Duplicated insertion mutation in the microtubule-associated protein Spag5 (astrin/MAP126) and defective proliferation of immature Sertoli cells in rat hypogonadic (*hgn/hgn*) testes

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Abstract

Male rats with hypogonadism (*hgn/hgn*) experience sterility from testicular dysplasia, which is controlled by a single recessive gene, *hgn*. The postnatal growth of the seminiferous tubules was severely affected. In this study, we localized the *hgn* locus to a 320 kb region on rat chromosome 10 and detected the insertion of a 25 bp duplication into the sixth exon of the sperm-associated antigen 5 (*Spag5*/*astrin*/*MAP126*) gene, which codes for a microtubule-associated protein. This mutation results in a truncated *Spag5* protein lacking the primary spindle-targeting domain at the C terminus. Immunological staining with antibodies to markers for Sertoli and germ cells during the early postnatal period indicated that the abnormal mitosis with dispersed chromosomes in *hgn/hgn* testes occurs in proliferating Sertoli cells. Therefore, apoptotic Sertoli cell death would result from the disorganization of the spindle apparatus caused by defective *Spag5*. These findings suggested that the *Spag5* is essential for testis development in rats and that the *hgn/hgn* rat is a unique animal model for studying the function of *Spag5*.

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Introduction

Spermatogenesis is a central event in male reproduction. During this process, spermatogonia differentiate into functional spermatozoa, which carry the male genetic information into oocytes. The Sertoli cell is a highly specialized cell type responsible for spermatogenesis. The cytoplasm of Sertoli cells is extended into the lumen of seminiferous tubules in order to nurse germ cells at various stages of development. Since each Sertoli cell can support a limited number of germ cells, making the establishment of the normal complement of Sertoli cells during testicular development is important for ensuring a normal level of sperm production during adulthood (Sharpe *et al.* 2003). During the period from the late embryonic to the early postnatal stage, Sertoli cells proliferate rapidly and form a palisade-like distribution along the basement membrane of seminiferous tubules (Clermont & Perey 1957). Once Sertoli cells become mature, they lose their proliferative activity (Sharpe *et al.* 2003). Despite the importance of Sertoli cells in the establishment and maintenance of testicular function, the mechanism regulating the differentiation of Sertoli cells is not fully understood. Especially, the molecular events

surrounding the proliferation and differentiation of Sertoli cells during the perinatal period are not exactly known.

Male hypogonadic (*hgn/hgn*) rats are characterized by male sterility resulting from testicular dysplasia (Hakamata *et al.* 1988, Suzuki *et al.* 1988, 1993, 1998, 2004b), a short female reproductive life resulting from ovarian hypoplasia (Suzuki *et al.* 1992), progressive renal insufficiency resulting from renal hypoplasia (Suzuki & Suzuki 1995, 1998, Suzuki *et al.* 1991, 2005, 2006), and body growth retardation (Suzuki *et al.* 1992). All of these effects are controlled by a single recessive allele (*hgn*) located on chromosome 10 (Suzuki *et al.* 1999, 2004a). In adult male *hgn/hgn* rats, the plasma testosterone level is low, the gonadotropin levels are high, and testis weight is about 1% the weight of a normal testis (Hakamata *et al.* 1988). Histologically, some undifferentiated seminiferous cords remain in fibrous interstitial tissue and no spermatogenesis occurs in adult *hgn/hgn* testes (Suzuki *et al.* 1988). Moreover, the growth of seminiferous tubules is inhibited during the early postnatal period and Sertoli cells fail to make a line along the basement membrane because of their severely reduced number (Suzuki *et al.* 1998, 2004b). In the peritubular tissue of *hgn/hgn* testis, a single layer of immature myoid cells is found around the

tubules, mesenchymal cells form multiple layers, and the accumulation of extracellular matrix into basement membrane is defective (Suzuki *et al.* 1998, 2004b). Since in *hgn/hgn* testes, Sertoli cells often undergo apoptosis during their proliferative period and lose the expression of follicle-stimulating hormone receptor mRNA after postnatal day (PD) 12 (Suzuki *et al.* 2004b), their failure to proliferate and differentiate during the perinatal period may be the primary defect in male hypogonadism (Yagi *et al.* 2006).

To determine the candidate *hgn* gene, we constructed a fine linkage map around the *hgn* locus. Using RNA samples from neonatal testes, we performed RT-PCR experiments to identify candidate *hgn* genes. We found that a 25 bp duplication was present in the sixth exon of the gene encoding sperm-associated antigen 5 (*Spag5/astirin/MAP126*) of the *hgn/hgn* genome. The complete cDNA sequence of rat *Spag5* determined by RACE-PCR method indicates that *hgn* allele produce a truncated product lacking almost all the coiled-coil domain. *Spag5* is a microtubule-associated protein expected as rat orthologue for human *astirin/MAP126* (Fitzgerald *et al.* 2006), which is essential for cell mitosis progression (Gruber *et al.* 2002). Histologically, we observed apoptotic cell death and abnormal mitotic metaphases in the Sertoli cells of *hgn/hgn* testes during postnatal testicular development. These results suggest that the defect of functional *Spag5* causes male hypogonadism and that *Spag5* is essential for normal testis development in rats.

Materials and Methods

Linkage analysis

To construct a fine linkage map around *hgn* locus, we genotyped the backcross progeny with a newly generated polymorphic markers (Table 1) using previously obtained genomic DNA (Suzuki *et al.* 1999, 2004a). PCRs, separation of PCR products, and gel staining, and data analysis were done as previously described (Suzuki *et al.* 2004a).

RT-PCR analysis of candidate genes

The *hgn/hgn* females were selected from the *hgn*-inbred strain derived from hypogonadism strain (HGN) by the laparotomy for the detection of renal hypoplasia at weaning (Suzuki *et al.* 1991). The *+ /hgn* and *hgn/hgn* neonates were obtained by mating between the *hgn/hgn* females and the *+ /hgn* males. The *+ /+* neonates were derived from *hgn*-free dominant homo inbred strain derived from the HGN strain (HDH). Total RNA was extracted from neonatal testes. Each 40 µl RT reaction mixture containing 1 µg total RNA, 0.25 µM oligo (dT) primer, 1 mM of each dNTP, and 10 U RNase inhibitor was incubated at 65 °C for 5 min and cooled on ice.

After adding 5 U ReveTra Ace (Toyobo, Osaka, Japan), the samples were reverse transcribed by incubation at 42 °C for 1 h and heated at 95 °C for 5 min to inactivate the reverse transcriptase. Each PCR was performed in 20 µl of a reaction mixture containing 1 µl cDNA, 1 mM MgSO₄, 0.3 µM of each primer (Table 1), 0.2 mM of each dNTP, 1 U KOD-Plus-DNA polymerase (Toyobo), and the reaction buffer supplied by the manufacturer. The amplification protocol consisted of 35 cycles of denaturation for 15 s at 94 °C, annealing for 30 s at 55 °C, and extension for 60 s at 68 °C, performed in a Thermal Cycler PTC-200 (MJ Research, Waltham, MA, USA). Gel electrophoresis, staining, and imaging were performed as described previously (Suzuki *et al.* 2004a,b).

RACE-PCR of *Spag5*

To determine the predicted amino acid sequence of rat *Spag5*, we performed 5'- and 3'-RACE using Cap Fishing Full-length cDNA Premix Kit (Seegene, Inc., Seoul, Korea). After heating the RNA samples at 75 °C for 3 min, 5'- or 3'-enriched cDNAs were synthesized in 20 µl mixture containing 3 µg total RNA, 10 U Super Script II RNase H⁻ RTase (Invitrogen) and 0.5 µM random hexamers (for 5'-RACE) or 0.5 µM oligo (dT) primer (for 3'-RACE) by incubation at 42 °C for 1 h. Following addition of 3 µl CapFishing adaptor and 60 U RTase, the mixture was further incubated at 42 °C for 30 min. The samples were heated at 95 °C for 5 min to inactivate the RTase, and diluted to 200 µl with water. Each 5'-RACE-PCR was performed in 25 µl of a reaction mixture containing 2.5 µl diluted first-stranded cDNA, 12.5 µl SeeAmp Taq Plus Master Mix, 0.2 µM 5'-RACE primer, and 0.2 µM 3'-target primer. 3'-RACE-PCR was performed in similar reaction mixture containing 0.2 µM 3'-RACE primer and 0.2 µM 5'-target primer (Table 1). The amplification protocol consisted of 35 cycles of denaturation for 40 s at 94 °C, annealing for 40 s at 58 °C, and extension for 60 s at 72 °C. The PCR products were electrophoresed on agarose gels as described above.

PCR detection of *Spag5* mutation in genomic DNA

Genomic DNA was isolated from the livers of the rats derived from the HDH, HGN (Suzuki *et al.* 1988), and HGNII (*hgn*-inbred strain derived from Wistar-Imamichi closed colony) (Suzuki *et al.* 2005) strains. All had been genotyped by test mating. Rats were assumed to be *+ /+* if, after mating with carriers, none of at least 11 pups was affected. PCR of genomic DNA and polyacrylamide gel electrophoresis were performed as described (Suzuki *et al.* 2004a). The sequence of the *Spag5* cDNA fragment containing the mutation site of the *hgn* allele was BLAST-searched against the rat genome (Map Viewer,

Table 1 The primer sequence of polymorphic markers, RT-PCR for candidate genes, and genotyping of mutation

Name	AC number	Position	Forward	Reverse
<i>Polymorphic markers</i>				
M1	AC095686	255143–255323	TGGCAAGACGACAGGTTGAA	AGTGTGCCACTCTCGGGACT
M2 (Supt6 h)	AC095686	172146–172446	GTGTGAGGCTTCCAGATGCC	GCTTCAGAATAAAACATTTCCCTCAT
M3 (Sdf2)	AC095686	149974–150194	GGTCCCTGCTTAAGGAGGACA	GGTGTGTGGTGGGTGTTCC
M4 (Slc 1 3a2)	AC097687	27624–27913	TCTACCATACTAAATGAAGAGAGACTCTG	GCTCAGGCAATTAACATCTGTCTGT
M5	AC097867	33351–39062	GAACCTCAAATGTTGTTGTTGGTGG	GAACCTCAAATGTTGTTGTTGGTGG
<i>RT-PCR</i>				
SDF2	XM213377	510–1113	CTGCACAGCCACCATTTCACT	ATGCCAAGCTCCTGTGGAGTT
LOC303280	XM220636	4977–5764	TGGACTTGCTGTTAGATGGC	ACTGCAGCGCCAATAAAATG
PIGS	BC083862	587–1258	CTTTGACAGAGGATGTGCTTGCT	GTCCTTCACTCTTCGGTCCTGA
SPAG5-1	XM340848	78–926	GAGCCTCTGGTGTGGTATGG	CTGGGCCAGGTATCTTGTGT
SPAG5-2	XM340848	697–1625	TTGACCATGTTGGTCAAGTGT	CGGGACATCCAAGTCTCTGA
SPAG5-3	XM340848	1441–2293	AGTTTTCTCGCACTCCCTGA	CTTTAAGGGCCATTTCTCTCC
SPAG5-4	XM340848	2183–3177	AGACCCTGGTTTCTCTCTGT	AGAATGCTGGTGCCTCTTCT
SPAG5-5	XM340848	2146–2440	CATGTGCAAGATGACAGAGTGA	CTGTGTGAAGCCCTATCAGTTG
G3PHD			ACCACAGTCCATGCCATCAC	TCCACCACCTGTTGCTGTA
5'-RACE				
3'-TARGET	XM340848	–1558		CCTCCTGGGGTCCCATTCTACTAGAC
3'-RACE				
5'-TARGET	XM340848	2970–	CATGGAGTTTGTAGATGAAGAGAGTCAAG	
SEQ-1	XM340848	2988–	AGAGAGTCAAGTTGCTCACCTG	
SEQ-2	XM340848	3569–	AAATCTGTGAAGTGCACACAAGAC	
<i>Genotyping</i>				
SPAG5-G	NW0433	5321196–5321606	CCTGCACAGATGTGATACCCT	CCTCACCTGTCCCTGACACAT

National Center for Biotechnology Information (NCBI) <http://www.ncbi.nlm.nih.gov/mapview/>). Primer sequences were subsequently designed to flank the mutation site of genomic DNA (SPAG5-G, Table 1).

Sequencing

Following gel electrophoresis of the first PCR products, a portion of each band was picked from the gel with a pipette tip and used for a second round of PCR. The resulting products were purified using QIAquick PCR purification Kit (Qiagen GmbH) and directly sequenced in both the sense and antisense directions by an outside supplier (Hitachi Instrument Service Co., Ltd). 5'-RACE-PCR samples were subsequently cloned into pGEM T-Easy Vector (Promega) and sequenced. 3'-RACE-PCR samples were directly sequenced using SEQ-1 and SEQ-2 primers (Table 1). The sequence data were integrated and analyzed using Genetyx-Mac Ver. 12 (Genetyx Co., Shibuya-ku, Tokyo, Japan).

Preparation of paraffin sections and periodic acid Schiff (PAS) staining

Male *hgn/hgn* rats and their phenotypically normal littermates (+/*hgn* and +/+) from the HGN strain were sacrificed on PD 0 and 1 by an overdose of ether, and their testes were fixed in Bouin's solution for 2 h. Paraffin sections were prepared as described (Suzuki *et al.* 2004b, Yagi *et al.* 2006) and serial sections were deparaffinized in xylene, hydrated in graded alcohol, and immersed in 0.01 M PBS (pH 7.4). To examine

mitotic cells in neonatal testes, tissue sections were stained with PAS stain and hematoxylin (Suzuki & Suzuki, 1995).

Immunohistochemistry and TUNEL staining

Tissue sections were processed in a microwave (3 min × 5) in 0.01 M citric acid buffer (pH 6.0) to reactivate their antigenicity, soaked in water, immersed in 2 M HCl for 20 min, and soaked in PBS. The sections were immersed in methanol containing 3% periodic acid to inactivate internal peroxidases and incubated in PBS containing 5% skim milk or 10% normal rabbit serum for 60 min to block non-specific antigen-antibody reactions. Sections were incubated overnight at 4 °C with antibodies against GATA-binding protein 4 (GATA-4; goat polyclonal, 1/500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Mullerian-inhibiting substance (MIS, goat polyclonal, 1/500 dilution; Santa Cruz Biotechnology), mouse VASA homolog (rabbit polyclonal, 1/1000 dilution; the kind gift of Dr T Noce) (Toyooka *et al.* 2000), and vimentin (VM, monoclonal, 1/3 dilution; clone V9, BMB, Mannheim, Germany). The sections were rinsed in PBS and incubated with Histofine Simple Stain Rat PO (MULTI) (Nichirei Co., Tokyo, Japan) for monoclonal primary antibodies, Hitofine SAB-PO (G) kit (Nichirei Co.) for goat polyclonal primary antibodies, and Histofine Simple Stain Rat MAX-PO (R) (Nichirei Co.) for rabbit polyclonal primary antibodies. Apoptotic cells were detected with an *in situ* apoptosis detection kit (TaKaRa Bio, Inc., Shiga, Japan). The slides were incubated with 3,3'-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin, and digital images were obtained as described (Suzuki *et al.* 2004b, Yagi *et al.* 2006).

Cell proliferation assay

Pups were intraperitoneally injected with 5-bromo-2'-deoxyuridine (BrdU 100 mg/kg) on PD 1. Two hours later, the rats were sacrificed and their testes were removed and fixed overnight at 4 °C in 4% buffered formalin. Paraffin sections were prepared as described above, and BrdU incorporated into S-phase cells was detected by immunohistochemistry as described (Sugihara *et al.* 1986). The sections were subsequently processed for anti-MIS immunostaining to detect BrdU-positive Sertoli cells. Briefly, after deparaffinization in xylene, the sections were incubated in 4 M HCl for 30 min and in 0.01% trypsin in PBS for 10 min and soaked in PBS. After the incubation in PBS containing 10% normal rabbit serum for 10 min to block non-specific antigen-antibody reactions, the sections were incubated with primary antibody (anti-BrdU MAB, clone BRD.3, Lab. Vision Corp., Fremont, CA, USA) for 30 min. Following immersion in PBS, primary antibodies were detected with streptavidin and biotin complex methods (Histofine SAB-AP kit; Nichirei Co.). A Fast Blue substrate kit (Nichirei Co.) was used to detect the activity of alkaline phosphatase conjugated with streptavidin. After stopping the reaction by immersion in tap water, the sections were incubated in 0.1 M EDTA for 40 min at 95 °C, cooled for 1 h at RT, and rinsed in tap water. Following immersion in PBS, MIS immunostaining was done as described above.

Animals

All rats were fed a certified commercial diet (CR-LPF; Oriental Yeast Co., Ltd, Tokyo, Japan) and maintained in a clean conventional animal facility (Suzuki *et al.* 2004a,b). All experimental procedures and care of animals were in accordance with the guidelines of the Animal Care and Use Committee of Nippon Veterinary and Animal Science University.

Results

Fine linkage mapping

We previously reported that the *hgn* locus was linked to aldorase C (*Aldoc*) and located in an 840 kb region between *D10Rat195* and *D10Rat68* (Suzuki *et al.* 2004a). This region includes an unsettled segment of rat chromosome 10, in which the order of loci is opposite to that reported in the homologous regions of mouse chromosome 11 and human chromosome 17. Thus, the positions of some of the markers mapped on our linkage map were inconsistent with those presumed from the rat physical map obtained from the NCBI (Map Viewer, NCBI, World Wide Web). Using BLAST, we searched BAC clones for the marker sequences on our fine linkage map against Baylor Rat Data (Rat Genome Project, Human Genome Sequence Center at Baylor College of

Medicine, <http://www.hgsc.bcm.tmc.edu/projects/rat/>). To narrow the region that includes the *hgn* locus, we identified fragments containing short tandem repeats in the sequences of the BAC clones AC095686 and AC097867, which covers most of the region containing the *hgn* locus. Using PCR primers designed to amplify these fragments, we identified five PCR fragments (*M1*–*M5*) (Table 1) that showed polymorphisms between HGN and Brown Norway (BN) strain. We, therefore, genotyped the backcross progeny with these markers using previously obtained genomic DNA (Suzuki *et al.* 1999, 2004a). The fine linkage map revealed that the *hgn* locus was linked to *M3* (located in an intron of stromal cell-derived factor 2 (*Sdf2*)), *Arb9* (*Aldoc*), *D10Rat69*, and *whn* (*Foxn1*), and was located between *M2* (*Supt6 h*) and *M4* (*Slc13a2*) (Fig. 1A). The order of these loci (i.e. *Supth6-sdf2-Aldoc-Foxn1-Slc13a*) in our fine linkage map was consistent with the order reported for the homologous regions of mouse chromosome 11 and human chromosome 17 (Suzuki *et al.* 2004a) indicating that, in the rat, the *hgn* locus was located between *Supth6 h* and *Slc3a*.

Positional candidate analysis

Seven genes with known or predicted function were mapped to this region (Fig. 1A). *Aldoc* is expressed in brain-specific manner (Kukita *et al.* 1988). *Unc119* (UNC-homology, HRG4) is exclusively expressed in photoreceptors, and a mutation of *Unc119* is related with retinal degeneration (Kobayashi *et al.* 2000). Nude rats with a mutation of *Foxn1* (Forkhead box N1) have a phenotype obviously different from that of *hgn/hgn* rats (Segre *et al.* 1995). These genes, therefore, could be excluded as a candidate gene. Phosphatidylinositol glycan, class S (*Pigs*) is involved in glycosyl phosphatidylinositol (GPI)-anchor biosynthesis in cell membrane (Ohishi *et al.* 2001). *Sdf2* shows ubiquitous expression pattern in adult mouse organs (Hamada *et al.* 1996) and its physiological function have not been determined. There has been no information about the physiological function of hypothetical protein, *LOC303280* gene (Map Viewer). Although the expression of *Spag5* (*astrin*) is detected in many human tissues and cell lines (Chang *et al.* 2001, Mack & Compton 2001), *Spag5* is abundantly expressed in testes (Shao *et al.* 2001), and the alternatively spliced variant of *Spag5* is highly expressed in human kidney (Chang *et al.* 2001). Therefore, *Spag5* is the most possible candidate gene as *hgn* mutation. Pathological studies performed in *hgn/hgn* rats have indicated that the gene responsible for *hgn* is expressed in perinatal testes themselves (Suzuki *et al.* 1993, 1998, 2004b). We, therefore, assayed the expression of the candidate genes in the neonatal testes of normal (+/+ and +/*hgn*) and *hgn/hgn* rats. Although *LOC303280*, *Spag5*, *Sdf2*, and *Pigs* were expressed in neonatal rat testes, the levels of expression of *LOC303280*,

Figure 1 (A) Fine linkage map showing the location of newly generated markers (M1–M5) in relation to other loci around the *hgn* locus. The *hgn* locus is located in the region between M2 and M4. The physical map shows the position of new markers close to the *hgn* locus relative to genes with known or inferred functions. The functional genes located within the region between M2 (*Supt6h*) and M4 (*Slc13a2*) are underlined, and their relative position, in kilobase, is shown in parentheses. BAC clones covering this region are also shown. (B) Expression of *Spag5* mRNA in neonatal +/+ (HDH), +/*hgn*, and *hgn/hgn* testes. Each RNA sample was reverse transcribed and amplified using the primers for SPAG5-3 and -4 shown in the Materials and methods section. As internal controls, a G3PDH fragment was amplified from the same RNA samples. The PCR products were electrophoresed on agarose gels. (C) Small segments of *Spag5* cDNA, which contain the region overlapped in SPAG5-3 and SPAG5-4, were amplified using SPAG5-5 primers, and electrophoresed on acrylamide gels. In addition to intense band observed in +/+ and +/*hgn* testes, the weak band with large molecular size was observed in +/*hgn* and *hgn/hgn* testes.

Sdf2, and *Pigs* were comparable in *hgn/hgn* and normal testes (data not shown). However, the level of *Spag5* mRNA expression was extremely lower in *hgn/hgn* neonatal testes than in normal (+/*hgn* and +/+) neonatal testes. Similar results were obtained when PCR were performed using SPAG5-3 and SPAG5-4 primers (Fig. 1B). Since the molecular sizes of the products of SPAG5-3 and SPAG5-4 appeared to be slightly larger in *hgn/hgn* testes than in normal testes, we amplified a small segment (SPAG5-5) containing the region overlapped in SPAG5-3 and SPAG5-4, and we separated the PCR product by non-denatured polyacrylamide gel electrophoresis. In addition to the intense band of which molecular size was identical

with that of a single product in +/+ testis, another weak band with large molecular size was detected in +/*hgn* testes. In *hgn/hgn* testis, only a single weak band with large molecular size was detected (Fig. 1C).

Determination of *Spag5* cDNA sequence

To confirm the *Spag5* mutation in *hgn/hgn* rats, we directly sequenced the PCR products of SPAG5-3 and SPAG5-4 in *hgn/hgn* and +/+ rats. The full-length sequence of rat *Spag5* cDNA has not been reported, and we could not detect any specific amplification in RT-PCR using the upstream primers, SPAG5-1 and -2. To predict the amino

acid sequence of the *Spag5* protein, we determined the 5'- and 3'-sequence of the *Spag5* cDNA using RACE-PCR method. The rat *Spag5* cDNA sequence (AB231661) determined by our sequence analysis was almost corresponding to the sequence after nt 980 of the automated computationally predicted cDNA sequence of rat *Spag5* (XM340848), although the sequence of nt 500–820 of AB231661 was not found in the sequence of XM340848. The partial cDNA sequence (AF111111) of rat *Spag5* (Shao *et al.* 2001) was corresponding to the sequence of nt 2429–3862 of AB231661. The 3561 bp ORF of AB231661 encodes a protein of 1187 amino acids (132 kDa). The amino acid sequence of rat *Spag5* protein was 63 and 85% homologous to human and mouse *Spag5* respectively. We found that the initial 60aa and the latter 500aa were highly conserved among these species (Fig. 2). The former is a sequence unique to *Spag5*, and the latter contains two large coiled-coil domains.

Identification of a mutation in cDNA and genomic *Spag5* sequences

When we compared the *Spag5* cDNA sequences of *hgn/hgn* and *+/+* testes, we found that the former contained a 25 bp fragment, a duplication of an adjacent sequence, inserted at position nt 1742. This insertion results in the addition of 15 amino acids after amino acid 562 of the wild-type protein, and the frame shift leads to the introduction of a stop codon, producing a truncated product lacking almost all of the coiled-coil domain (Fig. 3). This mutation was confirmed by amplifying the fragment surrounding the insertion in genomic DNA derived from *hgn/hgn*, *+/hgn*, and *+/+* rats (Fig. 4). The genotype of the *Spag5* allele was completely consistent with the *hgn* genotype in the HGN and HGNII strain (Fig. 4A). Since the *Spag5* mutation was not found in the rats of other strains (OCD (Suzuki *et al.* 2000) and HN (Suzuki *et al.* 1987)), derived from an original closed

A	1	MNRVKTLLNLGLSPSPQSGKPMRTPLRELKLQPEALADSCKGP-MFSSLTPLYRLDLKERNSSSPVNFVNAENSSPVDVNTENTSPV	89
B	1	MNRVKTLLNLGLSPSPQSGKPMRTPLRELKLQPEALADSCKGPMISALTPLYRLDLKERNSSSPVDFTI	71
C	1	MNRVKTLLNLGLSPSPQSGKPMRTPLRELKLQPEALADSCKGPMISALTPLYRLDLKERNSSSPVDFTI	72

	90	DFDNTENNFLSEQF SHPSEYIEACQRESDLTPESSSP-FNTLEKAVEAMDDFVAPIDDGIVKTMVLLPFSLGQQQDLMLQALDITTAER	178
	72	---NTENNFLSEQF SHPSEYIEACQRESDLTPESSSP-FHTLEEAETVDDFVDPDDDSIVE SMVLLPFSLGQQQDLMLQALDITTAER	157
	73	---NKRDTLSEEHF SHSSKWLCTCHESDEQLDPLPQISSTPKTSEE---AVDPLGNYMVKTI VLVPSP LGQQQDMIFEARLDTMAET	155

	179	NKSSLLKESLGLEDLVEKEAAPCEDSLPETVAIR-----PEQPI SQDPP-----LRDSN-----TEAAPAGSVPSSE-NV-LGFTV	246
	158	TKSSLLNESLGLEDLVGKEVAPCEVDSLTEIVAIR-----PEQPTFQDPP-----LGPSD-----TEDAPDVLVPSSE-NV-LNLSL	225
	156	NSISLNGPLRTDLDVRE EVAPGMGRFSEVAAVSEKPIFQESPSHLEESPPNPCS EQHLCSKESLSSTRTAEVREDLVPSESNAFLPSVS	245

	247	ACLSPSTVLTGDFSDVHDGPEETAENRVIQEVKSF LALPEEAGLGDQALAVNAEDVSSLYLSSSLVEMGPQEA PGPTAE DASRI PDL	336
	226	ARLSPSAVL AQDFSDVHDGPEETAENRVLQEMETSFPFPFEEAELGDQAPANAEEAVSPLYLTS SLVEMGP REAPGPTVEDASRI PDL	315
	246	LWLSPTALAADFRVNHVDPPEELIVEHGAMEREHRFPFTHPKESSETEDQALVSSVEDLSLCTLTPNLVEMESQEA PGPAVEDVGRILGSD	335

	337	SETWMSPLAWLEKGVNTSVMLQSLRQSLPFSKSVLQDAAVGNTPHSTYSVGTSTFPPVPLE-----VGTKDSSTSETRLLGCRP	416
	316	SETWMSPLAWLEKGVNTSVMLQSLRQSLSPFS-SVLQDAAVGNTPLATCSVGTSTFPPAPLE-----VGTKDSSTSETRLLGCRP	394
	336	SETWMSPLAWLEKGVNTSVMLQSLRQSLSLP-SMLRDAAGTTPFSTCSVGTWTFPSAPQEKSTNTSQGLVGTKHSITSETEQLLGG-RP	423

	417	PDLAALSRHDLLENLLNALVLEVLVSHQLQAWKSQLTVP SRQAQDSSTQDSSAAVVTKTPKHLQDSKEIRQALLQARNVHISWGLVSRD	506
	395	PDLATLSRHDLLENLLNSLVLEVLVSHQLQAWKSQLTVP HREARDSSTQDSSPCGVTKTPKHLQDSKEIRQALLQARNVHISWGLVSRD	484
	424	PDLATLSRHDLLENLLNSLVLEVLVSHQLQAWKSQLTVP HREARDSSTQDSSPCGVTKTPKHLQDSKEIRQALLQARNVHISWGLVSRD	513

	507	LMSLLHLSTHVQDDRRVTVSQESQRAETLVSSCSRVLLKRLAKLQSLKTEWEEARHREEMALRGKDAAEAVLEAFRAHASQRI SQLEQGI	596
	485	LLSLHLSTHVQEGRTVSQESQRSKTVLS SCSRVLLKRLAKLQSLKTECEEARHSKEMALRGKAAAEAVLEAFRAHASQRI SQLEQGL	574
	514	LISLLHLSTLHLEEDKTTVSQESQRAETLVCCFCDLLKRLAKLQSLKAEEREEARHREEMALRGKDAAEIVLEAFCAHASQRI SQLEQGL	603

	597	TSVQEFRGLLQEAQTQLIGLHTEQELAQHTVSLTSALQDDWTSVQNYGTWAA LLSRSRELTKLKTAKSRQALQERDAAIEEKQDVVKE	686
	575	TSVQEFRGLLQEAQTQLIGLHTEQELAQHTVSLSSALQDDWTSVQNYGTWAA LLSWSRELTKLKTAKSRQALQERDAAIEEKQDVVKE	664
	604	ASMRFRGLLQEAQTQLIGLHTEQELAQHTVSLTSLQDDWTSVQNYGTWAA LLSRSRELTKLKTAKSRQALQERDAAIEEKQDVVKE	693

	687	VEQVSAHLEDCKGQIEQLKLENSRLTADLSAQQLTLASTENELKELQSQHSHCVQDLAMKDELLCQLTQSNKEQAAQWQKEETELKHQA	776
	665	VEQVSAHLEDCKGQIEQLKLENSRLTADLSAQQLTLASTENELKELQSQHSHCVQDLAMKDELLCQLTQSNKEQATQWQKEEMELKHQA	754
	694	LEQVSAHLEDCKGQIEQLKLENSRLTADLSAQQLTLASTENELKELQSQHSHCVQDLAMKDELLCQLTQSNKEQAAQWQKEEMALKHQA	783

	777	ELLQQKAVLAKEVQDLRETFVDEESQVAHLELQGISQLKATLEVLRLRSQCELT KDVTESLRALASTEAKHEQQALEKTHQHSKE	866
	755	ELLQQKAVLAKEVQDLRETFVDEESQVAHLELQGISQLKATLEVLRLRSQCELT KDVTESLRALASTEAKHEQQALEKTHQHSKE	844
	784	ELLQQKAVLAKEVQDLRETFVDEESQVAHLELQGVQCQLKTLEVLRLRSQCELT KDVTESLRALASTEAKHEQQALEKTHQHSKE	873

	867	LCLLAEQLQSLTLFLAKLEENKAESEDIIPSTGCPAQEHPPSSDSSVSEQIPTAVDVEVPEPAPVPLLGSVKSATRVAS TAPFRPTE	956
	845	LRLLAELQLQSLTLFLAKLEENKAESEDIIPSTGCPAQEHPLSNDSSISEQTPTAAVDVEVPEPAPVPLLGSVKSATRVASMASFPQTE	934
	874	LGLLTELQLQSLTLFLAKLEENKAESEDIIPSTGCPAQEHPLSNDSSISEQTPTAAVDVEVPEPAPVPLLGSVKSATRVASMASFPQTE	962

	957	TPALEKSLAEMSAVLQELQRLCALLQESKEEAVGVLRQREICELHTRLQAAQEEHQEAQKAEADIEKLNQALCLRHKNKEKLELVIQKQN	1046
	935	TPDLKSLAEMSAVLQELQRLCALLQESKEEAVGVLRQREICELHTRLQAAQEEHQEAQKAEADIEKLNQALCLRHKNKEKLELVIQKQN	1024
	963	TPQMEELAEMSAVLQELQRLCALLQESKEEAVGVLRQREICELHTRLQAAQEEHQEAQKAEADIEKLNQALCLRYKNKEKLELVIQKQN	1052

	1047	EKILGQIDTSQGLINLREEVTQLTRLRRAETETKVLQEALEGGQVDPSCQLMATNWIQEKVFLSQEVNKL RAMFLVKNKKQLMDKYLS	1136
	1025	EKILGQIDTSQGLINLREEVTQLTRLRRAETETKVLQEALEGGQVDPSCQLMATNWIQEKVFLSQEVNKL RVMLFMKTEKEQLMDKYLS	1114
	1053	EKILEQIDTSQGLINLREEVTQLTRLRRAETETKVLQEALEGGQVDPSCQLMATNWIQEKVFLSQEVNKL RVMLFMKTEKEQLMDKYLS	1142

	1137	HRHILEENLRSDTELKKLDOTIHIQHYETLLSIPEVVKSKCKELQGLLEFLS	1187
	1115	HRHILEENLRSDTELKKLDOTIHIQHYETLLSIPEVVKSKCKELQGLLEFLS	1165
	1143	HRHILEENLRSDTELKKLDOTIHIQHYETLLSIPEVVKSKCKELQGLLEFLS	1193

Figure 2 Comparison of the amino acid sequences of rat (A), mouse (B), and human (C) *Spag5* proteins. The rat amino acid sequence was predicted from rat *Spag5* cDNA sequence determined by RACE-PCR method. Arrowhead indicates the position at 562aa.

Figure 3 Diagram of rat Spag5 cDNA and transcript showing the position of *hgn* mutation. cDNA sequence and predicted amino acid sequence of the wild type and *hgn* alleles of *Spag5* were shown. In the *hgn* allele, a 25 base duplication (Box) of a sequence present in the wild-type allele (underline) causes a frame shift mutation, leading to the insertion of a stop codon (*). The position of primers used in the RT-PCR and RACE experiments were also shown.

colony of Wistar–Imamichi rats, this mutation was unique for *hgn/hgn* rats of HGN and HGNII strains. Part (517–650 bp) of the *Spag5* cDNA could not be found in the *Spag5* sequence of rat genome database because of gaps in the draft sequence (Map Viewer, NCBI, World Wide Web). Comparing the exon–intron organization of rat *Spag5* gene with those of mouse and human *Spag5* genes indicate that the 25 bp fragment is inserted into the sixth exon of the *Spag5* gene (Fig. 4B).

Abnormal mitosis and apoptosis in *hgn/hgn* testes

Spag5 is a microtubule-associated protein, which is functionally related with the stability of the spindle apparatus in mitotic cells (Mack & Compton 2001, Chang *et al.* 2001, 2003, Gruber *et al.* 2002, Fitzgerald *et al.* 2006). In order to obtain histological evidences involved in the defect of the *Spag5* function in *hgn/hgn* rats, we characterized cell mitosis and apoptosis in the testes at PD 1. In the normal testes at PD 1, Sertoli cells form a line along the basement membrane of seminiferous tubules (Fig. 5A). These cells often undergo mitosis, with gonocytes located in the center of the tubules arresting during G0 (Fig. 5C). In the *hgn/hgn* testes, the number of Sertoli cells is very low, so they fail to make a line along the basement membrane (Fig. 5B). We often found abnormal mitotic metaphase cells, which contained large amount of cytoplasm and diffused chromosomes in the *hgn/hgn* tubules (Fig. 5D). Although no TUNEL-positive cells were found in normal testis at PD 1 (Fig. 6A), we often observed TUNEL-positive apoptotic cells and abnormal mitotic metaphases in *hgn/hgn* tubules (Fig. 6B). In disagreement with the increased appearance of mitosis, the number of MIS-positive Sertoli cells containing BrdU-positive nuclei was apparently low in *hgn/hgn* testes (Fig. 6D and F) compared with that in normal testes (Fig. 6C and E).

Identification of cell types undergoing mitosis and apoptosis

To determine the cell types undergoing abnormal mitosis and apoptosis, we stained tissue sections with antibodies specific for marker proteins of germ cells (VASA; Toyooka *et al.* 2000), Sertoli cells (MIS; Kuroda *et al.* 1990), Sertoli and Leydig cells (GATA-4; Ketola *et al.* 1999), and testicular somatic cells (VM; Paranko *et al.* 1986). In normal testes at PD 1, the cytoplasm of gonocytes, the mitotic arrest phase of germ cells, were specifically stained with anti-mouse VASA antibody (Fig. 7A and B). Sertoli cells were positive for GATA-4, MIS, and VM. GATA-4 staining was observed in the nuclei and cytoplasm (Fig. 7C and D), and MIS staining in the cytoplasm (Fig. 8A and B). VM immunostaining in Sertoli cells was observed in their cytoplasm under the nucleus (Fig. 8C and D). These marker proteins were detected in mitotic and apoptotic cells. In *hgn/hgn* testes, these proteins showed similar patterns of cell specificity, consistent with the relatively reduced number of Sertoli cells (reduction of total GATA-4 and MIS staining) and the relatively increased number of gonocytes (increased total VASA staining) in seminiferous tubules. Abnormal mitotic and apoptotic cells were both negative for VASA (Fig. 7B), but positive for GATA-4 (Fig. 7D) and MIS (Fig. 8B). VM immunostaining was located in the cell membrane of abnormal mitotic cells in *hgn/hgn* testes (Fig. 8D). These findings indicate that, in *hgn/hgn* testes, the abnormal mitosis with dispersed chromosomal distribution and apoptosis occurred predominantly in Sertoli cells rather than gonocytes.

Discussion

Male hypogonadic mutant rats were first detected in the fifth generation of a hydronephrotic strain derived from a closed colony of Wistar–Imamichi rats (Suzuki *et al.* 1988).

Figure 4 (A) PCR amplification of the *Spag5* *hgn* and wild-type alleles in genomic DNA from rats with the indicated genotype. HDH, *hgn*-free dominant homo inbred strain derived from the HGN strain; HGN, *hgn*-inbred strain derived from hydronephrosis (HN) strain; HGNII, *hgn*-inbred strain derived from Wistar-Imamichi closed colony. (B) Genomic sequence showing that the *hgn* allele contains an insertion of a 25 base fragment into the sixth exon of *Spag5* gene.

The HGN inbred strain has been maintained in our laboratory by sister-brother mating between carriers. We have described the pathophysiological characters of these *hgn/hgn* rats, in which testicular dysplasia was used to study testes development (Hakamata *et al.* 1988, Suzuki *et al.* 1988, 1993, 1998, 2004*b*, Yagi *et al.* 2006). In addition, we have used the renal hypoplasia occurring in these rats as a model of oligomeganephronia and chronic renal failure (Suzuki & Suzuki, 1995, 1998; Suzuki *et al.* 1991, 2005, 2006). Since no other knockout mouse or mutant rat showing a similar phenotype has been reported, we started linkage analysis in 1997 and located the responsible gene on rat chromosome 10 (Suzuki *et al.* 1999). Since *hgn/hgn* females can be distinguished from phenotypically normal females by the presence of renal hypoplasia (Suzuki *et al.* 1991, 1992), 126 *hgn/hgn* females were selected from the HGN inbred strain by the laparotomy for the detection of renal hypoplasia at

weaning. Five hundred and sixty-five male backcross progeny were obtained by mating between F₁ males (obtained by mating +/+ Brown Norway male rats with *hgn/hgn* HGN female rats) and *hgn/hgn* females, and these animals were genotyped by microsatellites around *hgn* locus. In our previously described fine linkage map, we restricted the region that included the *hgn* locus to an 840 kb region of rat chromosome 10 (Suzuki *et al.* 2004a). In this study, we used newly generated markers to narrow the region to 320 kb, which includes seven genes with known or predicted function. Semi-quantitative RT-PCR analysis revealed that the level of expression of *Spag5* was much lower in *hgn/hgn* than in normal (+/+ and +/*hgn*) testes. In consistent with these findings, the level of *Spag5* was extremely lower in *hgn/hgn* kidneys than in normal kidneys (data not shown). We found that, in *hgn/hgn* testes, there was a 25 bp insertion in the sixth exon of *Spag5* gene. The extremely low level of *Spag5* expression in *hgn/hgn*

Figure 5 Periodic acid Schiff staining in normal (A and C) and *hgn/hgn* (B and D) testes at PD 1 (A and B $\times 135$, C and D $\times 360$). In normal testes, Sertoli cells, of which nuclei are uniform in shape, form a palisade distribution along the basement membrane of the seminiferous tubules (A and C). Normal mitotic Sertoli cell was found (C, arrow). In *hgn/hgn* testes, Sertoli cells fail to form an alignment along the basement membrane because of their small number. The nuclei of most Sertoli cells are irregular, and a number of abnormal mitotic (arrows) and apoptotic (arrowheads) cells are observed (B and D).

testes may not be due to the reduced number of cells expressing *Spag5* but may be due to the rapid degradation of abnormal mRNA resulting from its instability, since the expression of abnormal mRNA derived from mutated allele was extremely low in $+/\text{hgn}$ testes.

Using a yeast two-hybrid system, rat *Spag5* was identified as a protein interacting with the outer dense fiber of sperm tails. *Spag5*, which has a molecular weight

of 200 kDa, was shown to be expressed in pachytene spermatocytes and spermatids, indicating its functional role in spermatid morphogenesis in rats (Shao *et al.* 2001). Although targeted disruption of the mouse *Spag5* protein was performed by the same group, they did not find any significant effect on spermatogenesis or fertility (Xue *et al.* 2002). In contrast, the other two groups independently identified *hMAP126* (human), *astrin*

Figure 6 TUNEL staining (A and B $\times 410$) and immunological detection of BrdU-incorporated Sertoli cells (C and D $\times 260$, E and F $\times 100$) in normal (A, C, and E) and *hgn/hgn* (B, D, and F) testes at PD 1. (A and B) TUNEL-positive (arrowheads) and mitotic cells (arrows) were frequently observed in *hgn/hgn* (B) but not in normal (A) seminiferous tubules. Compared with the normal mitosis observed in normal testes (A), the abnormal mitotic cells in *hgn/hgn* testes had more abundant cytoplasm containing diffuse chromosomes. (C–F) BrdU-incorporated nuclei were stained blue, and the MIS positive-cytoplasm of Sertoli cells were stained brown. BrdU is incorporated in Sertoli cells but not in gonocytes in both normal and *hgn/hgn* seminiferous tubules (C and D). There were fewer BrdU-positive Sertoli cells in *hgn/hgn* (F) than in normal (E) testes. G, gonocyte; S, Sertoli cell; M, myoid cell.

(human), and *mastrin* (mouse), all of which have amino acid sequences similar to that of *Deepest* and have a molecular weight of around 130 kDa, and these proteins were co-localized with the spindle apparatus in mitotic cells (Chang *et al.* 2001, 2003; Mack & Compton, 2001). *Astrin* has been found to be expressed in various organs and essential for mitosis progression, since silencing of *astrin* in HeLa cells by RNA interference resulted in mitotic arrest with formation of multipolar and highly disordered spindles (Gruber *et al.* 2002). Since, at present, the gene encoding *MAP126*, *astrin*, and *Deepest* has been mapped to the *Spag5* locus in the NCBI genome database (Map Viewer, NCBI, World Wide

Web), there is an obvious discrepancy between the results of knockout mice and RNA interference. In mouse, *Spag5* is composed of 24 exons, distributed over 24 kb of the genome in mouse (Chang *et al.* 2003). Although deleting a 0.7 kb genomic fragment containing the first exon of mouse *Spag5* extinguished a 200 kDa protein, two peptides (125 and 150 kDa) immunologically detectable with anti-*Spag5* antibody were present in the knockout mice (Xue *et al.* 2002). Recently, the same group has reported that rat *Spag5*, expected as an *astrin* orthologue, is expressed in various tissues and that rat testis contains several *Spag5* proteins with different molecular weights (Fitzgerald *et al.* 2006). Based on this

Figure 7 Identification of mitotic and apoptotic cells in the seminiferous tubules of normal (A and C) and *hgn/hgn* (B and D) rats at PD 1. Tissue samples were immunostained for the germ cell-specific marker VASA (A and B) and for the Sertoli cell-specific marker GATA-4 (C and D) ($\times 360$). Sertoli cells were negative for VASA, but positive for GATA-4, whereas gonocytes were positive for VASA, but negative for GATA-4. Mitotic cells in the seminiferous tubules of normal testes (arrows) were positive for Sertoli cell-specific markers (C). Cells showing abnormal mitotic metaphase (arrows) and apoptosis (arrowheads) were negative for VASA, but positive for GATA-4 (B and D).

situation, they mentioned the possibility that particular knockout event did not extinguish all functionality of *Spag5* proteins (Fitzgerald *et al.* 2006).

Our findings indicate that rat hypogonadism (*hgn/hgn*) is involved in a mutation in the *Spag5* gene. This gene was located in the appropriate chromosomal region restricted by our fine linkage mapping. The molecular weight of rat *Spag5* protein predicted by our RACE-PCR is estimated to be a 132 kDa, which is comparable with that of human

astrin (Mack & Compton 2001), but not with 200 kDa of rat *Spag5* (Shao *et al.* 2001). Moreover, the 25 bp insertion present in the first coiled-coil domain of *Spag5* was found to result in a truncated protein lacking the primary spindle-targeting domain at the C terminus (Mack & Compton 2001). The *hgn* mutation, therefore, might lose the *Spag5* function as a microtubule-associated protein. If we consider the functional importance of microtubules and its associated proteins in cell proliferation and

Figure 8 Identification of mitotic and apoptotic cells in the seminiferous tubules of normal (A and C) and *hgn/hgn* (B and D) rats at PD 1 (A and B) and PD 0 (C and D). Tissue samples were immunostained for the Sertoli cell-specific marker MIS (A and B) and for the somatic cell-specific marker VM (C and D) ($\times 360$). Sertoli cells were positive for MIS and VM, whereas gonocytes were negative for the markers. Mitotic cells in the seminiferous tubules of normal testes (arrows) were positive for MIS and VM (A and C). Cells showing abnormal mitotic metaphase (arrows) and apoptosis (arrowheads) were positive for MIS (B). In the apoptotic Sertoli cells, VM is located in their cell membranes (D, arrows).

differentiation, it is reasonable that the *hgn* phenotype involving defective cell mitosis and increased apoptosis (Suzuki *et al.* 2004b, Yagi *et al.* 2006) would be caused by the mutation in the *Spag5* gene. Although, at present, it is unclear why *Spag5* knockout mice have a normal phenotype, one of the possibilities is that the discrepancy between the *hgn/hgn* rats and the knockout mice may be due to the types of mutations. In *hgn/hgn* rats, since the

insertion mutation causes a frame-shift mutation, leading to the insertion of a stop codon, the *Spag5* protein is truncated and subsequently degraded. If the downstream sequence containing coiled-coil domain is shared with the variants, the compensation of function is not expected. Further investigations are required to reveal this possibility and the species differences in the requirement of functional *Spag5*.

We also provide pathological evidence supporting that the *hgn* phenotype is caused by deletion of *Spag5* function. In *hgn/hgn* testes of PD 1 rats, we often found abnormal mitotic metaphase cells with large amounts of cytoplasm. Previously, we suspected that these cells were abnormal gonocytes that failed to undergo mitotic arrest (Suzuki *et al.* 1993, 2004b). However, our immunohistochemical results have shown that these mitotic metaphase cells are Sertoli cells, since they are positive for GATA-4, MIS, and VM but negative for VASA. The defects in mitosis of Sertoli cells of *hgn/hgn* rats have features analogous to *in vitro* mitotic arrest in HeLa cells caused by *Spag5* RNA interference (Gruber *et al.* 2002). Low-mitotic activity in Sertoli cells of *hgn/hgn* testes, as shown by the low level of BrdU incorporation, indicates that increased abnormal mitosis is not accompanied by increased S-phase DNA synthesis, suggesting mitotic arrest or a prolonged period of mitotic metaphase. In addition, the dispersed distribution of chromosomes observed in mitotic Sertoli cells in *hgn/hgn* testes suggests the presence of disordered spindles. These abnormal mitotic cells are not thought to progress through the cell cycle by passing through mitotic checkpoints (Gruber *et al.* 2002). In agreement with findings in *Spag5* deficient-HeLa cells, we found that, in *hgn/hgn* testes, a considerable number of Sertoli cells undergo apoptotic cell death (Suzuki *et al.* 2004b, Yagi *et al.* 2006). These histological features suggest that the testicular phenotype in *hgn/hgn* rats is caused by a loss of *Spag5* function and that these effects appear predominantly in proliferating Sertoli cells. In *hgn/hgn* males, most of gonocytes fail to differentiate into spermatogonia and are likely to have degenerated before entering meiosis. Transplantation of *hgn/hgn* gonocyte into normal testis may be informative to know whether the *Spag5* mutation directly affect the germ cells development.

Fitzgerald and colleagues have reported that the preparation from rat total testis contains several *Spag5* proteins with different molecular weights (200, 130, 110, 58, and 31 kDa). Although 58 kDa *Spag5* protein has been localized in sperm tail, other *Spag5* proteins such as 130 kDa *Spag5*, which is recognized by anti-astrin antibodies and may be similar to 134 kDa human astrin, is absent from spermatids and sperm (Fitzgerald *et al.* 2006). Although other *Spag5* proteins and *astrin* have not yet been localized in testicular sections, which may be due to the low level of immunostaining with the antibody used (Shao *et al.* 2001, Chang *et al.* 2003), it is reasonable to consider that testicular somatic cells also express *Spag5* proteins (Fitzgerald *et al.* 2006). In our preliminary experiments, we detected a low level of defective *Spag5* mRNA expression in adult *hgn/hgn* testes (data not shown), which has no spermatogenesis (Suzuki *et al.* 2004b), suggesting that testicular somatic cells express *Spag5* mRNA. In *hgn/hgn* rats, differentiation or maturation is defective in all testicular cell

types during postnatal development. To distinguish the direct effect of *Spag5* mutation in other testicular cell types from the secondary effects caused by Sertoli cell dysfunction, it is necessary to show the spatial and temporal expression of *Spag5* in developing testes. Our observation of abnormal mitotic metaphase and apoptosis in postnatal Sertoli cells of *hgn/hgn* testes indicates that *Spag5* may be expressed in Sertoli cells or that Sertoli cells are most sensitive to the loss of *Spag5* function. *Spag5* may be involved in the synchronization of active proliferation and differentiation of Sertoli cells during the early postnatal period. Premature Sertoli cell must develop a large cytoplasm to support the considerable number of germ cells at various stages of differentiation during adulthood (Sharpe *et al.* 2003). This might cause subtle instability in chromosome distribution during mitosis progression. Furthermore, *Spag5*, which has a role in microtubule stabilization, is also expressed in interphase cells (Mack & Compton 2001, Fitzgerald *et al.* 2006). Microtubules are important for cytoskeletal networks as well as for the formation of mitotic spindles. Sertoli cells have an abundant amount of microtubule networks, which are involved in structural maintenance and polarization (Redenbach & Boekelheide 1994). Therefore, the deletion of *Spag5* may affect the physiological function of these microtubules.

Our RT-PCR experiments (data not shown) and gene expression data from NCBI (Map Viewer, NCBI, World Wide Web) indicate that *Spag5* mRNA is expressed in various embryonic tissues. In addition to severe testicular dysplasia, *hgn/hgn* rats show renal hypoplasia, ovarian hypoplasia, and growth retardation (Suzuki *et al.* 1991, 1992), all of which are detected by ED 16.5 (data not shown). Therefore, the *Spag5* defect may affect many cell types during early stages of embryonic development, and it may have different degrees of effects on cell types during organogenesis. To reveal the physiological function of *Spag5* in the embryonic development of ovaries, kidneys, and other organs, it is necessary to characterize cell mitosis and apoptosis in the embryonic pathogenesis of the hypoplastic organs in *hgn/hgn* rats. Basic research into the molecular events of the cell cycle is important for our understanding of embryonic development as well as for identifying target molecules to prevent cancer growth. Many proteins participating in the progression of mitosis have been identified in yeast, and RNA interference has been used to determine gene function during mammalian cell mitosis. There have been few animal models, however, showing the molecular events associated with defects in mitosis progression. To date there has been no other animal model in which the deletion of a MAP results in a defect of mitosis progression. The results presented here suggest that *Spag5* is involved in the *in vivo* mitosis progression of Sertoli cells and that the *hgn/hgn* rat is a unique animal model for studying the function of *Spag5*. In addition, although the basic machinery responsible for

mitosis progression is considered to be common in most eukaryotes, mitosis progression in mammalian cells, which have a large diversity in size and shape, might require additional factors, such as *Spag5*.

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