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Overexpression of PD-L1 causes germ cell failure and infertility via CRISP1/PD-L1 interaction in mouse epididymis

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Research Article

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Summary

Spermatogenesis is a highly complex process through which mature sperms are produced, and it requires three important stages; mitosis, meiosis and sperm formation. The expression of genes regulated by transcription factors at specific stages exerts important regulatory effects on the development process of germ cells. Male mice with overexpressed programmed death ligand 1 (PD-L1) (B7 homolog1) in the testis have infertility and abnormal sperm development, thereby exhibiting severe malformation and sloughing throughout spermatid maturation and collapsed and disorganized seminiferous epithelium structure. Furthermore, PD-L1 overexpression causes overexpression of cysteine-rich secretory protein 1 (CRISP1) in the epididymis and adversely affects or precludes sperm energization, sperm-pellucida binding and sperm-oocyte fusion. These findings suggest that CRISP1 and PD-L1 can interact with each other to induce male infertility and germ-cell dissociation.

Introduction

The process of spermatogenesis in the testicles is very complex and requires spermatogenesis cells, support cells and interstitial cells to interact at various stages, along with coordinated and orderly progression. It involves a variety of biological mechanisms, including the regulation of several hormones, cell cycle checks, cell adhesion and cell polarity maintenance (Skinner, 1991). In mammals, after entering the female reproductive tract, sperms must undergo several processes, such as energy gain, apical response and sperm-ovichomic membrane fusion, to fertilize the oocyte (Ikawa *et al.*, 2010; Da Ros *et al.*, 2015). Numerous *in vitro* experiments and knockout model studies have shown that cysteine-rich secretory protein 1 (CRISP1) in the epididymis is involved in the regulation of sperm energization, sperm-pellucida binding and sperm-oocyte fusion (Weigel *et al.*, 2018; Claw *et al.*, 2014; Matamoros *et al.*, 2018). Cameo first discovered in 1976 that the protein CRISPl, which is secreted only by the rat epididymis by polyacrylamide gel electrophoresis, which has two forms, called D protein and E protein, which are synthesized by epithelial cells at the proximal end of the epididymis in an androgen concentration-dependent manner and secreted into the lumen of the epididymis (Cameo and Blaquier, 1976). In rats, CRISPl is initially localized in the sperm head's parietal region, and with the occurrence of the apical reaction, the localization of the two forms of protein changes: the larger molecular-weight D protein binds to the sperm surface loosely and reversibly, and when the sperm migrates from the epididymis into the female reproductive tract, the D protein is released from the sperm surface. The D-form protein migrates to the equatorial region of the sperm acrosome during sperm fertilization, and since sperm-egg binding begins in this region, it is speculated that CRISP1 plays a role in sperm-egg fusion. In contrast, the E protein with lower molecular weight is strongly bound to the sperm surface, and as the acrosomal reaction occurs, it remains bound there and migrates to the sperm head's equatorial plate region (Roberts *et al.*, 2007). After the release of mouse sperms into the epididymis, mainly exposed to CRISP1 and CRISPP4, CRISP1 and CRISPP4 double knockout individuals show a low fertility phenotype with immune infiltrates in the epididymis, which indicates the function of CRISPs in the epididymis which is the capability to create an immune privilege environment that allows sperms to mature in the epididymis (Hu *et al.*, 2018)

The expression of CRISP1 is on the sperm's surface, which can inhibit the absorption of some ions, such as calcium (Hu *et al.*, 2018). Calcium ions are important for sperms to generate energy (Matamoros *et al.*, 2018), and CRISP1 proteins can inhibit tyrosine acidification, thereby preventing sperm energization and precluding the acrosomal reaction. The inhibition effect of CRISP1 is mainly reflected in the effect of exogenous cAMP, which plays a role in the early stage of the energy acquisition process (Jansen *et al.*, 2015; Ernesto *et al.*, 2015). In rats, CRISP1 causes inhibition of tyrosine phosphorylation in a way that is dependent on its concentration, thereby preventing sperm energization, which is reversible and can be overcome by exogenous cAMP, indicating that CRISP1 and similar proteins may have an inhibitory effect in the early stage of

energy acquisition by controlling certain ion channels on the surface of the sperm plasma membrane (Roberts *et al*., 2008).

Previously, programmed death ligand 1 (PD-L1)-transgenic mice were used to examine the involvement of PD-L1 in spermatogenesis. The examination results showed that overexpression of PD-L1 causes germ cells to slough from mouse seminiferous tubules via the PD-L1/PD-L1 interaction, which caused male mice infertility. PD-L1, also known as CD274 or B7-H1, is a member of the B7 family and is widely expressed in antigen-presenting cells, as well as in non-lymphoid tissues, such as vascular endothelial cells, astrocytes, keratinocytes and many types of tumour cells. Programmed death 1 is its recognized receptor that binds to PD-L1 to transmit inhibitory signals to T cells (Fang *et al*., 2022). Spermatids showed maturation defects during spermiogenesis-related stages, characterized by premature degeneration of spermatids, apoptosis and sloughing in the lumen, and a few spermatids were produced in the functional lumen that exhibited malformed heads with disorganized alignment. Although the number of sperms in the epididymis of transgenic mice showed a significant reduction in contrast with wild-type (WT) mice, a considerable number of long sperms were still visible. Therefore, the protein interaction in the epididymis of PD-L1 transgenic mice was the main focus of this study. The results showed a strong interaction between the PD-L1 and CRISPP1 proteins in the epididymis of PD-L1 transgenic mice, which affected sperm development in the epididymis. These findings suggest that PD-L1 and CRISP1 play a synergetic effect to result in male infertility and germ-cell dissociation.

Materials and methods

Animal

Experimental animal materials included male and female C57BL/6 (B6) mice purchased from the Shanghai Sleek Experimental Animal Centre. All experimental procedures were approved by the Institutional Animal Care and Use Committee. Transgenic mice and WT mice were fed in an specific pathogen free-grade animal laboratory and sacrificed at 60 days (As there was no significant difference in the weights between the PD-L1 group and control groups before the day 40, which was the time point when the mice's sexual maturation proceeded to the first sperm release Fang *et al*., 2022). WT mice and transgenic mice were divided into WT and PD-L1 groups, with five mice in each group. Testis and epididymis were immediately taken.

Construction of transgenic vectors and development of PD-L1-EGFP transgenic mice

The construction processes of PD-L1-EGFP transgenic vectors and mice were described previously (Fang *et al*., 2022). 4-week-old (C57BL/6) female mice were injected intraperitoneally to superovulated with 5 IU gestational mare serum gonadotropin (PMSG, Sigma-Aldrich, St. A. Brown). Louis, MO, USA). Forty-eight hours later, rice was followed by an injection of 5 IU of human chorionic gonadotropin. Mice are then crossed with 8-week-old (C57BL/6) male mice. The PD-L1 transgene fragment was diluted to 1 ng/ml in microinjection buffer (5 mM Tris, 0.1 mM EDTA, pH 7.4) and microinjected into the male pronuclei of embryos and cultured in microdroplet M16 medium (MR016, Sigma-Aldrich, USA) for 1 day. All solutions used for culture experiments were pre-equilibrated at 37°C and 5% CO2 and covered with mineral oil (M5904, Sigma-Aldrich, USA). The two-celled embryos are picked up and transferred to the pseudo-pregnant institute for cancer research adoptive mother (prepared as described by Fang *et al*., 2022). A total of 11 lines of PD-L1-EGFP transgenic founder mice were identified using PCR detection. Only the male mice with EGFP+ testes and epididymis were infertile. Because the male founder was infertile, the 60-day-old male offspring of line 9 female founders were used for the experiments.

Haematoxylin-eosin (HE) staining

HE staining of mouse testes and epididymis was performed as previously reported (Fang *et al*., 2022). Mouse testes and epididymis are fixed in Bouin's fixative solution (PH0976, Phygene, China) for 24 h at room temperature, then placed in 70% ethanol, and then the testes and epididymis are embedded in paraffin (76242, Sigma-Aldrich, USA). Paraffin-embedded mouse testis and epididymal tissue are cut into 5 μm thick testicular sections and mounted on normal glass slides. Then, the sections were deparaffinized in xylene (LM1330-20-7, LMAI Bio, China), hydrated in gradient alcohols (100, 95, 80 and 70%), and the slides were washed in running distilled water for 2 min before staining with haematoxylin and eosin (C0105M, Beyotime, China). The slices were taken with a light microscope (Leica, Germany) (prepared as described by Fang *et al*., 2022). The experiment was repeated three times.

GST pull down

Construction of GST-exPD-L1vector and induction of fusion protein expression

A GST-exPD-L1 (an extracellular fragment of PD-L1) vector was produced for GST pull down of PD-L1. Total ribonucleic acid was extracted from mouse spleen tissue, and cDNA for exPD-L1 was amplified using PCR by employing the primers mentioned below:

Forward 5′-GCGAATTCATGAGGATATTTGCTGGCATTATATTC-3′ and reverse 5′-GCGTCGACAGTCCTGTTCTGTGGAGGATGTG-3′ (synthesized by Shenggong Co. Shanghai, China). PCR products were purified using purification kits and analyzed by agarose gel electrophoresis. Cloning of cDNA was done into the TA cloning vector pGEX-4T-1 followed by its insertion between the *EcoR*Iand *Sal*Isite to construct the pGEX-4T-1-exPD-L1 plasmid. Host bacteria BL21 (D3) were converted through the constructed recombinant plasmid p GEX-4T-1-exPD-L1. Monoclonal inoculation was done into LB culture medium (50 μg/ml Ampicillin) and shock cultured at 37 °C overnight. The medium was inoculated the next day again using the same resistance at a ratio of 1:100, followed by culturing under the same conditions until an OD600 value of approximately 0.6–1.0 was reached. Then, 1.0 mmol/L IPTG was added for stimulation for four hours. A control treatment without IPTG was used, and bacteria transformed with empty plasmid pGEX-4T-1 were used as control. Centrifugation was done to collect the stimulated bacteria, followed by boiling in 2 × PAGE loading buffer and analysis via 10% SDS-PAGE electrophoresis. Coomassie brilliant blue R-250 staining was employed for the purpose of visualizing protein bands, and scanning analysis of the gels was performed. The experiment was repeated three times.

Purification and identification of GST-exPD-L1 fusion protein

Bacterial cells were collected by centrifugation of the bacterial solution. They were stimulated with 1.0 mmol/L IPTG for four hours, and each 1 g cell mass was washed using 10 mL cell lysate containing 10 mol/L Triton X-100, followed by centrifugation. The precipitate was the inclusion body. Each 1 g inclusion body was resuspended with 10 mL guanidine hydrochloride denaturation buffer (50 mmol/L Tris-HCl, pH 8.0; 2.0 mmol/L EDTA; 200 mmol/L NaCl; 7.0 mol/L guanidine hydrochloride). After

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fully dissolving under stirring at room temperature, the denatured inclusion body protein was added to the renaturation buffer (50 mmol/L Tris-HCl, pH 7.4; 1.0 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.2 mmol/L arginine) at a ratio of 1:100, after which renaturation was done at 4 °C for 24 hours.

The renatured protein was added to a polypropylene tube containing glutathione 4B agarose beads to facilitate the binding of the proteins to the beads. Afterwards, centrifugation was performed to collect the beads, followed by washing and boiling the beads in a 2 × PAGE loading buffer. Subsequently, the beads were analyzed through 10% SDS-PAGE electrophoresis. Coomassie brilliant blue R-250 staining was conducted for visualization of the protein bands, and a scanning analysis was performed on the gel.

After electrophoresis, the purified exPD-L fusion protein was transferred to a PVDF membrane and placed in tris-buffered saline with 5% skim milk powder for one hour. GST polyclonal antibody diluted 1:4,000 and horse radish peroxidase labelled rabbit secondary antibody diluted 1:10,000 were added. Afterwards, the developer was added, and film scanning was executed for analysis. The experiment was repeated three times.

GST pull-down detection

C57/BL6 background PD-L1 transgenic mice and WT mice were killed, and their testis and epididymis were removed and weighed. Afterwards, 1 mL non-determined protein lysate (20 mmol/L Tris-HCl, 2.0 mmol/L EDTA, 137 mmol/L NaCl, 100 mL/L glycerol, 10 mL/L NP-40, 1.0 mmol/L PMSF; pH 8.0) was added per 100 mg. The tissue was ground using an electric homogenizer for subsequent tissue protein extraction. The exPD-L1 fusion protein and glutathione 4B agarose beads were combined as described above. Beads were resuspended with 500 µL GST pull-down buffer (50 mmol/L Tris-HCl, 1.0 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 5 mL/L NP-40; pH 7.4). In the subsequent step, 200 µL was mixed with an equal amount of tissue protein, and the product was placed on an inverted shaker and mixed at 4 °C for 12 hours. Centrifugation was conducted to collect the beads, followed by washing, boiling in 2 × PAGE loading buffers, and analysis using 10% SDS-PAGE electrophoresis. The protein bands were subjected to visualization by performing Coomassie brilliant blue R-250 staining. The specific bands on the gel were cut out, destained, dried and trypsinized. The experiment was repeated three times.

LC-MS/MS mass spectrometry analysis

Significantly differentially expressed protein spots were removed from the bidirectional electrophoresis gel, enzymatically hydrolyzed with trypsin solution, and the samples were lyophilized. The sample's LC-MS/MS mass spectrometry analysis was performed at The Fourth Military Medical University. Secondary mass spectrometry data were used using Proteome Discoverer 2.2 to conduct a search. Amino acid sequence of all mouse proteins was downloaded from the Uni Port database. (http://www.uniprot.org/uniprot/).

Results

Overexpression of PD-L1 causes infertility in male mice

As described by Fang *et al.* (2022), infertility was confirmed in PD-L1 transgenic male mice, whereas the females exhibited normal fertility. To further examine the correlation between the PD-L1 expression site and the sterile phenotype, PD-L1-EGFP transgenic mice were produced. Of the 11 lines of PD-L1-EGFP transgenic founder mice, 7 lines were males and 4 lines were females

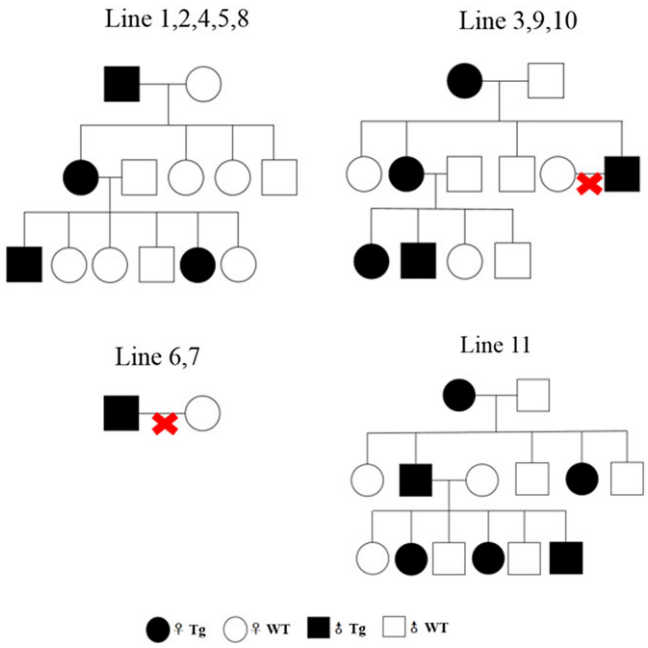

Figure 1. PD-L1 transgenic mouse reproductive lineage.

(Figure 1). Among those lines, 2 male mice expressing PD-L1 in the testicles and epididymis showed infertile phenotype; the other 5 mice with normal fertility. The male offspring of 3 female mice founders were infertile, which also expressed PD-L1 in the testicles and epididymis. The testicles and epididymis of the male offspring of the 11 female founders are EGFP (-), indicating normal fertility. The previous research also showed that only male mice expressing PD-L1 in testicular support cells and sperm cells at the same time were sterile (Fang *et al.*, 2022).

Overexpression of PD-L1 in male mice causes testicular cell sloughing and infertility with few mature sperms in the epididymis

Cross sections of testes and epididymis in WT and TgPD-L1 mice were used to examine the stages in the cycle of the seminiferous epithelium and the developmental processes of germ cells. The structure of the seminiferous epithelium in the testes of TgPD-L1 animals at the age of 60 days was clearly disorganized, with loose cell arrangement, and it lacked step VI of germ cell development followed by spermiation at stages VII-VIII in the seminiferous epithelium cycle (Fang *et al.*, 2022). Numerous sloughing spermatids were observed in HE-stained cross-sections of the testes of TgPD-L1 mice (Figure 2). PD-L1 transgenic mice carrying the green fluorescent protein fluorescence reporter gene showed that support cells and sperm cells co-express PD-L1 and are necessary to render PD-L1 transgenic mice sterile (Fang *et al.*, 2022). However, the epididymis structure was not markedly changed in TgPD-L1 mice. Some apparently normal sperms were seen inside the testes and the epididymis in TgPD-L1 mice, and few mature sperms were observed in the epididymis tail (Figure 2).

Expression of GST-exPD-L1fusion proteins

After 4-hour induction by IPTG, SDS-PAGE electrophoresis showed a distinct protein staining band at a size of 55 kD, and the 26 kD GST protein staining band was visible after induction of the air plasmid

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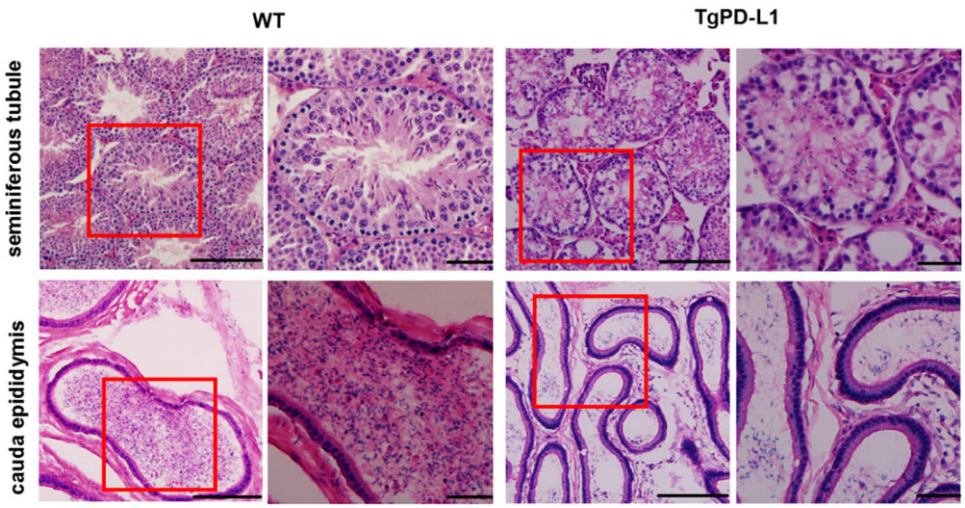

Figure 2. PD-L1 transgenic mice have abnormal testicles, Scar bars, the left of WT and TgPD-L1, 200 μm, the right, 40 μm.

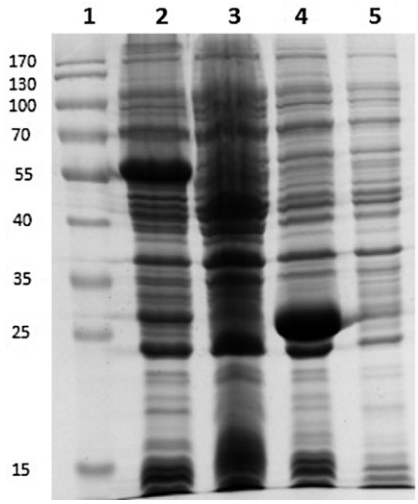

Figure 3. SDS-PAGE electrophoresis analyzes the expression of fusion proteins. 1. Protein pre-stain Marker, 2. Recombinant plasmid IPTG induction, 3. Recombinant plasmid unimulated, 4. pGEX-4T-1 IPTG induced, 5. pGEX-4T-1 is not stimulated.

control pGEX-4T-1. The unstimulated negative control did not produce bands of the corresponding size (Figure 3). Therefore, the 55 kD protein was preliminarily determined to be a pGEX-4T-1-exPD-L1 fusion protein. Most of the identified fusion proteins were present in the form of inclusion bodies.

Purification and identification of GST-exPD-L1 fusion proteins

After dilution, recombination and dissolution of the inclusions with a guanidine hydrochloride solution followed by purification with glutathione 4B agarose beads, SDS-PAGE electrophoresis analysis showed clear fusion protein bands at 55 kD (data not shown). The size was consistent with that of the expected protein. The purified exPD-L1 fusion protein was transferred to a PVDF membrane after SDS-PAGE electrophoresis, and after binding to the GST polyclonal antibody, the exPD-L1 fusion protein was detected at 55 kD (Figure 4), further confirming the successful expression of exPD-L1 fusion protein prokaryotic.

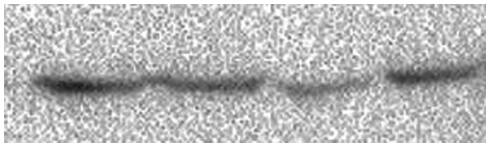

Figure 4. Western blot detects GST-exPD-L1 fusion protein.

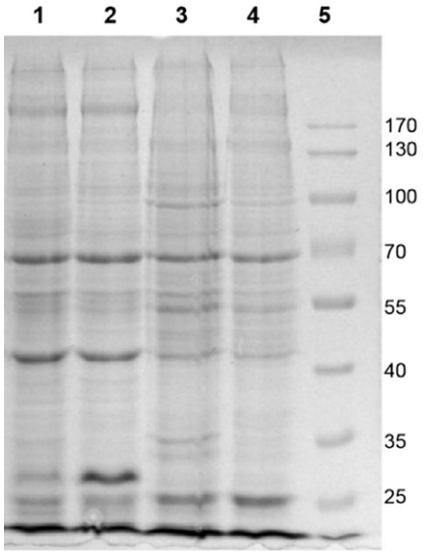

Figure 5. GST pull down detects proteins that interact with PD-L1. 1. WT epididymis, 2.TgPD-L1 epididymis, 3.WT testicles, 4.TgPD-L1 testicles, 5. Protein pre-stain marker.

GST pull down detected proteins that interact with PD-L1

TgPD-L1 and WT mouse proteins of testis and epididymis were extracted for GST-pull-down detection. The results showed no significantly different bands (1-170 KD) in WT and TgPD-L1 testicular histonein. However, the pull-down of epididymal tissue protein produced a band with a molecular weight of approximately 30 kD, indicating a marked difference between TgPD-L1 and WT (Figure 5). Mass spectrometry analysis of protein bands expressing differences.

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Table 1. Potential intercropping proteins with programmed death ligand 1 identified by GST pull down combined with mass spectrometry

IPI number	Molecular mass	Score	Description
00132195.3	65.98	748.26	Cysteine-rich secretory protein 1
00132195.109	37.14	127.47	14-3-3 protein zeta/delta
00132195.125	31.38	94.26	Actin, gamma-enteric smooth muscle
00132195.142	40.68	88.73	Actin, gamma, cytoplasmic 1
00132195.159	23.46	64.76	Isoform 2 of 14-3-3 protein theta

GST pull-down binding mass spectrometry identifies proteins that potentially interact with PD-L1

The GST pull-down assay was used to screen tissue proteins that potentially interact with PD-L1. 66 PD-L1-bound proteins were separated by SDS-PAGE and liquid chromatography-tandem mass spectrometry, including CRISP1 (Table 1).

Discussion

During normal mammalian physiology, sperm leave the testes when they cannot yet recognize and fertilize the oocyte. For the purpose of fertilization, sperms must go through various physiological changes as they move through the male and female reproductive tracts, including sperm maturation and capacitation, respectively (Arévalo *et al.*, 2020). CRISP has been a crucial tool for studying the molecular mechanisms regulating the interaction between mammalian gametes. The CRISP family of mammals consists of four members (CRISP1-4), primarily expressed in the male reproductive tract, present in spermatozoa, and capable of regulating Ca^{2+} channels (Giacomini *et al.*, 2020). Every CRISP protein takes part in more than one stage of gamete interaction (i.e., cumulus penetration, sperm-zona pellucida [ZP] binding, ZP penetration and gamete fusion) by either ligand-receptor interactions or the regulation of various capacitation-associated events, according to biochemical, molecular and genetic studies (Martinez *et al.*, 2020)

CRISP1 has been shown to be involved in multiple steps in the fertilization process, and its localization and expression in the gonads varies from species to species. There are two different forms of CRISP1, each with different mechanisms to bind to oocyte and participate in the regulation of sperm capacitation, sperm-zona pellucida binding and sperm-oocyte fusion. Rat CRISP1, the first member of the family, was detected with the help of a glycoprotein expressed by the principal epithelial cells in response to androgens and secreted into the lumen and showed an association with the sperm surface with two distinct affinities (Curci *et al.*, 2020). One population is tightly linked to the gamete and participates in sperm-ZP binding and gamete fusion via the mechanism of interacting with oocyte-complementary sites, whereas the other population is loosely connected to spermatozoa and acts as a decapacitating factor when capacitation occurs (Lv *et al.*, 2020; Gaikwad *et al.*, 2021). Scientific evidence is available on the ability of CRISP proteins to perform specialized roles crucial to fertility and their organization in function modules through independent pathways, along with their significance in successful fertilization (Sheng *et al.*, 2021; Nigro *et al.*, 2021). Epididymal CRISP1 is crucial for sperm maturation in the epididymis, which can interact and compensate for each other (Weigel *et al.*, 2018).

However, our previous studies have confirmed that the reproductive function of PD-L1/PD-1−/− transgenic mice has not been saved, suggesting that PD-L1 does not act by binding to the PD1 receptor in PD-L1 male sterile mice (Fang *et al.*, 2022). At the same time, since PD-L1 is a type I transmembrane protein, the extracellular region is linked to an intracellular domain by a hydrophobic transmembrane domain (Sun *et al.*, 2018; Freeman *et al.*, 2000). We performed a GST pull down experiment in the extracellular region of PD-L1 to look for proteins in the epididymis of PD-L1 transgenic mice that interact with it. With LC-MS/MS mass spectrometry analysis, we found for the first time that there is a high probability that CRISP1 will interact with PD-L1. As mentioned earlier, CRISP1 in the epididymis is a key protein in the process of sperm maturation and fertilization (Jorasia, *et al.*, 2021; Ernesto *et al.*, 2015), so we speculate that the high expression of PD-L1 not only causes severe malformation and sloughing during spermatid development in the testis, but also affects sperm maturation and capacitation and normal fertilization process through interaction with CRISP1, resulting in infertility in PD-L1 transgenic male mice.

In conclusion, this research shows that PD-L1 contributes to aberrant sperm development. The findings revealed that PD-L1 overexpression in mouse epididymis causes the male to become infertile. PD-L1 is capable of interacting with CRISP1, indicating its regulatory function in regulating sperm motility and orientation and in the Ca^{2+} signalling pathway, which required confirmation through further research.

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Competing interests. The authors declare no conflicts of interest.

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