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Apoptosis of porcine Sertoli cells is inhibited by QKI-5 via regulating *CASP8*

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Abstract: QKI, a KH domain containing RNA binding, is an RNA-binding protein that is involved in cell proliferation and apoptosis through binding to the QKI response element (QRE) site of its target mRNA. And Caspase 8 (*CASP8*) and Caspase 3 (*CASP3*) play important roles in the pathway of apoptosis. The purpose of this study was to investigate the effect of *QKI-5* on the apoptosis of Sertoli cells. The experimental results show that pig tissues contain *QKI-5*, *QKI-6* and *QKI-7*. Overexpression of QKI-5 significantly decreased the mRNA expression of *CASP8* ($P < 0.05$) and the protein expression of *CASP8* ($P < 0.05$). On the contrary, inhibiting *QKI-5* increased the expression of *CASP8* significantly. Overexpression of *QKI-5* significantly reduced the apoptosis of Sertoli cells and promoted cell growth ($P < 0.05$). Furthermore, *QKI-5* specifically reduced the stability of *CASP8* mRNA by binding QRE sites on *CASP8*. Our experiments provide preliminary evidence that QKI-5 induces Sertoli cells proliferation by inhibiting apoptosis, and this may be one of the factors promoting testicular development.

Keywords: pig; *QKI-6*; *QKI-7*; QRE; RNA-binding protein; stability

Sertoli cells are located at the base of testicular seminiferous tubules. These cells form a seminiferous tubule wall with spermatogenic cells. They form the wall of convoluted seminiferous tubules together with spermatogenic cells from various developmental states, which is important for spermatogonia proliferation and sperm differentiation (Zhang et al. 2013). Sertoli cells also ensure the normal production and maturation of spermatozoa by secreting androgen and other growth factors (Geens et al. 2011). Studies have shown that there is a significant positive correlation between the number of mature Sertoli cells and the number of normal sperm, and their morphological and functional characteristics are consistent (Aranha et al. 2006). The number of immature Sertoli cells is directly correlated with the number of mature Sertoli cells. Therefore, immature Sertoli cells also play an important role in testicular development and spermatogenesis.

QKI is an RNA-binding protein; it is a member of the STAR family of proteins, and is involved in cell proliferation, differentiation and apoptosis (Gavino and Richard 2011; Wang et al. 2013; Zong et al. 2014). QKI is relatively conserved throughout evolution, as most species express three subtypes of the protein: QKI-5, QKI-6 and QKI-7. Currently, only one verified mRNA sequence (NM_001007195.1) of pig *QKI* is available in the NCBI database. The protein structure of QKI contains a conserved KH

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domain that binds to a recognition sequence in the 3'UTR of RNA. Binding of QKI is involved in mRNA transport, splicing, translation, and stabilization (Zhao et al. 2010; Van der Veer et al. 2013). QKI recognizes the sequence ACUAAY-N(1-20)-UAAY, (Y = C or U); this sequence is known as the QKI response element (QRE) (Galarneau and Richard 2005). QKI has been shown to target mRNAs, including SRY-box 2 (*SOX2*) (Lu et al. 2014), WD repeat domain 1 (*WDR1*), heterogeneous nuclear ribonucleoprotein A1 (*HNRNPA1*) (Zearfoss et al. 2011), and argonaute RISC catalytic component 2 (*AGO2*) (Wang et al. 2010). Additionally, bioinformatics analysis showed that the 3'UTR of *CASP8* contains a QRE element, indicating that QKI might contribute to the cellular apoptosis by regulating the expression of *CASP8*.

The purpose of this study was to determine the effect of QKI-5 on the apoptosis of Sertoli cells. Therefore, we first identified the *QKI* isoforms studied by reverse transcription-PCR (RT-PCR). Then, when over-expressing or silencing QKI-5 in Sertoli cells, the mRNA expression of *CASP8* and *CASP3* was detected by Real-time Quantitative PCR (qPCR), the protein expression of CASP8 and CASP3 was detected by Western blot, and the effect of QKI-5 on Sertoli cells apoptosis was detected by flow cytometry. Finally, we found that QKI-5 binds to the 3'UTR of *CASP8* mRNA by the dual Luciferase reporter system and affects the stability of *CASP8* mRNA by qPCR.

MATERIAL AND METHODS

Ethic statement. The experimental protocol of the study underwent an ethical review process and was approved by The Institutional Animal Care and Use Committee of Jilin University, IACUC (permission No. 20170717). The pigs were euthanized by high-voltage electric shocks.

RT-PCR. The total RNA was extracted from the heart, liver, spleen, lung, muscle and adipose tissue of 2-month-old healthy pigs by innuPREP RNA Mini Kit (Analytik Jena AG, Germany) according to the manufacturer's instructions. The cDNA was synthesized from the RNA using a reverse transcription kit (TaKaRa, China). Specific primers for PCR (*QKI*-F/*QKI-5*-R, *QKI*-F/*QKI-6*-R, and *QKI*-F/*QKI-7*-R) were designed using the human *QKI-5*, *QKI-6* and *QKI-7* sequences, respectively. The PCR was performed using DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, USA) according to the instructions. The conditions were as follows: 2 µl of cDNA, 7.5 µl of PCR Master Mix, 0.5 µl of each primer, 4.5 µl of H_2O. The obtained PCR products were subjected to agarose gel electrophoresis and Sanger sequencing.

Gene cloning and vector construction. The construction of an overexpression vector for *QKI-5* (pEF1a-*QKI-5*) was performed as follows. The coding sequence (CDS) of porcine *QKI-5* was amplified by the primers *QKI-Nhe*I-F and *QKI-Sal*I-R. PCR was performed using TaKaRa LA Taq® (TaKaRa, Japan) according to the instructions. The conditions were as follows: 2 µl of cDNA, 1 µl of LA Taq, 5 µl of buffer, 1 µl of each primer, 8 µl of dNTP, 32 µl of H_2O. The coding sequence was then ligated into pEF1α-IRES-DsRed-Express2 using the *Nhe*I and *Sal*I (New England Biolabs, (Beijing), China) restriction enzyme sites of the plasmid. *QKI* was silenced using shRNA (sh*QKI*) with the sequence 5'-GCAGCTGATGAACGACAAGAA-3'. It was synthesized by Suzhou Jima Co., Ltd., China, and the primer sequences are shown in Table 1.

Cell culture and transfection. Porcine Sertoli cells, which were isolated from swine fetal testes of 80–90-day-old pigs, were purchased from Boster Biological Technology Company (China). Sertoli cells were cultured in DMEM high glucose medium (Gibco, USA) containing 10% foetal bovine serum (FBS; Gibco) and 1% double antibody (penicillin and streptomycin; HyClone, USA) at 37°C, 5% carbon dioxide. The percentage of cells confluency grew to 80%. The cells were digested with 0.25% trypsin (HyClone) and transferred into a 6-well plate. The plasmid was transfected into cells using FuGENE transfection reagent (Roche, Switzerland) according to the manufacturer's instructions. Transfection efficiency was observed by fluorescence microscopy at 24 h post-transfection.

MTS assay. A total of 5000 cells were plated in each well of a 96-well plate and cultured for 24 h. pEF1α-*QKI-5*, pEF1α-IRES, sh*QKI* and negative control plasmids were transfected as described above. The time point 24 h post-transfection was recorded as 0 h and the number of cells was measured at different time points. Then, 20 µl of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; Promega, USA) was added to each well and incubated at 37°C for 1 h. The

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Table 1. The primer design of PCR amplification fragments

Gene	Primer code	Primer sequence(5'-3')	Accession No.	Amplicon length (bp)	Annealing temperature (°C)
QKI	*QKI-Nhe*I-F	CTAGCTAGCGCCACCATGGTCGGGGAAATG-GAAACG	NM_001007195.1	1026	58
	*QKI-Sal*I-R	ACGCGTCGACTTAGTTGCCGGTGGCGGCTC			
CASP8	*CASP8-Xho*-F	CCGCTCGAGATTTCTGCGGACTGGATG	NM_001031779.2	401	55
	CASP8-Not-R	GAATGCGGCCGCTGTAATTAGTGTGTA-CAATAATTTGG			
CASP8	*CASP8*-Mut-F	CTTCTGGGACTACTTCCCAAATTATTGTA-CAC*GTGCTG*TAC	NM_001031779.2	404	55
	CASP8-Mut-R	GTACAGCACGTGTACAATAATTTGGGAAG-TAGTCCCAGAAG			
QKI	qPCR-*QKI*-F	CCAAACGGAACTCCTCACCC	NM_001007195.1	156	60
	qPCR-*QKI*-R	AGCCACCGCACCTAATACACC			
CASP8	qPCR-*CASP8*-F	CCTGGTATATCCAATCACTGTGC	NM_001031779.2	157	60
	qPCR-*CASP8*-R	CTCAGGGTGAAAGTAGGTTGTGG			
CASP3	qPCR-*CASP3*-F	TGGGATTGAGACGGACAGT	NM_214131.1	101	60
	qPCR-*CASP3*-R	AGTAACCAGGTGCTGTAGAAT			
ACTB	qPCR-*ACTB*-F	CCCAAAGCCAACCGTGAGA	ENSSS-CG00000007585	102	60
	qPCR-*ACTB*-R	AGAGGCGTACAGGGACAGCA			
	QKI-F	CTAGCTAGCGCCACCATGGTCGGGGAAATG-GAAACG	NM_006775.3		
QKI-5	*QKI-5*-R	ACGCGTCGACTTAGTTGCCGGTGGCGGCTC	NM_006775.3	1050	58
QKI-6	*QKI-6*-R	CTAGCTAGCTTAGCCTTTCGTTGGGAAAGCC	NM_206853.2	983	59
QKI-7	*QKI-7*-R	CTGCTGTATTCTAGTCCTTCATCC	NM_206854.2	1022	59
	sh*QKI*	GCAGCTGATGAACGACAAGAA	NM_001007195.1		

absorbance was then measured at a wavelength of 450 nm in a microplate reader.

Flow cytometry analysis. Sertoli cells were transfected with the plasmid. At 48 h post-transfection, cells were washed twice with phosphate buffer saline (PBS) and digested with trypsin without ethylenediaminetetraacetic acid (EDTA) until the cells were detached. The cells were aspirated into a 1.5 ml centrifuge tube, centrifuged at 1000 rpm for 5 min, and washed twice with PBS. A total of 250 µl of binding buffer, 5 µl of Annexin V-FITC and propidium iodide (PI) (KeyGEN, China) were added to the cells, and the reaction was incubated at room temperature for 15 min in the dark; the reaction was subjected to flow cytometry within 1 h.

qPCR. Sertoli cells were transfected with the plasmid. Total RNA was extracted using an innuPREP RNA Mini Kit (Analytik Jena) according to the manufacturer's instructions. The quality and concentration of the RNA were detected with a spectrophotometer (Thermo Scientific). cDNA was synthesized using a PrimeScript™ RT-PCR Kit (TaKaRa). Expression levels of *CASP8* and *CASP3* mRNA in the overexpression group, the silencing group, the pEF1α-IRES group and the shNC group were detected on an Eppendorf AG-5341 instrument (Eppendorf, Germany) using SYBR® Green I (TaKaRa). The reaction procedure was as follows: 95°C for 5 min, 95°C for 10 s, 60°C for 30 s. The conditions were as follows: 10 µl of SYBR® Green, 0.5 µl of each primer, 2 µl of cDNA, and 7 µl of H_2O. The C_t value of actin beta (*ACTB*) was used as an internal control, and the $2^{-\Delta\Delta Ct}$ method was used to analyse relative expression levels. Primers used for qPCR are listed in Table 1.

Western blot. Cells were lysed using RIPA Lysis Buffer (BOSTER, China) supplemented with protease inhibitor IV. Lysed cells were then centrifuged, and the supernatant was retained. Protein concentration was determined with a BCA protein quantitation assay kit (KeyGEN BioTECH, China). Samples were subjected to SDS-PAGE using 30–50 µg

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of protein then transferred to polyvinylidene fluoride (PVDF) membrane by semi-dry rotation. The membrane was immunoblotted with CASP8 (1 : 500; Sangon Biotech, China), CASP3 (1 : 500; Sangon Biotech) and ACTB (1 : 1500; Abcam, USA) primary antibodies overnight. ACTB was used as an internal control. Secondary antibodies (1 : 3000, Goat Anti-Rabbit IgG H&L, Abcam) were incubated for 1.5 h at room temperature. Finally, proteins were visualized using the Tanon 5200 detection system (Tanon, China).

Dual Luciferase reporter vector. The construction of a *CASP8* 3'UTR Luciferase reporter (*CASP8*-3'UTR-WT) was performed as follows. The 3'UTR of *CASP8* contains a QRE site between 1008 bp and 1015 bp. The *CASP8* gene was amplified using the following primers: *CASP8-Xho*I-F and *CASP8-Not*I-R. The *CASP8* gene was inserted into the pmiR-RB-Report Luciferase vector between the *Xho*I and *Not*I (New England Biolabs) restriction enzyme sites. To construct a mutant *CASP8* 3'UTR Luciferase reporter (*CASP8*-3'UTR-MUT), site-directed mutagenesis was performed with the primers *CASP8*-Mut-F and *CASP8*-Mut-R, using the wild type vector as a template. The product was digested with *Dpn*I (NEB, China), transformed, and a single clone was chosen. Final clones were verified by sequencing to obtain a mutant dual Luciferase vector.

Dual Luciferase assay. A total of 5000 Sertoli cells were plated per well in a 24-well plate and grown for 24 h. The *CASP8*-3'UTR-WT, *CASP8*-3'UTR-MUT and pmiR-RB-Report plasmids were each co-transfected with the pEF1α-*QKI-5* plas-

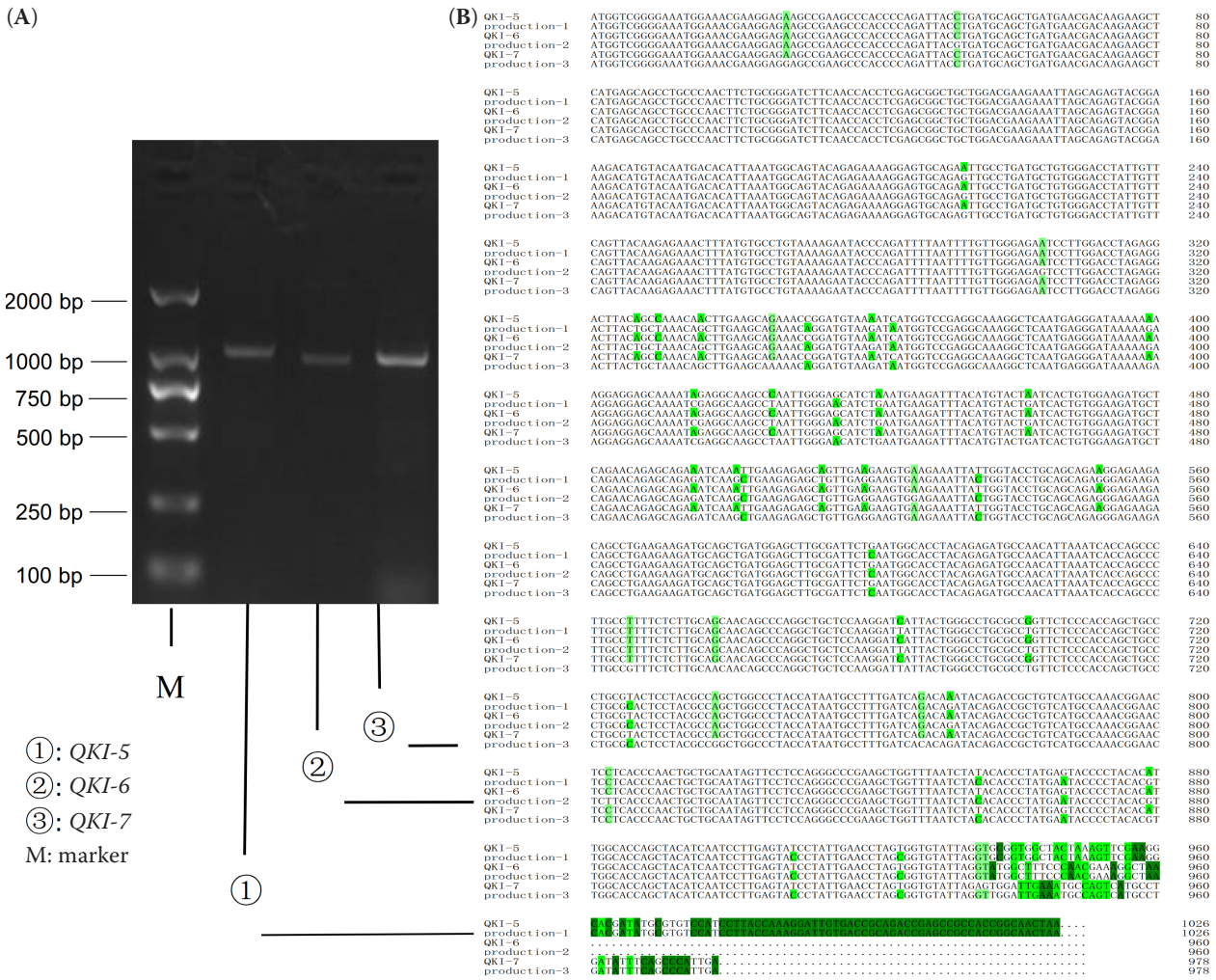

Figure 1. Electrophoresis and sequencing results of *QKI-5*, *QKI-6*, *QKI-7*: (**A**) Gel electrophoresis of PCR products, (**B**) sequence alignment of PCR products and human *QKI-5*, *QKI-6*, *QKI-7*. For detailed sequences comparison see Supplementary Figure S2 in Supplementary Online Material (SOM)

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mid. After 36 h, cells were washed twice with PBS and lysed with lysis buffer for 15 min. Luciferase activity was measured using the SpectraMax M5 Microplate Reader (Molecular Devices, USA) 48 h after transfection.

mRNA stability analyses. Cells plated in the 24-well plates were transfected with pEF1α-*QKI-5* and pEF1α-IRES plasmids. After 24 h, 5 μg/ml of actinomycin D was added. Cells were harvested, and total RNA was collected from cells at 0, 2, 4, 6, 8, and 12 h after the addition. The mRNA level of *CASP8* was analysed by qPCR.

Statistical analysis. The one-way ANOVA was used to conduct the comparison of the different optical density (OD) values, mRNA and Luciferase activity expression levels. All data were analysed using the SPSS 13.0 statistical software. All of the experiments were performed at least in triplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered statistically significant or very significant, respectively.

RESULTS

Isoforms of QKI in pig tissues. Total RNA was extracted from the above tissues and reverse transcribed into cDNA. cDNA from all tissues were mixed, and *QKI-5*, *QKI-6* and *QKI-7* were amplified using the mixed cDNA as a template. Agarose gel electrophoresis showed that three fragments could be amplified from pig tissues, and the sizes corresponded with the expected fragment sizes (Figure 1A). Sanger sequencing and sequence alignment showed that the three sequences have the same C-terminal sequence as human *QKI-5*, *QKI-6* and *QKI-7* (Figure 1B), and similarities of sequences were as high as 97.08, 96.46 and 96.11%.

Effect of QKI-5 on the proliferation of Sertoli cells. MTS assay was used to analyse the proliferation of Sertoli cells following overexpression or silencing of QKI-5. After overexpression of QKI-5 (pEF1a-*QKI-5*), the proliferation of cells was significantly increased compared to the control group (pEF1α-IRES), and the number of cells at 72 h increased significantly ($P < 0.01$) (Figure 2). At 72 h after silencing QKI-5 (sh*QKI*), the number of cells was significantly lower than that in the control group (shNC), and there was a significant difference ($P < 0.01$) (Figure 2).

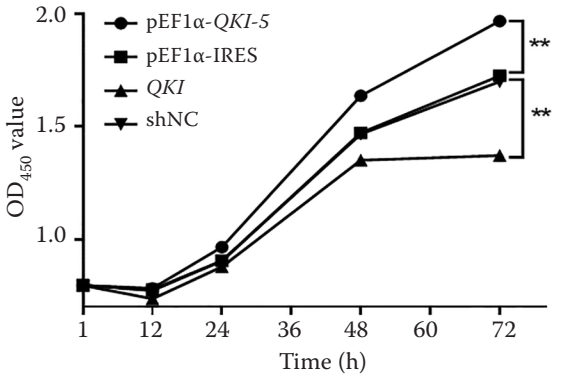

Figure 2. Proliferation of Sertoli cells was measured by the MTS assay. pEF1α-*QKI-5* is the overexpression group, and its control group was pEF1α-IRES. sh*QKI* is the knockdown group, and its control group is shNC. $n = 5$; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vector control

Effect of QKI-5 on the apoptosis of Sertoli cells. Sertoli cells transfected with pEF1a-*QKI-5*, pEF1a-IRES, sh*QKI* and shNC plasmids were processed with the Annexin V FITC/PI (KeyGEN BioTECH) according to the manufacturer's instructions and then were analyzed using a flow cytometer. The results showed that the apoptosis rate of Sertoli cells (pEF1a-*QKI-5*) was lower than that of the control group (pEF1α-IRES) following QKI-5 overexpression (Figure 3A, 3B). However, silencing of QKI-5 (sh*QKI*) caused that the apoptosis rate of Sertoli cells decreased (Figure 3C, 3D).

Effect of QKI-5 on the expression of CASP3 and CASP8. The mRNA and protein levels of *CASP3* and *CASP8* were measured by qPCR and Western blot. The results of qPCR (Figure 4A1, 4B1) showed that overexpression of QKI-5 in Sertoli cells (pEF1a-*QKI-5*) could significantly inhibited the mRNA levels of *CASP3* and *CASP8* ($P < 0.01$). Silencing QKI-5 (sh*QKI*) in Sertoli cells caused a significant increase in the mRNA expression of *CASP8*, but there was no significant difference in the mRNA of *CASP3*. The results of Western blot (Figure 4A2, 4B2) showed that overexpression of QKI-5 could significantly inhibit the protein expression of *CASP8.* Silencing of QKI-5 caused *CASP8* protein levels to increase significantly. The protein expression of *CASP3* showed a slight increase, but the change was not very significant ($P > 0.05$).

QKI-5 binds to the 3'UTR of CASP8. Dual Luciferase reporter vectors of *CASP8* were successfully constructed. The schematic diagram of its

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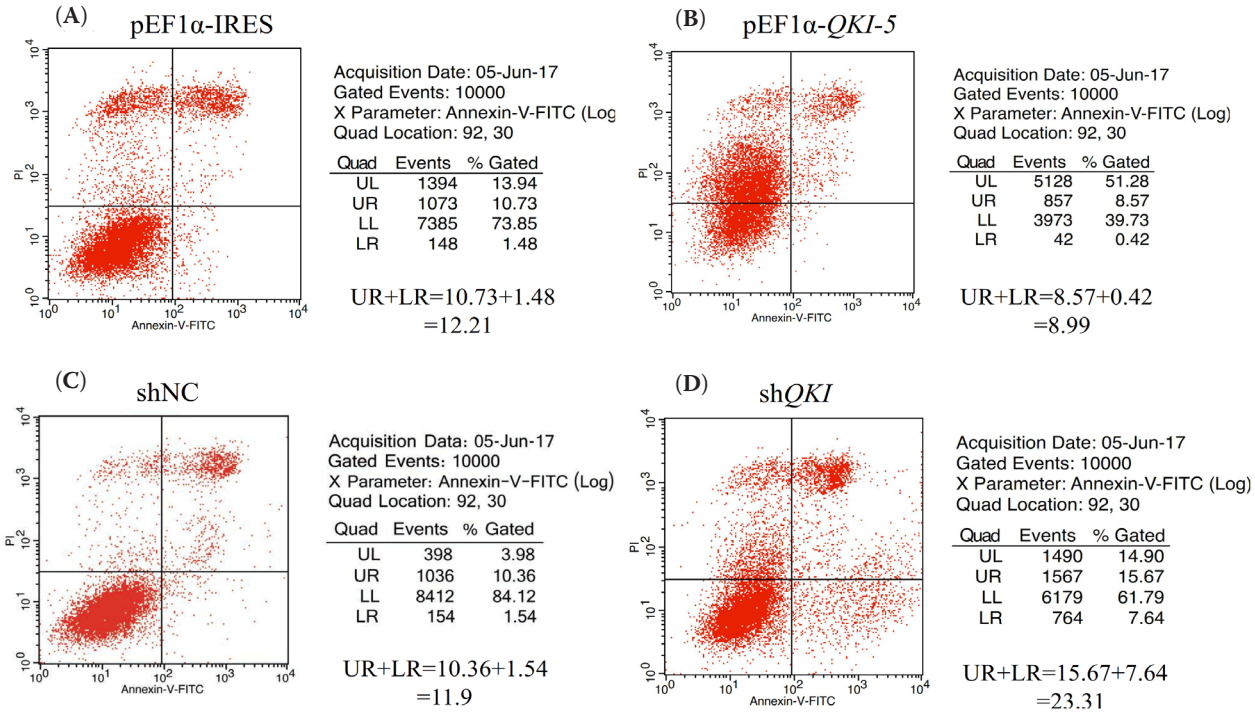

Figure 3. Flow cytometry analysis of apoptosis rate. (**A**) pEF1α-IRES is the control group of pEF1α-*QKI-5*, (**B**) pEF1α-*QKI-5* is the overexpressed group of QKI-5, (**C**) shNC is the control group of sh*QKI*, (**D**) sh*QKI* is the knockdown group of QKI-5. LR = early apoptosis rate, UR = rate of apoptosis in late stage

structure is shown in Figure 5A. pEF1a-*QKI-5* was co-transfected with each *CASP8*-3'UTR-WT and *CASP8*-3'UTR-MUT. The results of cotransfection (Figure 5B) showed that the fluorescence activity of *QKI* + *CASP8*-3'UTR-WT decreased significantly compared with *QKI* + Luciferase vector ($P < 0.01$). There was no significant difference between *QKI* + *CASP8*-3'UTR-MUT and *QKI* + Luciferase vector. These results confirmed that *CASP8* were target genes of QKI-5.

Effect of QKI-5 on the stability of CASP8 mRNA. Sertoli cells were transfected with pEF1a-*QKI-5* and pEF1a-IRES plasmids. The amount of *CASP8* mRNA was measured at different time points following addition of actinomycin D. The results (Figure 5C) showed that the *CASP8* mRNA expression in the over expression group was lower than that in the control group ($P < 0.05$) from 2 h to 8 h.

DISCUSSION

In this study, we first validated the presence of *QKI-5*, *QKI-6* and *QKI-7* in porcine tissues using specific primers. Then, the effects of QKI-5 on Sertoli cells were studied in detail. The results showed that QKI-5 could inhibit apoptosis and promote the proliferation of Sertoli cells. Finally, using molecular biological methods, we found that QKI-5 can reduce the stability of *CASP8* by binding its 3'UTR, thus inhibiting mRNA expression.

RNA binding protein QKI is highly conserved throughout evolution and has been shown to have high homology in humans and animals such as fruit flies, zebra fish, and rodents (Murata et al. 2005). *QKI* is mainly expressed as three isoforms. The size of each mRNA is 5 kb, 6 kb and 7 kb, and as such, they are named *QKI-5*, *QKI-6* and *QKI-7*, respectively. These isoforms differ only slightly at the C-terminus. Currently, the pig *QKI* (ID: 492277) has only one verified mRNA sequence (NM_001007195.1) in the NCBI database. To test the transcripts of *QKI* in pigs, we performed PCR amplification using porcine tissue cDNA as a template. The results showed that tissues of the pig contained mRNA from *QKI-5*, *QKI-6* and *QKI-7* (Figure 1).

QKI can express four subtypes during the development of chicken sperm (Mezquita et al. 1998), and quaking (qkv mutant) mice also show severe

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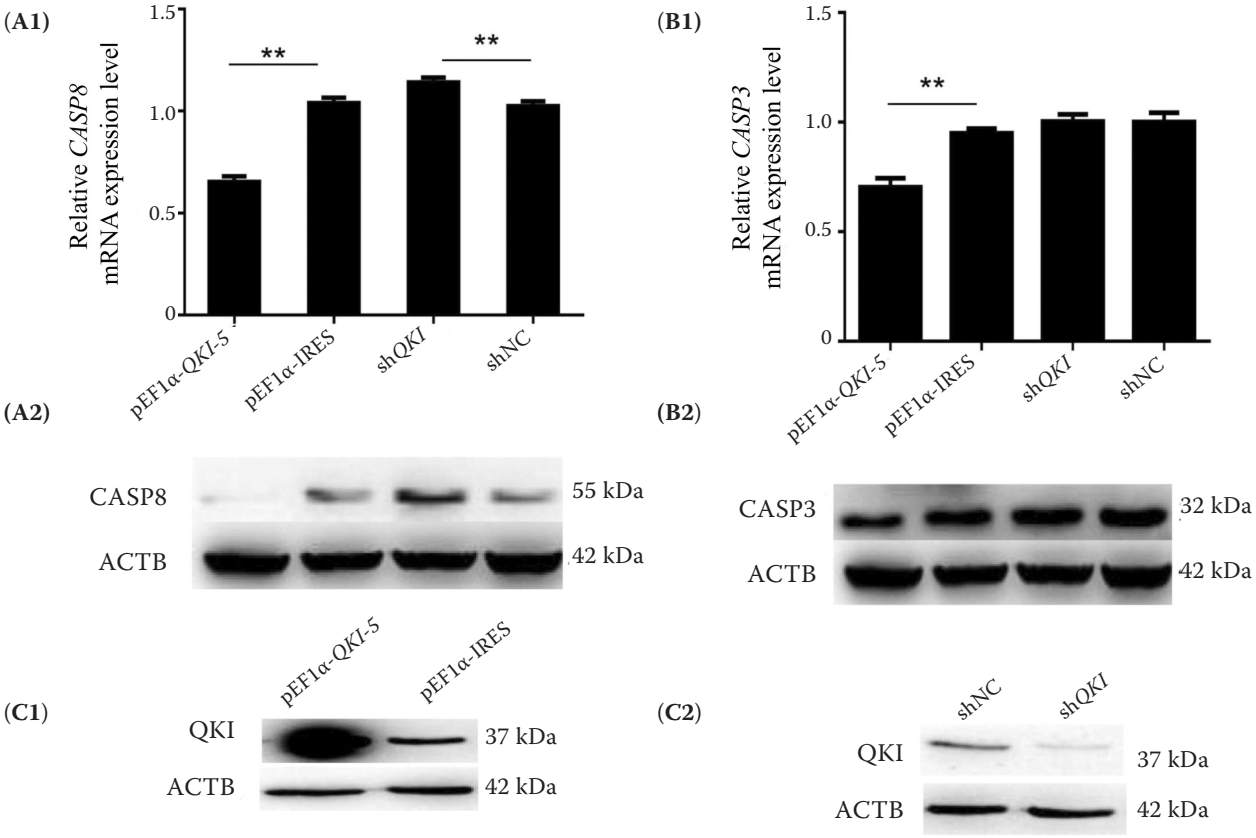

Figure 4. The mRNA levels of *CASP8* and *CASP3* were detected by qPCR and the protein levels of CASP8 and CASP3 were detected by Western blot (WB): (**A1**) mRNA levels of *CASP8* were detected by qPCR, (**B1**) mRNA levels of *CASP3* were detected by qPCR, (**A2**) protein levels of CASP8 were detected by WB, (**B2**) protein levels of CASP3 were detected by WB, (**C1**) after transfection, the protein levels of QKI-5 were detected by WB. pEF1α-*QKI-5* is the overexpression group, and its control group was pEF1α-IRES, (**C2**) sh*QKI* is the knockdown group, and its control group is shNC. $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vector control. Values were normalized using ACTB for gene expression assays. For RNA electropherogram see Supplementary Figure S1 in SOM

sperm maturation disorders. In addition, the study showed that QKI expression is increasing in 1- to 7-month-old boar's testes (Zhang et al. 2015). These results indicate that *QKI* plays an important role in the development of testes and in spermatogenesis. At the same time, Sertoli cells function to feed spermatogenic cells and help maintaining spermatogenesis, and they also play an important role in maintaining the reproductive capacity of male animals (Oliva et al. 2005; Zhang et al. 2013). In this study, overexpression and silencing of QKI-5 in porcine Sertoli cells showed that QKI-5 could promote the proliferation of Sertoli cells and inhibit apoptosis ($P < 0.01$) (Figures 2 and 3). QKI-5 was also found to inhibit the expression levels of *CASP3* and *CASP8* ($P < 0.01$) (Figure 4).

QKI is an RNA-binding protein; thus, it regulates the stability, transport, and localization of mRNA by binding to the QRE site within the 3'UTR of the target mRNA (Guo et al. 2011; Hall et al. 2013). QKI can inhibit the proliferation of breast cancer cells by binding to the 3'UTR of forkhead box O1 (*FOXO1*), reducing the stability of *FOXO1* mRNA (Yu et al. 2014). Our research used dual Luciferase reporter gene analysis, and the results showed that QKI-5 significantly inhibited the fluorescence activity of *CASP8*-3'UTR-WT compared with the control group and the mutant group. These results indicate that QKI-5 can specifically bind to the 3'UTR of *CASP8* (Figure 5). At the same time, the effect of QKI-5 on the stability of mRNA of *CASP8* was measured and showed that QKI-5 can significantly reduce the stability of *CASP8* mRNA (Figure 5C). The mechanism, by which *QKI* affects the stability of this mRNA, is not known and would require further study.

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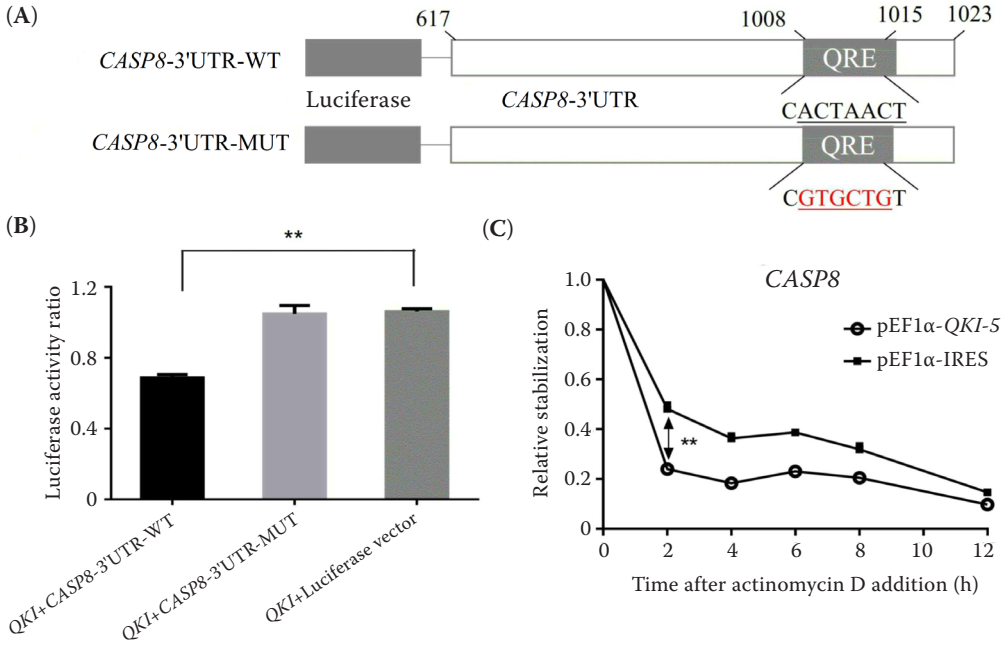

Figure 5. (**A**) Schematic diagram of the wild and mutant double Luciferase reporter genes of *CASP8*; (**B**) *QKI* + *CASP8*-3'UTR-WT: co-transfection of pEF1α-*QKI-5* and *CASP8*-3'UTR-WT, *QKI* + *CASP8*-3'UTR-MUT: co-transfection of pEF1α-*QKI-5* and *CASP8*-3'UTR-MUT, *QKI* + Luciferase vector: co-transfection of pEF1α-*QKI-5* and Luciferase vector; (**C**) results of *CASP8* mRNA stability. pEF1a-*QKI-5* is the overexpression group, and its control group was pEF1a-IRES. Values were normalized using ACTB for gene expression assays. $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vector control

CONCLUSION

QKI can be transcribed into three isoforms of mRNA: *QKI-5*, *QKI-6* and *QKI-7*. Moreover, *QKI-5* can reduce the stability of *CASP8* mRNA by recognizing and binding the QRE site in its 3'UTR. This dynamics directly reduces the expression level of *CASP8* and indirectly inhibits the expression of *CASP3*, thereby inhibiting the apoptosis of Sertoli cells.

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