Biochemical and Biophysical Research Communications 446 (2014) 167–172

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Interleukin 6 promotes endometrial cancer growth through an autocrine feedback loop involving ERK–NF-κB signaling pathway

Qi Che [a,1], Bin-Ya Liu [a,1], Fang-Yuan Wang [a], Yin-Yan He [a], Wen Lu [a], Yun Liao [a], Wei Gu [b,*], Xiao-Ping Wan [c,*]

[a] Department of Obstetrics and Gynecology, Shanghai First People's Hospital Affiliated to Shanghai Jiao Tong University, Shanghai, China
[b] Department of Obstetrics and Gynecology, International Peace Maternity & Child Health Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China
[c] Department of Obstetrics and Gynecology, Shanghai First Maternity and Infant Hospital Affiliated to Tong Ji University, Shanghai, China

ARTICLE INFO

Article history:
Received 11 February 2014
Available online 25 February 2014

Keywords:
Endometrial carcinoma
IL-6
Proliferation
Autocrine

ABSTRACT

Interleukin (IL)-6 as an inflammation factor, has been proved to promote cancer proliferation in several human cancers. However, its role in endometrial cancer has not been studied clearly. Previously, we demonstrated that IL-6 promoted endometrial cancer progression through local estrogen biosynthesis. In this study, we proved that IL-6 could directly stimulate endometrial cancer cells proliferation and an autocrine feedback loop increased its production even after the withdrawal of IL-6 from the medium. Next, we analyzed the mechanism underlying IL-6 production in the feedback loop and found that its production and IL-6-stimulated cell proliferation were effectively blocked by pharmacologic inhibitors of nuclear factor-kappa B (NF-κB) and extra-cellular signal-regulated kinase (ERK). Importantly, activation of ERK was upstream of the NF-κB pathways, revealing the hierarchy of this event. Finally, we used an orthotopic nude endometrial carcinoma model to confirm the effects of IL-6 on the tumor progression. Taken together, these data indicate that IL-6 promotes endometrial carcinoma growth through an expanded autocrine regulatory loop and implicate the ERK–NF-κB pathway as a critical mediator of IL-6 production, implying IL-6 to be an important therapeutic target in endometrial carcinoma.

1. Introduction

Endometrial carcinoma is the most common gynecologic malignancy in the United States [1]. The hallmarks of cancer originally reviewed by Hanahan and Weinberg have been recently extended to include inflammation [2]. Inflammatory processes accompany the regular growth, shedding, and repair of the endometrium over the course of the menstrual cycle, which are controlled by sex hormones and possibly also inflammatory mediators themselves [3]. Additionally, chronic inflammation may play a central role in the development of endometrial cancer through both intrinsic (tumor promoting) and extrinsic (tumor initiating) cancer pathways [4].

Interleukin (IL)-6 is a pleiotropic cytokine that plays an important role in multiple pathological and physiological processes. IL-6 regulates immune and inflammatory responses in physiological conditions, but recent reports suggest that IL-6 expression is implicated in the regulation of tumor growth and metastatic spread, including breast cancer and hepatoma [5,6]. Previously we demonstrated that IL-6 activation was associated with endometrial cancer development by inducing aromatase expression in intratumoral stromal cells [7]. Additionally, the clinical relevance of recent studies was demonstrated by the strong association between serum IL-6 levels and poor clinical outcome in endometrial cancer patients [8]. But the exact role of IL-6 in endometrial cancer tumorgenesis and progression remains elusive. IL-6 autocrine feedback loop has been reported to trigger cells proliferation and trastuzumab resistance in breast cancer [9,10]. And IL-6 links inflammation to malignant transformation in several malignant tumors by constitutively activating the nuclear factor-kappa B (NF-κB) pathway, which, in turn, drives further IL-6 production creating a positive feedback loop [11].

Here, we show that IL-6 promotes endometrial cancer cells proliferation by an autocrine feedback loop and this loop also involves NF-κB and extra-cellular signal-regulated kinase (ERK) signaling pathways. The regulatory circuit links inflammation to malignant tumor promotion and blocking this loop may provide alternative strategy to overcome endometrial cancer progression.

* Corresponding authors. Address: Department of Obstetrics and Gynecology, International Peace Maternity & Child Health Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, No. 910, Hengshan Road, Shanghai 200032, China. Fax: +86 21 64474645 (W. Gu). Address: Department of Obstetrics and Gynecology, Shanghai First Maternity and Infant Hospital Affiliated to Tong Ji University, No. 536, Changle Road, Shanghai 200080, China. Fax: +86 21 54032482 (X.P. Wan).

E-mail addresses: krisgu70@163.com (W. Gu), wanxp@sjtu.edu.cn (X.-P. Wan).

[1] The first two authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2014.02.080
0006-291X/

2. Materials and methods

2.1. Reagents and antibodies

Recombinant human IL-6 was purchased from Peprotech (Rocky Hill, NJ). Bay 11-7082 and PD98059 were from Selleck Chemicals (Houston, TX). Anti-p65, anti-p65 Phospho (Serine 529), anti-ERK, anti-ERK Phospho antibodies were from Epitomics (CA).

2.2. Cell culture and cell proliferation assay

Human endometrial carcinoma cell lines, Ishikawa and RL95-2 were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained according to the provider's instruction in DMEM/F12 (Gibco, Auckland, NZ) supplemented with 10% fetal bovine serum (FBS). To determine cell proliferation, cells were plated into 96-well plates with 2000 cells per well. At each indicated time, the number of metabolically active cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma; St. Louis, MO, USA) assay.

2.3. Enzyme-linked immunosorbent assay (ELISA)

IL-6 protein levels were detected in culture medium using solid phase sandwich ELISA assays according to the manufacturer's protocol (D6050, R&D Systems). The IL-6 assay sensitivity was 0.7 pg/ml, and the assay range was 3.12–300 pg/ml. For the statistical analysis, culture medium was collected three times independently.

2.4. Total RNA extraction and real-time reverse transcription-PCR

Total RNA from Ishikawa, RL95-2 cells was isolated by Trizol (15596-026, Invitrogen) and cDNA was prepared using the reverse transcriptase kit. Real-time reverse transcription (RT)-PCR was conducted using an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA) and performed with SYBR Green PCR Master Mix (Toyobo, Osaka, Japan). A comparative CT method ($2^{-\Delta\Delta CT}$) was used to analyze the relative changes in gene expression. The results are expressed relative to the number of GAPDH transcripts (internal control). For IL-6 mRNA real-time RT-PCR, the forward and reverse primers were 5′-GACATCATTGGCTGACACTTTC-3′ and 5′-TCCAGCAGAAAGAGAAGAGGAG-3′, and for GAPDH mRNA (control), the forward and reverse primers were 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′.

2.5. Immunofluorescence

Cultures growing on chamber slides or were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 for 5 min. Cells were incubated with the diluted primary antibodies for p65 or p-ERK for overnight at 4 °C. Cells were then treated with tetramethyl rhodamine isothiocyanate-conjugated or fluorescein isothiocyanate-conjugated secondary antibody (BD Biosciences, San Jose, CA) for 1 h. Nuclei were visualized by counterstaining with DAPI. Samples were then analyzed using a fluorescence microscope (Leica DMI 3000B, Solms, Germany). The control slides received PBS in place of the primary antibody.

2.6. Western blot

For Western blot analysis, cells were lysed in lysis buffer for 30 min at 4 °C. Total proteins were fractionated by SDS–PAGE and transferred onto nitrocellulose membrane. The membranes were then incubated with appropriate primary antibodies (p65, p-p65, ERK, p-ERK and β-actin), followed by incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz). The probed proteins were detected by enhanced chemiluminescent reagents. β-Actin was used as an internal control.

2.7. Cell stable transfection and orthotopic endometrial carcinoma model in nude mice

The IL-6 over-expressing plasmid was generated by GENECHEM (Shanghai, China), the transfection reagent was obtained from Qiagen (Shanghai, China). Transfection of Ishikawa cells with the IL-6 over-expressing plasmid was done according to the manufacturer's instructions. To obtain a stable cell line, selection pressure was maintained by supplementing the cultures with either G418 (300 μg/ml, Sigma, St. Louis, MO, USA) or puromycin (1.5 μg/ml, Sigma) for a period of 2–8 weeks. Clonal populations of cells derived from the clonal ancestor, were selected by isolating single colonies of cells from each well.

Athymic female nude mice (BALB/c, 4–6 week-old, $n = 5$ per group) were used. The animals were maintained in pathogen-free conditions. All mice were handled according to the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the Department of Laboratory Animal Science Shanghai Jiao Tong University School Of Medicine.

The surgical procedures were carried out as described previously [7]. Briefly, 5×10^6 Ishikawa cells were injected into the subscapular region of nude mice. After 4 weeks, the animals were killed and the subcutaneous tumors were taken off for orthotopic implantation. Mice were anesthetized with intraperitoneal 10% sodium pentobarbital. A transverse incision was performed and the uterus was exposed. A tumor sample of 1 mm^3 was immediately implanted onto the posterior face of the uterus and fixed with a 5–0 surgical suture. After 8 weeks, the animals were killed and the peritoneal cavity was examined macroscopically and microscopically. The tumors were measured using digital caliper, and tumor volumes were calculated according to the equation: tumor volume = $(\text{short diameter})^2 \times (\text{long diameter}) \times 0.5$.

2.8. Statistical analysis

Continuous variables were recorded as mean ± SD and analyzed with the Student's *t*-test. Correlation analysis was performed with Spearman's test. All statistical analyses were done using Statistical Package for the Social Sciences version 17.0 (SPSS, Chicago, IL). *P* values <0.05 were considered statistically significant. All experiments were performed at least three times.

3. Results

3.1. IL-6 increases in the growth of endometrial cancer cells and promotes its production through an autocrine feedback loop

We found that administration of IL-6 significantly increased cells growth of Ishikawa and RL95-2 cells (Fig. 1A and B). Furthermore, once exposed to IL-6 (20 ng/ml for 24 h), Ishikawa and RL95-2 cells also exhibited the increased growth ability compared with the control group, even after the withdrawal of IL-6 from the medium (Fig. 1A and B), thus signifying the importance of autocrine IL-6 signaling. Compared with untreated cells, the cells treated or pretreated with IL-6 displayed significantly increased proliferation ability at 5th day for Ishikawa cells and 3th day for RL95-2 cells. Then we examined the IL-6 protein concentration in the culture medium of the pretreated group and found that there was approximately a 2-fold increase in IL-6 secretion when cells were cultured

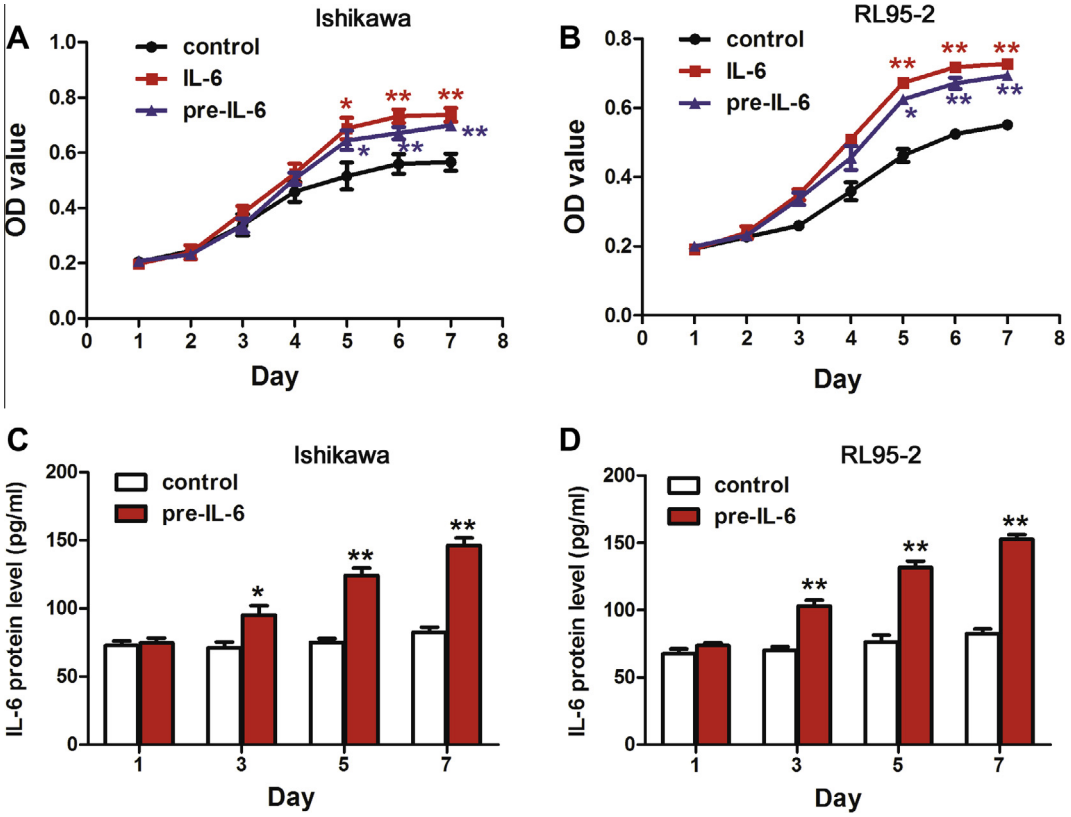

Fig. 1. IL-6 promotes endometrial cell lines proliferation and an autocrine loop increases IL-6 protein levels. (A, B) Growth curves of Ishikawa and RL95-2 cells treated and pretreated with IL-6 (20 ng/ml). Cell growth was measured by MTT assay. (C, D) IL-6 protein concentration was stepwise increased by pretreated with IL-6 in Ishikawa and RL95-2 cells culture media. IL-6 protein levels were measured by ELISA analysis. Pre-IL-6, pretreated with IL-6. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group.

for one week. Stepwise increase of IL-6 protein concentration over time of culture was observed in Fig. 1C and D.

3.2. NF-κB and ERK signaling inhibitors suppress IL-6 production and IL-6-induced endometrial cancer cells proliferation

Using IL-6 to stimulate Ishikawa and RL95-2 cells, we found that IL-6 significantly elevated its own mRNA levels after incubation for 18 h determined by real-time RT-PCR. The NF-κB transcription factor is known to transcribe a number of cytokine genes. Accordingly, we utilized NF-κB inhibitor Bay 11-7082 and ERK inhibitor PD98059 to determine their effects on IL-6 production in Ishikawa and RL95-2 cells. Simultaneous addition of Bay 11-7082 or PD98059 reduced the enhancement of IL-6 production in Ishikawa and RL95-2 cells (Fig. 2A and B).

Next, to investigate whether activation of the NF-κB and ERK is directly involved in IL-6-induced proliferation of endometrial carcinoma cells, we studied the effect of Bay 11-7082 and PD98059 on pretreated IL-6-induced stimulation of proliferation. As shown in Fig. 2C and D, administration of Bay 11-7082 or PD98059 in Ishikawa and RL95-2 cells inhibited their ability to proliferate. Thus, it was possible that NF-κB and ERK were capable of inhibiting cell growth by decreasing IL-6 production.

To observe the activation of p65 (a subunit of NF-κB) and p-ERK induced by IL-6, we examined the expression of p-ERK and the localization of p65 stimulated by IL-6 in Ishikawa cells using immunofluorescence. Increase in p-ERK immunoreactivity in plasma and p65 nuclear translocalization were observed after IL-6 treatment for 1 h (Fig. 2E).

3.3. The ERK–NF-κB signaling pathway axis mediates IL-6 production

To gain insight into the mechanism underlying the production of IL-6, we next examined the activation of NF-κB and ERK stimulated by IL-6. An increase in p65 and ERK phosphorylation was observed when cells were stimulated by IL-6 for 1 h (Fig. 3). Immunoblots were reprobed with antibodies against total p65 and ERK, showing that the increase in p65 and ERK phosphorylation was not due to the increased protein expression. Next, to investigate if activation relationship of the ERK and NF-κB pathway, we studied the effects of Bay 11-7082 and PD98059 on NF-κB and ERK activation. Treatment of cells with PD98059 decreased the phosphorylation of both ERK and p65 significantly, whereas Bay 11-7082 only decreased the phosphorylation of p65 without affecting levels of phosphorylated ERK (Fig. 3).

3.4. IL-6 promotes endometrial carcinoma growth in orthotopic tumor model

We then examined whether IL-6 could promote endometrial cancer growth *in vivo* using orthotopic endometrial carcinoma model. To ensure that IL-6 was stably overexpressed in Ishikawa cells, we established IL-6 stable transfected Ishikawa cell line and confirmed that IL-6 mRNA and protein levels in the transfected cells were significantly increased compared with the parental cells (Supplementary Fig. 1). As shown in Fig. 4A, tumor was obviously growing in the implantation area in the uterus. Histological examination showed that the tumor developed from the uterine cavity and was surrounded by normal endometrial glands (Fig. 4B). The

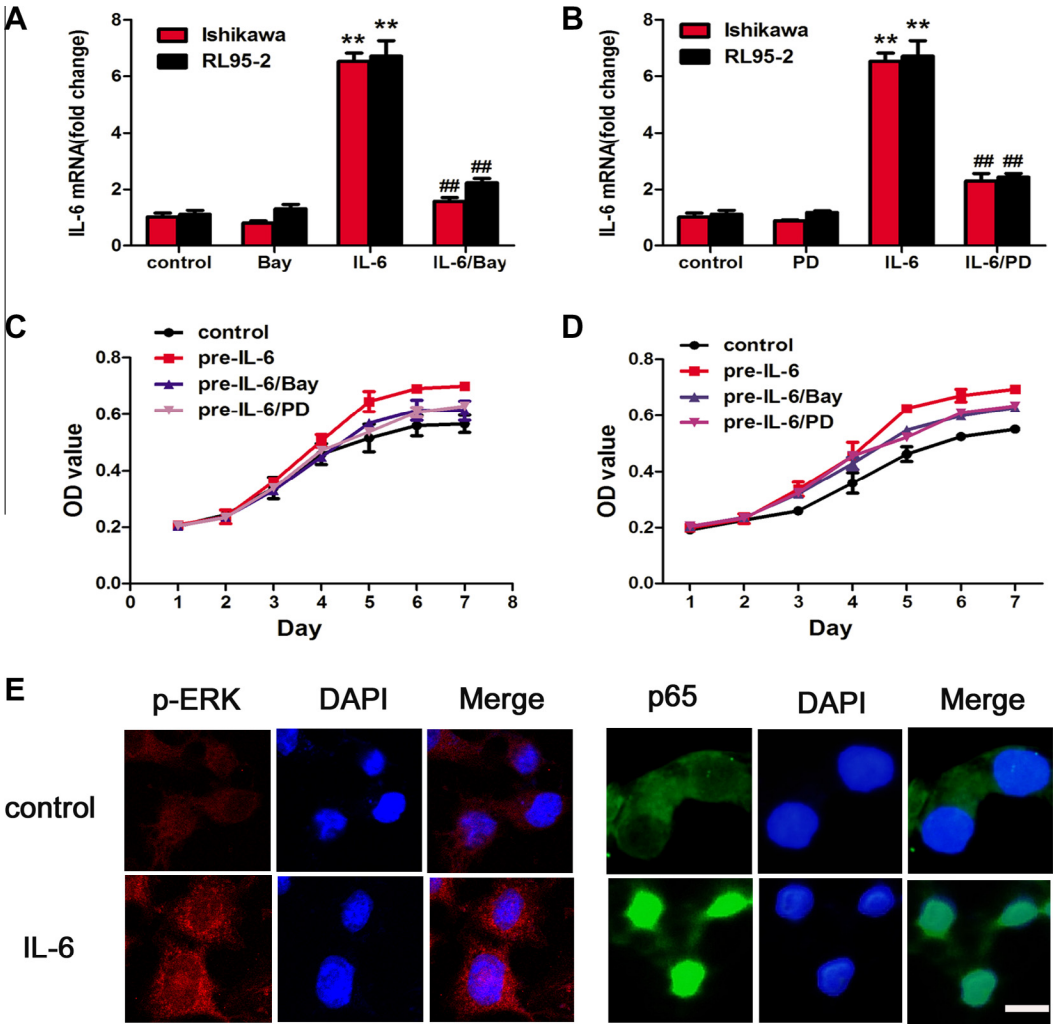

Fig. 2. NF-κB and ERK inhibitors decrease IL-6 mRNA levels and endometrial cancer cells growth ability. (A, B) Ishikawa and RL95-2 cells were stimulated with IL-6 (20 ng/ml) for 18 h with or without Bay 11-7082 (NF-κB inhibitor, 5 μM) and PD98059 (ERK inhibitor, 25 μM). The IL-6 mRNA levels were measured by real-time RT-PCR. Bay, Bay 11-7082; PD, PD98059; IL-6/Bay, IL-6 with Bay 11-7082; IL-6/PD, IL-6 with PD98059. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus IL-6-treated group. (C, D) Growth curves of Ishikawa and RL95-2 cells pretreated with IL-6 in the present or absent of Bay 11-7082 and PD98059. Cell growth was measured with the MTT assay. Pre-IL-6, pretreated with IL-6; pre-IL-6/Bay, pretreated with IL-6 and cultivated with Bay 11-7082; pre-IL-6/PD, pretreated with IL-6 and cultivated with PD98059. (E) Immunofluorescence images of Ishikawa cells showing the increase in p-ERK (*red*) and the nuclear translocalization of p65 (*green*) after IL-6 incubation. *Blue*, DAPI-stained nuclei. Bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mean volume of IL-6 overexpression tumors was significantly larger than the control group (Fig. 4C).

4. Discussion

Although it is well established that estrogens and insulin play important roles in endometrial carcinogenesis, the role of inflammation still has to be demonstrated. Several studies have contributed to look for biomarkers of endometrial cancer including inflammation factors tumor necrosis factor-alpha (TNF-α), C-reactive protein and IL-6 [8,12]. And there was also finding that high IL-6 serum levels in endometrial cancer patients are associated with endometrial carcinogenesis and cancer progression [13]. Previously, we proved that IL-6 localized in endometrial cancer cells and promoted cancer progression via a paracrine manner [7]. Herein we investigated the effect of IL-6 on the endometrial cancer cells proliferation and, for the first time, demonstrated that an autocrine feedback loop existed in the endometrial cancer cells.

In our study, we found that, in addition to IL-6-induced endometrial cancer cells proliferation, if pretreated with IL-6, the endometrial cancer cells still displayed increased growth ability compared with untreated cells, even one week after the withdrawal of IL-6 from the medium. Furthermore, once endometrial cells were exposed to IL-6, IL-6 concentration in the culture medium showed the stepwise upregulation during culture process. These results suggested that IL-6 autoregulation might perpetuate phenotypic changes caused by exposing endometrial cancer cells to IL-6. IL-6 production through an autocrine positive feedback loop has been demonstrated in breast cancer transformation [11], in which once the loop is activated, the regulatory circuit is sufficient to generate and maintain the chronic inflammatory state without the original signal, i.e., without IL-6 existence. Importantly, this inflammatory feedback loop has been proved to occur in diverse cancer cells including lung, hepatocellular, prostate, and colon cancer cells. Additionally, the role of an IL-6-mediated inflammatory loop in trastuzumab resistance has been proved in breast cancer [10]. Combined with our study, we reasoned that IL-6 might promote tumor carcinogenesis and progression through a regulatory circuit and stepwise amplification.

By combining with their ligand-specific binding subunit, IL-6 activates receptor-associated tyrosine kinases janus kinase (JAK)

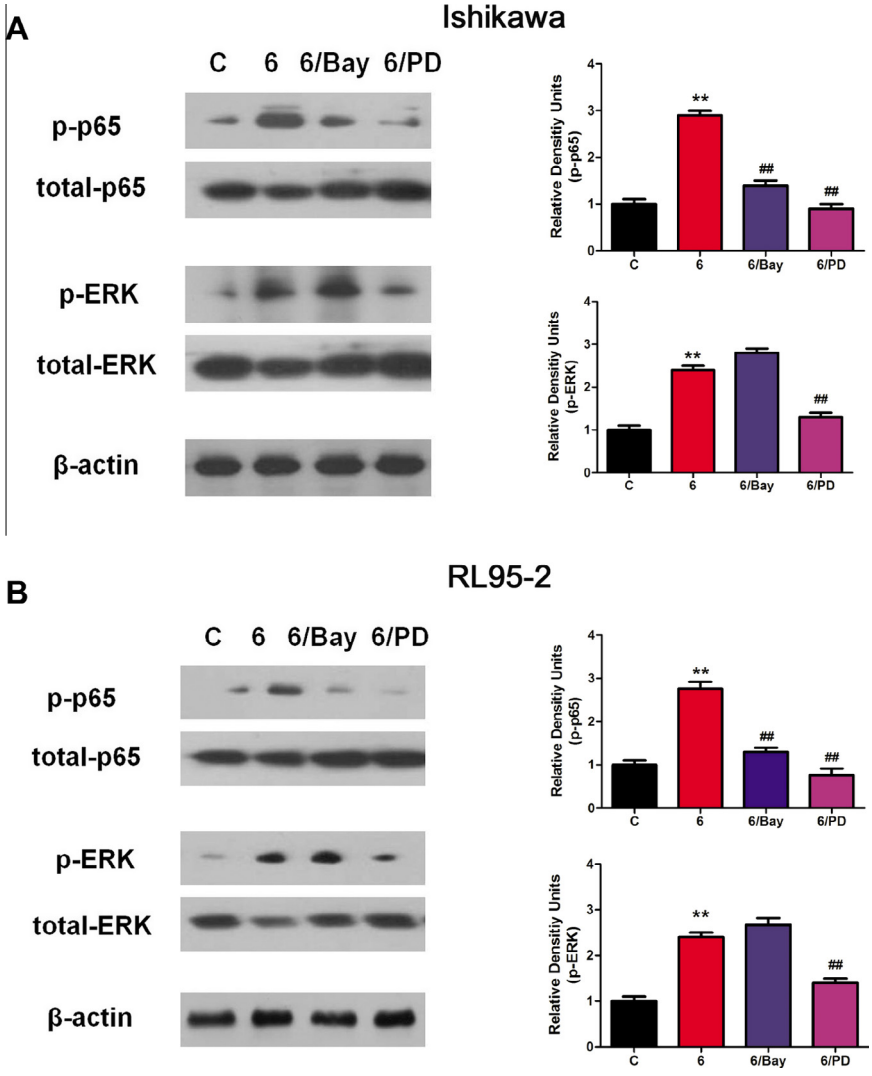

Fig. 3. Activation of ERK is upstream of the activation of the NF-κB pathways after stimulation with IL-6 in endometrial cancer cells. (A, B) Western blot analysis to measure the phosphorylated and total p65 and ERK levels in Ishikawa and RL95-2 cells after treatment with 20 ng/ml of IL-6 with or without Bay 11-7082 (NF-κB inhibitor, 5 μM) and PD98059 (ERK inhibitor, 25 μM). The representative histogram is the densitometric analysis of bands showing fold increase in levels of phosphorylated forms with respect to total protein. C, control; Bay, Bay 11-7082; PD, PD98059; 6, IL-6; IL-6/Bay, IL-6 with Bay 11-7082; IL-6/PD, IL-6 with PD98059. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus IL-6-treated group.

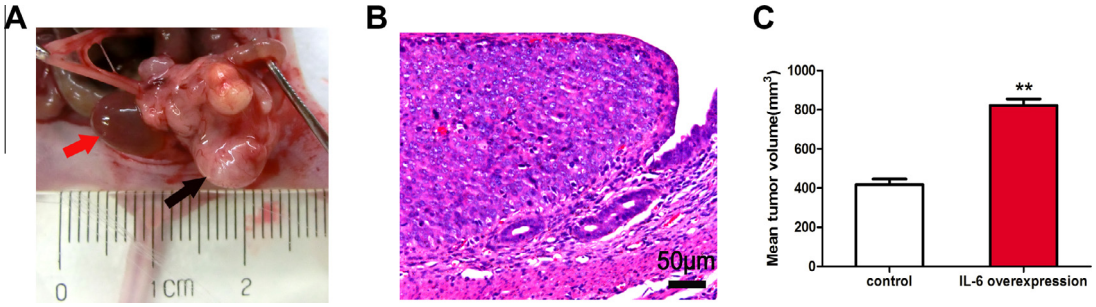

Fig. 4. IL-6 promotes tumor growth in the orthotopic endometrial cancer model. (A, B) Macroscopic and microscopic view of an orthotopic tumor. *Black arrow*, orthotopic tumor; *red arrow*, mouse bladder. Original magnification ×200, bar = 50 μm. (C) Effect of IL-6 overexpression on orthotopic endometrial cancer growth. $^{**}P < 0.01$ versus control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1, JAK2 and leads subsequently to phosphorylation of gp130 and recruitment and phosphorylation of signaling molecules, such as signal transducer and activator of transcription-3 (STAT3), mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K) [14,15]. NF-κB is the critical transcription factor that mediates the inflammatory response, and it stimulates IL-6 expression in response to inflammatory signals [16]. Furthermore, IL-6 feedback mediated by IL-6-activated NF-κB signaling has been reported in breast cancer [10,11]. Recently, there has been reported that ERK2 small interfering RNAs could reduce IL-6 levels [17].

Consistent with these studies, we found that IL-6 significantly elevated its own mRNA levels and simultaneous addition of NF-κB or ERK inhibitor reduced the enhancement, which indicated that NF-κB and ERK participated in the production of IL-6. Immunofluorescence analysis and Western blot confirmed two signaling pathways mentioned above were activated by IL-6. To reveal the hierarchy of these events, we observed the effect of inhibitors of signaling pathway on their activities respectively. NF-κB inhibitor had no effect on the activation of ERK but PD98059, an ERK inhibitor, could block NF-κB activity. These data suggest that activation of ERK is upstream of the NF-κB pathway and IL-6 induces its own production through the ERK–NF-κB signaling axis, paralleling by an enhancement of tumor cells proliferation. Inhibition of NF-κB or ERK signaling could decrease endometrial cancer cells growth ability, indicating that such features were dependent upon an autocrine IL-6 feedback loop. Finally, we confirmed the IL-6 action on endometrial cancer growth *in vivo*. In particular, we used the orthotopic xenograft models to elucidate the effect of IL-6 on the progression of tumor, which better mirrored the tumor growth environment.

In summary, we demonstrated exist of an autocrine positive feedback loop in which IL-6 promotes endometrial cancer cells proliferation and meanwhile it stimulates its own production through the ERK–NF-κB pathway. Once the loop is activated, the regulatory circuit is sufficient to maintain the tumor growth and progression. These findings highlight the important role of inflammation state in regulating malignant tumor growth and identify a novel carcinogenesis mechanism of endometrial carcinoma. We suggest that the IL-6–ERK–NF-κB signaling axis defined could prompt new therapeutic or prevention strategies for treatment of endometrial cancer.

Acknowledgments

We thank Hui Wang, Qin Huang (Center Laboratory of International Peace Maternity & Child Health Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China) and Jian-Zhang Jia (Forensic Department of Shanghai Medical College, Fudan University) for excellent technical assistance.

Conflicts of interest: The authors declare no conflict of interest.

National Natural Science Foundation of China (Nos. 81272885, 81172476, 81072139, 81001154). The Foundation Project of Shanghai Municipal Science and Technology Commission (No. 13JC1404500). Ph.D. Programs Foundation of Ministry of Education of China (No. 20120073110090).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.080.

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