EFFECTS OF Wnt / β -CATENIN SIGNALING PATHWAY AND STAR D7 ON TESTOSTERONE SYNTHESIS

T. Zhang, T. Zheng, C. Wang, W. Zhang, D. Jia, R. Wang, B. Qiao*

The First Affiliated Hospital of Zhengzhou University, Department of Urology, Zhengzhou, China

Abstract

Background. This study aimed to assess the mechanism through which Wnt/ β -catenin signaling pathway, and StarD7, promotes testosterone synthesis, and to explore a new pathway for the regulation of testosterone synthesis.

Animals and Methods. Leydig cells were isolated from male Sprague-Dawley rats divided into four groups and treated with Annexin 5 in concentration of 0, 0.1, 1 and 10 nmol/L. Testosterone secretion, expression of StarD7, StarD7 mRNA, β -catenin and changes of β -catenin localization in Leydig cells of testis of rats were tested in the four groups.

Results. mRNA and protein levels of StarD7 and β -catenin increased significantly, upon stimulation with 1 nmol/L annexin 5. Accumulation of β -catenin inside the cells and the nucleus, was observed by immunofluorescence staining, in cells treated with annexin 5. These findings indicate a possible role of StarD7 and β -catenin in the process of annexin5-mediated stimulation of testosterone synthesis.

Conclusions. Wnt/ β -catenin signaling pathway and StarD7 are involved in the process of annexin5 stimulation of testosterone synthesis. Activation of Wnt/ β -catenin signaling pathway by Annexin5, and increase in StarD7 expression lead to elevated expression of key regulatory enzymes in testosterone synthesis, thus promoting testosterone synthesis.

Key words: StarD7, β -catenin, Testosterone Synthesis.

INTRODUCTION

Annexin 5 is a calcium-dependent phospholipid-binding protein with approximate molecular weight of 36 kD. Due to lack of the TATA reading frame in the region 5' of the gene, annexin 5 is thought to be a housekeeper gene. It has a variety of biological functions, including activity as anticoagulant and inhibitor of protease C (1). Studies have shown that the effect of annexin 5 on testosterone synthesis is significantly dependent on time and dose (2). The reduced testosterone synthesis in men has been associated with metabolic syndrome and low bone mineral density (3).

The steroid-sensitive regulatory protein (StAR)-related lipid transport domain protein 7 (StarD7) is a novel protein encoded by 295 amino acid residues. StAR has a lipid transporter domain (START). The START family of proteins consists of 15 proteins (StarD1-StarD15). START proteins are involved in regulating several physiological functions. They participate mainly in lipid-mediated cell signaling, lipid transport and regulation, and lipid metabolism signaling pathways (4). Previous studies (7, 8), using two-dimensional electrophoresis proteomic techniques to establish differential protein profiles of Annexin 5-stimulated testosterone production in rat Leydig cells, revealed that StarD7 was up-regulated.

Binding of the extracellular Wnt protein to the membrane-specific receptor curl protein (Frz) and the helper receptor low-density lipoprotein receptor-associated protein 5/6 (LRP5/6), activates the dishevelled (Dvl) protein. Thus, β -catenin accumulates in the cytoplasm and enters the nucleus, where it interacts with the associated transcription factor T cell factor/lymphocyte enhancer (TCF/LEF), and activates the expression of the downstream-related target gene (7, 8). Rena *et al.* found that the Wnt/ β -catenin signaling pathway facilitates StarD7 transcription by modulating the promoter of the StarD7 gene. It is suggested that the transcription of StarD7 gene may be regulated by Wnt/ β -catenin signaling pathway (9).

The aim of this study was to investigate the correlation between expression of StarD7 and β -catenin at transcriptional and translational level and its role in the Annexin5-stimulated testosterone synthesis, in Leydig cells.

MATERIALS AND METHODS

Experimental animals

Healthy adult male Sprague-Dawley rat provided by the Experimental Animal Center of The

*Correspondence to: Baoping Qiao MD, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, China, E-mail: qiaobaopingqbp@163.com

First Affiliated Hospital of Zhengzhou University, was selected (one rat from which the interstitial glands cells were incubated). The body weight was 231.5g. Laboratory animal was housed under standard conditions of 25°C temperature and 60% relative humidity, and was sacrificed after an adaptation period of 7-14 days. The experiment had the Ethical Approval by the Institution Ethical Committee of First Affiliated Hospital of Zhengzhou University

Major instruments and materials

Western Blot electrophoresis and electric transfer device (Tanon Biotech), Fluorescence inverted microscope (TH4-200, OLYMPUS company), Biophotometer protein nucleic acid analyzer (Eppendorf company), High speed refrigerated centrifuge (Centrifuge 5417R, Eppendorf), Rabbit against rat StarD7 polyclonal antibody (Protein Tech Group Company), Rabbit anti-rat β -catenin polyclonal antibody (Cell Signaling Technology Company), Mouse anti-rat β -catenin monoclonal antibody (Wuhan Boster Company).

Experimental method

Isolation, purification and culture of Leydig cells from male Sprague-Dawley rats

First, the male Sprague-Dawley (SD) rat, with a body weight of about 231.5g, was sacrificed using three pentobarbital sodium injections of the anesthetic dosage. The abdominal cavity was opened. The testis, the membrane and the blood vessels were removed and placed in a sterilized petri dish (ice operation), then 5 mL of PBS was added to wash residual blood stains. The testicular transfer of the membrane and blood vessel were removed and placed in a 10 mL centrifuge tube. Collagen type I (0.5 mg/ mL) was added at 34°C and digested at 200 r/ min for 25 min. Equal volume of DMEM/ F12 medium was added and mixed to terminate the digestion, and the supernatant was allowed to stand. The supernatant was transferred to a 200um filter twice and the flow through was transferred to a new centrifuge tube and centrifuged at 2000 r/ min for 5 min. Percoll density gradient centrifugation liquid was gently added on top of the pellet. Finally, the third layer of cells were aspirated and diluted with equal volume of DMEM (Dulbecco's Modified Eagle Medium) / F12. After centrifugation, the residual Percoll solution was removed by adding DMEM / F12 medium to adjust the cell density and seeded in the cell culture plate. It was incubated in a 5% CO₂ incubator at 34°C for 24 h.

The extraction of cell total RNA and reverse transcription of StarD7 RNA

After the extraction of cell total RNA, the qRT-PCR (Quantitative Real-time polymerase chain reaction) method was used to determine the StarD7 mRNA expression.

The cultured testicular Leydig cells were treated with different concentrations of annexin 5, and cultured at 34°C in a 5% CO₂ incubator for 24 h. In the study were included 3 experimental groups and one control group. The control group received no treatment and the experimental groups were exposed to 0.1 nmol/L; 1 nmol/L and 10 nmol/L Annexin 5. The old culture solution was discarded, the cells were washed twice with precooled PBS, and 1000 μ L of TRIZOL was added to each well. The cell fluid was transferred to a new EP tube, and 200 μ L of chloroform was added to each EP (Eppendorf) tube, and the mixture was vigorously shaken to mix well. The samples were let at room temperature for about 5 min and then centrifuged at 12,000 rpm for 15 min in a 4°C cooling centrifuge. The samples formed three layers after centrifugation. The upper layer is a colorless aqueous phase (RNA is mainly present in this layer). The bottom layer is an organic phase and a mesophase. The aqueous phase containing RNA is transferred to a new EP tube (about 400 μ L). After adding an equal volume of isopropanol, the mixture is thoroughly shaken and allowed to stand at room temperature for about 10 min. It was centrifuged at 12°C for 15 min at 4°C. At the end of the centrifuge, the precipitate at the bottom of the centrifuge tube is RNA. The supernatant was discarded. The newly prepared 75% ethanol wash was added (prepared with Diethyl pyrocarbonate water) and centrifuged at 12°C for 5 min at 4°C. The supernatant was discarded and the pellet was dried at room temperature for about 10 minutes. After drying, 20 μ L of DEPC was added to dissolve the RNA. Take 5 μ L RNA diluted 30 times to determine the RNA concentration. The quality of the RNA was observed by 1.5% agarose gel electrophoresis.

The reverse transcription reaction was carried out by Fermentas first strand cDNA synthesis kit. The specific system is as follows: 2.0 μ L(10 mmol/L) dNTP); 4.0 μ L (5 $\times$ Reaction Buffer); 1.0 μ L (RNase inhibitors); 1.0 μ L (Olig(dT)18 primers); 1.0 μ L(M-MuLV reverse transcriptase (200 U / μ L)); Fill up to 20 μ L (No ribozyme water); 1.5 μ g(RNA).

The reverse transcription conditions: 42°C 60 min reaction, 70°C inactivation, 5 min reaction products placed in -20°C refrigerator to save.

Real-time fluorescence quantitative PCR

process performed as follows: PCR was carried out under the catalysis of Taq DNA polymerase using the cDNA obtained by reverse transcription. The Wnt4's upstream primer was 5'-CTGGAGAAGTGTGATGTGA-3', the down stream primer was 5'-GGACTGTGAGAAGGCTACGC-3', and the product length was 108bp. The β -catenin's upstream primer was 5'-GGCAGAGAGCTCAGGTATGG-3', the downstream primer was 5'-GAGCTTGCTTTTCCTGATTGC-3', the product length was 202bp. The TGF- β 1's upstream primer was 5'-TGAGTGGCTGTCTTTTGACG-3', downstream primer was 5'-ACTGAAGCGAAAGCCCTGTA-3', the product length was 82bp. The β -actin's upstream primer was 5'-GTCAGGTCATCACTATCGGCAAT-3', the down stream primer was 5'-AGAGGTCTTTACGGATGTCAACGT-3', the product length was 147bp. Primers were made by Shanghai Health and Biological Engineering Co., Ltd. The target gene and the internal parameters were expanded and grown in the same tube.

The PCR amplification reaction system was as follows: 2 μ L cDNA; 0.5 μ L upstream primer (15 uM); 0.5 μ L downstream primer (15 uM); 10 μ L Fast Start Universal SYBR Green Master (ROX); 7 μ L water without DNase and RNase. The reaction system totalled 20 μ L.

Determination of StarD7 protein expression by Western Blotting analysis

The control and experimental groups of testicular Leydig cells exposed to 0; 0.1 nmol/L; 1 nmol/L and respectively 10 nmol/L annexin 5 were incubated at 34 ° C in a 5% CO₂ incubator for 24 h. DAB immunohistochemistry kit was used for determination of StarD7 protein expression using western blotting analysis as described below.

Extraction of total cell protein: The culture medium was removed from the culture plate, rinsed twice with pre-cooled PBS (Phosphate-Buffered Saline), and 200 μ L of cell lysate was added containing PMSF (Phenylmethylsulfonyl Fluoride) at a final concentration of 1 mmol / L. After 2-3 min, the cells were scraped off the wells repeatedly with cell scraper and the adherent cells were lysed. It should be noted that the whole process is operated on ice to avoid protein degradation. The cell suspension was transferred into an EP tube and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant included the total protein lysate of Leydig cells and was quantified by BCA (Bicinchoninic Acid) protein quantification kit. **Boiling sample:** a quantitative protein sample was diluted with a 6 x sample buffer and boiled 5 min in a boiling bath. **Preparation of gelatin:** 12% of the separation of

plastic, 5% of the concentration of glue, the amount of each hole is about 40 μ L. **Electrophoresis:** constant voltage 80 V, about 2.5 h. **Transfer film:** PVDF film and filter paper, sponge pad soaked in the membrane buffer for about 30 min. In the gel transfer apparatus, from the negative to the positive order, placed: blackboard - sponge pad - three layers of filter paper - gel -PVDF film - three layers of filter paper - sponge pad - whiteboard. A pre-cooling new transfer membrane buffer is added in the transfer tank. In the correct polarity direction, the transfer device is placed in the rotary instrument. It was placed in an ice-water bath cooling system with a constant voltage of 85 V for 90 min. At the end of the rotating membrane, the membrane is removed. A suitable amount of sealing liquid is added and placed in a 37 DEG water bath for 1.5 h. **The preparation of primary antibody:** depending on the location of the protein marker, the mouse anti-rat beta-actin antibody (1: 400 dilution) or rabbit anti-rat StarD7 (1: 400 dilution), beta-catenin antibody (1: 800 dilution) (all primary antibodies were diluted with PBS containing 10% calf serum) were added and incubated overnight at 4 ° C. The next day, membranes were washed 3 times with closed liquid and TBST, for 5 min each time. **Preparation of secondary antibody:** HRP-labeled goat anti-rabbit IgG (1: 850 dilution) or goat anti-mouse IgG (1: 900 dilution) were placed in a 37°C water bath for 1h. It was washed 3 times with closed liquid and BST, 5 min each time. The DAB chromogenic kit was used for color rendering, and the color rendering time was about 1-5 min. The ddH₂O terminated the color reaction, and the strips were photographed for preservation. The quantity-one software is used to analyze. The relative content of StarD7 and β -catenin were expressed by the ratio of β -catenin and β -actin gray value, respectively.

Detection of testosterone secretion in testicular interstitial cells by chemiluminescence assay

After cells were cultured for 24 h, the old culture medium was removed and the final concentration was 0.1 nmol / L, 1 nmol / L, 10 nmol / L annexin 5 protein solution (dissolved in Tris-HCl solution at pH 8.0). The control was added to the Tris-HCl solution with the same volume of pH 8.0 for 24 h at 34°C. The medium was aspirated, and the testosterone content was determined by chemiluminescence method. Chemiluminescence Immunoassay (CLIA) was used to determine the testosterone. The Testosterone Chemiluminescence Immunoassay (Abbott Laboratories, Chicago, Illinois, United States) is based on the principle of competitive binding between Testosterone in the test specimen and Testosterone-HRP conjugate for a constant amount of mouse anti-Testosterone.

Determination of β -catenin protein expression by immunofluorescence

Cells were gently washed two times with PBS to avoid cell detachment. Then, paraformaldehyde was prepared with 4% PBS, incubated at room temperature for 30 min, to fix cells. Next, fixed cells were washed 3 times with PBS. The cells were prepared with 0.1% Tween-20 solution for 30 min. Then cells were washed 3 times with TBST (Tris-Buffered Saline-T), 15 min each time. 5% BSA was added at room temperature for 30 min. Anti- β -catenin (1:50, Cell Signaling Technology) was added overnight at 4°C, and TBST was used to wash 3 times for 15 min each time. Fluorescence secondary antibody Alexa-goat anti-rabbit IgG was added to the secondary antibody, 37°C water bath was incubated 2h, TBST wash 3 times, each 15min. Then, take DAPI (4',6-diamidino-2-phenylindole) dye 1 min, TBST wash 3 times, each 15 min, sealing tablets. Immunofluorescent labeling of the sample will be performed using the TH4-200 fluorescence inverted microscope (OLYMPUS). The Alexa Fluor 555 fluorescent dye was excited at a wavelength of 555 nm and was detected at a wavelength of 565 nm to emit red fluorescence. Immunofluorescence quantification was performed with ImageJ software. The image was split into three color channels (RGB Merge/split function) to obtain one image per channel. The measure option in the program was used to determine the average number of cells and to assess the integrated density value (IDV) for the blue channel. Elliptical selection tool was used to mark at least ten representative nuclei with different sizes and intensities. To determine the IDV for all the selected nuclei and to calculate the mean nucleus value the blue channel IDV was divided by the mean nucleus value. The resulting value corresponds to the average number of cells. Next, to avoid quantifying the membrane signal of β -catenin, the signal that colocalizes between β - and α -catenin is subtracted. This was done by opening the image calculator from the process menu and created a new image using the operator subtract (Image1: β -catenin, Image 2: α -catenin). A merged image is created combining the subtracted image with the blue channel image using the operator AND. The merged picture shows nuclear localized β -catenin. β -catenin content per nucleus was

calculated by measuring the IDV in this newly created merged image and divided it by the average number of cells calculated before.

Statistical analysis

SPSS 15.0 statistical software was used to analyze the data, expressed as mean $\pm$ standard deviation ($X \pm S$). The statistical methods of comparison between groups were analyzed by one-way ANOVA. The statistical methods were compared between the two groups using the least significant difference method (LSD) test. If the variance is missing, the Games-Howell method is used for data analysis. Pearson correlation analysis was used to analyze the correlation between the two groups. If $P < 0.05$, the difference was significant, and it was statistically significant. If $P < 0.01$, the difference was extremely significant.

RESULTS

Effects of different concentrations of annexin 5 on testosterone secretion of testis Leydig cells in rats

Testosterone secretion was significantly increased ($P < 0.05$) in the three groups, by 65.3% ($26.36 \pm 3.17, 14.98 \pm 0.63$), 108.4% ($33.64 \pm 2.28, 14.98 \pm 0.63$) and 101.5% ($35.86 \pm 3.25, 14.98 \pm 0.63$) respectively, compared to the control group. The results were shown in Table 1 and Figure 1.

Figure 1. The effect of annexin 5 on testosterone secretion in Leydig cells from rat testis.

Table 1. Testosterone levels in Leydig cells after annexin 5 exposure (Percentage changes)

Group	Testosterone level (percentage changes compared to control)	P values (Comparing to control group)
0.1 nmol/L annexin 5	+ 65.3%	0.03
1 nmol/L annexin 5	+ 108.4%	0.00
10 nmol/L annexin 5	+ 101.5%	0.03

Effects of different concentrations of annexin 5 on the expression of StarD7 and β -catenin protein in Leydig cells of testis of rats

The western blot's results show (Fig. 2 and Table 2) that, compared with the control group, the expression of StarD7 protein increased by 25.8% (1.35 ± 0.12 , 1.12 ± 0.04 , $P=0.03$), 41.9% (1.48 ± 0.04 , 1.12 ± 0.04 , $P=0.02$), 22.1% (1.31 ± 0.06 , 1.12 ± 0.04 , $P=0.03$) in the three experimental groups respectively. The unit for the protein expression is gray scale ratio to the control group. Accordingly, the expression of β -catenin protein was increased by 55.3% (1.62 ± 0.01 , 1.04 ± 0.01 , $P=0.02$) in cells treated with 1 nmol / L annexin 5. The other two groups showed a weak but not statistically significant increase.

Correlative analysis of expression of StarD7 and β -catenin protein in different concentrations of annexin 5 stimulated interstitial cells

Statistical analysis showed a significant positive correlation between StarD7 and β -catenin protein expression. The correlation coefficient r was 0.674 and the P value was 0.016 ($P=0.02$, $n=3$). The results are shown in Table 3.

Effects of different concentrations of annexin 5 on the expression of StarD7 mRNA in Leydig cells

mRNA levels of StarD7 were increased by 141% (2.41 ± 0.25 , 1.00 ± 0.00 , $P=0.03$), 279% (3.79 ± 0.37 , 1.00 ± 0.00 , $P=0.02$), 138% (2.38 ± 0.32 , 1.00 ± 0.00 , $P=0.02$) compared to control group (Fig. 3 and Table 4). The unit for the mRNA expression is gray scale ratio to the control group.

Changes of β -catenin localization in Leydig cells of rat testes by annexin 5

Immunofluorescence staining of cells demonstrated an accumulation of β catenin in the cytoplasm, while the nucleus was almost invisible in the control group. However, β -catenin was notably accumulated inside the nucleus, in the experimental group, while the core of β -catenin increased significantly. This finding is consistent with Western Blot results.

DISCUSSION

Recombinant annexin 5 can promote testosterone synthesis in rat Leydig cells in a time - and dose - dependent manner. At the same time, we suggest

Figure 2. A. The effect of annexin 5 on StarD7 protein expression in Leydig cells from rat testis. B. The effect of annexin 5 on β -catenin protein expression in Leydig cells from rat testis.

Table 2. StarD7 and β -catenin protein expression in Leydig cells after annexin 5 exposure (Percentage changes)

Group	StarD7 protein expression level (percentage changes compared to control)	P values (Comparing to control group)	β -catenin protein expression level (percentage changes compared to control)	P values (Comparing to control group)
0.1 nmol/L	+ 25.8%	0.03	+8.4	0.31
1 nmol/L	+ 41.9%	0.02	+54.9%	0.02
10 nmol/L	+ 22.1%	0.03	+10.1	0.16

Figure 3. The effect of annexin 5 on the expression of StarD7 mRNA in Leydig cells from rat testis.

that Wnt/ β -catenin signaling pathway is a key signaling pathway of annexin 5, regulating testosterone synthesis in rats. The expression of protein synthesis in testis Leydig cells, stimulated by annexin 5, was screened by two - dimensional electrophoresis. The analysis revealed that StarD 7 was up-regulated. Further analysis showed that StarD7 had a significant increase in mRNA and protein levels under the stimulation of 1 nmol/ L of annexin 5. These findings suggest that StarD7 may be another key factor in testosterone synthesis under the stimulation of annexin 5, though the exact mechanism remains unclear.

In this study, we investigated the role of StarD7 in testosterone synthesis in Leydig cells. The levels of testosterone and the expression of StarD7 and

Figure 4. Changes of β -catenin distribution determined by annexin 5 in Leydig cells from rat testis. Note: DAPI is 4',6-diamidino-2-phenylindole staining, merge is the combination of β -catenin and DAPI stainings.

Table 3. The correlation of StarD7 and β -catenin protein expression

Group	StarD7 protein expression level	β -catenin protein expression level
0.1 nmol/L annexin 5	1.35 $\pm$ 0.12	1.13 $\pm$ 0.02
1 nmol/L annexin 5	1.48 $\pm$ 0.04	1.62 $\pm$ 0.01
10 nmol/L annexin 5	1.31 $\pm$ 0.06	1.145 $\pm$ 0.01
Pearson correlation coefficient		0.674
P values		0.02

Table 4. StarD7 mRNA expression in Leydig cells after annexin 5 exposure (Percentage changes)

Group	Testosterone level (percentage changes compared to control)	P values (Comparing to control group)
0.1 nmol/L annexin 5	+ 141%	0.03
1 nmol/L annexin 5	+ 279%	0.02
10 nmol/L annexin 5	+ 138%	0.03

β -catenin increased significantly in 1 nmol/L Annexin 5 treated Leydig cells. This indicates a possible role of StarD7 and β -catenin in testosterone synthesis. Some studies on the key enzymes of testosterone synthesis have found that the expression of StAR was not affected by annexin 5. It was suggested that increase in the expression of StarD7 may not be achieved through the regulation of StAR. In the present study, the protein expression levels of StarD7 and β -catenin were analyzed by annexin 5 stimulation. It was found that there was a significant positive correlation between the two. This suggests that StarD7 expression may be involved in the regulation of testosterone synthesis through the Wnt/ β -catenin signaling pathway (10, 11). Subsequently, we identified the localization of β -catenin in Leydig cells by immunofluorescence staining. It was found that β -catenin accumulated in cells and inside the nucleus under the stimulation with annexin 5. Therefore, increased expression of StarD7 may be reduced by ubiquitination of β -catenin, leading to its accumulation in the cytoplasm and incorporation into the nucleus to promote the initiation of StarD7 expression (12-15). In a similar study, Rena V *et al.* measured the expression of StarD7 gene in trophoblast cells. It was found that the Wnt / β -catenin signaling pathway regulates the expression of StarD7 through the cis-acting elements TCF4 and SF-1 in the promoter region of StarD7 (9). In line with our experimental results, it can be concluded that StarD7 may regulate Leydig cell testosterone synthesis through the Wnt / β -catenin signaling pathway (16). Under the effect of 10 nmol/L annexin 5, the expression of StarD7 and β -catenin protein decreased to some extent. At 10 nmol/L, the mRNA expression of StarD7 also decreased under the stimulation of annexin 5, indicating that there are other cellular pathways regulating StarD7 and β -catenin at this dose.

Recently, many studies have shown that StarD7 is involved in the process of lipid, steroid transport and binding. Horibata's study indicated that mammals contain two StarD7 variants, namely, StarD7-I and StarD7-II (17-19). The N-terminus of the StarD7-I contains a leader-directed mitochondrial peptide sequence, whereas StarD7-II is more abundant in the cytoplasm. The expression of these two StarD7 proteins will favor phosphatidylcholine from the cytoplasm into mitochondria (20). Angeletti's study showed that StarD7 was involved in lipid transport during placental development (21). The effect of RNA interference in the reduction of StarD7 expression and decrease in phospholipid synthesis was observed by

Flores-Martin's. It has been demonstrated that StarD7 plays an important role in the synthesis and transport of lipids, and the underlying mechanism remains to be further investigated (22).

The Wnt signaling pathway is an open signal pathway consisting of multiple processes and multiple sites. It has been identified as one of the key signaling pathways in tumorigenesis. It is activated in a variety of human tumor cells, thus regulating the cell growth, migration and differentiation. However, at present, there are few reports on the regulation of testosterone synthesis by Wnt signaling pathways (28). In this study, it was shown that Wnt / β -catenin signaling pathway was involved in the regulation of StarD7 expression and promoted the physiological process of annexin 5-regulating testosterone synthesis in Leydig cells.

This study provides a theoretical basis for further research on the mechanism of Annexin 5 stimulation of testosterone synthesis in Leydig cells. The exact mechanism of StarD7 and β -catenin in the synthesis of cell testosterone and especially the role of Wnt/ β catenin in testosterone synthesis of Leydig cells, under the regulation of annexin 5, remains to be elucidated. There are several questions that need to be addressed including the association of related enzymes expression with the regulation of testosterone synthesis and the involvement of other signaling pathways to this process. The StarD7 and β -catenin expression under high concentration of annexin 5 is worth further study.

Conflict of interest

The authors declare that they have no conflict of interest.

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