

MicroRNA-33b-5p is overexpressed and inhibits GLUT4 by targeting HMGA2 in polycystic ovarian syndrome: An *in vivo* and *in vitro* study

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Abstract. Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disease, but its pathogenesis remains largely unknown. The present study explored the role of microRNA-33b-5p (miR-33b-5p) in PCOS pathogenesis, with a particular focus on its role in regulating glucose transporter 4 (GLUT4). A rat model of PCOS was developed by injecting female SD rats with insulin and HCG. miR-33b-5p, GLUT4, sterol regulatory element-binding protein 1 (SREBF1), and high mobility group A2 (HMGA2) expression in rat ovarian tissues was examined by qRT-PCR and immunohistochemistry. The effect of a high dose of either glucose or insulin on miR-33b-5p, GLUT4, SREBF1 and HMGA2 expression was also examined in cultured adipocytes by qRT-PCR and western blotting. Additionally, the luciferase reporter assay and chromatin immunoprecipitation (ChIP) were used to explore the role of miR-33b-5p in regulating HMGA2, SREBF-1 and/or GLUT4. Elevated levels of miR-33b-5p expression were detected in the ovarian tissues of insulin resistant PCOS rats, and those levels were negatively correlated with those of GLUT4, HMGA2 and SREBF1 expression ($P < 0.05$). Immunohistochemistry studies revealed that GLUT4, SREBF1, and HMGA2 expression levels in the ovarian tissues of insulin resistant PCOS rats were significantly lower than those in other groups of rats. In cultured

adipocytes, excess extracellular glucose or insulin increased miR-33b-5p expression but reduced GLUT4, SREBF1 and HMGA2 expression, whereas the levels of GLUT4, SREBF1 and HMGA2 were elevated by inhibition of miR-33b-5p. HMGA2 could directly bind to the 5'-promoter region of *GLUT4* and promote its expression, and could also promote SREBF1 expression. Moreover, SREBF1 could also directly bind to the 5'-promoter region of *GLUT4* and promote its expression. Our findings revealed that miR-33b-5p was overexpressed in the ovarian tissues of insulin resistant PCOS rats, and thus may play an important role in the development of insulin resistance in PCOS patients. miR-33b-5p can inhibit GLUT4 production by targeting *HMGA2*, and in addition, HMGA2 and SREBF1 are important molecules involved in modulating GLUT4 expression.

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disease affecting 5-20% of women of reproductive age worldwide (1). PCOS is characterized by high luteinizing hormone levels, polycystic ovarian morphology, hyperandrogenism, hyperinsulinemia, and insulin resistance. Excessive androgen production by the ovaries is the main feature of PCOS (1,2). The prevalence of PCOS has increased during the past decade, and its adverse effects on the health of women are becoming more serious (3,4). Moreover, women with PCOS are at increased risks for cardiovascular disease and diabetes (5-7). Therefore, when considering its increasing prevalence, more effective methods for preventing or treating PCOS are needed. Presently, the pathogenesis of PCOS is not fully understood, and requires further investigation. More than 50% of PCOS patients are insulin resistant (2,8), which can partly explain their increased risk for diabetes and cardiovascular disease. Furthermore, insulin resistance can also be the cause for hyperlipidemia and hyperinsulinemia in PCOS patients. Therefore, identifying key factors involved in the development of insulin resistance should provide us with a better understanding of PCOS pathogenesis. For example, glucose transporter 4 (GLUT4) is a protein that plays critical roles in

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controlling the translocation of glucose into adipocytes and regulating glucose homeostasis (9,10). GLUT4 is strongly associated with insulin sensitivity, and its dysfunction has been reported to cause insulin resistance in PCOS patients (11-13). High mobility group A2 (HMGA2), a non-histone chromosomal protein, is associated with lipomas, suggesting its roles in adipogenesis and insulin resistance (14,15). SREBF1, also known as sterol regulatory element-binding protein 1 (SREBP1), is a major regulator of genes involved in metabolic pathways and sterol biosynthesis (16-18), and its expression is significantly increased in the endometrium of PCOS patients (17). More importantly, the transcripts for the two hsa-miR-33 isoforms (a and b) are located in a non-coding region of the *SREBF* gene, suggesting a potential role for miR-33 in regulating SREBF1 expression (19). However, the underlying molecular mechanisms that regulate these key factors and lead to insulin resistance in PCOS patients remain poorly understood.

MicroRNAs (miRNAs) consist of a group of small non-coding RNAs ~20-24 nt in length (20). Previous studies have revealed that miRNAs play key roles in modulating the functions of human bodies (20-22). miRNAs mainly exert their effects by binding to the 3'-untranslated regions (3'-UTRs) of target genes and inhibiting transcription of the mRNAs of those genes (22). Recent studies have proposed that miRNAs are important gene regulators involved in human diseases, including endocrine and metabolic diseases (23,24). Some miRNAs have been found to play critical roles in modulating glucose homeostasis, and the aberrant expression of those miRNAs can result in insulin resistance that is often involved in the development of metabolic diseases (25,26). Several miRNAs (i.e. miR-93 and miR-320) have been implicated in the pathogenesis of PCOS (27-32). However, our understanding of their involvement in the development of PCOS is limited, and more studies are needed to clarify their roles in PCOS pathogenesis. In the present study, we examined the levels of miR-33b-5p expression in the ovarian tissues of PCOS rats with insulin resistance, and the correlations between miR-33b-5p levels and the expression of three different proteins: GLUT4, HMGA2 and SREBF1. We also investigated the possible role of miR-33b-5p in regulating GLUT4, HMGA2 and SREBF1 in cultured adipocytes.

Materials and methods

Animal model of PCOS. Twenty-four female Sprague-Dawley (SD) rats (180-250 g) were obtained from the Center of Laboratory Animal Science, Hubei, China. The rats were group-housed in a room with a regular 12-h light/dark cycle, and had free access to food and water. All animals were treated in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the study protocol was approved by the Animal Ethics Committee of Hubei University of Arts and Science.

The PCOS model was developed in female rats according to the method described by Poretsky *et al* (33). Female rats (70 to 85 days old) were subcutaneously injected with increasing doses of insulin (0.5 IU, 5.0 IU and 5.0 IU/day) for the first 10 days, and then with 5 IU insulin + 1.5 IU HCG from day 11 through day 22. The rats were fed a high fat diet for 6 weeks,

and their weights were measured weekly. Rats in the control groups were fed a normal diet every day. Finally, the PCOS model rats displayed typical characteristics of PCOS, such as hyperinsulinemia, insulin resistance, weight gain, and polycystic degeneration in the ovaries (33). Fasting blood glucose and fasting insulin levels were assessed and the homeostatic model assessment-insulin resistance (HOMA-IR) of each rat was calculated. Next, the rats were assigned to four different groups: a non-PCOS/non-IR group, a non-PCOS/IR group, a PCOS/non-IR group, and a PCOS/IR group. The rats were then anesthetized with 10% chloral hydrate, and laparotomies were performed to collect ovarian tissue specimens for further processing. Insulin resistance was assessed by the HOMA-IR index, and rats with a HOMA-IR ≥ 2.5 were considered insulin resistant (IR).

Hematoxylin and eosin (H&E) and immunohistochemical staining. The ovaries of each rat were removed, processed, and sectioned at 4-mm thickness; after which, the sections were stained with H&E and examined for evidence of morphological changes. Five sections were obtained from each ovary. Immunohistochemistry (IHC) was then used to examine the ovarian tissues for SREBF1, HMGA2 and GLUT4 expression. Tissue sections used for immunohistochemistry were first deparaffinized and hydrated, and endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in PBS. Next, the tissue sections were incubated in blocking buffer for 60 min, and then incubated overnight at 4°C with primary antibodies that included GLUT4 antibody (1:500; rabbit polyclonal; cat. no. PB0143; Boster Biological Technology, Wuhan, China), SREBF1 antibody (1:500; rabbit polyclonal; cat. no. 14088-1-AP; Proteintech; Wuhan Sanying Biotechnology, Wuhan, China), and HMGA2 antibody (1:500; rabbit polyclonal; cat. no. bs-0556R; Bioss Antibodies, Beijing, China). They were then incubated with horseradish peroxidase-conjugated secondary antibodies [1:5,000; goat anti-rabbit IgG H&L (HRP); cat. no. ab205718; Abcam, Cambridge, MA, USA] as per the manufacturer's instructions. Positively-labeled cells were visualized using a streptavidin-HRP conjugate and DAB chromogen. The tissue sections were then counterstained with hematoxylin, dehydrated, and mounted in DPX.

Culturing of human adipocytes in high glucose medium. Human adipocyte cells (ScienCell Research Laboratories, Inc., San Diego, CA, USA) were incubated with adipocyte maintenance medium at 37°C in a humidified incubator containing 5-10% CO₂. The adipocyte cells were then cultured in normal adipocyte maintenance medium (control group) or treated with different concentrations of high-glucose adipocyte maintenance medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) (0, 3.15, 9.45 or 28.35 g/l) for 24 h. After culture, the adipocytes were collected and their levels of miR-33b-5p, SREBF1, HMGA2 and GLUT4 were examined using real-time polymerase chain reaction (PCR) or and western blotting methods.

Culturing of human adipocytes in different concentrations of insulin. Human adipocytes were incubated with adipocyte maintenance medium at 37°C in a humidified incubator

Table I. PCR primer information.

ID	Sequence (5'-3')	Product length (bp)
U6 F	CTCGCTTCGGCAGCACA	
U6 R	AACGCTTCACGAATTTGCGT	
r-GAPDH F	CCTCGTCTCATAGACAAGATGGT	169
r-GAPDH R	GGGTAGAGTCATACTGGAACATG	
h-GAPDH F	TGTTTCGTTCATGGGTGTGAAC	154
h-GAPDH R	ATGGCATGGACTGTGGTCAT	
r-miR-33-5p RT	CTCAACTGGTGTCTGTTGGAGTC GGCAATTCAGTTGAGTGCAATGC	
r-miR-33-5p F	ACACTCCAGCTGGGGTGCATTGTAGTTGCAT	
h-miR-33b-5p RT	CTCAACTGGTGTCTGTTGGAGTC GGCAATTCAGTTGAGGCAATGCA	
h-miR-33b-5p F	ACACTCCAGCTGGGGTGCATTGCTGTTGCA	
ALL R	CTCAACTGGTGTCTGTTGGA	
r-SREBF1 F	TTACAGCACAGCAACCAGAA	132
r-SREBF1 R	CATGCCCTCCATAGACACAT	
h-SREBF1 F	GCAACACAGCAACCAGAAACT	191
h-SREBF1 R	GGAAAGGTGAGCCAGCATC	
r-HMGA2 F	CCTGTGAGCCCTCTCCTAA	107
r-HMGA2 R	CCGTTTTTCTCCAATGGTC	
h-HMGA2 F	AGCAGCAGCAAGAACCAAC	203
h-HMGA2 R	CCCAGGCAAGGCAACAT	
r-GLUT4 F	TGGTTGGGAAGGAAAAGG	191
r-GLUT4 R	AGATGAGTGGGGGCGAT	
h-GLUT4 F	GCCCCGCTACCTCTACAT	363
h-GLUT4 R	CCGCTCCACCAACAAC	

r, rats; h, humans; F, forward; R, reverse; RT, reverse transcription.

containing 5-10% CO₂. The adipocyte cells were then treated with different doses of insulin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) (0, 5, 50 or 500 nM) for 24 h; after which, they were collected and tested for their levels of miR-33b-5p, SREBF1, HMGA2 and GLUT4 expression using real-time PCR or western blotting methods.

Treatment of human adipocytes with different concentrations of miR-33b-5p inhibitor. Human adipocytes were cultured as aforementioned; after which, they were seeded into 6-well plates and grown to 60-80% confluence for use in transfection studies. A 9- μ l volume of Lipofectamine 2000 was mixed with 150 μ l of Opti-MEM medium (both from Thermo Fisher Scientific, Inc.). A miR-33b-5p inhibitor or NC inhibitor (Shanghai JiKai Gene Chemical Technology Co., Ltd., Shanghai, China) was diluted with Opti-MEM medium and then mixed with Lipofectamine-Opti-MEM medium at a ratio of 1:1 to achieve miR-33b-5p inhibitor concentrations of 0, 25, 50 and 100 nM, respectively. The sequence of miR-33b-5p inhibitor: 5'-GCAAUGCAACAGCA AUGCAC-3'. After allowing the cells to incubate at room temperature for 5 min, the miR-33b-5p inhibitor was added, and the cells were incubated with the different concentrations of miR-inhibitor complex for 48 h. Next, the adipocytes were collected, and their levels of

miR-33b-5p, SREBF1, HMGA2 and GLUT4 were determined using real-time PCR or western blotting methods.

RNA extraction and real-time PCR. A miRACLE isolation kit (Jinfiniti Biotech, LLC, Augusta, GA, USA) was used to extract total RNA from the ovarian tissues of rats and cultured adipocytes. The various cDNAs of mRNAs or miRNAs were synthesized using a cDNA reverse transcription kit. Real-time PCR was carried out using SYBR-Green Supermix and an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers used for PCR are listed in Table I. U6 and GAPDH were used as internal control genes. Real-time PCR was performed on target genes under the following conditions: 35 cycles each containing 94°C for 30 sec, 58°C for 45 sec and 72°C for 35 sec. The relative levels of mRNA and miR-33b-5p were calculated using the 2^{- $\Delta\Delta$ CT} method.

Western blotting studies. Proteins were extracted from cultured adipocytes, and then separated using 10% SDS-PAGE. The separated protein bands were transferred onto a PVDF membrane, which was then probed with rabbit monoclonal antibodies against SREBF1 (1:500; rabbit polyclonal; cat. no. 14088-1-AP; Proteintech); HMGA2 (1:1,000; rabbit

Table II. Serum fasting glucose and fasting insulin levels and HOMA-IR index in the PCOS rats.

Groups	N	Fasting glucose (mmol/l)	Fasting insulin (mIU/l)	HOMA-IR
PCOS	10	7.05±0.93 ^a	27.6±2.3 ^a	7.5±2.7 ^a
Control	10	5.51±0.58	11.6±1.9	2.7±0.3 ^a

^aP<0.05. PCOS, polycystic ovary syndrome; HOMA-IR, homeostatic model assessment-insulin resistance.

polyclonal; cat. no. bs-0556R; Bioss Antibodies); GLUT4 (1:1,000; rabbit polyclonal; cat. no. PB0143; Boster Biological Technology) or GAPDH (1:2,000; mouse monoclonal; cat. no. ab8245; Abcam) as an internal control overnight at 4°C. After incubation at 37°C for 1 h with horseradish peroxidase-conjugated secondary antibodies (1:5,000; goat polyclonal to HRP; cat. no. ab181658; Abcam), an Immobilon™ Western Chemiluminescent ECL kit was used to detect bound antibodies. Protein staining intensity was assessed using Image-Pro Plus 6.0 software.

Luciferase reporter assay. Wild-type and mutant type HMGA2 3'-UTRs were amplified and inserted into psiCHECK2 luciferase vectors (Promega Corporation, Madison, WI, USA). Next, the vectors with either the miR-33b-5p mimics or negative mimics (GenePharma Co., Ltd., Shanghai, China) (1.5 mg) were used to transfect 293T cells. Following 48 h of transfection, cellular luciferase activity was detected with a Microplate reader (Tecan GENios Pro; Tecan Group, Ltd., Mannedorf, Switzerland) according to the manufacturer's protocol.

HMG2 transcription factor binding sites for GLUT4 and SREBF1 5'-promoter luciferase vectors were amplified and inserted into pGL3 luciferase vectors (Promega Corporation). Next, the vectors with either the pcDNA3.0-HMGA2 overexpression plasmid or empty plasmid (0, 0.5, 1.5, 2.5 and 5.0 µg) were transfected into 293T cells. Luciferase activity was examined after 48 h of transfection. SREBF1 transcription factor binding sites were used to establish GLUT4 5'-promoter wild-type and mutant type luciferase vectors. Vectors with either the pcDNA3.0-SREBF1 overexpression plasmid or empty plasmid were transfected into 293T cells, and cellular luciferase activity was assessed.

Chromatin immunoprecipitation (ChIP). To analyze the binding of SREBF1/HMGA2 protein to the GLUT4 gene promoter, adipocyte cells transfected with either SREBF1 or HMGA2 antibodies (Abcam) were used for ChIP analyses that were performed using a Pierce Agarose Chip Kit (EpiGentek Group, Inc., Farmingdale, NY, USA). Normal rabbit IgG was used as a negative control. Non-precipitated genomic DNA input was amplified as an input control. After purification, the DNAs were used for PCR analyses that were performed using primers (Sangon Biotech Co., Ltd., Shanghai, China) that encompassed the GLUT4 promoter region. The conditions used for PCR were as follows: denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 20 sec, 58°C for 20 sec, and 72°C for 20 sec.

Statistical analysis. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 19.0 (IBM Corporation, Armonk, NY, USA). The levels of miR-33b-5p and gene expression are shown as the mean ± SD, and differences between groups were analyzed using Student's t-test. One-way ANOVA and Tukey's test were used to evaluate the levels of miR-33b-5p and gene expression in human adipocytes treated with different concentrations of glucose or insulin. The association between miR-33b-5p and SREBF1/HMGA2 expression was assessed using Pearson's correlation test. P-values <0.05 were regarded as statistically significant.

Results

Expression of miR-33b-5p, GLUT4, SREBF1 and HMGA2 in the ovarian tissues of PCOS rats. The PCOS rat model was successfully developed using the method described by Poretsky *et al.* (33). Insulin resistance in PCOS rats was indicated by their significantly increased insulin resistance index (HOMA-IR) values when compared with those of control rats (Table II). Based on their HOMA-IR score, the rats were then further classified into four groups: a non-PCOS/non-IR group, a non-PCOS/IR group, a PCOS/non-IR group, and a PCOS/IR group. The levels of miR-33b-5p expression in the ovarian tissues of non-PCOS/IR rats were significantly higher than those in the ovarian tissues of non-PCOS/non-IR rats (P<0.05). The PCOS/IR group also exhibited upregulated levels of miR-33b-5p expression when compared with the PCOS/non-IR group (Fig. 1A). Conversely, the levels of HMGA2, SREBF1, and GLUT4 expression in the ovarian tissues of non-PCOS/IR rats were significantly lower than those in the non-PCOS/non-IR rats (both P<0.05). PCOS/IR rats had lower HMG2 and GLUT4 levels when compared with PCOS-non-IR rats (Fig. 1B-D). Furthermore, the levels of HMG2 and GLUT4 expression were lower in the PCOS/non-IR group than in the non-PCOS/non-IR group. miR-33b-5p levels were negatively associated with SREBF1 and HMGA2 expression in the ovarian tissues of rats in all four groups (P<0.01), and miR-33b-5p levels were negatively correlated with GLUT4 levels (P<0.01) (Fig. 1E-G).

H&E staining of rat ovarian tissues revealed that ovary oocytes in the non-PCOS/ non-IR group were clear, and large numbers of granulosa cells were present. Ovarian tissues in the other three groups contained significantly fewer granulosa cells. The granulosa cells were loosely arranged, and the cortical stromal cells displayed obvious signs of hyperplasia (Fig. 2). Furthermore, immunohistochemistry was conducted to detect and compare the expression of GLUT4,

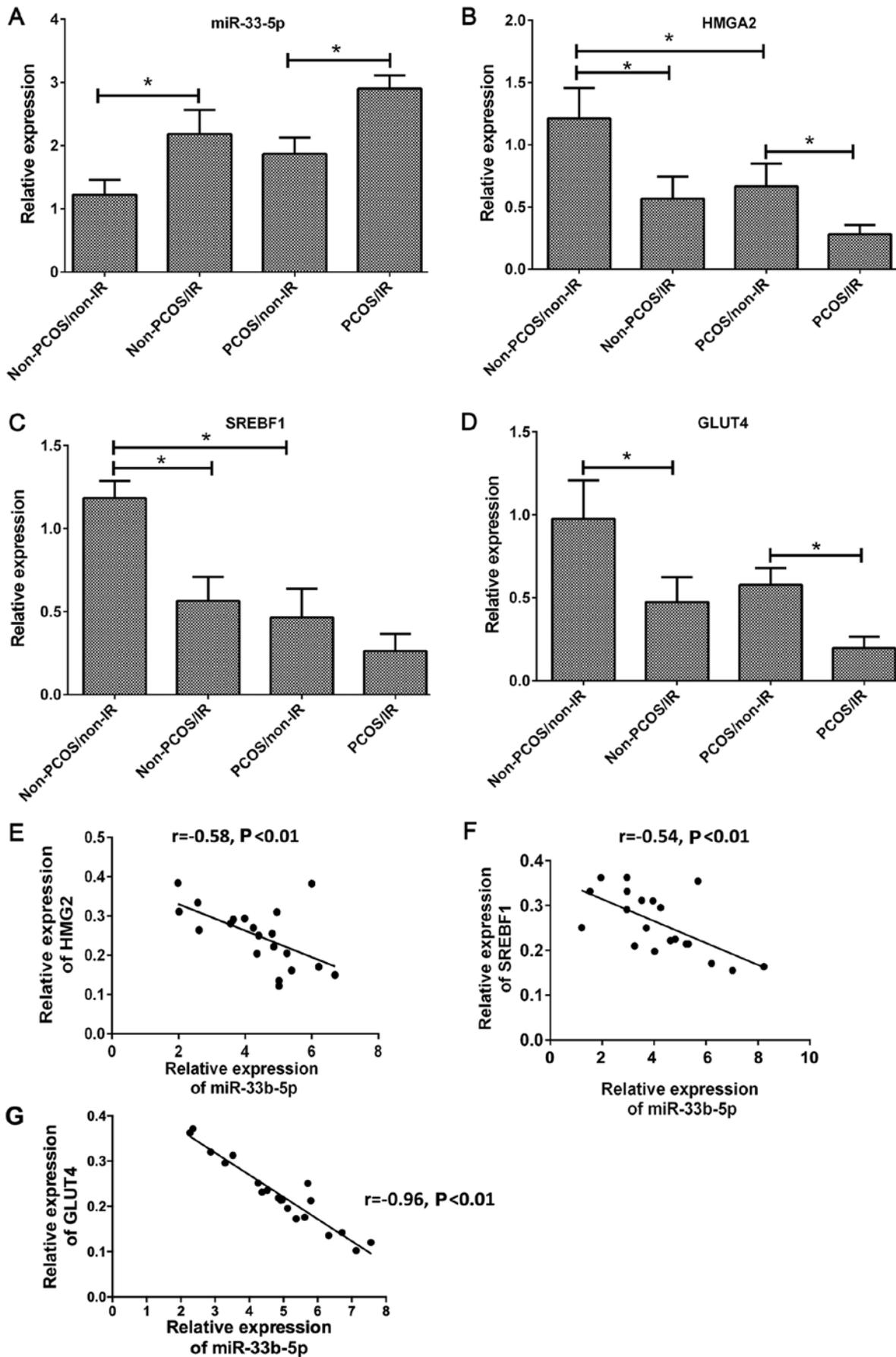


Figure 1. (A-D) The RNA levels and correlations of miR-33b-5p, GLUT4, SREBF1, and HMGA2 in rat ovarian tissues from the non-PCOS/non-IR group, non-PCOS/IR group, PCOS/non-IR group, and PCOS/IR group (U6 and GAPDH were used as internal controls for miR-33b-5p and the genes aforementioned, respectively). Relative expression levels of mRNAs and miRNAs were calculated using the $2^{-\Delta\Delta CT}$ method. (E-G) The respective correlations between miR-33b-5p and HMGA2, SREBF1 and GLUT4 in rat ovarian tissues, * $P < 0.05$. PCOS, polycystic ovary syndrome; IR, insulin resistance.

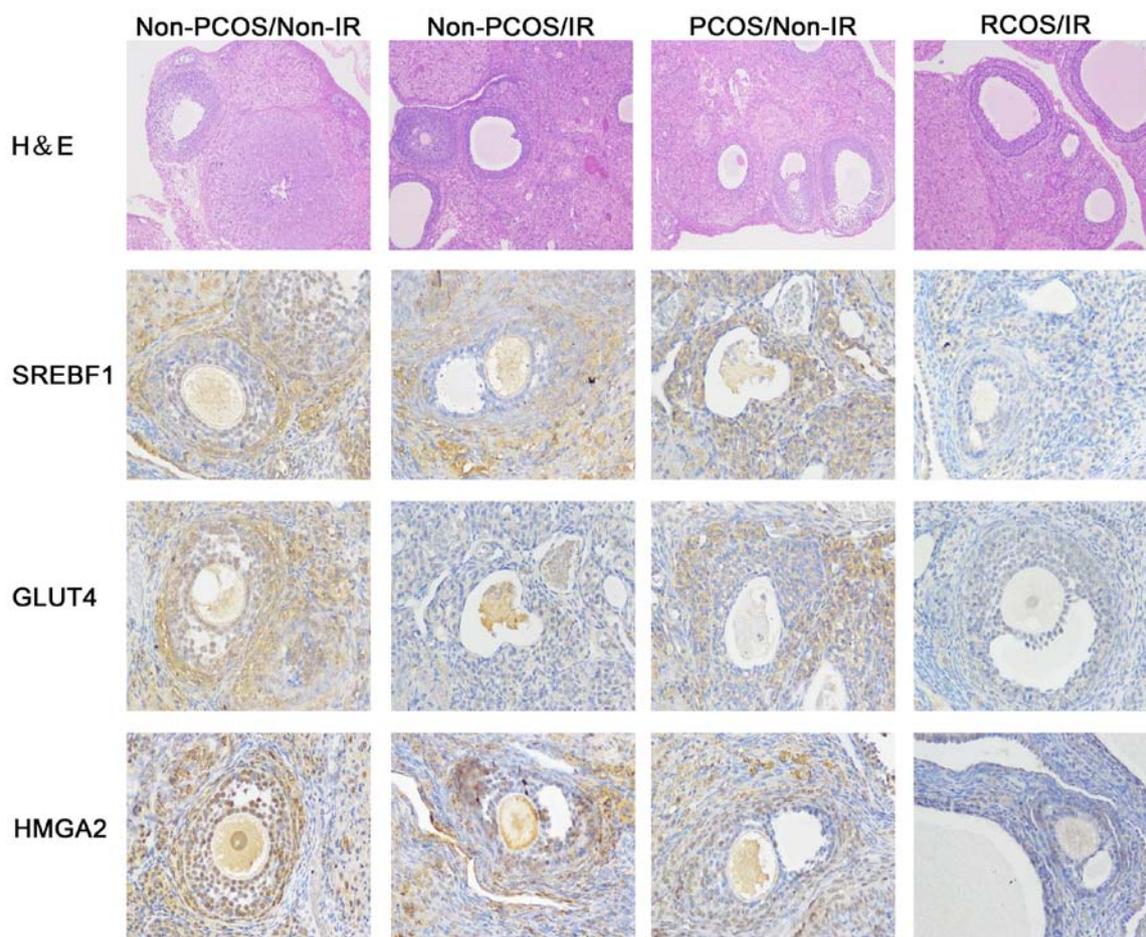


Figure 2. Hematoxylin and eosin (H&E) staining and immunohistochemistry results for GLUT4, SREBF1 and HMGA2 in rat ovarian tissues from the non-PCOS/non-IR group, non-PCOS/IR group, PCOS/non-IR group and PCOS/IR group. Relative expression comparison by color (yellow to colorless, the nucleus was stained blue) was performed among four groups. Immunohistochemistry revealed that GLUT4, SREBF1 and HMGA2 were highly expressed in ovarian tissues from the non-PCOS/non-IR group, and their levels were significantly decreased in the PCOS/IR group (x100, magnification). PCOS, polycystic ovary syndrome; IR, insulin resistance.

SREBF1 and HMGA2, which validated the data by real-time PCR in Fig. 1. Consistently, obvious positive expression of GLUT4, SREBF1 and HMGA2 (yellow color) were found in non-PCOS/non-IR group compared to that in the other three groups. Immunohistochemistry data revealed that GLUT4, SREBF1 and HMGA2 were all highly expressed in the ovarian tissues of rats in the non-PCOS/non-IR group, however, their expression levels were significantly decreased in the PCOS/IR group (Fig. 2).

Excess extracellular glucose increases miR-33b-5p expression and reduces GLUT4, SREBF1 and HMGA2 expression. As shown in Fig. 3, excess extracellular glucose increased miR-33b-5p mRNA expression and reduced GLUT4, SREBF1, and HMGA2 expression in adipocytes (Fig. 3A-D). These findings were further supported by western blotting data (Fig. 3E-F). These results revealed that excess glucose can alter the level of miR-33b-5p expression, which in turn, may further alter the expression of mRNAs for GLUT4, SREBF1 and HMGA2 in adipocytes.

Excess extracellular insulin increases miR-33b-5p expression and reduces the levels of GLUT4, SREBF1 and HMGA2

expression. We also assessed the effect of extracellular insulin on miR-33b-5p, GLUT4, SREBF1 and HMGA2 expression in adipocytes. We found that insulin produced a similar effect as glucose; it upregulated miR-33b-5p levels and down-regulated GLUT4, SREBF1 and HMGA2 mRNA and protein levels (Fig. 4). These results revealed that GLUT4, SREBF1 and HMGA2 expression in human adipocytes may also be regulated by changes in miR-33b-5p levels induced by excess insulin.

Downregulation of miR-33b-5p induces GLUT4, SREBF1 and HMGA2 expression. After determining that increased miR-33b-5p levels produced by treatment with either excess extracellular glucose or insulin suppressed GLUT4, SREBF1 and HMGA2 expression, we examined the effect of miR-33b-5p on GLUT4, SREBF1 and HMGA2 levels in adipocytes by reducing miR-33b-5p expression. Our data revealed that the expression levels miR-33b-5p gradually decreased as the concentration of miR-33b-5p inhibitor increased. In contrast, the GLUT4, SREBF1 and HMGA2 mRNA and protein levels increased in a concentration-dependent manner, suggesting that miR-33b-5p negatively regulates the levels of GLUT4, SREBF1 and HMGA2 (Fig. 5).

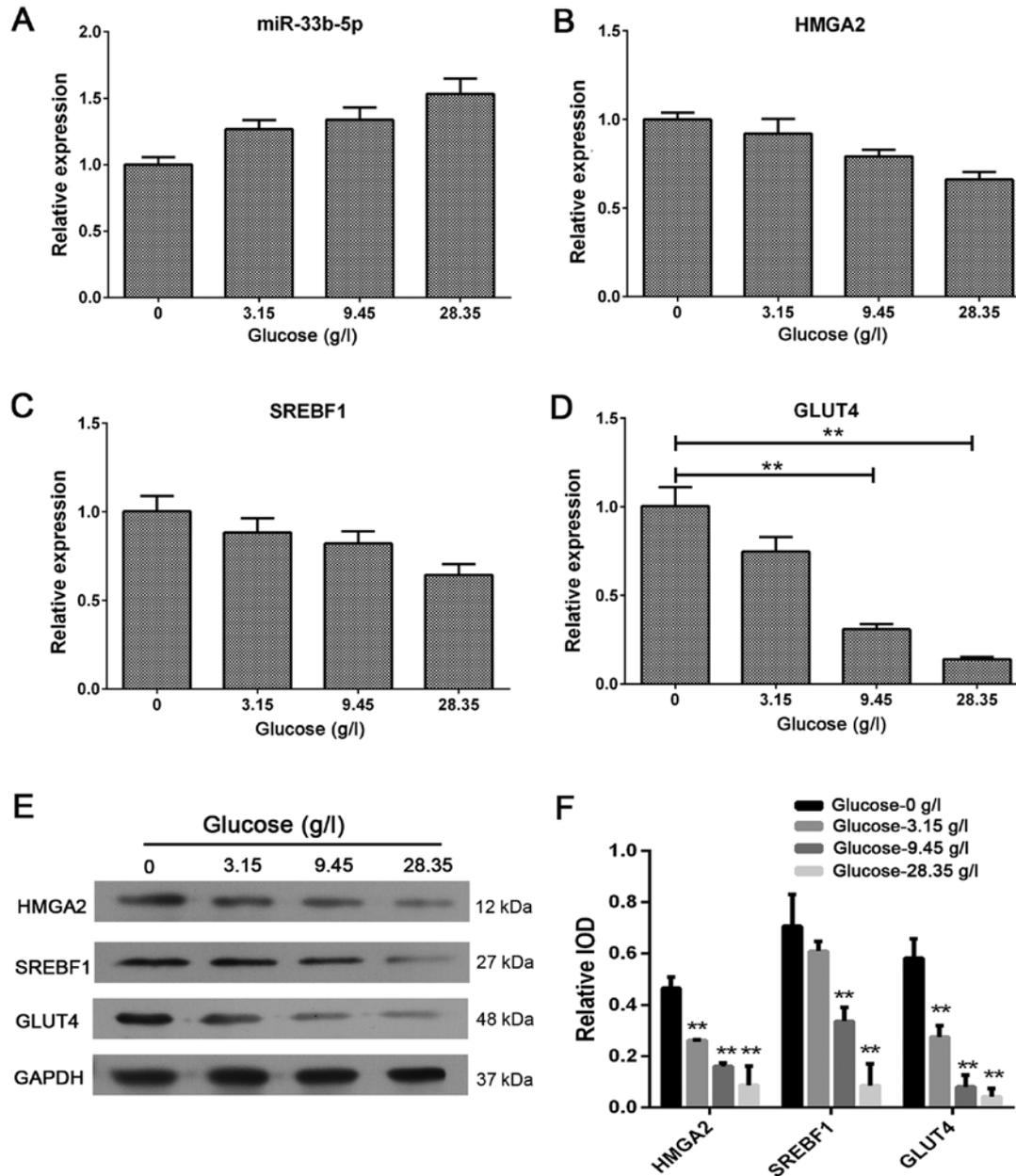


Figure 3. Changes in miR-33b-5p, GLUT4, SREBF1 and HMGA2 in glucose-treated adipocytes. (A-D) Changes in miR-33b-5p, GLUT4, SREBF1 and HMGA2 mRNA expression levels in adipocytes. (E-F) Western blotting results and quantification of these results revealing the GLUT4, SREBF1 and HMGA2 protein levels in the four groups treated with glucose at concentrations of 0, 3.15, 9.45 and 28.35 g/l, respectively. **P<0.01.

The role of miR-33b-5p in regulating HMGA2, SREBF-1 and GLUT4 expression. Transient co-transfection of the SREBF1 5'-promoter luciferase vector with either the pcDNA3.0-HMGA2 overexpression or empty plasmid revealed that pcDNA3.0-HMGA2 significantly increased luciferase activity in a dose-dependent manner, suggesting that HMGA2 could target SREBF1 and promote its expression (Fig. 6A). Chip data (Fig. 7A) revealed that HMGA2 directly binds to the 5'-promoter region of *SREBF1* and promotes its expression.

Transient co-transfection of the GLUT4 5'-promoter luciferase vector with either the pcDNA3.0-HMGA2 overexpression or empty plasmid revealed that pcDNA3.0-HMGA2 also increased luciferase activity in a dose-dependent manner (Fig. 6B). Chip experiments also demonstrated that HMGA2 could bind to the 5'-promoter region of *GLUT4* (Fig. 7B). These findings

revealed that HMGA2 can directly bind to the 5'-promoter region of *GLUT4* and promote its expression.

Transient co-transfection of the wild-type *GLUT4* 5'-promoter or mutant type vectors with either the pcDNA3.0-SREBF1 overexpression or empty plasmid revealed that SREBF1 could significantly increase luciferase activity in the wild-type *GLUT4* 5'-promoter group (Fig. 6C). Chip experiments also demonstrated that SREBF1 could bind to the 5'-promoter region of *GLUT4* (Fig. 7C). These findings indicated that SREBF1 can directly bind to the 5'-promoter region of *GLUT4* and promote its expression.

Transient co-transfection of the wild-type luciferase HMGA2 3'-UTR and mutant vectors with either miR-33b-5p mimics or NC mimics into 293T cells demonstrated direct binding of miR-33b-5p to the 3'-UTR of HMGA2, and resulted

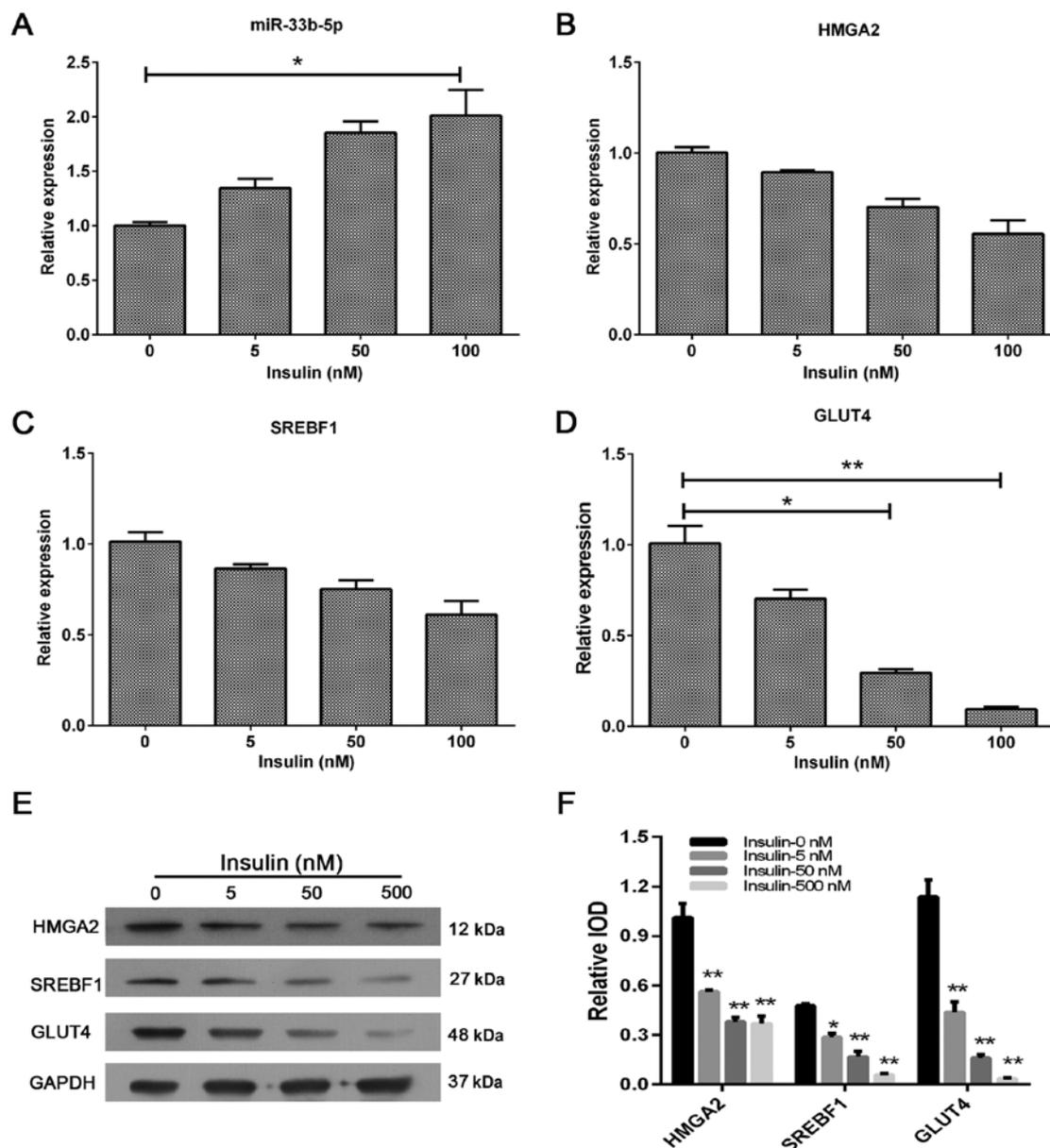


Figure 4. Insulin induces changes in miR-33b-5p, GLUT4, SREBF1 and HMGA2 in adipocytes. (A-D) Insulin induced changes in the miR-33b-5p, GLUT4, SREBF1 and HMGA2 mRNA levels in adipocyte cells. (E-F) Western blotting results and quantification of these results revealing the GLUT4, SREBF1 and HMGA2 protein levels in the four groups treated with insulin at concentrations of 0, 5, 50 and 500 nM, respectively. * $P < 0.01$ and ** $P < 0.01$.

in significant reductions in luciferase activity (Fig. 6D). These results revealed that miR-33b-5p could target the 3'-UTR of HMGA2 and inhibit its expression.

Discussion

The present study investigated the role of miR-33b-5p in PCOS, and particularly its role in regulating GLUT4. We determined that miR-33b-5p expression was higher in PCOS/IR rats, and correlated with SREBF1 and HMGA2 expression ($P < 0.05$). Results of luciferase reporter assays and ChIP studies performed with adipocytes revealed that miR-33b-5p could target the 3'-UTR of HMGA2 and inhibit its expression. Moreover, HMGA2 could directly bind to the 5'-promoter region of *GLUT4* and promote its expression, and could also promote SREBF1 expression. Finally, we determined

that SREBF1 could directly bind to the 5'-promoter region of *GLUT4* and promote its expression. Therefore, our study results demonstrated that miR-33b-5p was overexpressed in the ovarian tissues of PCOS/IR rats, and revealed that miR-33b-5p can inhibit *GLUT4* by targeting HMGA2 during the development of insulin resistance in PCOS patients. Our results also indicated that HMGA2 and SREBF1 are important molecules involved in modulating GLUT4 expression.

miRNAs consist of a group of small non-coding RNAs, some of which play key roles in modulating the functions of human bodies (25,34,35). Numerous miRNAs have been demonstrated to be involved in endocrine and metabolic diseases (36,37), and several miRNAs (e.g., miR-93 and miR-145) have been reported to be involved in the pathogenesis of PCOS (27-31,38-42). miRNA-93 was initially revealed to play an important role in modulating GLUT4 expression,

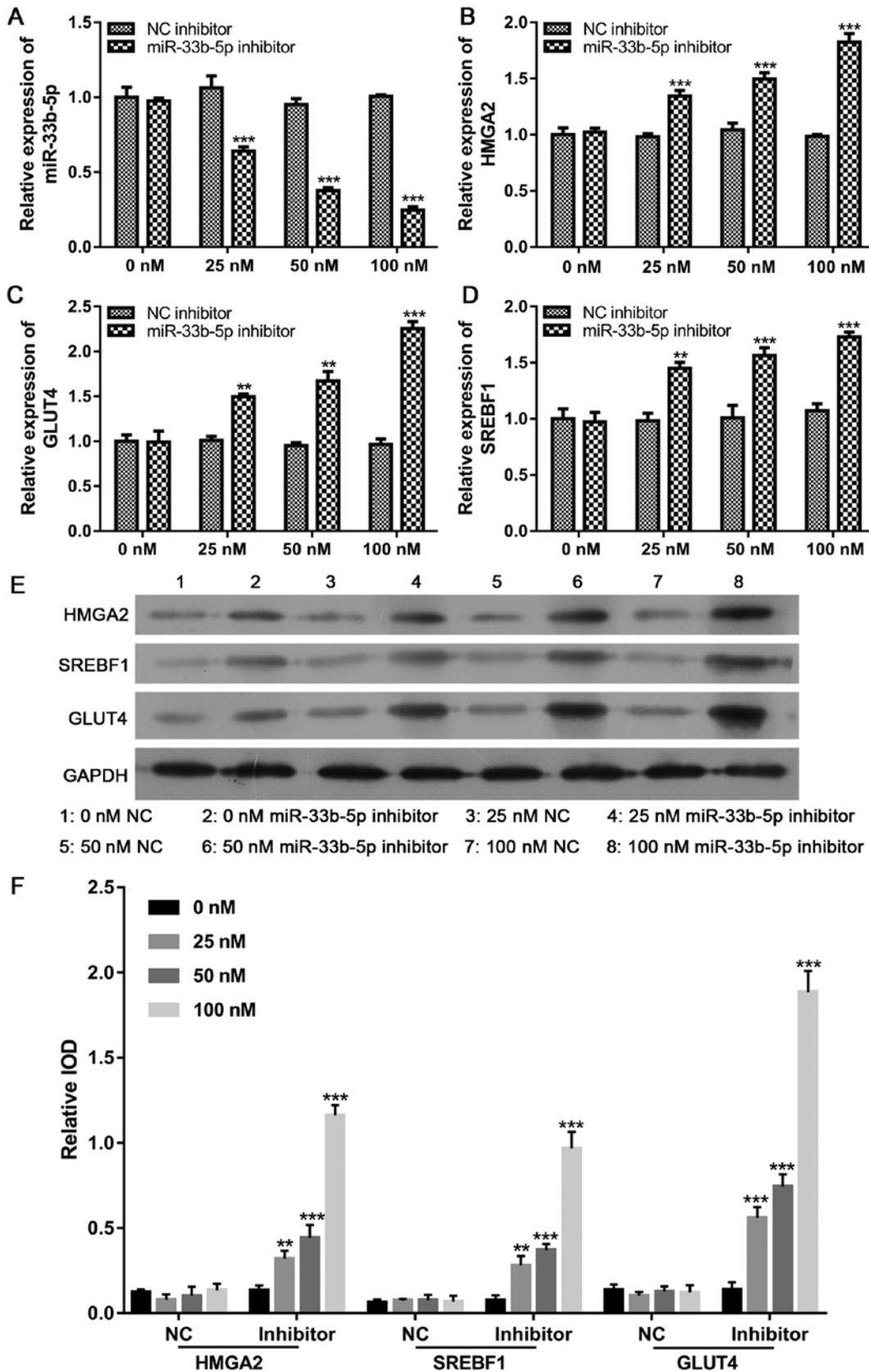


Figure 5. Effect of the miR-33b-5p inhibitor on miR-33b-5p, GLUT4, SREBF1 and HMGA2 in adipocytes (A-D) Levels of miR-33b-5p, GLUT4, SREBF1 and HMGA2 in the adipocytes. (E-F) Western blot results showing GLUT4, SREBF1 and HMGA2 protein levels in the eight groups treated with the control molecule or miR-33b-5p inhibitor at concentrations of 0, 25, 50 and 500 nM, respectively. **P<0.01 and ***P<0.001, when compared to treatment with the control.

and thus be involved in the development of insulin resistance in PCOS patients (27). miR-145 was identified as a molecular

target associated with granulosa cell dysfunction in PCOS, because it could inhibit IRS1 expression and the MAPK/ERK

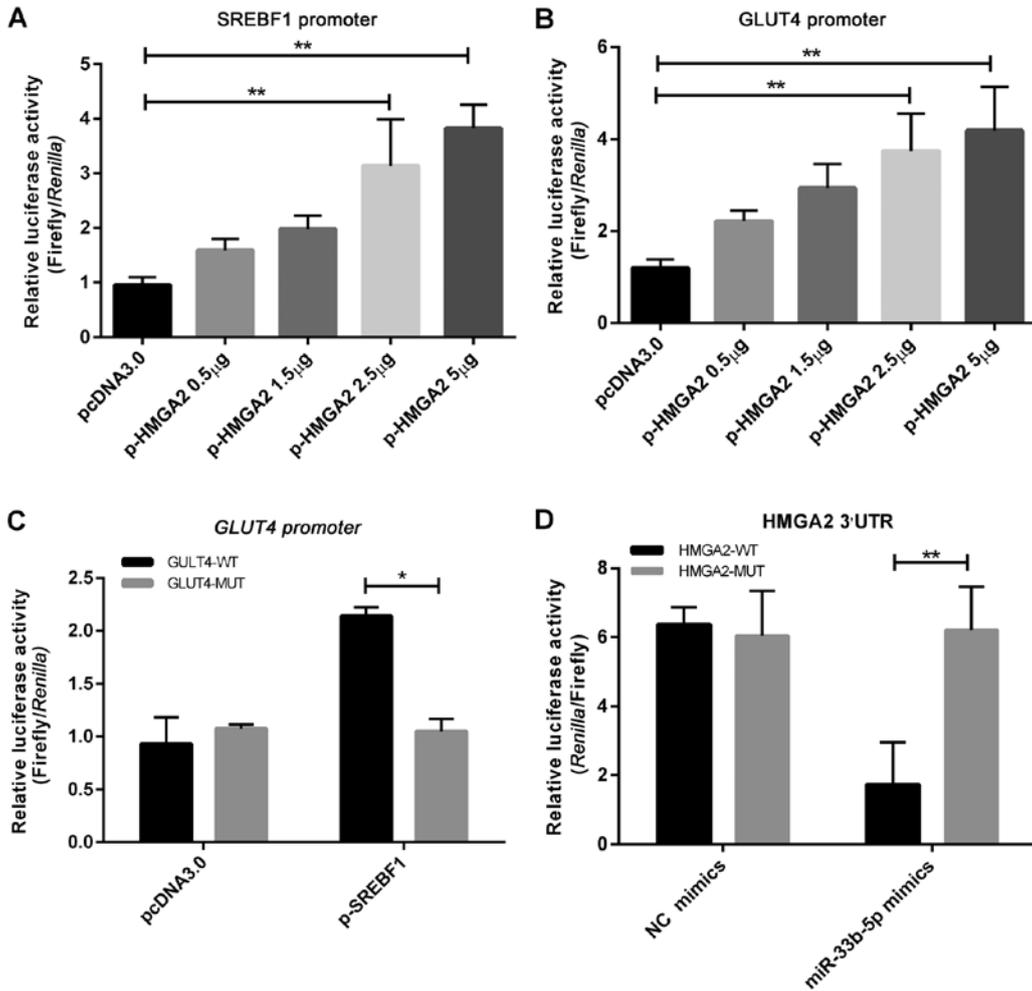


Figure 6. Findings from luciferase reporter assays. (A) pcDNA3.0-HMGGA2 significantly increased luciferase activity of SREBF1 5'-promoter in a dose-dependent manner. (B) pcDNA3.0-HMGGA2 also dose-dependently increased the luciferase activity of GLUT4 5'-promoter. (C) pcDNA3.0-SREBF1 significantly increased the luciferase activity of GLUT4 5'-promoter group. (D) Direct binding of miR-33b-5p to the 3'-UTR of HMGGA2 resulted in significant reductions in luciferase activity. *P<0.05 and **P<0.01.

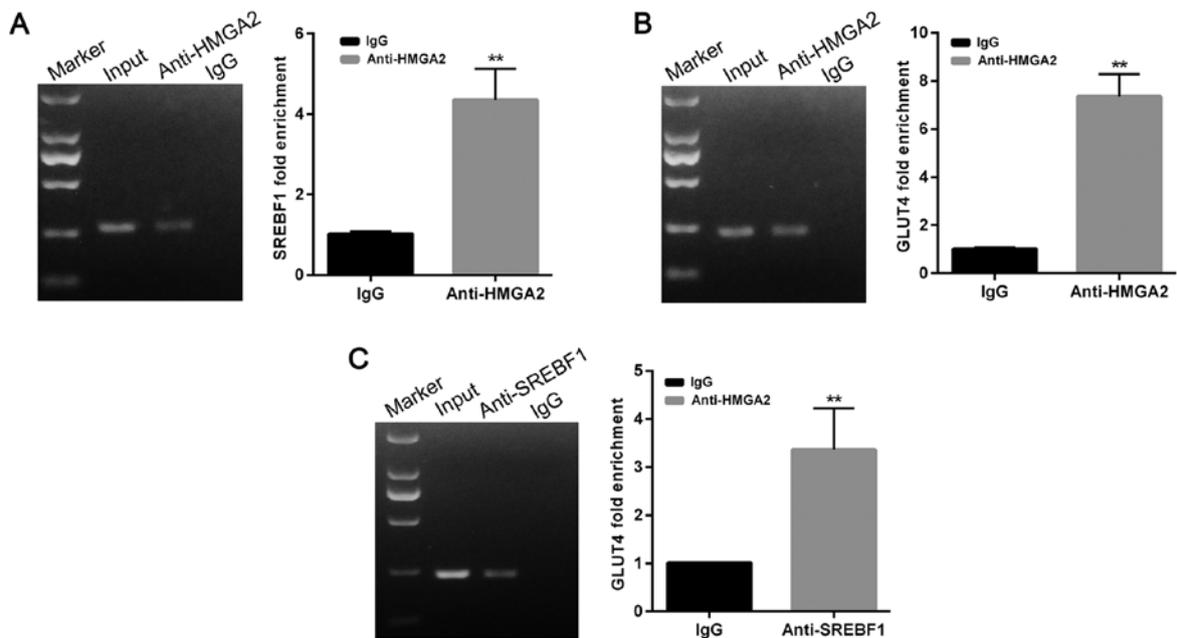


Figure 7. Outcomes from the ChIP experiments. (A) HMGGA2 could bind to the 5'-promoter region of SREBF1. (B) HMGGA2 could bind to the 5'-promoter region of GLUT4. (C) SREBF1 could bind to the 5'-promoter region of GLUT4. Left lane: representative ChIP images; right lane: quantification of PCR results. **P<0.01.

signaling pathways (43). The findings aforementioned revealed that some miRNAs indeed play key roles in the development of PCOS. In the present study, we found that miR-33b-5p was highly involved in the development of PCOS, and its overexpression was observed in our rat model of PCOS. The findings in our present study provide new additional evidence that miRNAs play key roles in the development of PCOS.

We further examined how miR-33b-5p affects GLUT4, SREBF1 and HMGA2 expression. Both excess extracellular glucose and insulin suppressed the levels of GLUT4, SREBF1 and HMGA2 by upregulating miR-33b-5p, whereas GLUT4, SREBF1 and HMGA2 levels became elevated when miR-33b-5p was inhibited, indicating the powerful effects that glucose and insulin exert in the regulation of GLUT4, SREBF1 and HMGA2 by miR-33b-5p. Insulin resistance is a main feature of PCOS, and can adversely affect a woman's health (8,44,45). GLUT4 is strongly linked to the development of insulin resistance in type 2 diabetes or PCOS (46,47). GLUT4 is a transporter molecule responsible for the uptake of glucose, and its dysfunction is observed in individuals with insulin resistance or PCOS (48). When blood glucose levels are low, GLUT4 is stored intracellularly in muscle and fat cells, preventing GLUT4 from reaching the cell surface and transporting glucose into cells (49). When blood glucose levels are high, GLUT4 is translocated to the cell surface after receiving an intracellular signal produced by insulin, resulting in glucose uptake (49). Therefore, adequate intracellular retention and a quick response to insulin stimulation are needed to maintain the function of GLUT4 in modulating glucose homeostasis. GLUT4 is currently regarded as an important molecule for regulating glucose homeostasis, and also a key factor involved in the development of insulin resistance (49). A previous study found decreased levels of GLUT4 expression in the endometrium of PCOS patients (13); however, the mechanism for that decrease is largely unclear. In the present study we found that GLUT4 expression could be regulated by HMGA2 and SREBF1, which offers a novel explanation for the aberrant expression of GLUT4 in PCOS patients. Our findings also provided some new promising therapeutic targets for PCOS, and suggest that increasing GLUT4 expression by inhibiting miR-33b-5p or increasing HMGA2 and SREBF1 levels may be a promising strategy for treating PCOS.

HMGA2 is a non-histone chromosomal protein with three DNA binding domains, and can act as a transcription regulating factor (50). HMGA2 can alter the structure of chromatin by forming a stereospecific complex with other proteins in the promoter or enhancer region of certain genes (51). It is also an essential component of the enhanceosome, and can promote the assembly of protein complexes and regulate the transcription of target genes (51). Previous studies have revealed that HMGA2 is associated with lipomas, indicating its roles in adipogenesis and insulin resistance (14,15). In the present study, we demonstrated that miR-33b-5p was responsible for regulating HMGA2 and HMGA2 could regulate *GLUT4* transcription. These findings explain the roles played by miR-33b-5p and GLUT4 in the pathogenesis of PCOS at a molecular level.

SREBF1 is a major regulator of genes involved in metabolic pathways and sterol biosynthesis, as it binds to the steroid regulatory element of the promoter or enhancer of those genes (16-18). Previous studies have revealed that SREBF1 is an important transcription factor that regulates certain genes

related to lipid metabolism, which is also associated with insulin resistance (52,53). In the present study, we determined that SREBF1 could directly regulate the target gene of GLUT4, which is a key gene involved in insulin resistance. We also determined that miR-33b-5p could target *SREBF1* and inhibit its expression. Moreover, we revealed that SREBF1 could regulate the expression of GLUT4 as a transcription factor, which further illustrates the key roles played by miR-33b-5p and GLUT4 in the development of PCOS.

In summary, the findings from this study revealed that miR-33b-5p was overexpressed in the ovarian tissues of PCOS rats with insulin resistance, and thus may play an important role in the development of insulin resistance in PCOS patients. miR-33b-5p could inhibit GLUT4 by targeting HMGA2 during the development of PCOS. Furthermore, HMGA2 and SREBF1 are important molecules involved in modulating GLUT4 expression. These findings enhanced our understanding of the molecular mechanisms that contribute to insulin resistance during the development of PCOS, and may assist in finding new therapeutic targets for PCOS. Future studies may determine whether it is feasible to treat PCOS by targeting miR-33b-5p.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YY, HJ and XZY conceived and designed the study. YY, HJ and LX performed the experiments. YY and XZY wrote the paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All tissue specimens were obtained with permission from the Affiliated Hospital of Hubei University. All participants have read and signed the written informed consent.

Consent for publication

All participants have read and signed the written informed consent for the publication.

Competing interests

The authors declare that they have no competing interests.

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