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RESEARCH ARTICLE

# Mechanism of miR-665 Regulating Luteal Function Via Targeting HPGDS

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## Abstract

Given few reports on the interaction of miRNA-665/target gene pair, we aimed to construct a dual luciferase reporter gene vector for 3′-untranslated region (3′-UTR) of the haematopoietic prostaglandin D synthase (HPGDS) gene and elucidate the underlying molecular mechanism of miR-665 regulation of HPGDS. Bioinformatics software was used to predict miR-665 targeting of 3′-UTR region of HPGDS gene. The reliability of the synthetic psiCHECK-HPGDS-w/m-3′-UTR was determined using double luciferase digestion method. Then, miR-665 mimic/negative control was separately co-transfected with sheep luteal cells, and then, luciferase activity and HPGDS expression were detected. Results showed that 3′-UTR wild-type (psiCHECK2-HPGDS-w-3′UTR) and mutant (psiCHECK2-HPGDS-m-3′UTR) expression vectors for HPGDS were successfully constructed, and dual luciferase reporter gene assay showed that the expression of relative luciferase activity was inhibited in the w-3′-UTR group, with 52% decrease compared to the blank/negative control group, and the difference was statistically significant ($P<0.05$). Whereas in luteal cells, compared to the blank/negative control group, RT-PCR and western blot assays showed lower HPGDS expression levels when miR-665 overexpression in miR-665-mimic group. Meanwhile, our result also showed that P4 concentration significantly increased using ELISA test in above group. In brief, our data verified that 3′-UTR wild-type dual luciferase reporter gene vector of HPGDS was successfully constructed, and miR-665 could target the gene by acting on the 3′-UTR region of HPGDS to regulate the ovine luteal cell function, providing a strong support to study the function and molecular mechanism of miR-665 in targeting HPGDS, especially in the ovarian-luteal tissue of sheep.

***Keywords:*** *3′-UTR, miRNA, Target gene, Luteal cells, Sheep*

# MiR-665'nin HPGDS Hedefli Luteal Fonksiyon Düzenleme Mekanizması

## Öz

MiRNA-665/hedef gen çiftinin etkileşimi hakkında az sayıdaki rapor göz önüne alındığında, hematopoietik prostaglandin D sentaz (HPGDS) geninin 3′-kodlanmayan bölgesi (3′-UTR) için bir çift lusiferaz raportör gen vektörünün oluşturulması ve HPGDS'nin miR-665 regülasyonunun moleküler mekanizmasının aydınlatılması amaçlandı. HPGDS geninin 3′-UTR bölgesini hedefleyen miR-665'nin saptanması için biyoinformatik yazılım kullanıldı. Sentetik psiCHECK-HPGDS-w/m-3′-UTR'nin güvenilirliği, dual lusiferaz sindirim yöntemi ile belirlendi. Daha sonra miR-665 mimik/negatif kontrolün, koyun luteal hücreleri ile ayrı ayrı ko-transfeksiyonu yapıldı ve ardından lusiferaz aktivitesi ve HPGDS ekspresyonu tespit edildi. Sonuçlar, HPGDS için 3′-UTR yabanıl tip (psiCHECK2-HPGDS-w-3′UTR) ve mutant (psiCHECK2-HPGDS-m-3′UTR) ekspresyon vektörlerinin başarıyla oluşturulduğunu ve dual lusiferaz raportör gen analizi, relatif lusiferaz aktivite ekspresyonunun, blank/negatif kontrol grubuna kıyasla %52'lik bir düşüşle w-3′-UTR grubunda inhibe edildiğini ve farkın istatistiksel olarak anlamlı olduğunu gösterdi ($P<0.05$). Luteal hücrelerde, blank/negatif kontrol grubu ile karşılaştırıldığında, RT-PCR ve western blot deneyleri, miR-665-mimik grubunda miR-665'in aşırı ekspresyonunda HPGDS'nin daha düşük oranda eksprese olduğunu gösterdi. Ayrıca, sonuçlarımız yukarıda bahsedilen grupta ELISA testi kullanılarak P4 konsantrasyonunun önemli ölçüde arttığını gösterdi. Özetle, verilerimiz özellikle koyunların yumurtalık luteal dokusunda HPGDS'yi hedeflemede miR-665'nin işlevini ve moleküler mekanizmasını incelemek için güçlü destek sağlayarak, HPGDS'nin 3′-UTR yabanıl tip dual lusiferaz raportör gen vektörünün başarıyla oluşturulduğunu ve miR-665'in, koyun luteal hücre fonksiyonunu düzenlemede HPGDS'nin 3′-UTR bölgesi üzerinde hareket ederek geni hedefleyebildiğini doğruladı.

***Anahtar sözcükler:*** *3′-UTR, MiRNA, Hedef gen, Luteal hücre, Koyun*

**How to cite this article?**

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## Introduction

Haematopoietic prostaglandin D synthase (HPGDS) belongs to the GST superfamily and the sigma family [1]. HPGDS contains GST C-terminal structural domain and GST N-terminal structural domain. HPGDS is a cytosolic enzyme that heterodimerises $PGH_2$ and is able to selectively and efficiently heterodimerise $PGH_2$ to $PGD_2$ [2], whereas other GST isoenzymes non-selectively catalyse the conversion of $PGH_2$ to $PGD_2$, $PGE_2$, and $PGF_{2\alpha}$ [3,4]. HPGDS is widely expressed in mast cells, macrophages, Th2 cells, and other leukocytes, and it is believed that it can promote $PGD_2$ synthesis and play an important regulatory role in most inflammatory responses [5,6]. Moreover, HPGDS can exist as a homodimer in living organisms and is highly expressed in the adipose tissue, placenta, ovary, and corpus luteum [7,8]. This also suggests that HPGDS may be actively involved in lipid synthesis and metabolism as well as in reproductive regulation (e.g., follicular development and luteal maintenance and degeneration) in animals.

MicroRNAs (miRNAs) are endogenous non-coding RNAs with regulatory functions in eukaryotic organisms, whose mature bodies recognise target mRNAs through base complementary pairing and direct silencing complexes to degrade target genes or block mRNA transcription depending on the degree of complementarity [8-10]. Accumulating evidence suggests that miRNA mediated post-transcriptional modification of genes can regulate many pathophysiological processes, such as inflammation, angiogenesis, fibrotic repair, apoptosis, and uterine adhesion [11]. miR-665 is a newly identified miRNA, which is closely associated with the functions of the reproductive system in females, playing a regulatory role in follicular-luteal transition [12]. This study aims to investigate the changes in miR-665 expression in luteal tissue after follicle-luteal transition in females and conduct experimental validation and correlation analyses using the bioinformatics target gene prediction results of our group, that is, the existence of target binding sites between miRNA-665 and HPGDS, to lay a solid foundation for an in-depth investigation of miR-665 role in luteal tissue and its targeted regulation of HPGDS in the molecular regulation mechanism of luteal cells.

## Material and Methods

### *Primary Cell Culture and Identification*

Mid-term luteal tissue of 20 healthy Kazakh nulliparous ewes was obtained from Shihezi Slaughterhouse in Xinjiang, China in September 2020, and brought back to the laboratory with fat and connective tissue removed. The tissue was cut into 1-$mm^3$ pieces, and the shredded luteal tissue was digested using collagenase II (Gibco, USA) at 37°C for 1 h. The resulting cell precipitate was then inoculated in DMEM/F12 medium containing 10% foetal bovine serum and cultured at 37°C in a 5% $CO_2$ incubator. Purified ewe luteal cells were obtained after 3-4 passages of the culture. Synaptophysin (SYP) (Bioss, China) immunocytochemical staining was used to identify ewe luteal cells.

### *MicroRNA-665 Target Gene Prediction*

Target scan *(http://www.targetscan.org/)* and RNA hybrid *(http://bibiserv.techfak.uni-bielefeld.de/)* target gene software were used simultaneously to predict the target gene of miR-665, namely HPGDS, and to obtain more plausible miR-665 and HPGDS-3′ sequence pairing sites through screening and analysis.

### *Construction and Characterisation of Wild-Type and Mutant psiCHECK-HPGDS-w/m-3′-UTR Vectors*

Our laboratory provided the vector psiCHECK-2 (Promega, USA) and sent it to Shanghai Bioengineering Co., Ltd. for direct synthesis of psiCHECK-HPGDS-w/m-3′-UTR, which contains Luc marker gene expression (the Appendix for information on the wild-type and mutant HPGDS sequences *(Table 1)*. To verify the reliability of the synthesised w/m-3¢-UTR vector, the target fragments were inserted into XhoI and NotI sites for enzymatic cleavage in the following system: w/m-3′-UTR vector (500 ng/μL) 4 μL, 10×H buffer 2 μL, XhoI 1 μL, NotI 1 μL, $ddH_2O$ 12 μL, in a constant temperature water bath at 37°C for 1 h, followed by addition of 1.5% agarose gel for double digestion. Then,

*Table 1. Wild-type HPGDS-3'UTR and Mutant-type HPGDS-3'UTR sequences*

| **Wild-type HPGDS-3'UTR Sequences** |
|---|
| TCACCAGAGTCTAGCAATAGCAAGATACTTGACCAGAAACACAGATTTGGCTGGAAAAACAGAACTTGAACAATGTCAAGTGGATG<br>CAATTGTGGACACACTGGATGATTTCATGTCTCGTTTTCCTTGGGCAGAGAAAAGACAAGATATAAAAAATCAGATATTTAAGGAGC<br>TACTTACCTGTGATGCACCTCCTCTTCTGCAAAGTTTGGACACATACTTAGGGGAAAACGAGTGGTTTATTGGTGACTCTGTAACTTG<br>GGCAGACTTCTACTGGGAAATTTGCAGTACCACACTTTTGGTCTTTAAACCTGATTTGTTGGACATCCACCCCAGGCTGGTGACATT<br>ACGGAAGAAAGTCCAAAGCATCCCTGCCATCGCTGACTGGATACTGCGAAGGCCCCAGACCAAACTCTAG |
| **Mutant-type HPGDS-3'UTR Sequences** |
| TCACCAGAGTCTAGCAATAGCAAGATACTTGACCAGAAACACAGATTTGGCTGGAAAAACAGAACTTGAACAATGTCAAGTGGATG<br>CAATTGTGGACACACTGGATGATTTCATGTCTCGTTTTCCTTGGGCAGAGAAAAGACAAGATATAAAAAATCAGATATTTAAGGAGC<br>TACTTACCTGTGATGCACCTCCTCTTCTGCAAAGTTTGGACACATACTTAGGGGAAAACGAGTGGTTTATTGGTGACTCTGTAACTTc<br>GtCAGACTTgTcCaGtGAAATTTGCAGTACCACACTTTTGGTCTTTAAACCTGATTTGTTGGACATCCACCCCAGGCTGGTGACATTAC<br>GGAAGAAAGTCCAAAGCATCCCTGCCATCGCTGACTGGATACTGCGAAGGCCCCAGACCAAACTCTAG |

w/m-3′-UTR was transformed into DH5α receptor cells for expansion and plasmid extraction. The concentration and purity of the plasmids were determined using NanoDrop 2000 (Thermo, USA), and the qualified plasmids were used for the later transfection assays.

### Cell Grouping and Transfection

Luteinising cells were inoculated in 12-well plates with $2\times10^6$ cells/well and incubated overnight. When the cell density reached 70%-80%, five groups were made, namely, blank control group (adding equal volume of blank liposome mixture, abbreviated BC), test group 1 (psiCHECK-HPGDS-w-3′UTR + miR-665 mimic, abbreviated w-3′-UTR), negative control group 1 (psiCHECK-HPGDS-w-3′UTR + miR-665 negative control, abbreviated w-NC), test group 2 (psiCHECK-HPGDS-m-3′UTR + miR-665 mimic, abbreviated m-3′-UTR), and negative control group 2 (psiCHECK-HPGDS-m-3′UTR + miR-665 negative control, abbreviated m-NC). Each group of 3 wells was replicated and transiently transfected with luteinising cells by lipofectamine 3000 (Invitrogen, USA), incubated at 37°C for 6 h, and then, the cell culture medium was changed. After 48 h of incubation, the wells were washed 3 times with PBS and 1 mL of total RNA lysate was added to each well and agitated on a shaker for sufficient lysis. The total RNA was extracted by centrifuging supernatant lysate and their concentration was measured, and then, it was stored at -80°C until further analysis.

### Dual Luciferase Reporter Gene Activity Assay

After 48 h of transfection, the instructions of the manufacturer of the dual luciferase reporter gene assay kit (Beyotime, China) were followed, and finally, the fluorescence values were read, and the relative fluorescence activity was calculated using Synergy H4 multifunctional enzyme marker (BioTek, USA).

### Changes in HPGDS and miR-665 Expression were Evaluated Using qRT-PCR

When the density of the luteinising cells in the culture dish reached 70%-80%, miR-665-mimic/negative control transfection test was continued and three groups were set up, namely, blank control group, miR-665-mimic group, and negative control group. After 48 h, total RNA and miRNA were extracted from each group and were subjected to reverse transcription using reverse transcription kit at 42°C for 15 min and 85°C for 5 min, and then stored at -20°C. The ploidy changes of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method according to SYBR Mixture kit (TaKaRa, Japan) instructions. All primers are shown in *Table 2*. Three parallel sets were set up for each test group. The test was repeated thrice and the results were recorded.

### Changes in HPGDS Expression were Evaluated Using Western Blot

Luteinising cells from each group transfected for 48 h were collected and total protein was extracted from each group using RIPA strong cell lysate. Protein from cells was extracted using RIPA lysis buffer, and the protein concentrations was detected using the BCA protein quantification kit (Abcam, Britain). 30 μg protein samples were performed SDS-PAGE, and transferred to PVDF membranes (Millipore, USA) after 1.5 h. 5% skimmed milk was used to block for 30 min at room temperature. The membranes were incubated with the primary antibodies overnight at 4°C (Rabbit anti-Bcl-2 (ab196495; 1:500), rabbit anti-caspase3 (ab90437; 1:1,000) and rabbit anti-β-actin (ab8227; 1:1,000), Abcam, Britain). After washing with PBS three times, the membranes were incubated with goat anti-rabbit immunoglobulin G (IgG) at room temperature for 2 h. Protein bands were visualized using an Electrochemiluminescence (ECL) chemiluminescence kit (WBULS0500; EMD Millipore, USA) and the bands intensity was quantified with Quantity One software.

***Table 2.*** *All primers for miR-665 and HPGDS*

| Name | Primers |
|---|---|
| miR-665 | ACCAGTAGGCCGAGGCC |
| U6 | CAAGGATGACACGCAAATTCG |
| HPGDS | F: ATGCCTAACTACAAACTGCTT |
| | R: CTAGAGTTTTGTCTGTGGCCT |
| β-actin | F: CAGCAGATGTGGATCAGCAAGCAG |
| | R: TTGTCAAGAAAAAGGGTGTAACGCA |

### Detection of Progesterone Concentration in Culture Medium Using ELISA

All culture medium from each group transfected for 48 h were collected, and then these samples were centrifuged and their upper part liquid were absorbed to detected using Sheep $P_4$ ELISA Kit according to the manufacturer's instruction. Three parallel sets were set up for each test group. The test was repeated thrice and the results were recorded.

### Statistical Analysis

Data were presented in figures. Bar graph with y-axis indicating mean and error bar as standard deviation in *Fig. 5, 6* and *7*. Student's t-test or One-way ANOVA followed by Tukey's test was used to analyse the statistical differences among groups (normally distributed and the group variances are homogenous) based on SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was regarded as a statistically significant difference.

## Results

### Culture and Identification of Corpus Luteal Cells

The corpus luteal cells isolated by collagenase II digestion were in a single dispersed state, and the cell bodies were transparent, round or polygonal cells with a relatively

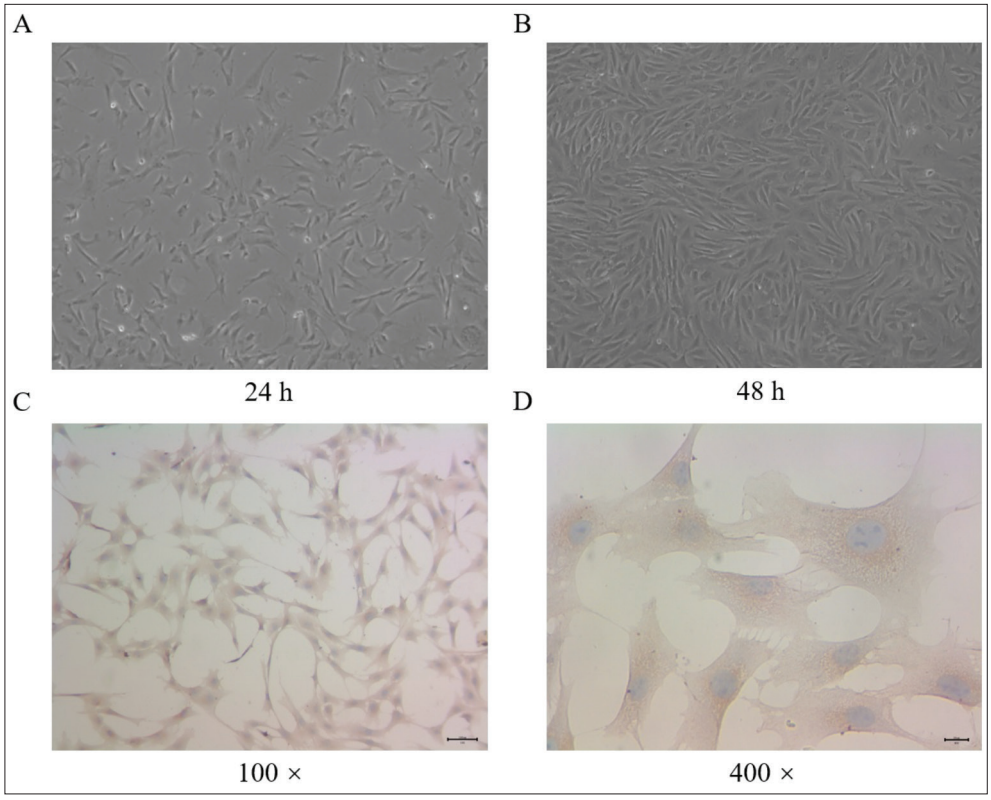


**Fig 1.** Primary luteal cells were cultured *in vitro* and its specific marker, the expressions of SYP protein was identified with immunohistochemistry. A and B were cultured for 24 and 48 h *in vitro,* respectively. C and D were cultured for 48 h, SYP protein was detected by immunocytochemistry

small volume. After 24 h of culture, the corpus luteal cells began to adhere to the wall, showing an irregular shape with a tendency to extend outward, and connections between adjacent cells began to be established *(Fig. 1-A)*. After culturing for 48 h, the cells entered the logarithmic growth phase, and the cells were completely attached to the wall, showing a fusiform or irregular shape *(Fig. 1-B)*. Mammalian corpus luteum belongs to the diffuse neuroendocrine system, and SYP is a more comprehensive neuroendocrine marker. The positive rate of corpus luteal cells identified by SYP cell immunohistochemistry was over 90% *(Fig. 1-C,D)*, indicating that the isolated cells were sheep corpus luteal cells.

### *Target Genes of miR-665 were Predicted Using Targetscan and miRDB Software*

The predictions from the two databases were amalgamated and potential binding sites between miR-665 and HPGDS were found to exist. The plausible target sites for HPGDS were obtained using the Targetscan software prediction, as shown in *Fig. 2*. Meanwhile, these sites (GGGC and CUACUGG) were mutated to CGUC and GUCCAGU in 3′-UTR mutant vector *(Fig. 2)*.

### *Identification of the Dual Luciferase Reporter Plasmid psiCHECK-HPGDS-w/m-3′-UTR*

To verify the reliability of synthesising psiCHECK-HPGDS-w/m-3′-UTR vector, the target fragments were inserted into XhoI and NotI sites for enzymatic cleavage, and the results here indicated that the target gene HPGDS had been inserted into psiCHECK successfully, as shown in *Fig. 3*. And then, the constructed psiCHECK-HPGDS-w/m-3′-UTR vectors were sent to Bioengineering (Shanghai) Co. for sequence determination, the sequencing results demonstrated that the sequence bases were correct *(Fig. 4-A,B)*, which could be used subsequently for large-scale extraction of plasmids from the constructed vectors and further transfection experiments using the endotoxin-free plasmid bulk extraction kit.

### *Dual Luciferase Reporter Test*

Luciferase reporter assays were performed, and total cellular RNA was extracted for qRT-PCR analysis. The results showed that transfection with miR-665 significantly reduced the relative fluorescence activity ratio (abbreviated Rluc/Luc) of luteal cells transfected with psiCHECK2-HPGDS-w-3′UTR compared to that of w-NC/m-NC group ($P<0.01$), whereas there was no significant change in Rluc/Luc of luteal cells transfected with psiCHECK2-HPGDS-m-3′UTR ($P>0.05$) *(Fig. 5)*.

### *miR-665 Negatively Regulates HPGDS Expression in Luteal Cells*

After miR-665 mimic/negative control transfection of luteal cells, total RNA, miRNA, and protein were extracted after

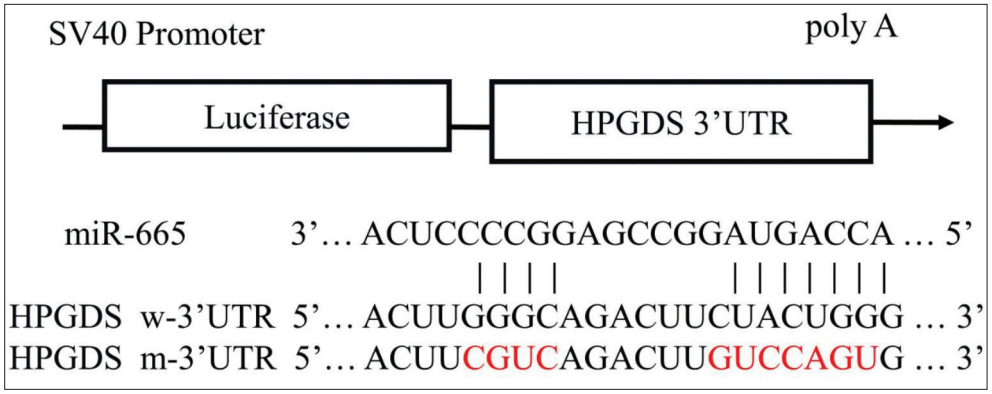


**Fig 2.** Schematic diagram of target binding site between miR-665 and HPGDS (in red font for mutation sites)

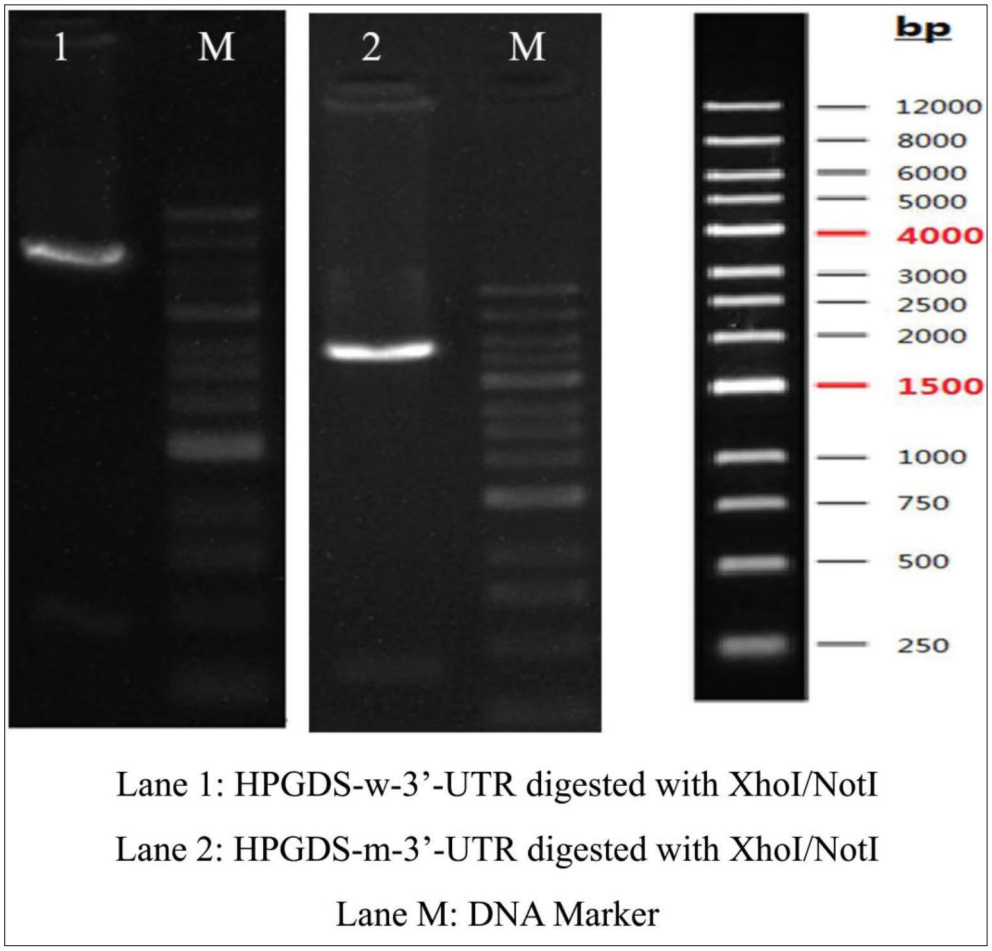


**Fig 3.** Expression vector psiCHECK-HPGDS-w/m-3'UTR was correctly constructed, identified with double-enzyme digestion

48 h of culture and the changes in HPGDS expression were detected using qRT-PCR and western blot. The results revealed that miR-665 mimic caused miR-665 overexpression, when compared with the miR-655 expression in the blank control and negative control groups *(Fig. 6-A)* ($P<0.001$). However, at the mRNA level, the results showed that HPGDS expression was significantly inhibited in luteal cells ($P<0.01$) *(Fig. 6-B)*; moreover, the results of western blotting showed a significant decrease in HPGDS protein levels ($P<0.01$) *(Fig. 6-C,D)*.

### *Progesterone Concentration Test*

Cell cultures from the different treatment groups in the previous section were collected and their $P_4$ concentrations were measured separately using ELISA method, as shown in *Fig. 7*. The results showed that $P_4$ concentration

A

| | |
|---|---|
| HPGDS-3'UTR | TCACCAGAGTCTAGCAATAGCAAGATACTTGACCAGAAACACAGATTTGGCTGGAAAAAC |
| Sequencing | CTCGAGTCACCAGAGTCTAGCAATAGCAAGATACTTGACCAGAAACACAGATTTGGCTGGAAAAAC |
| HPGDS-3'UTR | AGAACTTGAACAATGTCAAGTGGATGCAATTGTGGACACACTGGATGATTTCATGTCTCGTTTTCCT |
| Sequencing | AGAACTTGAACAATGTCAAGTGGATGCAATTGTGGACACACTGGATGATTTCATGTCTCGTTTTCCT |
| HPGDS-3'UTR | TGGGCAGAGAAAAGACAAGATATAAAAAATCAGATATTTAAGGAGCTACTTACCTGTGATGCACCT |
| Sequencing | TGGGCAGAGAAAAGACAAGATATAAAAAATCAGATATTTAAGGAGCTACTTACCTGTGATGCACCT |
| HPGDS-3'UTR | CCTCTTCTGCAAAGTTTGGACACATACTTAGGGGAAAACGAGTGGTTTATTGGTGACTCTGTAACTT |
| Sequencing | CCTCTTCTGCAAAGTTTGGACACATACTTAGGGGAAAACGAGTGGTTTATTGGTGACTCTGTAACTT |
| HPGDS-3'UTR | GGGCAGACTTCTACTGGGAAATTTGCAGTACCACACTTTTGGTCTTTAAACCTGATTTGTTGGACAT |
| Sequencing | GGGCAGACTTCTACTGGGAAATTTGCAGTACCACACTTTTGGTCTTTAAACCTGATTTGTTGGACAT |
| HPGDS-3'UTR | CCACCCCAGGCTGGTGACATTACGGAAGAAAGTCCAAAGCATCCCTGCCATCGCTGACTGGATACT |
| Sequencing | CCACCCCAGGCTGGTGACATTACGGAAGAAAGTCCAAAGCATCCCTGCCATCGCTGACTGGATACT |
| HPGDS-3'UTR | GCGAAGGCCCCAGACCAAACTCTAG |
| Sequencing | GCGAAGGCCCCAGACCAAACTCTAGGCGGCCGC |

B

| | |
|---|---|
| HPGDS-3'UTR-mut | TCACCAGAGTCTAGCAATAGCAAGATACTTGACCAGAAACACAGATTTGGCTGGAAAAAC |
| Sequencing | CTCGAGTCACCAGAGTCTAGCAATAGCAAGATACTTGACCAGAAACACAGATTTGGCTGGAAAAAC |
| HPGDS-3'UTR-mut | AGAACTTGAACAATGTCAAGTGGATGCAATTGTGGACACACTGGATGATTTCATGTCTCGTTTTCCT |
| Sequencing | AGAACTTGAACAATGTCAAGTGGATGCAATTGTGGACACACTGGATGATTTCATGTCTCGTTTTCCT |
| HPGDS-3'UTR-mut | TGGGCAGAGAAAAGACAAGATATAAAAAATCAGATATTTAAGGAGCTACTTACCTGTGATGCACCTCC |
| Sequencing | TGGGCAGAGAAAAGACAAGATATAAAAAATCAGATATTTAAGGAGCTACTTACCTGTGATGCACCTCC |
| HPGDS-3'UTR-mut | TCTTCTGCAAAGTTTGGACACATACTTAGGGGAAAACGAGTGGTTTATTGGTGACTCTGTAACTTCGTC |
| Sequencing | TCTTCTGCAAAGTTTGGACACATACTTAGGGGAAAACGAGTGGTTTATTGGTGACTCTGTAACTTCGTC |
| HPGDS-3'UTR-mut | AGACTTGTcCaGTGAAATTTGCAGTACCACACTTTTGGTCTTTAAACCTGATTTGTTGGACATCCACCCC |
| Sequencing | AGACTTGTcCaGTGAAATTTGCAGTACCACACTTTTGGTCTTTAAACCTGATTTGTTGGACATCCACCCC |
| HPGDS-3'UTR-mut | AGGCTGGTGACATTACGGAAGAAAGTCCAAAGCATCCCTGCCATCGCTGACTGGATACTGCGAAGGC |
| Sequencing | AGGCTGGTGACATTACGGAAGAAAGTCCAAAGCATCCCTGCCATCGCTGACTGGATACTGCGAAGGC |
| HPGDS-3'UTR-mut | CCCAGACCAAACTCTAG |
| Sequencing | CCCAGACCAAACTCTAGGCGGCCGC |

**Fig 4.** psiCHECK-HPGDS-w/m-3'UTR vector synthesis and sequencing sequences. A was psiCHECK-HPGDS-3'UTR vector synthesis and sequencing sequence; B was psiCHECK-HPGDS-3'UTR-mut vector synthesis and sequencing sequences. The sequence for cloning is shown in grey and the predicted binding site is shown in green

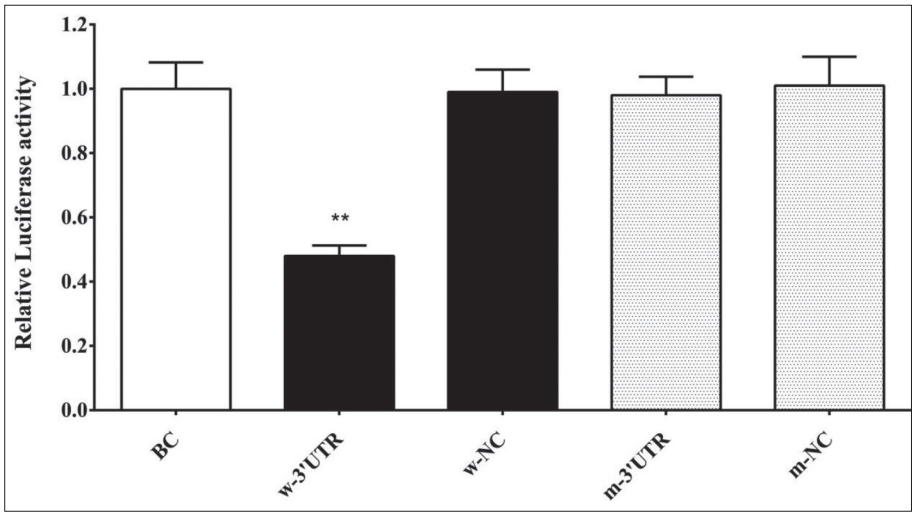


**Fig 5.** Analysis of dual relative luciferase activity of psi-CHECK2-HPGDS-w/m-3'UTR in luteal cells. Compared with BC and w-NC groups, Rluc/Luc of the w-3'UTR, ** P<0.01; Compared with BC and m-NC groups, *Rluc*/Luc of the m-3'UTR, not significant. P>0.05

significantly increased in the transfected miR-665 group compared to the blank control group (P<0.01), while $P_4$ concentration in the luteal cells transfected with negative control did not change significantly (P>0.05) *(Fig. 7)*.

## Discussion

miRNA mimic is a new technique and method of gene silencing that imitates the high expression levels of endo-

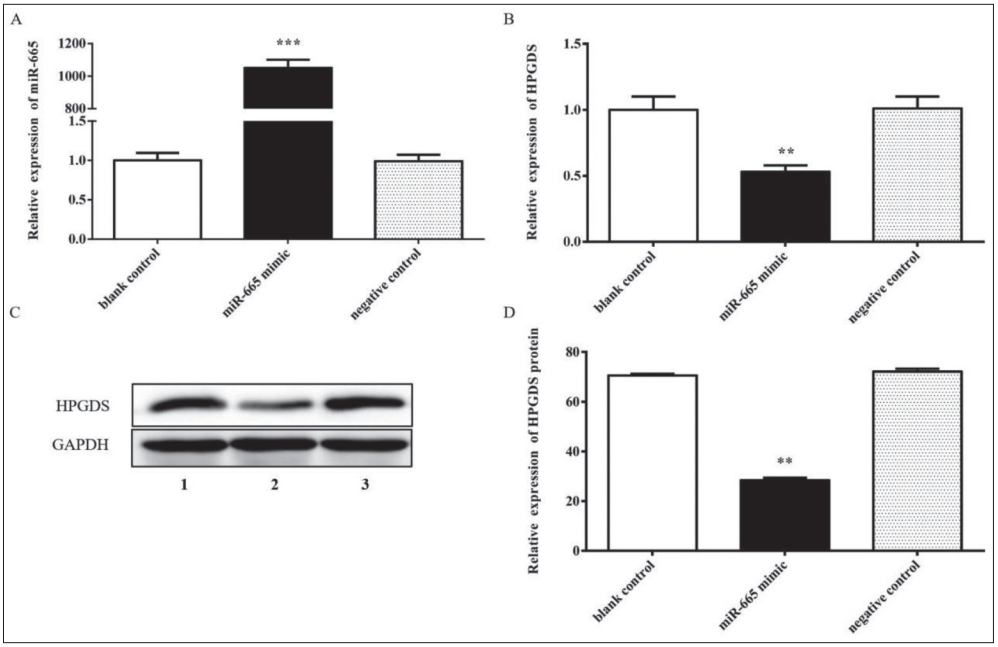


**Fig 6.** Expression of miR-665, HPGDS mRNA and protein in miR-665 mimic transfected luteal cells. A shows miR-665 expression, B and C represent HPGDS mRNA and protein expression and D indicates its protein expression using Image J software. Note: Compared with blank control and negative groups, expression of miR-665 in the w-3'UTR group, *** P<0.001; expression of HPGDS mRNA and protein in the w-3'UTR group, ** P<0.01. Each group was replicated three times

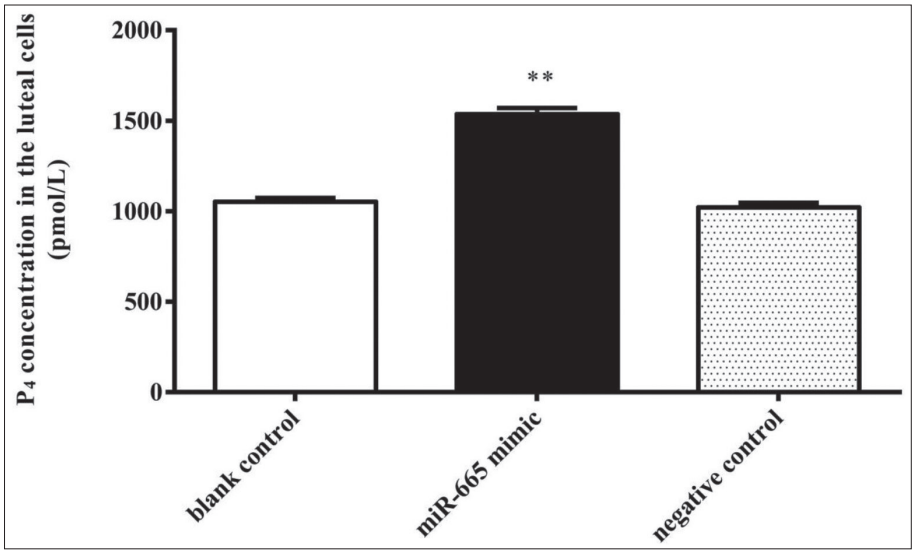


**Fig 7.** $P_4$ concentrations in miR-665 mimic transfected luteal cells. Compared with blank control and negative groups, $P_4$ concentrations in the w-3'UTR group, ** P<0.01

genous, mature miRNAs in cells to enhance their regulatory role. This method can generate unnatural double-stranded miRNA-like RNA fragments [13]. Such RNA fragments are designed to carry a motif at their 5'-end, which is partially complementary to the selected sequence in the unique 3'-UTR of the target gene. Once introduced into the cell, the mimic and its endogenous miRNA bind specifically to their target gene, causing a post-transcriptional repressive effect on that gene. miR-mimic approaches are "miRNA-targeting" and "miRNA-gain-of-function" strategies, mainly used as an exogenous tool to study gene function by targeting mRNAs in mammalian cells through miRNA-like actions [14]. For experiments, the cells are simply transfected with a transfection reagent package and ready for the next step, eliminating the need for cumbersome vector construction and the fear of viral infection.

Dual luciferase reporter gene detection system is designed for RNA interference, especially when using miRNA mimic for miRNA function study. It has become a convenient,

fast, and efficient method of detecting miRNA target sites for validation and is widely used in laboratory and clinical settings [15,16]. PsiCEHCK™-2 vector used in this pilot study included two luciferases, namely *Renilla* and firefly luciferase, which were used to detect and validate the target genes of miRNAs. The vector can be expressed in plant and animal cells with high efficiency. In this vector, the downstream polyclonal site of *Renilla* luciferase is after its stop codon, so that the target fragment to be detected can be inserted. Moreover, as no fusion protein is expressed, there is no concern regarding code shift mutations. *Renilla* luciferase and target fragment are expressed in a fused manner. After co-staining with RNAi vector and binding to the target site, the fusion product of *Renilla* luciferase and the target fragment were cleaved and degraded, thus attenuating *Renilla* luciferase signal [17-19].

In the present preliminary study, miR-665 was utilised to co-transfect luteinising cells with wild-type recombinant vector psiCHECK-HPGDS-w-3'UTR and mutant psiCHECK-HPGDS-m-3'UTR, respectively. A comparative analysis based on w-3'-UTR group (psiCHECK-HPGDS-w-3'UTR + miR-665 mimic) and the BC group revealed that the activity of *Renilla* luciferase significantly changed. In other words, the relative activity of Rluc/Fluc reduced by about 52% in the w-3'-UTR group (HPGDS) compared to that in the BC group, which indicated that HPGDS was the true target gene of miR-665. It has been recently shown that miR-665 is significantly upregulated in the inflamed mucosa of mice suffering from colitis and can participate in regulating inflammatory response process in animals [20,21]. Another study demonstrated that miR-665 not only inhibits cell proliferation but also participates in regulating apoptosis [20]; moreover, Engelsvold et al.[22] reported that miR-665 was also found to induce neovascularisation and promote the repair of cardiac function after myocardial ischaemia. In addition, one report showed that miRNA-665 could regulate cell proliferation and apoptosis through the Bcl-2 and Akt signalling pathways [23,24]. However, little has been reported about the function of miR-665 in the regulation of reproduction in females, especially the expression profile in the luteal tissue. Therefore, a dual-luciferase reporter approach was used to study miR-665 expression patterns and its regulatory functions in the luteal tissue of females, which is the first report of its kind. Based on this, our data also established that HPGDS was a bona fide target gene of miR-665. This gene was initially identified in rat spleen [25] and later in other tissues [26]. HPGDS is the only vertebrate sigma-like member of the GST superfamily and is widely distributed in antigen-presenting cells, Th2 lymphocytes, mast cells, and megakaryocytes [27]. It has been shown that in mouse models of asthma and allergic disease, HPGDS has significant pro-inflammatory effects, modulating many hallmark features, including eosinophilia, airway hyperresponsiveness, mucus production, and Th2 cytokine levels [28,29]. However, it has been demonstrated that HPGDS also exhibits significant anti-inflammatory effects in HPGDS knockout mouse assay [30]. This phenomenon is most likely related to the type of receptor in its downstream mechanism. HPGDS is a key synthase of the D member of the prostaglandin family, which catalyses the conversion of $PGH_2$ to $PGD_2$ and plays a role in the production of prostaglandin-like substances in target cells. Some studies have shown that HPGDS mRNA is highly expressed in female reproductive organs such as the fallopian tubes and the uterus [31], and the study conducted by Kanaoka et al.[2] indicated that HPGDS plays a key role in the regulation of inflammation in the fallopian tubes. Interestingly, Dozier et al.[4] found increased mRNA expression of HPGDS in the cultures of luteinised granulosa cells collected from rats after treatment with human chorionic gonadotropin, suggesting that HPGDS, like PGFS (synthase of PGF) or PGES (synthase of PGE), probably also plays an important regulatory role in the formation, maintenance, and degeneration of the corpus luteum, especially in the early stages of luteal formation [32]. Thus, the ovary is considered a potential site of action for HPGDS-catalysed synthesis of $PGD_2$, where HPGDS maintains the *in vivo* dynamic balance of the function and morphology of the female reproductive organs. This was further verified by Farhat et al.[8] who found that HPGDS-catalysed synthesis of $PGD_2$ could activate the expression of steroidogenic Cyp11A1 and StAR genes and subsequently promoted the secretion of progesterone, thereby playing a regulatory role in follicle growth by inhibiting the proliferation of granulosa cells. Based on the above results and analysis, we inferred that miR-665 and its target gene HPGDS probably also play important roles in the formation, maintenance, and degeneration of the corpus luteum in females, such as the proliferation and apoptosis of luteal cells involved in the formation and degeneration of the corpus luteum, which was in accordance with the functional mechanism of miR-665 reported so far. Furthermore, the target gene HPGDS and its synthesiser $PGD_2$ are members of the PGs family (which is recognised as a key factor involved in the regulation of luteal structure and function), suggesting that miR-665 is likely to be involved in the regulation of luteal function by targeting this PGs family members (HPGDS/$PGD_2$), which is also demonstrated in the present study, as shown by the increase in progesterone concentration after miR-665 transfection.

In conclusion, dual-luciferase reporter vector pisCHECK2-HPGDS-w/m-3'UTR was synthesised artificially for the first time, in this study, and the vector was verified to be successfully constructed using double digestion assay and sequencing. Moreover, after the upregulation of miR-665 expression, it was demonstrated that miR-665 could target the gene by acting on the 3'-UTR region of HPGDS to regulate the ovine luteal cell function, which provides a new perspective and material for studying the role of miRNAs in regulating the reproductive function in females, especially in the ovarian-luteal tissue of sheep.

## Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding authors (H. Yang and Z. Zhao) on reasonable request.

## Ethical Statement

This study was approved by the Shihezi University Animal Experiments Local Ethics Committee (Approval no: 2020108).

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## Conflict of Interest

The authors declared that there is no conflict of interest.

## Author Contributions

Y.S., L.F., H.Y. and Z.Z. conceived and designed the experiment and analyzed and interpreted the data. Y.S. wrote the manuscript. Y.S., L.F., H.Y. and Z.Z. collected samples and data. Z.T., Y.N. participated in RNA extraction and analysis. All authors have read and agreed to the published version of the manuscript.

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