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MAMMALIAN SPERM FLAGELLA AND CILIA

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To my family

ABSTRACT

In mammals, both sperm flagella and cilia are axoneme-containing organelles.

The sperm flagella are major parts of the spermatozoa that are male gametes produced in the testis from progenitor spermatogonia through a process called spermatogenesis. During last stage of spermatogenesis, also called spermiogenesis, an axoneme grows out from one of the two centrioles to form the flagellum. With the growth of the axoneme, accessory components including mitochondria, outer dense fibers, fibrous sheath and annulus are added to the flagellum to form its middle, principal and end pieces. The sperm flagellum is responsible for the sperm motility, and defects in it often cause male infertility.

Unlike sperm flagella, cilia contain only axonemes covered with the plasma membrane. Generally two types of cilia exist in mammals: multiple motile cilia and single primary cilia. All cilia extend from basal bodies that are derived from centrioles. Cilia play important physiological roles in the body. Defects in them cause a large number of genetic diseases, such as polycystic kidney disease, retinal degeneration, hydrocephalus, laterality defects, chronic respiratory problems, and even obesity and diabetes.

In this thesis, I characterized two proteins related to mammalian sperm flagellum development and ciliogenesis: DNAJB13 and SMC1.

Dnajb13 is a type II Hsp40 gene once reported to be highly expressed in testis. By multi-tissue RT-PCR, I found that it was also expressed in several ciliated tissues. In mouse testis, *Dnajb13* mRNA was detectable at postnatal week 1 while DNAJB13 was undetectable until postnatal week 4, indicating a translational control for this gene. Although being an Hsp40, DNAJB13 was not heat-inducible. In the cross-sections of seminiferous epithelium, DNAJB13 was first detectable in step2-3 spermatids, peaked at step9-10 and then gradually decreased afterwards. In a mature spermatozoon, DNAJB13 was present in the flagellum throughout the length. Its presence in normal sperm flagellum but not in SDS-resistant sperm tail indicates that it is axoneme-associated, which is further confirmed by its presence in motile cilia of airway and oviduct epithelia. By immunoelectron microscopy DNAJB13 was defined as a radial spoke protein.

DNAJB13 is also associated with the annulus spatiotemporally during mouse sperm flagellum development. This association started from the formation of annulus. The annular DNAJB13 increased with the maturation of annulus, and peaked when the annulus reaches the midpiece-principal piece junction of the flagellum, and then gradually disappeared during late spermiogenesis. In annulus-deficient spermatids, DNAJB13 still formed an annulus-like ring in the neck region. In vitro DNAJB13 was colocalized and interacted with an annulus constituent SEPT4. All the data suggest that DNAJB13 may be involved in the assembly and positioning of the annulus during mouse sperm flagellum development.

Cohesin protein SMC1 is well known for its roles in sister chromatid cohesion and DNA repair. It was also reported to be present in primary cilia. By immunofluorescence, I found that SMC1 also localized to centrosomes throughout the cell cycle in a microtubule-independent manner. In addition, SMC1 was associated with both centrioles of a centrosome. Biochemically, SMC1 was cofractionated with the centrosomal marker γ-tubulin in centrosomal preparation. In vivo SMC1 localized

to the basal bodies of motile cilia. These data suggest that SMC1 is also a centrosome/basal body protein and may play a role in ciliogenesis.

LIST OF PUBLICATIONS

This thesis is based on the following papers.

I. A heat-shock protein 40, DNAJB13, is an axoneme-associated component in mouse spermatozoa.

Jikui Guan, Li Yuan*
Mol Reprod Dev. 2008 Sep;75(9):1379-86.

II. DNAJB13 is a radial spoke protein of mouse '9+2' axoneme.

Jikui Guan, Emelie Ekwurtzel, Ulrik Kvist, Kjell Hultenby, Li Yuan*
Submitted, 2009

III. Spatiotemporal association of DNAJB13 with the annulus during mouse sperm flagellum development.

Jikui Guan, Makoto Kinoshita, Li Yuan*
BMC Dev Biol. 2009 Mar 19;9(1):23. [Epub ahead of print]

IV. Cohesin protein SMC1 is a centrosomal protein.

Jikui Guan, Emelie Ekwurtzel, Ulrik Kvist, Li Yuan*
Biochem Biophys Res Commun. 2008 Aug 8;372(4):761-4.

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LIST OF ABBREVIATIONS

AKAP	cAMP-dependent protein kinase anchoring protein
aPKC	Atypical protein kinase C
Arf	ADP-ribosylation factor
ATP	Adenosine triphosphate
BBS	Bardet-Biedl syndrome
cAMP	Cyclic adenosine monophosphate
Cep	Centrosomal protein
CP	Centrosomal protein
CRB	Crumbs homolog
DNA	Deocyribonucleic acid
Dnah(c)	Dynein axonemal heavy chain
DNAJB	DnaJ homolog subfamily B
EM	Electron microscopy
Foxj	Forkhead box J
FS	Fibrous sheath
GAPD-S	Spermatogenic cell-specific glyceraldehyde 3-phosphate dehydrogenase
GTP	Guanosine triphosphate
Gtrgeo	Gene trap ROSA b-geo
HDAC	Histone deacetylase
HFH	Hepatocyte Nuclear Factor 3/forkhead homologue
HSP	Heat shock protein
IB	Immunoblotting
IEM	Immunoelectron microscopy
IF	Immunofluorescence
IFT	Intraflagellar transport
IHC	Immunohistochemistry
Jund	Jun proto-oncogene-related gene d
KIF	Kinesin superfamily protein
KO	Knockout
LC	Longitudinal columns
Mkks	Mckusick-Kaufman syndrome
MS	Mitochondrial sheath

Neur	Neuralized
Nna	Nervous system nuclear protein induced by axotomy
ODF	Outer dense fiber
Pacrg	Park2 co-regulated
Pals	Protein associated with Lin-7
PAR	Partitioning-defective
PAS	Periodic acid-Schiff
PATJ	Pals1 associated tight junction protein
PF	Paralysed flagella
Pgk	Phosphoglycerate kinase
RSP	Radial spoke protein
Sar	Secretion-associated, ras-related
SDS	Sodium dodecyl sulfate
SEPT	Septin
Shh	Sonic hedgehog
SMC	Structural maintenance of chromosome protein
Spag	Sperm-associated antigen
TAT	Testis anion transporter
Vdac	Voltage-dependent anion channel
Wnt	Wingless-int

1. SPERMATOGENESIS

Spermatogenesis is a complex biological process in which progenitor spermatogonia develop into mature spermatozoa. In mammals this process occurs in the seminiferous tubules of the testes (**Figure 1**). It takes approximately 64 days in human beings and approximately 35 days in mice.

The seminiferous tubule consists of a specialised spermatogenic epithelium encircling a central lumen. Two distinct cell populations constitute the epithelium: the Sertoli cells and the spermatogenic cells including spermatogonia, spermatocytes and spermatids. Sertoli cells are somatic cells residing among the spermatogenic cells and supporting spermatogenesis. The spermatogonial stem cells located at the basal compartment of the tubule renew themselves by mitosis and simultaneously generate the proliferative spermatogonia that will undergo numerous rounds of mitoses and differentiate into primary spermatocytes. In mice, this spermatogonial proliferation takes about 10 days, followed by an ~11-day-long meiotic stage in which a spermatocyte undergoes two successive cell divisions and produces four round spermatids. As the genomic DNA only replicates once during this stage, the resultant spermatids are haploid. The spermatids then undergo a drastic transformation and mature into streamlined spermatozoa adapted for fertilization. This process is called spermiogenesis and takes about 14 days in mice (Russell et al., 1990).

Spermatogenesis occurs in a wave manner along the length of a seminiferous tubule, and it repeats at one location over time. To simplify its complexity, spermatogenesis is divided into different stages based on the cell association observed in each tubule cross-section (Leblond and Clermont, 1952).

1.1. Stages of spermatogenesis

In cross-sectioned seminiferous tubules (**Figure 1**), several types of germ cells are more or less concentrically layered with the support from Sertoli cells, and germ cells of a particular type appear developmentally relatively synchronized. In addition, the composition of germ cell types is relatively identical in all cross-sectioned seminiferous tubules at the same developmental progression.
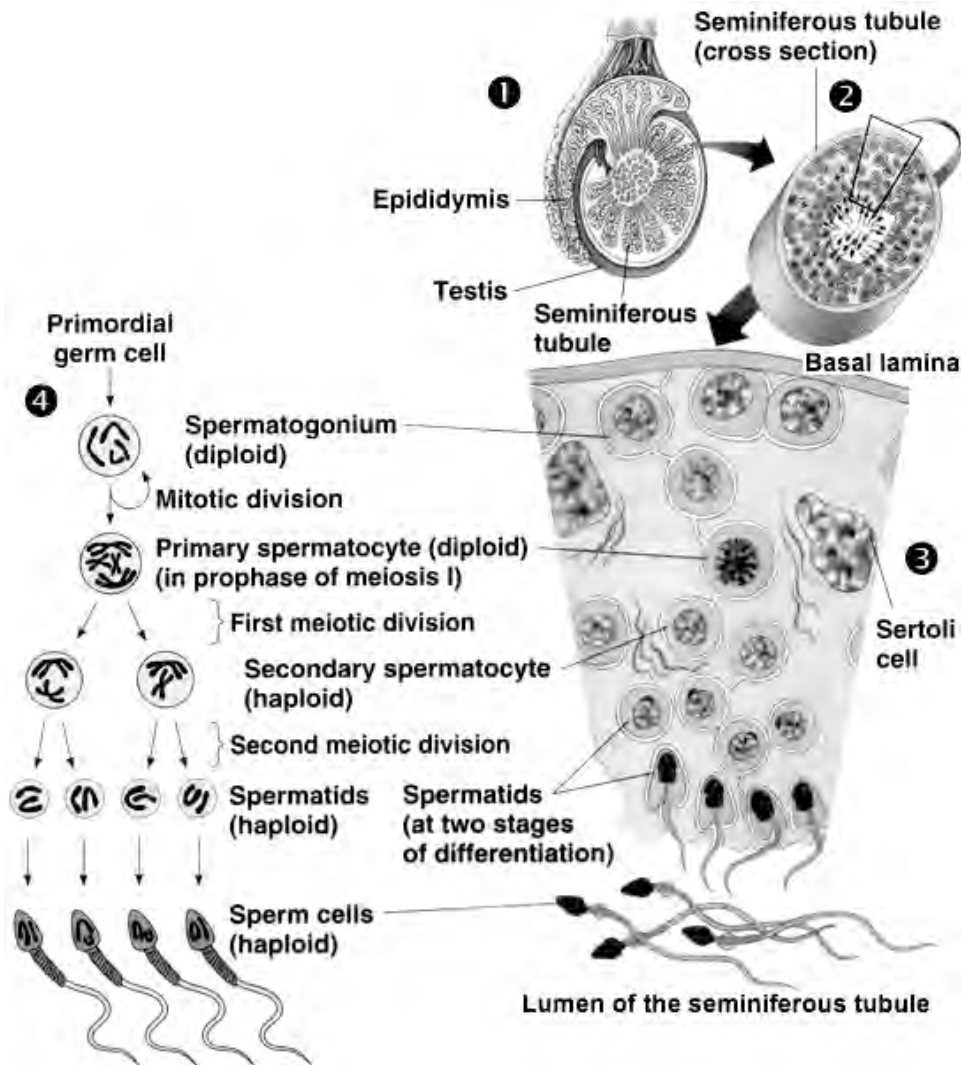
Figure 1. Schematic drawings of the testis, seminiferous tubules and spermatogenesis. The testis (❶) houses a network of seminiferous tubules (❷) in which spermatogenesis (❹) takes place. A detailed view of the seminiferous epithelium (❸) corresponding to the boxed region in ❷ shows its composition and arrangement of germ cells at different phases of spermatogenesis. [Picture downloaded from the Internet with some modification]

Based on the above facts, a **cell association** or **stage** is defined as a grouping of germ cell types at particular phases of development in cross-sectioned tubules (Leblond and Clermont, 1952; Russell et al., 1990). Conventionally, each stage is designated by a Roman numeral. A complete spermatogenesis contains 12 designated stages in mice (**Figure 2**).

1.2. Steps of spermiogenesis

Generally, young spermatids are used to define stages of spermatogenesis due to their most easily recognizable morphological features. With special staining like PAS staining and PAS-hematoxylin staining, changes in the acrosomal region of the spermatids and in their head shape are easily identified under the light microscope. A defined morphological entity of spermatid development is called a **step of spermiogenesis**. Steps of spermiogenesis are designated by Arabic numbers (**Figure 2**). There are 16 designated steps in mouse spermiogenesis (Leblond and Clermont, 1952; Russell et al., 1990).
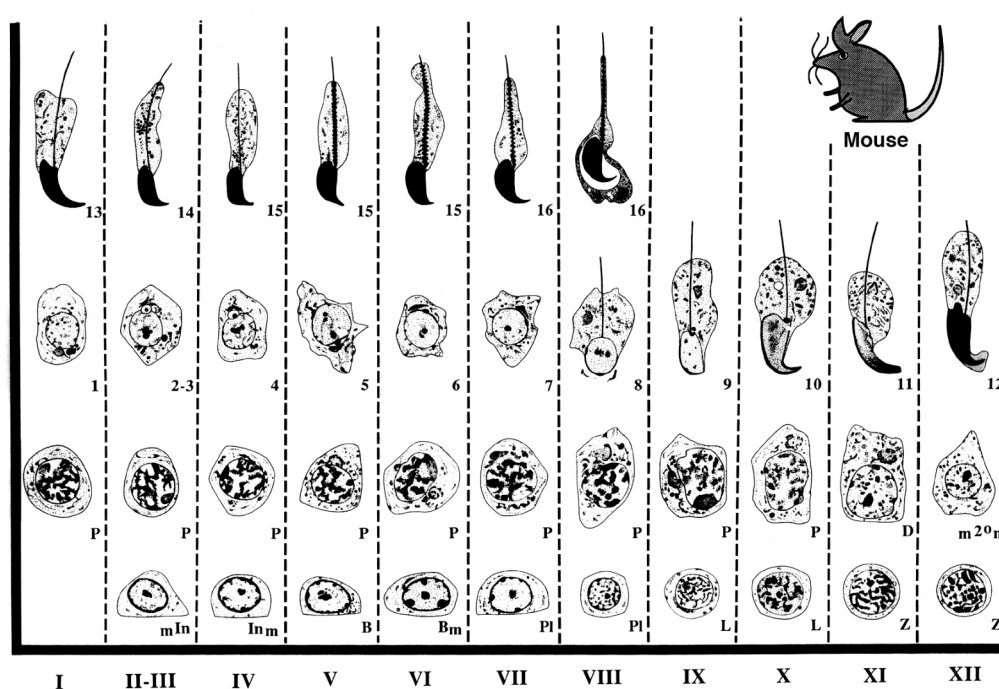

Figure 2. Stages of spermatogenesis and steps of spermiogenesis in the mouse. The vertical columns designated by the Roman numerals depict the stages (cell associations). The Arabic numbers depict the steps of spermiogenesis. The specific phases of cell development designated by the symbols are: type A spermatogonia in mitosis (m^{In}), intermediate spermatogonia in mitosis (In_m), type B spermatogonia (B), type B spermatogonia in mitosis (B_m), preleptotene spermatocytes (Pl), leptotene spermatocytes (L), zygotene spermatocytes (Z), pachytene spermatocytes (P), diplotene spermatocytes (D) and secondary spermatocytes in meiosis ($m^{2°}m$). [Adapted from (Russell et al., 1990)].

1.3. Spermiogenesis

During the last stage of spermatogenesis, round spermatids mature into streamlined spermatozoa that can swim through female reproductive ducts to fertilize eggs. This process is called **spermiogenesis**. It takes place in the adluminal compartment of the seminiferous tubule (**Figure 1**). During spermiogenesis, round spermatids undergo great morphological changes including development of the flagellum and the acrosome, nuclear shaping and condensation, and elimination of cytoplasm.

The sperm flagellum develops from the very start of spermiogenesis. In the very youngest spermatid, the centriole pair migrates to the cell surface and one of the two centrioles forms an axoneme that rapidly protrudes from the cell. The centriole pair forming the flagellar axonme then migrates back to the nucleus and finally indents it, which causes the plasma membrane attached to the centriole to become infolded. As the axoneme grows, accessory components including mitochondria, outer dense fibers, fibrous sheath and annulus are added to the flagellum to build its middle, principal and end pieces. The development of flagellum is a continuous process occurring throughout whole spermiogenesis (Russell et al., 1990).

The acrosome is a cap-like structure at the anterior end of the sperm head that releases digestive enzymes to aid the penetration of egg envelopes by the spermatozoon (Fawcett, 1975). During early spermiogenesis, the Golgi apparatus produces small vesicles and granules that eventually fuse into one large membrane bounded vesicle containing a single granule called acrosomal vesicle. Shortly after it contacts the nucleus, the acrosomal vesicle indents the nucleus somewhat and begins to flatten into a small cap over the nuclear surface. With the progress of spermiogenesis, the acrosome continues to spread over approximately 1/3 of the nuclear surface until the nucleus begins to elongate. Because the Golgi apparatus moves away, the acrosome appears not to grow further in mass but becomes dense gradually. During late spermiogenesis, the acrosome migrates over the ventral surface of the elongating nucleus, and the mature acrosome covers nearly all the nucleus except for the part connected to the flagellum (Russell et al., 1990).

Sperm of different species display their characteristic head shapes. Generally the mammalian sperm head shape is spatulate. However, sperm heads of rats and mice as well as other rodents are somewhat sickle-shaped. It is believed that the manchette, a transient microtubule structure formed around the nucleus, plays an important role in

nuclear shaping (Kierszenbaum et al., 2007; Kierszenbaum and Tres, 2004; Toshimori and Ito, 2003). But it is also argued that nuclear shape may be the result of an intrinsic nuclear condensation pattern (Fawcett et al., 1971; Swierstra et al., 1974). Nuclear condensation is accomplished by replacing somatic and testis-specific histones with transition proteins, and subsequently protamines (Dadoune et al., 2004). The high degree of nuclear DNA packing decreases the nuclear volume substantially. Through changes in size and shape, sperm heads become hydrodynamically streamlined (Russell et al., 1990).

Before sperm release, most of their cytoplasm is eliminated to make them smaller and more streamlined. This takes place in three phases. First, water is eliminated from the nucleus and cytoplasm during the elongation of the spermatid. Second, some cytoplasm is eliminated by tubulobulbar complexes shortly before sperm release. Third, a cytoplasmic package called residual body is separated from the spermatid at sperm release. The eliminated cytoplasm is phagocytosed and digested by Sertoli cells, and subsequently used by them as an energy source (Xiong et al., 2009). After cytoplasmic elimination, a small portion of cytoplasm remains around the neck of the spermatid called cytoplasmic droplet (Russell et al., 1990).

2. SPERM FLAGELLUM

The flagellum is the major part of a spermatozoon and is responsible for its movement. Structurally, it is divided into four pieces: the connecting piece, the middle piece, the principal piece and the end piece (Fawcett, 1975). The short connecting piece attaches the flagellum to the implantation fossa of the sperm head. The core of the flagellum is a microtubule structure called axoneme that extends from the connecting piece to the end of the flagellum. The axoneme is surrounded by nine outer dense fibers (ODFs) and a mitochondrial sheath (MS) in the middle piece. An annulus marks the termination of the middle piece and the start of the principal piece. In the principal piece, two longitudinal columns (LCs) of the fibrous sheath (FS) replace two of nine ODFs, reducing the ODF number to seven. The two LCs run the length of the principal piece and are stabilized by the circumferential ribs of the FS surrounding the ODFs. The end piece contains only the axoneme covered by the plasma membrane (**Figure 3**).
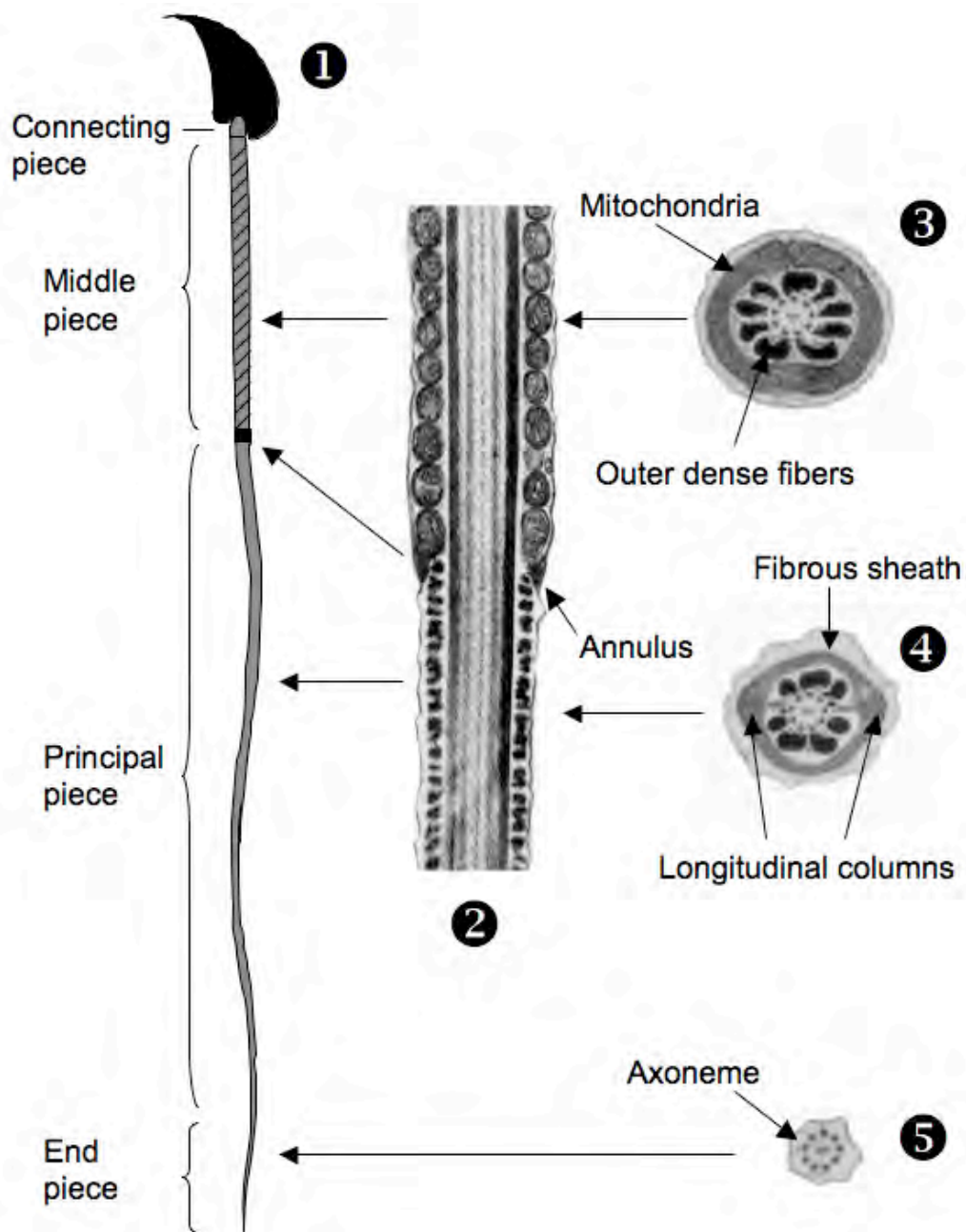
Figure 3. The sperm flagellum. (❶) A diagram of the spermatozoon whose flagellum is divided into four pieces: the connecting piece, the middle piece, the principal piece and the end piece. Electron microscopy (EM) of a longitudinal section (❷) and three cross sections (❸, ❹, ❺) of the flagellum showing the arrangement of axoneme, outer dense fibers, mitochondrial sheath, fibrous sheath and annulus in different pieces.

2.1. Axoneme

The motor apparatus of the sperm flagellum is the axoneme (Fawcett, 1975). The sperm axoneme is a '9+2' microtubule structure, in which nine doublet microtubules arranged in a ring surround two central singlet microtubules. Several other macromolecular complexes including outer and inner dynein arms, the dynein regulatory complex, nexin links, and radial spokes are anchored to the outer doublets (**Figure 4**).
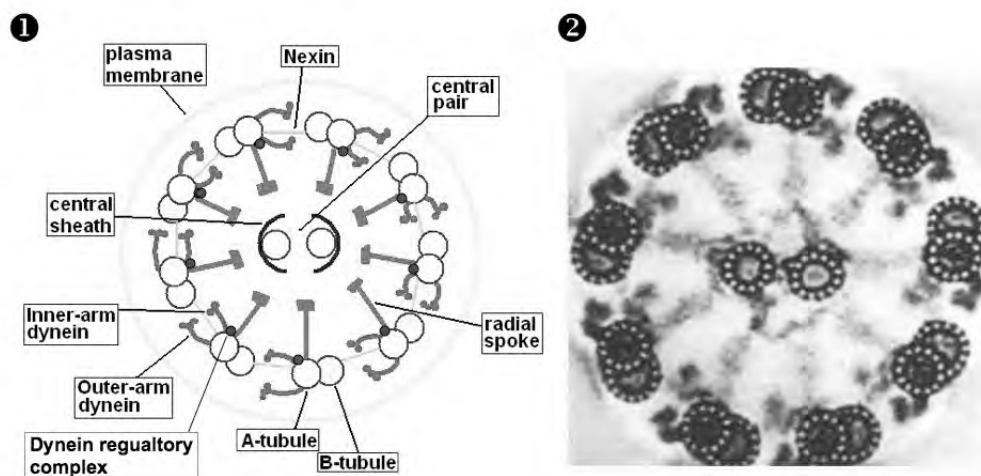

Figure 4. Typical '9+2' Axoneme. (❶) A schematic drawing of axoneme showing its structure and components; (❷) A transmission electron microscopic image of axoneme. [Pictures downloaded from the Internet with some modification]

The dynein arms are molecular motors generating the sliding force between adjacent doublet microtubules by hydrolysis of ATP (Gibbons, 1996). Both outer and inner dynein arms are macromolecular complexes consisting of heavy chains, intermediate chains and light chains (Inaba, 2007), of which the heavy chains have ATPase activity. The dynein regulatory complex is located at the junction between radial spokes and the inner dynein arms, and is thought to coordinate the dynein arm activity. Flexible nexin molecules link adjacent doublets together. Radial spokes are T-shaped structures (Qin et al., 2004; Yang et al., 2006) protruding towards the central pair and are believed to regulate the axonemal beating together with the central pair.

The '9+2' axoneme is a conserved structure in motile cilia and flagella from protozoa to metozoa (Inaba, 2007), and knowledge about it mainly comes from studies on the green algae *Chlamydomonas reinhardtii*. This fine machinery possesses a large number of proteins. With both traditional genetic approaches and modern proteomic tools, over 250 proteins are identified in *Chlamydomonas* flagellar axoneme (Luck, 1984; Pazour et al., 2005), and over 200 potential proteins are identified in human ciliary axoneme (Ostrowski et al., 2002). To function, the axoneme must be first assembled. It is now clear that intraflagellar transport (IFT), a conserved process among ciliated organisms, is required for the axonemal assembly (Baker et al., 2003; Cole et al., 1998; Kozminski et al., 1993; Pazour et al., 2002; Pazour et al., 2000). IFT particles target and then move axonemal components from the cell body to the tip of the flagellum where axonemal assembly occurs. To simplify the whole process, many axonemal components, like dynein arms and radial spokes that consist of multiple subunits, are pre-assembled in the cell body before being transported into the flagellum (Qin et al., 2004). In most cases, these components are added to the growing axonemal tip, but in the case of radial spokes, assembly can occur in a tip to base direction (Johnson and Rosenbaum, 1992).

As described previously, the sperm axoneme grows out from one of the two centrioles beneath the cell plasma membrane in the very youngest spermatids. With targeted gene disruption technology, a few genes involved in mouse sperm axoneme development have been identified (Escalier, 2006). BBS2 and BBS4, which stand for Bardet-Biedl syndrome (BBS) 2 and 4 respectively, localize to the basal bodies of cilia-related structures with potential roles in basal body dysfunction. Male *Bbs2* and *Bbs4* knockout (KO) mice are sterile because the spermatid fails to produce a flagellum (Mykytyn et al., 2004; Nishimura et al., 2004). *Mkks/Bbs6* null male mice give a similar phenotype (Fath et al., 2005). Genetic disruption of mouse *Dnahc7* (Neesen et al., 2001) and *Tektin-t* (Tanaka et al., 2004) causes defects in inner dynein arms. The sperm-associated antigen (Spag) 6 and 16 are orthologs of *Chlamydomonas* PF16 and PF20 respectively. Disruption of mouse *Spag6* and *Spag16* results in loss of the central pair and disorganization of outer microtubule doublets in the flagellar axoneme (Sapiro et al., 2002; Zhang et al., 2006). In *Neur1* (Vollrath et al., 2001) and *Vdac3* (Sampson et al., 2001) KO mouse models, the flagellar axoneme generally loses doublet No 7, while in *Gtrgeo22* KO mouse model (Campbell et al., 2002), it loses outer doublets and the central pair progressively. In addition, *Pacrg* (Lorenzetti et al., 2004), *Nna1*

(Fernandez-Gonzalez et al., 2002) and *Jund1* (Thepot et al., 2000), are also suggested to be involved in axonemal assembly because disruption of them causes disorganization of the axoneme. The above-mentioned genes encode a broad range of proteins, including transcription factors, ion channels, enzymes in addition to axonemal proteins themselves, suggesting that a complex regulating network underlying the axonemal development.

2.2. Outer dense fibers

The ODFs are hypothesized to provide a structural support for the long sperm tail (Fawcett, 1975; Turner, 2006). So far a few genes encoding ODF proteins and ODF-binding proteins have been cloned (Brohmann et al., 1997; Iida et al., 2006; Morales et al., 1994; O'Bryan et al., 2001; Petersen et al., 2002; Petersen et al., 1999; Salmon et al., 2006; Schalles et al., 1998; Shao et al., 1998; Shao et al., 1999; Shao et al., 2001; Tarnasky et al., 1998; Xue et al., 2002; Zarsky et al., 2003), but due to lack of available ODF-defective sperm models, the true role of ODFs is still not clear.

The development of ODFs has been well studied in rat spermatids by EM and autoradiography (Irons and Clermont, 1982a). Its whole developmental process can be divided into three phases. In the first phase (steps 8-14), nine very fine fibers (also called anlagen of the ODFs) develop in association with the microtubule doublets along the most proximal portion of the axoneme, and gradually increased in length in a proximal-to-distal direction. In the second phase (steps 15-16), the fibers suddenly increase in diameter, resulting from the deposition of electron-dense materials along the length of anlagen of the ODFs. In the last phase (steps 17-19), the fibers mature very slowly into their definitive forms.

2.3. Mitochondrial sheath

Formation of the MS begins at late spermiogenesis around step 15 in mice (Russell et al., 1990). To date, several models have been proposed to demonstrate this developmental process (Baradi and Rao, 1979; Ho and Wey, 2007; Otani et al., 1988; Woolley, 1970), among which the latest one divides it into four stages. In stage 1, spherical mitochondria line up regularly and attach on the ODFs around the middle piece, forming four dextral longitudinal arrays. In early stage 2, mitochondria elongate

laterally and become crescent-shaped. The elongating two ends stagger between the mitochondria at the adjacent arrays. In late stage 2, pairs of mitochondria from the opposing arrays contact each other forming a doughnut-shaped structure. In stage 3, mitochondria continue to elongate and stagger in a specific pattern, which transforms the MS into a sinistral double helical structure. In stage 4, crescent-shaped mitochondria become rod-shaped and abut end to end (Ho and Wey, 2007).

As in other cells, sperm mitochondria produce ATP by aerobic respiration (Turner, 2006). Therefore, the MS is believed to generate energy for sperm locomotion (Fawcett, 1975). However, the mitochondrial ATP is not the only energy source, because on one hand it is not sufficient to diffuse along the length of the flagellum to meet the energy requirement of the axonemal dyneins (Du et al., 1994), and on the other hand sperm still produce ATP in the case of a dysfunction of the mitochondria (Narisawa et al., 2002).

2.4. Fibrous sheath

The principal piece, which occupies about two-thirds of the length of the flagellum, is defined by the FS. The FS consists of two LCs connected by the circumferential ribs. As the flagellum tapers along its length, the columns attenuate and the ribs become more slender (Fawcett, 1975).

The development of the FS has been studied in detail in the rat (Clermont et al., 1990; Irons and Clermont, 1982b; Oko and Clermont, 1989). FS assembly proceeds from distal to proximal along the axoneme and spans almost whole period of spermiogenesis (Eddy et al., 2003). The LCs first appear in step 2 as thin rods near the distal end of the flagellum and increase in length until step 10. The nascent ribs originating from bundles of proteinaceous filamentous materials arise in step 11 and become evenly aligned along the plasma membrane of the principal piece, starting at the distal end. They associate with the columns in a distal to proximal direction during steps 12–14, and in steps 15 and 16 additional ribs are formed and the LCs thicken to complete FS development. FS assembly appears to occur in the same manner in the mouse (Sakai et al., 1986).

Like ODFs, the FS is thought to play a mechanical role in sperm motility by providing a rigid support for the flagellum and influencing its beat plan and shape (Fawcett, 1975; Turner, 2006). Recently, proteins present in or associated with the FS have been

identified and are mainly categorized into two groups: cAMP-dependent protein kinase anchoring proteins (AKAP), and glycolytic enzymes. The AKAPs, including AKAP4 that is the major constituent of the FS, function as scaffolds for signalling pathway components that regulate sperm motility. The glycolytic enzymes, including lactate dehydrogenase, the spermatogenic cell-specific glyceraldehyde 3-phosphate dehydrogenase (GAPD-S) and hexokinase 1, are responsible for localized ATP production that is required for hyperactivated sperm motility. Based on these new findings, it is now well accepted that the FS has more direct roles in regulation of sperm motility and ATP generation (Eddy, 2007; Eddy et al., 2003; Edwards and Scott, 2000; Turner, 2003; Turner, 2006). In addition, a spermatogenic cell-specific glutathione S-transferase has been identified able to inactivate cytotoxic substances formed due to oxidative stress. Therefore a new role of the FS is proposed in protecting sperm from oxidative stress (Fulcher et al., 1995).

2.5. Annulus

The sperm annulus is a ring-like structure existing in all mammalian spermatozoa. It was identified 100 years ago and formerly called "Jensen's ring". At low magnification, it appears dense and homogeneous, but at high magnification it shows to be composed of closely packed filamentous subunits oriented circumferentially (Fawcett, 1970). Recent studies show that it is a septin-based structure consisting of SEPT1, 4, 6, 7 and 12 (Ihara et al., 2005; Kissel et al., 2005; Steels et al., 2007).

The importance of annulus in maintaining the structural rigidity and integrity of mammalian spermatozoa is illustrated by the SEPT4-null mouse spermatozoa, which are immotile due to lack of the annulus and often show bent-back tail morphology at the midpiece-principal piece junction (Ihara et al., 2005; Kissel et al., 2005). TAT1-null mouse spermatozoa show similar structural abnormities as SEPT4-null spermatozoa. In this sperm model, the annulus is present but abnormally shaped and only linked to the MS, indicating the importance of a normal and intact annulus apparatus for male fertility (Toure et al., 2007). In addition, the annulus has been proposed to establish a gated diffusion barrier between the midpiece and the principal piece for membrane-bound proteins (Cesario and Bartles, 1994; Ihara et al., 2005; Kissel et al., 2005).

It has been shown by EM that the annulus can be detected shortly after the flagellum formation and migration toward the nucleus in the early stage of cat spermiogenesis

(Yasuzumi et al., 1972). In Chinese hamster spermiogenesis, the anlage of the annulus is first detected in spermatids in which the centriolar pair forming the flagellum has reached and impacted the nucleus (Fawcett et al., 1970). However, ultrastructural studies in rat and mouse spermatids show that the annulus cannot be clearly seen until step 9, and stays at the neck region from step 9 to early step 15 (Campbell et al., 2002; Russell et al., 1990). During late spermiogenesis, the annulus slips towards a more distal position followed by formation of the MS. In a mature spermatozoon, the annulus is located between the midpiece and the principal piece of the flagellum, and beneath the plasma membrane, firmly connecting them together (Fawcett, 1958; Fawcett, 1975; Fawcett et al., 1970).

3. CILIA

Cilia are membrane-bound and axoneme-containing projections from the cell surface found in eukaryotic cells. In the mammalian body, they occur on most cells (Alieva and Vorobjev, 2004) and exist generally in two types: motile cilia and primary cilia. Motile cilia contain the same '9+2' axoneme as the sperm flagella, while primary cilia contain the '9+0' axoneme, which lacks the central pair of singlet microtubules (**Figure 5**). Usually, primary cilia also lack the accessory structures responsible for ciliary movement, and therefore are non-motile. The only exception is the motile embryonic nodal cilia that are responsible for the left-right axis determination during development (Satir and Christensen, 2007).

In addition to the two types of cilia found on the surface of most mammalian cells, certain tissues contain specialised cilia, such as transient non-motile kinocilia of the inner ear and highly modified connecting cilia of the retina (Dawe et al., 2007).

3.1. Motile cilia

In mammals, motile cilia occur on the airway epithelium, along the female reproductive tract, and on the ependymal cells of the brain (Satir and Christensen, 2007). They are present in multiples on the surface of a cell. By beating in coordinated waves, they move mucus, fluid and even other cells over their surfaces. Dysfunction of motile cilia usually causes respiratory disease, ectopic pregnancy, and hydrocephalus.
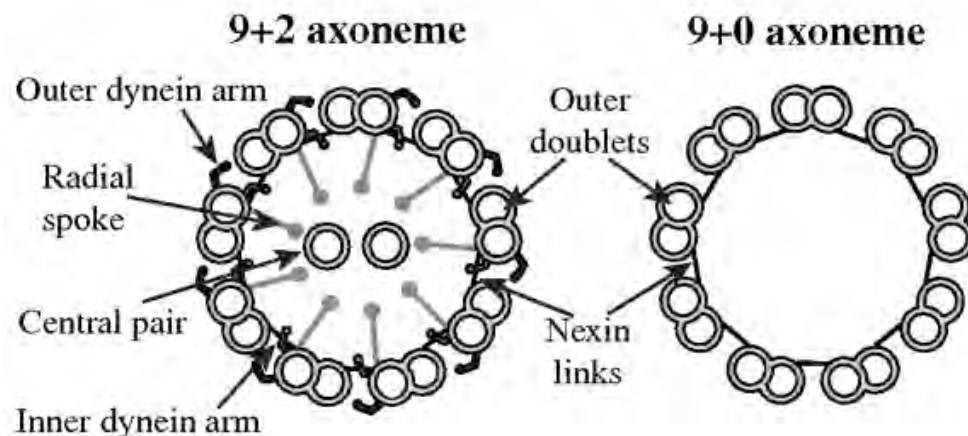
Figure 5. Two different axonemal patterns. Compared with the '9+2' axoneme, the '9+0' axoneme lacks the central pair as well as the accessory structures like the dynein arms and radial spokes. [Adapted from (Dawe et al., 2007) with permission from *J Cell Sci*].

3.2. Primary cilia

In contrast to motile cilia, primary cilia usually occur singly on cells. They are more common than motile cilia in the mammalian body, and also found in some quiescent cultured fibroblasts and epithelial cell lines (Alieva and Vorobjev, 2004; Wheatley, 1995; Wheatley et al., 1996). Primary cilia play pivotal roles in normal tissue homeostasis and embryonic development. Their ciliary membranes contain ion channel proteins and specific receptors like G protein-coupled receptors, making primary cilia function as mechano-sensors and chemo-sensors to initiate signalling pathways (Satir and Christensen, 2007). In addition to sensing environmental stimuli, primary cilia are also involved in intracellular signal transductions, such as Shh signal transduction and Wnt signalling (Singla and Reiter, 2006).

Defects in primary cilia are linked with a number of genetic diseases, such as polycystic kidney disease (Pazour et al., 2000), and BBS, a polygenic disorder featured by retinal degeneration, anosmia, hearing loss, renal abnormalities, polydactyly, obesity, diabetes, and hypertension (Singla and Reiter, 2006).

3.3. Ciliogenesis

Ciliogenesis is a complex sequence of events involving the generation and docking of basal bodies at the apical membrane, followed by ciliary axoneme generation.

All cilia extend from basal bodies (Dawe et al., 2007), which are converted by centrioles migrating and subsequently docking to the apical cell surface. In quiescent cultured cells and some differentiated cells, the single cilium, either a primary cilium or a specialised cilium like kinocilium, extends from a basal body converted by the pre-existing mature centriole of the centrosome in the cell. By contrast, in multi-ciliated epithelia, ciliogenesis begins with centriole multiplication that produces hundreds of centrioles. At least six pathways of ciliogenesis exist (**Figure 6**), which enables the production of many kinds of cilia, as well as sperm flagella (Dawe et al., 2007).

The migration and subsequent docking of centrioles to the cell cortex is the prerequisite for ciliary formation. However, mechanisms controlling this process remain unclear. In multi-ciliated cells, it is the actin-myosin network, rather than the microtubule cytoskeleton, that is involved in the process (Boisvieux-Ulrich et al., 1989; Boisvieux-Ulrich et al., 1990; Lemullois et al., 1988). Recently, HFH-4 (Brody et al., 2000) and Foxj1 (Pan et al., 2007; You et al., 2004), two transcription factors expressed in ciliated epithelia, were revealed to be implicated in centriole migration and/or apical membrane docking. Once a centriole has docked to the membrane, it is known as a basal body. Accessory structures, including transition fibers, striated rootlets and basal feet that define the difference between centrioles and basal bodies, are acquired by centrioles during their migration and/or after migration (Dawe et al., 2007).

As described previously, the IFT machinery is responsible for the growth and maintenance of cilia. IFT particles carrying their cargoes like axonemal precursors are moved to the tip by a kinesin II motor KIF3. After depositing the cargoes and picking up axonemal turnovers, they are moved back to the base by a special cytoplasmic dynein motor called dynein 2 or dynein 1b (Rosenbaum and Witman, 2002; Satir and Christensen, 2007). The ciliary membrane is selectively different from the rest of cell membrane in overall composition. Vesicular trafficking, a process regulated by small GTPases of the Rab and Sar1/Arf families (Takai et al., 2001), is essential for ciliary membrane biogenesis (Leroux, 2007). Recently a complex termed BBSome, composed of seven highly conserved BBS proteins, is required for vesicular trafficking to the cilium with the help from a small GTPase Rab8 (Nachury et al., 2007). In addition,

IFT20, which localizes to the Golgi complex as well as to the basal body and cilium, is shown to direct the movement of ciliary membrane proteins (Follit et al., 2006).
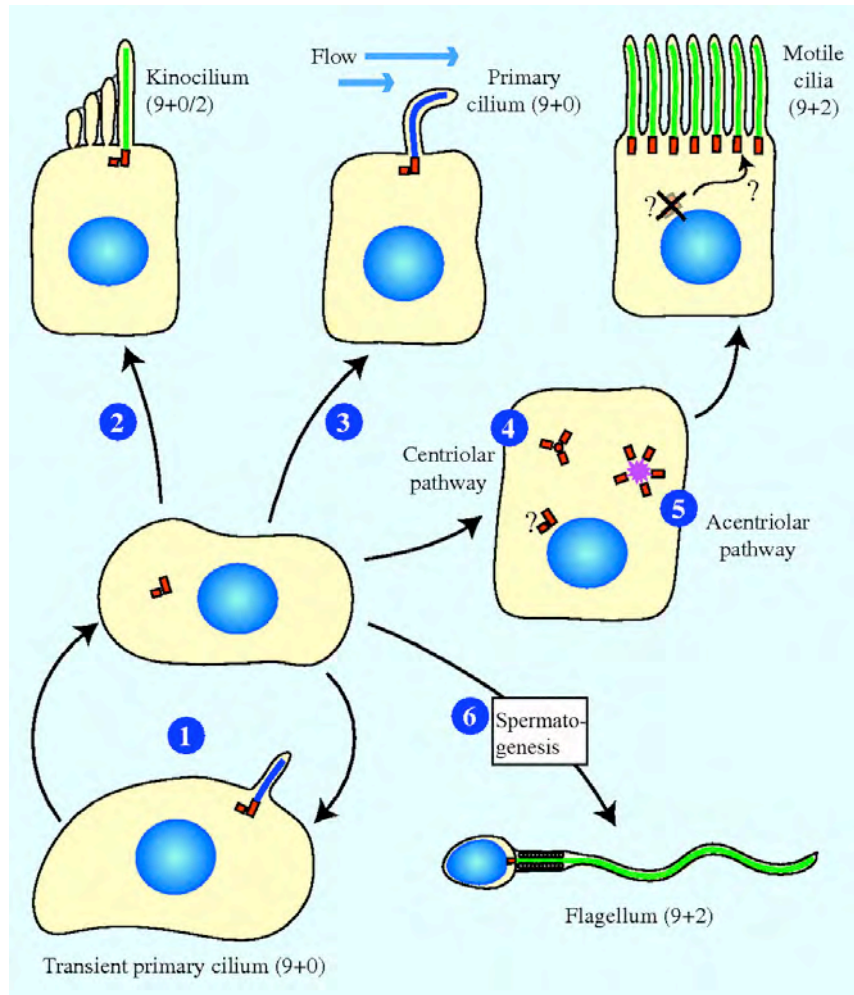

Figure 6. Multiple pathways of ciliogenesis. A quiescent somatic cell uses a pre-existing mature centriole to form a transient primary cilium, which is lost as the cell re-enters the cell cycle (❶). In differentiated cells, several different types of single cilia can be produced from a mature centriole, such as the temporary kinocilium (❷), which may or may not possess the central pair microtubules, and the primary cilium produced on the luminal epithelium of kidney tubules (❸). In multi-ciliated epithelial cells, ciliogenesis begins with centriole multiplication, in which hundreds of centrioles are produced, duplicated either using the pre-existing centriole as a template (❹), or formed via a non-templated method (❺). The sperm flagellum produced in spermatogenesis is depicted at ❻. Green denotes '9+2' axonemes; dark blue denotes '9+0' axonemes; centrioles are shown in red; the deuterosome is shown in purple. [Adapted from (Dawe et al., 2007) with permission from *J Cell Sci*].

Ciliogenesis must be regulated to ensure that the ciliary formation is restricted to stationary phases of the cell cycle. On one hand, aberrant formation of primary cilia at certain points of the cell cycle may alter the balance between growth stimulatory and inhibitory pathways and thus lead to uncontrolled proliferation. On the other hand, failure to resorb cilia prior to entry into mitosis could prevent centrosomes from participating in spindle pole formation and faithful chromosome segregation, leading to elevated genomic instability (Spektor et al., 2007). This depends on the regulation of the duality of centriole function in the context of centrosome and basal body. Now it is known that CP110, a centriolar protein, plays a key role in the regulation. CP110, recruited by Cep97 to the centrosome, inhibits primary cilium formation through interacting with CEP290, a protein that can promote ciliogenesis in cooperation with the small GTPase Rab8a (Spektor et al., 2007; Tsang et al., 2008). The protein level of CP110 is low during quiescence and increases upon cell cycle re-entry to suppress ciliogenesis, so that the duality of centriole function is regulated (Pearson et al., 2007). Upon cell cycle re-entry or prior to entry into mitosis, resorption of the primary cilium to liberate the centriole is essential for cell division (Pan and Snell, 2007). Recent studies show that the centrosomal mitotic kinase Aurora A regulates ciliary resorption. Activated Aurora A phosphorylates and activates the tubulin deacetylase HDAC6, thereby inducing ciliary resorption (Pugacheva et al., 2007).

Ciliogenesis relies on the establishment of cell polarity. The Rho GTPase cdc42 and the PAR3-PAR6-aPKC complex play a central role in cell polarization in diverse cell types (Assemat et al., 2008; Hartleben et al., 2008; Joberty et al., 2000; Koh et al., 2008; Schwamborn and Puschel, 2004; Welchman et al., 2007). Another polarity complex CRB3-Pals1-PATJ is also involved in mammalian epithelial cell polarization (Roh et al., 2003). Disruption of these complexes or their interacting proteins like the von Hippel-Lindau protein (Schermer et al., 2006) causes impaired ciliogenesis (Bossinger and Bachmann, 2004; Fan et al., 2004; Park et al., 2006), indicating an essential role of polarity proteins in ciliogenesis. However, the mechanisms on how those proteins control ciliogenesis remain obscure, despite the finding of an association between polarity complexes and the IFT motor KIF3 (Fan et al., 2004).

4. PRESENT INVESTIGATION

4.1. Aim of the present study

The overall aim of present study was to characterize proteins involved in mammalian sperm flagellum development and ciliogenesis. In particular, I focused on:

- Characterization of DNAJB13, a type II heat shock protein (HSP) 40, about its tissue-specific and developmental stage-specific expression patterns at both mRNA and protein levels; its distribution on tissue sections; and its subcellular and substructural localization in mouse sperm (**Papers I and II**)

- Relationship between DNAJB13 and the annulus during mouse sperm flagellum development (**Paper III**)

- Characterization of SMC1, the cohesin protein, about its novel centrosome/basal body localization (**Paper IV**)

4.2. DNAJB13 localizes to mouse sperm flagella and motile cilia as a radial spoke protein (Papers I and II)

DNAJB13 is a type II HSP40 (**Figure 7**). Its encoding gene *Dnajb13* was once reported to be highly expressed in testes (Liu et al., 2003). To define its function in mouse testis and other tissues, we generated a specific antibody against this protein. With this antibody, we demonstrated that DNAJB13 localized to both mouse sperm flagella and motile cilia as a radial spoke protein of the axoneme.
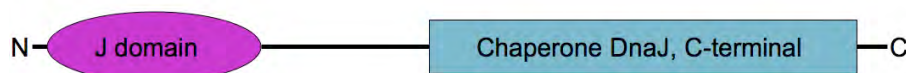

Figure 7. Structural diagram of DNAJB13. DNAJB13 has a J domain, which is a hallmark for all HSP40s and is known to mediate interaction with HSP70 chaperones, and a C-terminal domain for homodimerization and substrate capture.

In mouse testis, the expression of *Dnajb13* mRNA was detectable as early as at postnatal week 1, and developmentally up-regulated to postnatal week 4 and then kept

constant. However, the expression of DNAJB13 protein was undetectable until postnatal week 4. This asynchronous expression pattern suggests that the expression of DNAJB13 is under a translational control. Indeed, many germ cell-specific proteins like protamine and Pgk-2 are also under such a control, which is evolved to solve the conflict between a large demand for protein syntheses and a reduction in transcriptional activity during spermiogenesis (Schafer et al., 1995).

On mouse testis sections, distribution of DNAJB13 was restricted to the spermatid layer. The DNAJB13 immunosignal was present in both the cytoplasm and the developing flagella of spermatids. The cytoplasmic DNAJB13 was detectable from step 2–3, reached a peak in step 9 and 10, and decreased gradually afterward. Mouse round spermatids first appear around postnatal week 3, however, we did not see the expression of DNAJB13 at this time with immunoblotting (IB). This might be caused by the sensitivity difference between the two methodologies. Nevertheless, a burst of *Dnajb13* mRNA expression at postnatal week 3 might account for the initial translation of DNAJB13 in step 2–3 spermatids.

The expression of *Dnajb13* was reported to be significantly changed in experimental cryptorchidism (Liu et al., 2003). In addition, several heat shock factor-binding sites were predicted in the promoter region of *Dnajb13* gene by a transcription element search system. However, we found that both *Dnajb13* mRNA and DNAJB13 protein from heat-treated testes were indistinguishable from their corresponding controls. These data indicate that the expression of DNAJB13 is constitutive in the mouse testis and is not heat-inducible.

IB analysis showed that DNAJB13 was present in mature epididymal spermatozoa. Immunofluorescence (IF) analysis showed that DNAJB13 was colocalized with α-tubulin to the entire length of the sperm flagellum, indicating that DNAJB13 might be present in the axoneme. To examine our hypothesis, we made SDS-insoluble sperm flagella that contain no axonemes (Cao et al., 2006). As expected, DNAJB13 was not present in the SDS-insoluble sperm flagella. These data demonstrate that DNAJB13 is an axonemal component of the sperm flagellum. Further study with immunoelectron microscopy (IEM) defined it to be a radial spoke protein. Indeed, RSP16 (Yang et al., 2005) and CiAxHSP40 (Satouh et al., 2005), the orthologs of DNAJB13 in *Chlamydomonas* and *Ciona* respectively, also localize to the radial spokes, indicating that DNAJB13 is conserved during evolution.

Like sperm flagella, motile cilia also contain the '9+2' axoneme. Therefore DNAJB13 should also be present in motile cilia. To test our hypothesis, we first examined the expression pattern of *Dnajb13* gene in different mouse tissues. As predicted, *Dnajb13* was expressed not only in testis, but also in ciliated tissues like lung, oviduct, brain and ovary. This multi-tissue expression pattern is similar to those of the sperm flagellar genes including Spag6, Pf6, Pf20 and Dnah7, which encode axonemal proteins of the sperm flagella (Horowitz et al., 2005). Subsequent immunohistochemistry (IHC) performed on mouse trachea and oviduct sections demonstrated the presence of DNAJB13 in motile cilia.

It is interesting to know whether DNAJB13 functions as a cochaperone in sperm flagella and cilia. In *Chlamydomonas*, RSP16 is transported in a 6S complex separately from the major 12S radial spoke precursors, in addition, it can homodimerize, which is a prerequisite for HSP40s to recruit substrates, suggesting that RSP16 may function as a cochaperone in the assembly of radial spokes (Yang et al., 2005). But in a successive study, knockdown of RSP16 did not cause obvious defects in any axonemal complexes, except for flagellar motility. Moreover, this motility defect could be rescued by RSP16 without the J domain. It seems that RSP16 is not to help HSP70s but simply to bind and secure the radial spoke structures (Yang et al., 2008). Despite these findings, the roles of DNAJB13 in mammals still need to be elucidated, because the mammalian sperm flagella and cilia are much more complex than the *Chlamydomonas* flagella.

4.3. Spatiotemporal association of DNAJB13 with the annulus during mouse sperm flagellum development (Paper III)

The sperm annulus is a septin-based fibrous ring structure connecting the midpiece and the principal piece of the mammalian sperm flagellum (Fawcett et al., 1970; Ihara et al., 2005; Kissel et al., 2005). Although ultrastructural abnormalities and functional importance of the annulus have been addressed in *Sept4*-null mutant mice and a subset of human patients with asthenospermia syndrome, little is known about how the structure is assembled and positioned to the midpiece-principal piece junction during mammalian sperm flagellum development. In this study we demonstrate a spatiotemporal association of DNAJB13 with the annulus during mouse sperm flagellum development, which provides a clue to look into this puzzle.

DNAJB13 localized to the annulus only in developing spermatids, not in mature spermatozoa. In addition, a dot labelled with the DNAJB13 antibody was kept apart from the annular staining toward the distal end of the flagellum in a subset of spermatids. In detail, the annular DNAJB13 was first detected in step 9 spermatids, in synchronization with the appearance of annulus indicated by the antibody against SEPT4, an annular constituent. As spermiogenesis proceeded, the annular DNAJB13 increased until the annulus reached the midpiece-principal piece junction, and then gradually disappeared from it in late spermiogenesis. The DNAJB13 dot appeared around step 13-14, a time point slightly prior to annulus migration, advanced toward the midpiece-principal piece junction, and then stayed there. The annular DNAJB13 followed the dot downwards, and eventually merged with it at the midpiece-principal piece junction. These data indicate that the annular DNAJB13 and the DNAJB13 dot may be involved in positioning of the annulus.

In annulus-deficient *Sept4*-null mouse spermatids from step 9 to step 15, the annulus-like DNAJB13 was still able to be detected, albeit weaker, at the neck region of the flagellum. Perhaps due to lack of the annulus, it disappeared soon. These data indicate that the annulus-like DNAJB13 appears prior to formation of the septin-based annulus, and further that it may assist in assembling the septin-based annulus. As in wild type, the DNAJB13 dot was also present on flagella of the *Sept4*-null spermatids, and migrated towards the midpiece-principal piece junction. Therefore, its presence and migration seem to be independent of annulus. Since the MS is still formed in *Sept4*-null spermatids (Ihara et al., 2005; Kissel et al., 2005), the dot may play a role in MS orientation in the absence of annulus.

We also demonstrated that DNAJB13 was colocalized and interacted with SEPT4 in vitro. Based on all the data, we tentatively propose a model of how DNAJB13 is involved in the assembly and positioning of sperm annulus. In the early stage of sperm flagellum development, DNAJB13 forms an annular anlage, or attaches to a pre-existing annular anlage, and then recruits septins through the direct interaction with SEPT4 or SEPT4-containing septin complex to build up a genuine annulus. In the late stage of sperm flagellum development, DNAJB13 may assist the migration of the annulus, and meanwhile forms a dot on the flagellum to direct the annulus toward the midpiece-principal piece junction. As the annulus is positioned to the midpiece-principal piece junction, DNAJB13 disappears gradually with formation and maturation of the MS.

4.4. Cohesin protein SMC1 is a centrosomal protein (Paper IV)

Structural maintenance of chromosome protein 1 (SMC1) is well known for its roles in sister chromatid cohesion and DNA repair (Harvey et al., 2002; Strom and Sjogren, 2007). In addition to the nuclear localization, SMC1 was shown to be present in the cytoplasm (Gregson et al., 2001; Lam et al., 2005), although the function is unclear. It was also reported to localize to the connecting cilia of photoreceptors and the primary cilia of MDCK cells (Khanna et al., 2005). Based on these findings, we hypothesized that cytoplasmic SMC1 might be associated with ciliogenesis. In this study, we demonstrate novel localizations of SMC1 in the centrosome/basal body and the midbody.

By IF, we showed that SMC1 localized to centrosomes throughout the cell cycle in a microtubule-independent manner. Biochemically, SMC1 was cofractionated with the centrosomal protein γ-tubulin in centrosomal preparation. Furthermore, we showed that SMC1 was associated with both centrioles of a centrosome. These results demonstrate that SMC1 is a centrosomal protein, suggesting a possible involvement of SMC1 in centrosome-related events, such as organization of radial arrays of microtubules and mitotic spindles. Indeed, SMC1 has two long coiled-coil domains (Gruber et al., 2003), which are very common among the centrosomal proteins (Andersen et al., 2003) and are thought to form a lattice-like structure for docking regulatory components of microtubules, the cell cycle and so on (Bornens, 2002).We also found that SMC1 localized to the midbody, a transient structure formed during cytokinesis. A proteomic study of midbody also identified SMC1 as a putative component (Skop et al., 2004), which is consistent with our finding. The midbody localization of SMC1 suggests that it may function in cytokinesis. Finally, IHC and IEM performed on mouse trachea sections revealed that SMC1 localized to the basal bodies of cilia in ciliated epithelia. It is known that basal bodies are derived from centrioles and serve as the base for cilium formation. Therefore, SMC1 may also play a role in ciliogenesis.

5. PERSPECTIVES

As a conserved protein in a conserved structure, DNAJB13 may be preassembled in a 6S complex and transported separately like its *Chlamydomonas* ortholog RSP16 (Yang et al., 2005). Besides, it may function in the same way as RSP16 in regulate axoneme motility (Yang et al., 2008). However, it remains unclear whether DNAJB13 functions as a cochaperone in the assembly of the mammalian axoneme.

Recently an in vitro mouse tracheal epithelial cell culture system was established (You et al., 2002). This system, in combination with lentivirus-based RNA interference technology (Vladar and Stearns, 2007), makes it possible to investigate the functions of DNAJB13 in mammalian axonemal morphogenesis and motility. But in addition to axoneme, DNAJB13 is also associated with the annulus during mouse sperm flagellum development. Therefore, to elucidate complete functions of DNAJB13, generation of *Dnajb13* knockout mouse is ultimately required.

The novel localizations of SMC1 in the centrosome/basal body and the midbody widen our knowledge about this protein. But what are its novel functions? As a cohesin protein, SMC1 is critical for cell viability. Thus it is infeasible to study the functions of SMC1 with RNA interference. Antibody microinjection may be applied. Additionally, identification of cytoplasmic SMC1-interacting proteins may provide more clues.

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