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The role of the septin family in spermiogenesis

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Septins (full name: Septin; symbol name: SEPT) belong to a family of polymerizing GTP-binding proteins that are required for many cellular functions, including membrane compartmentalization, vesicle trafficking, mitosis and cytoskeletal remodeling. Two of the 14 family members in the mammalian species, *Septin12* and *14* are expressed specifically in the testis. In the mouse, knockout of *Septin4* and *Septin12* leads to male sterility with distinctive sperm pathology (defective annulus or bent neck). In humans, sperm with abnormal expression patterns of SEPT4, 7 and 12 are more prevalent in infertile men. How septin filament is assembled/dissembled and how the SEPT-related complex regulates spermatogenesis still await further investigation.

Septin Gene Family

SEPTs belong to a highly conserved family of polymerizing GTP binding proteins.[1] Bioinformatic prediction indicates several potential motifs of the human SEPTs.[1] The lengths of N-termini range from long (e.g., SEPT8 and SEPT9) to very short (SEPT1).[2] The central GTP-binding domain is highly conserved in all human septins.[1] Immediate N-terminal of the GTP-binding domain is a polybasic region which is also conserved in the eukaryotic phylogeny. Based on the C-termini sequences, human septins could be classified into four distinct groups. SEPT6, 7, 8, 10, 11, 13 and 14 have long stretch of coiled-coil sequences. They are further sub-divided into two groups: SEPT6 group (SEPT6, 8, 10, 11 and 14) and SEPT7 group (SEPT7 and SEPT13). SEPT 1, 2, 4 and 5 are similar to each other and have a short coiled-coil structure (SEPT2 group). Septins 3, 9 and 12 have no predicted C-terminal coiled-coil domain (SEPT3 group) (**Table 1**). It has been hypothesized that the coiled-coil domains is important for oligomerization of SEPT filament.[2,4]

Physiological Roles of the Septin Gene Family

Septins are required for the completion of cytokinesis in somatic cells. Loss of SEPT function results in multinuclear phenotypes from yeast to mammals, suggesting highly conservative role of septins during evolution. The budding yeast, *Saccharomyces cerevisiae*, has five septins: Cdc3p, Cdc10p, Cdc11p, Cdc12p and Shs1p/Sep7p. They are localized to the ring(s) to compartmentalize mother and daughter cells.[5] Mutations in any one of the five septins result in a distinctive phenotype with multi-nuclear and multi-cellular morphology.[5,6] The nematode, *Caenorhabditis elegans*, has two septins: *unc-59* and *unc-61*. Their mutants have normal early embryogenesis but show multiple defects, including abnormal morphogenesis of the vulva, male tail, sensory neurons and gonad in the larvae.[7] There are at least 14 septin genes in the mammalian species. Some septins are expressed ubiquitously, while some are expressed only in certain types of types (e.g., neuron or male germ cells).[1] In dividing cells, SEPT2, SEPT6, SEPT7 and SEPT9 have been implicated in the completion of cytokinesis, a highly conserved function during evolution.[8-10] In well-differentiated cells, septins are involved in vesicle trafficking and cytoskeletal remodeling.[11-13]

The Role of Septins in Reproduction

Drosophila has three SEPTs: *Pnut*, *Sep1* and *Sep2*. In Drosophila, septins are involved in the formation of ring canal structure between the intercellular bridge of male and female germ cells.[14] The function of septins in the intercellular bridge seems to be conserved across different species. In the mouse, SEPT2, 7 and 9 are co-localized with an intercellular bridge markers of male germ cells, TEX14 (testis-expressed gene14).[15] Loss of TEX14 in mice has been shown to cause disruption of intercellular bridge as well as increased apoptosis of germ cells.[16] SEPT4, along with other SEPTs (SEPT1, SETP6 and SEPT7), is located at the annulus, a ring-like structure between the midpiece and the tail region of mature spermatozoa.[12] During spermiogenesis, SEPT4 was found to be essential for the maintenance of proper mitochondrial architecture and establishment of the annulus. *Septin4* null (*Septin4*$^{-/-}$) mice were viable but sterile in male due to asthenozoospermia with bent neck.[12,13] The immotile sperm with defective annulus also showed dis-localization of SEPT1, SEPT6 and SEPT7 from the annulus.[12,13] The latter finding suggests septin complex in sperm may consist of SEPT1, 4, 6 and 7.[12] Recently, Kwitny et al. found domain confinement was lost in the sperm tail of *Septin4*$^{-/-}$ mice. Their finding provides strong evidence of a role of mammalian septin structure in establishing membrane diffusion barrier.[17] To address the function of *Septin12*, we knocked out the *Septin12* locus in the mouse.[18] All the chimeric mice were viable without obvious defects. The male chimeric mice were mated with C57BL/6 female mice but only few chimeric mice fathered black progeny (C57BL/6 genetic background). Most chimeric males were infertile. Semen analysis of the infertile chimeras showed

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Table 1. The human septins and relevant summary information

Gene name	Accession Number	Phylogeneic Relationships	Containing Domain*	Involved Diseases**
SEPTIN 1	NM_052838	Group 2	PR, GBD, CC	Alzheimer disease
SEPTIN 2	NM_004404	Group 2	PR, GBD, CC	MLL, Alzheimer disease, Brain tumor
SEPTIN 3	NM_145733	Group 3	PR, GBD	Alzheimer disease
SEPTIN 4	NM_004574	Group 2	PRD, PR, GBD, CC	Alzheimer disease, Male infertility, Down syndrome
SEPTIN 5	NM_002688	Group 2	PR, GBD, CC	MLL, Down syndrome, Parkinsonism
SEPTIN 6	NM_145799	Group 1	PR, GBD, CC	MLL
SEPTIN 7	NM_001788	Group 4	PR, GBD, CC	Male infertility
SEPTIN 8	NM_001098811.1	Group 1	PRD, PR, GBD, CC	
SEPTIN 9	NM_001113491.1	Group 3	PRD, PR, GBD	MLL, Ovarian cancer, Infectious disease, Breast cancer, Prostate cancer
SEPTIN 10	NM_144710.2	Group 1	PR, GBD, CC	–
SEPTIN 11	NM_018243.2	Group 1	PR, GBD, CC	MLL
SEPTIN 12	NM_144605	Group 3	PR, GBD	Male infertility
SEPTIN 13	SEPT7/Pseduo-gene 2	Group 4	PR, GBD, CC	–
SEPTIN 14	NM_207366.2	Group 1	PR, GBD, CC	–

*Domains: PRD = proline-rich domain; PB = polybasic region; GBD = GTP-binding domain; CC = coiled-coil domain. **Disease: MLL = mixed lineage leukemia. ***Table modified from references.[1-3]

decreased sperm counts, decreased sperm motility and spermatozoa with defects involving all subcellular compartments. Our findings suggested haploinsufficiency of *Septin12* could disrupt spermiogenesis. Mice whose both *Septin12* alleles are knocked out *(Septin12$^{-/-}$)* have not been generated.

Expression Pattern of SEPT7 and 12 in the Mouse

SEPT12 is exclusively expressed in the mouse testis.[18] Immunofluorescence staining showed SEPT7 and 12 expressions are confined to post-meitoic germ cells in the seminiferous tubules.[18-20] During spermiogenesis, SETP7 and 12 filaments start to appear around the acrosome at step 7 of spermiogenesis. At step 10–11 of spermiogenesis, SEPT7 and 12 form a circular structure between the edge of acrosome and the perinuclear mantle of the manchette. With the formation of mitochondria, SEPT7 and 12 start to be localized at the sperm neck and annulus. In mature spermatozoa, the SEPT7 and 12 signals are located at the sperm head, neck and midpiece with scanty signals at the tail.[18,19] Considering SEPT 7 and 12 are widely expressed and form ring-like structure in different locations of post-meiotic germ cells, including the peri-acrosome area, peri-nuclear area, midpiece and tail, we hypothesize they are structural proteins involved in the formation of subcellular compartments-head, neck, midpiece and tail, during terminal differentiation of male germ cells. Recent studies show that septins have a role in microtubule-dependent processes, such as karyokinesis, exocytosis and maintenance of cell shape. In addition, many members of the septin family have been shown to associate with the microtubule cytoskeleton. We speculate SEPTs, e.g., SEPT12, may participate in cytoskeleton remodeling during terminal differentiation of male germ line.[21,22]

Reproductive Phenotypes of *Septin4$^{-/-}$* and *Septin12$^{+/-}$* KO mice: Similarities and Differences

Septin4$^{-/-}$ male mice are sterile due to defective morphology and motility of the sperm flagellum. In *Septin4$^{-/-}$* null spermatozoa, the annulus is disrupted with by a fragile segment lacking cortical material.[12] In addition, *Septin4$^{-/-}$* mutant sperm showed defects in acrosome and mitochondrial architecture and retention of the cytoplasmic droplet.[12,13] *Septin12$^{+/-}$* chimeric mice shared some common features with the *Septin4$^{-/-}$* male: defects of annulus, mitochondrial architecture and acrosome. Retention of cytoplasmic droplet was also observed in *Septin4$^{-/-}$* as well as *Septin12$^{+/-}$* chimeric mice.[18] However, the phenotypic effect of SEPT12 deficiency seems to be more profound than SEPT4. First, deletion of a single allele of *Septin12* is sufficient to produce profound phenotypes. Second, immature germ cells exfoliated from the seminiferous tubules possibly due to maturation arrest at the round spermatid stage. Third, the acrosome defect is more apparent. There is also nuclear defect associated with haploinsufficiency of SEPT12. Electron microscopy examination of spermatozoa purified from the cauda epididymis revealed ultrastructural abnormalities only in the spermatozoa of *Septin12$^{+/-}$*chimeras, including misshapen nuclei and broken acrosome. Some vesicles were found in the sperm nuclei, suggesting nuclear damage. In the chimeric mice with high percentage of SEPT12 deficient cells, almost all germ cells found in the cauda epididymis were round spermatids without tail formation. These findings suggest expression level of SEPT12 is critical for the formation of four major compartments (acrosome, mitochondrial, tail and nucleus) during spermiogenesis.

SEPT12 and DNA Damage

Although *Septin4*$^{-/-}$ mutant sperm could not fertilize oocytes, the sterility could be rescued by injection of the mutant sperm into oocytes, demonstrating mutant sperm carry an intact haploid genome.[12,13] Consistently, oocytes fertilized by spermatozoa of *Septin12*$^{+/-}$ KO mice using intracytoplasmic sperm injection (ICSI) can't develop beyond the morula stage, possibly due to significant nuclear DNA damage.[20] Given that SEPT12 is expressed at the edge of the sperm nucleus in both humans and mice, we hypothesized the vital roles of *Septin12* in sperm head shaping, nuclear DNA condensation and early embryonic development.[20] This finding is in line with the observation of misshapen nuclei in the sperm of *Septin12*$^{+/-}$ KO mice.[18]

Expression Patterns of SEPTs in Infertile Men

In humans, SEPT12 is expressed at the edge of the sperm nucleus, sperm neck, mitochondria and annulus during spermiogenesis.[20] However, sperm with head, neck or tail abnormalities tended to lose their SETP12 and SEPT7 signals.[18,19] In humans, disorganized annulus/SEPTIN rings were also identified in a subset of human patients with asthenozoospermia.[12,23,24] These findings suggest defective synthesis, increased degradation or dysfunction of SEPTs may be causally related to both motility and morphological defects of sperm.

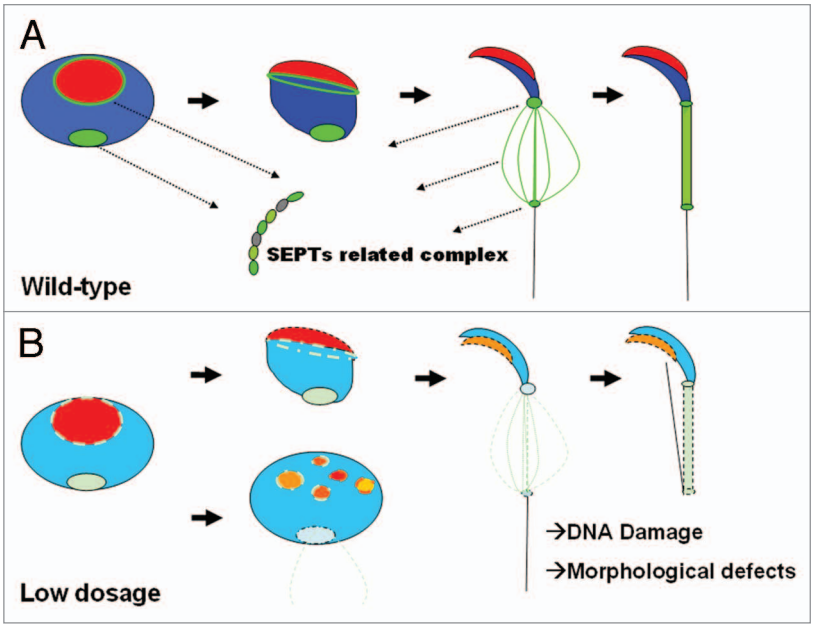

Figure 1. Working model of SEPT-related complex during mammalian spermatogenesis. (A) We reason that SEPT12-related complex consists of SEPT12, SEPT1, 4, 6 and 7. In the wild-type spermatozoa, SEPT12 coils around the arcosome and is concentrated at the neck of sperm. With the formation of mitochondria, SEPT12 starts to express at the neck and annulus. The SEPT 12 filaments also cover the mitochondrial area. (B) Decreased expression of SEPT12, results in maturation arrest at the spermatid stage, broken acrosome, bent tails, disorganized mitochondria and nuclear DNA damage.

Septins as Sterile Genes

The causes of infertility could not be identified in the majority of cases with spermatogenic defects.[25] The pathology of male infertility is diverse, including anatomic defects, gametogenesis dysfunction, endocrinopathies, immunologic problems, ejaculatory failure, environmental exposures and gene mutations.[26-29] Given the distinct reproductive phenotype of the *Septin12*$^{+/-}$ chimeric mice and unique expression pattern of SEPT12, *SEPTIN12* seems to be a good candidate for sterile gene in humans. Recently, Miyakawa et al. reasoned *SEPTIN12* as a good candidate gene for male infertility and chose cases with Sertoli-cell-only syndrome (SCOS) to test their hypothesis.[30] They compared genetic variants of 140 healthy men and 100 cases with SCOS and identified eight single-nucleotide polymorphisms (SNPs) in *SEPTIN12*. Among these SNPs, three synonymous variants were more prevalent in the SCOS patients, but their functional significance was not characterized. We also have found two missense mutations in men with infertility men compared with controls located in the predicted GTP-binding domain of *SEPTIN12*. Importantly, patients with mutations of SEPTIN12 were presented with oligo-astheno-teratozzoseprmia (OAT) and distinctive sperm morphology, including defective annulus and bent tail (unpublished data). Recently, SEPT14 was found to be a SEPT9-interacting partner and was exclusively expressed in the testis.[31] The role of *SEPTIN14* in spermatogenesis and male infertility also deserves further investigation.

SEPT Complex in Sperm

SEPTs usually mediate their cellular function through the formation of macromolecular and hetero-oligomeric filaments.[3,9] Biochemical methods have been used to isolate several SEPT complexes (e.g., SEPT2/6/7, SEPT7/9b/11 and SEPT4/5/8).[9,32,33] The filament-like structure was also observed in many SEPTs[8,10,34] and loss of a SEPT subunit may affect the stability of the complex.[9,10,35] The oligomeric core of SEPT-related complex in sperm remains to be uncovered. Since many SEPTs found in spermatids/spermatozoa are located at the annulus, there may be functional redundancy for some SEPTs. *Septin12*$^{+/-}$ KO mice shared similar phenotypes reminiscent of those for the *Septin4*$^{-/-}$ KO mice, but there are some obvious differences between mouse models of these two genes. SEPT12 has been found to interact with SEPT6 and SEPT11 and forms filaments in Hela cells.[36,37] It is highly likely that SEPT12 cooperates in interactions with fixed stoichiometry with other SEPTINS (e.g., SEPT1, 4, 6, 7 or 11). Considering its dosage-sensitive effect, SEPT12 may play a pivotal role in the complex formation during spermiogenesis (**Fig. 1**).

Proteins in the GTPase superfamily usually engineer molecular switch to promote GTP binding and hydrolysis. Because the

consensus GTPase domain exists in all SEPTs, it is expected that assembly of SEPT complex are mediated through GTPase signaling. So far, GTP binding/hydrolysis has not been demonstrated in the SEPT-related complex of sperm. However, we did observe decreased GTP binding/hydrolysis in two patients who carried missense mutations in the predicted GTP-binding domain of *SEPTIN12* (unpublished data). Some proteins located at sperm annulus may be involved in regulating assembly/disassembly or stability of SEPT-related complex, including a novel Male Germ Cells Rab GTPase-Activating Proteins (MgcRabGAP), DNAJB13, a type 2 heart shock protein 40 (HSP40) and also a component of mouse sperm axoneme and TAT1, a new family member of Slc26 family of anion transporters.[38-40] *Tat1* null males were sterile due to disorganization of the midpiece-principal piece junction and abnormal mitochondrial sheath assembly, a phenotype characteristic of SPET4 and SEPT12 deficiency.[40]

It is also intriguing how SEPT12 deficiency results in nuclear DNA damage. In yeast, all of five septins, Cdc3p, Cdc10p, Cdc11p, Cdc12p and Shs1p/Sep7p, in the SEPT complex interacted with FHA domain of Rad 53, an important DNA damage checkpoint kinase.[41] Shs1, one of these septins, appears to have an important role in the response to DNA replication stress.[41] Cdc3p also interacted with BUB2, which is important to maintain a mitotic arrest during kinetochore damage.[42] In mammalian cells, SEPT2/6/7 complexes regulate actin organization and are links to the DNA damage checkpoint by accumulation of adaptor protein, NCK, in nucleus.[43] Whether the SEPT/SCOS7/NCK pathway is conserved during evolution deserves further investigation.

Prospects

Of all family members, four septin genes have been targeted in the mouse. Except for *Sepint4* and *Septin12*, *Septin3, Septin5* and *Septin6* deficiencies do not cause overt phenotypes, suggesting a high degree of functional redundancy in the SEPTIN system.[12,13,18,44-46] SEPT12 thus becomes an ideal target for male contraception. Mature spermatozoa consist of four major compartments: acrosome, nucleus, midpiece and tail. How these compartments are formed during terminal differentiation still remains obscure. Studies on the septin gene family may provide further insights into the biochemical basis of intracellular compartmentalization during spermatogenesis.

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