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Original Paper

High Glucose Promotes Epithelial-Mesenchymal Transition of Uterus Endometrial Cancer Cells by Increasing ER/GLUT4-Mediated VEGF Secretion

Chun-Jie Gu[a,e] Feng Xie[b] Bing Zhang[a] Hui-Li Yang[a] Jiao Cheng[a] Yin-Yan He[c] Xiao-Yong Zhu[d] Da-Jin Li[a] Ming-Qing Li[a]

[a]NHC Key Lab of Reproduction Regulation (Shanghai Institute of Planned Parenthood Research), Hospital of Obstetrics and Gynecology, Fudan University, Shanghai, [b]Medical Center of Diagnosis and Treatment for Cervical Diseases, Hospital of Obstetrics and Gynecology, Fudan University, Shanghai, [c]Department of Obstetrics and Gynecology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, [d]Department of Gynecology, Hospital of Obstetrics and Gynecology, Fudan University, Shanghai, [e]Department of Obstetrics and Gynecology, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Key Words
High glucose • Uterus endometrial cancer • GLUT4 • epithelial-mesenchymal transition • Estrogen • Estrogen receptor • VEGF

Abstract
Background/Aims: Uterus endometrial cancer (UEC) is the common malignancy among gynecologic cancers, and most of them are type I estrogen-dependent UEC. Diabetes is well-known risk factor for the development of UEC. However, the underlying link between high glucose (HG) and the estrogen receptor in UEC remains unclear. Epithelial-mesenchymal transition (EMT) has also been shown to occur during the initiation of metastasis in cancer progression. The aim of this study was to determine the relationships and roles of HG, estrogen receptor and EMT in the growth and migration of UEC. ***Methods:*** The expression of glucose transport protein 4 (GLUT4) in the control endometrium and UEC tissues was detected by immunohistochemistry (IHC); the cell viability and invasion were analyzed through CCK-8 and Matrigel invasion assays; the transcriptional level of EMT-related genes was evaluated through real-time PCR; and the effect of HG and / or GLUT4 on estrogen receptors, vascular endothelial growth factor (VEGF) and its receptor VEGFR was analyzed through western blotting, ELISA and flow cytometry (FCM) assay, respectively. In addition, Ishikawa-xenografted nude mice were constructed and were used to analyze the effect of estrogen and GLUT4 on the growth of UEC *in vivo*. ***Results:*** Here, we found that exposure to HG led to a high level of viability

C.-J. Gu and F. Xie contributed equally to this work.

Ming-Qing Li NHC Key Lab of Reproduction Regulation (Shanghai Institute of Planned Parenthood Research), Hospital of Obstetrics and Gynecology, Fudan University, Shanghai 200011 (China)
Tel. 86-21-63457331, Fax 86-21-63457331, E-Mail mqli@fudan.edu.cn

and invasion of UEC cell lines (UECC, Ishikawa and RL95-2 cells). Compared with the normal endometrium, a higher level of GLUT4 was observed in UEC tissues. Silencing GLUT4 obviously inhibited the HG-promoted viability, invasion and expression of EMT-related genes (*TWIST*, *SNAIL* and *CTNNB1*) of UECC promoted by HG. Further analysis showed that HG and GLUT4 promoted the secretion of VEGF and expression of VEGFR in UECC. Treatment with HG led to the increase of estrogen receptor α (ERα) and β (ERβ) in UECC, blocking ERα or ERβ resulted in the decreases in GLUT4 expression, *TWIST*, *SNAIL* and *CTNNB1* transcription, and VEGF and VEGFR expression in UECC. Treatment with anti-human VEGF neutralizing antibody restricted the viability and invasion of UECC that was induced by HG and estrogen. Exposure to estrogen accelerated growth, VEGF production, and *TWIST* and *CTNNB1* expression in UEC in Ishikawa-xenografted nude mice, and silencing GLUT4 restricted these effects. ***Conclusion:*** These data suggest that HG increases GLUT4 and VEGF/VEGFR expression, further promotes EMT process and accelerates the development of UEC by up-regulating ER.

Introduction

Uterus endometrial carcinoma (UEC) is the most common malignancy among gynecologic cancers, and the incidence of UEC has been rising (a 2.3% annual increase) [1]. Estrogen controls various physiological and disease processes, especially reproduction, bone remodeling, and development of gynecologic cancer [2]. This increased occurrence of UEC has been attributed to the most common subtype of UEC, that is, type 1 estrogen-dependent UEC, which is comprised of grades 1 and 2 endometrioid adenocarcinoma [3, 4]. Estrogen receptors (ER) belong to the nuclear receptor superfamily, including estrogen receptor alpha (ERα), estrogen receptor beta (ERβ) and, estrogen-related receptors (ERRα, β, and γ) [2, 5, 6]. Estrogen triggers the proliferation, invasion and epithelial-mesenchymal transition (EMT) event of uterus endometrial cancer cells (UECC) and contributes to the pathogenesis and progression of UEC [7, 8].

Diabetes has been shown to independently increase the risk of endometrial cancer [8, 9]. As an early symptom of diabetes, an elevated serum glucose level may directly regulate tumor-related signaling pathways, especially meeting the high glucose need of cancer cells [9, 10]; however, little is known regarding the underlying mechanism. In the endometrium, glucose transporter (GLUT) expression and glucose transport are known to fluctuate throughout the menstrual cycle. It has been widely demonstrated that GLUT1 has a positive correlation with tumor aggressiveness in type I UEC, such as myometrial invasion or tumor grade [11]. However, the expression and role of GLUT4 in UEC are largely unknown.

During the tumor progression, EMT is associated with the loss of adherence junctions, tissue and organ morphological changes, and enhanced migratory and invasive capabilities of cancer cells [12, 13]. Recently, Rahn *et al*. reported that hyperglycemia promotes EMT and stem cell properties in pancreatic ductal epithelial cells [14]. However, it remains unknown whether high glucose (HG) is involved in regulating EMT of UECC.

Therefore, the aim of this study was to investigate the relationship between high glucose (HG), GLUT4 and estrogen, and the role and mechanism of these factors in EMT of UECC.

Materials and Methods

Patients and tissue samples

The protocol for this research was approved by the Human Research Ethics Committee of Obstetrics and Gynecology Hospital, Fudan University, and written informed consent was obtained from all participants. All the UEC tissues (10 cases) were obtained via laparoscopy from patients (mean age 44.3 years; range 35-57 years) at the Obstetrics and Gynecology Hospital of Fudan University. Normal endometrium was obtained through hysterectomy from patients with leiomyoma (10 cases) as normal control samples. All samples

were confirmed histologically. No patient received hormonal therapy or took any medications within six months prior to surgery.

Cell culture

The human endometrial cancer cell lines Ishikawa and RL95-2 cells were obtained from the cell bank of Chinese Academy of Science (Shanghai, China). Molecular authentication of each cell line was performed by Genetic Testing Biotechnology Corporation (Suzhou, China), and the passage numbers used for the experiments were within 15. Ishikawa was grown in RPMI-1640 medium (Gibco, USA) that was supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/mL penicillin and 100 mg/mL streptomycin. The RL95-2 cells were grown in DMEM/F12 (Gibco) that was supplemented with 5% FBS (HyClone), 100 U/mL penicillin, and 100 mg/mL streptomycin.

HG treatment

For HG treatment trials, the glucose concentration of the control medium was approximately 5 mmol/L. In the HG medium group, additional D-glucose (Sigma, Germany) was dissolved in normal medium up to a glucose concentration of 25 mmol/L.

IHC

Paraffin sections (5 uM) of normal endometrium (n=10) and UEC tissues (n=10) from the patients were dehydrated in graded ethanol and were then incubated with 3% hydrogen peroxide (H_2O_2) and 1% bovine serum albumin (BSA)/TBS to block endogenous peroxidase. The samples were then incubated with rabbit anti-human GLUT4 (1:500, Abcam, USA) or rabbit IgG isotype overnight at 4°C in a humid chamber. After washing three times with TBS wash buffer, the sections were overlaid with peroxidase-conjugated goat anti-rabbit IgG, and the reaction was developed using 3, 3-diaminobenzidine (DAB), and the sections were counterstained with hematoxylin.

Transfection

To obtain GLUT4 silenced Ishikawa and RL95-2 cells, the cells were transfected with an empty vector (NC) and *SLC2A4*/GLUT4-silenced (GLUT4i) plasmids (Genechem, Shanghai, China). For RNA interference studies, the Ishikawa and RL95-2 cells were transfected with predesigned small interfering RNAs (siRNAs). The siRNA sequences were as follows: *SLC2A4* sense strand: 5'-CCTTCTTAAGAGTACCTGAAA-3' and scrambled siRNA 5'-TTCTCCGAACGTGTCACGT-3', which did not target any gene, was used as the NC. The siRNA transfections were performed using Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. At 48 h post transfection, the cells were lysed and western blot analyses were performed.

Cell viability and invasion assays

Ishikawa and RL95-2 cells were cultured in the control medium or HG medium, transfected with NC or GLUT4i plasmids, and treated with 17β-estrogen (E_2, 10^{-7}M, Sigma-Aldrich Co. LLC., USA), recombinant human VEGF protein (rhVEGF, 10ng/ml, Peprotech, USA), anti-human VEGF neutralizing antibody (Anti-VEGF, 1 ug/ml, Peprotech, USA), HG plus anti-VEGF, or E2 plus anti-VEGF for 48 h. The viability and invasion of the Ishikawa and RL95-2 cells were analyzed via the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan) and Matrigel (BD, USA) invasion assay according to the manufacturer's specifications, as described previously [15].

RNA extraction and Real-time (RT)-PCR

Ishikawa and RL95-2 cells were cultured in the control medium or HG medium and were transfected with NC or GLUT4i plasmids. The cells were treated with the ERα antagonist MPP (10^{-6} M, Tocris Bioscience, Bristol, UK), ERβ antagonist PHTPP (10^{-6} M, Tocris Bioscience) or total ER antagonist-ICI182780 (10^{-6} M, Tocris Bioscience), E_2 (10^{-7}M, Sigma), rhVEGF (10 ng/ml, Peprotech), anti-VEGF (1 ug/ml, Peprotech), HG plus anti-VEGF, or E_2 plus anti-VEGF for 48h. The transcriptional levels of *TWIST, SNAIL*, and *CTNNB1* in these cells were analyzed via RT-PCR. Briefly, these cells were washed three times, and the total RNA was extracted using the TRIzol reagent (Invitrogen, Life Technologies). cDNA was reverse-transcribed from the total RNA using the Prime Script RT reagent Kit (Takara, Dalian, China). Real-time PCR was performed using

SYBR Premix Ex Taq (Takara, Dalian, PR China) and analyzed using an ABI Prism 7900 Fast Sequence Detection System. The primer sequences of these genes are described in Table 1. The fold change in the gene expression of the above genes was calculated using the change in cycle threshold value method (ΔΔ Ct). All values obtained were normalized to the values obtained for *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*).

Table 1. Sequences of primers used in the paper

Name of the primer	Sequence
Human TWIST1 sense primer	5′- GGCCAGGTACATCGACTTCC-3′
Human TWIST1 antisense primer	5′- CATCCTCCAGACCGAGAAGG-3′
Human SNAI1 sense primer	5′- GGCTCCTTCGTCCTTCTCCT-3′
Human SNAI1 antisense primer	5′- CTGGAGATCCTTGGCCTCAG-3′
Human CTNNB1 sense primer	5′- AGAAGGTCCGAGTGCTGCTC-3′
Human CTNNB1 antisense primer	5′- CTGAGCTGGCTGTTGACCAC-3′
Human GAPDH sense primer	5′- AGGTCCACCACTGACACGTT-3′
Human GAPDH antisense primer	5′- GCCTCAAGATCATCAGCAAT-3′

FCM assay

Ishikawa and RL95-2 cells were cultured with the control medium or HG medium and were treated with ERα, ERβ or total ER antagonists for 48h. The FCM assay was used to analyze the percentage of Ishikawa and RL95-2 cells that were positive for chemokine (C-C motif) ligand 2 (CCL2) (APC-conjugated anti-human CCL2 Abs, eBiosciences, USA), VEGF (allophycocyanin (APC)-conjugated anti-human VEGF Abs, eBiosciences), interleukin (IL)-6 (PE-Cy7-conjugated anti-human IL-6 Abs, eBiosciences), IL-8 (e-Flour™450- conjugated anti-human IL-8 Abs, eBiosciences), and VEGFR (APC-conjugated anti-human VEGFR Abs, eBiosciences). In parallel, isotype control antibodies (BioLegend) were used as controls. The samples were analyzed in a Beckman Cyan flow cytometer (Becton Dickinson, New York, NY, USA) using the Becton Dickinson CellQuest software (version 7.1; Becton Dickinson). Statistical analysis was conducted by using isotype matched controls as references.

Enzyme-linked immunosorbent assay (ELISA)

After culturing with the control medium or HG medium, or silencing GLUT4, the concentration of VEGF in the supernatants of the Ishikawa and RL95-2 cells was detected using a human VEGF ELISA Kit (Rockland, USA) according to the manufacturer's instructions.

Western blot analysis

Ishikawa and RL95-2 cells were treated with or without the HG medium, or ERα, ERβ or total ER antagonists for 48 h. The expression of ERα, ERβ and GLUT4 was detected by western blotting analysis. The cells were washed three times with PBS and were incubated with RIPA (Beyotime, shanghai, China) for 30 min at 4°C. The cells were scraped and centrifuged at 12000rpm for 25 min. The supernatant was collected, and the protein concentration was detected using the BCA Kit (Beyotime). Simultaneously, SDS-PAGE buffer was added to the supernatant, and the mixture was boiled at 95°C for 10 min. The total proteins (25 ug) were electrophoresed on SDS-PAGE gels (EpiZyme Scientific) using a Miniprotein III system (Bio-Rad, USA) and were transferred onto PVDF membranes (Millipore, 0.45 um) for 1.5 h. The PVDF membranes were blocked with 5% defatted milk for 2 h at room temperature and were incubated overnight with rabbit anti-human antibody against ERα, ERβ and GLUT4 and GAPDH at 4°C. Next, PVDF membranes were washed three times with TBST solution and were incubated with peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (1:5000; Bioworld Technology, co. Ltd. USA) at room temperature for 1 h. Next, the membranes were washed three times and were processed to analyze chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate Kit (Millipore).

In vivo experiment

Control or GLUT4-silenced Ishikawa cells (1×10^7 cells in 200 ul) were inoculated subcutaneously into the right scruff of 4-5 week old female nude mice on day 0. Starting from day 3, estrogen (100 mg/kg) or the vehicle were injected intraperitoneally once a day. Tumor growth was monitored via measuring the tumor volume every three days. The tumor volume was determined using the formula: volume (mm^3) = 1/2(length x width x height). After 19 days, the mice were euthanized, and the tumor tissues were collected for analyzing the expression of VEGF (rabbit anti-VEGF antibody, 1:500, Abcam) and the mRNA level of *TWIST* and *CTNNB1* via IHC and RT-PCR.

Statistical analysis

Data were presented as the means ± SEM and were analyzed using GraphPad Prism version 5 by the *t*-test or one-way ANOVA. Differences were considered statistically significant at $P<0.05$.

Results

The stimulatory effect of high glucose on cell viability and invasion of UECC is dependent on GLUT4

To verify the effect of high glucose on the growth and invasion of Ishikawa and RL95-2 cells, the cells were cultured with or without the HG medium for 48 hours. As shown in Fig. 1, HG led to an increase in viability (Fig. 1A) and invasion (Fig. 1B) of Ishikawa and RL95-2 cells *in vitro*.

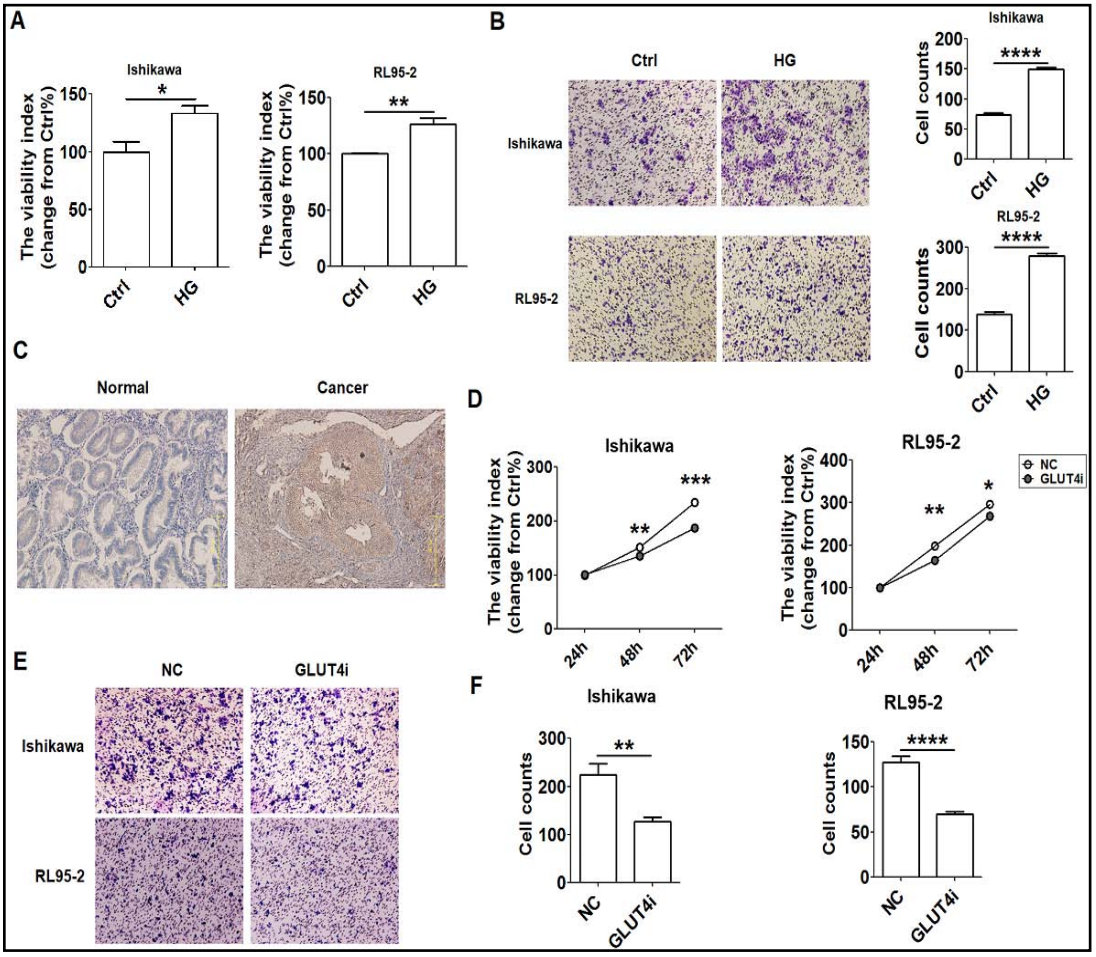

Fig. 1. High glucose and GLUT4 stimulate cell viability and invasion of UECC. (A, B) Ishikawa and RL95-2 cells were cultured with normal medium (5 mmol/L) or high glucose (HG, 25 mmol/L) for 48 hours, and the viability (A) and invasion (B) of Ishikawa and RL95-2 cells were detected by the CCK-8 assay and Matrigel invasion assay. (C) The expression of GLUT4 in normal endometrium (n=10) and UEC (n=10) tissues via immunohistochemical (IHC) staining. (D-F) After transfection with empty vector plasmid or GLUT4-silenced plasmid for 24, 48 or 72 hours, the viability (D) and invasion (E, F) of Ishikawa and RL95-2 cells were detected by the CCK-8 assay and Matrigel invasion assay (48 h). NC: Ishikawa and RL95-2 cells were transfected with empty vector plasmid; GLUT4i: Ishikawa and RL95-2 cells were transfected with the GLUT4-silenced plasmid. The data were expressed as the means ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 or ****P<0.0001.

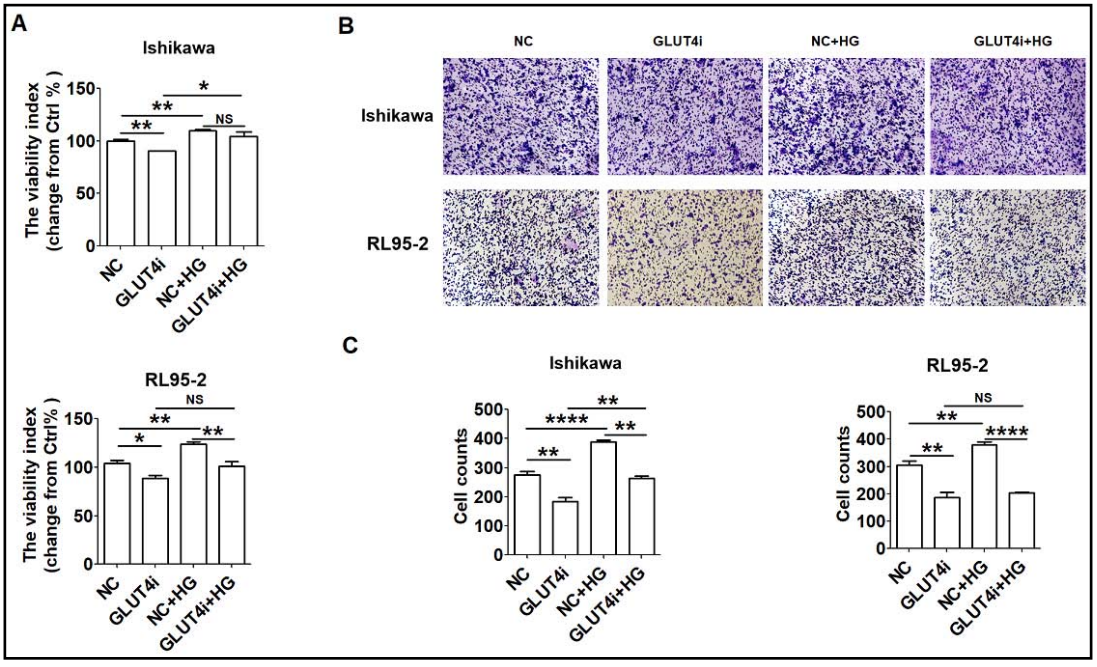

Fig. 2. The stimulatory effect of high glucose on cell viability and invasion of UECC is dependent on GLUT4. (A-C) Control and GLUT4- silenced Ishikawa and RL95-2 cells were cultured with normal medium (5 mmol/L) or HG (25 mmol/L) for 48 hours, and the cell viability (A) and invasion (B, C) were detected by the CCK-8 assay and Matrigel invasion assay. The data were expressed as the means ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 or ****P<0.0001. NS: no significant difference.

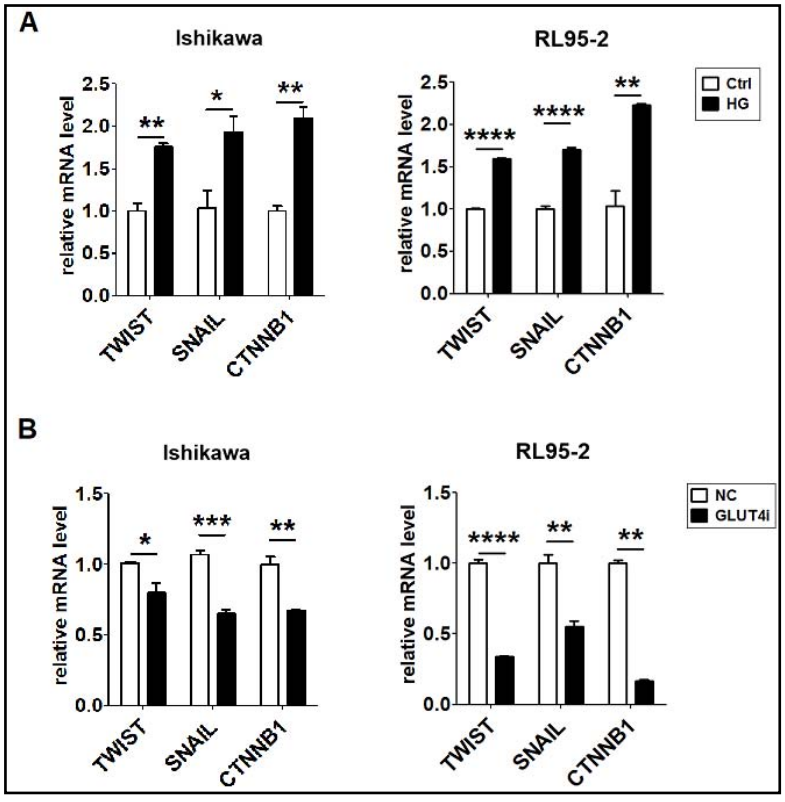

Fig. 3. HG and GLUT4 accelerate the EMT process in UECC. (A, B) Ishikawa and RL95-2 cells were cultured with normal medium (5 mmol/L) or HG (25 mmol/L) (A) or were transfected with empty vector plasmid or GLUT4-silenced plasmid (B) for 48 hours. The transcription of TWIST, SNAIL and CTNNB1 in Ishikawa and RL95-2 cells was analyzed by RT-PCR. The data were expressed as the means ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 or ****P<0.0001. NS: no significant difference.

To investigate the role of GLUT4 in HG-induced growth and invasion of UECC, first we analyzed the expression of GLUT4 in the UEC tissues and normal endometrium. As shown, there was a strong positive staining of GLUT4 in UEC tissues compared with normal endometrium (Fig. 1C). Subsequently, silencing GLUT4 directly resulted a decrease in the cell viability (Fig. 1D) and invasion (Fig. 1E and F) of the Ishikawa and RL95-2 cells and restricted the stimulatory effect of HG on the cell viability (Fig. 2A) and invasion (Fig. 2B and C) of the Ishikawa and RL95-2 cells. These data suggest that GLUT4 is involved in the regulation of UECC's viability and invasion, especially under HG condition.

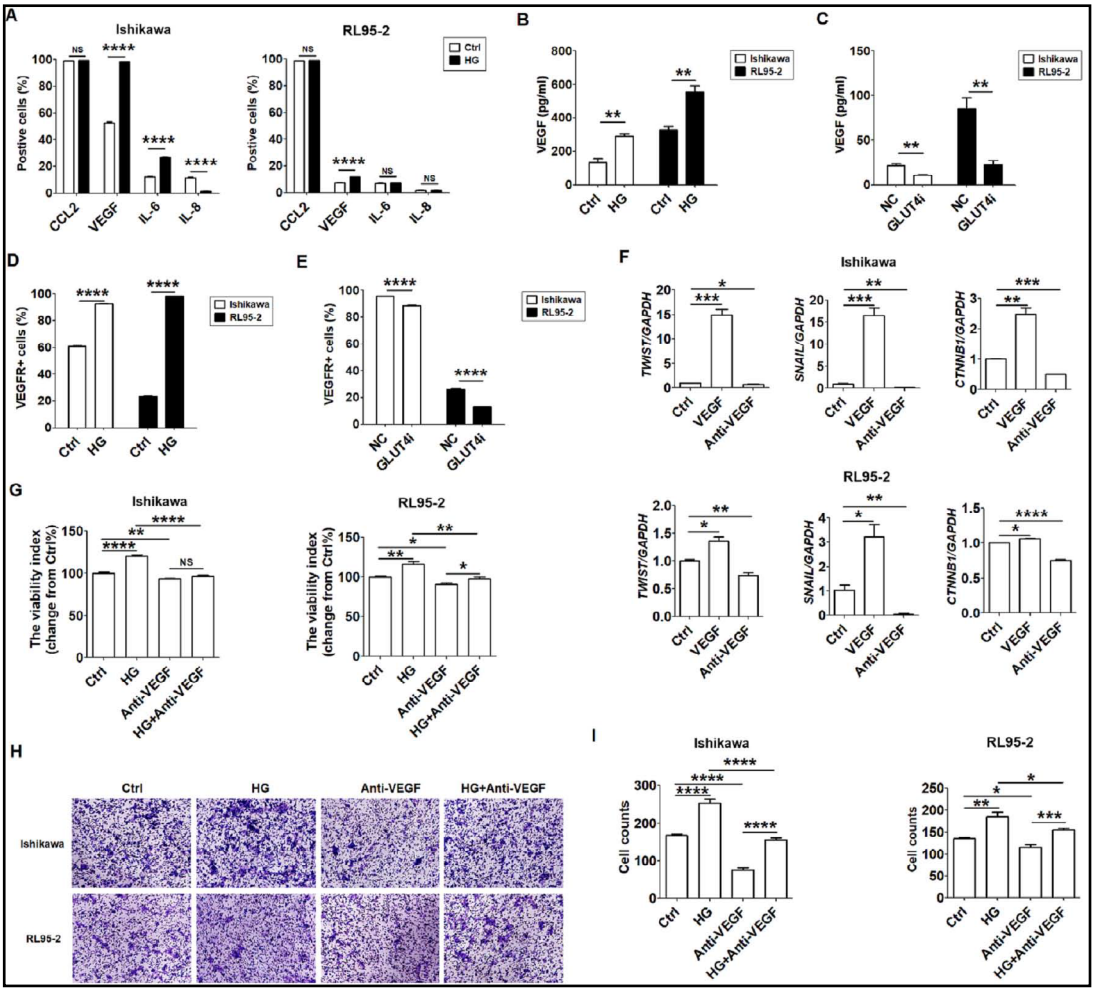

Fig. 4. HG and GLUT4 promote EMT, viability and invasion of UECC by up-regulating VEGF/VEGFR. (A) Ishikawa and RL95-2 cells were cultured with normal medium (5 mmol/L) or HG (25 mmol/L) for 48 hours, the percentage of $CCL2^+$, $VEGF^+$, $IL\text{-}6^+$ and $IL\text{-}8^+$ Ishikawa or RL95-2 cells were analyzed by flow cytometry (FCM) analysis. (B-E) The secretion of VEGF and expression of VEGFR by Ishikawa and RL95-2 cells as described in Fig. 3. were evaluated by ELISA and FCM. (F) Ishikawa and RL95-2 cells were treated with recombinant human VEGF protein (VEGF, 10 ng/mL) or anti-human VEGF neutralizing antibody (Anti-VEGF, 1 ug/mL) for 48 hours, and the transcription of TWIST, SNAIL and CTNNB1 in these cells was analyzed by RT-PCR. (G-I) Ishikawa and RL95-2 cells were cultured with or without HG and or Anti-VEGF for 48 hours, and the cell viability (G) and invasion (H, I) were detected by the CCK-8 assay and Matrigel invasion assay. The data were expressed as the means ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 or ****P<0.0001. NS: no significant difference.

HG and GLUT4 accelerate the EMT process in UECC

EMT occurs in various tumors, and it has been suggested to be crucial during epithelial cancer cell invasion. Early non-invasive tumor cells express epithelial markers such as E-cadherin. Induction of regulators of EMT, such as *TWIST*, *SLUG* and *SNAIL*, repress E-cadherin transcription in cancer cells, causing downregulation of other adhesive epithelial markers [13]. The expression of β-catenin/*CTNNB1* correlates with tumor grades, invasion and cancer progression, which is also an important regulator of EMT event [16]. Therefore, further analysis of the effect of HG and GLUT4 on EMT regulators showed that HG up-regulated the mRNA level of *TWIST*, *SNAIL* and *CTNNB1* in the Ishikawa and RL95-2 cells (Fig. 3A). In contrast, silencing GLUT4 significantly suppressed the transcription of *TWIST*, *SNAIL* and *CTNNB1* in the Ishikawa and RL95-2 cells (Fig. 3B). The above results suggest that HG and GLUT4 promote growth and invasion possibly by accelerating the EMT process.

HG and GLUT4 promote EMT, viability and invasion of UECC by up-regulating VEGF/VEGFR

It has been reported that there are high levels of cytokines, such as CCL2, VEGF, IL-6 and IL-8, in patients with diabetes [17, 18]. These cytokines also participate in the origin and development of UEC [19-21]. Therefore, we further investigate the role of HG and GLUT4 in the expression of CCL2, VEGF, IL-6 and IL-8 in UECC. As demonstrated in Fig. 4, HG obviously up-regulated the percentage of $VEGF^+$ and $IL\text{-}6^+$Ishikawa cells, and down-regulated $IL\text{-}8^+$ Ishikawa cells, however, HG only increased the percentage of $VEGF^+$ RL95-2 cells (Fig. 4A and B). Further analysis showed that exposure to HG led to a significant elevation of VEGF

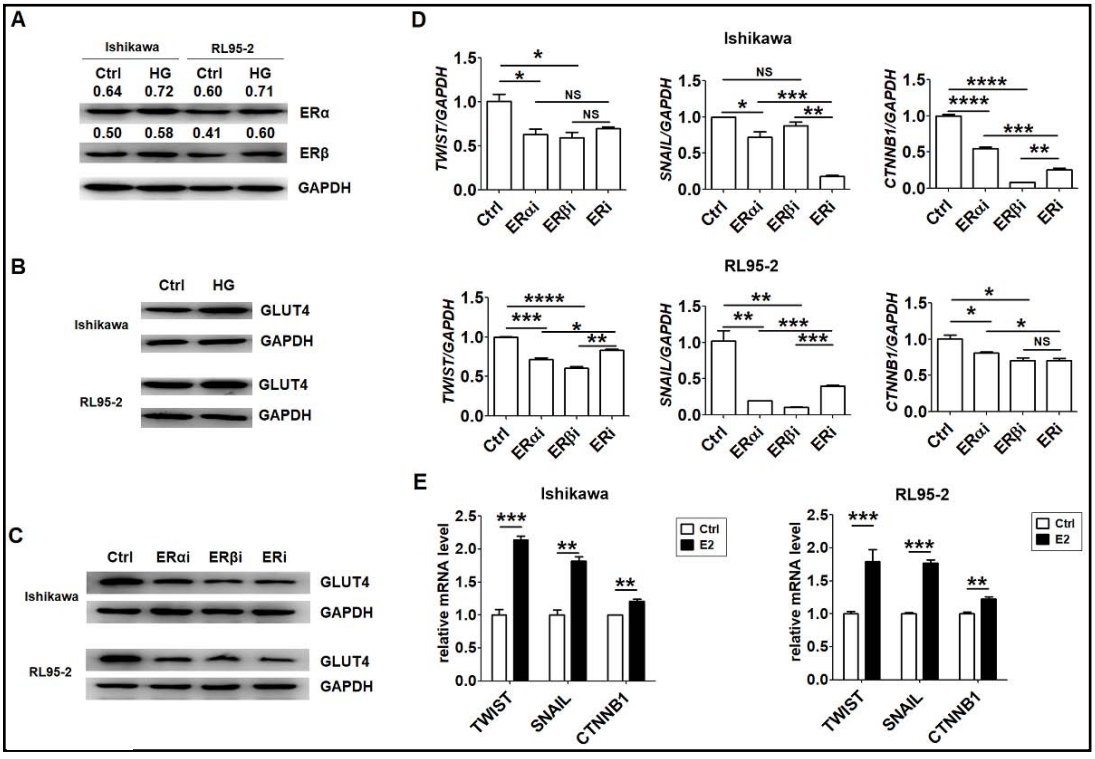

Fig. 5. HG up-regulates GLUT4 expression by up-regulating ER. (A, B) Ishikawa and RL95-2 cells were cultured with or without HG for 48 hours, the expression of ERα, ERβ and GLUT4 was detected by western blotting. (C, D) Ishikawa and RL95-2 cells were treated with the ERα antagonist MPP (10^{-6} M), ERβ antagonist PHTPP (10^{-6} M) or total ER antagonist-ICI182780 (10^{-6} M) for 48h, the expression of GLUT4 (C) and the mRNA levels of TWIST, SNAIL and CTNNB1 (D) in these cells were detected by western blotting and RT-PCR. (E) Ishikawa and RL95-2 cells were incubated with or without E_2 (10^{-7}M) for 48 h. The mRNA levels of TWIST, SNAIL and CTNNB1 were detected by RT-PCR. The data were expressed as the means ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 or ****P<0.0001. NS: no significant difference.

secretion and VEGFR expression by the Ishikawa and RL95-2 cells (Fig. 4B and D). Instead, silencing GLUT4i markedly down-regulated the expression of VEGF and VEGFR by the Ishikawa and RL95-2 cells (Fig. 4C and E).

To explore the potential role of VEGF signaling, we observed the expression of EMT-related genes in UECC after treatment with rhVEGF or anti-VEGF. As shown, stimulation with VEGF resulted in the up-regulation of *TWIST*, *SNAIL* and *CTNNB1* in the Ishikawa and RL95-2 cells (Fig. 4F); in contrast, blocking VEGF/VEGFR signaling with anti-VEGF led to the down-regulation of these genes (Fig. 4F). In addition, anti-VEGF significantly inhibited the viability and invasion of the Ishikawa and RL95-2 cells (Fig. 4G-I) and restricted the effect of HG on cell viability and invasion (Fig. 4G-I). These findings indicate that the stimulatory effect of HG and GLUT4 on the EMT process, viability and invasion of UECC is dependent on VEGF/VEGFR.

HG stimulates EMT, viability and invasion of UECC by up-regulating ER-mediated GLUT4 expression

To better understand the regulation relationship between HG, GLUT4 and ER, we treated the Ishikawa and RL95-2 cells with or without HG medium and found that HG increased the expression of ERα, ERβ (Fig. 5A) and GLUT4 (Fig. 5B) in the Ishikawa and RL95-2 cells. Additionally, blocking ERα and or ERβ with antagonists led to the obvious decrease of GLUT4 in the Ishikawa and RL95-2 cells (Fig. 5C), suggesting that HG elevates the expression of GLUT4 in UECC by up-regulating the ER expression. Compared with control group, the mRNA

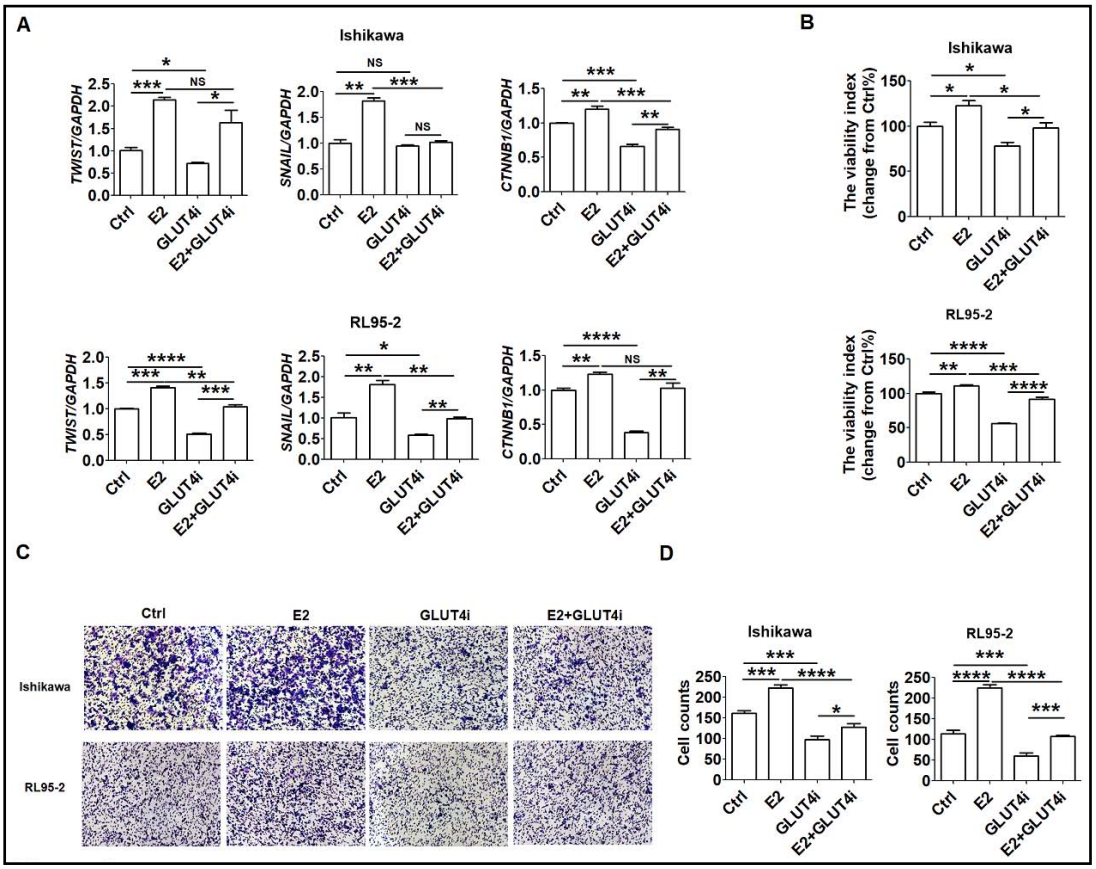

Fig. 6. HG stimulates EMT, viability and invasion of UECC by up-regulating ER-mediated GULT4 expression. (A-D) Control and GLUT4-silenced Ishikawa and RL95-2 cells were cultured with or without E_2 (10^{-7}M) for 48 h. The mRNA levels of TWIST, SNAIL and CTNNB1(A), cell viability (B) and invasion (C, D) were detected by RT-PCR, CCK-8 assay and Matrigel invasion assay, respectively. The data were expressed as the means ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 or ****P<0.0001. NS: no significant difference.

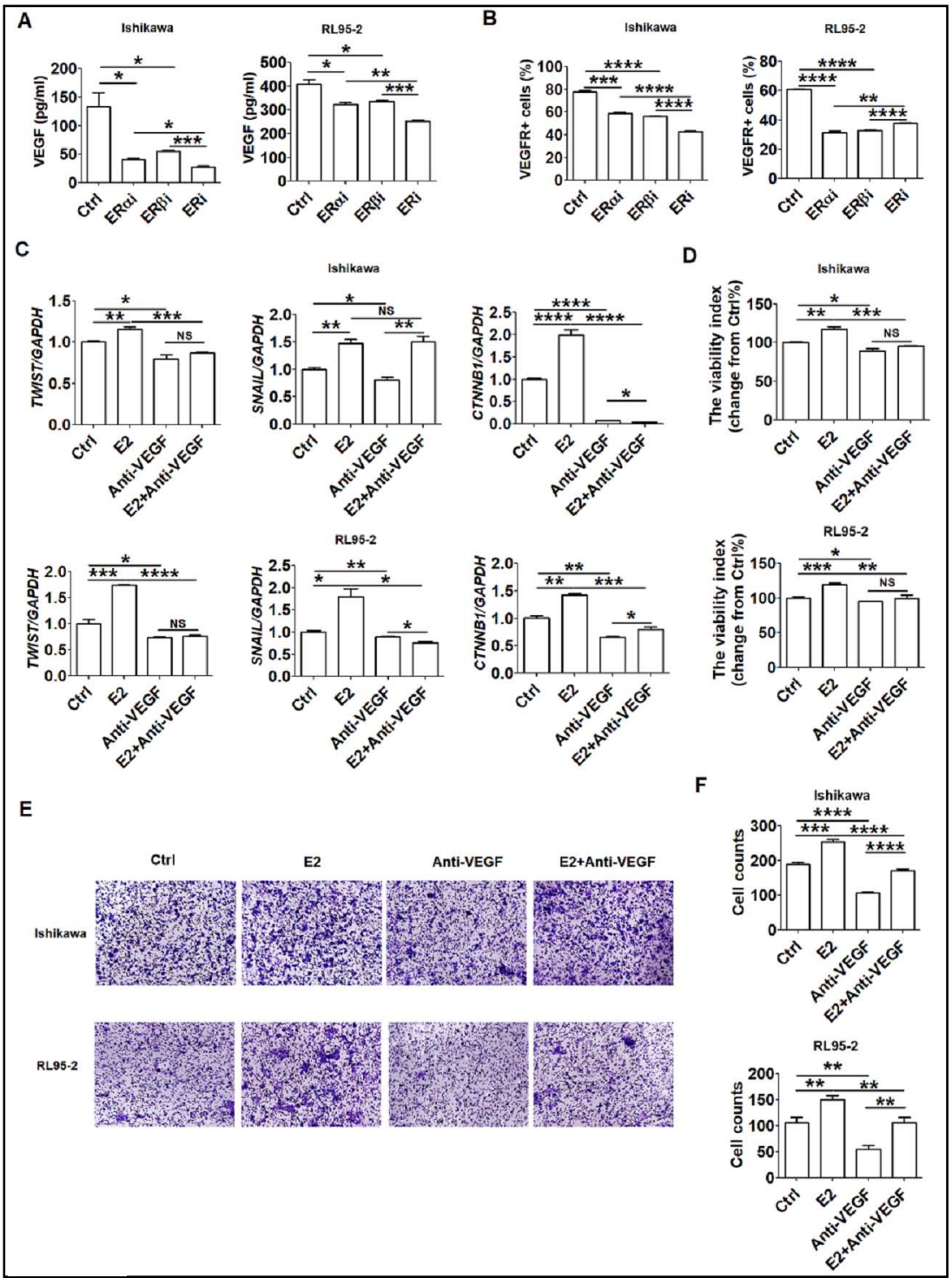

Fig. 7. Estrogen/ER promotes EMT of UECC partly through up-regulating VEGF/VEGFR. (A, B) Ishikawa and RL95-2 cells were treated with the ERα antagonist MPP (10^{-6} M), ERβ antagonist PHTPP (10^{-6} M) or total ER antagonist ICI182780 (10^{-6} M) for 48h, the secretion of VEGF (A) and the percentage of VEGFR$^+$ cells (B) were detected by ELISA and FCM. (C-F) Ishikawa and RL95-2 cells were cultured with or without E_2 (10^{-7}M) and / or Anti-VEGF for 48 h, and the mRNA levels of TWIST, SNAIL and CTNNB1 (C), cell viability (D) and invasion (E, F) were detected by RT-PCR, CCK-8 assay and Matrigel invasion assay, respectively. The data were expressed as the means ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 or ****P<0.0001. NS: no significant difference.

level of *TWIST*, *SNAIL* and *CTNNB1* in the Ishikawa and RL95-2 cells was markedly decreased in the ERα- and or ERβ-blocked groups (Fig. 5C). In contrast, exposure to E_2 resulted in the elevation of the *TWIST*, *SNAIL* and *CTNNB1* mRNA levels (Fig. 5E and 6A), cell viability (Fig. 6B) and invasion (Fig. 6C and 6D) of the Ishikawa and RL95-2 cells; however, silencing GLUT4 partly or completely reversed these effects on the viability and invasion of UECC that was induced by E_2 (Fig. 6B-D).

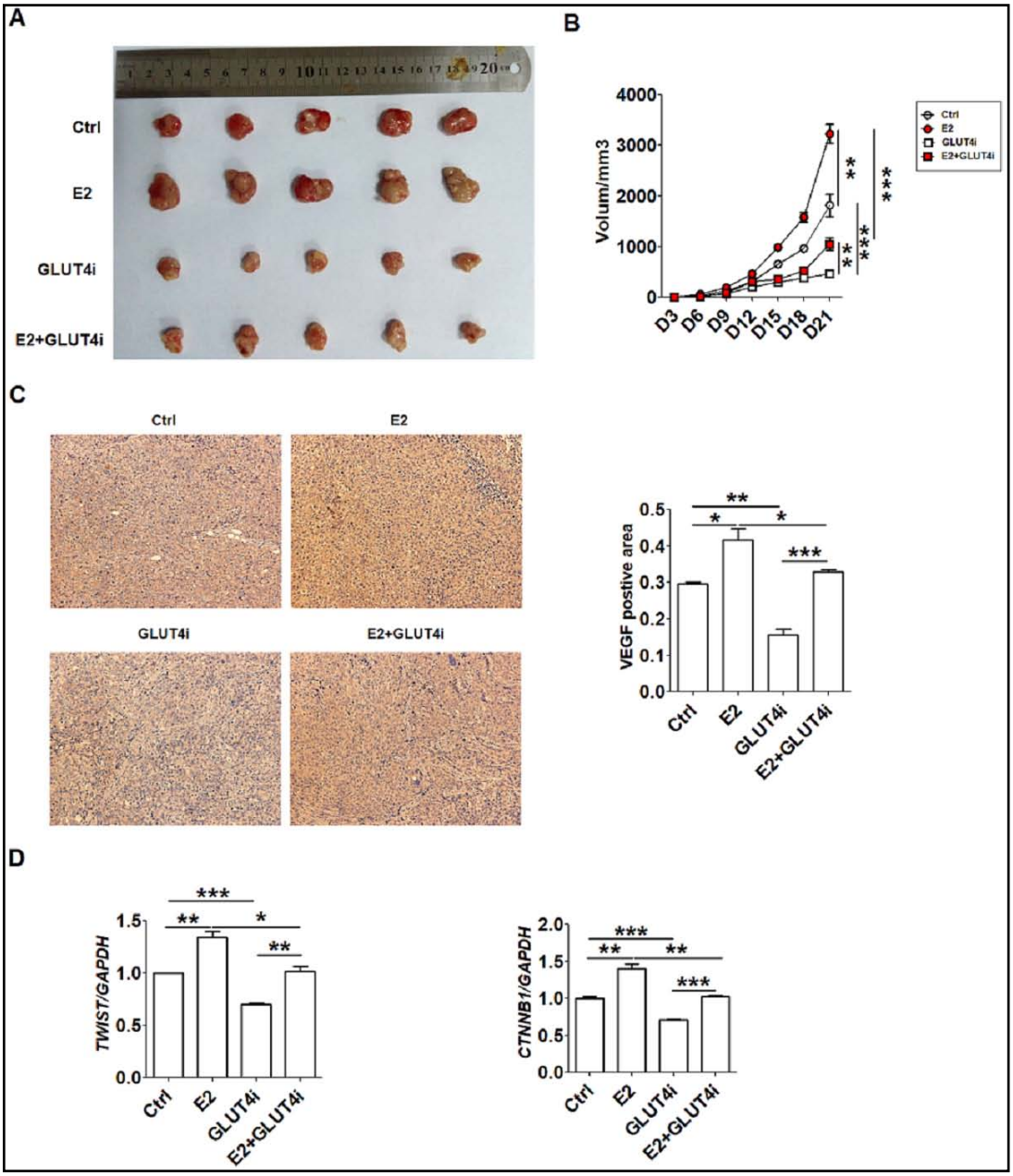

Fig. 8. Estrogen up-regulates GLUT4 and promotes EMT and growth of UEC in vivo. (A, B) The size and volume of cancer lesions were evaluated every three days for the control Ishikawa or GLUT4 silenced Ishikawa-xenografted nude mice (n=8 mice/group) after intraperitoneal injection with or without estrogen (100 mg/kg) once a day. In addition, the expression of VEGF (C) and the mRNA levels of TWIST and CTNNB1 (D) in the cancer lesions were analyzed by IHC and RT-PCR. The average positive area of VEGF from 10 randomly chosen views per sample was analyzed at a magnification of ×200 using the Image-Pro Plus image analysis software. The data were expressed as the means ± SEM (n=8). *P<0.05, **P<0.01, or ***P<0.001.

Estrogen/ER promotes EMT of UECC partly through up-regulating VEGF/VEGFR

To further investigate the relationship between estrogen/ER signaling and VEGF/VEGFR in UECC, we incubated the Ishikawa and RL95-2 cells with or without ERα and or ERβ inhibitors. As shown, we found that blocking ERα and or ERβ obviously decreased the secretion of VEGF and the expression of VEGFR (Fig. 7A and B). Further analysis showed that anti-VEGF significantly down-regulated the expression of *TWIST*, *SNAIL* and *CTNNB1* (Fig. 7C), cell viability (Fig. 7D) and invasion (Fig. 7E and F) of the Ishikawa and RL95-2 cells. In addition, blocking VEGF partly reversed the stimulatory effect of E_2 on *TWIST*, *SNAIL* and / or *CTNNB1* of the Ishikawa and RL95-2 cells (Fig. 7C and D) but not cell viability and invasion (Fig. 7E and F), suggesting that estrogen accelerates the EMT event of UECC, which should be dependent on VEGF/VEGFR.

Estrogen up-regulates GLUT4 and promotes EMT and growth of UEC in vivo

Finally, we analyzed the role of estrogen and GLUT4 on the EMT and growth of UEC in Ishikawa-xenografted nude mice. As shown, injection with E2 obviously accelerated the growth of UEC *in vivo* (Fig. 8A and B), and up-regulated the expression of VEGF (Fig. 8C), *TWIST* and *CTNNB1* (Fig. 8D) in UEC. However, compared with the control Ishikawa-xenografted nude mice, the tumor size and volume (Fig. 8A and B), the expression of VEGF (Fig. 8C), and the *TWIST* and *CTNNB1* expression (Fig. 8D) of UEC were significantly decreased in the GLUT4-silenced Ishikawa-xenografted nude mice.

Discussion

With the Westernization of lifestyle and economic development, the occurrence of UEC has also risen rapidly in Asian countries [22]. Excessive exposure to carcinogens, including insulin, estrogens, insulin-like growth factors, leptin, and adiponectin, may contribute to the development of UEC in diabetic and/or obese women. With increase in BMI, the incidence of UEC is increasing [23]. Diabetic patients are twice as likely to develop endometrial cancer [24]. Increased blood glucose levels contribute to the growth or carcinogenesis of UEC [25]. Here, we found that HG promoted the viability and invasion of UECC *in vitro*.

GLUT is essential to glucose intake. The GLUT family of proteins comprises 13 members [26]. According to the sequence homology and structural similarity, three subclasses of sugar transporters have been defined. Class I GLUTs (GLUTs 1–4) are glucose transporters; Class II GLUTs (GLUTs 5, 7, 9 and 11) are fructose transporters; and Class III GLUTs (GLUTs 6, 8, 10, 12 and HMIT1) are structurally atypical members of the GLUT family, which are poorly defined at present. Recent studies confirmed that GLUTs (GLUT1, GLUT3 and GLUT6) are upregulated in the malignant endometrium compared with nonmalignant counterparts [27, 28]. In this study, a high level of GLUT4 was observed in the UEC tissues. Further investigations showed that the effect of HG on UECC's viability and / or invasion is partly dependent on GLUT4.

Female sex hormone level is involved in influencing both BMI and endometrial cancer progress [29]. Of note, our findings showed that exposure to HG led to the increase of ERα, ERβ and GLUT4 in UECC and blocking ERα and or ERβ resulted in a decrease in GLUT4 in UECC, indicating that HG up-regulates GLUT4 expression and further promotes EMT, viability and invasion of UECC possibly through up-regulating the ER expression. However, the mechanism underlying the effect of ERα and ERβ on GLUT4 needs to be further researched.

VEGF is a critical growth factor involved in angiogenesis, which is a vital event for tumor proliferation and several pathological progressions [30]. It has been reported that VEGF takes part in the tumorigenesis and metastasis of UEC [31]. Moreover, VEGFRs are highly expressed in UEC, among these, VEGFR-3 is remarkably correlated with tumor stage and survival [32]. Here, we found that HG and estrogen promoted the expression of VEGF and VEGFR in UECC through up-regulation of GLUT4 and triggered the process of EMT, cell viability and invasion. However, the mechanism underlying the effect of GLUT4 on VEGF/

VEGFR is still unknown. It has been reported that HG promotes the expression of VEGF by activating STAT3 in non-small lung cancer cells [33], suggesting that the effects of GLUT4 on VEGF may also be dependent on STAT3 activation. Of note, our results suggest that the regulation of HG, estrogen and GLUT4 on VEGF may also contribute to the progression of UEC by stimulating angiogenesis. These questions need to be further studied.

The invasion and metastasis of epithelial cancer are closely related with the EMT process [34]. Loss of epithelial differentiation markers is an obvious feature of EMT [35]. The key EMT-related transcription factors *TWIST*, *SNAIL* and *CTTNB1* play a vital role in development and progression of cancer [36, 37]. Recently, VEGF/VEGFR signaling was found to be involved in the regulation of EMT in esophageal cancer [38]. In our study, the VEGF protein up-regulated the transcription of *TWIST*, *SNAIL* and *CTTNB1* in UECC; however, the specific mechanism underlying this regulation is unknown. HG induces the up-regulation of *CTNNB1* and the transcription of its target gene in UECC [39]. Here, we found that HG and estrogen accelerated the EMT process possibly through GLUT4-mediated up-regulation of VEGF/VEGFR.

Under HG condition and a high rate of aerobic glycolysis, more energy is generated. This is a major energy source for the growth of UECC [40]. In conclusion, based on our study and other research, it can be concluded that HG increases the expression of GLUT4 via up-regulating ER. On the one hand, GLUT4 promotes the EMT event, viability and invasion of UECC by up-regulating VEGF/VEGFR; on the other hand, it supplies more energy for the growth of UECC by increasing the glucose intake and finally accelerates the progression of UEC. Targeting glucose metabolism enzymes and / or GLUT4 may be a strategy for the clinical treatment of UEC. Further studies are required to explain the relationships among estrogen, glucose metabolism and GLUT4 and their underlying molecular mechanisms in the progression of UEC.

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Disclosure Statement

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