

Oleic acid promotes the development of endometrial cancer by up-regulating KLF4 expression

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Summary

Objective: To investigate the role and possible molecular mechanism of the transcription factor Krüppel-like factor 4 (KLF4) in the biological phenotype of endometrial cancer cells following induction by oleic acid (OA). **Materials and Methods:** Ishikawa endometrial cancer cells were grown *in vitro* with different concentrations of oleic acid. Cell proliferation was measured using the Counting Kit-8 (CCK-8) assay. Cell migration and invasion were evaluated by transwell chamber assay and scratch assay, respectively. The mRNA and protein expression levels of KLF4, ER α , Bcl₂, MMP1, MMP9, were evaluated by qRT-PCR and Western blot, respectively. **Results:** The expression levels of KLF4, ER α , Bcl₂, MMP1 and MMP9 increased significantly ($p < 0.05$) upon stimulation of the endometrial cancer cells with oleic acid (200 μ M), together with the migration and invasion abilities of the cells ($p < 0.05$). Up-regulation of KLF4 caused the expression of ER α , Bcl₂ and MMP9 to increase significantly ($p < 0.05$), whereas down-regulation of KLF4 caused the expression of ER α , Bcl₂, MMP1 and MMP9 to significantly decrease ($p < 0.05$). The increased expression levels of ER α , Bcl₂, MMP1 and MMP9 upon 200 μ M oleic acid stimulation were significantly blocked ($p < 0.05$) by down-regulation of KLF4. **Conclusion:** oleic acid promotes the expression of ER α , Bcl₂, MMP1 and MMP9 via up-regulation of KLF4, thus resulting in increased migration and invasion abilities of endometrial cancer cells.

Key words: OA; KLF4; Endometrial cancer; Migration; Invasion.

Introduction

Endometrial cancer accounts for 7% of female malignancies [1] and is the most common gynecological malignant tumor in developed countries such as the United States. It currently accounts for almost 50% of new gynecologic malignancies [2]. Because there is no early diagnosis method for endometrial cancer, many patients are diagnosed with a late stage of the disease. Therefore, early diagnosis is an important way to improve the prognosis of endometrial cancer. It has been reported that screening of women in the general population does not reduce the mortality rate from endometrial cancer [3]. The aim of the present study was to clarify possible mechanisms for one of the major risk factors associated with the development of endometrial cancer, thus providing a theoretical basis for improved diagnosis and prevention of this disease.

Obesity is a major risk factor for endometrial cancer and can lead to increased volume and quantity of fat cells, accumulation of visceral fat and enhanced lipolysis. The latter can increase the levels of free fatty acids (FFA) in plasma, eventually leading to lipid metabolism disorders [4]. FFA can act as a raw material for energy metabolism and as a signal transduction medium, resulting in the development of obesity-related tumors [5, 6]. FFA can be divided into saturated fatty acids and unsaturated fatty acids. Oleic acid (OA) is one of the most abundant monounsaturated FFA released into the serum by adipose tissue. It has been reported that OA promotes an aggressive phenotype in prostate can-

cer cells via FFA1/GPR40, calcium and PI3K/Akt signaling [7]. Dietary OA-induced CD36 promotes cervical cancer cell growth and metastasis via up-regulation of the Src/ERK pathway [8]. However, the role and mechanism of OA in obesity-related endometrial cancer has so far not been reported in the literature.

Kruppel-like factors (KLFs) are a transcription factor family composed of 17 members with a zinc finger structure. They are widely involved in cell proliferation, differentiation and the regulation of embryonic development. KLF4 was originally identified in the gastrointestinal tract and is one of the transcriptional regulation factors for adipocyte differentiation [9, 10]. It has been shown that KLF4 plays a dual role in promoting and suppressing cancer by regulating oncogenes or tumor suppressor genes, depending on the type of tumor and the stage of development [11]. The association between KLF4 and gynecologic tumors has been investigated in recent years. The expression of KLF4 in breast cancer tissue was significantly lower compared to normal tissue, resulting in low expression of the downstream target genes caspase-3 and Bax, and inhibition of tumor cell apoptosis [12]. Whether KLF4 is associated with the development of obesity-related endometrial carcinoma has not yet been reported. Therefore, the aim of this study was to investigate whether OA could be linked to the development of endometrial cancer through up-regulation of KLF4.

Table 1. — Primer sequences

Primer name	Sequence (5'→3')	Size (bp)
Human-KLF4-F	GGCACTACCGTAAACACACGC	140
Human-KLF4-R	CTGGCAGTGTGGGTCATATC	
Human-ER α -F	TGTGCAATGACTATGCTTCA	149
Human-ER α -R	GCTCTTCCTCCTGTTTTTA	
Human-Bcl $_2$ -F	TTCTTTGAGTTCGGTGGGGTC	304
Human-Bcl $_2$ -R	TGCATATTTGTTTGGGGCAGG	
Human-MMP1-F	AATGTGCTACACGGATACCC	214
Human-MMP1-R	CTTTGTGGCCAATTCCAGGA	
Human-MMP9-F	CGGAGTGGCAGGGGAAGATGCTG	256
Human-MMP9-R	GCAGGATGTCATAGGTCACGTAGC	
Human-GAPDH-F	GGTGGTCTCCTCTGACTTCAA	211
Human-GAPDH-R	TCTTCCTCTTGTGCTCTTGCT	

Materials and Methods

The human endometrial cancer cell line (Ishikawa) was purchased from Institute of basic medicine, Chinese Academy of Medical Sciences. Cells were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G sodium and 100 μ g/ml streptomycin sulfate at 37 °C in a humidified atmosphere containing 5% CO $_2$.

The overexpression plasmid and the small interfering RNA (siRNA) fragment of KLF4 were constructed by GenePharma company. The overexpression plasmid of mouse KLF4 is pcDNA3.1 (+) KLF4. The negative control plasmid was pcDNA3.1 (+) NC. Three kinds of siRNA fragments were used to inhibit KLF4 expression. The different siRNA sequence were:

- 1) si-1(5'-3'): CCGAGGAGUUCAACGAUCUTT, AGAUC GUUGAACUCCUCGGTT;
- 2) si-2(5'-3'): CCUACACAUGAAGAGGCATT, UGCCU CUUCAUGUGUAAGGTT;
- 3) si-3(5'-3'): CAGCCAGAAAGCACUACAATT, UUGUA GUGCUUUCUGGCUGTT;

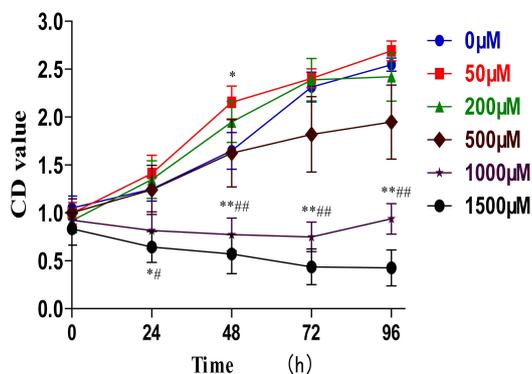


Figure 1. — The growth rate of Ishikawa cells was evaluated under different concentrations of OA. *50 μ M, 1000 μ M stimulation group vs. blank control group, #1500 μ M vs. blank control group, *,# p < 0.05, **,### p < 0.01.

4) The negative control siRNA sequence (si-NC) (5'-3') is: UUCUCCGAACGUGU-CACGUTT,ACGUGACACGUUCGGAGAATT.

The plasmid or siRNA was transfected into Ishikawa cells by using Lipofectamine 2000.

Ishikawa cells in the logarithmic growth phase were seeded into 96-well plates (1×10^4 cells/well) and after 24 h growth were stimulated by OA. They were divided into OA-stimulated group, blank control group, and cell-free control. Each group had four replicate wells. The cells were stimulated with OA for 24, 48, 72 and 96 h. Ten μ L of CCK8 solution was then added to each well and the cells placed in a 5% CO $_2$ incubator at 37 °C for 2 h. The absorbance (CD value) at 450 nm was detected by a microplate reader, allowing the growth curve of the different experimental groups of cells to be plotted continuously for five days.

Cell migration assays were performed in 24-well plates with 8 μ m poresized transwell chambers. The Ishikawa cells were grown overnight in serum-free medium. A total of 2×10^4 cells in serum-free medium were added to the upper chamber. RPMI1640 medium containing 20% FBS was added to the lower chamber and the cells were cultured for 24 h to observe migration. Migrated cells were stained with crystal violet for 15 minutes at room temperature. The stain was then removed from the chambers and the cells washed three times with phosphate buffer solution (PBS). Cells on the upper chamber of the membrane were scraped off using a cotton swab. The experiments were performed in triplicate for all conditions described. Cell migrations were evaluated using a microscope at 200-fold magnification.

Cell invasion assays were performed using a Bio-Coat Matrigel Invasion Chamber (Corning, Bedford, MA). The Ishikawa cells were grown overnight in serum-free medium. A total of 2×10^4 cells in serum-free medium were then added to the top invasion chambers of 24-well transwell plates containing a cell culture insert with an 8 μ m pore size. RPMI1640 (20% FBS) was added to the lower

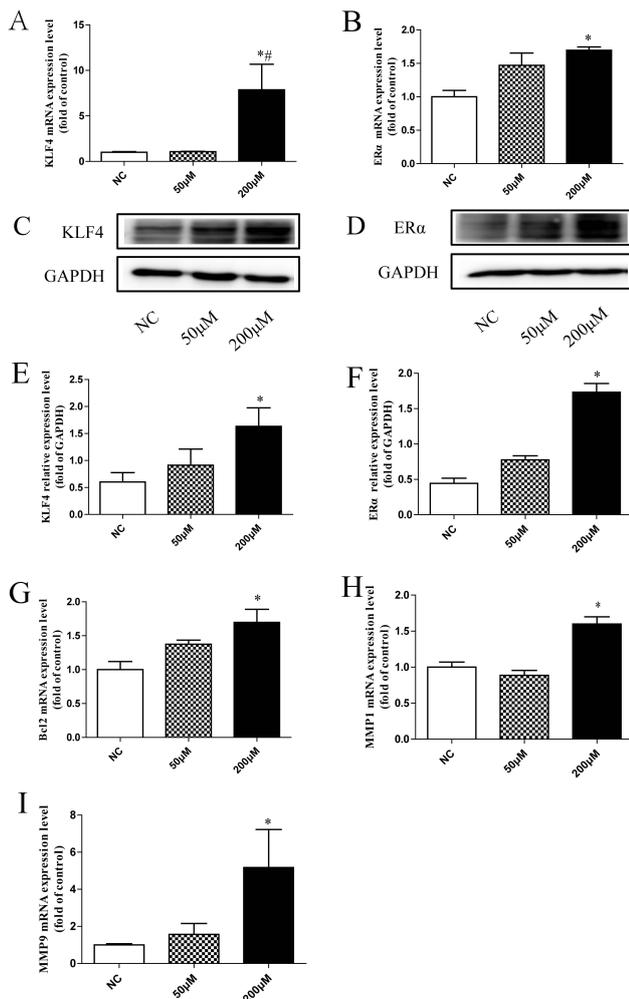


Figure 2. — OA stimulation of Ishikawa cells and its effects on cell migration, invasion and proliferation ability. A) 50 μM and 200 μM OA-stimulated Ishikawa cells for 0, 24, 48, 72, and 96 h. CCK-8 assay was used to assess cell proliferation. B, C) 50 μM and 200 μM OA-stimulated Ishikawa cells incubated for 0, 24, and 48 h. The area of scratch healing was assessed with a $\times 100$ microscope. D, E) Transwell migration experiment with 50 μM and 200 μM OA-stimulated Ishikawa cells grown for 24 h, $\times 200$ microscope; F, G) Transwell invasion test with 50 μM and 200 μM OA-stimulated Ishikawa cells grown for 48 h, $\times 200$ microscope; *NC vs. 50 μM ; #NC vs. 200 μM ; *, # $p < 0.05$, ** , ## $p < 0.01$.

chamber. Cells were grown in the culture insert for 48 h in order to observe invasion. The procedure after the crystal violet staining was the same as for the cell migration assay. Cytotoxic effects on the cells at concentrations above 500 μM . Therefore, 50 μM and 200 μM OA were selected for subsequent experiments.

After treatment with 50 μM OA for 48 h, the cell proliferation rate was significantly higher than that of the control group ($p < 0.05$) (Figure 2A). Following treatment with 50 μM and 200 μM OA for 24 h and 48 h, the area of scratch healing was measured. Healing in the 48 h group was sig-

nificantly better than in the control group ($p < 0.05$) (Figure 2B, 2C). After treatment with 50 μM and 200 μM OA for 24 h, cell migration was also significantly greater than in the control group ($p < 0.01$) (Figure 2D, 2E). After 50 μM and 200 μM OA treatment of Ishikawa cells for 48 h, cell invasion was significantly higher than in the control group ($p < 0.01$) (Figure 2F, 2G).

The expression levels of KLF4, ER α , MMP1 and MMP9 were evaluated after stimulation of Ishikawa cells for 24 h with 50 μM and 200 μM OA. The mRNA and protein expression levels of KLF4 and ER α in the 200 μM treatment group were found to be significantly higher than in the control group (Figures 3A-F).

Moreover, the mRNA expression levels of Bcl $_2$, MMP1 and MMP9 were significantly higher in the 200 μM OA treatment group compared to the controls (Figure 3G-I). These differences were all statistically significant ($p < 0.05$).

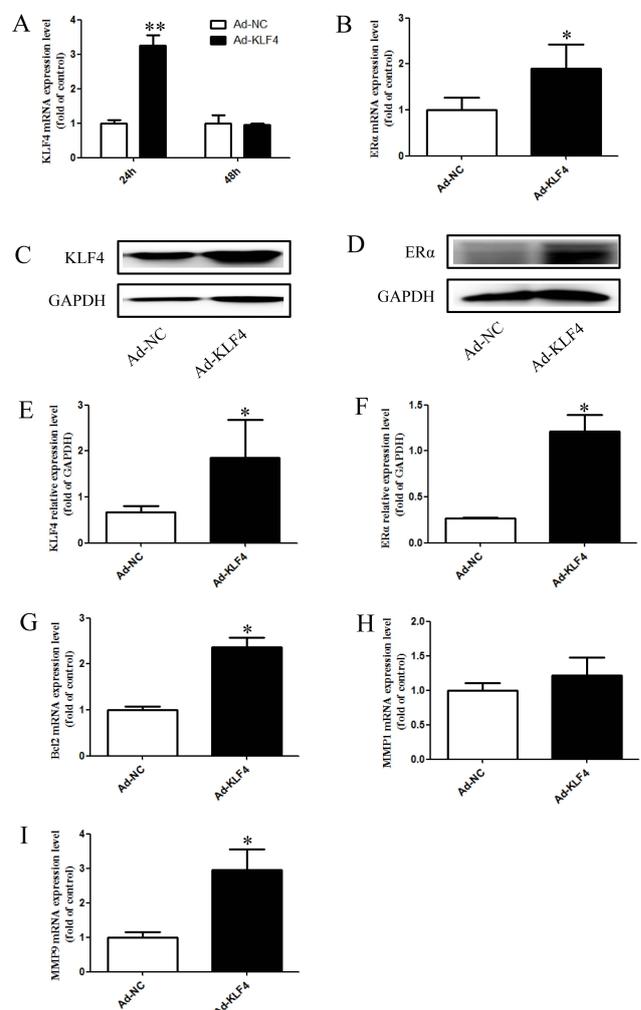


Figure 3. — Expression levels of ER α , Bcl $_2$, MMP1 and MMP9 in Ishikawa cells following stimulation with 50 μM and 200 μM OA for 24 h. A, B, G, H and I show mRNA expression. C, D, E and F show protein expression. *NC vs. 200 μM ; * $p < 0.05$.

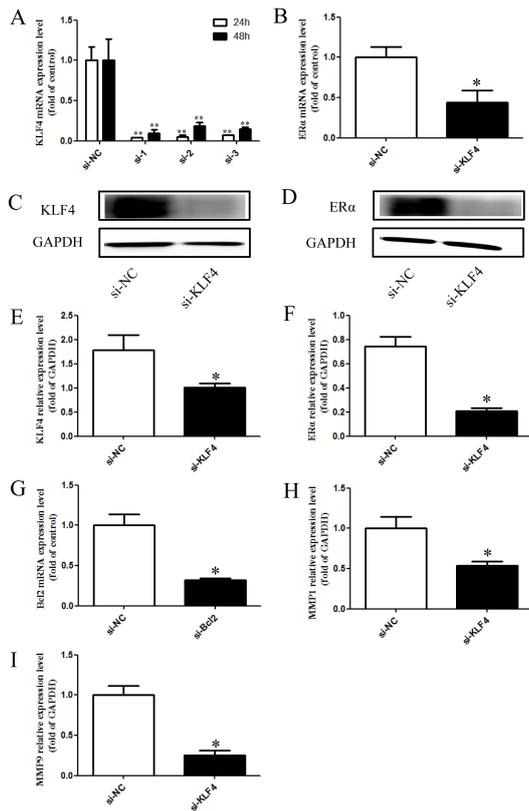


Figure 4. — Expression levels of ER α , Bcl $_2$, MMP1 and MMP9 following up-regulation of KLF4. A, B, G, H and I show mRNA expression. C, D, E and F show protein expression. *experimental group vs. control group; * $p < 0.05$.

Following transfection of a KLF4 overexpression plasmid into Ishikawa cells, the mRNA and protein levels for KLF4 increased significantly in the 24 h treatment group ($p < 0.05$) but not in the 48 h group ($p > 0.05$) (Figures 4A, 4C, 4E). Coincident with this up-regulation of KLF4 at 24 h, the mRNA and protein levels of ER α increased significantly ($p < 0.05$) (Figures 4B, 4D, 4F), while the mRNA levels of Bcl $_2$ and MMP9 also increased significantly ($p < 0.05$) (Figures 4G, 4I). The mRNA level of MMP1 increased but this was not significantly different ($p > 0.05$) (Figure 4H).

Three different KLF4 interference fragments (si-1, si-2, si-3) were transfected into Ishikawa cells for 24 h and 48 h. This resulted in significantly lower mRNA expression of KLF4 compared to controls ($p < 0.01$) (Figure 5A). The mRNA expression level was lowest when the interference fragment was si-1 and the transfection time was for 24 h.

A scratch wound healing assay was also performed to evaluate the migration ability of Ishikawa endometrial cancer cells. Briefly, cells were seeded into six-well plates at a density of 2×10^5 /well and grown until they reached 80% confluence. The scratch wounds were created in the monolayer of near confluent cells with a pipette tip. The width of the wounds was assessed to be the same at the beginning of the experiments. The wells were rinsed with PBS

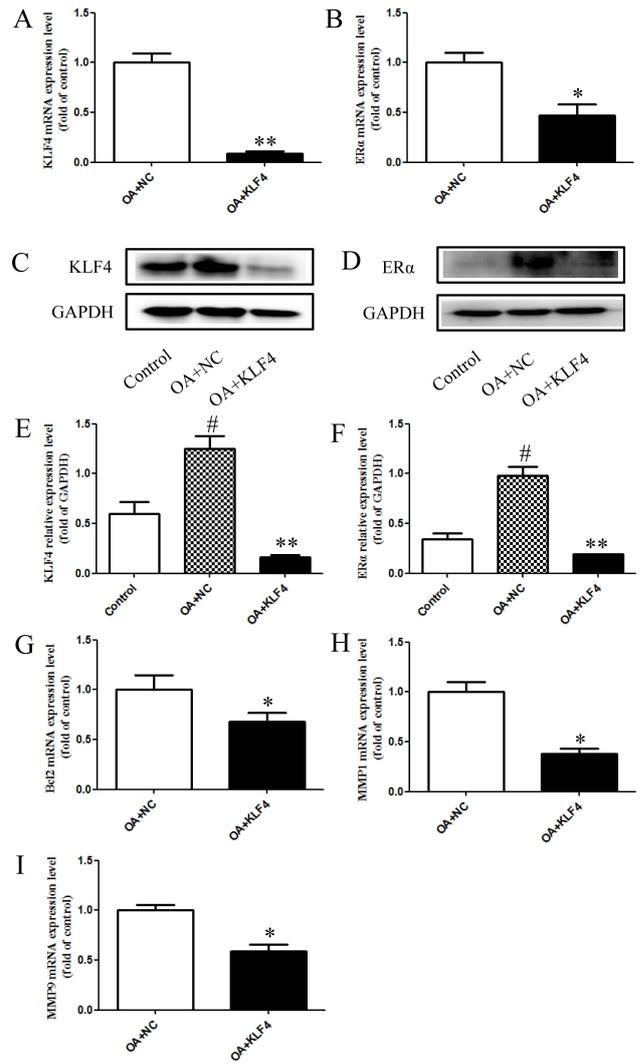


Figure 5. — Expression levels of ER α and oncogene following down-regulation of KLF4. A, B, G, H and I show mRNA expression. C, D, E and F show protein expression. *experimental group vs. control group, * $p < 0.05$, ** $p < 0.01$.

three times to remove floating cells and debris and the adherent cells treated according to different conditions. The cells were then incubated for 0–48 h at 37 °C and in 5% CO $_2$. Wound healing was measured and recorded photographically using phase-contrast microscopy at 0, 24 and 48 h.

Proteins were extracted from treated cells using the RIPA lysis buffer method and quantified using the BCA protein assay kit. Extracted proteins were loaded onto 10% SDS-polyacrylamide gels and electrophoresed fully. Subsequently, the separated proteins were transferred to a PVDF membrane using a wet transfer method. The membrane was blocked with 5% fat-free milk for 2–3 h and subsequently incubated with primary antibody (1 : 1,000 dilution) at 4 °C overnight. The membrane was then washed three times (45 minutes in total) with TBS-Tween buffer and incubated with secondary antibody (1 : 10,000 dilution) for 2 h at

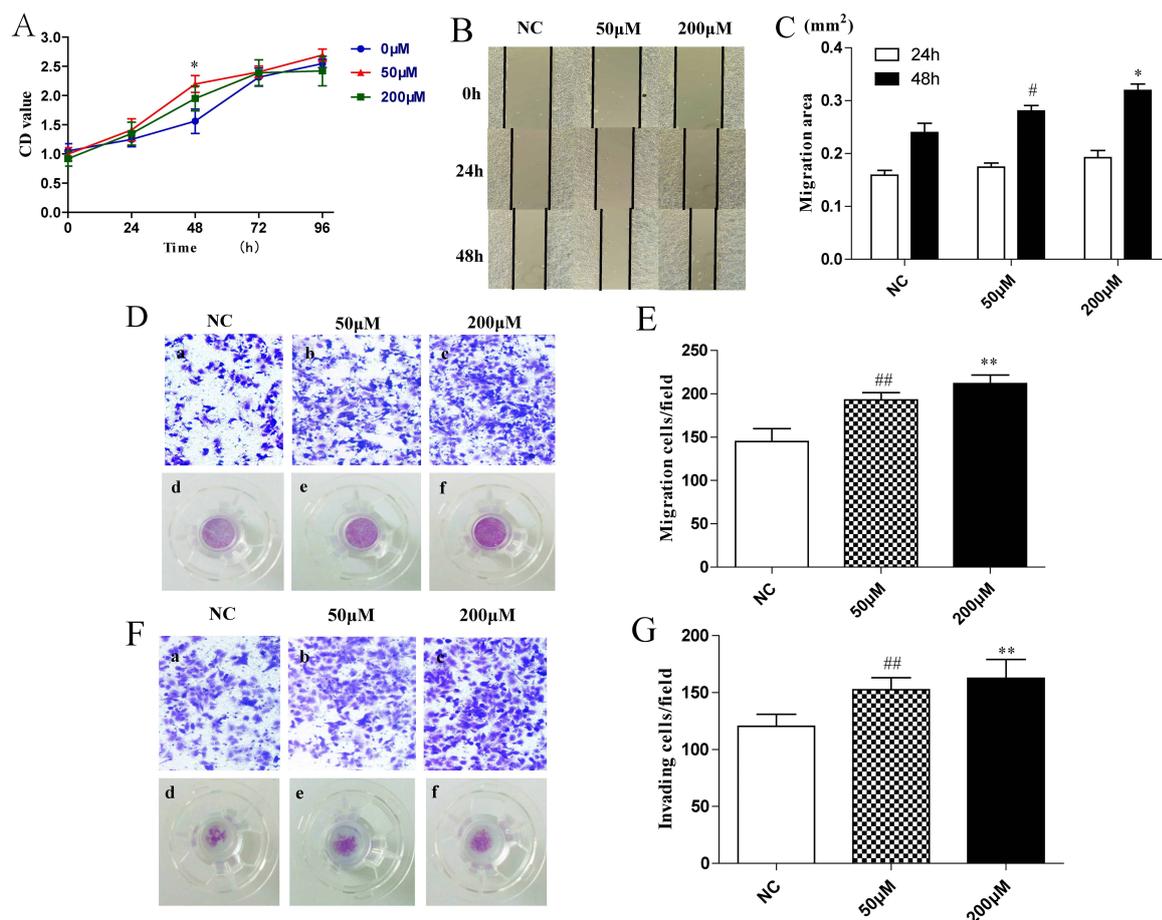


Figure 6. — ER α and oncogene expression following simultaneous OA stimulation and down-regulation of KLF4. A, B, G, H, and I show mRNA expression. D, E, and F show protein expression. *OA + NC vs. OA + KLF4; * $p < 0.05$, ** $p < 0.01$.

room temperature. After washing three times with TBS-Tween buffer, the protein level was detected with the enhanced chemiluminescence (ECL) system. The primary antibodies used were as follows: anti-GAPDH 36 kDa, anti-KLF4 62 kDa, anti-ER α 62 kDa.

Total RNA was extracted using Trizol according to the manufacturer's protocol. Complementary DNA was then synthesized using the Thermo Scientific TM Maxima TM RT reagent kit. Quantitative RT-PCR (qRT-PCR) was performed using SYBR Premix Ex Taq on an ABI 7900PRISM PCR system. The qRT-PCR procedure was: 95 °C for 5 minutes followed by 45 cycles of 95 °C for 10 seconds, 60 °C for 30 seconds, and 70 °C for 40 seconds. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences used are shown in Table 1.

All statistical analyses were carried out using SPSS 17.0 software. The data was expressed as the mean \pm standard deviation (SD) and analyzed for significance using the Student's *t*-test or one-way analysis of variance. $p < 0.05$ was considered statistically significant.

Institutional review board approval was provided by

the Medical Ethics Committee at the first Affiliated Hospital, Shihezi University School of Medicine (Reference no.2014LL22).

Results

Testing revealed no cytotoxicity at all time points (24, 48, 72, and 96 h) upon stimulation with 50 μ M and 200 μ M OA. However, the growth rate of Ishikawa cells in the 500 μ M OA treatment group was significantly lower than controls after 72 h and 96 h ($p < 0.05$). The growth of cells in the 1000 μ M and 1500 μ M OA groups was significantly lower than the control group at all time points ($p < 0.05$) (Figure 1). These results indicate that OA has KLF4 protein expression was significantly lower after 24 h compared to the si-NC group ($p < 0.05$) and hence the expression of related genes was evaluated under this condition. After down-regulating KLF4 for 24 h, both the mRNA and protein levels of ER α were significantly decreased ($p < 0.05$) (Figures 5B-F). In addition, the mRNA levels of Bcl₂, MMP1 and MMP9 were also significantly lower ($p < 0.05$) (Figures 5 G-I).

Under the condition of OA stimulation with coincident

down-regulation of KLF4 expression for 24 h, the mRNA and protein expression levels of both KLF4 and ER α were significantly lower than in the OA-stimulated group (Figures 6A-F). The mRNA expression levels of Bcl₂, MMP1 and MMP9 were also lower than in the OA stimulation group (Figures 6G-I), with all differences being statistically significant ($p < 0.05$).

Discussion

Obesity is caused by excess energy leading to high levels of FFA in plasma [13]. A growing number of studies have shown that FFA plays an important role in the development of cancer. For example, it has been reported the total FFA level in the serum of breast cancer patients is significantly higher than in non-cancer individuals [14]. Increased FFA in the plasma caused by diet is closely related to the occurrence of endometrial cancer [15]. Excessive FFA is essential for tumor cell growth and proliferation as an energy source and as a metabolically regulated signaling molecule for cell growth and gene expression [16, 17]. A decreased n-6/n-3 fatty acid ratio reduces the invasive potential of human lung cancer cells by down-regulating the expression of cell adhesion- and invasion-related genes such as MMP-1, integrin-alpha 2 and nm23-H4 [18]. In breast cancer cells, docosahexaenoic acid induces apoptosis via a transient increase in caspase-3 activity and the promotion of nuclear condensation [19].

OA is the most common mono-unsaturated fatty acid [20] and has been reported to promote the development of prostate and cervical cancers [7, 8]. Interestingly however, OA may have a protective role against bladder carcinogenesis [21]. It is not yet clear whether OA has a repressor or promotor effect for endometrial tumorigenesis. Therefore, in the present study we investigated the effect of OA on Ishikawa endometrial cancer cells *in vitro*. Our results showed that high concentrations of OA could promote the expression of ER α , Bcl₂, MMP1 and MMP9, as well as promoting the proliferation, migration and invasion of endometrial cancer cells. These results suggest the unsaturated fatty acid OA favors the development of endometrial cancer, however the specific mechanism of action is still unclear.

The transcription factor KLF4 plays an important role in regulating cell proliferation, differentiation and apoptosis [22]. KLF4 shows anti-cancer effects in some malignant tumors, including gastric, colorectal, bladder, lung and prostate cancers [23-27]. On the other hand, KLF4 can promote the development of skin squamous cell carcinoma, laryngeal squamous cell carcinoma and breast cancer [28]. The role of KLF4 in obesity-related endometrial cancer has not been reported to our knowledge. The present results showed that up-regulation of KLF4 could promote the expression of ER α , Bcl₂, MMP1 and MMP9. In contrast, down-regulation of KLF4 inhibited the expression of these proteins, all of which have a role in tumorigenesis.

It has been reported that high concentrations of the sat-

urated fatty acid palmitic acid has an inhibitory effect on the expression of KLF4 in adipocytes [29]. The effects of the unsaturated fatty acid OA on the expression of KLF4 has yet to be reported in the literature. The present results showed that high concentrations of OA could promote the expression of KLF4 in endometrial cancer cells. Moreover, OA stimulation of endometrial cancer cells with simultaneous down-regulation of KLF4 caused a significant decrease in the expression levels of ER α and Bcl₂, MMP1 and MMP9. This result suggests the unsaturated fatty acid OA promotes the development of endometrial cancer by up-regulating KLF4.

The mechanism by which OA up-regulates KLF4 expression has yet to be reported. Published studies have shown that FFA could affect the development of cancer via activation of G-protein coupled receptor (GPR) family members on the cell surface [30]. GPR family members include GPR40, GPR43, GPR41, GPR120 and GPR84. GPR41 and GPR43 are activated by short-chain FFA, while GPR40 and GPR120 are activated by medium and long-chain FFA [31]. Studies have shown that GPR40 and GPR120 can promote the proliferation and migration of breast cancer, prostate cancer, melanoma, pancreatic cancer and colon cancer cells [32-36]. The aforementioned studies indicate that FFAs could activate specific membrane receptor GPRs, thereby acting as extracellular signal molecules that favor cancer development. Whether OA promotion of KLF4 expression favors the development of endometrial cancer by regulating GPR receptors needs further investigation.

In conclusion, the present study showed the unsaturated fatty acid OA could promote the development of endometrial cancer by up-regulating the expression of KLF4.

Author contributions

Feng and J. Wu conducted the experiment. J. Zhang and C. Wang conceived and designed experimental method. J. Feng, J. Wu and M. Zhang analyzed data. J. Feng, J. Wu and C. Wang contributed writing—original draft preparation. J. Wang, K. Chen and X. Li contributed writing—review and editing. J. Xie, J. Zhang and C. Wang conducted supervision. All authors read and approved the manuscript.

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Conflicts of interest

The authors reports no conflicts of interest in the work.

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