

Role of metformin in functional endometrial hyperplasia and polycystic ovary syndrome involves the regulation of MEG3/miR-223/GLUT4 and SNHG20/miR-4486/GLUT4 signaling

JIE LIU¹, YANGCHUN ZHAO¹, LONG CHEN², RUILAN LI¹, YUMEI NING³ and XIUZHI ZHU¹

¹Department of Gynecology and Obstetrics; ²PCR Laboratory, Department of Laboratory Medicine, The Second Affiliated Hospital of Zhejiang Chinese Medical University; ³Department of Gynecology, Zhejiang Maternal and Child Health and Reproductive Health Center, Hangzhou, Zhejiang 310000, P.R. China

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Abstract. Metformin (MET) can effectively treat endometrial hyperplasia (EH), and the expression of glucose transporter type 4 insulin-responsive (GLUT4) is closely associated with the development of EH. The present study aimed to verify the effect of MET in functional EH and polycystic ovary syndrome (PCOS). H&E staining was performed to analyze the severity of EH, and immunohistochemistry was performed to evaluate the expression of GLUT4 in the endometrium of PCOS rats. Reverse transcription-quantitative PCR was used to calculate the expression of long non-coding (lnc)RNA-maternally expressed gene 3 (MEG3), lncRNA-small nucleolar RNA host gene 20 (SNHG20), GLUT4 mRNA, microRNA (miR)-223 and miR-4486. Sequence analysis and luciferase assays were performed to explore the regulatory relationship among certain lncRNAs, miRNAs and target genes. EH in PCOS rats was efficiently inhibited by MET administration. The increased expression of GLUT4 in PCOS rats was attenuated by MET treatment. Moreover, the expression levels of lncRNA-MEG3 and lncRNA-SNHG20 were significantly inhibited in the endometrium of PCOS rats. MET treatment also showed remarkable efficiency in restoring the expression of lncRNA-MEG3 and lncRNA-SNHG20. Meanwhile, the expression levels of miR-223 and miR-4486 were notably elevated in the endometrium of PCOS rats, while

MET treatment reduced the expression of miR-223 and miR-4486 in PCOS rats. Furthermore, a luciferase assay confirmed the inhibitory relationship between miR-223 and lncRNA-MEG3/GLUT4 expression, as well as between miR-4486 and lncRNA-SNHG20/GLUT4 expression. GLUT4 knockdown restored the decreased viability of HCC-94 cells induced by overexpression of lncRNA-MEG3. To conclude, MET exhibited a therapeutic effect in the treatment of EH by modulating the lncRNA-MEG3/miR-223/GLUT4 and lncRNA-SNHG20/miR-4486/GLUT4 signaling pathways. This work provides mechanistic insight into the development of EH.

Introduction

A common disorder caused by the imbalance of hormones, polycystic ovary syndrome (PCOS) can affect $\leq 20\%$ of women of reproductive age (1). PCOS is often associated with adverse effects on reproductive function in women, including infertility induced by irregular cycles of anovulation and recurrent pregnancy loss (1-3). Endometrial dysfunction has been attributed to the failure of reproduction in patients with PCOS (4). Reduced glucose transporter type 4 insulin-responsive (GLUT4) expression in the endometrium has been observed in women with PCOS (5-7). Endometrial hyperplasia (EH) can be triggered by exposure to an excessive level of estrogen in obese female patients as well as in female patients receiving estrogen replacement therapy (8). At present, it is hypothesized that the onset of cellular atypia increases the risk of uterine or endometrial cancer (EC) (9). As a premalignant disorder, EH can become invasive in $\leq 10\%$ of patients (10). If patients with PCOS are not treated properly by utilizing contraceptive steroids, $>30\%$ of patients with PCOS can eventually develop EH (11). In past research, nearly 2% of patients with PCOS eventually suffered from EC (12).

A type of long RNA transcript with no protein-coding ability, long non-coding (lnc)RNAs are speculated to have a diverse range of biological roles, including the regulation of target gene transcription in an epigenetic manner (13,14).

Correspondence to: Dr Xiuzhi Zhu, Department of Gynecology and Obstetrics, The Second Affiliated Hospital of Zhejiang Chinese Medical University, 318 Chaowang Road, Hangzhou, Zhejiang 310000, P.R. China
E-mail: jimmyliuzcmu@outlook.com

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Furthermore, lncRNAs can act as key regulators in inflammatory reactions and the development of a wide range of inflammatory disorders (15,16). On the other hand, microRNAs (miRNAs/miRs), a type of short RNA transcript with no protein-coding ability, participate in the control of a wide range of biological activities, including cellular development, survival, proliferation, tumorigenesis and inflammatory responses (17).

Metformin (MET) has clinical applications in the alleviation of metabolic disorders. MET can alleviate the severity of endometrial disorder by reducing the levels of androgen, thus attenuating endometrial diseases, including EH, especially in patients with PCOS who are also insulin resistant (18-22). Previous western blotting assays have demonstrated that the expression of GLUT4 in the endometrium is reduced in patients with PCOS. Furthermore, immunohistochemistry results have suggested that the presence of PCOS rather than EH acts as a key regulator of the expression level of GLUT4 in epithelial cells (2,23). In addition, previous data suggested that differences in GLUT4 expression levels may help to distinguish between patients with EH with and without PCOS (24). Downregulation of GLUT4 is associated with the development of EH during PCOS, and it has been reported that abnormal hormonal conditions, including PCOS, are associated with endometrial GLUT4 expression (24). Changes in the insulin receptor, as well as androgen-dependent alterations in androgen receptor expression, are involved in changes in endometrial GLUT4 (25). In addition, MET has been reported to alleviate uterine defects in PCOS animal models (26), and it is an effective treatment for endothelial cell pyroptosis by regulating lncRNA-maternally expressed gene 3 (MEG3) signaling in atherosclerosis (27). Furthermore, small nucleolar RNA host gene 20 (SNHG20) has been reported to target the expression of miR-4486 in the pathogenesis of glioma cell malignancy (28). In the present study, it was hypothesized that administration of MET regulates the expression of lncRNA-MEG3 and lncRNA-SNHG20, thereby influencing the expression of their competing endogenous RNAs (ceRNAs), such as miR-223 and miR-4486, which leads to upregulation of their target gene, GLUT4. To test this hypothesis, cultured cells were treated with MET to verify its effect on the lncRNA-MEG3/miR-223/GLUT4 and lncRNA-SNHG20/miR-4486/GLUT4 signaling pathways to confirm the role of MET in an animal model of PCOS.

Materials and methods

Animals and treatments. A PCOS rat model was established using a protocol described previously to determine the effect of MET administration on the endometrium (26). In brief, female SD rats (mean weight of 295 g, average age of 70 days, n=80) were acquired from the experimental animal center of Zhejiang Chinese Medical university laboratory animal research center and housed in the animal facility for 7 days of adaptation. In the experiment, all animals had unlimited access to drinking water and food. The environment in the SPF animal facility was controlled at a temperature of 23±1°C with a 12/12 h light/dark cycle and 50-75% humidity. All SD rats had a normal estrous cycle, which was confirmed by utilizing vaginal smear examinations performed under a conventional light microscope

prior to the study treatment. Then, the rats were randomly assigned into four groups with 20 rats in each group: i) SHAM (rats were treated with saline); ii) SHAM + MET (rats were treated with saline + MET); iii) PCOS [rats were treated with insulin + human chronic gonadotropin (hCG) to trigger hyperandrogenism and hyperinsulinemia]; and iv) PCOS + MET (PCOS rats were treated with MET). The establishment of animal models, doses and drug treatment protocols were performed according to a previous publication (29). In brief, treatment with insulin was started at 0.5 IU per day and then gradually increased to 6.0 IU per day to trigger insulin resistance and hyperinsulinemia. At the same time, 3.0 IU per day of hCG was used to trigger hyperandrogenism. All doses were administered by subcutaneous injections given twice a day until the experimental treatment was finished. On day 23 of the experiment, the rats in each group were further divided into two subgroups, with each subgroup containing 10 rats. For treatment with MET, the compound was mixed in saline before it was orally administered at a dose of 500 mg/kg utilizing a surgical cannula. Then, the trunk blood in each rat was collected, while the uteri in the rats were collected for analysis. The humane endpoints of this study were the point the uteri samples were collected from the rats. The whole experiment lasted for 30 days. A total of 80 animals were used and sacrificed at the end of this study. No rats were found dead during the study. The animal health and behavior was observed at a frequency of 2 days. No anesthetic agents were used for the collection of uteri samples, instead the rats were sacrificed by the i.p. administration of 100 mg/kg of sodium pentobarbital. This study was performed in line with the Guide for the Care and Use of Laboratory Animal by National Research Council (US) Committee (8th edition) (30) and the protocols were approved by the Animal Ethics Committee of Zhejiang Chinese Medical University Laboratory animal research center (approval no. ZSLL-2016-42; Hangzhou, China).

RNA isolation and reverse transcription-quantitative (RT-q)PCR. In this study, RT-qPCR was performed on endometrial tissues collected from rats in different experimental groups. In brief, the total RNA was isolated using a miRNeasy Mini kit (Qiagen GmbH) and evaluated using a Nanodrop 3000 spectrometer (Thermo Fisher Scientific, Inc.). Next, cDNA was synthesized from isolated total RNA (2 µg total RNA of each sample) utilizing a Transcriptor First Strand assay kit (Roche Diagnostics) in accordance with the manufacturer's instructions following a thermocycling protocol of 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and kept at 4°C. Then, 1 µl synthesized cDNA from each sample was utilized as a template to perform qPCR with SYBR Green Master Mix (Toyobo) using a LightCycler® 480 real-time PCR machine (Roche Diagnostics) in accordance with the manufacturer's instructions to assay the relative expression of MEG3 (forward, 5'-GGGAGCAGCTATGGATCACC-3' and reverse, 5'-ATAGCGCCCCCTATTCATGC-3'), SNHG20 (forward, 5'-CCTGTGTGCCTGGAAAGGAAT-3' and reverse, 5'-GGCACA GGAACCACAGAGTAT-3'), miR-223 (forward, 5'-CGTGTA TTTGACAAGCTGA-3' and reverse, 5'-GAACATGTCTGC GTATCTC-3'), miR-4486 (forward, 5'-ACACTCCAGCTG GGGCTGCGCGA-3' and reverse, 5'-TGGTGTCGTGGAGT CG-3'), antisense non-coding RNA in the INK4 locus (ANRIL;

forward, 5'-CTGGGACTACAGATGCACCAC-3' and reverse, 5'-GGAGGGAGCATGTCTGTTTCT-3'), down-regulated in hepatocellular carcinoma (DREH; forward, 5'-GUGCCUGUCACAAACAGAUTT-3' and reverse, 5'-AUCUGUUUGUGACAGGCACTT-3'), X inactive specific transcript regulator (FTX; forward, 5'-TATGCCACCTAGCCTTTCTACA-3' and reverse, 5'-ATCTCTTCAAAGCGGCATAAT-3'), H19 imprinted maternally expressed transcript (H19; forward, 5'-TACAACCACTGCACTACCTG-3' and reverse, 5'-TGG AATGCTTGAAGGCTGCT-3') and GLUT4 (forward, 5'-ATC CGGAACCTGGAGGGGCC-3' and reverse, 5'-CGGCCA GGCCCAACAGATGG-3') in each sample. Relative expression was calculated using the $2^{-\Delta\Delta C_q}$ method (31) with U6 (forward, 5'-GCATGACGTCTGCTTTGGA-3' and reverse, 5'-CCACAATCATTCTGCCATCA-3') and GAPDH (forward, 5'-GGGAGCCAAAAGGGTCAT-3' and reverse, 5'-GAG TCCTTCCACGATACCAA-3') used as the internal reference genes.

Cell culture and transfection. As EH is a medical condition characterized by deregulation of endometrial cells proliferation (8), HCC-94 cells, a cervical carcinoma cell line, were chosen for *in vitro* analysis. Other candidate cell lines, such as HELA and 293T cells, were also involved in the preliminary experiments, but the conditions were not appropriate for the assays (data not shown). To further explore the effect of MET treatment on expression levels of lncRNAs, the expression levels of six candidate lncRNAs were analyzed in HCC-94 cells treated with or without 100 μ M MET (Shanghai Squibb Pharmaceutical Co., Ltd., Shanghai, China) at room temperature for 24 h. In brief, HCC-94 cells were maintained in modified DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 1 g/l insulin (Gibco; Thermo Fisher Scientific, US), 2 mM glutamine (Gibco; Thermo Fisher Scientific, Inc.), 0.67 mg/l selenium (Sijiqing, Hangzhou, China), 10% FBS (Sijiqing, Hangzhou, China), 100 μ g/ml streptomycin, 100 U/ml penicillin and 0.55 g/l transferrin (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were cultured in a 37°C humidified incubator supplied with 5% CO₂. After reaching confluence, cells were deprived of serum for 24 h in a hypoxic environment consisting of 5% CO₂, 94% N₂ and 1% O₂. Next, the cells were cultured in complete medium under reoxygenation with 20% O₂. To determine the effects of MET and siRNAs on gene expression, the following six sets of experiments were performed using HCC-94 cells at the density of 1x10⁵/well. Experiment 1, HCC-94 cells were divided into two groups: i) Untreated (HCC-94 cells were cultured in normal medium); and ii) MET (HCC-94 cells were treated with MET). Experiment 2, HCC-94 cells were divided into three groups: i) Negative control (NC) small interfering (si)RNA (HCC-94 cells were transfected with scrambled siRNA as the NC); ii) MEG3 siRNA (HCC-94 cells were transfected with MEG3 siRNA); and iii) SNHG20 siRNA (HCC-94 cells were transfected with SNHG20 siRNA). Experiment 3, HCC-94 cells were divided into four groups: i) Untreated (HCC-94 cells were cultured in normal medium); ii) MET + NC siRNA (HCC-94 cells were treated with MET and scrambled siRNA as the NC); iii) MET + MEG3 siRNA (HCC-94 cells were treated with MET and MEG3 siRNA group); and iv) MET + SNHG20 siRNA (HCC-94 cells were

treated with MET and SNHG20 siRNA). Experiment 4, HCC-94 cells were divided into two groups: i) NC siRNA (HCC-94 cells transfected with scrambled siRNA as the NC); and ii) GLUT4 siRNA (HCC-94 cells transfected with GLUT4 siRNA). Experiment 5, HCC-94 cells were divided into four groups: i) NC (HCC-94 cells were cultured in normal medium); ii) lncRNA-MEG3 (HCC-94 cells transfected with lncRNA-MEG3); iii) lncRNA-MEG3 + NC siRNA (HCC-94 cells transfected with lncRNA-MEG3 and scrambled siRNA as the NC); and iv) lncRNA-MEG3 + GLUT4 siRNA (HCC-94 cells transfected with lncRNA-MEG3 and GLUT4 siRNA). Experiment 6, HCC-94 cells were divided into four groups: i) NC (HCC-94 cells were cultured in normal medium); ii) lncRNA-SNHG20 (HCC-94 cells transfected with lncRNA-SNHG20); iii) lncRNA-SNHG20 + NC siRNA (HCC-94 cells transfected with lncRNA-SNHG20 and scrambled siRNA as the NC); and iv) lncRNA-SNHG20 + GLUT4 siRNA (HCC-94 cells transfected with lncRNA-SNHG20 and GLUT4 siRNA). The mass of nucleic acid used was 50 nM, and the sequences (generated by Thermo Fisher Scientific, Inc) used for transfection were as follows: Scrambled siRNA (NC), 5'-UUGUACUACACAAAAGUACUG-3'; MEG3 siRNA, 5'-GAUCCCACCAACAUACAAATT-3'; SNHG20 siRNA, 5'-CCUGUUUGUACCUCCAUTT-3'; and GLUT4 siRNA, 5'-AGCTACAATGCAACTTGGCTGGGTA-3'. All transfections were performed using 1x10⁵ cells/well using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 4°C overnight in accordance with the manufacturer's instructions. The cells were treated for 48 h before they were collected for subsequent experiments.

Vector construction, mutagenesis and luciferase assay. In this study, luciferase assays were performed to confirm the regulatory relationships between SNHG20 and miR-4486, miR-4486 and GLUT4, MEG3 and miR-223 and miR-223 and GLUT4. In brief, the sequences of GLUT4, MEG3 and SNHG20 containing the binding sites for miR-4486 or miR-223 were cloned into pcDNA 3.1 vectors (Promega Corporation) to generate wild-type (WT) vectors of GLUT4, MEG3 and SNHG20. At the same time, site-directed mutagenesis was performed using a Quick Change mutagenesis assay kit (Stratagene; Agilent Technologies, Inc.) in accordance with the manufacturer's instructions. Next, the mutated sequences of GLUT4, MEG3 and SNHG20 containing the mutated binding sites for miR-4486 and miR-223 were also cloned into pcDNA 3.1 vectors to generate mutant type (MUT) vectors of GLUT4, MEG3 and SNHG20. Then, 1x10⁵/well HCC-94 cells were co-transfected with MUT/WT GLUT4 vectors in conjunction with miR-4486 or miR-223 mimics (Thermo Fisher Scientific, Inc.), MEG3 vectors in conjunction with miR-223 mimics (Thermo Fisher Scientific, Inc.), as well as SNHG20 vectors in conjunction with miR-4486 mimics (Thermo Fisher Scientific, Inc.) using Lipofectamine 3000. The sequences of miR-4486 mimics were 5'-GCUGGGCGAGGCUGGCA-3' and the sequences of miR-223 mimics was 5'-ACCCCAUAAACU GUUUGACUGU-3'. The luciferase activities in transfected cells were measured 48 h after transfection using a Bright Glo assay kit (Promega Corporation) and normalized to Renilla luciferase activity in accordance with the manufacturer's instructions.

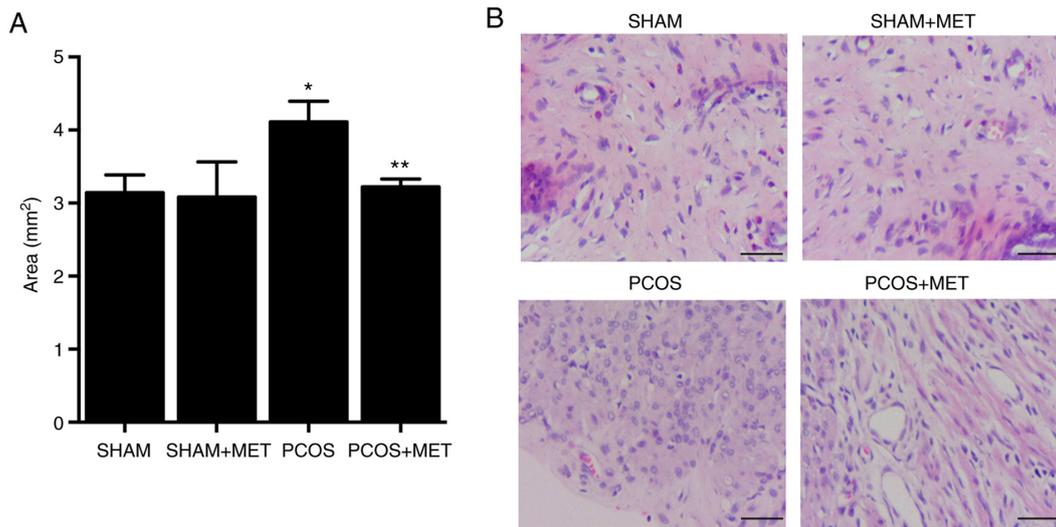


Figure 1. EH is increased in PCOS rats and MET treatment reverses EH. (A) The area of endometrium in PCOS rats was increased compared with the SHAM rats, and MET treatment decreased the elevated area of endometrium in PCOS rats. * $P < 0.05$ vs. SHAM rats; ** $P < 0.05$ vs. PCOS rats. (B) The administration of MET reversed EH in PCOS rats (scale bar, 25 μm). EH, endometrium hyperplasia; PCOS, polycystic ovary syndrome; MET, metformin.

Western blot analysis. Protein expression of GLUT4 in HCC-94 cells was measured using western blotting. In brief, the cells were first lysed and the protein was extracted in RIPA buffer (MilliporeSigma), and the concentration of GLUT4 protein was determined using a BCA assay kit (Thermo Fisher Scientific, Inc.). Subsequently, proteins in the supernatant were resolved via 10% SDS-PAGE and subsequently blotted onto a PVDF membrane (MilliporeSigma) in accordance with the manufacturer's instructions (50 $\mu\text{g}/\text{lane}$). Next, the membrane was blocked with 5% non-fat milk at room temperature for 1 h and incubated at 4°C overnight with anti-GLUT4 primary antibody (1:1,000; catalogue number ab188317; Abcam) and then further incubated for 1 h at room temperature with HRP-tagged secondary antibody (dilution: 1:5,000; catalogue number ab6721; Abcam) using β -actin as an internal reference protein (1:1,000; cat. no. ab8226; Abcam). Finally, after treatment with ECL reagent (Pierce; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions, the relative expression of GLUT4 was calculated using Quantity One 1-D Analysis software (V4.6.8, Bio-Rad Laboratories, Inc.).

Immunohistochemistry. Expression of GLUT4 protein in collected endometrial tissues was measured using routine immunohistochemistry assays. In brief, the tissues were fixed at room temperature for 20 min in 4% paraformaldehyde, dehydrated, embedded in paraffin and sliced into 5 μm sections, permeabilized using PBS containing 0.5% Triton X-100, blocked at room temperature for 1 h in 10% FBS, and then incubated in succession with anti-GLUT4 primary antibodies (1:500; cat. no. ab216661; Abcam) at room temperature for 1 h and HRP-tagged secondary antibodies (1:1,000; Cat. no. ab150077; Abcam) at room temperature for 30 min. Finally, after counterstaining with DAPI for 3 min at room temperature to stain the nuclei, the cells were visualized under a fluorescence microscope to analyze GLUT4 expression. The quantification was performed with a fluorescence microscope (IX71; Olympus Corporation) by using cellSens Software (V1.16, Olympus Corporation).

H&E. The degree of EH was determined in endometrial tissues (5 μm sections) by H&E staining using an H&E Staining Kit (Abcam) in accordance with the manufacturer's instructions. The tissues were fixed with 4% formalin for 24 h at room temperature. The areas of endometrium were measured in three sections from each rat at x2 magnification under an Olympus light microscope (Olympus Corporation), and the areas of endometrium were calculated using Micro Image (V2.5, Olympus Corporation).

Cell viability assay. HCC-94 cells were collected 48 h after transfection and subsequently seeded into a 96-well plate at a density of 1×10^4 cells per well in 100 μl DMEM. After the cells reached 80% confluence, they were cultured with Cell Counting Kit-8 (CCK-8) assay reagent (Abcam) for 2 h in the dark. Finally, the optical density value (450 nm) was measured using a microplate reader.

Statistical analysis. All experimental results are presented as the mean \pm standard deviation (SD). Each experiment was repeated at least 3 times. One-way ANOVA was utilized for comparisons between groups, and Tukey's test was used as the post hoc study. All statistical analyses were performed using SPSS version 22.0 (IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

MET treatment prevents EH in PCOS rats. The endometrial area was significantly increased in PCOS rats compared with the SHAM rats. Administration of MET significantly decreased the endometrial area in PCOS rats by 21.65% compared with the PCOS group, but did not change the endometrial area in the SHAM + MET rats (Fig. 1A). H&E staining analysis revealed that EH in PCOS rats was efficiently inhibited by MET treatment (Fig. 1B). These results indicated that MET treatment exerted a notable therapeutic effect on PCOS.

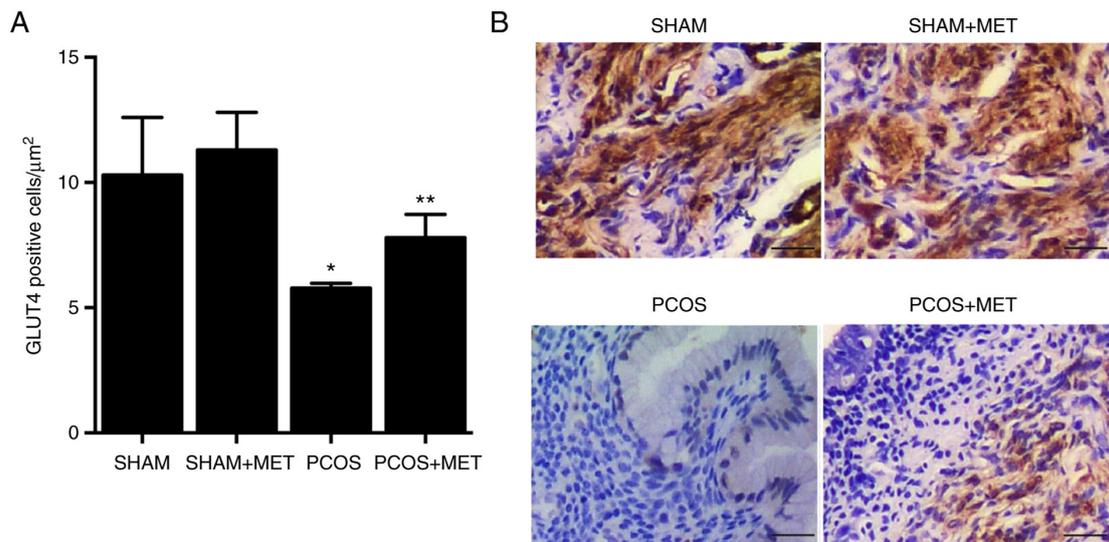


Figure 2. Repressed expression of GLUT4 in the endometrium of PCOS rats is partially restored by MET treatment as determined via immunohistochemistry analysis and quantification. (A) Quantification of immunohistochemistry analysis showed that the repressed GLUT4 expression in the endometrium was restored by MET treatment in PCOS rats. *P<0.05 vs. SHAM rats; **P<0.05 vs. PCOS rats. (B) Representative images of immunohistochemistry analysis of GLUT4 expression in all animal model groups (scale bar, 50 μm). PCOS, polycystic ovary syndrome; MET, metformin; GLUT4, glucose transporter type 4 insulin-responsive.

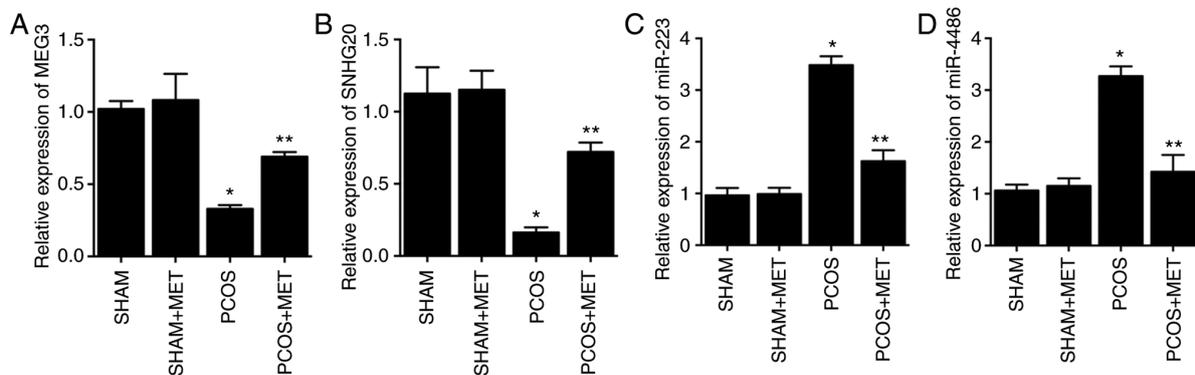


Figure 3. MET treatment partially restores the normal expression of lncRNA-MEG3, lncRNA-SNHG20, miR-223 and miR-4486 in the endometrium of PCOS rats as determined via reverse transcription-quantitative PCR. (A) The expression of lncRNA-MEG3 was repressed in the endometrium of PCOS rats, while MET treatment recovered the repressed lncRNA-MEG3 expression in PCOS rats. (B) The expression of lncRNA-SNHG20 was repressed in the endometrium of PCOS rats, while MET treatment recovered the repressed lncRNA-MEG3 expression in PCOS rats. (C) The expression of miR-223 was increased in the endometrium of PCOS rats, and it was restored by MET treatment in PCOS rats. (D) The expression of miR-4486 was promoted in the endometrium of PCOS rats, and it was restored by MET treatment in PCOS rats. *P<0.05 vs. SHAM rats; **P<0.05 vs. PCOS rats. PCOS, polycystic ovary syndrome; MET, metformin; lnc, long non-coding; MEG3, maternally expressed gene 3; SNHG20, small nucleolar RNA host gene 20; miR, microRNA.

MET treatment rescues the repressed expression of GLUT4 in the endometrium of PCOS rats. Immunohistochemistry was performed to evaluate the expression of GLUT4 in the endometrium of PCOS rats in response to different treatments. Expression of GLUT4 in the endometrium of PCOS rats was significantly reduced by 43.89% compared with the SHAM group. Treatment with MET in the PCOS + MET group significantly rescued the expression of GLUT4 by 42.33% compared with the PCOS group. Meanwhile, treatment of SHAM rats with MET had no effect on the expression of GLUT4 in the endometrium (Fig. 2A and B).

MET treatment alleviates the dysregulation of lncRNA-MEG3, lncRNA-SNHG20, miR-223 and miR-4486 in the endometrium of PCOS rats. In the present study, qPCR was

performed to analyze the expression of lncRNA-MEG3 and lncRNA-SNHG20 in the endometrium of PCOS rats in response to different treatments. Compared with the SHAM group, the expression levels of lncRNA-MEG3 (Fig. 3A) and lncRNA-SNHG20 (Fig. 3B) were significantly inhibited in the endometrium of PCOS rats by 67.65 and 85.57%, respectively. Administration of MET significantly upregulated the expression of lncRNA-MEG3 (Fig. 3A) and lncRNA-SNHG20 (Fig. 3B) by 109.09 and 343.62%, respectively, in the endometrium of PCOS rats. Expression of lncRNA-MEG3 and lncRNA-SNHG20 in SHAM + MET rats remained unchanged after MET administration. Moreover, the expression of miR-223 and miR-4486 exhibited the opposite pattern. Compared with the SHAM group, expression levels of miR-223 and miR-4486 were significantly elevated in the endometrium of PCOS rats by

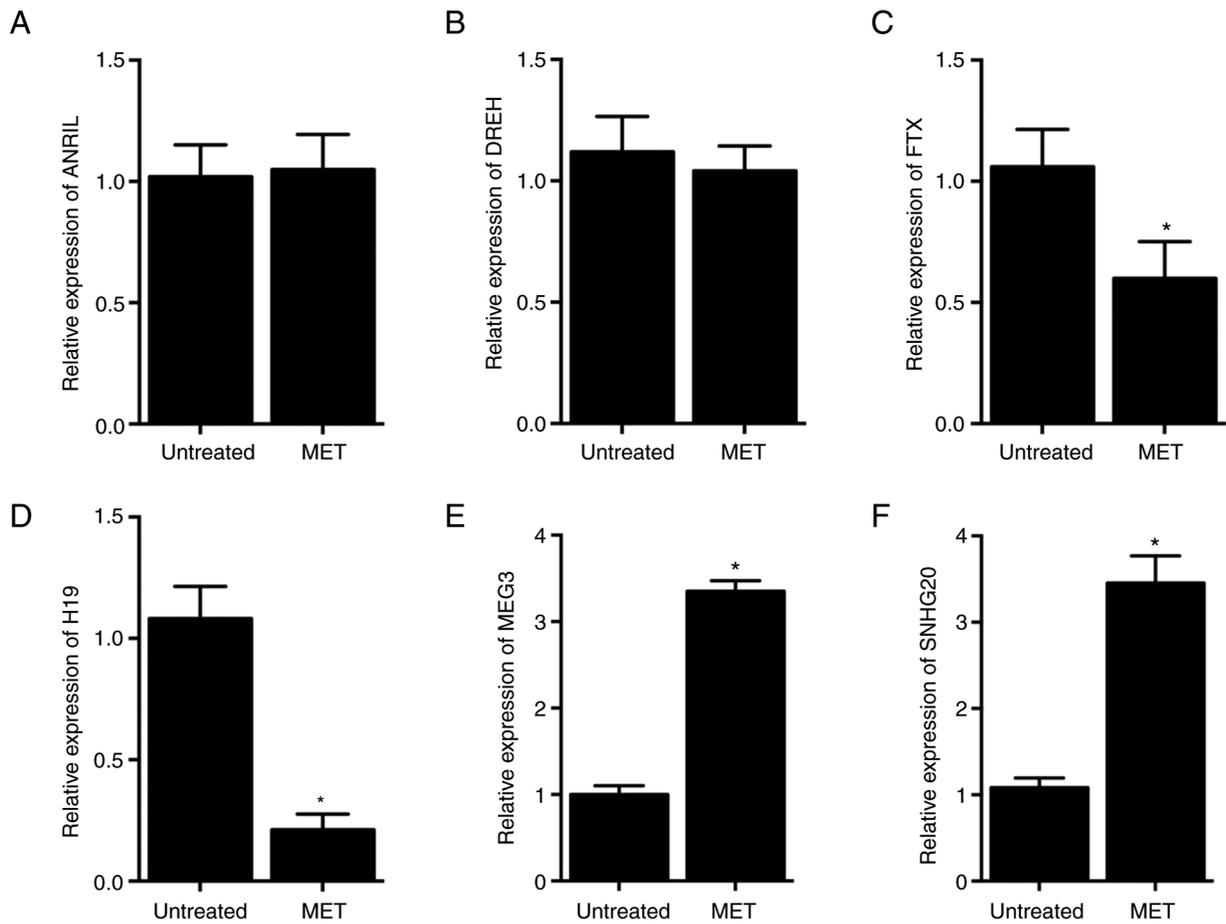


Figure 4. Expression of lncRNA-MEG3 and lncRNA-SNHG20 is promoted by MET treatment in HCC-94 cells as determined via reverse transcription-quantitative PCR. (A) The expression of lncRNA-ANRIL was not altered by MET treatment in HCC-94 cells. (B) The expression of lncRNA-DREH was not altered in MET-treated HCC-94 cells. (C) The expression of lncRNA-FTX was decreased by MET treatment in HCC-94 cells. (D) The expression of lncRNA-H19 was reduced in MET-treated HCC-94 cells. (E) The expression of lncRNA-MEG3 was promoted by MET treatment in HCC-94 cells. (F) The expression of lncRNA-SNHG20 was upregulated in MET-treated HCC-94 cells. * $P < 0.05$ vs. untreated cells. MET, metformin; lnc, long non-coding; MEG3, maternally expressed gene 3; SNHG20, small nucleolar RNA host gene 20; ANRIL, antisense non-coding RNA in the INK4 locus.

360.88 and 308.55%, respectively, while treatment with MET significantly reduced the expression of miR-223 (Fig. 3C) and miR-4486 (Fig. 3D) by 53.34 and 87.00%, respectively, in the endometrium of PCOS rats.

Differential effects of MET treatment on the expression of lncRNAs in HCC-94 cells. In this study, the effect of MET treatment on the expression of six lncRNAs was analyzed in HCC-94 cells. No significant difference was observed in the expression of lncRNA-ANRIL (Fig. 4A) or lncRNA-DREH (Fig. 4B) in HCC-94 cells in response to MET treatment. By contrast, expression of lncRNA-FTX (Fig. 4C) and lncRNA-H19 (Fig. 4D) was significantly inhibited by 43.40 and 80.46%, respectively, while expression of lncRNA-MEG3 (Fig. 4E) and lncRNA-SNHG20 (Fig. 4F) was significantly increased by 235.15 and 219.10%, respectively, in HCC-94 cells undergoing MET treatment.

Knockdown of MEG3 and SNHG20 inhibits GLUT4 mRNA expression via upregulating miR-223 and miR-4486 expression, respectively, in HCC-94 cells. As shown by the results, compared with the NC siRNA group, MEG3 expression was significantly suppressed by the transfection of MEG3 siRNA

(Fig. 5A), while the expression of SNHG20 was decreased by the transfection of SNHG20 siRNA (Fig. 5B), thus verifying the successful transfection of MEG3 siRNA and SNHG20 siRNA. Moreover, the expression of miR-223 (Fig. 5C) and miR-4486 (Fig. 5D) was promoted by transfection with MEG3 siRNA and SNHG20 siRNA, respectively. However, the gene expression of GLUT4 was both significantly inhibited in HCC-94 cells transfected with MEG3 siRNA or SNHG20 siRNA compared with the NC siRNA group (Fig. 5E).

Knockdown of MEG3 and SNHG20 alters the effects of MET on the expression of their competing miRNAs and GLUT mRNA and protein expression in HCC-94 cells. Expression of lncRNA-MEG3 was significantly upregulated by 205.10% in response to MET treatment alone. Compared with the MET + NC siRNA group, MET + lncRNA-MEG3 siRNA significantly reduced the upregulated expression of lncRNA-MEG3 by 59.67%, while MET + lncRNA-SNHG20 siRNA into HCC-94 cells had no effect on the expression of lncRNA-MEG3 induced by MET treatment (Fig. 6A). Moreover, the expression of miR-223 was significantly repressed by 76.85% upon MET treatment, and lncRNA-MEG3 siRNA rather than lncRNA-SNHG20

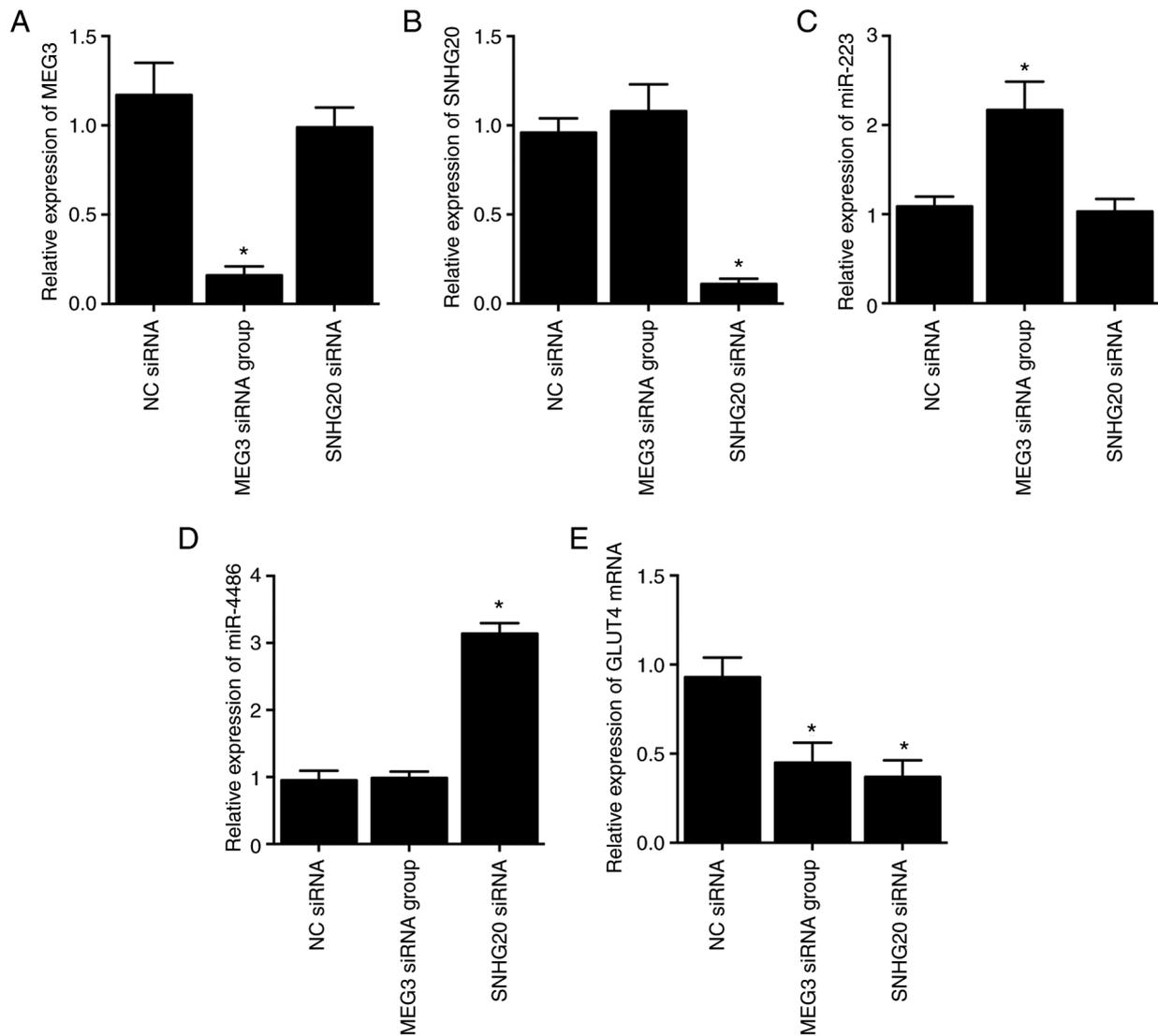


Figure 5. Knockdown of lncRNA-MEG3 and lncRNA-SNHG20 upregulates the expression of miR-223 and miR-4486, respectively, and inhibits the expression of GLUT4 in HCC-94 cells as determined via reverse transcription-quantitative PCR. (A) The successful transfection of lncRNA-MEG3 siRNA significantly reduced the expression of lncRNA-MEG3 in HCC-94 cells. (B) The successful transfection of lncRNA-SNHG20 siRNA significantly reduced the expression of lncRNA-SNHG20 in HCC-94 cells. (C) The knockdown of lncRNA-MEG3 significantly promoted the relative expression of miR-223 in HCC-94 cells. (D) The knockdown of lncRNA-SNHG20 significantly increased the expression of miR-4486 in HCC-94 cells. (E) The knockdown of lncRNA-MEG3 and lncRNA-SNHG20 significantly inhibited the expression of GLUT4 mRNA in HCC-94 cells. *P<0.05 vs. NC siRNA group. lnc, long non-coding; MEG3, maternally expressed gene 3; SNHG20, small nucleolar RNA host gene 20; miR, microRNA; GLUT4, glucose transporter type 4 insulin-responsive; siRNA, small interfering RNA; NC, negative control.

showed considerable effects in restoring the expression of miR-223, which was upregulated by 190.44% in the MET + lncRNA-MEG3 siRNA group (Fig. 6B). Similarly, the expression of lncRNA-SNHG20 and miR-4486 was also restored by the transfection of lncRNA-SNHG20 siRNA in HCC-94 cells treated with MET (Fig. 6C and D). Subsequently, the expression levels of GLUT4 mRNA and protein were analyzed via qPCR and western blotting, respectively. Expression of GLUT4 mRNA (Fig. 6E) and protein (Fig. 6F) was notably increased by 225.10 and 221.26%, respectively, in HCC-94 cells treated with MET. The transfection of lncRNA-MEG3 and lncRNA-SNHG20 siRNAs restored the normal expression of GLUT4 mRNA and protein increased by MET. These results indicated that expression of GLUT4 was associated with that of lncRNA-MEG3 and lncRNA-SNHG20.

Luciferase activities of lncRNAs and GLUT4 are inhibited by their targeting miRNAs. Luciferase assays were performed to confirm the regulatory relationship among lncRNA-SNHG20-miR-4486 (Fig. 7A), miR-4486-GLUT4 (Fig. 7B), lncRNA-MEG3-miR-223 (Fig. 7C) and miR-223-GLUT4 (Fig. 7D). WT and MUT luciferase vectors containing target sequences of the above miRNAs were constructed and then transfected into HCC-94 cells with corresponding miRNA mimics. As shown in Fig. 7, the luciferase activities of lncRNA-SNHG20 WT (Fig. 7A) and GLUT4 WT (Fig. 7B) were significantly inhibited by miR-4486. The luciferase activities of lncRNA-MEG3 WT (Fig. 7C) and GLUT4 WT (Fig. 7D) were significantly repressed by miR-223.

Knockdown of GLUT4 increases the viability of HCC-94 cells. HCC-94 cells were then transfected with GLUT4 siRNA, and successful transfection was validated by significantly

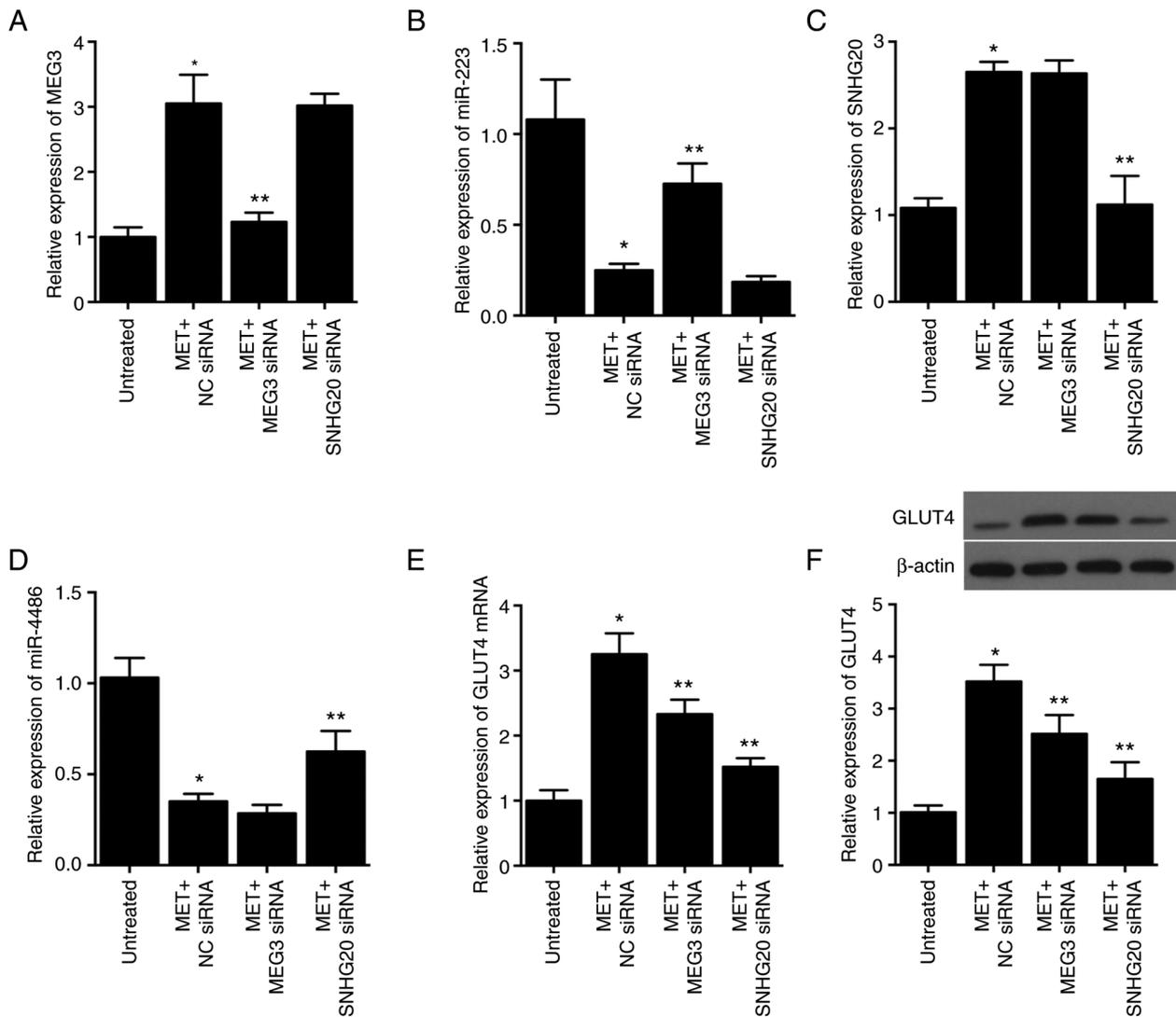


Figure 6. MET treatment upregulates the expression of lncRNA-MEG3, lncRNA-SNHG20 and GLUT4, and downregulates the expression of miR-223 and miR-4486 in HCC-94 cells, while the effect of MET is reversed by siRNAs targeting lncRNA-MEG3 and lncRNA-SNHG20 as determined via RT-qPCR and Western blotting. (A) MET treatment upregulated the expression of lncRNA-MEG3 in HCC-94 cells, and the successful transfection of lncRNA-MEG3 siRNA was validated by the significantly reduced lncRNA-MEG3 level in MET-treated HCC-94 cells. (B) MET treatment led to the downregulation of miR-223 in HCC-94 cells, and the suppressive effect of MET was reversed by the transfection of lncRNA-MEG3 siRNA. (C) MET treatment upregulated the expression of lncRNA-SNHG20 in HCC-94 cells, and successful transfection of lncRNA-SNHG20 siRNA was validated by the significantly reduced lncRNA-SNHG20 level in MET-treated HCC-94 cells. (D) MET treatment significantly suppressed the expression of miR-4486 in HCC-94 cells, and the inhibitory effect of MET was partially reversed by the transfection of lncRNA-SNHG20 siRNA. (E) RT-qPCR showed that the MET treatment upregulated the expression of GLUT4 mRNA in HCC-94 cells, and the effect of MET was partially reversed by siRNAs targeting lncRNA-MEG3 and lncRNA-SNHG20. (F) The results of western blotting and semi-quantification showed that MET treatment upregulated the expression of GLUT4 protein in HCC-94 cells, and the effect of MET was partially reversed by siRNAs targeting lncRNA-MEG3 and lncRNA-SNHG20. * $P < 0.05$ vs. untreated cells; ** $P < 0.05$ vs. MET + NC siRNA group. MET, metformin; lnc, long non-coding; MEG3, maternally expressed gene 3; SNHG20, small nucleolar RNA host gene 20; miR, microRNA; GLUT4, glucose transporter type 4 insulin-responsive; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control.

reduced expression of GLUT4 mRNA compared with the NC siRNA group (Fig. 8A). Then, lncRNA-MEG3 (Fig. 8B) and lncRNA-SNHG20 (Fig. 8C) were overexpressed in HCC-94 cells. Accordingly, it was found that the overexpression of lncRNA-MEG3 reduced cell viability (Fig. 8D), while the overexpression of lncRNA-SNHG20 exhibited no effect on the viability of HCC-94 cells (Fig. 8E). Moreover, when lncRNA-MEG3-overexpressing cells were transfected with GLUT4 siRNA, the reduced cell viability was clearly restored (Fig. 8D). Moreover, transfection with GLUT4 siRNA in HCC-94 cells overexpressing lncRNA-SNHG20 also increased the viability of HCC-94 cells (Fig. 8E).

Discussion

In the present study, a PCOS rat model was established to analyze the effect of MET treatment on EH. It was found that EH in PCOS rats was inhibited by MET treatment. In addition, immunohistochemistry was performed to evaluate the expression of GLUT4 in the endometrium of PCOS rats treated in response to different conditions. Also, the diminished expression of GLUT4 could be contributed to a complicated network of GLUT4 regulation, including potential functions of lncRNAs and miRNAs, which was not been extensively studied. In the endometrium of PCOS rats, the deregulation

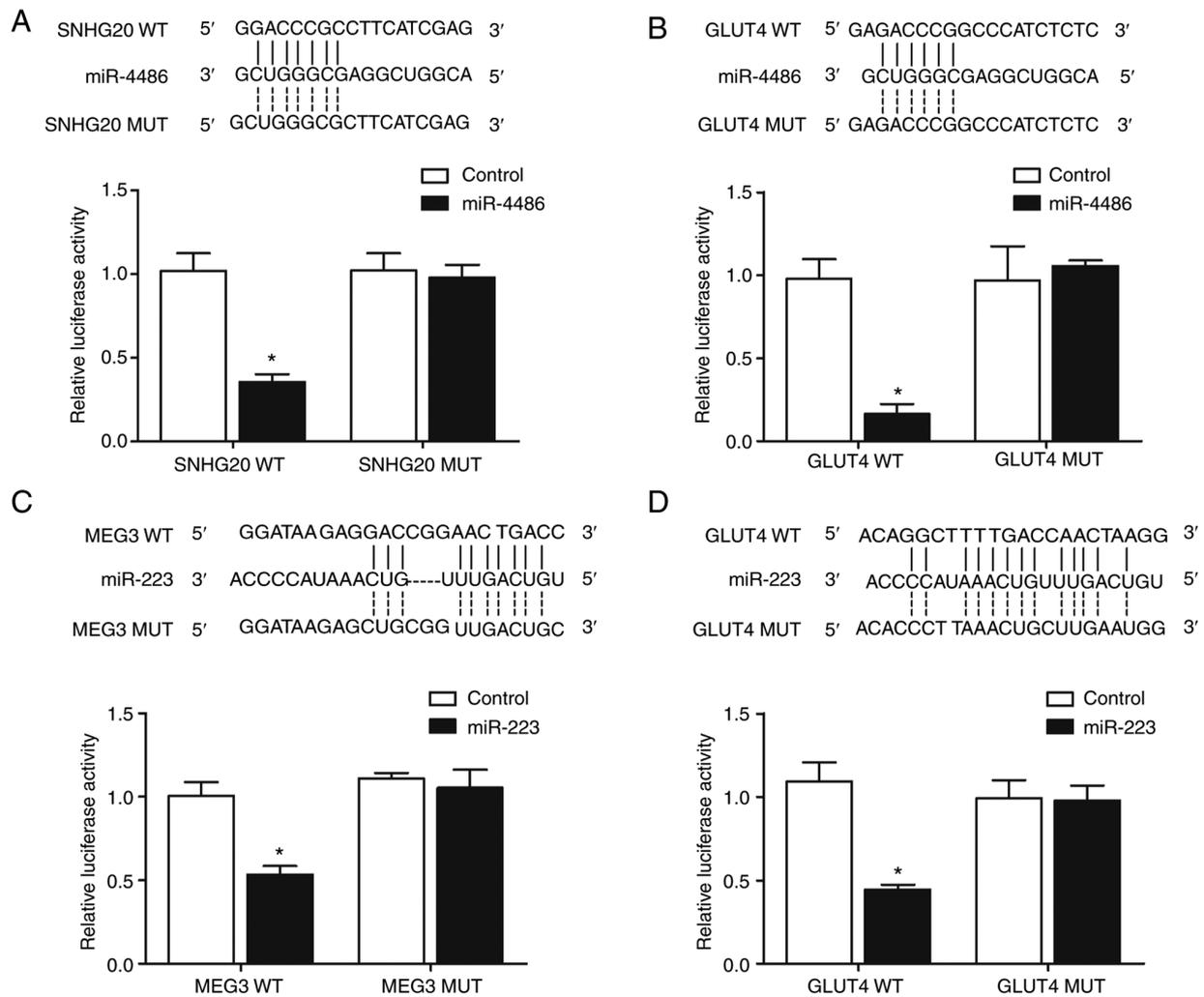


Figure 7. Targeting relationship between miR-4486 and lncRNA-SNHG20, miR-4486 and GLUT4, miR-223 and lncRNA-MEG3, as well as between miR-223 and GLUT4 as determined via a luciferase assay and sequence analysis. (A) Sequence analysis indicated the potential binding between miR-4486 and lncRNA-SNHG20, which was proved by the reduced luciferase activity in cells co-transfected with lncRNA-SNHG20 WT and miR-4486. (B) Sequence analysis indicated the potential binding between miR-4486 and GLUT4, which was proved by the reduced luciferase activity in cells co-transfected with GLUT4 WT and miR-4486. (C) Sequence analysis indicated the potential binding between miR-223 and lncRNA-MEG3, which was proved by the reduced luciferase activity in cells co-transfected with lncRNA-MEG3 WT and miR-223. (D) Sequence analysis indicated the potential binding between miR-223 and GLUT4, which was proved by the reduced luciferase activity in cells co-transfected with GLUT4 WT and miR-223. *P<0.05 vs. control group. miR, microRNA; lnc, long non-coding; SNHG20, small nucleolar RNA host gene 20; GLUT4, glucose transporter type 4 insulin-responsive; MEG3, maternally expressed gene 3; WT, wild-type; MUT, mutant.

of GLUT4 was restored by MET treatment. Meanwhile, qPCR was performed to measure the differential expression of lncRNA-MEG3, lncRNA-SNHG20, miR-223 and miR-4486 under different conditions. The decreased expression of lncRNA-MEG3/lncRNA-SNHG20 and increased expression of miR-223/miR-4486 in the endometrium of PCOS rats were recovered by MET treatment.

MET is a derivative of galegine, which is a natural compound synthesized by the herb *Galega officinalis* (7). MET is the most frequently used oral antidiabetic drug and improves serum lipid profiles, influences the process of hemostasis and exhibits anti-inflammatory effects (32). In the 1920s, initially discovered as derivatives of galegine, MET and phenformin were successfully isolated, although their uses in clinical applications were not confirmed until later on (33).

MEG3 was first discovered as a compound similar to that encoded by the Glycosyltransferase-like protein (Gtl2) gene

in mice, and it is highly expressed in tissues in normal human organs, especially in pituitary and other brain tissues (34). Nevertheless, the mRNA of MEG3 is not expressed in tumors of the pituitary gland or in cancer cells of humans and a number of other mammalian species (35). More importantly, as one of several isoforms of MEG3 proteins, the MEG3a protein acts as a powerful inhibitor of cell growth (35). In addition, the colony formation assay showed that MEG3a induced an ~60% reduction in the number of cell colonies in response to MEG3a transfection into cancer cells, including HeLa cervical carcinoma, H4 neuroglioma cancer and MCF7 breast adenocarcinoma cancer cells. The MEG3 gene is located at locus 14q32.3 of the human genome. Of note, the 14q32.3 locus contains a gene encoding a tumor suppressor that participates in the pathogenesis and metastasis of several tumors, including nasopharyngeal carcinoma, meningiomas, colorectal carcinoma and leukemia (36,37). In the present study, HCC-94 cells

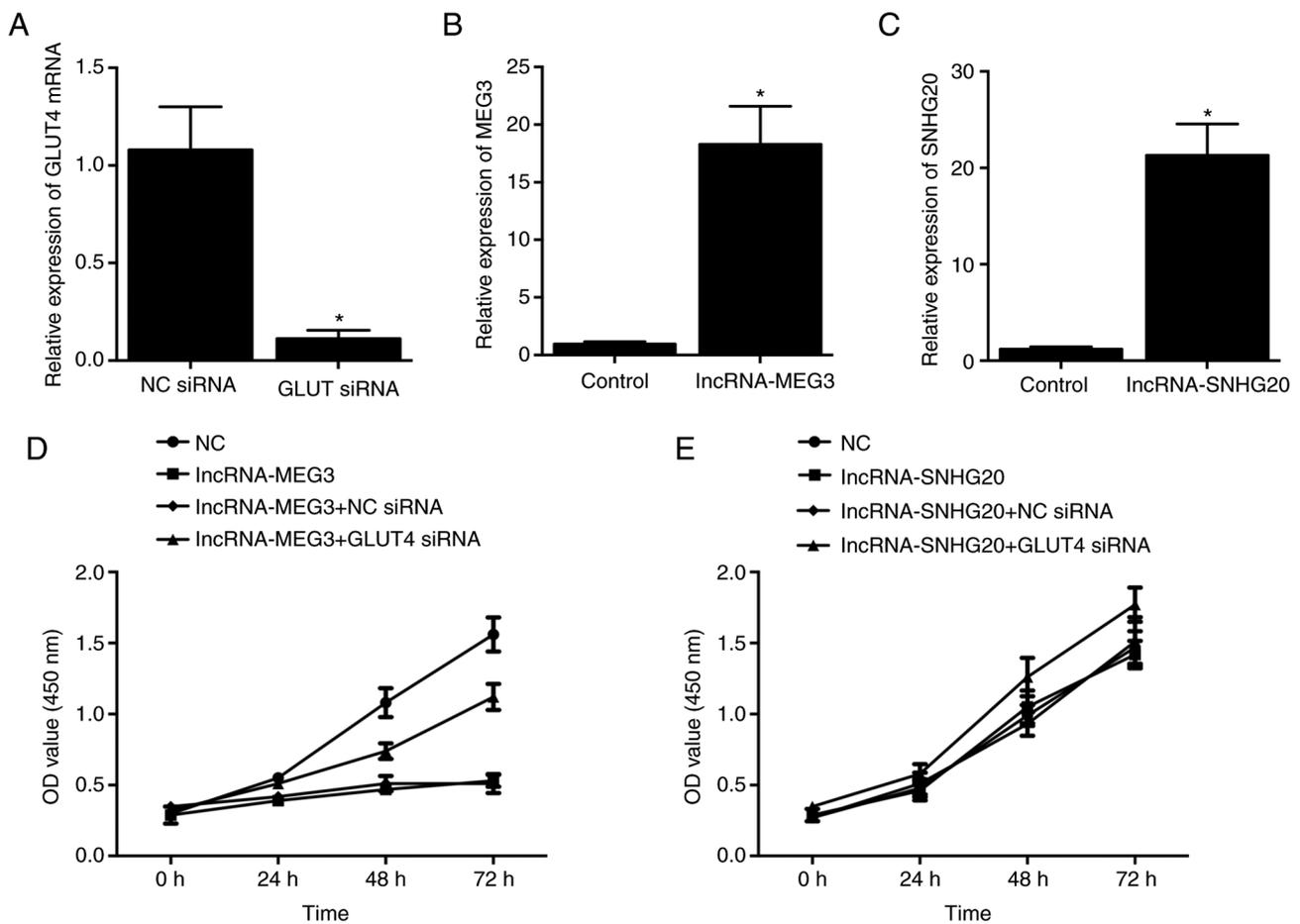


Figure 8. Successful transfection of GLUT4 siRNA in HCC-94 cells as validated via reverse transcription-quantitative PCR and the overexpression of lncRNA-MEG3 suppresses viability of HCC-94 cells, while the knockdown of GLUT4 mRNA increases the viability of HCC-94 cells. (A) The successful transfection of GLUT4 siRNA significantly reduced the expression of GLUT mRNA in HCC-94 cells. * $P < 0.05$ vs. NC siRNA group. (B) lncRNA-MEG3 was overexpressed in HCC-94 cells transfected with lncRNA-MEG3. (C) lncRNA-SNHG20 was overexpressed in HCC-94 cells transfected with lncRNA-SNHG20. * $P < 0.05$ vs. control. (D) The knockdown of GLUT4 mRNA restored cell viability suppressed by the overexpression of lncRNA-MEG3. (E) The knockdown of GLUT4 mRNA increased cell viability, while the overexpression of lncRNA-SNHG20 exhibited no effect on the cell viability. GLUT4, glucose transporter type 4 insulin-responsive; siRNA, small interfering RNA; lnc, long non-coding; MEG3, maternally expressed gene 3; NC, negative control; SNHG20, small nucleolar RNA host gene 20.

were transfected with lncRNA-MEG3/SNHG20 siRNAs and the expression of lncRNAs, their competing miRNAs and GLUT4 were analyzed. It was found that MET upregulated the expression of lncRNAs/GLUT4 and downregulated the expression of their competing miRNAs.

lncRNA MEG3 enhances pyroptosis in HAEC cells. In addition, MEG3 acts as a sponge of miR-223 based on the complementarity between the sequence of MEG3 and miR-223, reducing expression levels of miR-223, while increasing expression levels of NACHT, LRR and PYD domains-containing protein 3 to enhance levels of pyroptosis. Moreover, suppression of the expression level of miR-223 also reduces the activity of melatonin in inhibiting the pyroptosis of HAEC cells in the presence of oxidized low-density lipoprotein (27).

SNHG20 is an lncRNA initially discovered in liver carcinoma cells (38). Increased levels of SNHG20 expression are considered a valuable biomarker for the prognosis of hepatocellular carcinoma (38). In addition, an oncogenic role of SNHG20 is also present in colorectal cancer due to high expression levels of SNHG20 being closely associated with the metastasis of colorectal cancer (39).

Decreased levels of SNHG20 expression were shown to reduce the migration and proliferation of glioma cells as SNHG20 acts as a ceRNAs to reduce the levels of miR-4486 expression (28). In the present study, luciferase assays were performed to explore the inhibitory role of miRNAs on their targeting lncRNAs and the GLUT4 gene. The luciferase activities of lncRNA-SNHG20 WT and GLUT4 WT were significantly inhibited by miR-4486, while the luciferase activities of lncRNA-MEG3 WT and GLUT4 WT were remarkably repressed by miR-223.

GLUT4 is highly expressed in tissues with a high level of insulin expression (40). Therefore, the presence of GLUT4 enhances glucose uptake, while the inhibition of GLUT4 signaling results in insulin resistance (41). Furthermore, celastrol regulates the expression of some miRNAs involved in the regulation of insulin (42,43). For example, celastrol reverses the role of Palmitic acid in inhibiting expression levels of GLUT4 (44). More recently, it was demonstrated that MET treatment increases expression levels of GLUT4 in the endometrium of patients with PCOS and EH (7). Furthermore, the oral dose of MET promotes the protein expression of

myocyte-specific enhancer factor 2A and AMPK (7). These results clearly demonstrated that MET alleviates insulin resistance in endometrial tissues by increasing levels of GLUT4 expression, thus improving the conditions of patients with PCOS (7).

In summary, the findings of the current study demonstrated that the knockdown of GLUT4 was associated with the development of EH during PCOS, while MET was effective for the treatment of EH by upregulating the expression of GLUT4 via the lncRNA-MEG3/miR-223/GLUT4 and lncRNA-SNHG20/miR-4486/GLUT4 signaling pathways. Specifically, MET administration upregulated the expression of lncRNA-MEG3 and lncRNA-SNHG20, thereby downregulating the expression of miR-223 and miR-4486, respectively, leading to an increase in GLUT4 expression.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XZZ and JL conceived and designed the study. JL, YCZ and LC collected the literature, JL, YCZ, LC, RLL, YMN and XZZ performed the experiments, JL, YCZ and LC collected and analyzed the data. XZZ and JL confirm the authenticity of all the raw data. JL, XZZ and YCZ drafted the article. RLL and YMN revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was performed in line with the Guide for the Care and Use of Laboratory Animal by National Research Council (US) Committee (8th edition) and the protocols were approved by the Animal Ethics Committee of Zhejiang Chinese Medical University Laboratory animal research center (approval no. ZSLL-2016-42; Hangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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