Original Article
Cancer-associated fibroblasts promote endometrial cancer growth via activation of interleukin-6/STAT-3/c-Myc pathway

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Abstract: Cancer-associated fibroblasts (CAFs) secrete various pro-tumorigenic cytokines, yet the role of these cytokines in the progression of endometrial cancer remains unclear. We found that CAFs isolated from human endometrial cancer (EC) tissues secreted high levels of interleukin-6 (IL-6), which promotes EC cell proliferation in vitro. Neutralizing IL-6 in CAF-conditioned media reduced (47% inhibition) while IL-6 recombinant protein increased cell proliferation (~2.4 fold) of both EC cell lines and primary cultures. IL-6 receptors (IL-6R and gp130) were expressed only in EC epithelial cells but not in CAF, indicating a one-way paracrine signaling. In the presence of CAF-conditioned media, Janus kinase/signal transducers and activators of transcription (JAK/STAT3) pathway was activated in EC cells. Treatment with JAK and STAT3 specific inhibitors, AD412 and STATTIC, respectively, significantly abrogated CAF-mediated cell proliferation, indicating the role of IL-6 activation in EC cell proliferation. We further showed that one of STAT-3 target genes, c-Myc, was highly induced in EC cells after exposure to CAF-conditioned medium at both mRNA (>105-fold vs. control) and protein level (>2-fold vs. control). EC cell proliferation was dependent on c-Myc expression, as RNAi-mediated c-Myc down-regulation led to a significant 46% reduction in cell viability when compared with scrambled control. Interestingly, CAF-conditioned media failed to promote proliferation in EC cells with reduced c-Myc expression, suggesting that CAF-mediated cell proliferation was also dependent on c-Myc expression. Subcutaneous tumor xenograft model showed that EC cells grew at least 1.4 times larger when co-injected with CAF, when compared to those injected with EC cells alone. Mice injected with EC cells with down-regulated c-Myc expression, however, showed at least 2.5 times smaller tumor compared to those in control group. Notably, there was no increase of tumor size when co-injected with CAF, when compared to those injected with EC cells alone. Mice injected with EC cells with down-regulated c-Myc expression, however, showed at least 2.5 times smaller tumor compared to those in control group. Notably, there was no increase of tumor size when co-injected with CAFs. Further immunohistochemical staining on human tissues showed positive expression of IL-6 receptors, phosphorylated-STAT3 and c-Myc in human EC tissues with less signals in benign endometrium. Taken together, our data suggests that IL-6 secreted by CAF induces c-Myc expression to promote EC proliferation in vitro and in vivo. IL-6 pathway can be a potential target to disrupt tumor-stroma interaction in endometrial cancer progression.

Keywords: Uterine cancer, epithelial-stroma interaction, cytokine actions, tumor microenvironment, JAK/STAT3, c-Myc activation

Introduction
Endometrial cancer (EC) is the most common gynecological related carcinoma among women worldwide. The latest global EC incidence recorded 319,605 cases in 2012 which accounts for approximately 5% of all new cases for cancer in women [1]. Both EC incidence and death rates in the United States increased 21% since 2008, and increased more than 100% since the last two decades [2]. It is estimated in the recent GLOBOCAN 2012 report, that by 2025, half a million cases of EC would be diagnosed worldwide, emphasizing the exponential rate of this disease (http://globocan.iarc.fr). This increasing trend has been mainly attributed to the increasing rate of obesity among women and the use of tamoxifen as a mainstay treatment in breast cancer who later tend to develop EC [3]. Hysterectomy is the main treatment for patients suffering from EC and this disease is highly curable in early stages [1]. However, resistance to post-surgery treatment and disease recurrence tends to occur in later
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stages of this disease, rendering significant challenge in further treatment.

Recently, hormone (progesterone) treatment was shown to reduce the progression of EC through the action of the stroma in the EC tumor microenvironment [4]. This finding implies that the tumor microenvironment could be targeted to overcome treatment resistance and to improve the overall disease outcome. The tumor microenvironment, consisting of non-tumorigenic components such as cancer-associated fibroblasts (CAFs), macrophages, natural killer cells as well as lymphocytes, has been shown to sustain the growth and survival of tumors [5, 6]. Oncogenic signaling from fibroblast cells surrounding tumor lesion affect the growth of tumor cells, particularly in prostate and pancreatic cancer [6-8]. In epithelial ovarian cancer, CAFs are highly abundant in FIGO stages III-IV compared to earlier stages [9, 10], and have been shown to play a role in metastatic disease.

In endometrial cancer, we have previously shown that secretion from CAFs promoted the growth while those from benign fibroblasts inhibited the proliferation of EC cells in vitro. Cytokine profiling performed on these secretions showed high levels of interleukin-6 (IL-6) in CAFs compared to those in benign fibroblasts [11]. High levels of IL-6 are present in the serums of EC patients and are correlated with poor prognostic outcome in these patients [12]. While studies on the role of CAFs are quite abundant in other cancer types, their mechanism in EC is relatively understudied, especially on the tumorigenic role of the secreted IL-6. Hence, we aim to investigate the role of CAF-secreted IL-6 in the EC progression.

In the current study, we demonstrated that IL-6 secreted by CAFs is responsible for promoting EC cell proliferation. Through IL6-R and gp130, IL-6 activated JAK/STAT3 signaling pathway, leading to induction of proto-oncogene c-Myc expression. Down-regulation of c-Myc expression significantly inhibited EC proliferation, and subsequently abrogated CAF-mediated cell proliferation. The dependence on IL-6 pathway was also observed in vivo in a tumor xenograft model. In addition, we showed that IL-6 downstream molecules including IL-6 receptors, phosphorylated-STAT3 and c-Myc are highly expressed in human EC tissues but not in benign endometrial tissues. Our data strongly suggest that IL-6 pathway is activated in EC tumor cells following interaction with CAFs, leading to sustained cell proliferation. Hence, molecules activated in IL-6 pathway may potentially be targeted when designing novel therapeutic options for women with EC.

Materials and methods

Reagents and antibodies

LEAF™ purified anti-human IL-6 antibody, LEAF™ purified rat IgG1, κ Isotype control antibody and recombinant human IL-6 (carrier free) were purchased from Biolegend (CA, USA). STAT3 inhibitor V (STATIC) and JAK3 inhibitor VII (AD412) were purchased from Santa Cruz Biotechnology (CA, USA), and c-Myc inhibitor, 10058-F4 was purchased from Sigma-Aldrich (MO, USA).

Ethics statement

Fresh EC tissues were obtained for establishment of primary culture from patients undergoing surgery at University of Malaya Medical Center. Endometrium formalin-fixed paraffin blocks for both benign and cancer conditions were obtained for immunohistochemistry work from the Biobank Unit of the University of Malaya. This study was approved by the University of Malaya Medical Center Ethics committee (Ref No. 865.19). Written informed consent was obtained from all participants.

Human endometrial cell lines and primary cultures establishment

Cell lines: Human endometrial cancer cell lines, ECC-1 (CRL-2923) and HEC-1A (HTB-112) and immortalized human normal endometrial fibroblast cell line, T-HESC (CRL-4003) were purchased from American Type Culture Collection (MD, USA) and were cultured in media according to manufacturer’s protocol supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Primary cultures: All cultured primary cells obtained from surgical tissues were subjected to epithelial and stromal cell isolation using human CD326 (EpCAM) magnetic microbeads antibody and human anti-fibroblast magnetic microbeads (Miltenyi Biotec, Cologne, Germany), respectively as described previously [11].
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**Table 1.** Primer sequences used for quantitative real time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primers (5'-3')</th>
<th>Reverse Primers (5'-3')</th>
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<td>ATT GTG GTT CTT AAG GAG GC</td>
<td>AAA GGA CAG GAT GTT CCA GG</td>
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<td>c-Myc</td>
<td>GTC GTT TCC GCA ACA AGT CCT TCT C</td>
<td>AAT GAA AAG GGC CCC AAG GTA GTT ATC C</td>
<td>[42]</td>
</tr>
<tr>
<td>TIMP-1</td>
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<td>AAT TGT GTG CTG TGG ATA</td>
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<tr>
<td>PIM1</td>
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<td>TAC CAT GCC AAC TGT ACA CAC</td>
<td>[44]</td>
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<tr>
<td>SOCS-3</td>
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<td>ACC AGC TTG ATG ACA CAG TCG</td>
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<tr>
<td>NFkB1</td>
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<td>CGT GAA AGA CCC TCC TGT TC</td>
<td>AGA GCG AGA TCC TGG GAC GGA</td>
<td>[46]</td>
</tr>
</tbody>
</table>

**Establishment of ECC-1 cell line with low c-Myc expression:** ECC-1 cell and CAF cells (EC11-Fib) were transduced with red fluorescent protein (RFP) and green fluorescent protein (GFP) respectively (Gentarget, CA, USA). Selection was maintained by supplementing the cultures with puromycin with final concentration of 1 µg/ml (Sigma-Aldrich, MO, USA) for a period of 2 weeks. Consequently, the ECC-1 cell line was transfected with short hairpin RNA (shRNA) vector targeting c-Myc. GIPZ MYC shRNA viral particle kit was purchased from Dharmaco (CO, USA). Puromycin-resistant clones were selected in the presence of 1 μg/mL puromycin (Sigma-Aldrich).

**Preparation of conditioned media from fibroblast cells**

Fibroblast cells were seeded and cultured in complete media for 24 hours, before being cultured in media containing 2% FBS for the following 72 hours. Conditioned medium was collected using Amicon ultra centrifugal filters (Merck Milipore, MA, USA) by centrifugation at 5000 x g at 4°C for 1 hour. Protein in the concentrated media was quantified using Bradford assay (Biorad, CA, USA).

**Enzyme-linked immunosorbent assay (ELISA)**

Biolegend Human IL-6 and ELISA MCP-1/CCL2 MAX™ Deluxe (CA, USA) and Raybiotech #ELH-RANTES, #ELH-VEGF (GA, USA) were used to quantitate levels of these cytokines in conditioned media of CAFs. Briefly, 96 well plates were coated overnight with capture antibody. After blocking the plates with blocking buffer for 2 hours, conditioned media from ten different CAFs and control fibroblasts were added into the plate for another 2 hours, before addition of detection antibody for 1 hour, secondary antibody for 30 minutes and 3,3’,5,5’-tetramethylbenzidine (TMB) substrate for 15 minutes. Reactions were terminated with STOP solution, before being analyzed at 450 nm wavelength using spectrophotometer. Assay sensitivity was between 2 to 10 pg/ml.

**Methyl thiazolyl tetrazolium (MTT)**

Proliferation of endometrial cancer cells was assessed by methyl thiazolyl tetrazolium (MTT) test. Briefly, cells were seeded in complete media at 1-3 x 10^4 cells/well in 96-well plates. At 24 hours post seeding, the cells were treated with either complete media, media with 2% FBS, fibroblast-conditioned media, neutralizing antibodies and/or inhibitors for 72 hours. At the end of treatment, 20 µl of MTT solution (5 mg/ml) was added to each well. Following 4 hours incubation at 37°C, 100 µl of 10% sodium dodecyl sulfate were added to dissolve the formazan crystals by additional 4 hours incubation at 37°C. Absorbance was measured using spectrophotometer at 575 nm with reference of 650 nm.

**Quantitative real time polymerase chain reaction (qRT-PCR)**

Total RNA were extracted from cultured cells using TRIzol (Invitrogen, CA, USA) and 1 µg RNA was converted into cDNA using DyNAmo cDNA synthesis kit (Finzymes, Vantaa, Finland). Primer sequence used to detect IL-6 receptors and IL-6 downstream target genes are listed in Table 1. qRT-PCR was performed using ABI StepOne Plus (Applied Biosystem, CA, USA) in 35 cycles using 5 x HOT FIREPol EvaGreen qPCR Mix (Solis Biodyne, Tartu, Estonia), 10 pmol/µl forward and reverse primer, 10 ng/µl cDNA template and PCR grade H2O.
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Total protein extraction and western blotting

ECC-1 cells were seeded at $1 \times 10^4$ cells/well in 6-well plates in complete media. At 24 hours post seeding, the cells were treated with either complete media, media with 2% FBS, fibroblasts-conditioned media, neutralizing antibodies and/or inhibitors for 72 hours. Protein lysates were collected by scraping the cells in cold lysis buffer containing final concentration of 0.1% Triton-X, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 1 x phosphatase and 1 x protease inhibitors. Protein concentration was quantified using Bradford assay (Biorad, CA, USA). Approximately 30 μg of protein were resolved on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis before being transferred onto polyvinylidene difluoride membrane. Antibodies used were rabbit anti-human phospho-JAK3, JAK3, phospho-STAT3, c-myc, mouse anti-human STAT3 and β-actin (Cell Signaling Technology, MA, USA). Blots were visualized using ECL prime western blotting detection reagent (Amersham, GE Healthcare Lifesciences, Sweden) using gel documentation system (Biospectrum 410, UVP) and Vision Works LS software (CA, USA).

Immunohistochemical analysis

Tumors removed during debulking surgery were fixed overnight in neutral buffered formalin prior to paraffin wax professing and embedding. Tissue sections were cut at 4 μm size. For immunohistochemical analysis, endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 10 minutes. Antigen was retrieved using sodium citrate buffer method by heating at 100°C for 30 minutes. Slides were then incubated with one of the following antibodies for 1 hour, diluted according to the manufacturer protocol: IL-6Rα and gp130 (Santa Cruz Biotechnology, CA, USA), p-STAT3 and c-Myc (Cell Signalling Technology, MA, USA). A labelled streptavidin biotin-system with a horse-radish peroxidase label (DAKO Corp., CA, USA) was used to detect the primary antibodies and visualized by incubation with 3,3’-diaminobenzidine chromogen and hydrogen peroxide substrate (DAKO Corp., CA, USA) for 10 minutes. The slides were then counterstained with hematoxylin and mounted in dibutyl phathalate xylene (Sigma, MO, USA). Images were viewed and captured using Nikon Eclipse 2000 and Nikon ES-Fi1 respectively (Nikon GamBH, Dusseldorf, Germany).

EC tumor xenograft model

Athymic female nude mice (BALB/c, 4 weeks-old) were obtained from Taconic (Cambridge, MA, USA) and were quarantined for 2 weeks. The mice were divided into 7 groups (n=8/group) and were given subcutaneous injection of either ECC-1 cells (5,000 cells) alone or in combination with fibroblast cells (20,000 cells). The groups were Group 1-ECC-1 only; Group 2-EC11-Fib only; Group 3-combination of ECC-1 and EC11-Fib; Group 4-ECC-1 transfected with c-Myc scramble shRNA; Group 5-combination of ECC-1 transfected with c-Myc scramble shRNA and EC11-Fib; Group 6-ECC-1 transfected with c-Myc knockdown shRNA construct; Group 7-combination of ECC-1 transfected with c-Myc knockdown shRNA construct and EC11-Fib. Mice were monitored every other day and tumor size was measured twice weekly using calipers. Tumor volume was calculated according to equation: tumor volume = length (L) × width (W)^2/2. Tumors were also imaged once a week using Carestream MS FX-PRO small rodent imager (Molecular Imaging, CT, USA). Animals were maintained in specific pathogen free conditions in an AALAAC-accredited ani-

**Figure 1.** Differential levels of cytokines secreted by cancer and benign-associated fibroblasts. Endometrial cancer and benign-associated fibroblasts were cultured for 72 hours in media containing 2% fetal bovine serum, prior to collection of conditioned media. Cytokines levels were measured using ELISA. Data shown are representative of two independent experiments. Data, average; error bar, S.E.M. *P<0.05.
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Figure 2. IL-6 secreted by CAFs mediates EC cell proliferation. A, B. ECC-1 cell line and EC6-Ep primary cell were treated with IL-6 neutralizing antibody and its IgG control in the presence of either 1 mg/ml EC7-Fib (left) or EC11-Fib (right) conditioned media. C. ECC-1 and EC6-Ep cells were treated similarly with IL-6 neutralizing antibody and its IgG control but without the presence of CAFs conditioned media. Black bars indicate ECC-1 cells in complete media without any treatment. D. ECC-1 and EC6-Ep cells were treated with IL-6 recombinant protein without the presence of conditioned media. Cell viability was examined using MTT assay and normalized to control media (media containing 2% FBS). Data, average; error bars, S.E.M. Data shown are representative of three independent experiments.

Statistical analysis

Statistical analysis that assessed the differences between means of control and test group was performed using Student’s t-test on GraphPad-Prism (GraphPad Software, CA, USA). A

\[ P \text{-value} < 0.05 \]

was considered to be statistically significant.

Results

CAFs secrete higher levels of inflammatory cytokines compared to control fibroblasts

We previously showed that CAFs secrete high level of inflammatory cytokines compared to the benign counterparts [11]. We further validated this finding in twenty primary fibroblast

*mal housing facility, and were fed with Teklad Global 19% protein extruded rodent diet (Harlan Laboratories, WI, USA). All mice procedures were approved by IACUC committee of University of Malaya (FAR/27/07/2012/IC (R)).

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Cultures established from benign and malignant endometrial tissues. Using ELISA, we observed that IL-6 level was at least 10-fold higher in CAFs (897.6 pg/ml) than in benign fibroblasts (87.89 pg/ml) secretion (Figure 1). Other than IL-6, there were also higher levels of MCP-1 (5.6-fold), VEGF (3.3-fold) and RANTES (3-fold) in CAFs secretion than in those from benign fibroblasts (Figure 1).

**Activation of IL-6 signaling cascade in EC upon CAFs secretion**

To determine if IL-6 mediates EC proliferation, we treated endometrial cancer cell line, ECC-1 and primary endometrial cancer cell, EC6-Ep with IL-6 neutralizing antibody in the presence of CAFs conditioned media. While there were no changes in cell viability in cells treated with isotype control, increasing concentrations of IL-6 neutralizing antibody led to a remarkable inhibitory effect of almost 50% (Figure 2A, 2B). Without CAFs conditioned media, both IL-6 neutralizing antibody and isotype control caused only 5% inhibition in cell proliferation compared to control (non-treated cells) (Figure 2C). Additionally, ECC-1 and EC-6 Ep cells treated with IL-6 recombinant protein without the presence of CAFs conditioned media showed dose-response increase in cell proliferation (Figure 2D). This indicates that IL-6 was present in CAFs secretion and had directly induced EC cell proliferation.

We further showed that IL-6 receptors, IL-6R and gp-130, were expressed only in EC epithe-
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Lial cells but not in CAFs, at mRNA and protein levels (Figure 3A). Activation of IL-6 receptors by CAF secretion resulted in ~2-fold elevation of both phospho-JAK3 and phospho-STAT3 protein levels in ECC-1 cells (Figure 3B). This activation was abrogated by IL-6 neutralizing antibody, even in the presence of CAFs conditioned media (Figure 3C). Treatment of ECC-1 cells with AD412, a JAK3 selective inhibitor and STAT3, a STAT3 selective inhibitor in the presence CAFs conditioned media, significantly down-regulated ECC-1 cell proliferation, to about 50% inhibition at the lowest dose of 10 µM (P<0.0001) (Figure 3D). Cells were minimally affected after treatment with vehicle and inhibitors alone. Taken together, our data indicates that IL-6 secreted by CAFs induced paracrine signaling in EC cells, resulting in activation of IL-6 receptor pathway.

c-Myc regulates CAFs-mediated EC cell proliferation

To determine the induction of STAT3 further downstream genes, we measured the expression of six STAT3 target genes that are related...
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Compared to non-treated control, c-Myc was significantly induced in ECC-1 cells, with at least 100-folds at mRNA, and ~2-4 folds at protein levels (Figure 4A, 4B). Similarly, TIMP-1 and SOCS-3 mRNA expressions were higher in the treated ECC-1 cells compared to control. Interestingly, PIM1 was the only gene down-regulated in ECC-1 cells after treatment with different CAFs (<0.5 fold vs. control) (Figure 4A).

To determine if CAFs-induced c-Myc mediates EC cell proliferation, we down-regulated its expression in ECC-1 cells using three individual shRNA constructs. Expression of c-Myc protein was found to be downregulated in ECC-1 cells upon transfection with c-Myc specific shRNA constructs (Figure 4C). Cells with reduced c-Myc expression showed a significant decrement in cell proliferation (54% inhibition), when compared to those transfected with c-Myc non-targeting control (~10% inhibition). Treatment of CAFs conditioned media in these cells only marginally increased the proliferation (~11%) (Figure 4D).

We further treated ECC-1 cells with a c-Myc small molecule compound (10058-F4) in the presence of CAFs. This led to a dose-dependent suppression of cell proliferation (Figure 4E). Notably, about 80% inhibition of cell proliferation was observed at 100 µM in the presence of CAF conditioned media. Treatment of 10058-F4 alone (100 µM), however, reduced ECC-1 proliferation by only about 20% (Figure 4E), suggesting that presence of CAF secretion was necessary for the anti-proliferative action of c-Myc inhibitor. Taken together, we demon-

Figure 5. In vivo growth of EC tumor with c-Myc downregulation. (A) Stable clones of ECC-1 cells expressing c-Myc scramble or shRNA A constructs were established prior to subcutaneous injection into the right flank of Balb/c nude mice. These cells were injected with or without EC11Fib cells, in a ratio of 1:4 (tumor:fibroblast). The growth of tumor cells was measured using caliper. (B) At the end of the experiment, the animals were imaged using fluorescence imaging system, followed by tumor excision (C).
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<table>
<thead>
<tr>
<th></th>
<th>IL-6R</th>
<th>gp-130</th>
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CAFs contribute to endometrial cancer cell proliferation via IL-6 pathway

strated that c-Myc was upregulated by IL-6 secreted by CAFs, and c-Myc activation was required for CAF-mediated EC cell proliferation.

*CAFs mediated c-Myc induction promotes EC cell growth in vivo*

We further investigated the role of c-Myc expression in EC tumor-CAF crosstalk in a subcutaneous human tumor xenograft mouse model. Seven weeks post cell inoculation, ECC-1 tumors were about $736.8 \pm 11.1 \text{ mm}^3$ while those mice injected with CAFs alone (EC11-Fib cells) alone did not show any signs of cell growth. Mice injected with a combination of ECC-1 and EC11F (1:4 ratio) showed at least 1.4 times greater tumor size ($1042.2 \pm 27 \text{ mm}^3$) compared to those injected with ECC-1 cells alone ($P<0.0001$). Interestingly, mice injected with ECC-1 with c-Myc knockdown showed at least 2.5 times smaller tumor size ($293.9 \pm 7 \text{ mm}^3$) when compared to those in the scramble group ($P<0.0001$). Co-injection with CAF failed to induce greater tumor growth in this group ($361.9 \pm 13.7 \text{ mm}^3$) ($P=0.013$) (Figure 5A). Notably, there was a small but significant difference in growth rate of ECC-1 transfected with scramble shRNA, with and without EC11-Fib ($P=0.0118$). The tumor size between the groups were also analyzed using *in vivo* fluorescence imaging, and was closely correlated to the tumor volume measured at the end of experiment (Figure 5C). Our data indicated that c-Myc has a significant role in CAFs-mediated EC cell proliferation *in vitro* and *in vivo*.

*Activation of IL-6 signaling markers in cancer tissues*

We further validated our finding by analyzing IL-6/STAT3/c-Myc pathway in human endometrial benign and malignant tissues. We analyzed the expression of IL-6R, gp130, phospho-STAT3 and c-Myc in five benign and five cancerous tissues of the human endometrium. Overall, positive staining patterns were observed to be intense and uniform in the glandular linings of the cancer tissues compared to the benign tissues when observed at $10 \times$ magnification. Stronger IL-6R and gp130 staining were seen in the epithelial compartment of the can-

![Figure 6.](image)
ecious tissues than in benign tissues. Phospho-
STAT3 and c-Myc staining were also evident
and predominantly confined to the nucleus in
the cancer tissues; although a rather weak and
diffuse staining was seen in the cytoplasm of
the benign tissues (Figure 6). Our data demon-
strated that human endometrial cancer tissues
expressed high levels of IL-6R, gp130, phos-
pho-STAT3 and c-Myc proteins which were mini-
maly expressed in benign tissues. This indi-
cates that IL-6/STAT3/c-Myc pathway could be
an important player in the pathogenesis of
endometrial cancer and could potentially be
targeted for future treatments.

Discussion

While CAFs have been implicated as a key player
in cancer progression [6, 13], its role in EC is
relatively understudied. Our study suggests
that IL-6 secreted by CAFs could have a role in
regulating EC cell proliferation in vitro and in
vivo. Elevated levels of IL-6 in the tumor micro-
environment led to activation of JAK/STAT3
pathway in tumor cells, which resulted in in-
creased cell proliferation, probably through in-
duction of c-Myc protein. Inhibition of this path-
way significantly abrogated the tumor-promot-
ing effects of CAFs. Moreover, IL-6 pathway
inhibitors were only effective when CAFs were
present, indicating the importance of tumor
microenvironment as an element in effective
drug screening, which otherwise may be over-
looked in conventional screening using only the
tumor cells.

It has long been recognized that crosstalk
between tumor cells and their microenviron-
ment alters the mechanistic property of tumor
cells in proliferation, motility and metastasis
[14]. Paracrine signaling regulated by CAFs are
shown to contribute to many cancer progres-
sion, such as IGF-II, HGF and SDF-1 signaling in
lung cancer [15], TGF-β signaling in breast can-
cer [16] and hedgehog signaling in pancreatic
cancer [17]. In pancreatic, prostate, melanoma,
multiple myeloma and breast cancers, IL-6 was
linked to increased sustainability and survival
of tumors [18-20]. In endometrial cancer, how-
ever, it was not obvious how the microenviron-
ment affects tumor progression. It was report-
ed that IL-6 levels were elevated in the serum
of EC patients [21] and were associated with
chemotherapy resistance and poor prognostic
outcome [12]. Yet, it is not clear if the presence
of IL-6 was due to CAFs residing in the tumor
microenvironment. In this study, we demon-
strated a role of CAF in promoting EC cell prolif-
eration, and our data showed that this effect
was specific to the one-way paracrine signaling
by IL-6 secreted by CAFs to activate the recep-
tors expressed only by tumor cells. This in turn
translates to CAF's role as a key player in the
tumor microenvironment, modulating the be-
havior of cancer cells through its secretion of
inflammatory cytokines to communicate within
the cancer environment.

Aberrant activation of JAK/STAT downstream of
IL-6 pathway has been identified as an underly-
ing factor mediating tumor progression and
metastasis in various cancers [22], yet its impli-
cation and role in EC is unclear. In our study, we
found that STAT3 was induced by IL-6 secreted
by CAFs, similar to Bromberg's observation in
other cancers [23-25]. Subsequent activation
of STAT3 leads to induction of various onco-
genic proteins, including c-Myc and was shown
to be a pre-requisite to mediate the rapid activa-
tion of c-Myc gene [26]. c-Myc is found activat-
ed in many human tumors with poor disease
prognosis outcome, including EC [27-29]. We
observed increased c-Myc expression in EC tis-
ues compared to benign endometrial tissues.
Additionally, knockdown of c-Myc in EC cells sig-
nificantly reduced the proliferation of tumor
cells both in vitro and in vivo, despite exposure
to CAFs secretion. It is likely that activation of
c-Myc via IL-6/STAT3 acts as a “on-switch” in
EC cells to progress into a more aggressive
stage, as in multiple myeloma [30, 31]. Our
work may provide one of the first few evidences
that c-Myc can be induced via IL-6R/STAT3
pathway following activation by IL-6 from CAF
secretion in endometrial cancer. Inhibitors tar-
geting IL-6R downstream molecules were only
effective in exerting their anti-tumor effects
when in the presence of CAFs secretions. This
strongly suggests that IL-6R/STAT3/JAK/c-Myc
pathway can be targeted for EC treatment and
our study also implies the importance of CAFs
in the tumor microenvironment to provide the
paracrine stimulants.

IL-6 pathway may not be the only mediator for
CAFs to affect EC progression. This pathway is
shown to communicate with other signaling
pathways to achieve significant pro-tumorigenic
effects. It was shown that IL-6 in the microen-
vironment stimulated crosstalk between colorec-
tal cancer cells and immune cells via miRNAs
miR-21 and miR-29b, to sustain inflammation
CAFs contribute to endometrial cancer cell proliferation via IL-6 pathway

and to promote prometastatic behavior [32]. In addition, gastric cancer-derived mesenchymal stem cells was shown to induce chemotaxis of neutrophils, leading to protection against spontaneous apoptosis and activation of cancer cell motility via IL-6-STAT3-ERK1/2 signaling cascade [33]. Moreover, IL-6 was also shown to stimulate receptor activator of NF-κB ligand (RANKL) expression in bone cells, resulting in a direct paracrine-autocrine signaling between osteoblast and cancer cells to enhance the growth of metastatic breast cancers within the bone [34]. Hence, it is possible that IL-6 has further pro-tumorigenic effects in EC when acts in combination with other inflammatory cytokines present in the tumor microenvironment milieu. Further investigations on the crosstalk are crucial to understand the role of CAFs in EC, to design better therapy approaches for this disease.

With increasing role of IL-6 pathway in angiogenesis and cancer progression [35], it may be worthwhile to target this pathway for new treatment for oncology. IL-6 pathway inhibitors which were previously developed mainly for inflammatory conditions are currently tested in oncology settings. For example, tocilizumab, a humanized IL-6R specific monoclonal antibody, was previously approved for rheumatoid arthritis but is now proposed to alleviate cancer cachexia [36, 37]. REGN88, a fully humanized IL-6R monoclonal antibody, is currently undergoing phase III clinical trial in rheumatoid arthritis and ankylosing spondilitis with future indication possibility in solid tumor [38]. Additionally, in phase I/II clinical trial in advance or refractory solid tumors, siltuximab or NT28 that neutralizes IL-6, demonstrates promising safety and tolerability outcome [39] as well as a multitude of other indications [38]. Our study suggests IL-6 pathway as a novel target for EC, and hence application of these inhibitors may provide new avenue for treating aggressive EC.

Conclusion

This study shows that IL-6 secreted by cancer-associated fibroblasts in the tumor microenvironment promotes EC proliferation via activation of JAK/STAT3/c-Myc pathway in vitro and in vivo. Targeting IL-6, either through a single agent or by combination with co-activated inflammatory cytokines may be a novel therapeutic approach for women with EC.

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Disclosure of conflict of interest

None.

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