PAZ Domain is Critical for PIWIL1 Function During Spermatogenesis in Chicken

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Research

Keywords: PIWIL1, PAZ domain, chicken, embryogenesis, CRISPR/Cas9

Posted Date: July 17th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-42228/v1

Abstract

Background: Piwi-like protein 1 (*PIWIL1*) plays a crucial role in stem cell proliferation, embryogenesis, growth, and development. The aim of this study was to reveal the function of *PIWIL1* and its PAZ (Piwi/Argonaute/Zwille) domain in chicken embryogenesis.

Results: PIWI1 expression was analyzed in different stages of spermatogenesis by RT-PCR and the PAZ domain was mutated based on its 3D structure model using the CRISPR/Cas9 technology. The results indicated that *PIWIL1* mRNA was specifically expressed in spermatogonium cells undergoing meiosis. After targeting the PAZ domain (300–370 amino acid residues), we obtained two mutant DF-1 cell clones with 23-bp and 8-bp deletions. Injection of the pCMV-Cas9-puro-sgRNA-2 construct into 2.5-day embryos resulted in generation of 19 different PAZ mutants (13 males and 6 females), which had delayed hatching, reduced quality of semen, and decreased expression of *PIWIL1* and *SOX2* at embryonic days 5 and 18. However, we could not obtain PAZ double knockout (KO) chickens by crossing of the F0 generation, suggesting that PAZ double KO may halt embryonic development.

Conclusions: Our results indicate that *PIWIL1* plays an important role in meiosis and that PAZ mutations can lead to decreased sperm quality, whereas its double KO may arrest embryogenesis in chicken.

Introduction

Spermatogenesis is the process of sperm development from primordial germ cells (PGCs) through four consecutive stages: mitosis, meiosis I, meiosis II, and spermiation, which involve distinct cell types[1]. Therefore, spermatogenesis depends on the stage-specific expression of several genes encoding regulatory factors, including P element-induced wimpy testis (Piwi)1[2]. Piwi was first discovered in Drosophila, where it was shown to regulate asymmetric division of germline stem cells[3, 4]. Later, it has been established that Piwi proteins are highly conserved among plants and animals. During spermatogenesis, three Piwi proteins, Piwil1, Piwil2, and Piwil4 are strongly expressed in two stages: the gonocyte stage and the pachytene spermatocyte to round spermatid stage[5]. *PIWIL1* and *PIWIL2* are expressed in mouse pachytene spermatocytes and round spermatids, where they bind to pachytene Piwi-interacting RNA (piRNA), which requires *PIWIL1* to degrade mRNAs and lncRNAs and which depends, at least in part, on *PIWIL1* slicer activity. *PIWIL1* belongs to the Piwi subfamily of the Argonaute protein family specific to Metazoa, where it is expressed in gonads. The structure of Piwi proteins is similar to that of other Argonaute family members and includes four domains: the N-terminal region, Piwi-Argonaute-Zwille (PAZ) domain, MID domain, and Piwi domain[6]. The PAZ domain contains a hydrophobic pocket with an oligosaccharide-binding-like fold and is involved in the binding to the 3' end of piRNA[7–9], whereas the MID domain anchors the piRNA 5´end; as a result, the piRNA-Piwi protein complex called piRNA-induced silencing complex (pi-RISC) is formed. The association between Piwi proteins and piRNAs is known to suppress transposable elements and protect the integrity of the genome in germ cells[10]. We and other researchers have recently shown that PIWIL1 plays an important role in spermatogenesis[4]. Even though many studies have shown that each domain of Piwi protein plays an

important role in the process of germ cell development. However, the effect of knocking out the PAZ domain on the body and germ cells is still unclear. Therefore, in order to determine the effect of PAZ domain knockout on chicken sperm, this study first determined the expression period of Piwil1 gene, and further verified the expression period of *Piwil1* by RA induction. Through the structural analysis of Piwil1 protein 2D and 3D, the PAZ domain was determined, and the PAZ domain knockout model was constructed based on CRISPR/Cas. Through analysis, we further determined that the PAZ domain is essential for spermatogenesis. At the same time, we found After knocking out the PAZ domain, it may lead to embryonic lethality. It is reminiscent of the fact that PAZ knockout may cause piRNA to be blocked, resulting in the destruction of genomic stability, resulting in embryonic development process stagnation or death. These findings further prove the importance of PAZ, and also show that piRNA also plays an important role in embryo development.

Materials And Methods

Animals

Ten 28-week-old Langshan roosters (*Gallus gallus*) and 550 hatching eggs were purchased from the Poultry institute, Chinese Academy of Agricultural Sciences (Yangzhou, China). PGCs were isolated form 300 eggs incubated for 4.5 days and SSCs were obtained from 100 18-day-old embryos; PGCs and SSCs were separated by density gradient equilibrium centrifugation and differential adhesion[3]. spermatogonium cells (SaCs) and sperm were obtained from 10 roosters as previously described[11].

Germline cells identify, cell culture, and induction with retinoic acid (RA)

PGC colonies were identified using a mouse anti-chicken c-KIT antibody (1:50) as a primary antibody and goat-anti-mouse FITC-conjugated IgM (1:50) as a secondary antibody (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA). The identified PGCs were subjected to periodic acid Schiff (PAS) staining as previously described[12]. Briefly, PGCs were fixed in 4% paraformaldehyde for 5 min, rinsed twice with 1 × PBS (Hyclone, South Logan, UT, USA), immersed in periodic acid solution for 5 min, washed with 1 × PBS, immersed in Schiff's solution for 15 min, washed twice in 1 × PBS, and observed under an inverted microscope. Cells were stained for alkaline phosphatase using the 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium (BCIP/NBT) alkaline phosphatase substrate kit (Wuhan, booster, Wuhan, China)[13]. The c-KIT and ITGA6 antibodies were used to label PGCs and SSCs, respectively, for FACS analysis performed using a FACSAria flow cytometer (BD Biosciences, San Jose, CA, USA). SaCs were sorted as previously described.[11]

The medium for KO experiments consisted of DMEM (Gibco, Life Technologies, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) (Gibco), 2.5% chicken serum (Gibco), 2 mM L-glutamine (Gibco), 1 × MEM nonessential amino acids (WISENT, St. Bruno, Quebec, Canada), 2 mM sodium pyruvate (Gibco), 1 × HEPES (WISENT), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, Shanghai, China), 5 ng/ml

human stem cell factor (hSCF) (Sigma-Aldrich), 10 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich), and 10 ng/ ml mouse leukemia inhibitory factor (Sigma-Aldrich). The cells were cultured in an incubator at 37 °C with 5% atmospheric CO_2 and 60–70% relative humidity was used to culture PGCs and SSCs. DF-1which stored in our laboratory, chicken fibroblast cells were cultured in DMEM supplemented with 10% FBS.

For RA induction, PGCs were seeded into 24-well plates (1×10^5 cells/mL), treated with 10^{-5} mmol/L RA (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) for 24, 96, or 144 h and used for RNA extraction. Cell treated with RA for 144 h were stained with propidium iodide (PI) (Sigma-Aldrich, China) and analyzed by flow cytometry for karyotype changes.

Sg-RNA design and construction of CRISPR plasmids

The coding nucleotide and protein sequences of *PIWIL1* (Accession number: NC_006102.5) were obtained from NCBI. To determine the active site, we analyzed hydrophobicity/hydrophilicity of the PIWIL1 protein using ProScale. PI values of PIWIL1 and its PAZ domain were predicted using ExPASy, and the secondary structures were predicted using Jpred 4, SOMPA, and PredicProtein online tools. PIWIL1 conserved domains were analyzed using the NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?INPUT_TYPE=precalc&SEQUEN

CE = 149642713). Three-dimensional models of PIWIL1 and PAZ were generated with the Swiss-Model automated protein modeling server (http://swissmodel.expasy.org) using silkworm PIWI (PDB: 5GUH) and the MIWI PAZ domain (PDN: 2XFM) as models.

Based on the structure analysis and identification of conserved domains, we chose the PAZ domain as an sgRNA target site. Three sgRNAs targeting the 300–375-residue region at the PAZ domain were designed using the CRISPR design tool (http://crispr.mit.edu/): sgRNA-1 (5'-CAGAGTTGATGACATCGACTGGG-3'), sgRNA-2 (5'-TGTACTTTTAGACGAGCAGATGG-3'), and sgRNA-3 (5'-GCTATATAGACTACTACAAAAGG-3'); protospacer adjacent motifs (PAMs)[14] are underlined. SgRNAs with expect the first nucleotide PAM were cloned into pCMV-Cas9-puro vectors (YSY Biotech, Nanjing, China) by inverse PCR; sgRNA primers are shown in Supplementary Table 1.

The sgRNA-pCMV-Cas9-puro plasmids were purified with the EndoFree Maxi Plasmid Kit (TIANGEN, Beijing, China) and DF-1 cells were transfected with the plasmids using Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. After 48 h, cells were collected, DNA extracted, and cloned sequences were identified using the T7E1 enzyme (Viewsold Biotech, Beijing, China). DF-1 cells were again transfected with the confirmed plasmids and selected for 72 h with puromycin (11 μg/mL); the cells were seeded into 96-well plates after limited dilution at the proportion of $V_{cells\ (\mu L)}/V_{medium\ (\mu L)} = 1/1000$ and single cell-containing wells were selected under a microscope to obtain pure cultures. After reaching 50% confluence, cells were tested using the Tissue Direct PCR kit (Invitrogen, Shanghai, China); DNA integrity and concentration were determined using agarose gel electrophoresis and spectrophotometry, respectively.

The purified plasmids were wrapped in PEI (Sigma-Aldrich, China). To determine the optimal plasmid/PEI packaging ratio, 1 μg of the Cas9-PIWIL1 plasmid was diluted in a 200-μL centrifuge tube to obtain plasmid (μg/μL) to PEI (μg/μL) ratios of 1:1, 1:1.5, and 1:2. After incubation at room temperature (25℃) for 10 min, the samples were tested by electrophoresis in 1.0% agarose gels.

Hatching eggs at 2.5 embryo-days were injected with 1 μL transfection solution through an opening in the air chamber (Fig. 3A) and sealed with sterile medical breathable tape; the control group received the same volume of 4% trypan blue. Eggs were then placed in the incubator to continue hatching; genital ridges and hearts were collected at day 5 and hearts and testes – at day 18 after hatching for DNA and RNA extraction and verification of the KO effect.

Sperm quality index

To determine sperm quality, we analyzed semen appearance, sperm motility, and sperm density. Sperm was acquired by dorso-abdominal massage and evaluated for color, smell, and cloudiness. Sperm motility was analyzed by eosin-nigrosin staining. Sperm density was determined microscopically at 400 × magnification using a hemocytometer (XB-K-25, Anxin®; Shanghai, China): 10 μL of sperm was diluted in 490 μL of 0.9% NaCl preheated to 37 °C and 10 μL of dilution was added to the chamber; the counting was performed five times and the average value was used to calculate the concentration of sperm:

sperm number/mL = average value × 400 × 1,000 × 50.

RT-PCR

Total RNA was extracted using the RNA Simple Total RNA Kit (Tiangen, Beijing, China) according to the manufacturer's protocol and 1 μg of total RNA was reverse-transcribed to cDNA using the Fast Quant RT Kit (with gDNase) (Tiangen). Gene expression was quantitatively analyzed by RT-PCR in a QuantStudio™ 5 Real-Time PCR System using AceQ qPCR SYBR Green Master Mix (Low ROX Premixed; (Vazyme, Nanjing, China), gene-specific primers (Supplementary Table 2), and cDNA as a template. Cycling conditions were: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Ct values were obtained using the default analysis settings and relative mRNA expression of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method after normalization to actin-encoding mRNA.

Statistical analysis

The data are presented as the mean ± standard error. The significance of the difference between two groups was analyzed by *t*-test using R 3.3.2 software; P-value less than 0.05 was considered to indicate statistical significance.

Results

PIWIL1 plays an important role in meiosis during spermatogenesis

To determine the function of the *PIWIL1* gene during chicken spermatogenesis, we analyzed gene expression in PGCs ((Fig. 1A), spermatogonial stem cells (SSCs) (Fig. 1B), SaCs (Fig. 1C) and sperm (Fig. 1D). PGCs and SSCs were identified by c-KIT and integrin alpha 6 (ITGA6) expression, respectively (Fig. 1A, B). Flow cytometry was used for SaC separation. Analysis of stage-specific gene expression profiles indicated that *PIWIL1* expression was significantly upregulated in the SaC stage, whereas *c-KIT* was mostly expressed in SSCs and sperm stages, and *SOX2* – in SaC and sperm stages (Fig. 1F). Recent studies indicate that the exposure to retinoic acid (RA) can stimulate mouse fetal germ cells to undergo meiosis[11]. Our results showed that the proportion of meiotic PGCs increased with the time of RA exposure; thus, flow cytometry analysis revealed that 58.1% PGCs entered meiosis after 144 h (Fig. 1E), and RT-PCR demonstrated that *PIWIL1*, *c-KIT*, and *STA8* mRNA expression was upregulated by RA in a time-dependent manner (Fig. 1G–I).

Structure analysis of the PIWIL1 protein

To determine the key functional site of PIWIL1, we analyzed its secondary and tertiary structures. Structure homology modeling using the templates for the PAZ domain (PDB: 2XFM, sequence identity 0.53) and PIWIL1 (PDB: 5GUH) revealed that PIWIL1 had a structure similar to that of other Argonaute proteins, comprising N-terminal and MID regions, and PAZ and PIWI domains[15] (Supplementary Figure S1A) characteristic for the PIWI-like superfamily and PAZ superfamily (Fig. 2A). The PAZ domain was found in two protein families involved in post-transcriptional gene silencing. Protein sequence alignment showed that the PAZ domain of PIWIL1 was conserved (Supplementary Figure S1B). PIWIL1 and PAZ had grand average of hydropathicity (GRAVY) values of 0.381 and − 0.575 and theoretical isoelectric point (pI) values of 9.28 and 5.47, respectively (Supplementary Figure S1C, D). The secondary structure of PAZ comprised 63 alpha helices, 31 extended strands, and 34 random coils (Supplementary Figure S1E) and that of PIWIL1 included 335 alpha helices, 160 extended strands, 81 beta turns, and 291 random coils (Supplementary Figure S1F).

PIWIL1 gene editing using CRISPR/CAS9

To determine the function of PIWIL1 in spermatogenesis, we edited the *PIWIL1* gene using the CRISPR/Cas9 technology. Based on the structure of the PAZ domain, we selected three sites for sgRNA design[16] (Fig. 2B, C). The results of sgRNA annealing and sequencing indicated that the three sgRNAs could be used as knockout for alternative sgRNAs (Fig. 2D). The electrophoretic mobility shift assay showed that the plasmid/polyethylenimine (PEI) ratio of 1:1 was the best for packaging. To verify the activity of the pCMV-Cas9-puro-sgRNA plasmids, we used them to transfect DF-1 cells. PCR analysis confirmed that the three sgRNAs were active. Further selection using T7E1 digestion showed that the pCMV-Cas9-puro-sgRNA-2 was an active plasmid (Fig. 2E). DF-1 cells transfected with pCMV-Cas9-puro-

sgRNA-2 produced two positive mutant clones, in which 23-bp and 8-bp portions were deleted, respectively (Fig. 2F). The knockout (KO) efficiency of the CRISPR/Cas9 technology in DF-1 cells was 3.44%.

PAZ KO decreased semen quality

To verify the KO effect *in vivo*, we injected pCMV-Cas9-puro-sgRNA-2 into 2.5-day embryos (Fig. 3A) and found that PAZ KO caused a delay in embryonic development at days 5 and 18 (Fig. 3B, C). The results of the T7E1 assay showed that the mutant protein was expressed in heart and germline tissues of 5- and 18-day embryos, respectively (Fig. 3E). The expression of *PIWIL1* (Fig. 3G, I) and *SOX2* (Fig. 3H, J) was decreased by PAZ KO in tissues of both 5- and 18-day embryos. Among the 50 experimental chickens, 19 (13 males and 6 females) were mutants (Fig. 3K). To analyze the effect of the KO on sperm quality, we examined semen appearance and sperm motility, viability, and density (Table 1) and found that sperm motility was significantly lower in the KO group than in the control group (Fig. 4A). There was no significant difference in body weight between the KO and control groups (Supplementary Table S3 and Fig. 4B).

Generation of PAZ$^{-/-}$ mutants

To obtain PAZ double KO mutants, we performed crossing between F0 mutants and analyzed the offsprings by sequencing. The results showed there were no PAZ$^{-/-}$ KO chickens (Fig. 4C). Among the offsprings, six had a deletion of 3 bp (176–178 bp) and four had mutations at different sites (Fig. 4D). Overall, these data suggested that PAZ double KO could cause abnormal development or death (Fig. 5).

Discussion

Despite the recent interest in the function of PIWIL1, its dynamic regulation and biological role, particularly in spermatogenesis of mammals and birds, remain largely uncharacterized. PIWIL1 and piRNA have been shown to regulate the expression of lncRNA and mRNA[17] and recent data suggest that PIWIL1 plays an important role in meiosis. In this study, we showed that *PIWIL1* was strongly expressed in the SaC stage and upregulated by RA. Structural and biochemical analyses indicate that PIWIL1 has two domains (PAZ and Piwi), two regions (N-terminal and MID), and a motif (D-box). It was shown that PAZ domains of Argonaute proteins from different mammals and birds bind RNA, indicating a conserved function of PAZ[8]. It is known that the PAZ domain specifically recognizes and binds the 2'-O-methylated 3' end of piRNAs[17], whereas the MID region is required for the recognition of uridine in the first position of piRNAs[18]. The present study confirmed the conserved nature of the PAZ domain in chicken by multiple DNA sequence alignment and comparative 3D modeling. These results suggest that PAZ plays an important function during spermatogenesis in chicken.

CRISPR/Cas9 is a research tool to study gene function, which has been extensively used to generate and correct mutations in human[19], rodent[20], mouse[21] and zebrafish[22] cells *in vitro* and *in vivo*. With the CRISPR/Cas9 system, the spatiotemporal function of genes can be studied *in vivo*, provided that

selection is not required. Here, we used CRISPR/Cas9 to generate PAZ mutant chickens. Although we could not obtain $PAZ^{-/-}$ birds either in F0 generation or by F0 x F0 crossing, we observed stunted embryo growth, suggesting that PAZ double KO causes abnormal embryonic development and results in fetal death. Consistent with our findings, another study showed that PIWIL1 KO caused embryo growth retardation[22]. We speculate that the reason of stunted embryo growth is that PIWIL1 KO upregulates the expression of lethal genes and downregulates that of development-related genes. Previous studies show that piRNA regulates development by controlling the expression of transposable elements and that human PIWI (HIWI) expression is associated with cancer prognosis[23, 24]. Furthermore, our results indicated that the deregulation of PIWI1 function significantly decreased sperm quality, which is consistent with the finding that piRNAs are abundant in the spermatocyte and sperm stages[25] and that piRNA levels in spermatozoa correlate with sperm concentration and the fertilization rate after intracytoplasmic sperm injection[26].

Conclusions

In conclusion, we showed that PIWIL1 was significantly expressed in meiosis and that the PAZ domain of PIWIL1 is important in maintaining the sperm quality in chicken. Unfortunately, we could not obtain PIWIL1 double KO mutants and determine whether the disruption of the PAZ domain leads to embryonic death. Despite its preliminary character, this study clearly shows the role of PIWIL1 in spermatogenesis of chicken.

Abbreviations

PGCs
primordial germ cells; Piwi:P element-induced wimpy testis; piRNA:Piwi-interacting RNA; PAZ:Piwi-Argonaute-Zwille; CRISPR:Clustered Regularly Interspaced Short Palindromic Repeats; Cas:CRISPR-associated; SSCs:spermatogonial stem cells; SaCs:spermatogonium cells; ITGA6:integrin alpha 6; RA:retinoic acid; PEI:polyethylenimine; KO:knockout

Declarations

Acknowledgements

Authors' contributions

QXG and LX performed to the experiments; GBC was conceived and designed the experiments, QXG, LX and XYY was contribution to feeding chicken, YJ, HB and GBC contributed to interpretation of data and reviewing of the manuscript. All authors submitted comments on the draft, read, and approved the final manuscript.

Funding

This research was funded by the National Natural Science Foundation of China (31372297) and National Science & Technology Pillar Program of China (2015BAD03B03).

Ethics approval and consent to participate

All experimental procedures were performed in accordance with the Regulations on The Administration of Experimental Animals issued by the Ministry of Science and Technology in 1988 (last modified in 2001, Beijing, China). All animal experiments were approved and guided by the Animal Care and Use Committee of Yangzhou University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

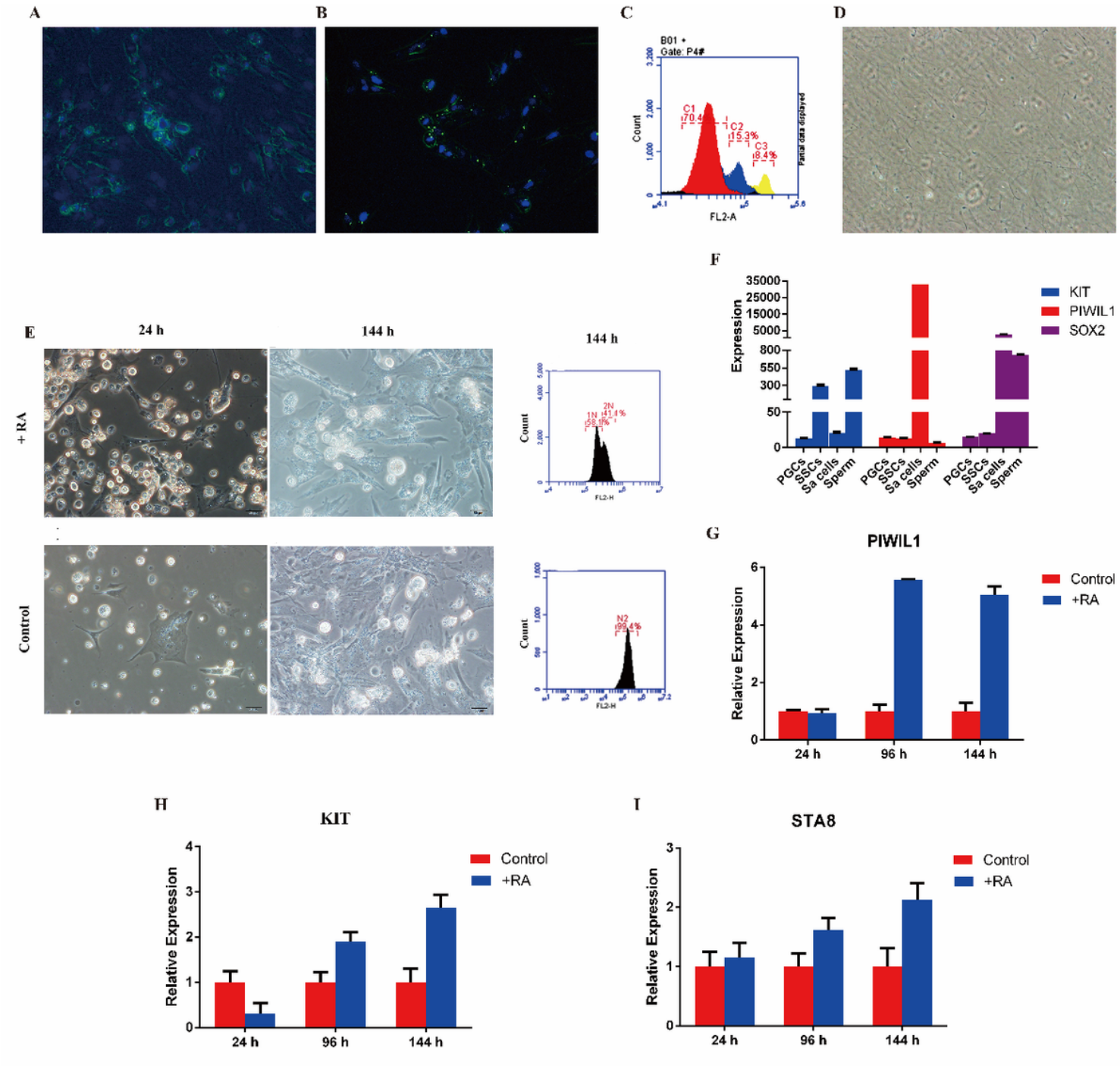

Figure 1

PIWIL1 is expression in the SSCs stage. A) The immunofluorescence result of PGCs；B) The immunofluorescence result of SSCs；C) Flow sorting result of spermatogonium; D) Pure sperm; E) RA induce the PGCs; D) The genes of PIWIL1, KIT and SOX2 expression during spermatogenesis; G,H and I) Piwil1, KIT and STA8 expression of PGCs by RA induced.

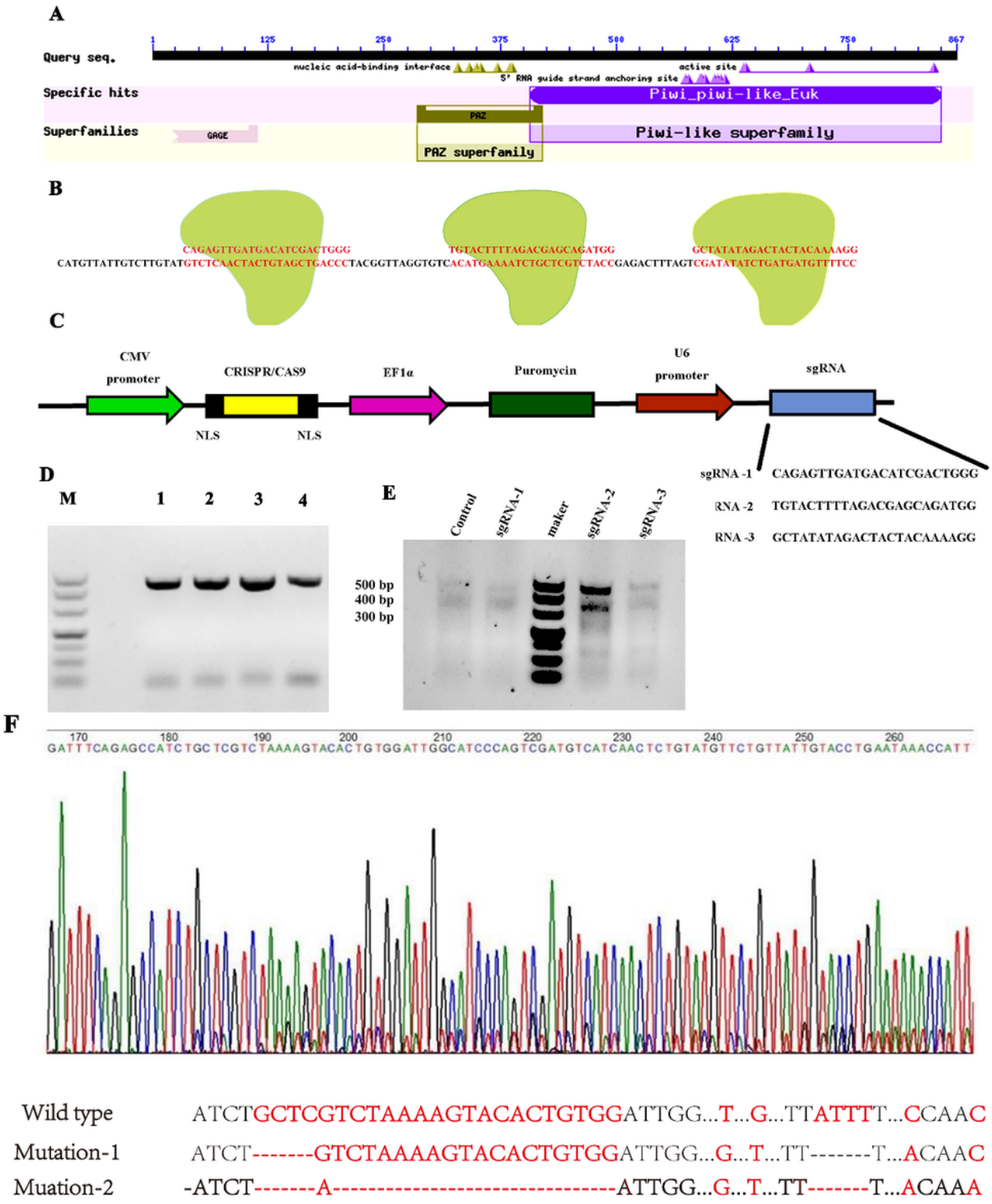

Figure 2

CRISPR/Cas system design and activity verification. A) the structure of Piwil1 protein; B) Sequence alignment of 3 sgRNAs. ; C) CRISPR knockout vector pattern diagram; D) agarose map for PCR identification of sgRNA; E) T7E1 digestion identification results; F) clone sequencing verification results

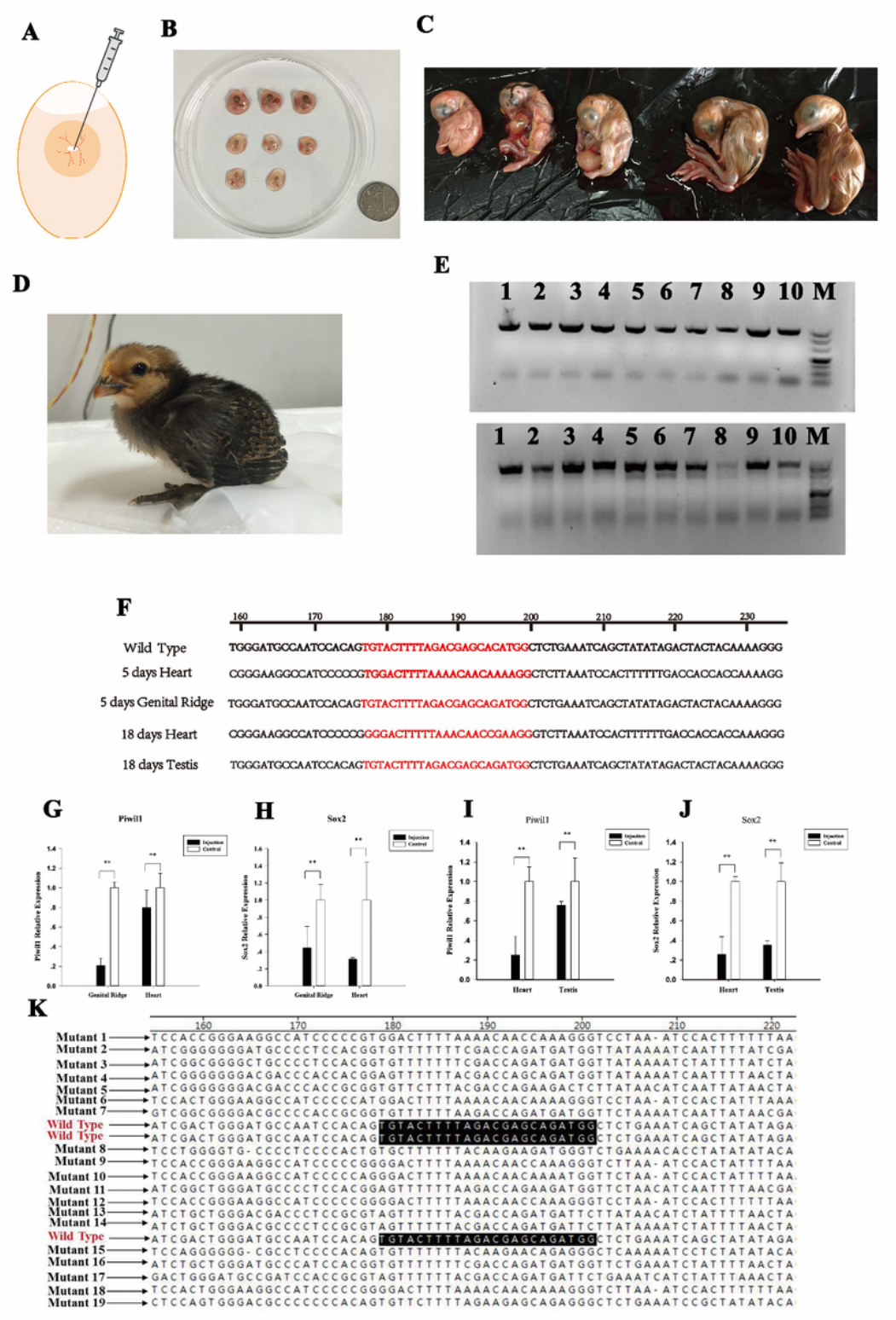

Figure 3

PAZ domain knockout model contracture. A) Chicken embryo injection pattern diagram; B) E5 embryo, the first line is the normal group, and the second and third lines are the knockout groups; C) E18 embryo. The first three are knockout groups and the second two are normal groups; D) knocked chick; E) For PCR amplification of target sites, lanes 1~6 are 18-day-old samples, where T is testis, H is heart, TC is testis control group, HC is heart control group; lanes 7~10 7~10 are 5 days old Samples, in which lane 7 is the

reproductive crest of the control group, lane 8 is the heart of the control group, lane 9 is the reproductive crest of the experimental group, and lane 10 is the heart of the experimental group; Figure B is the result of T7E1 digestion, the sequence number in the figure is consistent with A; F) Sequence alignments result; G and I) The expression changes in various tissues after the knockout of Piwil1 in E5 and E18; H and J) The expression changes in various tissues after the knockout of Sox2 in E5 and E18; K) Mutation detection of F0 knockout chicken.

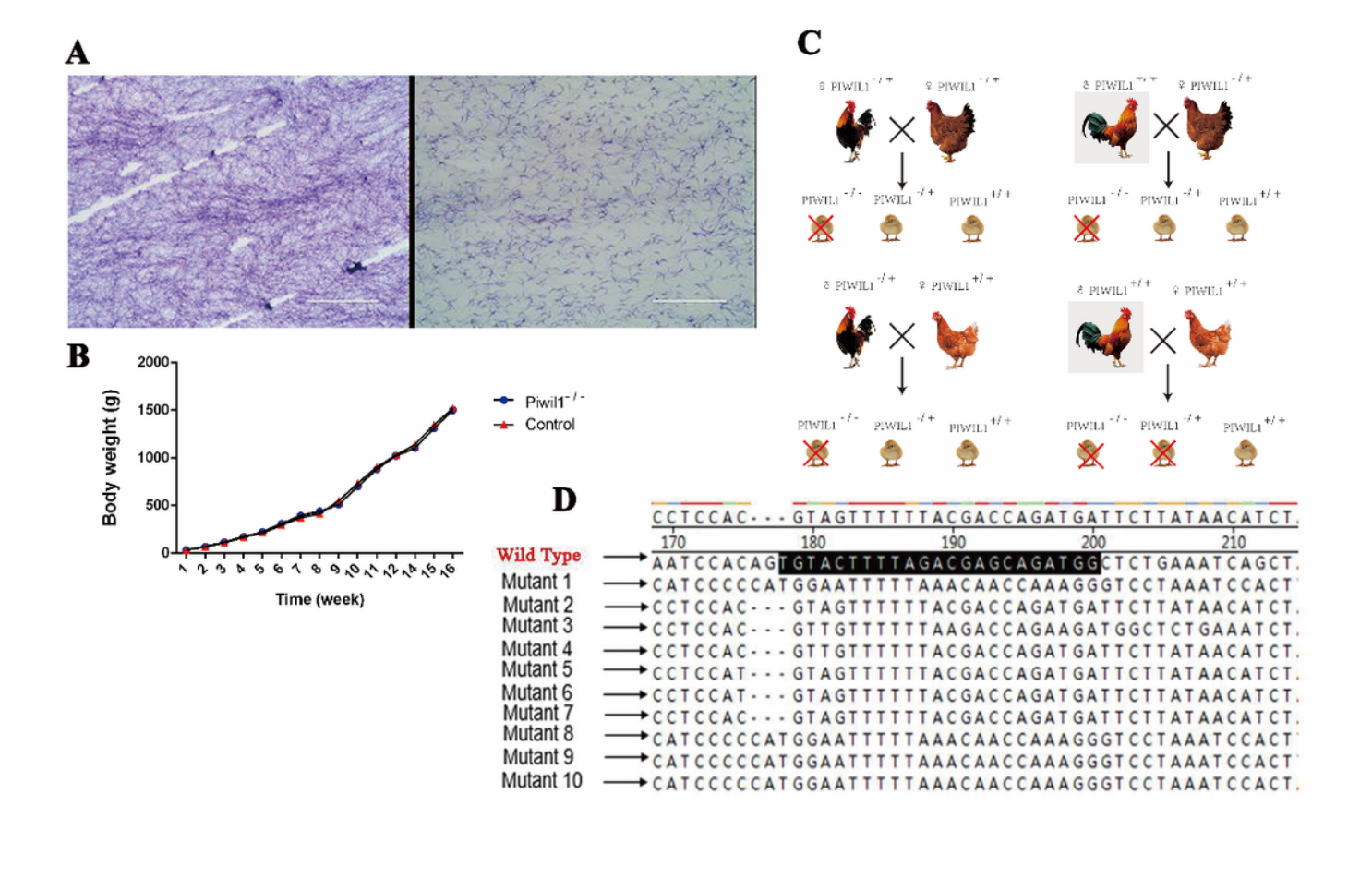

Figure 4

Piwil1 PAZ Mutation Knockout Chicken Show Male Sterility and embryonic lethality. A) sperm of Piwil1 PAZ mutation chicken; B) Statistics of body weight of Piwil1-/- and normal chicken; C) Mating pattern diagram; D) Sequencing alignment of F1 generate chicken.

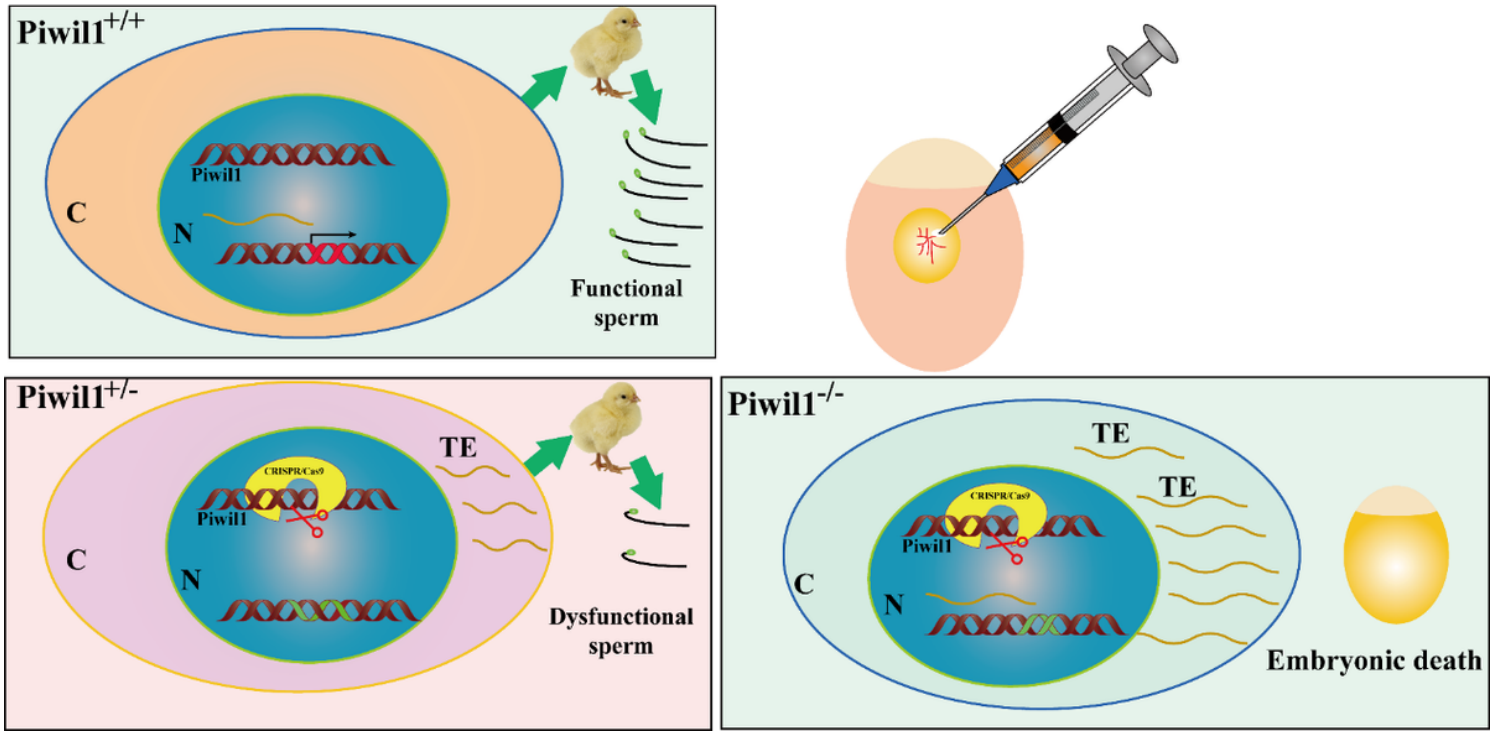

Figure 5

Figure 5. The pattern diagram of PAZ domain normal, single and double knockout affect chickens.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure.docx
- SupplementaryFigure.docx
- SupplementaryTable.xlsx
- SupplementaryTable.xlsx