Dal Maso, M. Montella, C. Pelucchi, E. Negri, S. Franceschi, C. La Vecchia, Metabolic syndrome and endometrial cancer risk, Ann. Oncol. 22 (2011) 884–889 https://academic.oup.com/annonc/article-lookup/doi/10.1093/annonc/mdq464.

[3] C.K. Glass, J.M. Olefsky, Inflammation and lipid signaling in the etiology of insulin resistance, Cell Metabol. 15 (2012) 635–645, https://doi.org/10.1016/j.cmet.2012.04.001.

[4] S.I. Grivennikov, F.R. Greten, M. Karin, Immunity, inflammation, and cancer, Cell 140 (2010) 883–899, https://doi.org/10.1016/j.cell.2010.01.025.

[5] Q. Che, B.-Y. Liu, Y. Liao, H.-J. Zhang, T.-T. Yang, Y.-Y. He, Y.-H. Xia, W. Lu, X.-Y. He, Z. Chen, F.-Y. Wang, X.-P. Wan, Activation of a positive feedback loop involving IL-6 and aromatase promotes intratumoral 17beta-estradiol biosynthesis in endometrial carcinoma microenvironment, Int. J. Canc. 135 (2014) 282–294, https://doi.org/10.1002/ijc.28679.

[6] C. Ning, B. Xie, L. Zhang, C. Li, W. Shan, B. Yang, X. Luo, C. Gu, Q. He, H. Jin, X. Chen, Z. Zhang, Y. Feng, Infiltrating macrophages induce ERalpha expression through an IL17A-mediated epigenetic mechanism to sensitize endometrial cancer cells to estrogen, Cancer Res. 76 (2016) 1354–1366, https://doi.org/10.1158/0008-5472.CAN-15-1260.

[7] X. Wang, W.-J. Lin, K. Izumi, Q. Jiang, K.-P. Lai, D. Xu, L.-Y. Fang, T. Lu, L. Li, S. Xia, C. Chang, Increased infiltrated macrophages in benign prostatic hyperplasia (BPH): role of stromal androgen receptor in macrophage-induced prostate stromal cell proliferation, J. Biol. Chem. 287 (2012) 18376–18385, https://doi.org/10.1074/jbc.M112.355164.

[8] Q.-Y. Lv, B.-Y. Xie, B.-Y. Yang, C.-C. Ning, W.-W. Shan, C. Gu, X.-Z. Luo, X.-J. Chen, Z.-B. Zhang, Y.-J. Feng, Increased TET1 expression in inflammatory microenvironment of hyperinsulinemia enhances the response of endometrial cancer to estrogen by epigenetic modulation of GPER, J. Canc. 8 (2017) 894–902, https://doi.org/10.7150/jca.17064.

[9] P. De Marco, E. Romeo, A. Vivacqua, R. Malaguarnera, S. Abonante, F. Romeo, V. Pezzi, A. Belfiore, M. Maggiolini, GPER1 is regulated by insulin in cancer cells

and cancer-associated fibroblasts, Endocr. Relat. Canc. 21 (2014) 739–753, https://doi.org/10.1530/ERC-14-0245.
[10] T. Chanmee, P. Ontong, K. Konno, N. Itano, Tumor-associated macrophages as major players in the tumor microenvironment, Cancers 6 (2014) 1670–1690, https://doi.org/10.3390/cancers6031670.
[11] S. Soeda, N. Nakamura, T. Ozeki, H. Nishiyama, H. Hojo, H. Yamada, M. Abe, A. Sato, Tumor-associated macrophages correlate with vascular space invasion and myometrial invasion in endometrial carcinoma, Gynecol. Oncol. 109 (2008) 122–128, https://doi.org/10.1016/j.ygyno.2007.12.033.
[12] S. Ohno, Y. Ohno, N. Suzuki, T. Kamei, K. Koike, H. Inagawa, C. Kohchi, G.-I. Soma, M. Inoue, Correlation of histological localization of tumor-associated macrophages with clinicopathological features in endometrial cancer, Anticancer Res. 24 (2004) 3335–3342.
[13] I. Shabo, O. Stal, H. Olsson, S. Dore, J. Svanvik, Breast cancer expression of CD163, a macrophage scavenger receptor, is related to early distant recurrence and reduced patient survival, Int. J. Canc. 123 (2008) 780–786, https://doi.org/10.1002/ijc.23527.
[14] Y. Gong, K. Yan, F. Lin, K. Anderson, C. Sotiriou, F. Andre, F.A. Holmes, V. Valero, D. Booser, J.E.J. Pippen, S. Vukelja, H. Gomez, J. Mejia, L.J. Barajas, K.R. Hess, N. Sneige, G.N. Hortobagyi, L. Pusztai, W.F. Symmans, Determination of oestrogen-receptor status and ERBB2 status of breast carcinoma: a gene-expression profiling study, Lancet Oncol. 8 (2007) 203–211, https://doi.org/10.1016/S1470-2045(07)70042-6.
[15] J.M. Bordeaux, H. Cheng, A.W. Welsh, B.G. Haffty, D.R. Lannin, X. Wu, N. Su, X.-J. Ma, Y. Luo, D.L. Rimm, Quantitative in situ measurement of estrogen receptor mRNA predicts response to tamoxifen, PloS One 7 (2012) e36559, , https://doi.org/10.1371/journal.pone.0036559.
[16] W. Zhou, J.M. Slingerland, Links between oestrogen receptor activation and proteolysis: relevance to hormone-regulated cancer therapy, Nat. Rev. Canc. 14 (2014) 26–38.
[17] C.M. Klinge, Estrogen receptor interaction with estrogen response elements, Nucleic Acids Res. 29 (2001) 2905–2919.
[18] J. Sun, W. Zhou, K. Kaliappan, Z. Nawaz, J.M. Slingerland, ERalpha phosphorylation at Y537 by Src triggers E6-AP-ERalpha binding, ERalpha ubiquitylation, promoter occupancy, and target gene expression, Mol. Endocrinol. 26 (2012) 1567–1577, https://doi.org/10.1210/me.2012-1140.
[19] S. Bhatt, Z. Xiao, Z. Meng, B.S. Katzenellenbogen, Phosphorylation by p38 mitogen-activated protein kinase promotes estrogen receptor alpha turnover and functional activity via the SCF(Skp2) proteasomal complex, Mol. Cell Biol. 32 (2012) 1928–1943, https://doi.org/10.1128/MCB.06561-11.
[20] P. Rajbhandari, K.A. Schalper, N.M. Solodin, S.J. Ellison-Zelski, K. Ping Lu, D.L. Rimm, E.T. Alarid, Pin1 modulates ERalpha levels in breast cancer through inhibition of phosphorylation-dependent ubiquitination and degradation, Oncogene 33 (2014) 1438–1447, https://doi.org/10.1038/onc.2013.78.
[21] P. Zhang, K. Gao, X. Jin, J. Ma, J. Peng, R. Wumaier, Y. Tang, Y. Zhang, J. An, Q. Yan, Y. Dong, H. Huang, L. Yu, C. Wang, Endometrial cancer-associated mutants of SPOP are defective in regulating estrogen receptor-alpha protein turnover, Cell Death Dis. 6 (2015) e1687, https://doi.org/10.1038/cddis.2015.47.
[22] N. Shembade, E.W. Harhaj, Regulation of NF-kappaB signaling by the A20 deubiquitinase, Cell. Mol. Immunol. 9 (2012) 123–130, https://doi.org/10.1038/cmi.2011.59.
[23] C. Wei, J. Mei, L. Tang, Y. Liu, D. Li, M. Li, X. Zhu, 1-Methyl-tryptophan attenuates regulatory T cells differentiation due to the inhibition of estrogen-Ido1-MRC2 axis in endometriosis, Cell Death Dis. 7 (2016) e2489, https://doi.org/10.1038/cddis.2016.375.
[24] M. Mojallal, Y. Zheng, S. Hultin, S. Audebert, T. van Harn, P. Johnsson, C. Lenander, N. Fritz, C. Mieth, M. Corcoran, F. Lembo, M. Hallstrom, J. Hartman, N.M. Mazure, T. Weide, D. Grander, J.-P. Borg, P. Uhlen, L. Holmgren, AmotL2 disrupts apical-basal cell polarity and promotes tumour invasion, Nat. Commun. 5 (2014) 4557, https://doi.org/10.1038/ncomms5557.
[25] I.E. Wertz, K.M. O'Rourke, H. Zhou, M. Eby, L. Aravind, S. Seshagiri, P. Wu, C. Wiesmann, R. Baker, D.L. Boone, A. Ma, E. V Koonin, V.M. Dixit, De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling, Nature 430 (2004) 694–699, https://doi.org/10.1038/nature02794.
[26] P. Zhang, D. Wang, Y. Zhao, S. Ren, K. Gao, Z. Ye, S. Wang, C.-W. Pan, Y. Zhu, Y. Yan, Y. Yang, D. Wu, Y. He, J. Zhang, D. Lu, X. Liu, L. Yu, S. Zhao, Y. Li, D. Lin, Y. Wang, L. Wang, Y. Chen, Y. Sun, C. Wang, H. Huang, Intrinsic BET inhibitor resistance in SPOP-mutated prostate cancer is mediated by BET protein stabilization and AKT-mTORC1 activation, Nat. Med. 23 (2017) 1055–1062, https://doi.org/10.1038/nm.4379.
[27] Y. Zheng, Y. Zeng, R. Qiu, R. Liu, W. Huang, Y. Hou, S. Wang, S. Leng, D. Feng, Y. Yang, Y. Wang, The homeotic protein SIX3 suppresses carcinogenesis and metastasis through recruiting the LSD1/NuRD(MTA3) complex, Theranostics 8 (2018) 972–989, https://doi.org/10.7150/thno.22328.
[28] J. Su, H. Liang, W. Yao, N. Wang, S. Zhang, X. Yan, H. Feng, W. Pang, Y. Wang, X. Wang, Z. Fu, Y. Liu, C. Zhao, J. Zhang, C.-Y. Zhang, K. Zen, X. Chen, Y. Wang, MiR-143 and MiR-145 regulate IGF1R to suppress cell proliferation in colorectal cancer, PloS One 9 (2014), https://doi.org/10.1371/journal.pone.0114420 e114420.
[29] V. Krishnan, B. Schaar, S. Tallapragada, O. Dorigo, Tumor associated macrophages in gynecologic cancers, Gynecol. Oncol. 149 (2018) 205–213, https://doi.org/10.1016/j.ygyno.2018.01.014.
[30] H.W. Bedell, J.K. Hermann, M. Ravikumar, S. Lin, A. Rein, X. Li, E. Molinich, P.D. Smith, S.M. Selkirk, R.H. Miller, S. Sidik, D.M. Taylor, J.R. Capadona, Targeting CD14 on blood derived cells improves intracortical microelectrode performance, Biomaterials 163 (2018) 163–173, https://doi.org/10.1016/j.biomaterials.2018.02.014.
[31] X.-M. Yuan, L.J. Ward, C. Forssell, N. Siraj, W. Li, Carotid atheroma from men has significantly higher levels of inflammation and iron metabolism enabled by macrophages, Stroke 49 (2018) 419–425, https://doi.org/10.1161/STROKEAHA.117.018724.
[32] J. Du, K. Paz, R. Flynn, A. Vulic, T.M. Robinson, K.E. Lineburg, K.A. Alexander, J. Meng, S. Roy, A. Panoskaltsis-Mortari, M. Loschi, G.R. Hill, J.S. Serody, I. Maillard, D. Miklos, J. Koreth, C.S. Cutler, J.H. Antin, J. Ritz, K.P. MacDonald, T.W. Schacker, L. Luznik, B.R. Blazar, Pirfenidone ameliorates murine chronic GVHD through inhibition of macrophage infiltration and TGF-beta production, Blood 129 (2017) 2570–2580, https://doi.org/10.1182/blood-2017-01-758854.
[33] L.W. Kappel, G.L. Goldberg, C.G. King, D.Y. Suh, O.M. Smith, C. Ligh, A.M. Holland, J. Grubin, N.M. Mark, C. Liu, Y. Iwakura, G. Heller, M.R.M. van den Brink, IL-17 contributes to CD4-mediated graft-versus-host disease, Blood 113 (2009) 945–952, https://doi.org/10.1182/blood-2008-08-172155.
[34] S.G. Silverberg, Problems in the differential diagnosis of endometrial hyperplasia and carcinoma, Mod. Pathol. an Off. J. United States Can. Acad. Pathol. Inc. 13 (2000) 309–327, https://doi.org/10.1038/modpathol.3880053.
[35] R.J. Kurman, P.F. Kaminski, H.J. Norris, The behavior of endometrial hyperplasia. A long-term study of "untreated" hyperplasia in 170 patients, Cancer 56 (1985) 403–412.
[36] S. Zhao, X. Chen, X. Lu, Y. Yu, Y. Feng, Epidermal growth factor receptor signaling enhanced by long-term medroxyprogesterone acetate treatment in endometrial carcinoma, Gynecol. Oncol. 105 (2007) 45–54, https://doi.org/10.1016/j.ygyno.2006.12.014.
[37] I. Espinosa, M. Jose Carnicer, L. Catasus, B. Canet, E. D'angelo, G.F. Zannoni, J. Prat, Myometrial invasion and lymph node metastasis in endometrioid carcinomas: tumor-associated macrophages, microvessel density, and HIF1A have a crucial role, Am. J. Surg. Pathol. 34 (2010) 1708–1714, https://doi.org/10.1097/PAS.0b013e3181f32168.
[38] J. Wang, Z. Cao, X.-M. Zhang, M. Nakamura, M. Sun, J. Hartman, R.A. Harris, Y. Sun, Y. Cao, Novel mechanism of macrophage-mediated metastasis revealed in a zebrafish model of tumor development, Cancer Res. 75 (2015) 306–315, https://doi.org/10.1158/0008-5472.CAN-14-2819.
[39] P. De Marco, V. Bartella, A. Vivacqua, R. Lappano, M.F. Santolla, A. Morcavallo, V. Pezzi, A. Belfiore, M. Maggiolini, Insulin-like growth factor-I regulates GPER expression and function in cancer cells, Oncogene 32 (2013) 678–688, https://doi.org/10.1038/onc.2012.97.
[40] J. Zhu, C. Zhao, A. Kharman-Biz, T. Zhuang, P. Jonsson, N. Liang, C. Williams, C.-Y. Lin, Y. Qiao, K. Zendehdel, S. Stromblad, E. Treuter, K. Dahlman-Wright, The atypical ubiquitin ligase RNF31 stabilizes estrogen receptor alpha and modulates estrogen-stimulated breast cancer cell proliferation, Oncogene 33 (2014) 4340–4351, https://doi.org/10.1038/onc.2013.573.
[41] X. Wei, H. Xu, D. Kufe, MUC1 oncoprotein stabilizes and activates estrogen receptor alpha, Mol. Cell. 21 (2006) 295–305, https://doi.org/10.1016/j.molcel.2005.11.030.
[42] V. Stanisic, A. Malovannaya, J. Qin, D.M. Lonard, B.W. O'Malley, OTU Domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1) deubiquitinates estrogen receptor (ER) alpha and affects ERalpha transcriptional activity, J. Biol. Chem. 284 (2009) 16135–16145, https://doi.org/10.1074/jbc.M109.007484.
[43] X. Pan, T. Zhou, Y.-H. Tai, C. Wang, J. Zhao, Y. Cao, Y. Chen, P.-J. Zhang, M. Yu, C. Zhen, R. Mu, Z.-F. Bai, H.-Y. Li, A.-L. Li, B. Liang, Z. Jian, W.-N. Zhang, J.-H. Man, Y.-F. Gao, W.-L. Gong, L.-X. Wei, X.-M. Zhang, Elevated expression of CUEDC2 protein confers endocrine resistance in breast cancer, Nat. Med. 17 (2011) 708–714, https://doi.org/10.1038/nm.2369.
[44] H. Masuyama, Y. Hiramatsu, Involvement of suppressor for Gal 1 in the ubiquitin/proteasome-mediated degradation of estrogen receptors, J. Biol. Chem. 279 (2004) 12020–12026, https://doi.org/10.1074/jbc.M312762200.
[45] J.A. Vendrell, S. Ghayad, S. Ben-Larbi, C. Dumontet, N. Mechti, P.A. Cohen, A20/TNFAIP3, a new estrogen-regulated gene that confers tamoxifen resistance in breast cancer cells, Oncogene 26 (2007) 4656–4667, https://doi.org/10.1038/sj.onc.1210269.
[46] J.-H. Lee, S.M. Jung, K.-M. Yang, E. Bae, S.G. Ahn, J.S. Park, D. Seo, M. Kim, J. Ha, J. Lee, J.-H. Kim, J.H. Kim, A. Ooshima, J. Park, D. Shin, Y.S. Lee, S. Lee, G. van Loo, J. Jeong, S.-J. Kim, S.H. Park, A20 promotes metastasis of aggressive basal-like breast cancers through multi-monoubiquitylation of Snail1, Nat. Cell Biol. 19 (2017) 1260–1273, https://doi.org/10.1038/ncb3609.
[47] S.T. Grey, M.B. Arvelo, W. Hasenkamp, F.H. Bach, C. Ferran, A20 inhibits cytokine-induced apoptosis and nuclear factor kappaB-dependent gene activation in islets, J. Exp. Med. 190 (1999) 1135–1146.
[48] R.U. Janicke, F.H. Lee, A.G. Porter, Nuclear c-Myc plays an important role in the cytotoxicity of tumor necrosis factor alpha in tumor cells, Mol. Cell Biol. 14 (1994) 5661–5670.
[49] B. Yang, L. Xie, H. Zhang, Q. Zhu, Y. Du, X. Luo, X. Chen, Insulin resistance and overweight prolonged fertility-sparing treatment duration in endometrial atypical hyperplasia patients, J. Gynecol. Oncol. 29 (2018) e35, https://doi.org/10.3802/jgo.2018.29.e35.
[50] N.M. Iyengar, A. Gucalp, A.J. Dannenberg, C.A. Hudis, Obesity and cancer mechanisms: tumor microenvironment and inflammation, J. Clin. Oncol. 34 (2016) 4270–4276, https://doi.org/10.1200/JCO.2016.67.4283.
[51] F.M. Wensveen, V. Jelencic, S. Valentic, M. Sestan, T.T. Wensveen, S. Theurich, A. Glasner, D. Mendrila, D. Stimac, F.T. Wunderlich, J.C. Bruning, O. Mandelboim, B. Polic, NK cells link obesity-induced adipose stress to inflammation and insulin resistance, Nat. Immunol. 16 (2015) 376–385, https://doi.org/10.1038/ni.3120.