An azoospermic factor gene, Ddx3y and its paralog, Ddx3x are dispensable in germ cells for male fertility

Takafumi MATSUMURA¹, 2), Tsutomu ENDO¹, 3), Ayako ISOTANI¹, 5), Masaki OGAWA¹) and Masahito IKAWA¹–4)

¹)Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan
²)Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan
³)Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan
⁴)The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan
⁵)Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara 630-0192, Japan

Abstract. About 10% of male infertile patients show abnormalities in spermatogenesis. The microdeletion of azoospermia factor a (AZFa) region of the Y chromosome is thought to be a cause of spermatogenic failure. However, candidate gene responsible for the spermatogenic failure in AZFa deleted patients has not been elucidated yet. Using mice, we explored the function of Ddx3y, a strong candidate gene in the AZFa region, and Ddx3x, a Ddx3y paralog on the X chromosome, in spermatogenesis. We first generated Ddx3y KO male mice using CRISPR/Cas9 and found that the Ddx3y KO male mice show normal spermatogenesis, produce morphologically normal spermatozoa, and sire healthy offspring. Because Ddx3x KO males were embryonic lethal, we next generated chimeric mice, which contain Ddx3x and Ddx3y double KO (dKO) germ cells, and found that the dKO germ cells can differentiate into spermatozoa and transmit their mutant alleles to offspring by normal mating. We conclude that Ddx3x and Ddx3y are dispensable for spermatogenesis at least in mice. Unlike human, mice have an additional Ddx3y paralog D1pas1, that has been reported to be essential for spermatogenesis. These findings suggest that human and mouse DDX3 related proteins have distinct differences in their functions.

Key words: Azoospermia factor region, Chimeric analysis, CRISPR/Cas9, Male infertility, Y chromosome

Received: December 6, 2018
Accepted: December 13, 2018
Published online in J-STAGE: January 7, 2019
©2019 by the Society for Reproduction and Development

Correspondence: M Ikawa (e-mail: ikawa@biken.osaka-u.ac.jp)
This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International (CC BY 4.0: https://creativecommons.org/licenses/by/4.0/)
homology at the amino acid and nucleotide sequence are 91% and 88%, respectively. In mice, homologies between Ddx3y and Ddx3x in amino acid sequences and nucleotides are 90% and 84%, respectively. DDX3X protein is expressed in the brain, kidney, ovary, and testis [21]. Interestingly, it is suggested that mouse DDX3X protein is localized in germ cells of the testes, including spermatogonia [22]. At present, functions of DDX3Y and DDX3X in spermatogenesis remains unclear.

In this study, we analyzed the functions of Ddx3y and Ddx3x in spermatogenesis, using mice. We first generated Ddx3y KO male mice by CRISPR/Cas9 [23, 24] and analyzed spermatogenesis. Because Ddx3x KO males show embryonic lethality, we next generated chimeric mice, which contain spermatogenic cells derived from ES cells mutated for both Ddx3x and Ddx3y, and analyzed spermatogenesis in the double KO (dKO) ES chimeric mice.

Materials and Methods

Animals

All animal experiments were conducted in accordance with the guidelines of “Animal experiment rules” established by the Research Institute for Microbial Diseases, Osaka University, and were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University (#Biken-AP-H25-02). B6D2F1, ICR, and C57BL/6NCr mice were purchased from CLEA (Tokyo, Japan) or SLC (Shizuoka, Japan).

Plasmid construction and genotyping

Construction of sgRNA/CAS9 expressing plasmids, pX330 (#42230, Addgene, Cambridge, MA, USA), were performed by ligating oligos into the BbsI site of each plasmid as described previously [25, 26]. Potential off-target sites were searched using Bowtie software (http://bowtie-bio.sourceforge.net/index.shtml) as described previously [25]. We chose gRNAs whose 14 bases at the 3’ end, plus the NGG site, only matched the target site. The sgRNA target sequences for generating Ddx3y KO mice were 5’-GTCTGTGATAAGGACAGTTC -3’ (sgRNA #1) and 5’- TATTTCAATGTGCGTGGAAAG -3’ (sgRNA#2). The sgRNA target sequence for generating Ddx3x KO mice was 5’- GTGCGATGGGAAAATGCCTC -3’. The following primer sets were used for genotyping PCR of Ddx3y: Forward primer 5’- CATGCCCTCATCATCATAATCCCATAAGTG -3’ and Reverse primer 5’- GGATAGCCATTGTTGAGACTTGTTGACA -3’. The following primer sets were used for genotyping PCR of Ddx3x: Forward primer 5’- CCAAGGCTTCTTTATGACCCGACG -3’ and Reverse primer 5’- CCACTCGCGGCTCATAATCAAC -3’.

Pronuclear injection of mouse fertilized eggs

B6D2F1 superovulated mice were fertilized with Mated B6D2F1 males, then fertilized eggs were collected 20 h after hCG. Circular pX330 plasmids were injected into one of the pronuclei at 5 ng/μl [25, 26]. The eggs were cultured in KSOM overnight [27], and the two-cell stage embryos were transferred into the oviducts of pseudopregnant ICR females.

Genome editing in mouse ES cells and generation of chimeric mice

The EGR-G01 ES cells [28] (1 × 10^3) were seeded on mouse embryonic fibroblasts (MEF) in a 6-well plate and transfected with pX330 (total 1.0 μg) using Lipofectamine LTX & PLUS technology (Thermo Fisher Scientific, MA, USA). A pPGK-puro plasmid (0.1 μg) was co-transfected. After 14–18 h, the cells were selected with puromycin (0.1 μg/ml) for 48 h, then grown for 5 to 6 more days, picked, and transferred onto MEF cells in 96-well plates. After 48–72 h of culture, each ES cell clone was split in duplicate, for freezing, and DNA harvesting. After PCR amplification and direct sequencing, the positive clones were thawed and expanded to analyze their karyotypes. The mutant ES cell clones with normal karyotypes were injected into 8-cell ICR embryos, and the chimeric blastocysts were transplanted into the uteri of 2.5 dpc pseudopregnant females [29].

cDNA synthesis and RT-PCR

Testes were collected from C57BL/6NCr and Ddx3y KO male mice. These samples were homogenized in TRIzol (Thermo Fisher Scientific). The total RNA was reverse-transcribed to cDNA using SuperScript III First Strand Synthesis System for RT-PCR (Thermo Fisher Scientific). Five ng of cDNA was used for PCR with primer sets and KOD DNA Polymerase (KOD-Fx Neo, TOYOBO, Osaka, Japan). The following primer sets were used for detecting Ddx3y: Forward primer 5’- ATGAGTCAGTGGAAGCAGGGG -3’ and Reverse primer 5’- TCAATTGCCCAACACGATCTGACG -3’. Prediction of domain structure

The predicted amino acid sequences of wild-type DDX3Y and DDX3X, or mutant DDX3Y and DDX3X were submitted to SMART web tool (http://smart.embl-heidelberg.de/).

Morphology analysis of spermatocytes

The spermatozoa collected from cauda epididymis of male mice were incubated in TYH medium [30] for 10 min and dispersed in PBS.

Morphology analysis of embryos

Ddx3x heterozygous females were mated with Ddx3x KO males. Mouse embryos were collected at embryonic day 10.5 (E10.5), with the visualization of the coital plug considered to be E0.5. Genotype of Ddx3x was verified by PCR and DNA sequencing. To determine the sex of embryos, primers amplifying Uba1 (ubiquitin-like modifier activating enzyme 1) (X chromosome) and Uba1y (ubiquitin-like modifier activating enzyme 1) (Y chromosome) were used. The size of the PCR product for Uba1 or Uba1y is 211-bp or 183-bp, respectively. The primers used were 5’- TGGTCTGGACCCAAACGCTGTCCACA -3’ and 5’- GGATAGCTAAGTGAGGGGCGG -3’.
DDX3Y is dispensable for spermatogenesis

Histological analysis of testis

After mating test, males were sacrificed by cervical dislocation following anesthesia. Testes were fixed in 4% paraformaldehyde in PBS and were processed for plastic sectioning using Technovit® 8100 (Mitsui chemicals, Tokyo, Japan) according to the manufacturer’s instruction. Briefly, fixed testes were washed in PBS at 4°C for an hour, dehydrated in acetone at 4°C for an hour, infiltrated with mixed solution of Technovit 8100 basic solution and hardener 1 (1.5 ml of basic solution plus 9 mg of hardener 1 per sample) at 4°C for 2–6 h, and then embedded after adding 50 µl of hardener 2. For analysis of Ddx3x KO mouse testes, 5 µm sections were treated with 1% periodic acid for 10 min, followed by treatment with Schiff’s reagent (Wako, Osaka, Japan) for 20 min. The sections were stained with Mayer’s hematoxylin prior to imaging, and observed under a microscope. For analysis of EGF-labeled spermatozoa in Ddx3x and Ddx3y dKO ES chimera, 5 µm sections were stained with 65 µM Hoechst 33342 (Life Technologies) in PBS for 5 min, and observed under a fluorescence microscope.

Statistical analysis

All values are shown as the mean ± SD of at least three independent experiments. Statistical analyses were performed using Student’s t-test for comparing the number of pups (litter size) derived from wild-type females mated with wild-type or Ddx3x KO males, and using Tukey HSD test for comparing litter size derived from Ddx3x heterozygous females mated with Ddx3x KO males.

Results

Generation of Ddx3y deficient mice

In order to generate Ddx3y KO mice, we performed genome editing by pronuclear injection of fertilized eggs using the CRISPR/Cas9 system. Mouse Ddx3y consists of 17 exons. We designed 2 single guide RNAs (sgRNAs) targeting the fourth exon of Ddx3y (#1 and #2) (Fig. 1A). We performed pronuclear injection of a combination of pX330s expressing human codon-optimized Cas9 and sgRNA #1 or #2. Mutations of Ddx3y in founder mice (F0) were confirmed by PCR and sequencing. From 94 injected eggs, 2 out of 24 offspring (8.3%), a male and a female, had a mutation in Ddx3x. The male had an in-frame mutation (12-bp deletion) in Ddx3x and no mutation in Ddx3y (X\(^{Ddx3x-em1}/Y\)). The female had a frame shift mutation (8-bp deletion) in one allele of Ddx3x (X\(^{Ddx3x-em1}/X^{WT}\)), and produced female offspring (F1) with the same mutant allele (Fig. 2B). Sequencing of the allele identified a premature stop codon before the DEXDe sequences (Fig. 2C and Supplementary Fig. 2A: online only), suggesting that only a non-functional protein without an RNA helicase domain can be translated. Therefore, X/Y\(^{Ddx3y-em2}\) males were considered KO mice.

Ddx3y deficient male mice have normal spermatozoa and sire offspring

There is a possibility that the founder Ddx3y KO mice (F0) generated by pronuclear injection were genotypically mosaic [31]. Thus, we used the male offspring (F1) for phenotypic analysis. To analyze spermatogenesis in Ddx3y KO mice (F1), the testis histology was observed by Hematoxylin-PAS staining. Ddx3y KO males showed no abnormality in spermatogenesis at 15 weeks of age (Fig. 1E left), suggesting that Ddx3y is not required for spermatogenesis or the maintenance of spermatogonial stem cells. We next collected spermatozoa from cauda epididymis of Ddx3y KO mice and found that the KO spermatozoa showed normal morphology (Fig. 1E right). Indeed, the litter size derived from Ddx3y KO males was comparable to that from WT males (Fig. 1F).

Ddx3x is required for development

Mouse Ddx3x also consists of 17 exons. We designed sgRNA #3 targeting the first exon of Ddx3x (Fig. 2A) and performed pronuclear injection of pX330s targeting Ddx3y (#2) and Ddx3x (#3) simultaneously. From 119 injected eggs, 2 out of 24 offspring (8.3%), a male and a female, had a mutation in Ddx3x. The male had a frame shift mutation (8-bp deletion) in one allele of Ddx3x (X\(^{Ddx3x-em1}/X^{WT}\)), and produced female offspring (F1) with the same mutant allele (Fig. 2B). Sequencing of the allele identified a premature stop codon before the DEXDe sequences (Fig. 2C and Supplementary Fig. 2A: online only), suggesting that only a non-functional protein without an RNA helicase domain can be translated. The F1 X\(^{Ddx3x-em1}/X^{WT}\) females were mated with X\(^{WT}\)/Y males, and dKO males (X\(^{Ddx3x-em1}/Y^{Ddx3y-em2}\)) were never obtained (Fig. 2D), suggesting the importance of Ddx3x in mouse embryonic development. To determine whether Ddx3x is important for male embryonic development, we analyzed embryos at E10.5 and found only one dKO male out of 34 embryos (2.9%), which was lower than the Mendelian ratio (25.0%), and the dKO male was also smaller than wild-type embryos (Fig. S2B and Supplementary Fig. 2C). These results are consistent with a recent report showing that Ddx3x KO males exhibit embryonic lethality because of abnormal embryogenesis and placental dysfunction [32].

Establishing Ddx3x and Ddx3y double KO ES cells using CRISPR/Cas9

In order to bypass the embryonic lethality of Ddx3x KO males, we utilized chimeric analysis (Fig. 3A). In 2016, we reported that chimeric mice derived from ES cells carrying a biallelic mutation in a lethal gene can overcome embryonic lethality due to the presence of wild-type cells [29]. This chimeric approach is a useful tool to analyze the function of lethal genes in spermatogenesis [29, 33]. We first transfected ES cells with pX330s, targeting Ddx3x (#3) and Ddx3y (#2) (Fig. 1A and 2A), into EGR-G01 ES cells [28]. The EGR-G01 ES cells were used as they ubiquitously express EGFP in the cytoplasm of all cell types and the acrosome of spermatozoa [28]. All ES cell clones (8/8) had mutations in both Ddx3x and Ddx3y. To generate chimeric mice, we used one ES cell line, which had frame
Fig. 1.

Fig. 2.
**Fig. 3.** Establishment of the Ddx3x and Ddx3y double KO ES cell clones using CRISPR/Cas9 system. (A) Schematic of chimeric male mice generation containing Ddx3x and Ddx3y double KO cells (EGFP positive). The image is adapted from ref. [29]. (B) (Upper region) Genomic sequence of wild-type and Ddx3x KO (X^{Ddx3x-em2}) alleles; 29-bp is deleted (dashed lines). (Lower region) Genomic sequences of wild-type and Ddx3y KO (Y^{Ddx3y-em3}) alleles; 125-bp is deleted (dashed lines). Orange box in Ddx3y-em3 allele indicates 1-bp (cytosine) insertion. Exons are in upper case letters and introns in lower case. (C) Male and female chimeric pups and ICR pups delivered from foster mothers. ES cell contribution is judged by coat color of mice: agouti (ES cells) and white (ICR). (D) Adult male chimera with more than 90% of coat color derived from ES cells. (E) Viability of male chimeras from various ES cell contribution rates. All 13 chimeras are viable after 4 weeks of age (wean) with a high contribution of ES cells.

**Fig. 1.** Ddx3y deficient male mice have normal spermatozoa and sire offspring. (A) Design of sgRNAs for generating Ddx3y KO mice. Red arrows indicate the location of sgRNAs. Black arrows indicate primers used for genotyping. White and gray boxes show untranslated region and coding sequences, respectively. (B) Genomic sequence of wild-type and Ddx3y KO (Y^{Ddx3y-em2}) alleles; 10-bp and 6-bp are deleted (dashed lines). (C) RT-PCR of full length transcripts of Ddx3y from wild-type and Ddx3y KO testis cDNA. Actb (actin beta) was used as control. (D) Predicted protein product of the wild-type and Ddx3y KO allele. Orange and pink boxes represent the DEXDc and HELICc RNA-helicase domains respectively. The gray region in Ddx3y KO indicates amino acid sequence differing from wild-type. (E) Testicular sections (Left) and sperm morphology (Right) of 15-week-old wild-type and Ddx3y KO mice. Testicular sections were stained with hematoxylin and PAS. Scale bar: 50 µm (Left). Sperm morphology of wild-type and Ddx3y KO. Scale bars: 20 µm (Right). (F) Average litter size derived from wild-type and Ddx3y KO males. Error bars represent standard deviation (SD).

**Fig. 2.** Ddx3x and Ddx3y double KO mice are embryonic lethal. (A) Design of the sgRNA for generating Ddx3x KO mice. Red arrows indicate the location of the sgRNA. Black arrows indicate primers for genotyping. White and gray boxes show untranslated region and coding sequences, respectively. (B) Genomic sequence of wild-type and Ddx3x KO (X^{Ddx3x-em1}) alleles; 8-bp is deleted (dashed lines). (C) Predicted protein product of the wild-type and Ddx3x KO allele. Orange and pink boxes represent the DEXDc and HELICc RNA-helicase domains respectively. The gray region in Ddx3x KO indicates amino acid sequence differing from wild-type. (D) Average litter size delivered from Ddx3x heterozygous mutant females mated with Ddx3y hemizygous mutant males. Error bars represent SD.
shift mutations in both Ddx3x (29-bp deletion) and Ddx3y (125-bp deletion and 1-bp insertion) (X\(^{WT}Y^{Ddx3y-em3}\)) (Fig. 3B). The obtained chimeric male mice were all viable (13/13) even though ES cell contribution rate, judged by coat color, was 90% or higher (Fig. 3C–E). We decided to use the mice showing high contribution rate (> 90%) for the analysis of spermatogenesis.

Ddx3x and Ddx3y double KO germ cells in chimeric males produce functional spermatozoa. (A) (Left) Testicular sections of Ddx3x and Ddx3y double KO ES chimeric males at postnatal day 10 (P10). Asterisks (*) indicate seminiferous tubules which contain ES cell contributed germ cells (EGFP positive). Scale bar: 50 µm. (Middle) Testicular sections of Ddx3x and Ddx3y double KO ES chimeric males at P36. Asterisks (*) indicate seminiferous tubules which contain ES cell contributed germ cells (EGFP positive). (Right) Magnified images of middle panel. White arrowhead indicates a Sertoli cell labeled with EGF. Scale bars: 50 µm. (B) Testicular sections of double KO ES chimeric males at 18 months of age. Asterisks (*) indicate seminiferous tubules which contain ES cell contributed germ cells (EGFP positive). Scale bar: 50 µm. (C) Sperm morphology of double KO ES chimeric males. White arrowheads indicate spermatozoa derived from ES cells (EGFP positive heads of spermatozoa). Scale bar: 10 µm. (D) Genotyping of F1 females (Left; X\(^{WT}Y^{Ddx3x-em2}\)) and males (Right; X\(^{WT}Y^{Ddx3y-em3}\)) derived from chimeras. Ddx3x KO (29-bp deletion) band is slightly smaller than the wild-type. Ddx3y KO (125-bp deletion and 1-bp insertion) band is slightly smaller than the wild-type.

To confirm the contribution of Ddx3x and Ddx3y dKO ES cells to the germ lineage, we generated chimeric mice with the dKO ES clone and examined testicular sections at postnatal day 10 (P10). Germ cells were labeled with EGFP, indicating the contribution of dKO ES cells to germ cells (Fig. 4A). At P36, spermatids, spermatozoa, and...
Sertoli cells were labeled with EGFP in some seminiferous tubules (Fig. 4A). Spermatозoa labeled with EGFP were also present in 18-month-old chimeric mouse testes (Fig. 4B), indicating that Ddx3x and Ddx3y is not cell autonomously required for spermatogenesis or maintenance of spermatogonial stem cells. No morphological abnormalities were observed in EGFP-labeled spermatозoa collected from cauda epididymis of 8-week-old chimeric mice (Fig. 4C). Finally, mating these chimeric male mice with wild-type female mice showed successful transmission of Ddx3x or Ddx3y mutant allele to the next generation of female or male mice, respectively (Fig. 4D).

**Discussion**

We investigated the function of mouse Ddx3y, one of three genes present in the AZFa region, and mouse Ddx3x, an X chromosomal gene homologous to Ddx3y, in spermatogenesis. It has been difficult to knock out Y chromosome related genes by conventional gene targeting with homologous recombination in ES cells because the Y chromosome is composed of a variety of repetitive DNA sequences [34, 35]. Recently developed genome editing techniques, such as TALEN and CRISPR/Cas9 systems enable us to target genes with specific 20–30 bp short sequences. Here, we succeeded in generating Ddx3y KO male mice by using CRISPR/Cas9 system. We found that Ddx3y KO male mice show no abnormality in spermatogenesis, produce normal morphology of spermatозoa, and sire offspring. We conclude that Ddx3y is dispensable for spermatogenesis at least in mice. In addition, because Ddx3x KO males were found to be embryonic lethal, we generated chimeric mouse, which contain Ddx3x and Ddx3y dKO germ cells, and found that these dKO germ cells can differentiate into spermatозoa and transmit their alleles to offspring. Because both Ddx3x and Ddx3y mRNAs are expressed in germ cells [36] and are predicted as non-secretory proteins lacking signal peptides, it is unlikely that wild-type germ or somatic cells present in chimeric mice compensate for the function of Ddx3x and Ddx3y in dKO germ cells. Thus, we conclude that the both Ddx3x and Ddx3y are not required for spermatogenesis at least in mice.

Recently it was reported that when human iPSC cell lines with AZFa deletions were xenotransplanted to mouse testes, spermatogenic failure of the derived germ cell-like cells were partially rescued by introduction of DDX3Y [37]. This suggests that DDX3Y is functional during spermatogenesis in human. Interestingly, in mice, Ddx3y has an autosomal paralog on chromosome 1, D1pas1, which is thought to be pseudogene in human [38, 39]. D1pas1 has 87% and 80% homology with Ddx3y at the amino acid and nucleotide sequence level respectively, respectively, and the mRNA expression is tissues specific [36]. Based on our finding, we hypothesize that D1pas1 has similar functions to Ddx3y, and thus can mask the phenotype of Ddx3y KO mice. Consistent with our hypothesis, it has been recently reported that spermatogenic failure is observed in D1pas1 KO mice, indicating the requirement of D1pas1 for spermatogenesis [40]. Because we showed no spermatogenic failure in Ddx3y KO mice, D1pas1 might overcome Ddx3y deficiency. However, D1pas1 mRNA is expressed in spermatocytes, but not in spermatogonia [36], and D1pas1 KO germ cells arrest in late pachytene spermatocytes [40], indicating that the spermatogenic failure in D1pas1 KO mice is milder than that in AZFa-deleted SCOS patients. These reports suggest that mouse D1pas1 alone is not sufficient to function as a substitute for human AZFa genes. Thus, it will be useful to conduct studies to determine whether Ddx3y and D1pas1 compensate for each other in mice by generating Ddx3y and D1pas1 dKO mice.

**Acknowledgments**

We thank Dr Julio M. Castaneda for critical reading of the manuscript; the member of both Department of Experimental Genome Research and NPO for Biotechnology Research and Development for experimental assistance. This research was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT)/Japan Society for the Promotion of Science (JSPS) KAKENHI Grants (JP17J09669 to TM, JP25112007 and JP17H13194 to MI), AMED under Grant Number JP18fk0210006h0003 and JP18gm5010001h0002 to MI, Takeda Science Foundation Grants to MI, NIH grant P01HD087157 and R01HD088412 to MI, and The Bill & Melinda Gates Foundation (Grand Challenges Explorations grant OPP1160866) to MI.

**References**

1. Kumar N, Singh AK. Trends of male factor infertility, an important cause of infertility: A review of literature. J Hum Reprod Sci 2015; 8: 191–196. [Medline] [CrossRef]
2. Kumar R. Medical management of non-obstructive azoospermia. Clinics (Sao Paulo) 2013; 68(Suppl 1): 75–79. [Medline] [CrossRef]
3. Palermo G, Joris H, Devroye P, Van Steirteghem AC. Precocious after intratubal injection of single spermatозoa into an oocyte. Lancet 1992; 340: 17–18. [Medline] [CrossRef]
4. Tesarik J, Mendoza C. Spermatid injection into human oocytes. I. Laboratory techniques and special features of extracytoplasmic injection of single spermatозoa into an oocyte. Hum Reprod 1996; 11: 772–779. [Medline] [CrossRef]
5. Fraiella R, Zylberstein DS, Estes SC. Hypogonadotrophic hypogonadism revisited. Clinics (Sao Paulo) 2013; 68(Suppl 1): 81–88. [Medline] [CrossRef]
6. Tietpolo L, Zaffardi O. Localization of factors controlling spermatogenesis in the non-luminal portion of the human Y chromosome long arm. Hum Genet 1976; 34: 119–124. [Medline] [CrossRef]
7. Vogt PH, Edelmann A, Kirch S, Henegarui O, Hirschmann P, Kiesewetter F, Kühn FM, Schil WB, Farah S, Ramos C, Hartmann M, Hartschuh W, Méschede D, Behre HM, Castel A, Nieschlag E, Weidner W, Gröne JJ, Jung A, Engel W, Haidl G. Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. Hum Mol Genet 1996; 5: 933–943. [Medline] [CrossRef]
8. Kamp C, Huetten K, Fernandez S, Sousa M, Schlegel PN, Mielnik A, Kleinman S, Yavetz H, Krause W, Köpker W, Johannißen R, Schülke W, Weidner W, Barros A, Vogt PH. High deletion frequency of the complete AZFa sequence in men with Sertoli-cell-only syndrome. MHR: Basic science of reproductive medicine 2001; 7: 987–994. [Medline] [CrossRef]
9. Lahn BT, Page DC. Functional coherence of the human Y chromosome. Science 1997; 278: 675–680. [Medline] [CrossRef]
10. Jones MJ, Furlong RA, Burtkin H, Chalmers IJ, Brown GM, Khwaja O, Affara NA. The Drosophila developmental gene fat facets has a human homologue in Xp11.4 which escapes X-inactivation and has related sequences on Yq11. Hum Mol Genet 1996; 5: 1695–1701. [Medline] [CrossRef]
11. Brown GM, Furlong RA, Sargent CA, Erickson RP, Longepied G, Mitchell M, Jones MH, Hargreave TB, Cooke HJ, Affara NA. Characterisation of the coding sequence and fine mapping of the human DFFRY gene and comparative expression analysis and mapping to the Sxr6 interval of the mouse Y chromosome of the Dffy gene. Hum Mol Genet 1998; 7: 97–107. [Medline] [CrossRef]
12. Sargent CA, Boucher CA, Kirch S, Brown G, Weiss B, Trundley A, Burgsyne P, Saut N, Durand C, Levy N, Terriou P, Hargreave T, Cooke H, Mitchell M, Rappold GA, Affara NA. The critical region of overlap defining the AZFa male infertility interval on proximal Yq contains three transcribed sequences. J Med Genet 1999; 36: 670–677. [Medline] [CrossRef]
13. Mazeryat S, Saut N, Sargent CA, Grimmond S, Longepied G, Ehrmann IE, Ellis PS, Greenfield A, Affara NA, Mitchell MJ. The human Y chromosome interval necessary for...
spematogonial proliferation is gene dense with syntenic homology to the human AZFa region. Hum Mol Genet 1998; 7: 1713–1724. [Medline] [CrossRef]

14. Krausz C, Degiannocetti S, Nuti F, Morelli A, Fehfli F, Sansone M, Varriale G, Forti G. Natural transmission of USP9Y gene mutations: a new perspective on the role of AZFa genes in male fertility. Hum Mol Genet 2009; 10: 360–365. [Medline] [CrossRef]

15. Luddi A, Margiotta C, Gambaer L, Serafini F, Cioni M, De Leo V, Balestri P, Piomboni P. Stereogenesis in a man with complete deletion of USP9Y. N Engl J Med 2009; 360: 885–891. [Medline] [CrossRef]

16. Boch J, Scholze H, Schramm S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A, Bonas U. Breaking the code of DNA binding specificity of TAL-type III effectors. Science 2009; 326: 1509–1512. [Medline] [CrossRef]

17. Moscow MJ, Bogdanove AJ. A simple ciprofloxin DNA recognition by TAL effectors. Science 2009; 326: 1510. [Medline] [CrossRef]

18. Shpargel KB, Sengoku T, Yokoyama S, Magnussen T, UT, and UTY demonstrate histone demethylase-independent function in mouse embryonic development. PLoS Genet 2012; 8: e1002964. [Medline] [CrossRef]

19. O’Flynn O’Brien KL, Varghese AC, Agarwal A. The genetic causes of male factor infertility: a review. Fertil Steril 2010; 93: 1–12. [Medline] [CrossRef]

20. Foresta C, Ferlin A, Moro E. Deletion and expression analysis of AZFa genes on the human Y chromosome revealed a major role for DBY in male infertility. Hum Mol Genet 2009; 15: 1161–1169. [Medline] [CrossRef]

21. Dittos HJ, Zimmer J, Kamp C, Hajbert-De Meyts E, Vogt PH. The AZFa gene DBY (DDX3Y) is widely transcribed but the protein is limited to the male germ cells by translation control. Hum Mol Genet 2004; 13: 2333–2341. [Medline] [CrossRef]

22. Sekiguchi T, Iida H, Fukumura J, Niimato H. Human DDX3Y, the Y-encoded isoform of RNA helicase DDX3, rescues a hamster temperature-sensitive ET24 mutant cell line with a DDX3X mutation. Exp Cell Res 2004; 306: 215–222. [Medline] [CrossRef]

23. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. Science 2013; 339: 819–823. [Medline] [CrossRef]

24. Wang H, Yang H, Shvaila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R. One-step generation of mice carrying multiple mutations in single genes by CRISPR/Cas-mediated genome engineering. Cell 2013; 155: 910–918. [Medline] [CrossRef]

25. Mashiko D, Fujihara Y, Sato M, Miyata H, Isotani A, Ikawa M. Generation of mutant mice by pronuclear injection of circular plasmid expressing Cas9 and single guided RNA. Sci Rep 2013; 3: 3355. [Medline] [CrossRef]

26. Abbasi F, Miyata H, Ikawa M, Revolutionizing male fertility factor research in mice by using the genome editing tool CRISPR/Cas9. Reprod Med Biol 2017; 17: 3–10. [Medline] [CrossRef]

27. Ho Y, Wigglesworth K, Eppig JJ, Schultz RM. Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. Mol Reprod Dev 1995; 41: 232–238. [Medline] [CrossRef]

28. Fujihara Y, Kaseda K, Inoue N, Ikawa M, Okabe M. Production of mouse pups from germline transmission-failed knockout chimeras. Transgenic Res 2013; 22: 195–200. [Medline] [CrossRef]

29. Oji A, Noda T, Fujihara Y, Miyata H, Kim YJ, Muto M, Nocaua K, Matsumura T, Isotani A, Ikawa M. CRISPR/Cas9-mediated genome editing in ES cells and its application for chimeric analysis in mice. Sci Rep 2016; 6: 31666. [Medline] [CrossRef]

30. Toyoda Y, Yokoyama M, Hoshi T, Studies on fertilization of mouse eggs in vitro. Jpn J Anim Reiprod 1971; 16: 152–157. [CrossRef]

31. Yen ST, Zhang M, Deng JM, Usman S, Smith CN, Parker-Thornburg J, Swionot PG, Martin JF, Behringer RR. Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. Dev Biol 2014; 393: 3–9. [Medline] [CrossRef]

32. Chen CY, Chan CH, Chen CM, Tsai VS, Tsai YJ, Wu Lee YH, You LR. Targeted inactivation of murine Ddx3x: essential roles of Ddx3x in placentation and embryogenesis. Hum Mol Genet 2016; 25: 2905–2922. [Medline] [CrossRef]

33. Oura S, Miyata H, Noda T, Shimada K, Matsumura T, Morohoshi A, Isotani A, Ikawa M. Chimeric analysis with newly established EGFP/DsRed2-tagged ES cells identify HYDIN as essential for spermiogenesis in mice. Exp Anim 2018 (in press). [Medline] [CrossRef]

34. Reijo R, Lee TY, Sato P, Alagappan R, Brown LG, Rosenberg M, Rozen S, Jaffe T, Strauss D, Hovatta O, et al. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. Nat Genet 1995; 10: 383–393. [Medline] [CrossRef]

35. Quintanana-Marei L, Fellous M. The human Y chromosome: the biological role of a "functional wasteland". J Biomed Biotechnol 2001; 1: 18–24. [Medline] [CrossRef]

36. Yong QP, Li Y, Lau YF, Dym M, Khenert OM, Chan WY. Structural characterization and expression studies of Dby and its homologs in the mouse. J Androl 2006; 27: 653–661. [Medline] [CrossRef]

37. Ramathai C, Angulo B, Sukhwani M, Cui J, Durruthy-Durruthy J, Fang F, Schanes P, Turek PJ, Orwig KE, Reijo Pera R. DDX3Y gene rescue of a Y chromosome AZFa deletion restores germ cell formation and transcriptional programs. Sci Rep 2015; 5: 15041. [Medline] [CrossRef]

38. Session BR, Lee GS, Wolgemuth DJ. Characterization of D1Pas1, a mouse autosomal homologue of the human AZFa region DBY, as a nuclear protein in spermatogenic cells. Fertil Steril 2001; 76: 804–811. [Medline] [CrossRef]

39. Kim YS, Lee SG, Park SH, Song K. Gene structure of the human DDX3 and chromosome mapping of its related sequences. Mol Cells 2001; 12: 209–214. [Medline] [CrossRef]

40. Inoue H, Ogouski N, Hirose M, Hatanaka Y, Matoba S, Chuma S, Kobayashi K, Wakana S, Noguchi J, Isotani K, Tanemura K, Ogura A. Mouse D1Pas1, a DEAD-box RNA helicase, is required for the completion of first meiotic prophase in male germ cells. Biochem Biophys Res Commun 2016; 478: 592–598. [Medline] [CrossRef]