Targeted disruption of the mouse testis-enriched gene Znf230 does not affect spermatogenesis or fertility

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Abstract

The mouse testis-enriched Znf230 gene, which encodes a type of RING finger protein, is present primarily in the nuclei of spermatogonia, the acrosome and the tail of spermatozoa. To investigate the role of Znf230 in spermatogenesis, we generated Znf230-deficient mice by disrupting Znf230 exon-5 and exon-6 using homologous recombination. The homozygous Znf230-knockout (KO) mice did not exhibit Znf230 mRNA expression and Znf230 protein production. Znf230 KO mice exhibited no obvious impairment in body growth or fertility. Male Znf230 KO mice had integral reproductive systems and mature sperm that were regular in number and shape. The developmental stages of male germ cells of Znf230 KO mice were also normal. We further examined variations in the transcriptomes of testicular tissue between Znf230 KO and wild-type mice through microarray analysis. The results showed that the mRNA level of one unclassified transcript 4921513I08Rik was increased and that the mRNA levels of three other transcripts, i.e., 4930448A20Rik, 4931431B13Rik and potassium channel tetramerisation domain containing 14(Kctd14), were reduced more than two-fold in Znf230 KO mice compared with wild-type mice. Using our current examination techniques, these findings suggested that Znf230 deficiency in mice may not affect growth, fertility or spermatogenesis.

Keywords: Znf230, knockout mice, spermatogenesis, Kctd14.

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Introduction

Mammalian spermatozoa development is a complex process that involves the renewal and differentiation of spermatogonia, the meiosis of spermatocytes, and drastic morphological changes accompanying the development from round spermatids to mature spermatozoa (Russell et al., 1990). Many environmental, behavioral and genetic factors affect male fertility. An estimated 50% of human infertility has been attributed to genetic abnormalities (Hwang et al., 2011). Two previous microarray studies (Schultz et al., 2003; Schlecht et al., 2004) showed that haploid germ cells express a large number of germ cell-specific genes (approximately 4% of mammalian genes). Therefore, it is necessary to identify these unique genes and characterize the precisely programmed cell- and stage-specific gene expression that occurs during the regulation of the developmental spermatogenesis process.

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The human gene ZNF230 (also named RNF141), which encodes a type of RING (Really Interesting New Gene) finger protein, was first identified in our laboratory to be restrictively expressed in the testicular tissue of fertile men (Zhang et al., 2001). RING finger proteins, a sub-family of zinc finger proteins (ZFP), often contain the cysteine-rich CX2CX(9-39)CX(1-3)HX(2-3)CX2CX(4-48)CX2C domain and are involved in a variety of biological processes, including transcriptional regulation, signal transduction, cell apoptosis and protein ubiquitination (Borden, 2000; Joazeiro and Weissman, 2000). The mouse homolog of Znf230 was also identified in our laboratory (Qiu et al., 2003). The expression of the mouse Znf230 gene is developmentally regulated, and the Znf230 protein functions as an activator module in transcription. Additionally, the mouse Znf230 protein is primarily expressed in the nuclei of spermatogonia but has subsequent expression in the acrosome system and the tails of developing spermatids and spermatozoa (Song et al., 2008). Hence, we wondered whether Znf230 may play a role in mammalian spermatogenesis.

Animal models have defined key signaling pathways that are involved in reproductive physiology (Li et al., 2001). To date, over 400 genes that are essential for male
fertility have been identified using transgenic, chemically induced, point mutants and KO/knock-in/gene-trap mouse models (Yatsenko et al., 2010; Jamsai and O’Bryan, 2011).

To investigate the role of Znf230 in mouse spermatogenesis, we used a targeted gene KO strategy to generate Znf230-deficient mice. We had previously constructed a gene-targeting vector based on a modified pPNT vector and generated mutant mice with exon-2 of the Znf230 gene disrupted (Liu et al., 2013). However, a partial sequence from the pPNT vector acted as an alternative exon-2, thus allowing a new Znf230 transcript to be produced in the mutant mice and a new protein product, possessing a C-terminal amino acid sequence with a RING finger motif similar to that of the wild-type Znf230 protein, to be generated. Thus, Znf230 function in the mutant mice was not entirely inactivated. In the current study, we changed the targeting strategy such that the region of exon-5 and exon-6, which encode the essential RING finger domain of the Znf230 protein, to be generated. Thus, Znf230 KO mice in the mutant mice was not entirely inactivated. In the current study, we changed the targeting strategy such that the region of exon-5 and exon-6, which encodes the essential RING finger domain of the Znf230 protein, was directly disrupted. This strategy successfully generated Znf230-null mice for use in this study.

Materials and Methods

Construction of Znf230 KO targeting vector and generation of Znf230 KO mice

Using a highly efficient recombineering-based method that has been previously described (Liu et al., 2003; Chan et al., 2007), the Znf230 KO targeting vector was constructed from a genomic DNA fragment derived from the C57BL/6J bacterial artificial chromosome clone bMQ-291L21. The targeting construct, which had a 4864 bp left arm containing introns 2 and 3 of the Znf230 gene and a 2902 bp right arm containing the partial untranslated region of exon-6, was inserted into the ABRLF-pBR32 vector (Figure 1).

Thirty micrograms of the targeting vector was linearized with Not I and transfected into CJ7 (derived from 129SV/J mice) embryonic stem cells (ESCs) by electroporation. Ninety-six ESC clones were selected with 300 μg/mL G418 (Geneticin, Sigma-Aldrich Co., St. Louis, MO, USA). Among these neomycin-resistant cells, 12 ESCs that had undergone homologous recombination were identified by long polymerase chain reaction (L-PCR) analysis with two pairs of primers P1F: 5'-acctgctggcttttaacatgtc-3', P1R: 5'-ggctcactacgcttcattgc-3' and P2F: 5'-ccgctgctccattgcctggc-3', P2R: 5'-cagcagcttattacccaggtg-3'.

Two correctly targeted ESC clones were microinjected into C57BL/6J blastocysts to generate chimeras that were then crossed into a C57BL/6J genetic background. The offspring were screened by L-PCR analysis of their genomic DNA using P1F/R and P2F/P2R primers.

The Znf230 KO mice were generated at the Shanghai Research Center for Model Organisms, Shanghai, China.
RIPA lysis buffer containing a 1 μL/mL protease inhibitor cocktail (Sigma) to obtain cell lysates. After centrifugation, the supernatants were examined for protein concentration, subjected to 12% SDS-PAGE and transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA, USA). The membranes were incubated with anti-Znf230 primary antibody (Catalog number: ab4542, Abcam, Cambridge, MA, USA) at 4 °C overnight, washed with 1 X PBS containing 0.1% Triton X-100, and incubated with horseradish peroxidase-linked secondary antibodies (1:10,000, Boster, Wuhan, China) at room temperature for 1 h. The respective bands were visualized using an immunostaining kit (Millipore). The anti-Znf230 primary antibody recognizes amino acids 218-230 in the C-terminal of the Znf230 protein. The quality of the antibody was validated by the manufacturer and our previous report (Song et al., 2008). The western blot analysis was repeated again.

Morphological examination

Testes from three mice per post-natal day (4, 8, and 15 weeks of age) were necropsied for histopathological examination. The tissues were routinely processed, embedded in paraffin and stained with hematoxylin and eosin (H&E). Three slides per testis were visualized using a Zeiss Axio Imager Z2 microscope (Carl Zeiss, Jena, Germany).

Analysis of sperm characteristics

Cauda epididymides from five 15-week-old mice per genotype were dissected and immediately minced in 1 X PBS solution (pH 7.4). Sperm were squeezed out with fine forceps and allowed to disperse in PBS at room temperature for 15 min, followed by repeated pipetting. Thereafter, the sperm remaining as a mono-dispersed suspension were counted using a hemocytometer. Sperm counting was performed four times for each sample.

In addition, the sperm were washed three times in 1 X PBS by centrifugation at 500 g for 5 min and then air-dried onto microscope slides. The slides were then stained with H&E and visualized using a Zeiss Axio Imager Z2 microscope.

Serum sex hormone assay

Blood was taken from five 15-week-old male mice per genotype that were generated from different breeding pairs and housed singly in separate cages. The serum testosterone, follicle stimulating hormone (FSH), and luteinizing hormone (LH) levels were measured using an ELISA kit (Uscn Life Science Inc., Wuhan, China), according to the manufacturer’s instructions.

Microarray analysis

One hundred micrograms of total RNA from testis tissues from three Znf230 KO and three C57BL/6J wild-type mice was used to generate biotin-labeled cRNA by using a Message Amp Premier RNA Amplification Kit (Ambion, Austin, TX, USA). Following fragmentation, the labeled cRNA of each sample was hybridized to Affymetrix GeneChip® Mouse Genome 430 2.0 Arrays (Santa Clara, CA, USA) and stained according to the manufacturer’s instructions.

Processed arrays were scanned using a GeneChip® Scanner 3000 (Affymetrix). The Affymetrix GeneChip® Command Console® Software was used to perform quality assessments and produce data reports. Differences in the expression patterns between groups were analyzed using the R program for significance analysis of microarrays (Irizarry et al., 2003).

Microarray analysis was performed at the CapitalBio Corporation, Beijing, China.

Quantitative real time PCR (qRT-PCR)

qRT-PCR analyses were performed with cDNA using a SYBR® Premix Ex Taq™ II kit (Takara, Dalan, China) and 10 mM of the corresponding set of sense and antisense primers of the Kctd14 gene: 5’-atgggccacccgtgaagc-3’ (Forward) and 5’-gcgcagcgcagcagtgctc-3’ (Reverse). The reactions were run on a Bio-Rad MyiQ Cycler (Hercules, CA, USA) using the following parameters: 40 cycles of 95°C for 1 min, 95°C for 10 s and 60°C for 30 s. Three independent assays were performed in duplicate on each sample. The CT values from each run were averaged per sample. The ΔΔCT method was applied for data analysis. To do so, the CT data for the Kctd14 mRNA samples were normalized with that of β-actin (Primers: 5’-aacagtgctcgctgaaga-3’ (Forward) and 5’-gcgtgcatctctgtaagcc-3’ (Reverse)). All qRT-PCR data are shown as the mean ± standard deviation (S.D.). The testis sample from Znf230 KO mice was set at 1 as arbitrary unit or 100%.

Statistical analysis

Student’s t-test was used to compare data between wild-type and mutant mice. A p < 0.05 indicated significance. All data analyses were performed using SPSS v17.0.

Results

Generation of Znf230 KO mice

A gene-targeting vector was constructed to delete the genomic segment of Znf230 that includes exon-5 and exon-6, which encode the RING finger motif of Znf230 (Figure 1). The targeting vector was introduced into ESCs. ESCs with successful homologous recombination were confirmed by two L-PCR analyses with primers flanking the targeting region (Figure 2A). The chimeric mice derived from the targeted ESCs transmitted the disrupted Znf230 allele to their offspring (Figure 2B-D).

RT-PCR analysis was performed to evaluate Znf230 gene expression, which should be disrupted in Znf230 KO mice. As shown in Figure 2E, the targeting region, i.e.,
exon-5 and exon-6, of the Znf230 gene was not amplified from total RNA of the testes in Znf230 KO mice using the specific primers, in contrast to wild-type mice. Furthermore, western blot analysis with anti-Znf230 antibody showed that the 26-kDa protein representing Znf230 was present in the testes of wild-type mice, as expected, but it was absent in the testes of Znf230 KO mice (Figure 2F). Thus, the Znf230 gene was indeed disrupted in Znf230 KO mice.

Znf230 KO mice appeared to be normal in growth and fertility

Znf230 KO mice exhibited no obvious impairment in body growth and development because no significant differences were observed in the weights of body or organs, including brain, lung, heart, liver, spleen and kidney, or life spans between Znf230 KO and wild-type mice (Table 1). Because Znf230 was identified as a testis-enriched gene that likely plays a role in male fertility, we focused our investigation on phenotypes related to male fertility. However, the fertility of Znf230 KO mice appeared to be normal and the offspring of Znf230 KO intercrosses were born at the expected Mendelian ratios. Compared with wild-type mice, Znf230 KO mice displayed no detectable differences in the male reproductive system, including the testis, seminal vesicle, prostate and bladder. There were also no differences in serum testosterone, FSH or LH levels between Znf230 KO and wild-type mice (Table 1, Figure 3A, B). H&E staining demonstrated that the testicular tissue of Znf230 KO mice was intact and that each developmental stage of male germ cells was normal (Figure 3C-H). No significant difference was detected in the shape and number of sperm isolated from the epididymides of Znf230 KO and C57BL/6J wild-type mice (Figure 3I, J and Table 1).

Changes in the mRNA expression profile of Znf230 KO mice

Our previous report proposed that Znf230 was a DNA-binding protein that may function as a transcriptional activator (Qiu et al., 2003). We therefore investigated the differences between the transcriptomes of testes from Znf230 KO and wild-type mice using Affymetrix Mouse Genome 430 2.0 Arrays. The expression levels of over 34,000 genes were assessed, and transcripts with fold changes greater than 2 or less than 0.5 between the two groups were analyzed (Table 2). Znf230 had the most down-regulated expression level of the analyzed transcripts, thereby confirming the validity of the experimental system. The mRNA level of one unclassified transcript 4921513I08Rik (GenBank No. AK014883) was detected to be more than two-fold higher, and the mRNA levels of three transcripts including two protein coding genes Kctd14 (GenBank No. NM_001136235) and 4930448A20Rik (GenBank No. Ak015411) and a non-coding RNA 4931431B13Rik (GenBank No. NR_045183) were detected to be more than two-fold lower in Znf230 KO mice compared with C57BL/6J wild-type mice. Because the Kctd14 gene may encode a functional protein, we performed qRT-PCR analysis to verify the expression changes of Kctd14 between Znf230 KO and wild-type mice. The results showed that the mRNA level of Kctd14...
was reduced by more than 10-fold in Znf230 KO mice compared with wild-type mice (Figure 4).

**Discussion**

The present work was undertaken in an effort to define the physiological role of the testis-enriched gene Znf230 in mammalian spermatogenesis. We generated a null mutation in the Znf230 gene by homologous recombination in mouse ESCs, which were used to produce homozygous Znf230 KO mice. Mice that were homozygous for the mutation lacked the intact mRNA and protein in germ cells, but did not exhibit any detectable abnormality in body growth or spermatogenesis. The absence of abnormality in Znf230-null testes was unexpected because of the testis-enriched expression pattern of Znf230. However, several explanations may account for the lack of a clear phenotype in Znf230 KO mice. First, the function of Znf230 may be dispensable for male fertility. As an example, H1t is an H1 histone variant that is unique to late spermatocytes and round spermatids, but H1t-null mice have no discernible phenotype (Fantz et al., 2001). Another example is SPAG5, which is an Odf1-interacting protein that is specifically expressed during meiosis. The disruption of SPAG5 does not affect spermatogenesis or fertility (Xue et al., 2002). Other examples include testicular orphan nuclear receptor 2 gene (Shyr et al., 2002), testicular haploid expressed gene (Mannan et al., 2003), transition protein 2, proacrosin and histone H1.1 genes (Nayernia et al., 2003), the tumor suppressor LRP1b gene (Marschang et al., 2004), the UBC4-testis gene (Bedard et al., 2005) and the testis-enriched

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**Table 1** - Comparison of phenotypes between Znf230 KO and C57BL/6J mice.

|                          | Znf230 KO       | C57BL/6J      | P*         |
|--------------------------|----------------|--------------|------------|
| Total body weight (g)b   | 30.18 ± 1.45   | 30.31 ± 2.07 | NS         |
| Organ weight (mg)c,d      |                |              |            |
| Brain                    | 434.55 ± 30.71 | 405.38 ± 44.78 | NS         |
| Heart                    | 151.8 ± 16.08  | 139.54 ± 8.48  | NS         |
| Lung                     | 171.21 ± 12.93 | 166.14 ± 11.21 | NS         |
| Liver                    | 1382.53 ± 82.92 | 1386.72 ± 192.7 | NS         |
| Kidney                   | 212.32 ± 25.95 | 204.1 ± 21.18  | NS         |
| Spleen                   | 60.37 ± 7.17   | 63.64 ± 8.29  | NS         |
| Seminal vesicle          | 268.2 ± 14.91  | 236.1 ± 40.69  | NS         |
| Prostate and Bladder     | 127 ± 25.22    | 127.5 ± 22.3  | NS         |
| Testis                   | 108.5 ± 15.46  | 109.6 ± 23.67  | NS         |
| Total no. of sperm (x10^6) | 24.4 ± 3.6    | 23.7 ± 2.8   | NS         |
| Serum sex hormone (ng/mL)b |       |              |            |
| Testosterone             | 1.290.25       | 1.2 ± 0.34   | NS         |
| Luteinizing hormone (LH) | 8.02 ± 1.37    | 8.21 ± 1.72   | NS         |
| Follicle-stimulating hormone (FSH) | 10.2 ± 3.05 | 9.56 ± 2.45  | NS         |
| Litter size d            | 7.5 ± 1.4      | 8.1 ± 1.2    | NS         |
| Life Span (days) e       | 713.2 ± 147.6  | 722.5 ± 168.5 | NS         |

*aStatistical analysis was carried out by Student’s t test, NS: Not significant.

bFifteen-week-old mice were examine, n = 5 per group, Values are means ± S.D.

cWet weights of paired organs were averaged for each mouse, and the single value was used to calculate mean ± S.D among same genotype.

dData are mean values derived from six breeding pairs for each genotype.

eData are mean values of 10 mice (5 male plus 5 female) per group.

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**Table 2** - Genes differentially expressed in the testes of Znf230 KO mice

| Gene ID | Gene symbol | Gene name                                             | Fold change a | ProbeSet ID b |
|---------|-------------|-------------------------------------------------------|---------------|--------------|
| 70875   | 4921513108Rik | RIKEN cDNA 492151308 gene                              | 2.9705        | 1432299_at   |
| 67150   | Znf230      | ring finger protein 141                                | 0.445/0.1841/0.158 | 1449086_at/1433655_at/1449087_at |
| 233529  | Kcnel14     | potassium channel tetramerisation domain containing 14 | 0.4276/0.4054 | 1426632_at/1426633_at |
| 70971   | 4931431B13Rik | RIKEN cDNA 4931431B13 gene                             | 0.4054        | 1430416_at   |
| 73993   | 4930448A20Rik | RIKEN cDNA 4930448A20 gene                             | 0.2394        | 1454205_at   |

aThe ratio of signal values between Znf230 KO and Wild-type mice detected by every probe. Statistical p-values < 0.001.
bAll probes hit one known transcript.
histone demethylase KDM4D gene (Iwamori et al., 2011). One explanation for such phenomena is that an unidentified protein may compensate for the loss of function of these genes. It is reasonable that other RING finger proteins may compensate for the loss of \textit{Znf230} function because RING finger proteins belong to one of the largest zinc finger protein families. Second, the defects in \textit{Znf230} KO mice may be too small to be detected using the techniques employed here. A detailed ultrastructural examination of testicular tissues and spermatozoa may be needed to confirm eventual minor changes in the mutant mice. Third, because spermatogenesis is a very complex process that involves many genes, the inactivation of one gene may not be sufficient to produce a detectable phenotype. For example, \textit{Tyro3}, \textit{Axl}, and \textit{Mer} encode three structurally related receptors that possess tyrosine kinase activity. Mice that lack any single receptor or any combination of two receptors are viable and fertile, but males that lack all three receptors produce no mature sperm (Lu et al., 1999). Therefore, it will be of interest to explore the interaction between \textit{Znf230} and its related proteins by ablating their network and dissecting the resulting phenotypes to help clarify the biological role of the \textit{Znf230} gene in male fertility.

Indeed, we found that the mRNA levels of four transcripts were changed more than two-fold in \textit{Znf230} KO mice compared with \textit{C57BL/6J} wild-type mice. Three of the four transcripts were unclassified. However, \textit{Kctd14} encodes a putative member of the KCTD protein family that contains the bric-a-brac/tramtrak/broad (BTB) complex domain, which resembles the tetramerization domain of voltage-gated potassium channels. The KCTD protein family, which comprises 22 members, has been implicated in many important biological processes (Schwenk et al., 2010; Seddik et al., 2012; Cao-Ehiker et al., 2013; Skoblov et al., 2013). However, because no report has yet described the biological function of the \textit{Kctd14} protein, \textit{Znf230} KO mice may provide a clue for investigating the pathways in which these unclassified transcripts are involved.
Conversely, the Znf230 protein may act as a transcriptional factor, and the disruption of the Znf230 protein may cause the related transcriptional complex to be destroyed, this directly affecting the expression of the four transcripts. It is interesting to note that Znf230 and the four transcripts are all located on mouse chromosome 7 (Chr7). As shown in Figure S1, the transcripts of 492151308Rik and Kctd14 are located upstream of Znf230, while the other two transcripts are located downstream of Znf230. It is thus possible that cis-acting elements may have been destroyed during the targeted disruption of the Znf230 gene. In addition, changes in the chromatin structure in the KO region of Chr7 may be the cause for the changes in the expression levels of the four transcripts.

In conclusion, we generated Znf230-deficient mice that exhibited normal body growth and fertility based on our current examination techniques. Using microarray analysis to compare the transcriptomes of testicular tissue from Znf230 KO and wild-type mice, we observed changes in the expression levels of four transcripts in Znf230 KO mice. In the future, the Znf230 KO mouse model may be used to uncover the biological roles and explore the interaction between Znf230 and its related transcripts.

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**Supplementary Material**

The following online material is available for this article:

Figure S1- The locations of the transcripts of *Kctd14*, *Znf230*, *4921513I08Rik*, *4930448A20Rik* and *4931431B13Rik*.

This material is available as part of the online article from http://www.scielo.br/gmb.

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