Specific deficiency of Plzf paralog, Zbtb20, in Sertoli cells does not affect spermatogenesis and fertility in mice

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Zbtb20 is a POK family transcription factor and primarily functions through its conserved C2H2 Krüppel type zinc finger and BTB/POZ domains. The present study was designed to define the function of the Zbtb20, in vivo, during mouse spermatogenesis. Immunohistochemical studies revealed that ZBTB20 protein was localized specifically in the nuclei of Sertoli cells in seminiferous tubules. To investigate its role during spermatogenesis, we crossed Amh-Cre transgenic mice with Zbtb20 floxp mice to generate conditionally knockout mice (cKO) in which Zbtb20 was specifically deleted in Sertoli cells. The cKO mice were fertile and did not show any detectable abnormalities in spermatogenesis. Taken together, though specific deletion of transcription factor Zbtb20 in Sertoli cells has no apparent influence on spermatogenesis, its specific localization in Sertoli cells makes Zbtb20 a useful marker for the identification of Sertoli cells in seminiferous tubules.

Transcription factors are regulatory proteins that are mainly involved in transcription activation (or rarely in inhibition) by binding to DNA sequences1,2. They are considered to be the most important regulators in gene expression1,2. Transcription factors work in germ and somatic cells (especially in Sertoli cells) to control spermatogenesis in mammals3,4. So far, only relatively few transcription factors have been identified in spermatogenesis. Studies of all the transcription factors functioning in spermatogenesis indicate that transcription factors expressing in germ cells are more likely to function in specifically developmental stages of germ cells, while transcription factors expressing in Sertoli cells are often required during all the stages of gametogenesis in seminiferous tubules5–6. ZBTB [zinc finger and BTB (Broad complex, Tramtrack, and Bric-a-brac)] proteins, also known as Poxvirus and zinc finger (POZ) and Krüppel-type (POK) proteins, are a family of transcription factors that play critical roles in development, differentiation and oncogenesis6–12. POK/ZBTB proteins exhibit C-terminal zinc fingers and an N-terminal BTB/POZ domain. The former recognizes and binds to specific DNA sequences, while the BTB/POZ domain mediates homodimerization and/or heterodimerization and interacts with other proteins13–15. In the mouse genome, 44 genes encode different POK/ZBTB proteins and a few of them have been reported to be essential for spermatogenesis. For example, Zbtb16, also known as Plzf (promyelocytic leukemia zinc finger), expresses in gonocytes and undifferentiated spermatogonia and it is the first gene reported to be essential for spermatogonial stem cell self-renewal in mouse testes13,16.

Besides Zbtb16, functional roles of other POK/ZBTB genes, such as Zbtb20, remain unclear during spermatogenesis. Zbtb20 overexpression in transgenic mice under the control of a forebrain-specific promoter (D6 promoter) leads to the formation of hippocampus-like neuronal structures and behavioral abnormalities17, which indicates that Zbtb20 can control the organ development and differentiation. Another study shows that Zbtb20 null mice were fertile, however, the underlying mechanisms remained unknown due to pleiotropic defects such as growth retardation, prolonged hypoglycemia and postnatal lethality before 12 weeks of age of these mice18.

The aim of this study was to define the function of the Zbtb20 in vivo during spermatogenesis of mice. We found that ZBTB20 specifically expressed in the nuclei of Sertoli cells, making it a useful marker for the identification of Sertoli cells in seminiferous tubes. However, mice homozygous for a null mutation of Zbtb20, specifically in Sertoli cells, do not exhibit any detectable abnormalities in spermatogenesis and fertility, indicating that Zbtb20 is dispensable for spermatogenesis and male fertility in mice.
Sertoli cell isolation. A modified previously described method was used to isolate Sertoli cells from the testes of 21-dpp mice\(^{27,28}\). Briefly, testes were decapsulated under a dissection microscope. The seminiferous tubules were pooled and washed with phosphate-buffered saline (PBS) thrice and incubated with 2 mg/ml collagenase I (Sigma, C7661, MO, USA) and 0.5 mg/ml DNase I (Sigma, D4527) in DMEM: F12 (HyClone, SH30073-01, UT, USA) for 15 minutes at 37°C on a shaker, then washed twice with DMEM: F12 and further digested with 2 mg/ml collagenase I, 0.5 mg/ml DNase I and 1 mg/ml hyaluronidase (Sigma, H3506) for 15 minutes at 37°C. The tubules were allowed to settle and then were washed twice with DMEM: F12 before being digested with 2 mg/ml collagenase I, 0.5 mg/ml DNase I, 2 mg/ml hyaluronidase and 1 mg/ml trypsin (Sigma, T8003) for 30 minutes at 37°C. These dispersed cells were then washed twice with DMEM: F12 medium and placed into culture dishes in DMEM: F12 containing 10% fetal bovine serum (HyClone, SV30087-02) and were incubated at 37°C with 5% CO\(_2\). After 1 day of culturing, the solution was removed, and the cells were treated with a hypotonic solution (20 mM Tris, pH 7.4) for 1 minute for the removal of remaining germ cells, if any, and harvested for further analyses.

Sperm counting. The unilateral epididymides and vasa deferentia were removed from 70-dpp control and cKO mice, incised several times and incubated in 1 ml buffer containing 75 mM NaCl, 24 mM EDTA and 0.4% bovine serum albumin (Sigma, A2058) at 37°C with 5% CO\(_2\) for 30 minutes to allow sperm release from the epididymides. Sperm were collected after a nylon-mesh filtration and counted with a haemocytometer.

Histological examination and immunohistochemistry. The control and cKO mice were euthanized by cervical dislocation and testes were immediately fixed in Bouin’s solution for hematoxylin and eosin (H & E) staining or in 4% paraformaldehyde in PBS for immunohistochemistry. For immunohistochemistry, anti-SOX9 (1:100; Millipore, AB5535, MA, USA), anti-3\(^\beta\)-HSD (1:100; Santa Cruz, SC-30820, CA, USA), anti-\(\alpha\)-ACTIN2 (1:100; Abcam, AB13840, Cambridge, UK) were co-incubated with anti-ZBTB20 (1:500) and incubated at 4°C overnight. The control and cKO mice were used as negative controls. The images shown are representative images of experiments that were repeated thrice using samples from different sets of testes and yielded similar results. Scale bars, 50 \(\mu\)m.

Real-time PCR. RNA isolation and real-time PCR were performed as previously described\(^{24}\). All PCR primers used are listed in Supplementary Table 1. For real-time PCR analysis, \(C_{T}\) values of samples were normalized to the corresponding \(C_{T}\) values of Gapdh. Quantification of the fold change in gene expression was determined by the comparative \(C_{T}\) method.

Western blot. Protein samples from Sertoli cells were prepared in lysis buffer (50 mM Tris/HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100) supplemented with protease inhibitors (Roche). Western blot was carried out as described previously\(^{24}\) and primary antibodies against ZBTB20 (1:500) and GAPDH (1:1000; Millipore, MAB374, MA, USA) were used to detect the protein bands.

Statistical analysis. Protein and mRNA levels, testis weights, testis/body weight ratios, sperm number and litter size between control and cKO mice were analyzed by using the Student’s t-test. Results are presented as mean ± SEM. Statistical significance was set at \(P < 0.05\).

Results

Expression of ZBTB20 in mouse testes. In seminiferous tubules of adult testes (70-dpp) (Fig. 1A), ZBTB20 protein was detected only in the nuclei of Sertoli cells, as it was co-localized with SOX9, a Sertoli cell nucleus specific marker (Fig. 1A) and it was not detected in germ cells as ZBTB20 staining was absent in the cells stained positive by DDX4, a germ cell specific marker (Fig. 1B). In order to more precisely evaluate ZBTB20 expression in Sertoli cells during testicular development, we performed immunohistochemical analysis of ZBTB20 in testes during early developmental stages (7- and 14-dpp) and observed that ZBTB20 was expressed at all the studied stages in Sertoli cells (Fig. 1A). Besides in Sertoli cells, ZBTB20 was also found in interstitial regions between seminiferous tubules in testis (Fig. 1A). Co-immunostaining of ZBTB20 with 3\(^\beta\)-HSD, a Leydig cell cytoplasm specific marker, revealed that ZBTB20 was localized in Leydig cells (Supplementary Fig. S1A). Additionally, co-immunostaining of ZBTB20 with \(\alpha\)-ACTIN2, a peritubular myoid cell marker, indicated that ZBTB20 also localized in some peritubular myoid cells (Supplementary Fig. S1B).

Zbtb20 deletion in Sertoli cells. In order to assess the Zbtb20 functions in Sertoli cells during spermatogenesis, we generated mice in which the Zbtb20 gene was specifically disrupted in testicular Sertoli cells. We combined a conditional flox Zbtb20 allele with the Cre-expressing line Amh-Cre (Fig. 2A). To study the CRE recombinase activity, Amh-Cre mice were crossed with fluorescence reporter mice (mT/mG), in which Amh-Cre mediated recombination resulted in excision of the RFP cassette and expression of the GFP reporter\(^{14}\). Based on the analysis of Amh-Cre recombinant mT/mG reporter mice, we confirmed that the Amh-Cre mice had strong recombinase activities in Sertoli cells (Supplementary Fig. S2). Zbtb20 deletion efficiency in Sertoli cells was analyzed by detecting the Zbtb20 mRNA and protein levels in the isolated Sertoli cells (Supplementary Fig. S3). We found that the
Zbtb20 disruption resulted in a drastic reduction in Zbtb20 mRNA and protein levels in the cKO Sertoli cells (Supplementary Fig. S4 and Fig. 2B–C). As shown in Fig. 2D, immunofluorescent analysis of the cKO testes revealed that the localization of ZBTB20 in Sertoli cell was abolished (Fig. 2D) without affecting its localization in Leydig cells (Supplementary Fig. S5), which further confirms the efficient and specific disruption of Zbtb20 in Sertoli cells.

Normal spermatogenesis in Zbtb20 cKO mice. After successfully generating a Sertoli cell Zbtb20 knockout mouse model, we investigated spermatogenesis in the cKO mice. The testes from control and cKO litters of different ages were analysed. We did not find any significant differences in testis morphology, weight and testis/body weight ratio between the two groups (Fig. 3A–B; Supplementary Fig. S6). As shown in Fig. 3C, both cKO and control testes exhibited typical seminiferous tubule morphology with all stages of spermatogenic cells (from spermatogonia to spermatozoa), suggesting that spermatogenesis was normal in cKO mice. We also analyzed the sperm in epididymides and found that cKO and control mice produced similar number of sperm (Fig. 3C–D). Finally, the breeding performance of the cKO males was found similar to that of control males as litters produced by mating cKO males with wild type females showed a size similar to those produced by control males (Fig. 3E). Taken together, these results indicated that Zbtb20 conditional knockout in Sertoli cells does not affect spermatogenesis or fertility of mice.

Discussion
Our present study describes the expression of transcription factor Zbtb20 in mouse testis. We have observed that ZBTB20 protein was specifically localized in the nuclei of Sertoli cells located within seminiferous tubules along with Leydig and some peritubular myoid cells present between seminiferous tubes in mouse testes. As compared to its paralog gene, Zbtb16, which is restricted to gonocytes and undifferentiated spermatagonia, Zbtb20 was specifically expressed in testicular somatic cells (Fig. 1A–B; Supplementary Fig. S1). In contrast, all Sertoli cells showed a constant and intense expression of Zbtb20 in all seminiferous tubules independent of the age of animals, which makes it a useful molecular marker for the identification of Sertoli cells (Fig. 1A–B). This expression pattern was in agreement with the expression of previously described Sertoli cell transcriptional factors, such as SOX9 and GATA4, which have been shown to play essential roles in spermatogenesis.
However, the phenotype of the Zbtb20 deficient mice indicates that Zbtb20 in Sertoli cells was not required for spermatogenesis (Fig. 3A–E; Supplementary Fig. S6).

The lack of phenotype in mice that Zbtb20 was specifically disrupted in Sertoli cells may result from functional redundancy in the Zbtb family. In this scenario, expression of Zbtb20 target genes in...
Zbtb20 null Sertoli cells would be regulated by other Zbtb factors, such as Zbtb32, Zbtb37 and Zbtb44, which are highly expressed in testes\(^*\). We thus hypothesize that some of the unidentified ZBTB proteins may have compensated for the loss of Zbtb20 function in Sertoli cells. ZBTB20 was also expressed in Leydig and peritubular myoid cells, however, the significance of this protein in these cells remains unclear. Thus, future studies on Leydig- or peritubular myoid cell-specific knockout animals for Zbtb20 may further shed light on the role of this transcription factor in the testes.

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Author contributions
Conceived and designed the experiments: Q.S., X.J., H.Z. and W.Y. Performed the experiments: X.J., H.Z., S.Y., Y.Z., W.Z., L.W. and Z.W. Analyzed the data: X.J., S.Y. and W.Y. Paper wrote up: Q.S. and X.J. Modification of the manuscript: I.B. F.I. and H.J.C.

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