Estrogen promotes fat mass and obesity-associated protein nuclear localization and enhances endometrial cancer cell proliferation via the mTOR signaling pathway

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Received October 15, 2015; Accepted January 11, 2016
DOI: 10.3892/or.2016.4613

Abstract. Extensive exposure to estrogen is generally acknowledged as a risk factor for endometrial cancer. Given that the accumulation of adipocytes also contributes to the increased production of estrogen, in the present study, we evaluated the expression of the fat mass and obesity-associated (FTO) gene in endometrial tumor tissues and further explored the mechanism of how estrogen facilitates FTO nuclear localization and promotes endometrial cancer cell proliferation. Immunohistochemical (IHC) staining assay was used to detect the FTO expression in endometrial tumor samples. Western blotting was performed to investigate the mechanism of estrogen-induced FTO nuclear localization. siRNA was used to knock down ERα and further explore its role in FTO nuclear localization. MTT assay was carried out to determine cell proliferation. We found that FTO was overexpressed in endometrial carcinoma tissues and served as a poor prognostic marker. Additionally, estrogen induced FTO nuclear accumulation via the mTOR signaling pathway and the nuclear localization was ERα-dependent, which contributed to enhanced proliferative activity. Therefore, the present study provides new insight into the mechanisms of estrogen-driven FTO nuclear localization, implying the possibility of using FTO as a potential therapeutic target for the treatment of endometrial cancer.

Introduction
Endometrial cancer is one of the most common gynecologic malignancies worldwide (1,2). There are two different clinicopathological types of endometrial carcinoma, including type I and II endometrial carcinoma (3). In clinical practice, over 80% of endometrial cancers are type I, which commonly comprise low-grade carcinomas. These tumors are often preceded by endometrial premalignant disease and are always estrogen nuclear receptor (ER) and progesterone receptor (PR)-positive, which frequently arise during excessive estrogen exposure. Recently, much effort has been made to confirm the involvement of aberrant estrogen metabolism in dysregulated endometrial cancer cell growth and malignant metastasis (4-6). We previously suggested that estrogen promoted endometrial cancer cell proliferation and invasion by fat mass and obesity-associated (FTO) gene (7), however, the molecular mechanism of how FTO regulates cellular growth by estrogen remains obscure.

FTO was identified as an oncoprotein frequently overexpressed in several types of cancer, including endometrial, breast and pancreatic cancer (8-13). FTO is a protein involved in energy homeostasis by controlling energy expenditure. Depletion of FTO in mice was found to result in growth retardation, adipose tissue reduction and lean body mass (14). Since estrogen-driven endometrial cancer is strongly and definitively linked to obesity, two studies previously examined the relationship between FTO gene polymorphism and the incidence of endometrial cancer (9,13). In our previous study, we also found that estrogen stimulation resulted in FTO accumulation in the nucleus (7), however, the mechanism of estrogen-driven FTO nuclear localization is not clear.

Although less than 0.1% of the total cellular protein, kinase and phosphatase enzymes play a pivotal role in conducting signals to control cell growth or invasion, abundant signaling pathways have been reported to be involved in estrogen-driven endometrial cancer. Inhibition of the PI3K/AKT pathway was found to lead to a decrease in proliferative and invasive activities. Blocking MAPK signaling also resulted in similar effects. These results are consistent with our previous study (7). Another important signaling molecule, mTOR is an atypical serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and is involved in energy metabolism (15,16). mTOR has been implicated in the development and progression of various types of cancers including melanoma, lung and endometrial cancer (17-27). Recent findings suggest that the mTOR pathway may play an important role in endometrial cancer.
progestin resistance (28). However, as a molecule controlling energy expenditure, whether estrogen-driven FTO nuclear localization is mediated by the mTOR signaling pathway has not been studied. Therefore, the aim of the present study was to investigate whether estrogen enhances FTO nuclear localization and promotes endometrial cancer cell growth via the mTOR signaling pathway.

Materials and methods

Sample collection. Forty-nine samples were obtained from the Tissue Bank of the Department of Obstetrics and Gynecology of the Shanghai First People’s Hospital affiliated to Shanghai Jiao Tong University, which were comprised of 18 normal endometrial tissues and 31 cases of type I endometrial carcinoma. None of the patients in the study had a history of prior radiotherapy or chemotherapy. Any patient with a known history of hormone replacement was excluded. The use of these specimens was approved by the Ethics Committee of the Medical College of Shanghai Jiao Tong University, China.

Immunohistochemical (IHC) staining and analysis. IHC analysis of FTO protein expression was performed as previously described and assessed using a semi-quantitative method. Briefly, specimens were deparaffinized in xylene and rehydrated in a graded series of ethanol and subsequently endogenous peroxidase activity was blocked by a 10-min treatment with 3.0% hydrogen peroxide. Subsequently, the sections were subjected to antigen retrieval by boiling in citrate buffer (pH 6.0) and incubated for 30 min with 0.01% Trixon and then incubated for 20 min with 5% bovine serum albumin (BSA). The sections were incubated overnight with a rabbit anti-human FTO primary antibody at 4˚C in a humidity chamber, followed by a 50-min incubation with a biotinylated secondary antibody. The sections were washed and blocked in 3% BSA (1 h) and then incubation with an anti-human FTO primary antibody (1:100, overnight). After incubation with a FITC-labeled secondary antibody (1 h at room temperature), the cells were photographed.

Subcellular fractionation. Ishikawa cells treated with estrogen, rapamycin or transfected with siERα were harvested and lysed with cytoplasmic extraction buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl2, 1 mM dithiothreitol, 0.2% Nonidet P-40, 1 mM NaF, 1 mM Na3VO4 and protease inhibitor cocktail). After being centrifuged at 14,000 rpm at 4˚C for 5 min, the cytoplasmic fraction was collected. Then, the pellet was re-suspended in nuclear extraction buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 0.1 mM EDTA pH 8.0, 1.5 mM MgCl2, 1 mM dithiothreitol, 0.2% Triton-X 100, 1 mM NaF, 1 mM Na3VO4 and protease inhibitor cocktail), and the nuclear fraction was collected after a 5-min centrifugation.

Immunocytochemistry. Ishikawa cells treated with 10⁻⁹ M E2 for 48 h were cultured on coverslips before fixation with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) (10 min), permeabilization with 0.2% Triton X-100 (10 min), blocking in 3% BSA (1 h) and then incubation with an anti-human FTO primary antibody (1:100, overnight). After incubation with a FITC-labeled secondary antibody (1 h at room temperature), the cells were photographed.

Small interfering RNA (siRNA) transfection and hormone stimulation. The acute knockdown of siERα was performed as previously described. Briefly, Ishikawa cells were seeded in 5 ml of growth medium in 6-cm dishes without antibiotics, and grown to 30-50% confluence 24 h prior to transfection with 200 pmol ERα siRNA (Shanghai GenePharma Co., Ltd.) using DharmaFECT (Thermo Scientific). siRNA-treated and untreated Ishikawa cells were exposed to 1 nM E2 for a further 48 h before being collected for western blot analysis. The FTO knockdown was performed in a 96-well with the incubated Ishikawa cells, and the cell proliferation was determined by MTT assay.

MTT assay. To investigate the proliferative activity of endometrial cancer cells after various treatments, the MTT assay was performed. Briefly, Ishikawa cells were plated in a 96-well plate (2,000 cells/well) and incubated for 24 h. The culture medium was then changed to serum-free DMEM/F-12 (1:1) medium for 24 h. In order to determine the effect of ICI or Rap on cellular growth, cells were pre-treated with ICI or Rap for 1 h after stimulation with 1 nM E2 or dimethyl sulfoxide (DMSO) for 48 h. Similarly, prior to 1 nM E2 stimulation, the cells were transiently transfected with FTO siRNA to investigate its role in cell proliferation. MTT solution (20 µl of 5 mg/ml MTT in PBS) was added to the cells. After 4 h of incubation at 37°C, the culture medium was removed, and 150 µl of DMSO was added to dissolve the formazan. Finally, the acute knockdown of siERα was performed as previously described. Briefly, Ishikawa cells were seeded in 5 ml of growth medium in 6-cm dishes without antibiotics, and grown to 30-50% confluence 24 h prior to transfection with 200 pmol ERα siRNA (Shanghai GenePharma Co., Ltd.) using DharmaFECT (Thermo Scientific). siRNA-treated and untreated Ishikawa cells were exposed to 1 nM E2 for a further 48 h before being collected for western blot analysis. The FTO knockdown was performed in a 96-well with the incubated Ishikawa cells, and the cell proliferation was determined by MTT assay.
absorbance at 490 nm was measured with a GENios multifunction reader (Tecan, Zurich, Switzerland).

**Statistical analysis.** The statistical significance of the differences in the IHC staining in endometrial tissues was calculated using the Chi-square test. The differences in various protein levels and cell proliferation between groups were analyzed using the Student’s t-test. A two-sided test with P<0.05 was considered statistically significant. All statistical analyses were performed using SPSS 11.0 (SPSS, Inc., Chicago, IL, USA).

**Results**

*FTO is overexpressed in endometrial cancer and serves as a marker for poor prognosis.* To understand the role of FTO in endometrial cancer development, we first examined the expression of FTO in endometrial carcinoma tissues. As shown in Fig. 1A, little positive staining was detected in the normal endometrial tissues while strong positive staining was observed in type I endometrial carcinomas. The significant difference is summarized in Fig. 1B. Although we reported
that FTO expression was not associated with age, stage, grade, invasion and lymph node metastasis, we still found that higher FTO expression correlated with poor prognosis and early relapse (Fig. 1C and D).

**Estrogen promotes FTO nuclear localization.** In our previous study, increasing nuclear expression of FTO was observed after estrogen stimulation in endometrial cancer cells (7). Consistent with this result, we found that estrogen promoted FTO expression in a dose-dependent manner. The major stimulating peak was observed with 1-10 nM estrogen treatment (Fig. 2A). Moreover, estrogen also induced FTO expression in a time-dependent manner. After treatment with 1 nM estrogen for 48 h, a significant stimulating effect was observed (Fig. 2B). We further isolated the nuclear and cytosolic proteins. Estrogen-induced FTO was detected, however, there was no significant alteration of FTO expression in the cytosol following estrogen treatment (Fig. 1C and D).

**Estrogen controls FTO nuclear localization through the mTOR signaling pathway.** To understand the mechanism of estrogen-induced FTO nuclear localization, we scanned the signaling pathways which may be involve in this event. As shown in Fig. 3A, estrogen increased the phosphorylation of mTOR in a dose-dependent manner. Pretreatment with rapamycin potently blocked the estrogen-activated mTOR signaling pathway (Fig. 3B). After combined treatment with estrogen and rapamycin, we found that estrogen-induced FTO nuclear localization was attenuated. The FTO expression pattern in the nucleus following estrogen plus rapamycin treatment was lower than that with estrogen stimulation alone (Fig. 3C and D).
ERα is required for estrogen-induced FTO nuclear localization. ERα plays an important role in estrogen-mediated bio-functions. Therefore, we investigated the effect of ERα on estrogen-induced FTO nuclear localization. As shown in Fig. 4A, acute transfection of siERα resulted in a marked decrease in ERα protein, which had no effect on FTO nuclear expression. However, estrogen-induced FTO expression in the nucleus was blocked by deletion of ERα (Fig. 4B). Immunocytochemistry assay demonstrated that estrogen stimulation accumulated FTO protein in the nucleus, however, the increased FTO protein dot in the nucleus was abolished by 1 µM fulvestrant (ICI 182,780; purchased from Sigma-Aldrich) treatment, a selective estrogen receptor antagonist (Fig. 4C).

These data suggested that ERα is required for estrogen-induced FTO nuclear localization.

Estrogen-induced FTO nuclear localization promotes endometrial cancer cell proliferation. To understand FTO nuclear localization in endometrial cancer proliferation, we carried out an MTT assay. As shown in Fig. 5, pretreatment with ICI, to block the ERα signaling pathway, attenuated Ishikawa cell proliferative activity. Moreover, ICI treatment also blocked estrogen-induced cell growth. Similarly, blocking the mTOR signaling pathway with rapamycin also abolished the estrogen-stimulated cell proliferation. Targeting FTO by siFTO directly resulted in decreases proliferative activity, whereas estrogen treatment could not rescue the cellular growth.

Discussion

In the present study, we found that the fat mass and obesity-associated (FTO) gene was overexpressed in endometrial carcinoma tissues and estrogen induced FTO nuclear localization, which facilitated endometrial cancer proliferation through the mTOR signaling pathway.

Most endometrial adenocarcinomas are characterized by positive nuclear estrogen receptor (ER) expression and responsiveness to hormone stimulation. Increasing evidence indicates that prolonged estrogen exposure is associated with initiation of type 1 endometrioid cancers (29-31). Estrogen exposure was found to result in an overall physiological response within several hours by a genomic mechanism which depends on estrogen binding to nuclear ER resulting in mRNA transcription and protein synthesis of target genes. Obesity is a well-established risk factor for endometrial cancer since obesity in post-menopausal women has been shown to increase circulating estrogen levels by upregulating the expression of aromatase and enhancing aromatization of androstenedione in
Adipose tissue. We documented that the FTO gene is involved in estrogen-driven endometrial cancer development. However, the detail molecular mechanism remains to be clarified.

An investigation with a larger sample size confirmed that the obesity-associated polymorphism FTO rs9939609 is strongly associated with endometrial cancer risk in non-Hispanic white women (13). In the present study, we re-evaluated the expression of FTO in endometrial cancer and the association with prognosis. The higher expression in endometrial carcinoma tissues was observed compared with the normal endometrial tissues. In our previous study, we found that there is no association between FTO expression and age, stage, grade, invasion and lymph node metastasis (7). Notably, we found that higher FTO expression was related with poor prognosis and early relapse. These data imply that FTO plays an important role in endometrial cancer development. It was observed that estrogen stimulation enhanced FTO protein accumulation in endometrial cancer cell nuclei. Yet, limited information is available concerning its mechanism and its effect on cellular growth. Consistent with our previous study (7), estrogen upregulated FTO protein in a dose- and time-dependent manner (Fig. 2A and B). We further isolated the nuclear and cytosolic proteins and found that in fact estrogen did not increase the FTO protein level in the cytoplasm, whereas elevated FTO protein by estrogen in the nucleus was observed. It is known that protein is synthesized in the cytoplasm, but we did not detect any alteration in FTO protein in the cytoplasm after estrogen treatment, which suggests that estrogen induces FTO to transfer into the nucleus.

A previous study demonstrated that mTOR signaling is involved in estrogen-driven endometrial cancer development (32). In the present study, we provided evidence that mTOR signaling controls estrogen-induced FTO protein nuclear localization. As shown in Fig. 3A and B, estrogen activated mTOR signaling and this activation was inhibited by rapamycin, an mTOR specific inhibitor. Most importantly, rapamycin blocked the accumulation of FTO in the nucleus with or without estrogen stimulation (Fig. 3C and D). These data suggest that activation of mTOR signaling is necessary for FTO nuclear localization.

Given that ERα mediated estrogen-induced multiple functions, we further investigated the role of ERα in FTO nuclear localization. In the present study, we found that knockdown of ERα had a slight effect on FTO protein nuclear localization. However, deletion of ERα blocked estrogen-induced FTO nuclear accumulation. We considered that ERα has no effect on FTO expression, but estrogen-mediated function is ERα-dependent. Therefore, depletion of ERα resulted in the inability of estrogen to bind with ERα, in turn leading to estrogen-driven FTO nuclear accumulation failure. These data imply that ERα is required for FTO nuclear localization. Although we gathered significant data in the present study, to the best of my knowledge, ERα was also located in the nucleus and served as a transcription factor. Yet, we still raised the question of how ERα mediates FTO nuclear accumulation. We hypothesized that ERα may recruit some molecules and construct a protein complex, which in turn helps FTO to transfer into the nucleus. Various proteins have different functions when they present in different cellular localizations, such as caveolin. In the present study, we found that FTO nuclear localization promoted cell proliferation, whereas blocking the nuclear accumulation by ICI or Rap pretreatment, even by direct deletion of FTO by siRNA, resulted in attenuated proliferative activity. The decreased proliferation could not be rescued by estrogen stimulation.

In conclusion, the present study suggests that overexpression of FTO in endometrial carcinoma may be a poor prognostic marker. Importantly, FTO nuclear accumulation may be an essential step for estrogen-driven endometrial tumor formation and progression. Our findings may provide new insight into the mechanisms underlying E2-induced proliferation. Additionally, the present study further supports the possibility of using FTO as a target for the treatment of endometrial cancer.

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (NSFC no. 81372795).

References


