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Mice Deficient in the Axonemal Protein Tektin-t Exhibit Male Infertility and Immotile-Cilium Syndrome Due to Impaired Inner Arm Dynein Function

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The haploid germ cell-specific Tektin-t protein is a member of the Tektin family of proteins that form filaments in flagellar, ciliary, and axonemal microtubules. To investigate the physiological role of Tektin-t, we generated mice with a mutation in the *tektin-t* gene. The homozygous mutant males were infertile, while the females were fully fertile. Sperm morphology and function were abnormal, with frequent bending of the sperm flagella and marked defects in motility. In vitro fertilization assays showed that the defective spermatozoa were able to fertilize eggs. Electron microscopic examination showed that the dynein inner arm structure was disrupted in the sperm flagella of *tektin-t*-deficient mice. Furthermore, homozygous mutant mice had functionally defective tracheal cilia, as evidenced by altered dynein arm morphology. These results indicate that Tektin-t participates in dynein inner arm formation or attachment and that the loss of Tektin-t results in impaired motility of both flagella and cilia. Therefore, the *tektin-t* gene is one of the causal genes for immotile-cilium syndrome/primary ciliary dyskinesia.

Tektins A, B, and C are stable protofilament components of biochemically fractionated sea urchin sperm flagella (10, 11, 22). Tektins are found in the axonemes of flagella, which are composed of heterodimers of tubulins α and β (31), dynein (5, 17), and other components (24). Tektins are the constitutive proteins of microtubules in cilia, flagella, basal bodies, and centrioles (9, 12, 21, 28) and are thought to contribute to the stability and structural complexity of axonemal microtubules. A biochemical analysis of tektins has shown that tektins A and B form heterodimeric protofilaments, while tektin C assembles onto the periphery of these core protofilaments or forms a separate homodimeric tektin filament (26). Tektins may act as templates and rulers for generating the three-dimensional organization of the axoneme (2, 13, 14, 23).

Recently, tektins were identified in various mammals, including mice (7, 21) and humans (8, 33, 34), as well as in *Drosophila melanogaster* (4) and silkworms (25). Tektin 1 is expressed during embryogenesis exclusively in the adult testis (21, 34). Tektin-t is expressed in the sperm tail (7, 8, 33). These tektins are conserved in animals and are thought to play an important role in the formation and movement of flagella and cilia (20, 22, 26).

To investigate the physiological role of Tektin-t, we studied *tektin-t* mutant mice generated by Lexicon Genetics Incorporated, Woodlands, Tex., from embryonic stem (ES) cells that corresponded to OST12401 (OmniBank sequence tag) and were targeted by gene trapping. Tektin-t-deficient mice are viable, and females are fertile but males are infertile. Defects in sperm and tracheal cilium motility result from defective dynein function.

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MATERIALS AND METHODS

Targeting construct and generation of mutant mice. The *tektin-t* mutant mice were generated by Lexicon Genetics Incorporated from ES cells that corresponded to OST12401 (OmniBank sequence tag) and that were targeted by gene trapping. The gene trap vector contained a retroviral 5′-end long terminal repeat (LTR), a splice acceptor sequence, a β-galactosidase-neomycin phosphotransferase fusion gene (BetaGeo), and the puromycin *N*-acetyltransferase gene (*pac*), which was regulated by the 3-phosphoglycerate kinase 1 (PGK-1) gene promoter, a splice donor sequence, and a 3′-LTR. Retroviral infection, selection, and screening of the ES cells were performed as previously described (36, 37). The gene trapping vector was inserted at the ninth intron of the *tektin-t* gene in ES cells (corresponding to OST12401), as detected by direct sequencing of the products from a 3′ rapid amplification of cDNA ends analysis. ES cells were selected for blastocyst injection into C57BL/6 mice to produce chimeric mice. Chimeric males with a high frequency of agouti coat color were mated with C57BL/6 females for germ line transmission of the targeted allele. Heterozygous F_1 males were then crossed with C57BL/6 females to obtain heterozygous F_2 animals, which were then bred to homozygosity, but siblings were never used as mating pairs. Homozygous animals were analyzed along with littermate control animals.

Southern blot and PCR analyses. Genomic DNAs were extracted from mouse tails by standard protocols (27). Southern blot analyses were used to determine the site of integration of the gene trap sequence in the *tektin-t* gene locus and to genotype the mice. A 1.6-kb probe for genomic Southern blotting was generated by PCR amplification from mouse genomic DNA. Genomic DNA samples (10 μg) were digested with BamHI and electrophoresed in 1.0% agarose gels. Southern blot hybridization was performed according to standard protocols (27). Mice were genotyped by PCRs using two sets of primers. Primers 5′-CCTCAGAAGAACTCGTCAAGAAG-3′ and 5′-ATCTGGACGAAGAGCATCAGGGG-3′ were used to amplify the BetaGeo gene, and primers 5′-ACACAGTATGGCCTCATTGATGAAGT-3′ and 5′-CCTCCCACCAATCTTGTGTCTTCCC-3′ were used for the *tektin-t* gene.

Northern and Western blot analyses. Total RNAs were extracted from mouse testes by the use of RNAzol B (Invitrogen, Valencia, Calif.) according to the manufacturer's recommendations. Ten-microgram samples of RNA were size fractionated by electrophoresis in a 1% agarose gel containing formaldehyde and then were transferred to a nylon membrane. The membrane was hybridized with a ^{32}P-labeled *tektin-t* cDNA fragment (7) and then rehybridized with glyceraldehyde-3-phosphate dehydrogenase to ensure equal loading.

Protein samples from various organs of each genotype of adult mice were lysed in RIPA buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride), centrifuged, and quantified by the Bradford protein assay (Nacalai Tesque, Kyoto, Japan). Mouse sperm were taken from the epididymis. Approximately 50 μg of protein per lane was separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride filter (Millipore, Bedford, Mass.). The filters were blocked with 5% skim milk in TBS (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 50 mM KCl) and incubated with an anti-Tektin-t antibody (8) diluted in TBS (1:300). The filters were washed with TBS containing 0.2% Tween 20 and then treated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Amersham Biosciences, Tokyo, Japan). After extensive washes, the filters were developed by use of a POD staining kit (Wako, Osaka, Japan).

Fertility test. For an investigation of the fertility of the *tektin-t*-deficient males, 10 sexually mature males from each genotype were mated, each with 2 females, for 3 months. The females were checked for the presence of vaginal plugs and pregnancy. Pregnant females were removed to holding cages to give birth. The numbers and sizes of litters sired by each group of males in a 3-month mating period were determined. In addition, 10 females of each genotype were caged with C57BL/6 male mice for mating, and the number of pups in each cage was counted within a week of birth.

Sperm counting. Cauda epididymal sperm were counted in a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) by the use of HTM-IVOS, v. 10, software (Hamilton Thorne Research, Beverly, Mass.). Six hours after mating, the uteri and oviducts were transferred to 500 and 50 μl of TYH medium (32), respectively, on plastic petri dishes and then cut into small fragments with a blade. The medium recovered from the uterus was transferred to a blood corpuscle-counting chamber and sperm were counted under a light microscope. All sperm in the medium recovered from the oviducts were counted under a light microscope.

In vitro fertilization (IVF) assays. Female C57BL/6 mice were superovulated by intraperitoneal injections of 5 IU of pregnant mare serum gonadotropin followed by an injection of 5 IU of human chorionic gonadotropin after 48 h. The eggs were recovered 24 h after human chorionic gonadotropin injection, and oocytes with cumulus cells were collected. Spermatozoa were isolated from the cauda epididymis of each male, capacitated in TYH medium at 37°C for 1 h, added to intact oocytes, and incubated for 6 h at 37°C in 5% CO_2 covered with mineral oil. After 24 h, the fertilized eggs were counted and transplanted to the uteri of pseudopregnant females.

Motility assays of sperm. Sperm motility was examined by computer-assisted sperm analysis (CASA). At the end of a 2-h incubation in TYH medium in the presence or absence of 1.7 mM Ca^{2+}, 10-μl aliquots of each sample were transferred to the Makler Counting Chamber (Sefi Medical Instruments) by use of a pipette to analyze the sperm movement with HTM-IVOS, v. 10, software (Hamilton Thorne Research). At least 200 sperm were counted, and the following parameters were evaluated: percentage of progressively motile spermatozoa, straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement, and linearity (18). The sperm movement analysis was performed by a standard CASA method with the following manufacturer-recommended operating parameters: temperature, 37°C; negative phase-contrast optics; frames acquired, 30; frame rate, 60 Hz; minimum contrast, 60; minimum cell size, 3 pixels; minimum static contrast, 30; low average path velocity (VAP) cutoff, 5.0 μm/s; static head size, 0.32 to 2.99; static head intensity, 0.42 to 1.60; and magnification, ×1.95. During the analysis, the playback feature was used to delete spermatozoa that had obviously mistracked due to collision. Tracks of fewer than 16 points were eliminated.

Ciliary function assay by exclusion of carbon particles. To examine tracheal ciliary function, we measured the migration of India ink particles in the mouse trachea. Three male and three female homozygous mutant mice and five female and eight male wild-type mice were examined. After each mouse was killed by exsanguination, the chest was opened at the throat (hyaline cartilage) to locate the bifurcation of the trachea. Fifteen minutes after the injection of 2 μl of India ink at the tracheal bifurcation, the trachea was cut and opened longitudinally with scissors from the throat to the bifurcation. The movement of carbon particles from the bottom (tracheal bifurcation) to the top of the trachea was scored relative to the numbering of the tracheal cartilage rings from the bifurcation (no. 1) to the hyaline cartilage (no. 13).

Electron microscopy. Testes and epididymides were fixed with 5% glutaraldehyde in 0.2 M phosphate buffer, postfixed with 2% osmium tetroxide, and embedded in epoxy (Epon) resin. Selected areas were thin sectioned and examined by electron microscopy.

RESULTS

Generation and analysis of *tektin-t* mutant mice. To investigate the physiological role of Tektin-t, we studied *tektin-t* mutant mice generated by Lexicon Genetics Incorporated from ES cells that corresponded to OST12401 (OmniBank sequence tag) and were targeted by gene trapping. Construction of the trapping vector (Fig. 1A) and the production and detection of mutant mice by Southern blot analysis and PCR are shown in Fig. 1. A Northern blot analysis did not detect *tektin-t* mRNA in homozygous mutant testes (Fig. 1D). The anti-Tektin-t antiserum recognized the 46-kDa Tektin-t protein in the testes of normal adult mice but did not detect any positive signals for Tektin-t protein in *tektin-t* homozygous mutant testes (Fig. 1E).

The proportions of wild-type, heterozygous, and homozygous offspring were not significantly different from the expected Mendelian pattern of inheritance. The weights and growth rates of the newborn pups and the weights of the testes and seminal vesicles in adulthood were not significantly different for mice with a mutation in the *tektin-t* gene than for wild-type mice. These results indicate that *tektin-t* mutant mice express neither the transcripts nor the translation products of *tektin-t*.

Infertility of *tektin-t* homozygous mutant males. Matings of *tektin-t*-deficient males with wild-type females produced no pregnancies after >3 months of continuous cohabitation, despite frequent observations of vaginal plugs in the females (Table 1). The *tektin-t* heterozygous mutant male mice were all fertile, producing as many offspring per pregnancy as their wild-type littermates. Furthermore, female homozygous *tektin-t* mutant mice showed normal reproduction capabilities in that they achieved the same number of pregnancies as wild-type control female mice during the observation period.

Histological analyses. Macroscopic observations of the testes did not show any significant differences among homozygous, heterozygous, and wild-type mice (data not shown). Observations by light microscopy revealed that >70% of the homozygous mutant sperm had abnormally bent flagella (Fig. 2A). Electron microscopy showed a partial deficiency or loss of the inner dynein arm structures in the flagellar axonemes of sperm from all four of the homozygous mutant mice tested and none of the four heterozygous mutants tested (Fig. 2B to E).

Sperm analysis of *tektin-t* mutant males. To elucidate the cause of male infertility, we examined the numbers of sperm that were recovered from the cauda epididymides and from the uteri and oviducts of females mated with mutant males. Although there was no difference in the levels of sperm recovery from the epididymides of *tektin-t* heterozygous or homozygous mutants, no sperm was found in the oviducts of females mated with homozygous mutant males (Table 2).

To examine sperm motility, we performed CASA after a 2-h incubation of the sperm in TYH medium. Homozygous *tektin-t* mutant mice showed reduced sperm motility and progressive

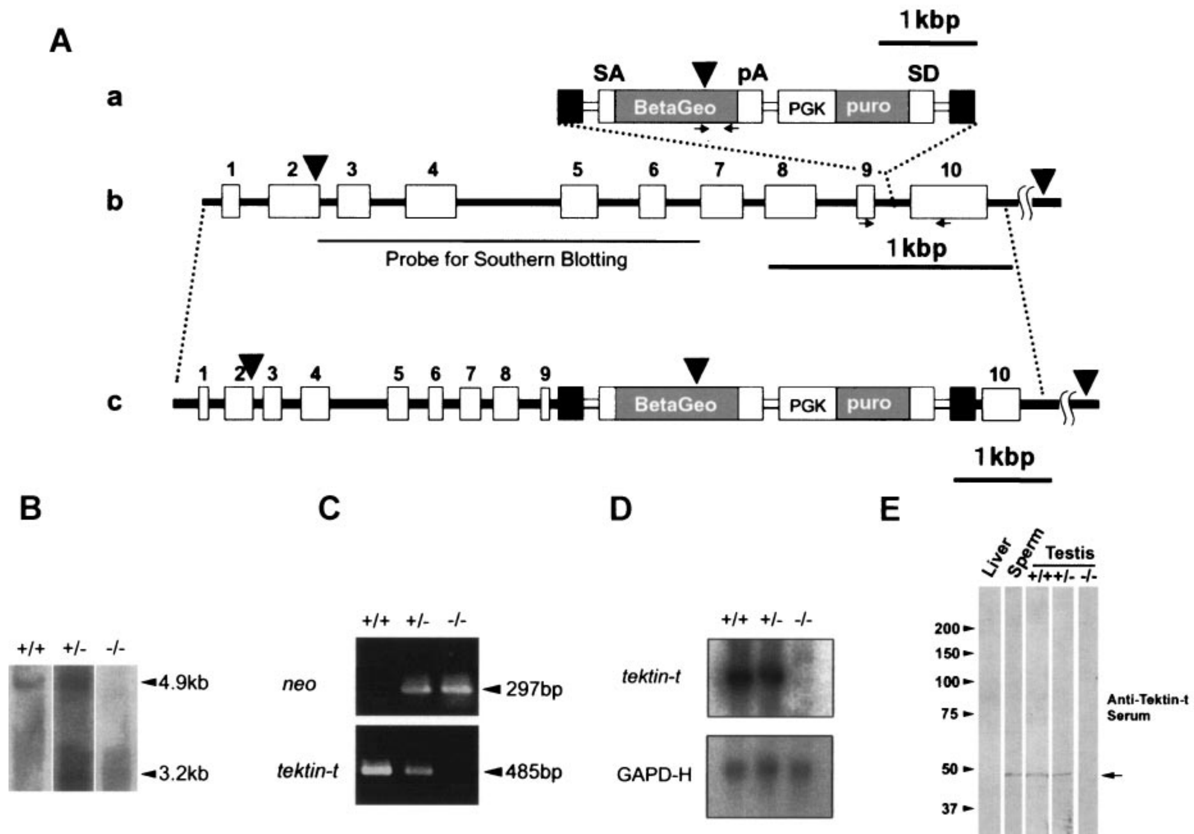

FIG. 1. Targeted mutagenesis of *tektin-t* gene. (A) Schematic representation of the strategy that was used for gene trapping. (a) Construction of gene-trapping vector. (b) Construction of *tektin-t* genomic locus. (c) Construction of mutated *tektin-t* genomic locus. The numbers indicate the exons. The gene trap construct contains a retroviral 5′-LTR (black box), a splice acceptor sequence (SA), the BetaGeo gene, and the puromycin *N*-acetyltransferase (*puro*) gene, which is regulated by the PGK-1 gene promoter, a splice donor sequence (SD), and a retroviral 3′-LTR (black box). Arrowheads indicate the BamHI restriction sites. Arrows indicate the PCR primers used for genotyping. (B) Southern blot analysis of BamHI-digested genomic DNAs from wild-type, heterozygous, and homozygous mice (arrowheads). The 1,647-bp genomic DNA fragment from introns 2 to 6 of the *tektin-t* gene was used as a probe for the Southern blot analysis. The endogenous and targeted alleles were recovered as 4.9- and 3.2-kb DNA fragments, respectively. (C) Genotyping by PCR. The wild-type allele yields a 485-bp amplicon that is absent from homozygous mutant mice. The 297-bp amplicon that represents the BetaGeo cassette was detected only in targeted alleles. (D) Northern blot analysis. Transcripts of the *tektin-t* gene were not detectable in the testes of *tektin-t* homozygous mice. The same filter was rehybridized with glyceraldehyde-3-phosphate dehydrogenase cDNA as a control. (E) Western blot analysis with anti-Tektin-t serum. The Tektin-t protein was not detected in the testicular lysates of homozygous mice.

movement compared to wild-type mice, and all of the CASA parameters, with the exception of beat cross frequency (BCF), were decreased ($P < 0.01$) (Fig. 3). CASA showed that the proportion of motile sperm in *tektin-t* mutant mice was reduced and that the sperm velocity, including the VAP, VSL, and VCL, was decreased. Nevertheless, the beat frequencies of the flagella were not changed in motile *tektin-t*-deficient sperm. These results indicate that the energy metabolism pathway is active in Tektin-t-deficient mice but that the molecular machinery for normal sperm movement does not function effectively. The loss of Tektin-t leads to a deficiency in flagellar construction and produces mice with immotile sperm.

We performed IVF experiments to examine whether sperm from the Tektin-t-deficient mice could fertilize eggs, and we found that homozygous mutant sperm had the ability to fertilize eggs which developed to adulthood. These results indicate that although the spermatozoa of *tektin-t* mutant mice are defective in sperm motility, and consequently unable to reach

TABLE 1. Fertility rates of mutant mice

Genotype	Male fertility (no. of fertile females/no. of vaginal plugs)	Litter size (avg no. of newborn pups)	Female fertility (no. of fertile females/no. of vaginal plugs)	Litter size (avg no. of newborn pups)
+/+	10/10	8.1	10/10	7.4
+/−	10/10	7.8	10/10	7.7
−/−	0/10	0	10/10	8.3

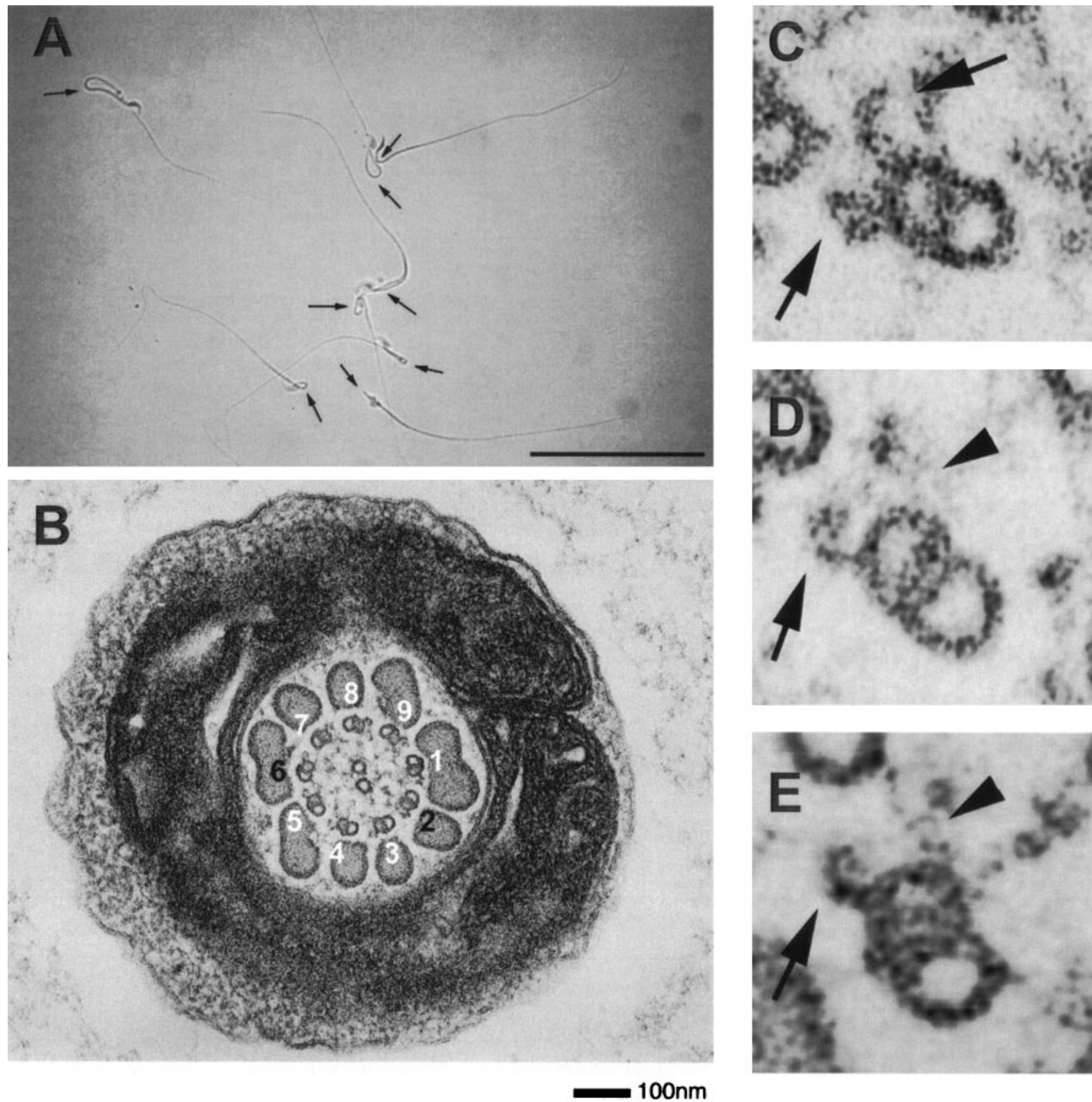

FIG. 2. Microscopic observations of homozygous *tektin-t* mutant sperm. (A) Bright field microscopy. Many of the homozygous sperm had bent tails (arrows). Bar = 100 μm. (B) Electron microscopic observation of the midpiece of the sperm tail. The numbers indicate the microtubule doublet positions. The inner dynein arms are lacking at positions 2 and 6. (C to E) Higher magnification pictures of microtubule doublets of the sperm tail. (C) Wild-type sperm. (D and E) Homozygous *tektin-t* mutant sperm. Arrows indicate inner dynein arms, and arrowheads indicate positions lacking inner dynein arms. Bar = 10 nm.

the oviducts in the female genital tract, they are able to fertilize eggs under IVF conditions involving the insemination of oocytes in TYH medium (Table 2).

These results indicate that defects in the dynein arms of the flagellar axonemal microtubules (Fig. 2) are the main cause of the abnormal sperm motility (Fig. 3) and that the mutant sperm are unable to reach the oviducts (Table 2) and oocytes in the female genital tract.

TABLE 2. Number of sperm recovered from epididymides or from females mated with tektin heterozygous or homozygous male mice and results of IVF[a]

Genotype	No. of recovered sperm			IVF result (% of fertilized oocytes resulting in pups)
	Epididymis (10^6)	Uterus (10^6)	Oviduct	
+/−	13.1 ± 3.4	0.7 ± 0.2	110 ± 50	90 ± 1.7
−/−	10.7 ± 8.0	0.7 ± 0.2	0	48 ± 16.0

[a] Results are means ± standard deviations.

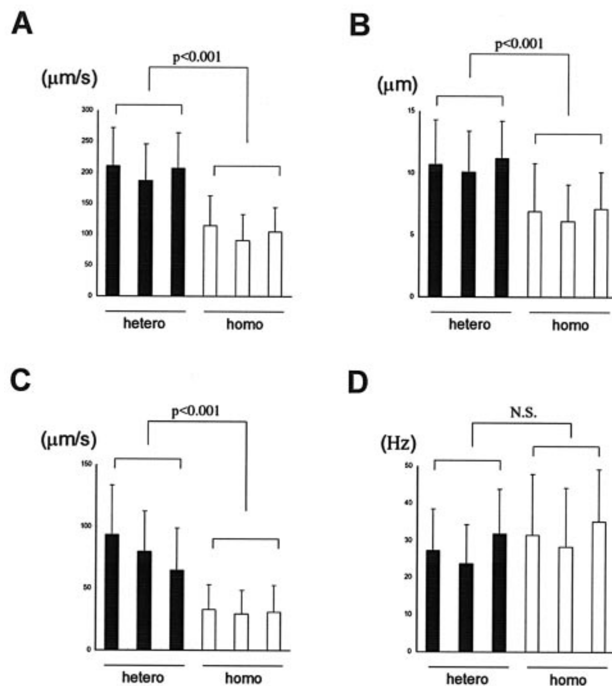

FIG. 3. Computer-assisted analysis of sperm motility. The results of analyses of heterozygous (black bars) and homozygous (white bars) sperm are shown. Sperm movement was assessed by CASA with cells that were incubated in medium for 2 h. The following CASA parameters were recorded and found to be representative: VCL (A), amplitude of lateral head displacement (B), VSL (C), and BCF (in hertz) (D). The standard deviations of the means were calculated with the Mann-Whitney U test. The *tektin-t* homozygous mutant sperm showed significant reductions in all parameters, except for BCF.

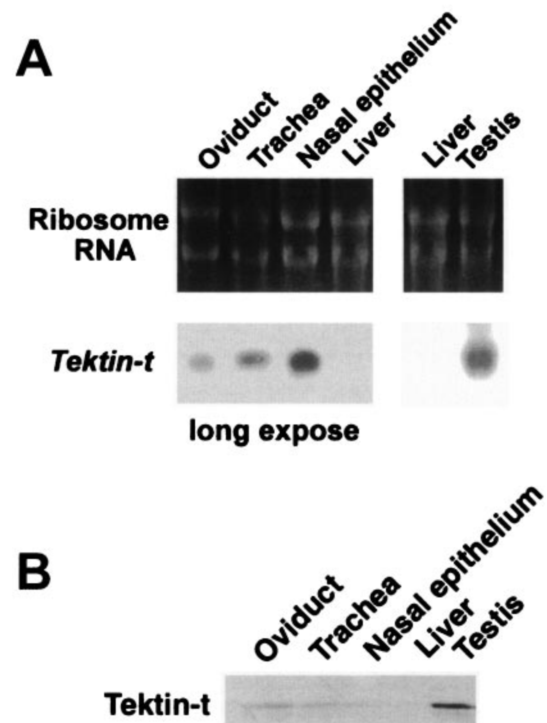

FIG. 4. Northern (A) and Western (B) blot analyses of Tektin-t in various organs containing ciliated epithelia in the wild-type mouse. Positive signals were evident in the oviduct, trachea, and nasal epithelium in addition to the testis.

Ciliary analyses of *tektin-t* mutant mice. We used Northern and Western blotting to examine whether the Tektin-t protein was expressed on ciliated cells other than sperm. Tektin-t was detected in the tracheas of male mice (data not shown) and in the tracheas and oviducts of female wild-type mice (Fig. 4). Electron microscopy showed that the ciliary morphology was abnormal in *tektin-t*-deficient mice (Fig. 5A) compared with wild-type mice. The functional activity of the cilia of *tektin-t* mutant mice showed severe defects in terms of carbon particle exclusion from the bottom of the trachea (Fig. 5B). There were no significant differences between each male and female in this experiment (data not shown). These results indicate that a deficiency of the *tektin-t* gene is a causative factor in immotile-cilium syndrome/primary ciliary dyskinesia (ICS/PCD).

DISCUSSION

Spermatozoa use flagellar movement to reach oocytes in the female genital tract. The core component of both cilia and flagella is the axoneme, and its structure is conserved in all eukaryotic organisms. The most prominent components of the axoneme are the central pair of microtubules and nine outer doublet microtubules. Each doublet microtubule is composed of an A and a B tubule, which are composed of 13 and 10 or 11 protofilaments, respectively (20). These filaments are made of tubulins and are linked to outer and inner dynein arms, radial spokes, and nexin links. At least one of the protofilaments in flagellar microtubules is composed of tektins (20). Tektins were originally isolated from sea urchin sperm as a set of proteins, namely, tektins A, B, and C (10, 22). Recently, Tektin-t and Tektin 1 from mice and humans were cloned (7, 8, 21, 33, 34). Computer-assisted analysis of the mouse genomic database (NCBI accession number AK016178) showed that Tektin 3 exists in mice and humans (EMBL/DDBJ/GenBank accession number NM_031898) and that both species express the gene in the testis. Analyses of these *tektin* genes showed that tektin proteins have fundamental roles in sperm flagella and are conserved from sea urchins to mammals.

To determine the physiological role of Tektin-t in mammals, we generated mice that were homozygous for an inactivating mutation of the *tektin-t* gene. The insertion point of the gene-trapping vector was in the ninth intron of the *tektin-t* gene in ES cells. We did not find any expression of truncated transcripts of the *tektin-t* gene from the endogenous promoter. An anti-Tektin-t antiserum recognized an immunoreactive band corresponding to the Tektin-t homomeric molecule (46 kDa). These results indicate that the gene trap mutagenesis resulted in nonfunctional *tektin-t* genes due to the instability of truncated transcripts, as suggested by the manufacturer (Lexicon Genetics Incorporated).

Microtubules in flagella are constructed of A and B tubules. Nojima et al. (20) showed that one protofilament in the A tubule is composed of tektins and that the tektin protofilament is near the binding sites for radial spokes, inner dynein arms, and nexin links. In contrast, we did not find any abnormal construction in flagellar microtubules by electron microscopy.

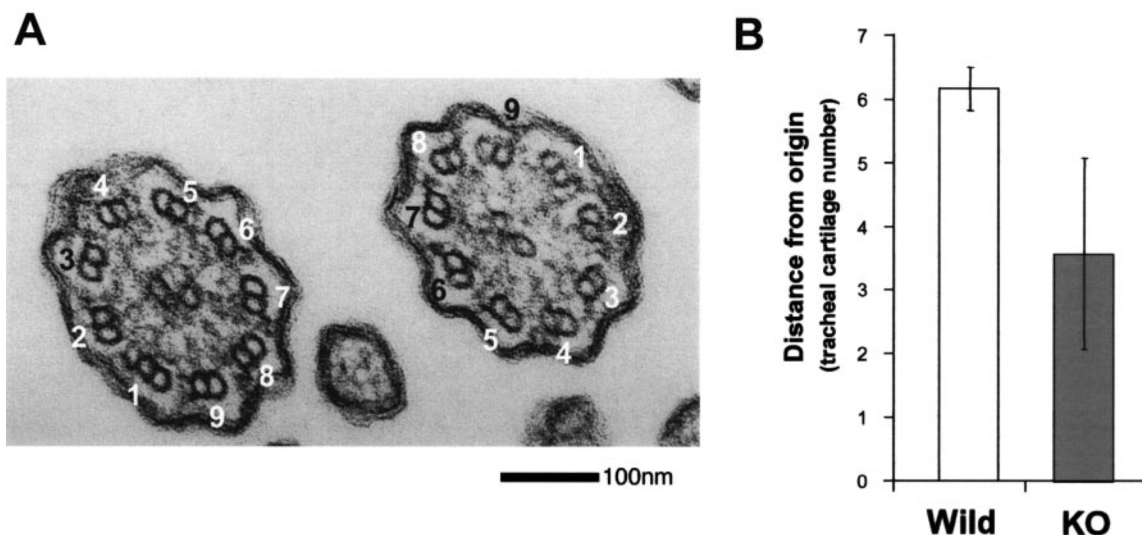

FIG. 5. Analyses of tracheas of *tektin-t* mutant mice. (A) Cross sections of tracheal cilia. The inner dynein arms are lacking at position 3 (left) and at positions 6, 7, and 9 (right). (B) Function of the ciliated epithelium of the trachea in excluding exogenous particles. Data on the exclusion of carbon particles due to ciliary motility in the trachea are shown as means ± standard deviations for 13 wild-type and 6 homozygous *tektin-t* (KO) mice.

The loss of Tektin-t may be partly compensated for by other tektin proteins in order to form tektin protofilaments, which are incorporated into microtubules. Microscopic observations showed that sperm flagella are frequently bent or curved in homozygous *tektin-t* mutant mice. An electron microscopic analysis of *tektin-t* mutant sperm showed that just the inner dynein arm structures were partially or fully lost. Tektin protofilaments without Tektin-t might distort the primary construction of the A tubule in the microtubule structure. An inappropriate organization of the microtubule and dynein might affect the construction of the inner dynein arm. These arguments are compatible with biochemical fractionation, which suggested a tektin-ribbon-inner arm dynein association (15, 29, 30). When this work was submitted, it was reported that a tektin homologue in *Chlamydomonas* was diminished in mutant axonemes that lack inner arm dyneins (35). The specific loss of the inner dynein arm without any other prominent morphological abnormalities in flagellar axonemes suggests that Tektin-t is a dynein arm constituent, or more likely, that it plays some key role in the assembly or attachment of the inner arm to microtubules.

CASA showed that the ratio of motile sperm from *tektin-t* homozygous mutant mice to those from wild-type mice was reduced and that the velocities (VAP, VSL, and VCL) were decreased, although the beat frequencies of the flagella were unchanged in motile *tektin-t*-deficient sperm. The loss of Tektin-t led to a deficiency in flagellar construction and should occasionally induce a serious phenotype, immotile sperm. These results indicate that the energy metabolism pathway was active but that the molecular machinery for normal sperm movement was not effective.

The male infertility associated with the chronic respiratory disease Kartagener syndrome has been attributed to a genetic deficiency in the construction of the dynein structures in the axonemes of sperm and respiratory cilia (1, 16). These patients suffer from ICS, which was recently renamed PCD. Although they exhibit deficiencies in sperm movement, some of these patients have partial or residual sperm motility (3). The immotile sperm of PCD/ICS patients are morphologically normal, although the flagella appear to be stiff under light microscopy (3). Ultrastructural investigations have revealed either that both dynein arms are lacking in the peripheral doublets or that just one of the dynein arms is missing, and there are no structural defects in the microtubules, radial spokes, axonemes, or fibrous sheaths. The phenotype of *tektin-t* mutant sperm is similar to that of ICS/PCD sperm.

We used Northern and Western blotting to examine whether the Tektin-t protein was expressed by ciliated cells other than sperm. Tektin-t was detected in the tracheas and oviducts of wild-type mice, and *tektin-t*-deficient mice had abnormal ciliary morphology by electron microscopy. The ciliary activity in *tektin-t* mutant mice was severely defective in terms of carbon particle exclusion from the bottom of the trachea. Female homozygous *tektin-t* mutant mice showed normal reproduction capabilities. The results may imply that coordinated ciliary movements are not necessary in the oviduct for pregnancy to occur, assuming that homozygous mutant females lack *tektin-t* and dynein inner arms. Although these results imply that the deficiency of the *tektin-t* gene is a causative factor in ICS/PCD, note that the phenotypic changes in ICS/PCD patients are complex and that this disease may be linked to mutations other than those in the *tektin-t* and dynein-encoding genes (6, 19).

Our results indicate that Tektin-t is essential for dynein arm integrity in sperm flagella and that a Tektin-t deficiency causes male infertility by debilitating sperm motility. Furthermore, the *tektin-t* gene appears to be one of the causal genes for ICS/PCD.

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