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Ruminants

Glucose Transport by Follicle-Stimulating Hormone Is Mediated Through the Akt/FOXO1 Pathway in Ovine Granulosa Cells

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ABSTRACT

Glycolysis in granulosa cells (GCs) is the primary location of energy metabolism and its substrates in oocytes and is closely related to follicular development in mammals. The complex morphological structure and physiological functions of GCs are regulated by follicle-stimulating hormone (FSH), but little is known about how FSH regulates glycolysis in GCs, and its mechanism remains unclear. The purpose of this study is to investigate the mechanism by which FSH activates the Akt/FOXO1 pathway, thereby regulating glucose metabolism in ovine GCs. Granulosa cells were cultured in the presence of different concentrations of FSH and a CCK-8 assay was used to measure the proliferation of the treated cells. Next, qRT-PCR was performed to measure the transcription of the target genes, glucose transporter (GLUT). Western-blot analysis of the phospho-Akt and -FOXO1 levels induced by FSH through the glucose signalling pathway, in addition to the effects of these proteins on the expression levels of the downstream GLUT genes, was measured. Results showed that the addition of 10ng/mL FSH to the culture medium increased the viability of granulosa cells. Transcription of GLUT1 and 4 was significantly up-regulated in FSH-treated cells through activation of the Akt/FOXO1 phosphorylation pathway, thereby affecting glucose metabolism. This study contributes to the current understanding of the metabolic features of and the associated developmental pathways of ovine follicular GCs.

1 | Introduction

Glucose is the main energy substrate of GCs in antral follicles and plays an important role in maintaining cell metabolism. On one hand, glucose is a key source of metabolic energy for cell metabolism and for life itself, and on the other hand, extremely high or low levels of glucose are potentially harmful to protein function. Hypoglycaemia will quickly become life-threatening, while long-term hyperglycaemia will lead to protein dysfunction and abnormal functional activity through non-enzymatic glycosylation and advanced glycation end-products (AGEs) of proteins (Wautier and Schmidt 2004). Therefore, mammals have evolved balancing mechanisms to maintain blood and tissue glucose concentrations within a very narrow physiological range.

Xin Xu and Ruotong Wang contributed equally to this work.

Glucose is a hydrophilic molecule that cannot permeate the plasma membrane. Thus, its uptake is mediated by several sugar transporters with different substrate specificities, kinetic properties and tissue distribution patterns (Wood and Trayhurn 2003). Published results have shown that multiple glucose transporters (GLUTs) exist in ovarian tissues, but that their expression patterns vary greatly among different species and tissues. GLUT1 and GLUT4 are expressed in ovine follicles (Williams et al. 2001), while GLUTs 1, 3 and 4 are expressed in bovine corpus lutea and follicles (Nishimoto et al. 2006). The expression of GLUTs 1, 3 and 4 has also been detected in rat ovaries (Kodaman and Behrman 1999). The expression of these GLUTs is regulated by intra-ovarian factors involved in follicle development, maturation and ovulation, such as E2, IGF-I and interleukin-1β (Kol et al. 1997), and gonadotrophins (Kodaman and Behrman 1999). Stimulation of rat ovaries with human chorionic gonadotropin (hCG) resulted in significantly increased levels of GLUT3 mRNA (Zhang et al. 2012). In bovine ovaries, glucose concentration in follicular fluid was negatively correlated with the levels of GLUT 1 and 3 mRNAs in GCs (Nishimoto et al. 2006). These findings suggest that the hormonal environment of the follicle was able to influence glucose uptake and that the ovary regulates glucose uptake by ovarian cells through some reproductive mechanism.

Numerous studies have shown that glucose is widely present in the follicular fluid of humans (Józwik et al. 2007), macaques (Brogan et al. 2011), buffaloes (Khan et al. 2011), sheep (Nandi et al. 2008), cattle (Nishimoto et al. 2009), pigs (Bertoldo et al. 2013), camels (El-Bahr et al. 2015) and horses (Salazar-Ortiz et al. 2014), and thus follicular fluid is the presumed source of glucose for cumulus cells. Many studies have shown that the glucose concentrations in follicular fluid are lower than those in serum or plasma. In addition, there are significant differences in the glucose concentrations in different follicles of the same animal (Nandi et al. 2007), suggesting that some local factor (such as hormones) regulates glucose entry into the follicle or its utilization by the follicle. Although the intrafollicular glucose concentration was increased after ovulatory stimulus in macaques, glucose is not utilized by mural GCs but is left to the cumulus-oocyte complex to provide essential energy for oocytes (Downs et al. 2002; Sutton-McDowall et al. 2004; Ratchford et al. 2008; Brogan et al. 2011). Thus, the intra-follicular glucose concentration appears to be regulated to a certain extent by intrafollicular hormones (Nandi et al. 2007, 2008; Shabankareh et al. 2013). In recent years, it has been shown that FSH is involved in regulating the metabolism of glucose in rat GCs (Liu et al. 2013). Several studies have demonstrated that increased FSH levels enhance the transport capacity of GLUT, which enhances glucose metabolism through the AKT/FOXO1 signalling pathway (Mohan et al. 2010; Gonzalez et al. 2011; Chen et al. 2019).

In sheep GCs, glucose, as the preferred energy substrate, is metabolized to lactic acid under anaerobic conditions to support gonadotropin-induced differentiation of GCs (Campbell et al. 2010) and theca cells cultured in vitro (Campbell et al. 2014). However, the available data do not fully unravel this phenomenon, and there are many contradictory reports describing the relationships between intra-follicular glucose, follicle size and follicle status. Further research is therefore required to elucidate how the concentration of glucose in follicles is regulated by hormones (such as FSH) during follicle development and its significance in fertility (Foong et al. 2006).

We hypothesize that the glucose uptake of ovine GCs is regulated by FSH through the AKT pathway and, therefore, in the present study, the FSH-induction of granulosa cell GLUT expression and the role of glucose in the process was investigated. This work will serve to elucidate the mechanism by which glucose is regulated in mammalian oocytes, spurring further research into this field.

2 | Materials and Methods

2.1 | Reagents and Antibodies

The Dulbecco's modified eagle medium (DMEM, 11995065), Fetal bovine serum (FBS, 16140071), penicillin-streptomycin (15140122) and phosphate-buffered saline (PBS, 10010023) were purchased from Gibco, USA. Ovine follicle-stimulating hormone (FSH) was purchased from Sioux Biochemical (No. 915, Sioux Center, IA). The glucose inhibitor, azaserine (HY-B0919) and the AKT inhibitor AT7867 (HY-12059) were purchased from MedChemexpress (MCE, New Jersey, USA). The 2-deoxyglucose (2-DG) uptake measurement kit was purchased from Cosmo Bio (Tokyo, Japan) and the Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). The Apoptosis assay kit was purchased from BD Biosciences (San Jose, CA) while the Revert Aid First Strand cDNA Synthesis Kit, with DNase I (cat: K16215) and TRIzol reagent, were both obtained from Thermo Fisher Scientific (Waltham, MA). The SYBR Green Supermix (172-5274) was purchased from Bio-Rad and the primers for the amplification of GLUT1-4 were synthesized by Sangon Biotech (Shanghai, China). The Cytoplasmic Protein Extraction Kit (P0027) and the BCA Protein Assay Kit (P0011) were purchased from Beyotime Biotech, Shanghai, China. Acrylamide (electrophoresis grade) was purchased from Sioux Biochemical, while PVDF membranes (0.45 μm, IPVH00010) were purchased from Millipore, USA. Pre-stained protein markers were purchased from Beijing Quanjin Biotechnology Co., Ltd., China and the enhanced chemiluminescence detection kit was obtained from Amersham Life Science (Oakville, ON, Canada). The polyclonal antibodies against GLUT-1 (ab652), GLUT-2 (ab95256), GLUT-3 (ab41525), GLUT-4 (ab654), anti-β-actin (ab8227) and horseradish peroxidase-conjugated secondary antibody (ab205718) were purchased from Abcam (San Francisco, CA, USA). The Akt antibody (9272), phospho-Akt (Ser473) antibody (9271), FoxO1 antibody (9454) and phospho-FoxO1 antibody (2486) were purchased from Cell Signaling Technologies (Danvers, MA).

2.2 | Ethical Procedures and Animal Treatments

All experiments were approved by the Institutional Animal Care and Use Committee of the Tarim University, China. The local abattoir obtained the ovaries after slaughter, removed residual tissues, and washed the ovaries thrice with physiological saline pre-warmed to 37°C. Samples were placed in a specimen container filled with physiological saline at 37°C for transport to the laboratory. Once back at the laboratory, the ovaries were washed three times with physiological saline containing

penicillin-streptomycin and pre-heated to 37°C. The ovaries were then incubated in a pre-warmed sterile saline solution.

2.3 | Ovine Granulosa Cell Isolation and Culture

Granulosa cells were aseptically harvested from ovarian antrum follicles. Briefly, the ovarian follicles were carefully separated using sterile ophthalmic scissors and the tissue around the follicles was cleaned as much as possible. Follicles with a diameter of 4–6 mm were selected for further analyses and were gently dissected using ophthalmic scissors in a concave dish containing PBS. The cells along the inner wall of the follicle were delicately scraped off with a sterile spatula, and placed in a DMEM solution containing 100 IU/mL penicillin/streptomycin. The cells were then washed with PBS three times, then re-suspended in complete DMEM medium (supplemented with HEPES 10 mM, 0.3% BSA, 10 % FBS and 100 IU/mL penicillin/streptomycin) and seeded in 6-well plates. The cells were detached from the six well plates by incubating with 0.05% trypsin for 2∼5 min. Quantification of live cells was carried out with a TC20 automated cell counter (BIO-RAD, California, USA). The cells were then seeded in 96, 12 or 6-well plates at specific densities for subsequent experiments. Before treatment, the complete DMEM medium was replaced with a serum-free medium and the cells were incubated in a serum-free medium for 12 h. Cells were maintained at 37°C under a humidified 5% CO_2 atmosphere. Each treatment had three experimental replicates.

2.4 | Analysis of GC Proliferation

The CCK-8 assay kit was used to assess the proportions of viable cells, by measuring the dehydrogenase activity in viable cells. The cells were seeded in 96-well plates at a density of 1×10^4 cells/well, and treated with different concentrations (0, 5, 10, 20 or 40 ng/mL) of FSH for 144 h, when the cells reached ∼60% confluence, to determine the optimal action time and concentration of FSH. To determine the effect of azaserine on GC proliferation, the cells were then seeded in 96-well plates at a density of 1×10^4 cells/well, and pre-treated with the glucose inhibitor azaserine (5 μM) for 2 h when the cells reached ∼80% confluence, followed by FSH treatment (10 ng/mL) for 24 h. After incubation, 10 μL of the CCK-8 solution was added to each well and the plates were incubated for an additional 2 h at 37°C. The OD values were recorded using a microplate reader (VARIOSKAN LUX, Thermo, USA) at 450 nm. The mean OD values for each treatment were considered the number of viable cells for each treatment.

2.5 | Glucose Uptake Assay

Glucose uptake levels were determined using a 2-DG measurement kit according to the manufacturer's protocol. Briefly, GCs were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured with the FSH either in the presence or absence of azaserine (5 μM), as described above. Then the cells were seeded in 96-well plates at the density of 1×10^4 cells/well, and pre-treated with the AKT inhibitor AKT7867 (10 μM) for 2 h when the cells reached ∼80% confluence, followed by FSH treatment (10 ng/mL) for 30 min to determine the effect of AKT7867 on GC glucose uptake. Then, the treated cells were detached from the 96 cell plates by incubating with 0.05 trypsin for 2∼5 min. The digested cells were suspended in a 1.5 mL tube and counted using an automated cell counter. The density of the cells to be tested was adjusted to 2×10^4 cells/mL and after three washes with PBS, the cells were incubated in the 2-DG solution at 37°C for 20 min. The 2-DC solution was removed, and the cells were gently washed three times. Cells were disrupted by a microtip sonicator (JY92-IIDN, China), and the cell lysates were collected. The cell lysates were then heated at 80°C for 15 min and centrifuged at 4°C and 15000 g for 20 min. The OD of each cell lysate was measured using the same microplate reader at 420 nm. The concentration of each sample was measured in triplicate and was calculated by comparing the data to that of the standard curve supplied with the kit.

2.6 | RNA Extraction and qRT-PCR Analysis

The cells were seeded in 12-well plates at a density of 1×10^6 cells/well. When the cells reached ∼80% confluence, they were treated with FSH (10 ng/mL) for 24 h. Total RNA from the cultured GCs was extracted using TRRIzol reagent according to the manufacturer's protocol, washed in 75% ethanol, and then dissolved in RNase-free distilled water. An aliquot of the total RNA of each sample was used to measure the total RNA concentration by using a nucleic acid quantifier (NanoDrop ND-2000, Thermo, USA) and 0.2 μg was used to perform the first strand cDNA synthesis using the Revert Aid First Strand cDNA Synthesis Kit, with DNase I. Next, qRT-PCR was performed to measure mRNA expression, which was normalized to the *β-actin* RNA. The cDNA of the GCs without FSH treatment served as the positive control and water served as the negative control. Amplification was performed at a final volume of 20 μL containing 1 μL cDNA, 2 μL of each primer diluted to 10 μM, 10 μL 2X SYBR Green SuperMix and 5 μL ddH_2O. The thermal cycle conditions used for real-time PCR were: 50°C for 2 min; and 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The specific primer pairs used in the experiments are listed in Table 1, and data were analysed by the $2^{-\Delta\Delta CT}$ method.

2.7 | Western Blot Analysis

The cells were seeded in 6-well plates at a density of 2×10^6 cells/well, and treated with FSH (10 ng/mL) for 24 h when the cells reached ∼80% confluence to determine the effect of FHS on GLUT expression. Cells were pre-treated with or without the AKT inhibitor AKT7867 (10 μM) for 2 h when the cells reached ∼80% confluence, followed by FSH treatment (10 ng/mL) for 30 min to determine the phosphorylation degree of AKT and FOXO1 and the expression of GLUT1 and GLUT4. The collected GCs were subjected to total protein extraction with a Cytoplasmic Protein Extraction Kit according to the manufacturer's instructions and quantified using the BCA Protein Assay Kit. A total of 15–80 μg of each protein sample (depending on the experiment) was separated by 12.5% SDS-PAGE and transferred into a PVDF membrane. The membranes were then blocked in TBST buffer (20 μM Tris-HCl, 150 mM NaCl, 0.05 % Tween 20; pH 7.5) containing 5% dehydrated non-fat milk at room temperature for 1 h. The membranes were subsequently incubated overnight at 4°C with the diluted primary antibody [polyclonal anti-GLUT-1 (1:500), polyclonal

TABLE 1 | Primer sequences used for qRT-PCR.

Target genes	GenBank accession no.	Primer sequence (5′–3′)	Product size (bp)
GLUT1	XM_015091913.1	Forward: GTGGGAGGCATGATTGGTTC Reverse: CGCAGTACACACCGATGATG	181
GLUT2	XM_004003162.3	Forward: TGGGACTTGTGCTCCTGAAT Reverse: TGAAATTGCCCGTCCAGTTG	192
GLUT3	NM_001009770.1	Forward: GATCTGTGGCCTCTACTGCT Reverse: TCCGCATACTCTCGTCCTTC	202
GLUT4	ID: 101118797	Forward: TCTTCTATTCGCGGTCCTCC Reverse: GTCATTCTCATCCGGCCCTA	178
β-actin	LOC100885765	Forward: CTGGACTTCGAGCAGGAGAT Reverse: TAGTTTCGTGAATGCCGCAG	172

anti-GLUT-2 (1:1000), polyclonal anti-GLUT-3 (1:1000), polyclonal anti-GLUT-4 (1:2000), polyclonal anti-Akt (1:2000), polyclonal anti-phospho-Akt (1:2000), polyclonal anti-FoxO1 (1:1000), polyclonal anti-phospho-FoxO1 (1:1000), or *β*-actin (1:5000)], followed by the horseradish peroxidase-conjugated secondary antibody (1:1000–1:10000 for 1.5 h at room temperature). Peroxidase activity was visualized using an enhanced chemiluminescence kit according to the manufacturer's instructions. Protein concentrations were determined by densitometrically scanning the exposed x-ray film. Immunoreaction signal intensities were analysed using the gel-pro Analyzer 4.0.

2.8 | Statistical Analysis

The experiments were repeated three times, all tests performed in SPSS statistics 26, IBM and experimental data are presented as mean ± standard deviation. An unpaired *t*-test was used for comparison between two groups and two-way ANOVA accompanied by an LSD post hoc test was used for multiple comparisons. Differences were considered significant when $p < 0.05$ or $p < 0.01$.

3 | Results

3.1 | Effects of FSH on GC Proliferation

Results showed no effect on GC density of 5, 20 or 40 ng/mL of FSH over time ($p > 0.05$) (Figure 1). Only the density of GCs in the 10 ng/mL FSH treatment group increased significantly after 24 h and was significantly higher than that of the other treatment groups ($p < 0.01$) (Figure 1). The optimal concentration of FSH was determined to be 10 ng/mL, and the FSH treatment time was set at 24 h for the subsequent experiments.

3.2 | FSH Enhanced Glucose Uptake in GCs

To determine whether FSH regulates the uptake of glucose, 2-DG was used to quantify the glucose content. Data showed that the addition of 10 ng/mL FSH to the cell-containing medium resulted in a significant increase in GC glucose uptake ($p < 0.01$) (Figure 2).

3.3 | Effects of FSH on GLUT Expression

The qRT-PCR results showed that during FSH treatment, the changes in GLUT mRNA transcription levels were consistent with the changes in protein expression in GCs cultured in vitro. Here, FSH significantly increased the transcription of GLUT1 and GLUT4 (Figure 3A,D), but not GLUT2 and GLUT3 (Figure 3B,C). Also, WB analysis showed that FSH could significantly increase the expression of GLUT1 and GLUT4 compared to the control group (Figure 3E,H), but did not increase the expression of GLUT2 and GLUT3 significantly (Figure 3F,G). This suggests that FSH significantly increases GLUT1 and 4 mRNA and protein expression during the development of sheep GCs.

3.4 | Glucose Mechanism of Action on GCs Through GLUT

To study the effect of glucose on the development of GCs as mediated by GLUT, the glucose inhibitor azaserine (5 μM) was added to GC cultures, which eliminated the FSH-induced glucose uptake capacity of the GCs ($p < 0.05$, Figure 4A). Furthermore, azaserine significantly reduced the viability of the GCs (Figure 4B).

3.5 | In GCs, FSH Acts Through the AKT/FOXO1 Signal Pathway

To detect the phosphorylation of AKT in GCs cultured in vitro with FSH treatment, GCs were collected at 5, 10, 20 and 30 min, respectively, and the proteins extracted to analyse the expression of phosphorylated AKT (p-AKT) and AKT (Figure 5A). The results showed that the concentration of p-AKT and AKT increased from 10 min, reached a maximum at 20 min, and then decreased to control values after 30 min of FSH exposure (Figure 5B,C). The protein levels of p-AKT, AKT and the ratio of p-AKT/AKT at 20 min was significantly higher than that in the control group (Figure 5B,C,D). Also, the expression of p-AKT and AKT in the GCs without FSH treatment did not change significantly from 5 to 30 min, nor did the ratio of p-AKT/AKT (Figure 5B,C,D). This suggests that FSH promotes the upregulation of AKT protein expression, as well as the activation of the protein through phosphorylation.

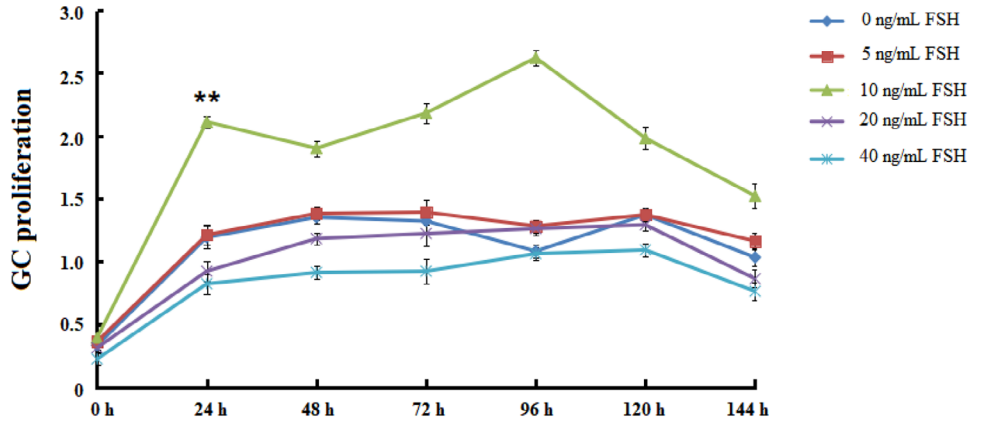

Figure 1. The effects of FSH on GCs proliferation.

FIGURE 1 | The effects of FSH on GCs proliferation. The mean OD values for each treatment were considered the numbers of viable cells for each treatment. ** on the shoulder mark indicates a significant difference ($p < 0.01$).

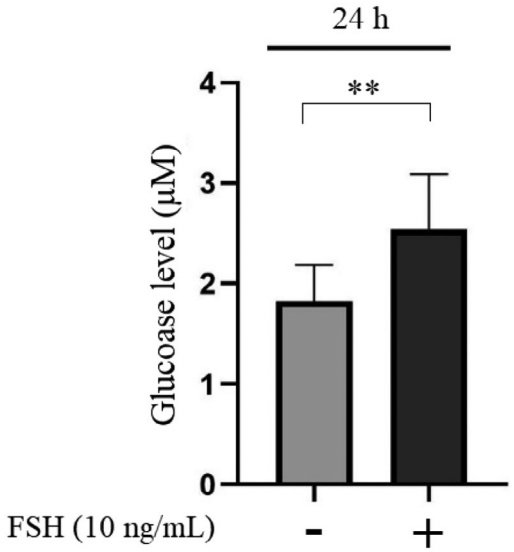

FIGURE 2 | Effect of FSH on glucose uptake in GCs cultured in vitro. Data are presented as mean ± SD of three independent experiments **, $p < 0.01$.

FIGURE 3 | Effect of FSH on expression of GLUTs in GCs cultured in vitro. Transcription levels of *GLUT 1* (A), *GLUT 2* (B), *GLUT 3* (C) and *GLUT 4* (D) mRNAs. Protein levels of GLUT 1 (E), GLUT 2 (F), GLUT 3 (G) and GLUT 4 (H). Data are presented as mean ± SD for 3 independent experiments. **, $p < 0.01$. *, $p < 0.05$. ns, no significance.

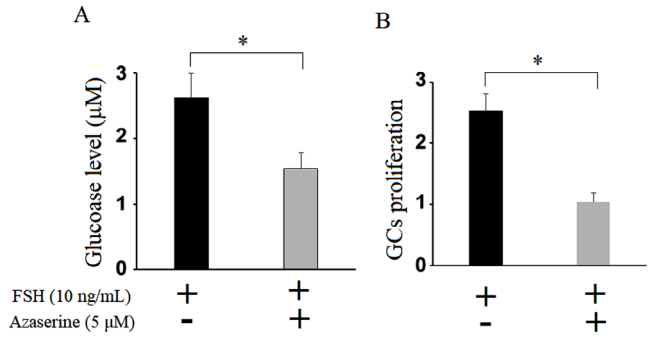

FIGURE 4 | Effect of GLUT inhibitor on glucose uptake and in vitro development of GCs. (A) Glucose uptake and (B) cell viability were analysed by 2-DG measurement and CCK-8 assay, respectively. Data are presented as mean ± SD of three independent experiments. *, $p < 0.05$.

3.6 | FSH Regulates GLUT Expression and Glucose Uptake in GCs via the AKT/FOXO1 Signalling Pathway

To investigate whether FOXO1 activation was mediated by AKT induced by FSH in ovine GCs cultured in vitro, the AKT inhibitor AT7867 was used to block the AKT signalling. When ovine GCs were cultured in the presence of AT7867 (10 μM) and FSH (10 ng/mL) for 20 min, the amount of p-FOXO1 increased significantly ($p < 0.05$) (Figure 6A), but the concentration of the total FOXO1 in the GCs remained unchanged, indicating that AKT regulated the phosphorylation of FOXO1 and activated its function in ovine GCs cultured in vitro.

Then, AT7867 was used to block the AKT/FOXO1 signalling pathway, while detecting the glucose uptake and the expression of GLUT1 and GLUT4 in GC cultured in vitro. Data showed that the glucose level in the GCs treated with FSH and AT7867 was significantly lower than that in the GCs treated with FSH only ($p < 0.05$) (Figure 6B), suggesting that AT7867 was able to inhibit the FSH-induced uptake of glucose in the GCs. Consistent with the above change in glucose uptake, the expression of GLUT1 and GLUT4 in the GCs treated with FHS and AT7867 was significantly lower than that in GCs treated with FSH only ($p < 0.05$) (Figure 6C,D). This indicates that the levels of FOXO1 and p-FOXO1 are enhanced by the AKT inhibitor AT7867 (10 μM) and that glucose uptake was reduced.

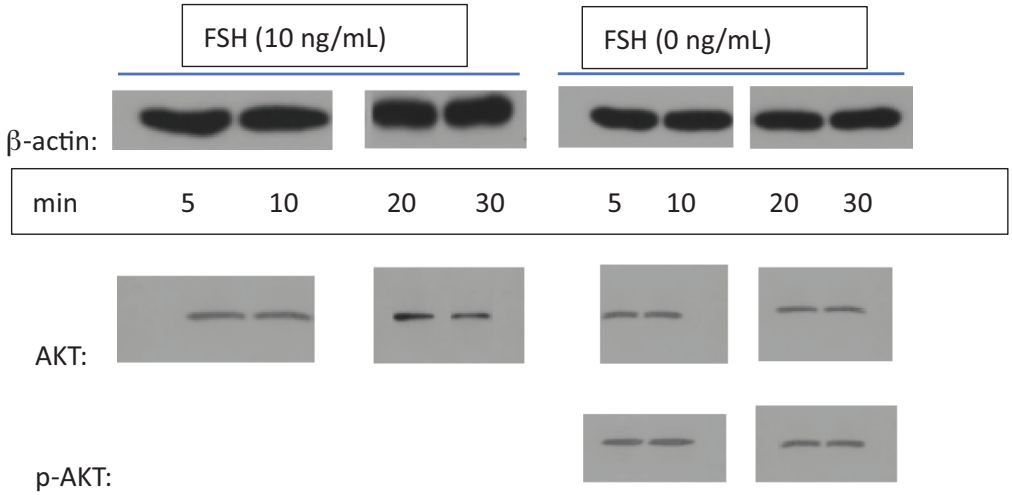

FIGURE 5 | Expression of p-AKT and AKT upon FSH treatment in GCs cultured in vitro. (A)Expression of p-AKT and AKT as determined by western blot analysis. The p-AKT (B) and AKT (C) expression were firstly normalized to β-actin expression, respectively, and then p-AKT expression was normalized to AKT expression (D). Data are represented as Mean ± SD ($n = 3$). *, $p < 0.05$. **, $p < 0.01$.

FIGURE 6 | Involvement of AKT/FOXO1 signalling in FSH induced GLUT expression and glucose uptake. GCs were cultured in the presence of AT7867 (10 μM) for 2 h prior to addition of FSH. Expression of FOXO1, GLUT1 and GLUT4 were assessed by western blot analysis. (A) Expression of p-FOXO1 and total FOXO1. (B) FSH-induced glucose uptake. Expression of GLUT1 (C) and GLUT4 (D). Data are represented as Mean ± SD ($n = 3$). **, $p < 0.01$.

4 | Discussion

Research indicates that exogenous FSH can significantly inhibit GC apoptosis and follicle atresia and exhibits the highest efficiency of all tested gonadotropins (Blanco et al. 2011; Parsanezhad et al. 2017). However, different species and different environmental conditions may result in different concentrations of FSH satisfaction required. In this study on goat ovaries, 5, 20 or 40 ng/mL of FSH had no effect on GC density over time, and only 10 ng/mL FSH could significantly improve the proliferation of GCs. This could be because high concentrations of FSH will increase the concentration of cAMP in GCs, and this increase in cAMP concentration will inhibit the maturation of GCs, resulting in a decline in cell proliferation and differentiation. In the process of in vitro culture of GCs, this inhibitory effect could not be eliminated fast enough and a high concentration of FSH would always inhibit cell proliferation and differentiation. Thus the cells showed a decreased proliferation ability and decreased activity.

The maintenance of GC proliferation and differentiation mainly depends on macromolecular substances such as glucose and while glucose cannot directly permeate the plasma membrane, cellular uptake is mediated by GLUTs (Hosoe et al. 1999; Tian et al. 2018). A negative correlation between glucose concentration in the follicular fluid and GLUT1 and GLUT3 mRNA levels in the GCs was observed in bovine follicles, suggesting that some local glucose environment (hormonal) affects the ability of follicles to uptake glucose. Here, GLUT1 and GLUT3 are mainly responsible for glucose transport in GCs (Nishimoto et al. 2006). The above experimental results suggest that different isoforms of GLUT exist in different species, whereas in the current study, FSH-induced effects were observed only for GLUT1 and GLUT4. As a result, glucose uptake was increased in sheep GCs, revealing that FSH promotes follicular production and GC development in mammals.

As a direct downstream signalling molecule of the PI3K/AKT signalling pathway, FOXO1 exerts various biological activities by transcribing and transducing various growth factors and cytokine signals (Xing et al. 2018). In mouse GCs, FSH rapidly phosphorylates FOXO1 by activating the PI3k/AKT signalling pathway and induces the translocation of FOXO1 from the nucleus to the cytoplasm when the rate of FOXO1-dependent apoptosis is reduced (Shen et al. 2014). Furthermore, deep sequencing results have shown that FOXO1-regulated genes in GCs are also FSH target genes (Herndon et al. 2016). In this study, 10 ng/mL of FSH significantly increased the concentration of p-AKT in GCs cultured in vitro, indicating that the AKT signalling pathway in ovine GCs is activated by FSH (Figure 4). Blocking AKT signalling with the AKT inhibitor AKT7867 showed that the protein level of p-FOXO1 was significantly increased, and the p-FOXO1/FOXO1 ratio was also increased (Figure 5), suggesting that FSH could activate the AKT/FOXO1 signalling pathway in ovine GCs cultured in vitro. Under inhibition of AKT, the expression of GLUT1 and GLUT4 were significantly decreased, even in the presence of FSH, suggesting that the AKT/FOXO1 signalling pathway induced by FSH may be involved in the regulation of glucose uptake in ovine GCs cultured in vitro. It is worth noting that the effect of FSH on glucose uptake identified here could be only one of the possible pathways through which FSH regulates glucose uptake, and that there might be relevant downstream mechanisms that contribute to the uptake of glucose as well (Chen and Downs 2008; Xu et al. 2019).

5 | Conclusion

The main objective of this study was to demonstrate that the molecular mechanism regulating the proliferation of sheep GCs is mediated by FSH. Data showed that FSH was able to activate the AKT/FOXO1 signalling pathway, inducing the up-regulation of GLUT1 and 4 expression, which promotes glucose binding and uptake by GCs, ultimately enhancing the biological activities of the GCs. The results of this study further the current understanding of the molecular mechanism of FSH activation of the AKT/FOXO1 signalling pathway in ovine GCs. We also propose a reference theory for future mechanistic analyses of metabolomics during follicular development in mammals.

Author Contributions

Xin Xu: investigation, methodology, software, writing – review and editing. **Ruotong Wang**: investigation, visualization, writing – original draft. **Linlin Pei**: formal analysis, visualization. **Quanfeng Wang**: data curation, resources. **Chunjie Liu**: conceptualization, funding acquisition, methodology, project administration, supervision, validation, writing – review and editing.

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Ethics Statement

All experiments were approved by the Institutional Animal Care and Use Committee of the Tarim University.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Peer Review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/vms3.70294

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