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Non-nuclear activation of Signal transducer and activator of transcription 3 by 17β- estradiol in endometrial cancer cells

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Why estrogen hyperstimulation can lead to endometrial carcinogenesis has not been fully clear yet. Non-nuclear action of estrogen has arised much attention of many experts. Signal transducer and activator of transcription 3 is a very important signal molecule, which plays vital role in endometrial canver. The present study is oriented to the problem whether estrogen can activate STAT3 by non-nuclear action in endometrial cancer cells. So, the levels of phosphorylated STAT3 (P-STAT3) and total STAT3 were examined by western blot in endometrial cancer cells including Ishikawa with rich-expressed estrogen receptor (ER) and HEC-1A with poor-expressed ER after stimulation with 1μM estradiol (E2) at different time points and at varied doses of E2 for optimal time. Inhibitory role of AG490 on activation of STAT3 induced by E2 was also tested. P-STAT3/STAT3 was used as a measure of activation of STAT3. We found that maximum P-STAT3/STAT3 took place at 15min in both Ishikawa cells and HEC-1A cells. The activation of STAT3 elicited gradually with increasing doses of E2. AG490 stopped the activating STAT3 in the same dose-dependent manner in both endometrial cancer cells. The results demonstrate that E2 is able to activate STAT3 in both Ishikawa with rich-expressed ER and HEC-1A with poor-expressed ER endometrial cancer cells by non-nuclear action, which provides the preliminary laboratory basis for the probability of endometrial adenocarcinoma treatment with blockage of STAT3 signaling, especially for ER-poor endometrial adenocarcinoma.

Key words: endometrial cancer, signal transducer and activator of transcription 3, estrogen, non-nuclear action, carcinogenesis, estrogen receptor

Endometrial carcinoma is one of the most common female genital tract malignancies. It is well-known that risk for endometrial adenocarcinoma increases in patients with high estrogen levels that are unopposed by progestins. Estrogen has been shown to exhibit growth-promoting properties in endometrial cancer cell [1]. The mechanism responsible for this promoting growth effect of estrogen involves 'classical' or 'genomic' mechanism, estrogen molecules penetrate into the cell and bind to the ER, which are members of the nuclear hormone receptors, and interact with the estrogen response element located in the regulatory region of target genes. The resulting fluctuations in mRNAs and the proteins they encode underlie series of responses that take place within hours following estrogen exposure. But the 'genomic' mechanism can't explain the truth that why some ER-positive endometrial carcinomas have no responses to endocrinal therapy while some ER-negative ones have responses. There must be other mechanisms involved. Indeed, there are rapid biochemical and physiological responses to estrogen occurring more rapidly (within seconds to minutes) than gene transcription events attributed to the ER (over the course of several hours) that cannot be accounted for by changes in gene expression mediated by nuclear ER. Our former data demonstrated that 17β-estradiol, by non-nuclear action, can activate phosphatidylinositol 3-kinase (PI3K) /Akt signaling pathway in endometrial carcinoma cell lines [2] and blockage of PI3K/Akt pathway can antagonize estrogen-induced endometrial cancer proliferation [3]. Moreover, in breast cancer[4], lung cancer [5], colon cancer[6], and prostate cancer [7], mechanism of non-nuclear action is also involved.

As we all know, Leptin as well as estrogen is closely associated with genesis and progression of endometrial carcinoma. Growth of malignant cells could be regulated by leptin-induced second messengers like STAT3 (signal transducers and activators of transcription 3). STAT3 seem to be involved in aromatase expression, generation of estrogens and activation of estrogen receptor α (ERα) in malignant breast[8] and endometrial epithelium[9]. Whatsmore, during the process of Leptin- in-

duced hepatocellular carcinoma cell invasion and migration, JAK/STAT, PI3K/Akt and Erk signaling are all Involved. STAT3, as a target of estrogen signaling in cells, has been reported[10]. There is a cross-talk between estrogen and leptin signaling relating to STAT3 activation in the hypothalamus[11]. STAT3 and phosphorylated STAT3 protein can be induced in ob/ob mouse liver after long-term estrogen treatment. It has been reported that STAT3 can be rapidly induced by estradiol in mouse livers and that STAT3 is a direct target gene for estradiol[12]. 17beta-Estradiol-activated estrogen receptor (ER) are able to induce transcriptional activation of STAT-regulated promoters via cytoplasmic signal transduction pathways by non-nuclear action [13]. STAT3 activation was involved in cancer cell proliferation and migration. Our previous study found that STAT3 is abnormally activated in endometrial carcinoma tissues and that STAT3 activation is involved in endometrial tumorigenisis. STAT3 activation is associated positively with Ki-67, a variable of cell proliferation in endometrial carcinoma[14]. But it has not been certain yet if estrogen can activate STAT3 in endometrial cancer cells by non-nuclear action. So, in the present study, we observe if estrogen can activate STAT3 by non-nuclear action in endometrial cancer cells including Ishikawa with rich-expressed estrogen receptor (ER) and HEC-1A with poor-expressed ER. By doing so, we can further understand the mechanism of estrogen-induced endometrial carcinogenesis and provide the preliminary laboratory basis for the probability of endometrial adenocarcinoma treatment with blockage of STAT3 signaling.

Materials and methods

Materials. Water-soluble 17beta-estradiol(estrogen, E2), serum replacement (SR2) containing no steroids, no growth factors, were purchased from Sigma Chemical Co.(St. Louis, MO). The antibodies against phospho-STAT3 at Tyr705 (P-STAT3) and STAT3, Anti-rabbit IgG, HRP-linked antibody, Enhanced Chemiluminescent detection system and AG490 were all from Cell Signaling Technology (CST, Beverly, MA).

Methods. Cell culture and treatment. Endometrial adenocarcinoma cell line Ishikawa, which bears rich estrogen receptor(ER)[2], a kind gift from Professor Li-Hui Wei, Peking university, and HEC-1A cell line, which bears poor ER[2], obtained from ATCC (the American Type Culture Collection, Manassas, VA, USA) 5 months ago, were maintained in Phenol red-free RPMI 1640 or DMEM (Dulbecco's Modified Eagle Medium) medium, respectively, supplemented with 10% fetal calf serum (FCS), 100U/ml penicillin and 100μg/ml streptomycin and incubated with 5% CO2 at 37 °C. Cell line cultured in serum-free medium was cultured in RPMI 1640 or DMEM containing 0.5% the defined, estradiol-free and growth factor-free serum replacement (SR2, Sigma).Cells (5×10^5 or 1×10^6) were seeded in 25 cm^2 flasks (or 100mm plates) in Phenol red-free RPMI 1640 or DMEM containing 5% steroid-stripped FCS (DCC-FCS) (using dextran-coated charcoal) for 24 h. The medium was replaced with RPMI 1640 or DMEM containing 0.5% (v/v) DCC- FCS and after 48 h, the cells were washed and incubated in RPMI 1640 or DMEM containing 0.5% (v/v) SR2 for 24 h before stimulation. One micromolar water-soluble 17β-estradiol (estrogen, E2, Sigma Chemical Co.) was used for incubation at indicated time points (0min, 15min, 30min, 1 h, 2 h) to observe the optimal time for STAT3 activation. Then, different concentrations of estrogen (vehicle, 100, 1, 0.01, 0.0001μM) were used to treat Ishikawa or HEC-1A cells for 15min (optimal time for the two cell lines). For treatment with inhibitor, the cells were pretreated with the respective inhibitor for 1 h and cotreated with estrogen for an additional 15min. In these experiments, different doses of AG490 (25, 50, 75, 100μmol/L) (CST, Beverly, MA, USA) were added alone or combined with 1μM estrogen in 0.5% SR2. All experiments were repeated three times.

Western blot. Western blot was performed according to the manufacturer's protocol with some modifications. Briefly, cells were harvested and lysed in 1× SDS sample buffer (62.5mM Tris–HCl, 2% SDS, 10% Glycerol, 50mM DTT, 0.01% bromophenol blue) on ice, sonicated for 15–20 s and microcentrifuged for 15min. Twenty microliters of cell lysates were loaded onto 10% SDS-polyacrylamide gels and separated by electrophoresis. The separated proteins were electrotransferred to nitrocellulose membrane and probed with the antibody against phosphorylated STAT3 at Tyr705 (P-STAT3) (1:1000 dilution) (CST, Beverly, MA, USA) overnight after being blocked at room temperature for 1 h. The membrane was then incubated with HRP-conjugated secondary antibody (1:2000) (CST, Beverly, MA, USA) and HRP-conjugated anti-biotin antibody (1:1000) (CST, Beverly, MA, USA) to detect biotinylated protein markers for 1 h at room temperature. The proteins were detected with the enhanced chemiluninecence (ECL) system (CST, Beverly, MA, USA).The membrane was sequentially exposed to X-Kodak film for 30 s and then processed. Following the Phototope-HRP chemiluminescent detection (after film exposure), the membrane was stripped in Stripping Buffer [31mMTris–HCl, 2% (w/v) SDS and 0.7% (v/v) 2-mercaptoethanol] at 50 °C for 30min and reprobed with the primary antibody against STAT3 (1:1000) (CST, Beverly, MA, USA). The above procedures were repeated for Western blot analysis. STAT3 activation was defined as the ratio of the P- STAT3 to STAT3 and quantitatively evaluated after the measurement of the optical density of the protein bands.

Statistical analysis. The signals of P-STAT3 and STAT3 were quantified after measurement of the optical density of the protein bands. STAT3 activation level was expressed as ratio of P-STAT3/STAT3. The data were expressed as mean± standard deviation calculated from three seperate experiments. Statistical analysis was performed using Student's t-test or t-test, with statistical significance defined as $P<0.05$.

Results

Effect of estrogen on activation of STAT3. We examined STAT3 activation in the two cell lines in response to treatment with estrogen. An activated function of STAT3 is associated with phosphorylated STAT3 at Tyr705. It is believed that STAT3 is

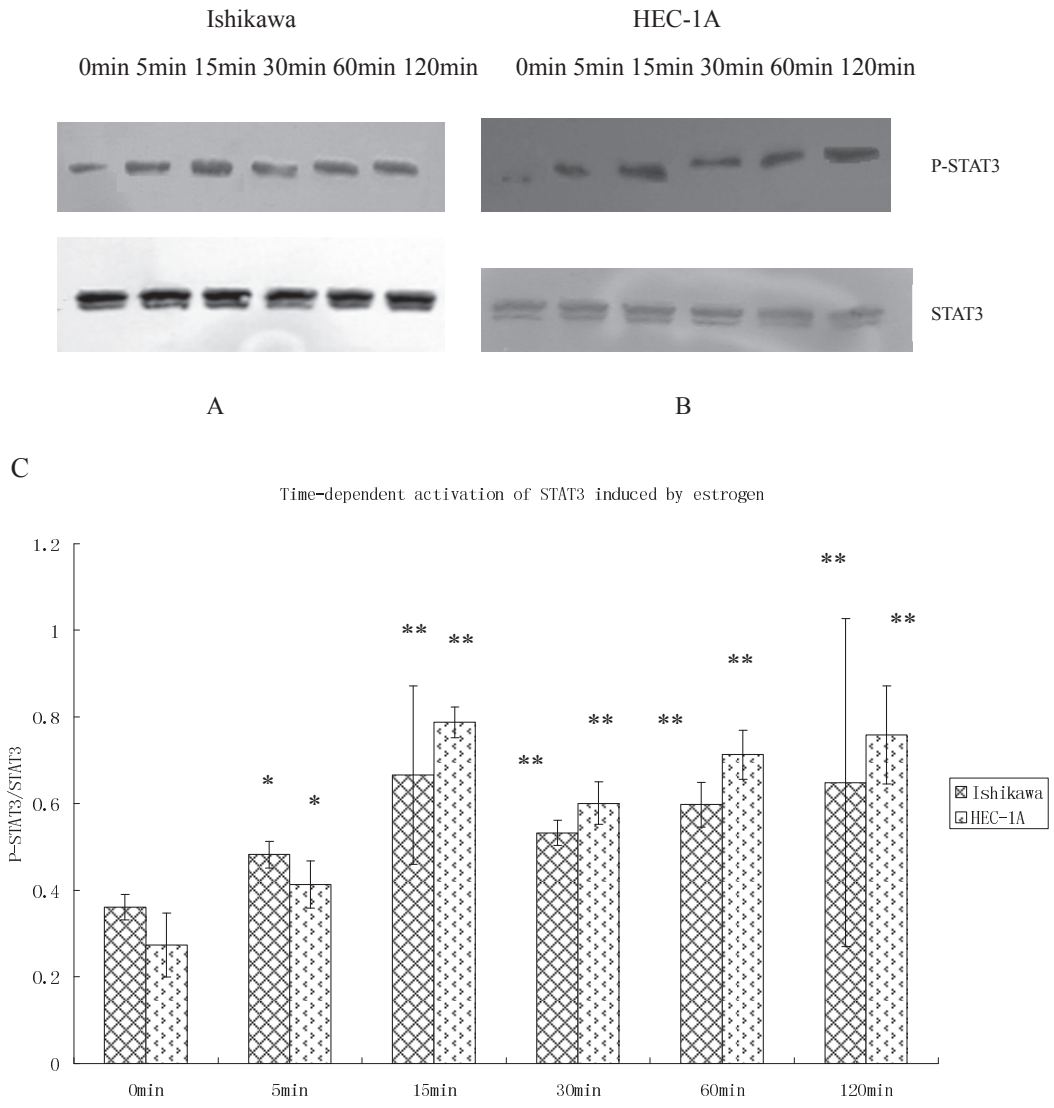

Figure 1. Time-dependent activation of STAT3 by estrogen. Cells were treated with 1 μM E2 and incubated for differentt durations of time as indicated before the cells harvested for lysis. The signals of P-STAT3 (phospho-STAT3 at Tyr705) and STAT3 were quantified after measurement of the optical density of the protein bands, and the ratios of phosphorylated STAT3:total STAT3 are shown in the bar diagram.*p < 0.05 vs. control;p < 0.001 vs. control. (A) Expression of P-STAT3, STAT3 in Ishikawa; (B) expression of P-STAT3, STAT3 in HEC-1A; (C) bar diagram of the ratios of P-STAT3:total STAT3 in the two endometrial cancer cells.**

fully activated by phosphorylation of Tyr705. The ratio of P-STAT3/STAT3 was used as levels of activation of STAT3. Using phospho-STAT3 (Tyr705) polyclonal antibody on western blot, STAT3 activation stimulated by 1μM estrogen after exposure at different lengths of time and that by varied concentrations of estrogen were investigated in two endometrial carcinoma cell lines. A rapid activation of STAT3 was observed by this steroid. Within 15min, estrogen induced a significant increase of STAT3 phosphorylation (Tyr705) and the peak level of P-STAT3 could be observed at 15min and persisted for at least 2 h in both cell lines (Fig. 1). STAT3 activation increased gradually with increased concentrations of the estrogen showing a dose-dependent manner in both cells (Fig. 2).

Effect of AG490 on estrogen-induced activation of STAT3. STAT3 activation decreased with increasing doses of AG490 in a dose-dependent manner (Fig. 3). More interestingly, trends of AG490 inhibition of STAT3 activation in the two different ER status endometrial cancer cells are same.

Discussion

Here, we reported for the first time that 17β-estradiol, through non-nuclear action, can activate promptly STAT3 signaling pathway in endometrial cancer cells Ishikawa and HEC-1A.

Estrogen can activate STAT3 by non-nuclear mechanism in endometrial cancer cells. According to the traditional

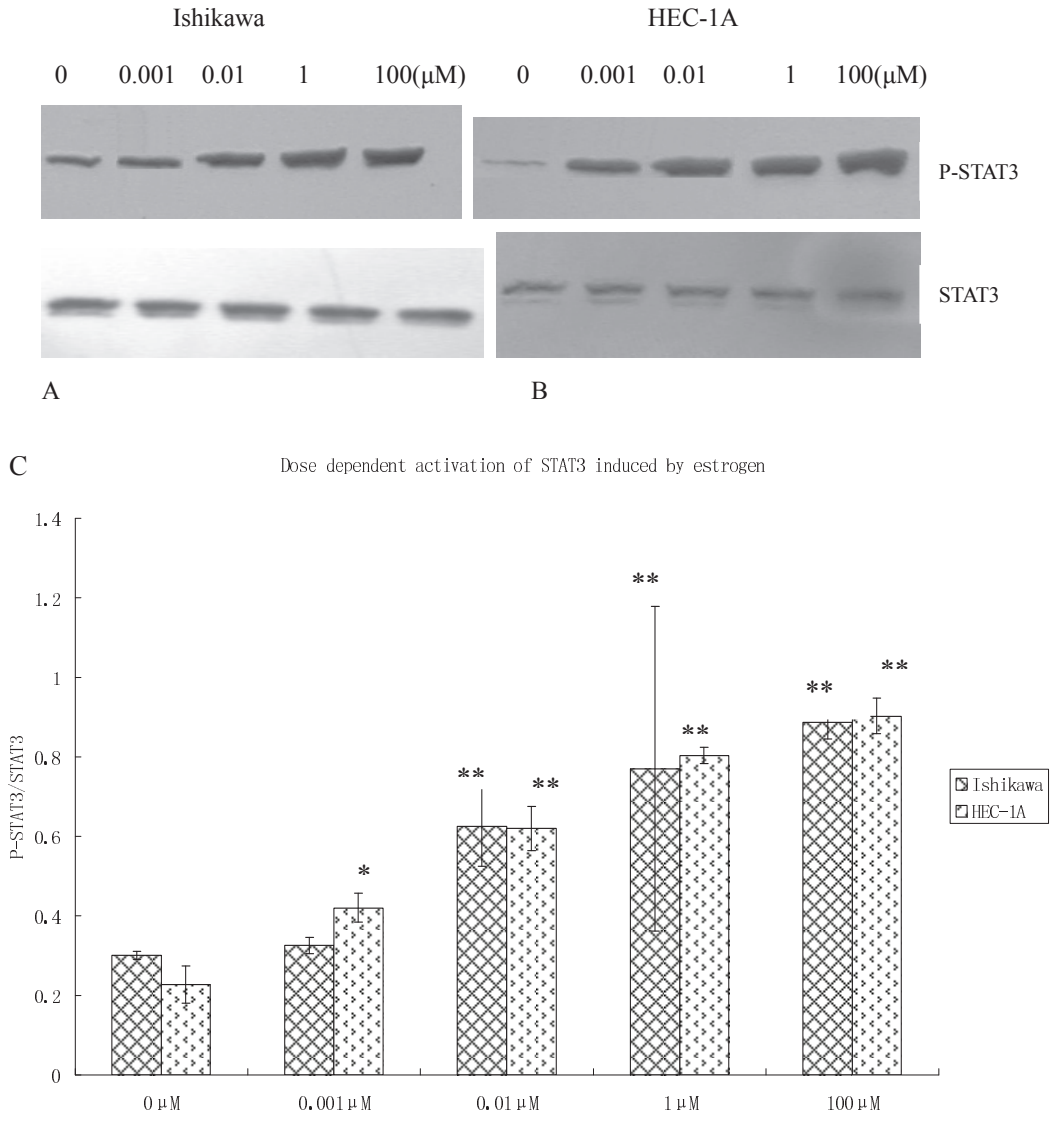

Figure 2. Dose-dependent activation of STAT3 by estrogen. Cells were treated with different concentrations (μM) of estrogen as indicated for15min in Ishikawa and in HEC-1A. The relative ratio of phosphorylated STAT3:total STAT3 are shown in the bar diagram.*$p < 0.05$ vs. control; **$p < 0.001$ vs. control. (A) Expression of P-STAT3, STAT3 in Ishikawa; (B) expression of P-STAT3, STAT3 in HEC-1A; (C) bar diagram of the ratios of P-STAT3:total STAT3 in the two endometrial cancer cells.

model, steroid hormones including estrogen bind to intracellular receptors and subsequently modulate transcription and protein synthesis, thus triggering genomic events finally responsible for delayed effects. Because of nucleus involved this traditional effect is also called nuclear effect. In addition, very rapid effects of steroids affecting mainly the intracellular signal transduction pathways have been widely recognized clearly found to be incompatible with the nuclear model. In contrast to the nuclear steroid action, non-nuclear steroid effects are principally characterized by their insensitivity to inhibitors of transcription and protein synthesis, and by their rapid onset of action (within seconds to minutes) [15]. It is believed that nuclear and non-nuclear effects of steroid hormones coordinately regulate cell processes. These rapid, non-nuclear steroid actions are likely to be transmitted via specific membrane receptors. Recent evidence of the G protein-coupled estrogen receptor (GPER) which takes part in non-nuclear steroid actions has begun to emerge[16]. For example, estrogenic G protein-coupled receptor 30 signaling is involved in activation of the MEK/ERK mitogen-activated protein kinase pathway [17]. In our study, activation of STAT3 by estrogen is a relatively fast response. Within 5min, estrogen induces a significant increase of STAT3 phosphorylation with maximal activation at 15min both in Ishikawa and HEC-1A and they persisted for at least 2 h. The possibility of the involvement of classical estrogen nuclear mechanisms like the transcriptional induction of growth

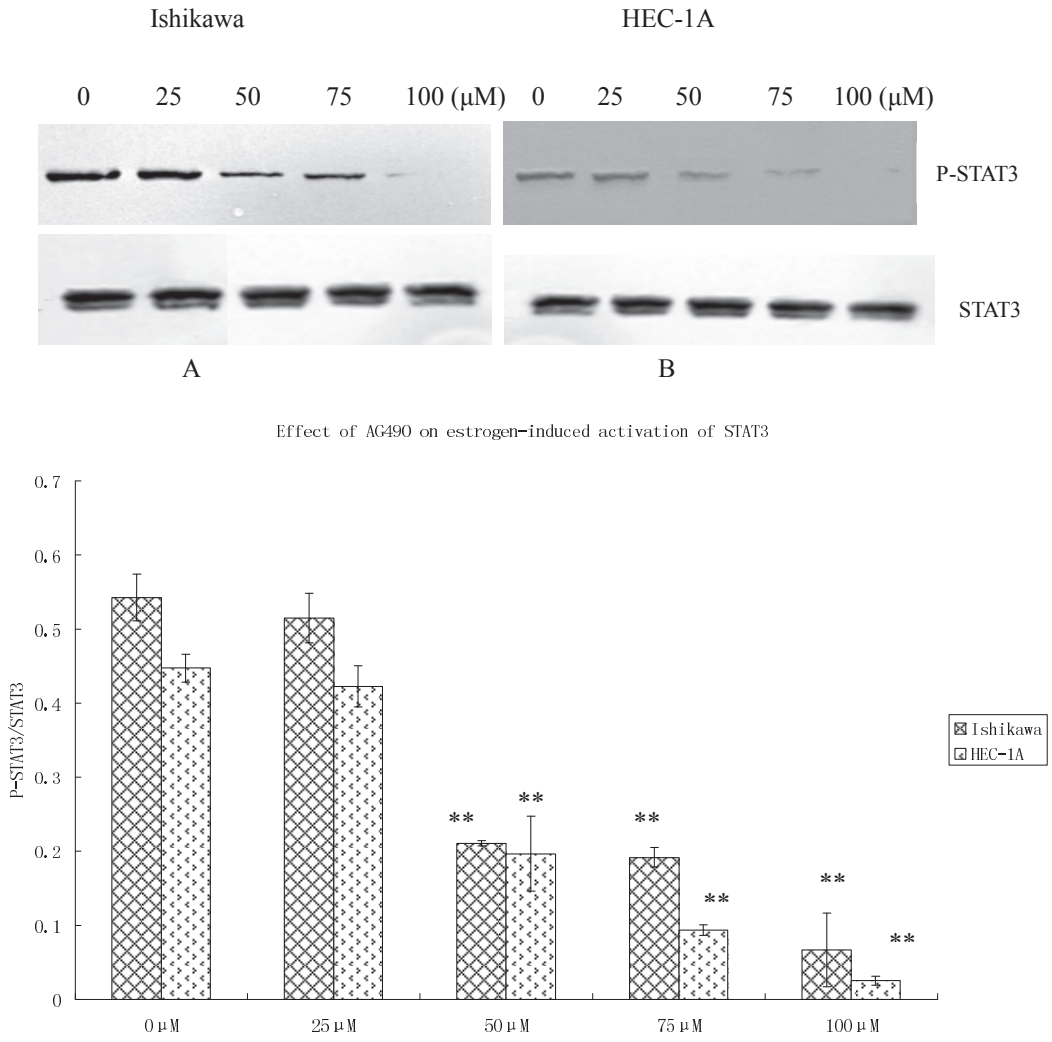

Figure 3. Effect of AG490 on estrogen-induced activation of STAT3. Cells were pretreated 1 h with different doses ofAG490 (0, 25, 50, 75,100 μM) alone respectively, and then cotreated with 1μM E2 for 15min. The relative ratio of phosphorylated STAT3:total STAT3 detected by Western blot are shown in the bar diagram.*$p < 0.05$ vs. control;$p < 0.001$ vs. control. (A) Expression of P-STAT3 and STAT3 in Ishikawa; (B) expression of P-STAT3 and STAT3 in HEC-1A; (C) bar diagram of the ratios of P-STAT3:total STAT3 in the two endometrial cancer cells.**

factor expression followed by activation of STAT3 pathway can be ruled out because of the short time period between the estrogen stimulus and STAT3 activation which supports non-nuclear effects. Our data suggests that this effect of estrogen resulted from non-nuclear effects, like those of involvement of GPER[18,19]. The time needed to activate STAT3 and the duration of maintenance varied with different stimulators and cell lines, as fluctuating from 5min to 1 h for the STAT3 activation. We further studied the relationship between STAT3 activation and estrogen dosage response by stimulating endometrial cells with different doses of estrogen for 15min. A dose-dependent fashion was found for activation of STAT3, where it elevated gradually with increasing doses of estrogen, as in accordance with the clinical fact that high-level estrogen state increases the risk for endometrial carcinoma.

STAT3 signaling pathway inhibitor, AG490 can block estrogen –induced rapid activation of STAT3 in both different ER status endometrial cancer cells. We further observed the effect of STAT3 signaling pathway specific inhibitor, AG490, on activation of STAT3 by estrogen. The induction of STAT3 by estrogen was blocked by the inhibitors, which strengthened that estrogen can activate STAT3 signaling pathway in both endometrial cancer cells. AG490 can block activation of STAT3 by not only estrogen as we suggested from our current study but also many other factors such as leptin, IGF, EGF, insulin, etc.[20-23]. As STAT3 promotes both cell survival and proliferation and suppresses cell apoptosis, the specific inhibition of its activity may be a good therapeutic strategy for tumors with amplification of STAT3 including poor-expressed ones, which always has

no response to hormone therapy. We will verify this point further by in vitro and in vivo experiments.

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