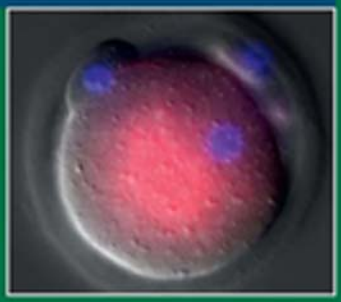
ADVANCES IN ANATOMY, EMBRYOLOGY AND CELL BIOLOGY

Kiyotaka Toshimori

Dynamics of the Mammalian Sperm Head

Modifications and Maturation Events from Spermatogenesis to Egg Activation



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With 36 Figures

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Summary

Mammalian spermatozoa have complex structures. The structure–function relationship of sperm has been studied from various viewpoints. Accumulated evidence has shown that the sperm components undergo sequential changes from the beginning of spermatogenesis to the time of fertilization/embryogenesis. Structural analyses have been performed using various new techniques of light and electron microscopy as well as immunohistochemistry and immunocytochemistry in combination with specific probes such as antibodies against sperm components. Recently developed gene-manipulation techniques have accelerated investigations on the events that govern the relationship between the structure and the molecular components of sperm. In addition, animal models with gene manipulations have been shown to exhibit various morphological and functional abnormalities that lead to infertility.

In humans, male infertility is caused by a number of factors, such as the external environment, nutrient changes, genotoxins, and mutagens. These factors affect not only the development of germ cells during spermatogenesis but also the functions of mature sperm, ultimately impairing fertilization or embryogenesis. Typical phenotypes of impaired fertilization or embryogenesis are visible in conditions such as azoospermia, oligozoospermia, and teratozoospermia, which have been induced in model animals with gene deficiencies. Thus, comparative analyses of the phenotypes expressed by model animals carrying gene mutations and infertile human patients should be performed in relation to the normal (natural) fertilization process to clarify the etiology of infertility due to male factors.

In this book, I discuss the events that occur in the normal sperm head and govern the structure–function relationship from the time of spermatogenesis to that of fertilization or egg activation. In this regard, I describe dynamic modifications and maturation events that occur in sperm-head components and compare the outcomes of these events with the outcomes of their failure.

Abbreviations Used in the Figure Legends

A	acrosome
AA	anterior acrosome
AF	axial filaments
AP	acrosome plate
BP	basal plate
CD	cytoplasmic droplet
CG	cortical granule
CP	connecting piece
DIC	differential interference contrast
EF	E-face
EP	end piece
ER	endoplasmic reticulum
ES	equatorial segment
ES–PM	plasma membrane covering the equatorial segment
FFR	freeze-fracture replica
FS	fibrous sheath
H	head
IAM	inner acrosomal membrane
IGS	immunogold staining
IIF	indirect immunofluorescence
KO	knock-out
M	microvilli
MP	middle piece
MS	mitochondrial sheath
N	nucleus
Ne	neck
NE	nuclear envelope
NP	nuclear plate
O	oocyte
OAM	outer acrosomal membrane
ODF	outer dense fiber
P	perforatorium
PA	posterior acrosome

PAR	postacrosomal region
PAS	postacrosomal sheath or paracrystalline sheet
PC	proximal centriole
PF	P-face
PM	plasma membrane
PP	principal piece
PR	posterior ring
PT	perinuclear theca
PVS	perivitelline space
RNE	redundant nuclear envelope
TEM	transmission electron microscope
Z	zona pellucida

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TEM: transmission electron microscope
Z: zona pellucida

Chapter 1
Introduction

I have been studying mammalian spermatozoa, fertilization, and early embryonic development for more than 30 years (i.e., from 1975 to the present), and the scope of my studies has ranged from basic research to its clinical applications. Recently, I have been involved in combination studies involving gene manipulation and ultrastructural, biochemical, and physiological approaches and have conducted these studies on both laboratory animals and clinical materials. My research has been focused not only on the continuously induced modifications in the sperm-head components that lead to sperm maturation but also on fertilization failure and infertility that can occur because of inappropriate sperm-component modifications.

A mature spermatozoon is a highly differentiated haploid cell with a head and a flagellum (tail). Although the shape of the sperm head varies among species, it can generally be classified into two major categories: (1) the sickle-shaped (falciform) head, as seen in rodents, and (2) the paddle-shaped (spatulate, fan-like) head, as seen in many species, including humans (Fig. 1.1). In most animals, the head appears flat in the ventrodorsal view. The sperm head comprises a nucleus, an acrosome, and a small volume of cytoplasm in the form of cytoplasmic layers (Fig. 1.2). The nucleus contains the paternal genome, consisting of DNA strands that are uniquely organized, along with the nuclear proteins protamines 1 and 2. The mature sperm acrosome is a cap-like saccule that covers the proximal region of the head; it is entirely covered by an acrosomal membrane that encloses hydrolyzing enzymes and matrix proteins. The cytoplasm between the nucleus and overlying plasma membrane becomes thin with narrow layers (spaces) toward the plasma membrane (Fig. 1.3).

The tail contains motility-related apparatus, mitochondria, an axoneme, and cytoskeletal structures such as outer dense fibers (ODFs) and fibrous sheaths (FSs) (Fig. 1.2). These components have recently been found to associate with unique cytoplasmic substances. Structurally, the tail is divided into four major regions (domains); the neck, middle piece (midpiece), principal piece, and end piece. The neck region is structurally complex: its major components are the basal plate, a redundant nuclear envelope, 2–3 mitochondria (neck mitochondria), and a connecting piece (comprising the capitulum, centrosome, and segmented columns connected to the ODFs). Centrosomes contain the proximal centriole and various

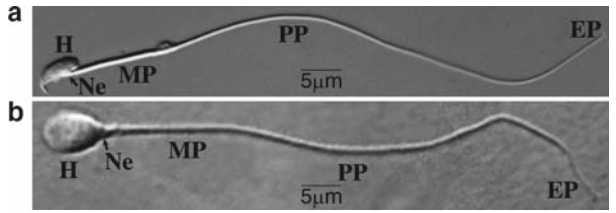

Fig. 1.1a–b Light micrographs showing mammalian sperm head and tail. DIC image. **a** Mouse. **b** Human

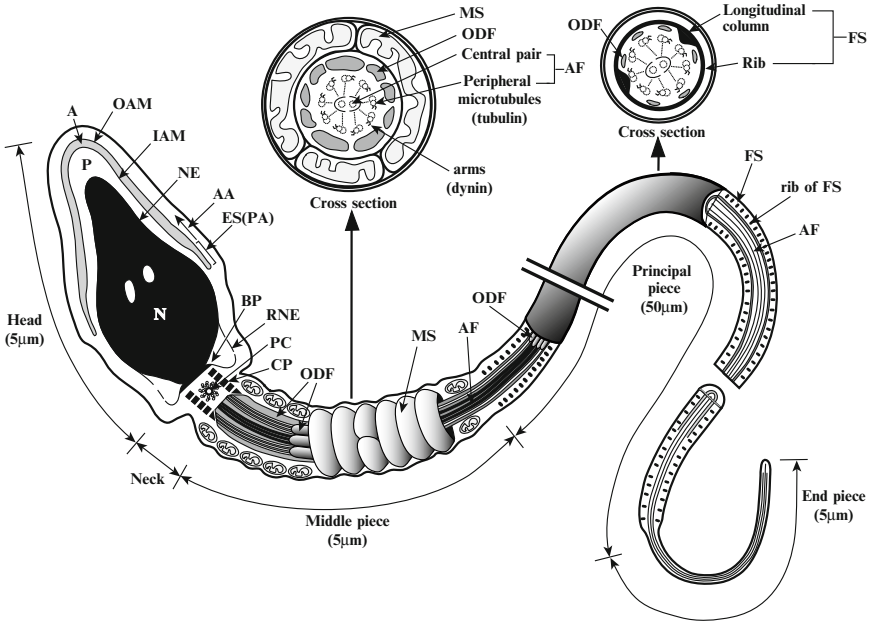

Fig. 1.2 Scheme of sperm head and tail components

pericentriolar matrix proteins. They are involved in microtubule formation when the sperm reaches the oocyte, and they morphologically develop into the sperm aster during fertilization in nonrodent mammals, including humans. The distal centriole is transformed into the proximal part of the axoneme.

In this book, I will primarily discuss the events related to sperm-head modifications and I will also discuss the sperm-tail structures and their involvement in these events. Events involving the tail components have previously been discussed in many comprehensive reviews (e.g., Eddy 2007).

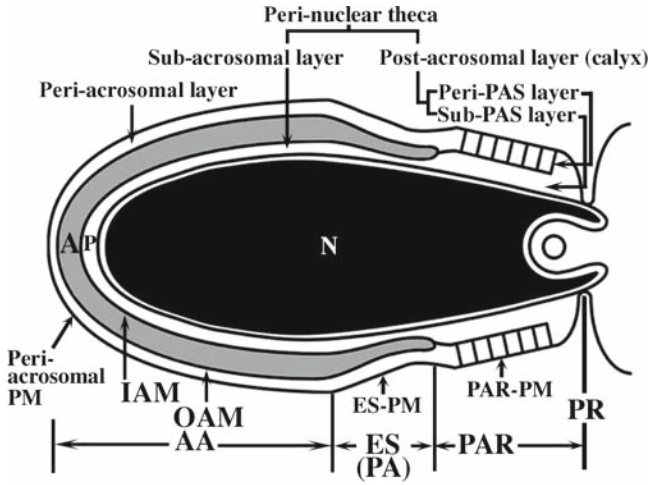

Fig. 1.3 Scheme of sperm-head subdomains and cytoplasmic layers

The function of mammalian spermatozoa is not only to carry the paternal genome to the oocyte but also to activate the oocyte arrested at the metaphase of the second meiosis (MII). For these purposes, elaborate structures develop in the sperm head during spermatogenesis.

Spermatogenesis is conserved throughout evolution and is maintained throughout the adult lives of most mammals. It is a highly programmed differentiation process involving developmental regulation with the stage-specific expression of genes and their transcript variants. Sertoli cells structurally and functionally support germ cells in the seminiferous epithelium under the control of testosterone (androgen) secreted by the Leydig cells and the follicle-stimulating hormone (FSH) secreted by the pituitary gland. The function of the Leydig cells is further controlled by a pituitary hormone—the luteinizing hormone or interstitial cell-stimulating hormone (ICSH)—in the hypothalamic–pituitary–gonadal axis. Spermatogenesis is completed in about 35 days in mice (approximately 11 days for the mitotic phase, 10 for the meiotic phase, and 14 for the postmeiotic phase), and about 74 days in humans (Clermont and Trott 1969). Mature spermatozoa produced by spermatogenesis are released from the Sertoli cells. The testicular spermatozoa are mature at the genomic level. In fact, spermatozoa can produce embryos when injected directly into an oocyte in the form of a testicular sperm extraction–intracytoplasmic sperm injection (TESE–ICSI). TESE–ICSI is an artificial reproductive technique (ART) that has recently been developed for the treatment of infertility.

Under natural (normal) conditions, spermiated spermatozoa pass through the rete testis, conduit canals, and the ductus efferentes, en route to the epididymis.

Spermatozoa that enter the caput epididymidis are functionally immature; they do not possess the properties of forward motility and zona pellucida recognition. On leaving the testes, spermatozoa mature by undergoing functional and morphological modifications during their passage through the epididymis. With these modifications, the sperm components not only gain several functional properties such as forward motility and zona pellucida recognition but they also develop the ability to protect the spermatozoa; collectively, these modifications are called "maturation in the epididymis" (Bedford 1967; Cooper 1998; Jones 1991; Orgebin-Crist 1967; Orgebin-Crist et al. 1987; Toshimori 1998; Tulsiani 2006; Turner 1979; Yanagimachi 1994). Spermatozoa are modified under the influence of products secreted by the epithelial cells lining the male reproductive tract. In most mammals, spermatozoa take 2–10 days to pass through the epididymis, regardless of the total length of the epididymal duct; in humans, this process takes 2–3 days. Spermatozoa that have achieved structural and functional maturation are stored in the cauda epididymidis and vas deferens until ejaculation; the cauda epididymidis is poorly developed in humans as compared to other mammals.

During ejaculation, spermatozoa are further coated with materials secreted by the seminal vesicle and prostate gland. In general, human semen contains 10^8 spermatozoa per milliliter (approximately 1% of the ejaculate in terms of weight), along with components of the prostate fluid (approximately 20% by weight; zinc, acid phosphatase, fibrinolysin precursors, polyamines, etc.) and products of the seminal vesicle (40–80%; fructose, ascorbic acid, fibrinogen, prostaglandin, cholesterol, etc.). These components contain many biological elements that spermatozoa require to execute various functions.

When ejaculated spermatozoa enter the female reproductive tract, they encounter several obstacles. First, they must pass through the viscous mucous at the junction between the vagina and the uterus (i.e., the cervix), and that between the uterus and the fallopian tube (oviduct; UT junction). At the UT junction, spermatozoa temporarily associate with the oviductal epithelium (Oh-Oka et al. 2003; Yanagimachi 1994) and are considered to undergo further unknown modifications; the duration of this association varies depending on the estrous cycle (e.g., the association is retained for six months in the case of bats). After leaving the UT junction, but before reaching the ampullary region, spermatozoa execute a major part of their capacitation function; the physiological (functional) changes that render ascending spermatozoa competent to penetrate the eggs (oocytes) are collectively termed "capacitation."

Spermatozoa that have undergone capacitation exhibit vigorous (whiplash) tail movement, i.e., hyperactivation. Hyperactivation is a visible sign that the spermatozoa have undergone all the physiological changes necessary for sperm–egg interaction. Under normal conditions, fully capacitated spermatozoa successfully pass through the cumulus matrix to bind to the zona pellucida. The binding of the spermatozoa to the zona pellucida is classified into two steps—primary binding and secondary binding. After primary binding, but before secondary binding, the spermatozoa undergo the acrosome reaction. The acrosome

reaction is the process of exocytosing acrosomal enzymes and associated matrix molecules from the spermatozoa; its biological significance is twofold. First, the acrosome reaction assists the passage of spermatozoa through the egg vestments via hydrolysis or modification of the zona pellucida components. Second, the reaction per se triggers the modification of pre-existing molecules on the sperm plasma membrane. This event is considered important for enabling the part of the plasma membrane over the equatorial segment (ES, i.e., the equatorial plasma membrane) to fuse with the oolemma, and it can be called "priming for gamete membrane fusion." The manner in which sperm-surface modifications are induced in vivo and in vitro appears to be considerably more complex than was originally thought. Eventually, only spermatozoa that have completed the acrosome reaction are able to penetrate the zona pellucida.

After the spermatozoa pass through the zona pellucida, only one acrosome-reacted spermatozoon binds to and fuses with the oolemma. During or immediately after the fusion of the two gamete membranes, the oocyte is activated. This activation triggers the oocyte arrested at MII to complete the second meiosis, and it induces Ca^{2+} oscillation, during which functional molecules are expressed in a timely and precise manner, synchronous with the structural changes in the sperm-head components. Oocyte activation is imperative for normal embryonic development.

The entire process of sperm maturation, from spermatogenesis to egg activation, involves sequential structural and biochemical modifications that occur on the sperm head and tail. Thus, this process is easily impaired by genetic disruption or by exogenous exposure to hazardous chemicals containing toxins such as endocrine disruptors (Escalier 2001; 2006; O'Bryan and de Kretser 2006; Russell et al. 1994; Toshimori et al. 2004). Using recently developed gene-deletion techniques such as gene-knockout (KO) systems, such drastic impairments in the sperm-maturation process can be mimicked to investigate the various conditions that lead to infertility, such as maturation arrest (azoospermia), the development of morphologically abnormal spermatozoa (teratozoospermia), combinations of abnormalities (oligoasthenoteratozoospermia), and functional disorders.

The purpose of this book is to discuss the dynamics of the modifications that occur in the mammalian sperm head, from the beginning of spermatogenesis to egg activation, and ultimately lead to normal fertilization and embryonic development. In addition, I compare such normal events with the abnormal events that occur under the deficient conditions induced by gene mutations in mouse models. The objective of this book is not only to improve the general understanding of sperm biology but also to discuss clinical treatment options for infertile human patients and animals.

Chapter 2
Mammalian Sperm Head

In mammals, spermatozoa complete meiosis before spermiation, while oocytes do not complete meiosis before ovulation. A mature spermatozoon weighs only picograms, and its head length is approximately 5 μm. On the other hand, a mature oocyte weighs 20–40 ng and is approximately 100 μm in width. Since spermatozoa have to cover a large distance to reach the oocyte, the sperm nucleus (containing the genome) must remain in a protected and safe condition; in fact, it is thoroughly embedded in the nuclear matrix. Approximately 2.5×10^7 of such structurally complex spermatozoa are produced daily in the human testes; in comparison, only a few oocytes (generally 1 cell per menstrual cycle in humans and about 10 per estrous cycle in rodents) develop in the ovaries and are ovulated into the fallopian tube.

2.1
Structures, Domains, and Related Functions

Mammalian sperm heads are divided into two major regions (domains): the acrosomal region and the postacrosomal region (PAR); these regions can be clearly visualized by using specific antibodies. In my studies, I have used the antiacrosomal antibody MN9 (antiEquatorin antibody) and the anti-PAR antibody MN13 for this purpose (Fig. 2.1). These regions are further divided into substructures and subdomains. The acrosome region contains two subdomains: the anterior acrosome and the posterior acrosome or the ES. The anterior acrosome is involved in the acrosome reaction, one of the most intriguing events of which is the release of acrosomal enzymes and matrix proteins. The ES appears to be involved in gamete membrane fusion. The PAR extends from the end of the ES and the posterior ring located at the distal-most end of the head, and it forms the border between the head and the flagellum or neck region (connecting piece). The posterior ring exhibits a belt-like constricted zone of plasma membrane that fuses with the underlying nuclear envelope. The proximal part of the PAR is also presumed to be involved in egg activation.

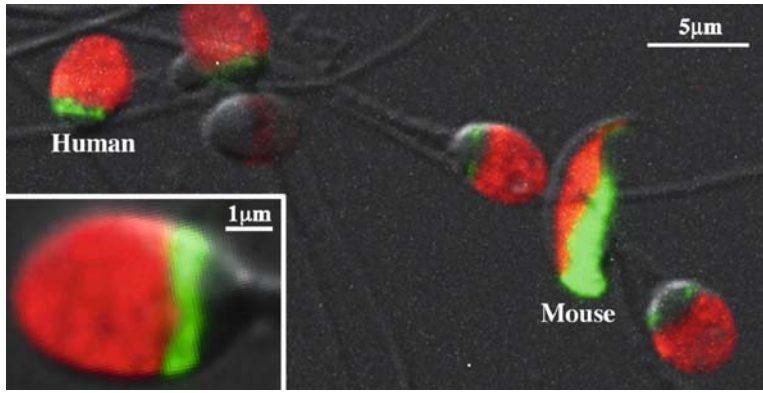

Fig. 2.1 IIF image showing sperm subdomains; acrosome (*red*) and postacrosomal region (*green*). Acrosome is specifically recognized by antiEquatorin (MN9) antibody, while the postacrosomal region is recognized by antipostacrosome substance (MN13) antibody

2.2 Cytoplasmic Layers

Cytoplasmic layers are the spaces formed between the membrane systems in the sperm head (Fig. 1.3). The layers develop as flat and narrow structures and exhibit dense accumulation of cytoskeletal substances and functional molecules. In mature spermatozoa, the acrosome, which is a membrane-bound organelle, is interposed between the plasma membrane and the underlying nucleus at the anterior head. Thus, the molecules accumulated in the cytoplasmic layers are likely to play important roles in the fertilization processes, and it is important to analyze these molecules. The cytoplasm can be differentiated into at least three layers (spaces): the periacrosomal, subacrosomal, and postacrosomal layers.

2.2.1 Periacrosomal and Subacrosomal Layers

The periacrosomal layer is the cytoplasmic layer present between the outer acrosomal membrane and the overlying periacrosomal plasma membrane, while the subacrosomal layer is the narrow layer of cytoplasm that lies between the inner acrosomal membrane and the nuclear envelope. The periacrosomal layer is reported to contain actin filaments (Hernández-González et al. 2000) and the actin-regulatory protein gelsolin (Cabello-Agüeros et al. 2003). These components are considered to be involved in the initial step of the acrosome reaction and subsequently in sperm–egg fusion. The outer surface of the apical segment of the subacrosomal layer forms a prominent cytoskeletal structure around the nucleus; this structure is called the perforatorium, and it protrudes toward the

rostral region in a triangular conformation. Ultrastructurally, the perforatorium is composed of an electron-dense substance (perinuclear theca substance). The peri- and subacrosomal layers are lost during the acrosome reaction, but the cytoplasm that extends to the ES remains structurally unchanged even after the acrosome reaction.

2.2.2 Postacrosomal Layer

The plasma membrane at the PAR is tightly bound to the underlying postacrosomal sheath (PAS) via a paracrystalline sheet (Longo et al. 1987; Toshimori et al. 1991b). The paracrystalline sheet consists of regularly arranged crossbridges (filaments) with a diameter of 10–14 nm, and the MN13 antigen is localized on these crossbridges (Figs. 2.2 and 2.3). The paracrystalline sheet can be visualized only after treatment with a mild detergent such as 0.1% Triton X-100. The postacrosomal layer can be further divided into the peri-PAS layer and the sub-PAS layer. The peri-PAS layer corresponds to the cytoplasm between the PAS and the overlying peri-PAS plasma membrane, while the sub-PAS layer corresponds to that between the PAS and the underlying nuclear envelope.

Since the substances in the sub- and postacrosomal layers can only be isolated collectively, these combined layers are referred to as the perinuclear theca or postnuclear cap (Bellvé et al. 1992). The part of the perinuclear theca at the PAR is also known as the calyx (Longo et al. 1987). The perinuclear theca is presumed to play a role in maintaining the conformation of the sperm nucleus or that of the sperm head per se and in storing many molecules required for egg activation, as discussed later.

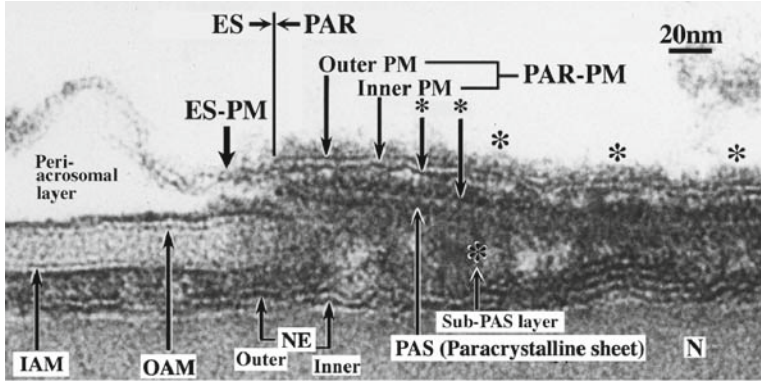

Fig. 2.2 TEM image showing sperm structural components at the border between the equatorial segment (ES) and the postacrosomal region (PAR). Tannic acid fixation. Note the periodic ladder-like striations (*arrows with asterisks*) between the postacrosomal sheath and the overlying plasma membrane, and the glycocalyx-like substance on the plasma membrane covering the postacrosomal region (*asterisks*)

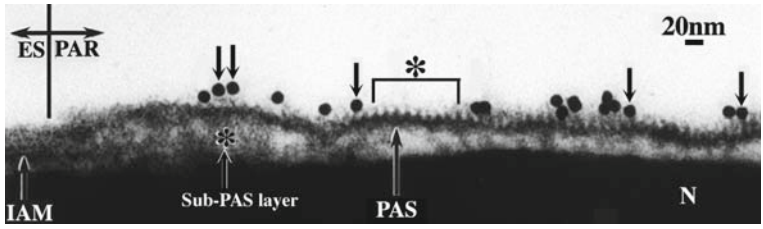

Fig. 2.3 Electron-microscopic IGS image to show the localization of MN13 (*arrows*). Plasma membrane is removed in this figure by Triton X-100 treatment. Immunogold particles (10 nm) are localized on the top of filamentous ladder-like striations (*arrows with asterisks*) between the postacrosomal sheath and the overlying plasma membrane; this image corresponds to Fig. 2.2

2.3 Membrane System

Many functional molecules necessary for spermatogenesis and fertilization are organized into the sperm membrane system. Here, I demonstrate the distinct nature of the membrane system, which has been clarified using the freeze-fracture (FFR) method. FFR performed using the antibiotic filipin as a specific marker is particularly helpful for studying the differences in the nature of the membrane components, since filipin has an affinity for membrane sterol. Cholesterol is present in abundance on the equatorial plasma membrane and the membrane at the anterior acrosome region, but it appears to be deficient or absent in the nuclear envelope and the outer and inner acrosomal membranes (Fig. 2.4) (Toshimori et al. 1987). The distal part of the redundant nuclear envelope is also rich in cholesterol.

Plasma membrane: The sperm plasma membrane is effectively modified (Flesch and Gadella 2000). Structurally, the plasma membrane covers the whole surface of the sperm head and tail prior to the acrosome reaction. The postacrosomal plasma membrane expresses a glycocalyx-like substance on its surface (Fig. 2.2); this substance is presumed to contain various unknown molecules that are involved in the events induced by capacitation and the acrosome reaction until the spermatozoon fuses with the oolemma.

As mentioned above, the periacrosomal plasma membrane can fuse with the outer acrosomal membrane during the acrosome reaction, while the equatorial plasma membrane can fuse with the oolemma during gamete membrane fusion. This suggests that after the acrosome reaction, the fusogenic molecule(s) is present in both the heterogeneous membranes (i.e., the periacrosomal plasma membrane and the outer acrosomal membrane). Similar fusion-related molecules can be identified on the equatorial plasma membrane and the microvilli of the oolemma during gamete membrane fusion. Recent evidence has demonstrated the importance of raft formation on the sperm plasma membrane prior to gamete membrane fusion. It is of particular interest to investigate how variations in the cholesterol

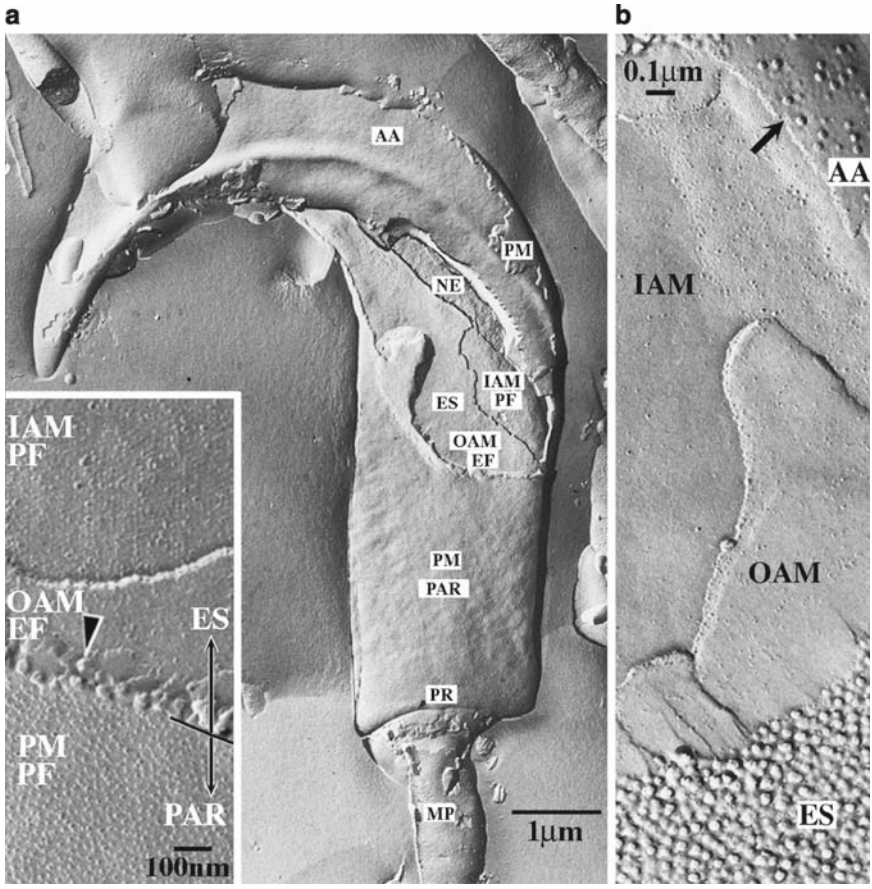

Fig. 2.4a–b FFR images of the sperm head showing the different nature of the membrane system with the aid of impregnation of a specific probe for cholesterol, filipin. **a** Entire image of the hamster sperm head. **b** Acrosomal region of the mouse sperm head. Cholesterols are scarce on the plasma membrane at the postacrosomal region and on the inner acrosomal membrane and outer acrosomal membranes (*inset of* **a**). **b** Cholesterols are abundant on the plasma membrane over the equatorial segment and present at medium levels or sporadically on the plasma membrane over the anterior acrosome (AA)

content of the plasma membrane are related to the raft formation induced by the acrosome reaction.

Acrosomal membrane: The outer and inner acrosomal membranes are differentiated from the proacrosomal granule-derived membrane that originates from the Golgi apparatus (Fig. 5.2). This differentiation appears to occur at the stage of late elongating spermatids, which approximately corresponds to step 14 in mice and the late Sd step in humans.

Outer acrosomal membrane: Peanut agglutinin (PNA), a plant lectin isolated from *Arachis hypogaea*, specifically binds to the β-galactose (1–3)-*N*-acetyl-galactosamine (β-Gal(1–3)-GalNAc) linkage of *O*-linked glycoproteins expressed on the outer acrosomal membrane (Huang and Yanagimachi 1985). Accumulated evidence has proven that the nature of the outer acrosomal membrane is more complex than thought previously.

The equatorial outer acrosomal membrane is not involved in the acrosome reaction; it is strongly attached to the inner acrosomal membrane via crossbridges separated from each other by a distance of approximately 7 nm. Since these bridges are embedded deep in the matrix, they are not clearly visible in mature spermatozoa but become distinct with the gradual release of the matrix substances during sperm–egg interaction (Manandhar and Toshimori 2003). Thus, the equatorial acrosomal membrane matrix contains tenacious cytoskeleton element(s) in its outer and inner membranes.

Inner acrosomal membrane: The inner acrosomal membrane is remarkably resilient, exhibiting resistance to damage induced by detergents and sonication, and it is not involved in the acrosome reaction. After the acrosome reaction, the inner acrosomal membrane remains intact and can be recognized by the plant lectin concanavalin A (Con-A), which has a specific affinity for glycoproteins with a high mannose content (Holden et al. 1990). Upon fertilization, the inner acrosomal membrane is internalized by the ooplasm via phagocytosis, as described later. The intramembranous particles are densely packed and resemble paracrystalline arrays (Fig. 2.4). Many molecules are localized in the inner acrosomal membrane, presumably in the form of a functional molecular complex, as discussed later.

Nuclear envelope: The sperm nucleus is dormant in terms of DNA synthesis. In fact, the nuclear envelope lacks pores, and the head cytoplasm lacks the machinery required for protein synthesis.

Chapter 3
Sperm-Head Formation and Factors Affecting It

Various types of sperm-head anomalies can be observed in patients with teratozoospermia or oligozoospermia. The presence of such abnormalities is one of the primary causes of infertility in males. Here, I discuss these abnormalities in relation to spermatogenesis.

Prior to their entry into the first meiosis, germ-line stem cells proliferate at the spermatogonial stage, undergoing self-renewal as type A spermatogonia (A1–A3 in the rat). Type A spermatogonia differentiate into type B spermatogonia, and these in turn differentiate into primary spermatocytes that subsequently undergo meiosis (Figs. 3.1 and 3.2). Prior to the first meiotic division, primary spermatocytes duplicate their DNA/chromosomes. A typical feature of the first meiosis is genetic recombination; at this stage, a synaptonemal complex containing recombination nodules is formed in the pachytene-phase spermatocytes. Immediately after meiosis (within approximately 8 h in the case of humans), the secondary spermatocytes immediately develop into haploid spermatids. Thus, almost all of the molecules/proteins required for sperm-head formation are presumed to be prepared at the gene level in primary spermatocytes. The gene expression of spermatids is referred to as postmeiotic gene expression. These steps are followed by four major events of spermiogenesis: nuclear modification, acrosome biogenesis, cytoplasmic trimming, and flagellum formation. The first three events are involved in head formation.

Sperm-head formation is triggered by the initiation of acrosome biogenesis during spermiogenesis. Head formation involves acrosome biogenesis, nuclear condensation and elongation (flattening), and the formation of cytoplasmic layers. Cytoplasmic molecules/substances gradually accumulate to form the perinuclear theca. Some of these essential molecules are transported along manchettes (a manchette is an organized microtubular structure that girdles the posterior region of the spermatid nucleus) and are organized into the various structural components. This organization occurs most actively in elongating spermatids. In particular, drastic changes occur from the mid-stage to the final step of spermiogenesis. The cytoplasm released by the germ cells is phagocytosed by the Sertoli cells before spermiation. Thus, germ cells produce proteins essential for fertilization, and these proteins are organized into the head and tail structures or their components in a time-sensitive manner.

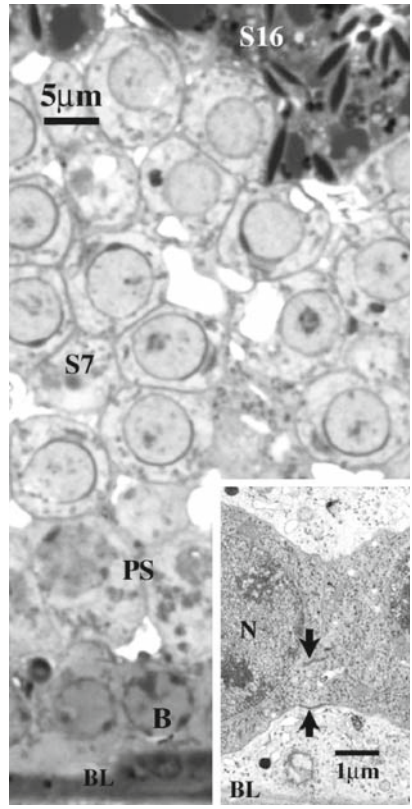

Fig. 3.1 Light micrograph showing stage VII of rat spermatogenesis. *Inset.* An intercellular bridge (*arrows*) between spermatogonia. *S7* and *S16*, step 7 and step 16 spermatids, respectively; B, type B spermatogonium. BL basal lamina. PS primary spermatocytes

3.1 Cellular Interactions

Various cellular interactions influence the process of sperm-head formation during spermatogenesis.

3.1.1 Stem-Cell Factor, Steel Factor, and the c-kit Receptor

Germ cells express the tyrosine kinase receptor c-kit, which is produced by a gene on the W locus. The ligand for this receptor is a c-kit ligand—the Steel factor—which is a proliferating factor or a stem-cell factor (SCF). The Steel factor signal

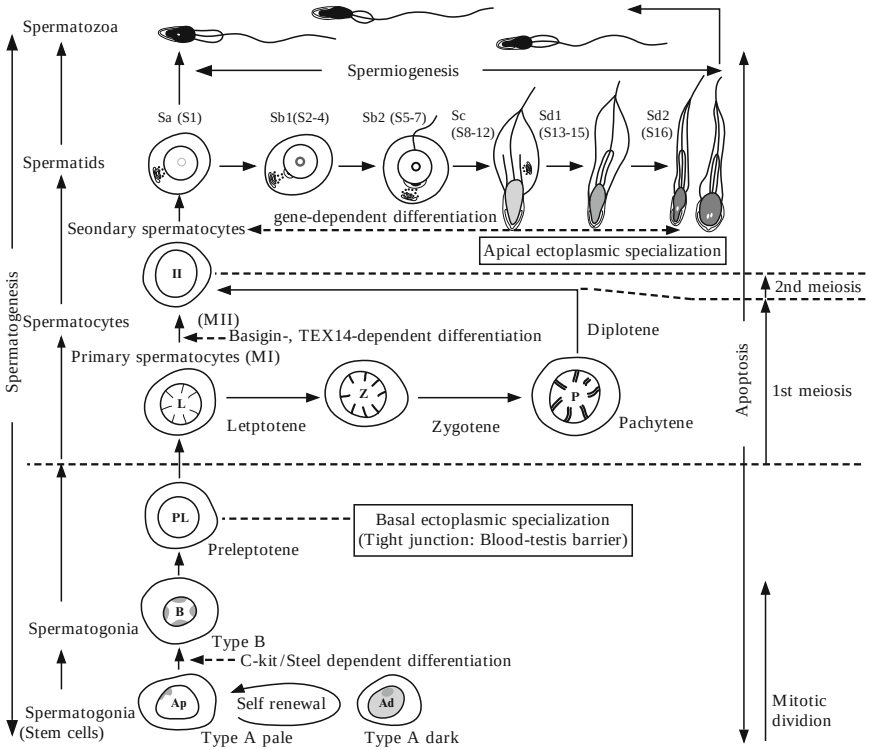

Fig. 3.2 Scheme of spermatogenesis, including various factors that affect spermatogenesis

released by Sertoli cell is transduced to its c-kit counterpart on germ cells (Fig. 3.3). Both of these molecules are required for the survival and proliferation of type A spermatogonia (Chabot et al. 1988; Dolci et al. 1991; Geissler et al. 1988; Witte 1990). If one of these genes is mutated or artificially deleted, germ cells are absent, and the mutant individual exhibits the Sertoli-cell-only syndrome. Typical cases of this condition have been demonstrated in Sl/Sl^{d} (Steel-deficient mutant mice) (Tajima et al. 1991) and W/W^{v} mice (*c-kit*-deficient mutant mice) (Koshimizu et al. 1991; Yoshinaga et al. 1991).

In addition to c-kit, tr-kit, a postmeiotic specific alternative *c-kit* gene product, is also formed (Fig. 3.3). Interestingly, tr-kit is localized in the sperm head and has been shown to be involved in egg activation when it is microinjected into the cytoplasm of mouse oocytes arrested at MII (Sette et al. 1998) (reviewed in Rossi et al. 2003). This topic is discussed in greater detail in the section on egg activation.

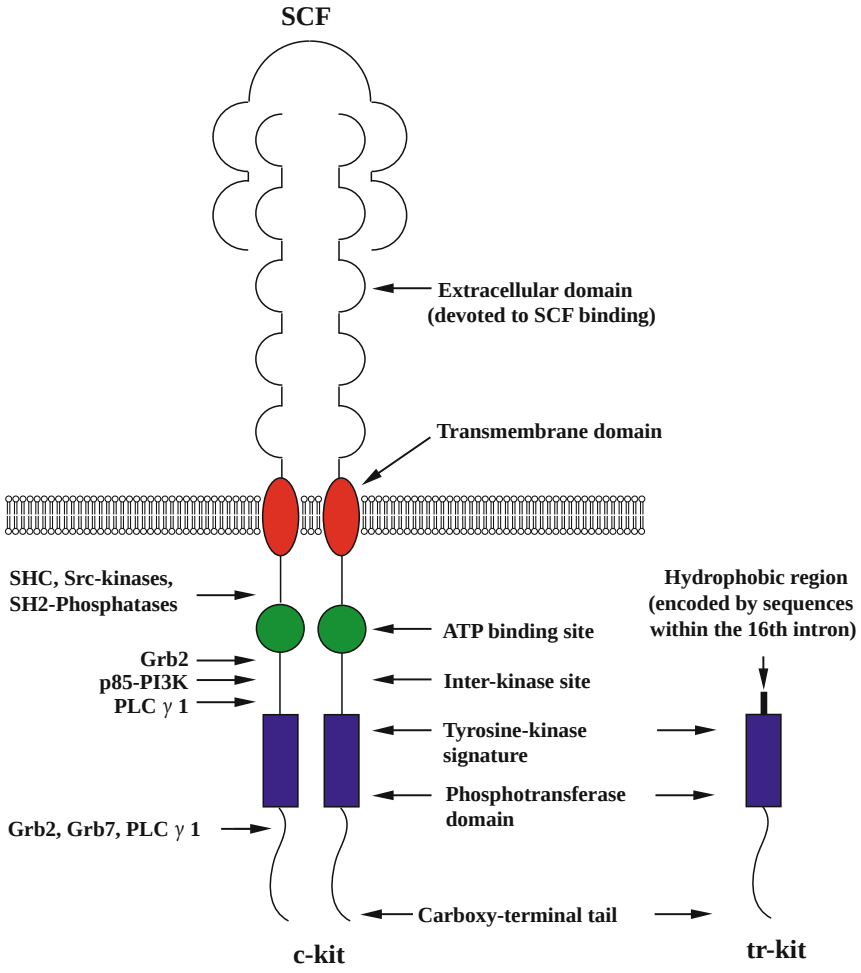

Fig. 3.3 Scheme showing the domains of c-kit and tr-kit. Redrawn after modifications of the original scheme presented by Sette et al. (1997)

3.1.2 Intercellular Bridges

Intercellular bridges are formed between developing germ cells (Figs. 3.4 and 3.5). They connect hundreds of germ cells that are derived from a single stem cell and develop as a huge syncytium. In mice (Fig. 3.4) and rats (Weber and Russell 1987), the diameter of the bridges connecting spermatogonia is reported to be approximately

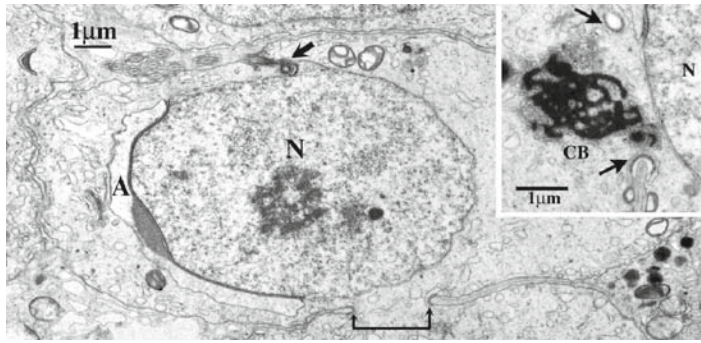

Fig. 3.4 TEM image showing a step 6 spermatid with round nucleus. Note the acrosome and developing tail extending from the centriole (*arrow*). Adjacent germ cells are connected by an intercellular bridge (*between arrows*). *Inset*: A migrating chromatoid body (CB) localized at the intercellular bridge

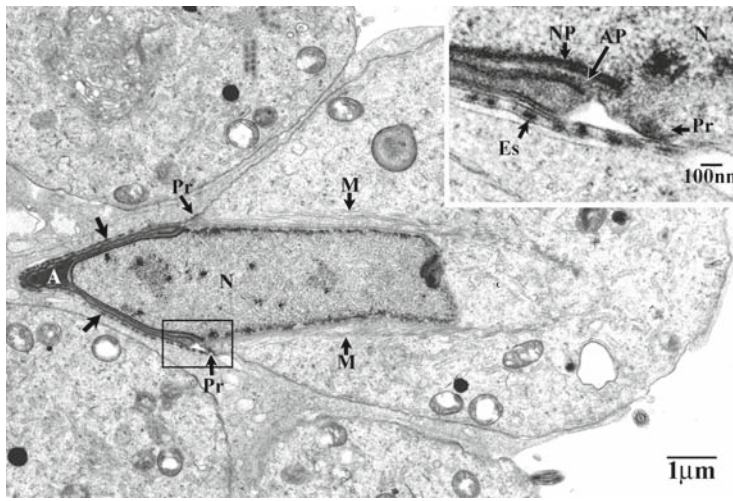

Fig. 3.5 TEM image showing step 9 spermatids that have elongating but not condensed nuclei. Note the apical ectoplasmic specialization (*Es with arrows*) surrounding the head region and the acroplaxomes that are formed at the distal end of acrosome (*boxed region and inset*). The perinuclear ring (*Pr*) that emanates the microtubule bundles (i.e., manchettes, *M*) is indicated by *arrows*. *Inset*: Higher magnification of the rectangular area (the acroplaxome area). Modified from a photograph shown by Toshimori and Ito (2003)

1 μm, and it increases to approximately 1.5 μm in the case of spermatocytes and 2–3 μm in that of spermatids. The bridge functions for haploid-cell communication and synchronization and for chromosome-dosage compensation (Ventelä et al. 2003).

The importance of intercellular bridges has been demonstrated in mutant mice carrying a deletion for testis-expressed gene 14 (*TEX14*). TEX14 transfers the cytokinesis machinery of somatic cells to other germ-cell proteins to form stable intercellular bridges (Greenbaum et al. 2007). In *TEX14*-deficient male mice, these intercellular bridges are not formed, and the spermatogenesis pathway is arrested before completion of the first meiotic division. TEX14 colocalizes with the centralspindlin complex, mitotic kinesin-like protein 1 (MKLP1), and the male germ cell Rac GTPase-activating protein (MgcRac GAP), and converts these midbody matrix proteins into stable components of the intercellular bridges. Intercellular bridges are formed in the midbody in a TEX14-dependent manner. Thus, TEX14 plays an essential role in the maintenance of spermatogenesis.

3.1.3 Ectoplasmic Specialization

As the head-surface domains develop in early elongating spermatids, the plasma membrane of the Sertoli cells is maintained in close contact with that covering the developing spermatid head, where the ectoplasmic specialization is formed. The ectoplasmic specialization is a specific interactive structure formed between spermatids and Sertoli cells; it is specifically localized in the adluminal compartment of seminiferous tubules (Fig. 3.2). This structure that anchors the adluminal compartment is also referred to as the apical ectoplasmic specialization, in contrast to the basal ectoplasmic specialization that develops between adjacent Sertoli cells and forms the blood–testis barrier. The ectoplasmic specializations as well as some other junctions formed in the testis have been reviewed previously (Mruk and Cheng 2004a; Yan et al. 2007).

Structurally, the apical ectoplasmic specialization is composed of unique cytoplasmic organelles derived from Sertoli cells, a series of smooth endoplasmic reticula, and aggregates of actin bundles organized into hexagonal arrays (Fig. 3.5). The ectoplasmic specializations face the developing acrosome of spermatids during steps 9–15 of spermatogenesis, shortly prior to spermiation. Since the ectoplasmic specializations function in maintaining spermatogenesis by positioning the developing head regions, spermatogenesis is readily affected by the deletion of genes encoding ectoplasmic specialization-related proteins. This has been demonstrated in KO mice lacking GOPC (Ito et al. 2004; Toshimori and Ito 2003; Yao et al. 2002) and genes encoding immunoglobulin-superfamily (IgSF) proteins such as nectin-2 (Mueller et al. 2003) and RA175 (Fujita et al. 2006). Recent evidence has demonstrated that deletion of the *Rap1* gene that encodes a small guanosine triphosphatase (GTPase) of the Ras family impairs the cell–cell adhesion mediated by VE-cadherin (CD144) at the ectoplasmic specialization. This eventually causes the premature release of spermatids with deformed heads that are hammer-shaped or ovoid (Aivatiadou et al. 2007). Here, I present the general findings of studies on IgSF-related proteins and genes and discuss their role in the impairment of sperm-head formation.

3.2 IgSF Proteins

IgSF proteins are uniquely expressed on the surfaces of Sertoli cells and germ cells (Table 3.1).

Sertoli cells express nectin-2/CD155, an IgSF protein that is classified as a cell adhesion molecule. Germ cells also express many other IgSF proteins, namely

Table 3.1 IgSF proteins expressed in male germ cells and spermatozoa

Cells/Molecule	Role	Phenotype of gene knockout	References
Sertoli cells			
Nectin-2/CD155	Cell adhesion	–	1, 2
		Teratozoospermia (failure of sperm–egg interaction)	3
Germ cells			
Basigin (Bsg)	–	–	4, 5, 6, 7
	Maintenance of meiosis	Azoospermia	8
CE9 (rat Bsg)	–	–	9, 10, 11
MC31 (rat Bsg)	–	–	12, 13, 14
IgSF4	Cell adhesion	–	15
RA175		Oligoteratozoospermia	16, 17
SGIgSF		–	18,19
Nectin-3		–	2
IgSF11		–	20
Ly9/CD150	–	–	21
JAM-C	Cell/spermatid polarization	Azoospermia	22
Necl-2	Cell adhesion	–	23
BT-IgSF		–	24
Spermatozoa			
Basigin (Bsg)	Sperm–egg interaction	–	7
CE9 (rat Bsg)	–	–	9, 10, 11
MC31 (rat Bsg)	Sperm–egg interaction	–	13
OBF13	–	–	25
Izumo (OBF13)	Sperm–egg fusion	Normozoospermia (failure of sperm–egg fusion)	26

References: *1*, Bouchard et al. (2000); *2*, Ozaki-Kuroda et al. (2002); *3*, Mueller et al. (2003); *4*, Miyauchi et al. (1990); *5*, Maekawa et al. (1998); *6*, Yuasa et al. (2001); *7*, Saxena et al. (2002); *8*, Igakura et al. (1998); *9*, Petruszak et al. (1991); *10*, Nehme et al. (1993); *11*, Cesario and Bartles (1994); *12*, Toshimori et al. (1992b); *13*, Wakayama et al. (2000); *14*, Saxena and Toshimori (2004); *15*, Gomyo et al. (1999); *16*, Urase et al. (2001); *17*, Fujita et al. (2006); *18*, Wakayama et al. (2001); *19*, Wakayama et al. (2003); *20*, Watanabe et al. (2005); *21*, Tovar et al. (2002); *22*, Gliki et al. (2004); *23*, Shingai et al. (2003); *24*, Harada et al. (2005); *25*, Okabe et al. (1987); *26*, Inoue et al. (2005).

basigin(BSG), CE9, MC31, IgSF4, RA175, Sg-IGSF, nectin-3, IgSF11, Ly9/CD150, JAM-C, nectin-like molecule 2 (Necl-2), and BT-IgSF. I personally have studied RA175 (Fujita et al. 2006) and mouse basigin (Saxena et al. 2002), which is homologous to rat CE9 (Nehme et al. 1993; Petruszak et al. 1991) and MC31 (Toshimori et al. 1992b; Wakayama et al. 2000). IgSF4 is a cell adhesion molecule identical to RA175, Sg-IGSF, Necl-2, TSLC1, and SynCAM1. These proteins are expressed on the surfaces of not only germ cells but also many epithelial cells. Although many of them function as cell adhesion molecules for the maintenance of spermatogenesis, IgSF proteins localized on the ectoplasmic specialization are specifically involved in the shaping of the sperm head.

3.2.1 IgSF Proteins Expressed on Sertoli Cells

Nectin, an IgSF protein, is localized on the ectoplasmic specialization formed by Sertoli cells, and it affects the shaping of the sperm head.

Nectin-2 (CD155): Nectin-2 is encoded by the member of the poliovirus receptor gene family found in mice, monkeys, and humans. It is localized on the ectoplasmic specialization and is ubiquitously distributed as a component of the cell–cell adherence junction, where it interacts with I-afadin, an F-actin-binding protein. The association between the C-terminal regions of nectins and afadins (including actin-binding proteins) is required for the assembly of actin filaments. Nectin-2 is considered to interact with nectin-3 on germ cells via heterophilic trans-dimer interactions (Ozaki-Kuroda et al. 2002). Since the ectoplasmic specialization functions as a scaffold stabilizing the adhesive domains of proteins expressed on the plasma membrane of Sertoli cells, it is considered to transmit signals from the Sertoli cells to germ cells.

When the *nectin-2* gene is deleted, the ectoplasmic specialization is disorganized and its adhesiveness is reduced because of dysfunction of the F-actin scaffold or the mislocalization of afadin. This eventually affects spermatogenesis and causes teratozoospermia, which is characterized by spermatozoa with randomly deformed heads and midpieces (Mueller et al. 2003). *nectin-2*$^{-/-}$ mice are found to be infertile, producing spermatozoa that are fewer in number and abnormally shaped; nevertheless, the produced spermatozoa are able to migrate to the oviduct, bind to the zona pellucida, and fuse with the oolemma. Although the unusual fertilization events noted to occur in these mice are the secondary effects of the sperm-head deformities, it is evident that nectin-2 plays an essential role in spermiogenesis, specifically in the cytoskeletal organization and reorganization at the apical ectoplasmic specialization.

3.2.2 IgSF Proteins Expressed on Germ Cells

Many IgSF proteins are associated with germ cells.

IgSF4: IgSF4 is a cell adhesion molecule and was formerly recognized as a candidate target molecule for a tumor-suppressor gene associated with loss of heterozygosity

on chromosome 11q23.2. IgSF4 is identical to RA175, Sg-IGSF, TSLC1, SynCAM1, and Necl-2, which form a group of cell adhesion molecules (CADM). RA175 is strongly expressed during the neuronal differentiation of embryonic carcinoma cells. Sg-IGSF is expressed in early spermatogenic cells. TSLC1 is a tumor-suppressor molecule that functions against nonsmall-cell lung cancer in humans. SynCAM1 is a brain-specific synaptic adhesion molecule that stimulates Ca^{2+}-independent homophilic cell–cell adhesion. SgIGSF and TSLC1 are ubiquitously expressed, and their expression has been visualized by northern blotting.

IgSF4/Necl-2 is predicted to contain V- and C2-type Ig domains as well as a hydrophobic signal sequence, a single transmembrane region, and a cytoplasmic domain. Ly9/CD150 is expressed at the mRNA level in the testis.

Nectin-3 is localized in the spermatid head region facing the apical ectoplasmic specialization. Proteins of the nectin family play a role in the organization of various cell–cell junctions such as adherent junctions and tight junctions in epithelial cells and synaptic junctions in neurons. The signal-proliferation pathways of these molecules have been recently identified in many tissue types.

Next, I will describe the typical findings in male mice carrying deletions for the JAM-C and RA175 genes.

JAM-C: JAM-C consists of two extracellular Ig domains, a single transmembrane region, and a short cytoplasmic domain; the extracellular Ig domains contain 1 VH-type domain and 1 C2-type domain, and the C2 fold contains two extra cysteine residues and a potential N-glycosylation site. JAM-C is involved in the assembly of tight junctions in endothelial and epithelial cells. In the testis, JAM-C is expressed in round and elongated spermatids (Gliki et al. 2004). In the nascent head region of elongated spermatids, its expression is confined to the junctional plaques. JAM-C alters the adhesion structures of spermatozoa by polarizing the localization of PAR-3 and anchoring spermatids to the Sertoli cells. When the *JAM-C* gene is deleted, the mutant spermatids are found to lack acrosomal structures and to become defective in polarizing the adhesion structure; this eventually leads to the production of spermatozoa with round nuclei. These round-nucleated spermatids fail to differentiate into elongated spermatids, and this dysfunction causes teratozoospermia, characterized by spermatozoa with deformed heads.

RA175: RA175 is a protein of the nectin family and is identical to Necl-2. All nectins analyzed thus far have been found to associate with the actin-like cytoskeleton via afadin, an F-actin- and nectin-binding protein. *RA175*$^{-/-}$ male mice develop severe oligoteratozoospermia, and their sperm production is dramatically reduced (Fujita et al. 2006). Interestingly, spermatogenesis proceeds rather normally in *RA175*-deficient mice until the early round-spermatid stage, but when spermatids reach the elongation stage, they begin to detach from the Sertoli cells. Consequently, very few spermatozoa reach the spermiation stage, and even those that do exhibit morphological abnormalities in the head and tail. Presumably, the signal transduction required for the association of nectins with actin via afadin at the apical ectoplasmic specialization is not induced in *RA175*$^{-/-}$ male mice, since RA175 is reported to interact with nectin-3 (Takai et al. 2003).

Chapter 4
Post-testicular Modifications and Maturation Events Occurring in the Sperm Head

In general, post-testicular maturation events occurring in sperm are controlled by factors expressed in the luminal fluid within the epididymis. This indicates that the sperm interacts with the ductal epithelial cells and that these interactions are continuously maintained throughout the passage of the sperm along the male and female reproductive tracts until the time of fertilization. The sperm maturation-related molecules secreted in the epididymis have recently been designated as epididymosomes (Sullivan et al. 2007). The direct and indirect interactions between sperm and the epithelial cells help to protect and stabilize the sperm components, and they suppress early activation of sperm motility and membrane fusion. These interactions facilitate the functional maturation of the sperm, leading to capacitation and the acrosome reaction. Functional impairment of the epithelial cells lining the male and female reproductive tracts causes obstruction of the epididymal passage and restricts the migration ability of the sperm; this has been demonstrated in cases of failed expression of the carnitine transporter (OCTN2) gene (Toshimori et al. 1999).

4.1
In the Epididymis

Epithelial cells lining the male reproductive tract actively secrete a large number of molecules. These molecules affect the sperm-head components and alter the nature of the cell surface in aging sperm.

Many proteins associate with the sperm head and tail. Here, I will first discuss the plasma-membrane glycoproteins and subsequently the acrosome reaction and nuclear modifications. Membrane glycoproteins, including glycan-modifying enzymes such as glycohydrolases (glycosidases) and glycosyltransferases, directly affect the glycoproteins expressed on the sperm surface.

Glycohydrolases (glycosidases) form a class of hydrolytic enzymes that are capable of cleaving sugar residues from existing glycoconjugates. Glycosidases include proteases, phosphatases, lipidases, phospholipases, and sulfatases. Hydrolytic

enzymes are collectively referred to as acid hydrolases; they are optimally active at an acidic pH within lysosomes (de Duve 1963), and primarily exhibit N-linked glycan regions. Such hydrolytic enzymes are also present in the epididymal tract fluid (Skudlarek et al. 1992) and in the female reproductive tract (Tulsiani et al. 1996). However, their substrate specificity appears to be considerably limited to the different classes of hydrolytic enzymes (Tulsiani 2006). Glycohydrolases and glycosyltransferases in the luminal fluid are most active in the soluble form (exhibiting more than 90% and 81–92% activity respectively). Only 10–19% of the total activity of these enzymes is associated with spermatozoa.

Glycosyltransferases are a group of synthetic enzymes that can transfer sugar residues from donor species to the acceptor site(s) on existing molecule(s). Glycosyltransferases such as galactosyltransferase and sialyltransferase are present both on the sperm surface and in the luminal fluid and thus modify the surface proteins of spermatozoa during their passage through the epididymis (Dacheux et al. 2003; Dacheux et al. 1998; Tulsiani 2006).

The carbohydrate and peptide regions of sperm-surface glycoproteins can be cleaved by the action of hydrolases such as sheddase. The IgSF proteins basigin (mouse) and MC31 (rat; identical to rat CE9) are found to reduce in size during sperm maturation from 60 kDa (when expressed on testicular germ cells) to 25 kDa (when expressed on mature spermatozoa); however, the mechanism responsible for this finding remains unclear. This size reduction involves deglycosylation and will be discussed in detail in the section on post-testicular maturation of the sperm plasma membrane.

In the epididymis, sperm components are protected by the cell-surface glycoproteins (e.g., by sialic acid residues), in various ways. Spermatozoa are protected from agglutination although they are densely packed in the epididymis and vas deferens. When epididymal spermatozoa are treated with exogenous sialidases, neuraminic acid residues are released from the sperm surface, and this results in sperm agglutination (Toshimori et al. 1991). In addition, there has been evidence to show that sialic acid expressed on the sperm surface is capable of masking the epitopes of the sperm antigenic molecules: the epitope of a sperm antigenic molecule—a 54-kDa sialoglycoprotein on the tail surface, recognized by a monoclonal T21 antibody—cannot be exposed unless the sperm cells are treated with a neuraminidase (Toshimori et al. 1990). Thus, sialic acid residue(s) on the T21 antigen molecule functions to inhibit tail-to-tail agglutination.

Sialic acid residues are actively secreted by the epididymal epithelial cells, and they bind to the surface of the maturing sperm. In ram and bulls, the net surface negative charge on testicular spermatozoa is low but becomes high as the spermatozoa pass through the epididymis (Bedford 1963; Holt 1970; Yanagimachi 1994). In rats, the sialyl transferase activity is very high in the proximal caput region but becomes low in the cauda epididymidis (Tulsiani 2006; Tulsiani et al. 1993). Thus, sialic acids lend a negative charge to the surface of mature sperm.

4.2 In the Female Reproductive Tract

Capacitation and the acrosome reaction: Capacitation leads to hyperactivation of the spermatozoa (characterized by whiplash movement) and subsequently to the acrosome reaction. The periacrosomal plasma membrane fuses with the underlying outer acrosomal membrane, and this step is imperative for the acrosomal reaction to proceed normally. Recent evidence suggests that proteins of the nerve transmission-related soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) family that are expressed on the sperm head are involved in membrane fusion during the acrosome reaction, as described later.

Priming of the sperm plasma membrane for gamete fusion: After the acrosome reaction, the inner acrosome membrane is exposed to the external environment and becomes a limiting membrane. The plasma membrane of the sperm head is retained in the ES and the PAR. Recent evidence indicates that the membrane molecules undergo spatial and biochemical modifications during capacitation and the acrosome reaction, as a result of which the equatorial plasma membrane develops the ability to fuse with the oolemma. This event represents the "priming of the plasma membrane for gamete fusion," and it leads to sperm–egg fusion. During this step, a new fusogenic molecule(s) is expressed on the ES surface; this is discussed in detail later.

Chapter 5
Dynamics of the Acrosome

The acrosome has several structural subdomains in which specific molecules are organized, depending on the species. Acrosomes are dynamically modified and mature during the life of the sperm. The events that bring about structural and biochemical modifications in the acrosomal membrane and matrix proteins are collectively known as the "post-testicular maturation of the acrosome." Here, I focus on this topic.

5.1
Acrosome Biogenesis During Spermatogenesis

The acrosome is formed by the fusion of Golgi-derived secretory vesicles containing various hydrolytic enzymes and some unidentified molecules. These vesicles are present in either a soluble form or an insoluble form. The insoluble substances are considered to be anchored to the acrosomal matrix substances.

In terms of the acrosome morphology, its biogenesis is classically divided into four steps: the Golgi, cap, elongation (acrosome), and maturation phases. This classification was established more than 50 years ago (Clermont and Leblond 1955), mainly through light microscopic analyses of periodic acid–Schiff (PAS)-stained testicular sections (Fig. 5.1) and many ultrastructural analyses that have been performed thus far.

Many markers that can serve as probes for acrosomal substances have recently been discovered. I myself have developed several antiacrosomal antibodies, such as: the monoclonal antibody MN7 that recognizes the MN7 antigen on the anterior region of the acrosome (Oh-Oka et al. 2001; Tanii et al. 1994); MC101 that recognizes an antigenic molecule on the acrosomal cortex (Toshimori et al. 1995); and MN9 that recognizes the antigenic molecule Equatorin, which is localized throughout the acrosome and predominantly on the ES after the acrosome reaction (Manandhar and Toshimori 2001; Toshimori et al. 1992a). In particular, MN9 (Equatorin) is a useful marker not only for determining the progress of acrosome biogenesis (i.e., identifying the step) during spermatogenesis but also for evaluating the post-testicular maturation of the acrosome and the acrosome reaction.

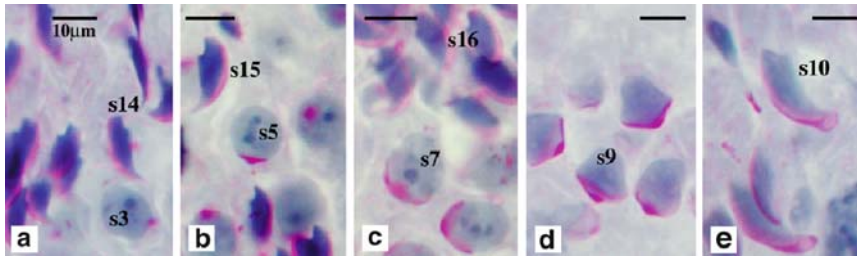

Fig. 5.1 Light micrograph showing the normal process of acrosome formation. Periodic acid Shiff (PAS) staining. **a–e** Developing spermatids (s) at stage III, V, VII, IX and X, respectively. Modified from a photograph shown by Ito et al. (2004). Compare this figure to Fig. 5.5 showing abnormal acrorome biogenesis

5.1.1 Golgi Phase (Steps 1–3 in Mice, Sa in Humans)

The Golgi apparatus is located near the developing acrosome cap. Proacrosomal vesicles derived from the trans-Golgi network fuse to form a single large acrosomal vacuole (Fig. 5.2). The vacuole contains the acrosomal granule, which is closely associated with the nuclear envelope at the center and with the proximal part of the perinuclear theca at the periphery. This structural organization suggests that specific molecules necessary for the biogenesis of head structures such as the perforatorium and PAS are probably transported appropriately, concomitant with the accumulation of proacrosomal vesicles.

5.1.2 Cap Phase (Steps 4–7 in Mice, Sb1–2 in Humans)

As spermatids develop, the acrosomal vesicle (cap) gradually becomes flattened and spreads over the nucleus, while the Golgi apparatus simultaneously migrates distally toward the nascent neck region. At this stage, round spermatids express more than 200 specific transcripts (Griswold lab homepage; http://www.wsu.edu/~griswold/microarray).

Since one of the critical checkpoints in acrosome biogenesis is the transition from the cap phase to the elongation (acrosome) phase, spermiogenesis is readily disrupted by gene deletion at this stage. This has been demonstrated in mice carrying deletions for *Herb* (Kang-Decker et al. 2001) and *GOPC* (Yao et al. 2002), as described later. In these mutants, the Golgi-derived vesicles fail to fuse, and the acrosomal granules are completely detached from the nuclei as the spermatids grow.

The recently termed "acroplaxome," a structure that was previously known to exist but has recently been characterized (Kierszenbaum et al. 2003), is found at the distal end of the acrosome (Fig. 3.5). The acroplaxome consists of a marginal ring containing keratin 5, 10-nm-thick filaments, and F-actin; this complex is called the

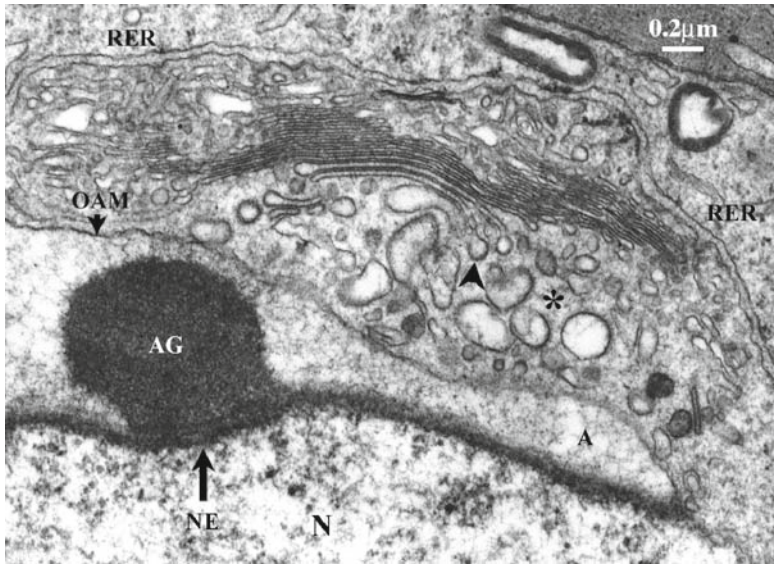

Fig. 5.2 TEM image showing the Golgi apparatus that abutts to the developing acrosome. *AG* Acrosomal granule. Transport vesicles or proacrosomal granules RER rough endoplasmic reticulum (*asterisk*) are derived from the cisterns at the trans region of the Golgi apparatus (*arrowheads*)

"acrosomal plate." The ring is closely associated with the leading edge of the acrosome (acrosomal plate) and with the nuclear envelope (nuclear plate) during elongation of the spermatid head. The acroplaxome is assembled in the subacrosomal space of spermatids; this space is a cytoskeletal scaffold containing F-actin and Sak57 (keratin 5), which is a keratin ortholog. Thus, the acroplaxome connects the acrosome and the nucleus and ensures that the developing acrosome cap remains anchored to the elongating nuclear envelope.

Since the acroplaxome plays an important role in shaping the head of early elongating spermatids, the failure of acrosome formation results in the development of an incomplete acroplaxome, which eventually produces spermatozoa with deformed heads showing the ta phenotype (i.e., the condition of teratozoospermia).

5.1.3 Elongation Phase or Acrosome Phase (Steps 8–12 in Mice, Sc in Humans)

In mice, steps 8–12 correspond to the period from the early stage to the midstage of spermatid elongation. As the spermatid cytoplasm becomes thin, the acrosome is gradually oriented to face the overlying plasma membrane, and manchettes begin to develop from the nuclear ring region in the elongating spermatids. The manchette microtubular bundles develop in spermatids during steps 8–15 in mice. The acrosomal contents gradually condense into an electron-dense matrix while the acrosomal

cap elongates. The next checkpoint is the period of transition from the elongation (acrosome) phase to the maturation phase. As mentioned above, deletion of the gene encoding the IgSF protein RA175 causes the disruption of spermiogenesis at approximately the stage of early elongating spermatids, resulting in oligoteratozoospermia.

5.1.4
Maturation Phase (Steps 13–16 in Mice; Sd1–2 In Humans)

In mice, steps 13–16 correspond to the stages in which late elongating to elongated spermatids can be observed. The dense material (acrosomal granule) in the acrosomal vesicles spreads over the entire acrosomal membrane, eventually differentiating the acrosome into the anterior acrosome and the posterior acrosome (ES). During this period, fundamental molecules appear to be translocated and organized into the anterior acrosome and the ES. At around step 15 in mice, the posterior acrosome develops in the spermatids, and bulb-like structures or acrosome bulbs become evident at the distal end of the acrosome.

Post-translational modifications of acrosomal matrix proteins can be observed in late spermatids (i.e., at step 16); in fact, the expression of the acrosomal antigen MC41 suddenly becomes visible in late spermatids at step 16 (Tanii et al. 1992). The mechanism responsible for this phenomenon is unclear. Immediately prior to spermiation, most of the cytoplasm and organelles of the spermatids are discarded in the cytoplasmic droplet. The residual bodies that are released from spermatids are eventually engulfed by the Sertoli cells.

5.2
Pathways for the Transport of Acrosomal Molecules

In general, acrosomal molecules are translocated from the rough endoplasmic reticulum (ER) to the developing acrosome system via the Golgi apparatus (Clermont and Tang 1985; Fawcett and Hollenberg 1963). However, there may be several other pathways by which these (acrosomal) materials are transported to the developing acrosome. I have discussed this in a previous report, where I suggested the existence of an extra-Golgi tract as well as a Golgi tract, the latter being further divided into a Golgi-acrosomal granule tract and a Golgi-head cap tract (Toshimori 1998).

In addition, four putative extra-Golgi transport routes have recently been suggested on the basis of the expression of the acrosome formation-associated factor (Afaf) (Li et al. 2006). A schematic representation of these pathways is shown in Fig. 3.5. However, before I describe these pathways, I will provide a brief summary of the Afaf molecule.

Afaf: Afaf is reported to belong to the family of type Ia membrane proteins, which are predicted to produce two transcripts (long and short transcripts) in mice. The putative Afaf protein in both mice and humans possesses a signal peptide at the N-terminus and a transmembrane domain with a leucine zipper structure at the

C-terminus. The putative signals for sorting and internalization, namely ETRFLL and DHQLLL, are presumably present in the Afaf sequences of mice and rats. The Afaf protein is abundantly expressed in round spermatids and is reported to be localized in the inner and outer membranes of developing acrosomes; however, the Afaf content of the acrosome is reported to decline as spermatids develop. In transfected HeLa cells, the Afaf protein is found to be localized on the plasma membrane, in early endosomal antigen 1 (EEA1)-positive endosomes, and occasionally in the nuclei. This evidence has led to the hypothesis that Afaf is directly transported from the plasma membrane to the acrosome via the early endosomes (Fig. 5.3) (Li et al. 2006). This hypothesis, if accurate, could provide an explanation for the existence of plasma-membrane molecules on the acrosome (Song et al. 2007). However, extensive evaluation is required to fully validate this hypothesis. The four above-mentioned putative extra-Golgi transport routes are as follows (Li et al. 2006):

Path 1: The ER-to-acrosome pathway; this direct pathway has been suggested on the basis of the MC41 antigen expression (Tanii et al. 1992; Toshimori 1998)
Path 2: Direct transport from the plasma membrane to the acrosome via the early endosome
Path 3: A pathway from the late endosome to the acrosome
Path 4: A transport route from a lysosome to the acrosome

Paths 3 and 4 have been proposed based on studies on rab7.

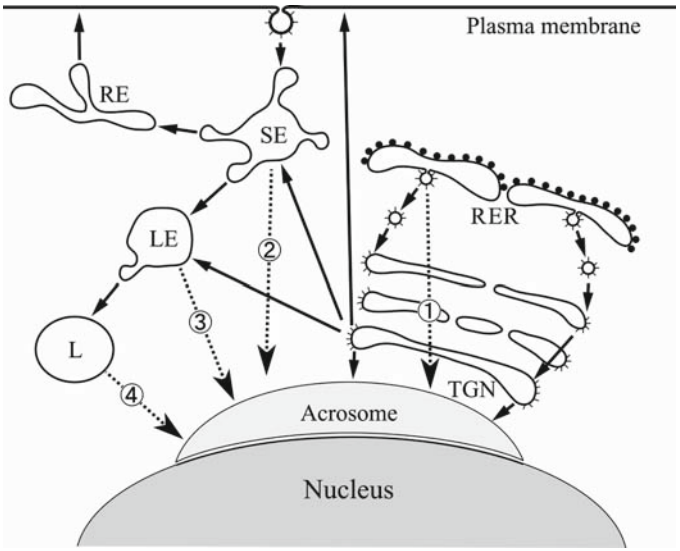

Fig. 5.3 Scheme of possible four Extra-Golgi transport pathways to acrosome. See the text for explanation of the pathway. *SE* secondary endosome; *RE* resorbed endosome; *LE* lysosomal endosome; *L* lysosome; *TGN* trans Golgi network; *RER* rough endoplasmic reticulum

5.3 Microenvironment of the Acrosome

The intra-acrosomal pH is maintained at approximately 5.3 before the acrosome reaction occurs. This acidic pH is maintained with the aid of an H^+ pump. However, after the acrosome reaction, the intra-acrosomal pH increases to approximately 6.2, as has been determined by GFP-assisted flow cytometry in the case of transgenic mice (Nakanishi et al. 2001). Hydrolytic enzymes in the epididymal fluid are functionally active in the pH range of 3.5–5.0 and even remain active up to a pH of 6.2 (Tulsiani 2006). The acrosomal matrix and membranes are known to contain many glycoproteins; however, it is unclear as to whether carbohydrates of acrosomal proteins are present in the acidic microenvironment of the acrosome.

5.4 Post-testicular Modifications and Maturation Events

The process of acrosome maturation is not completed within the testis but continues post-testicularly, during the sperm transit through the epididymal duct. This process has been studied in some mammals such as mice (Toshimori et al. 1995), hamsters (Olson et al., 2003) and guinea pigs (Westbrook-Case et al. 1994; Yoshinaga and Toshimori 2003). Subcompartments of the acrosome are formed during sperm maturation in the epididymis.

5.4.1 Acrosomal Membrane

The outer acrosomal membrane expands anterolaterally toward the rostral region, while the inner membrane is maintained in close contact with the nuclear envelope almost throughout the anterior region of the acrosome.

The paracrystalline cytoskeletal complex is assembled in the outer acrosomal membrane during the post-testicular maturation of the acrosome in the epididymis (Olson et al. 2004). The intra-acrosomal structure adjacent to the inner acrosomal membrane is called the inner acrosomal matrix, and it contains various hydrolases. The acrosomal matrix appears to play roles in the maintenance of many molecules within the inner acrosomal membrane. I will now discuss several interesting molecules organized within the acrosomal matrix.

5.4.2 Acrosomal Matrix

During post-testicular maturation, the acrosome resembles a structurally polarized membrane-bounded organelle containing a stable acrosomal matrix. The matrix is generally a detergent-resistant insoluble framework that remains in close association with the sperm head after the acrosome reaction, while other highly soluble acrosomal proteins are readily released during the acrosome reaction (Hardy et al.

1991). Thus, the acrosome of a mature spermatozoon functions as a regulated secretory granule that is involved in critical processes such as zona pellucida binding and the acrosome reaction. In addition, acrosomal molecules that are portioned into the acrosomal subcompartments are differentially released during the acrosome reaction, and some of these molecules remain functionally active. In fact, the released acrosomal molecules are relocated into the next domain, where they play roles in the subsequent processes of the acrosome reaction and membrane fusion. These processes are involved in the priming of the sperm plasma membrane for gamete fusion.

5.4.3 Subcompartments

Light microscopy as well as general transmission electron microscopy has revealed that the acrosomal matrix is generally homogeneous. However, it is evident that the sperm acrosomes in some mammals (at least in mice, hamsters, and guinea pigs) contain subdomains.

The cortex and medulla in the mouse acrosome: The mouse acrosome contains at least two subcompartments: the cortex and the medulla. These regions differ in terms of their molecular components, as has been evidenced by immunoelectron microscopy. The MC101 antigen is expressed only in the cortex region, and its antigenicity increases during the sperm transit through the epididymis (Fig. 5.4) (Toshimori et al. 1995).

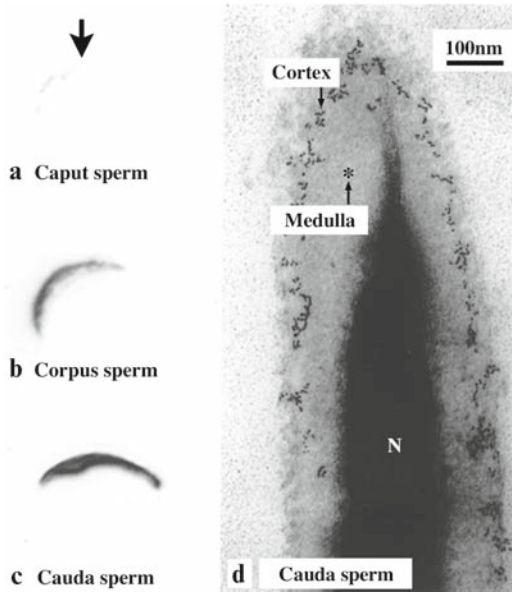

Fig. 5.4a–d Post-testicular modification, showing the change of the immunostaining pattern of intra-acrosomal antigen MC101 during epididymal maturation. **a–c** Immunoperoxidase staining. **d** Immunogold staining. **a–c** The antigenicity of MC101 increases during passing in the epididymis, which is a part of epididymal maturation. **d** Gold particles are localized at the cortex region

M1, M2, and M3 in the acrosome of guinea pigs: The acrosome of guinea pigs contains at least three distinct subdomains in the acrosomal matrix; these subdomains are designated M1, M2, and M3 (Westbrook-Case et al. 1994; Yoshinaga and Toshimori 2003). The spatial location of the acrosomal molecules varies during sperm maturation in the epididymis.

The significance of these subdomains remains to be clarified, although they appear to be related to sperm maturation.

5.5 Acrosomal Enzymes and Other Molecules

Acrosomal enzymes include many hydrolytic enzymes, such as hyaluronidase, proteinases (acrosin), esterases, neuraminidases, acid phosphatases, phospholipases, glycohydrolases, beta-N-acetylglucosaminidase, aryl sulfatases, and collagenase (Yanagimachi 1981). TESP5, a recently identified protein, is a glycosylphosphatidylinositol (GPI)-anchored protein and is presumed to be a testis-specific serine protease (Honda et al. 2002). In the intact acrosome, acrosin is biologically inactive. Other proteins expressed in the acrosome are the ES protein (ESP), Equatorin (MN9 antigen), SP-10, zonadhesin, acrogranin, SP56/AM67, PH-20, etc. Although the functions of these molecules remain largely unclear, I will discuss some of them in greater detail.

Hyaluronidase (Hyal5): Hyal5 is a single-chain hyaluronidase expressed on the plasma and acrosomal membranes, presumably as a GPI-anchored protein. Hyal5 is enzymatically active at pH 5–7 but inactive at pH 3–4, as determined by zymography analysis for hyaluron. Spermatozoa of mice homozygous for a PH-20 deletion are compensated with Hyal5-enriched PH-20-free soluble protein extracts and are rendered capable of dispersing cumulus cells. The cumulus-cell dispersal is strongly inhibited in the presence of the hyaluronidase inhibitor apigenin. Therefore, Hyal5 is presumed to function as a cumulus matrix depolymerase during the process of sperm penetration through the cumulus mass and during the local hyaluronan hydrolysis near or on the surface of the egg zona pellucida.

Proacrosin/acrosin: Proacrosin/acrosin is a hydrolytic enzyme expressed in the acrosomal matrix (Töpfer-Petersen and Henschen 1988b; Tulsiani et al. 1998; Urch and Patel 1991). Proacrosin-deficient mice remain fertile, and this indicates that the hydrolytic function of proacrosin/acrosin is not essential for spermatozoa to penetrate the zona pellucida; however, the zona-pellucida binding and penetration ability of these spermatozoa are found to be reduced in vitro (Baba et al. 2002).

ESP (SP-ESP): The sperm ESP is a 349-amino acid Con-A-binding protein that is predominantly localized in the ES of ejaculated human spermatozoa with intact acrosomes (Wolkowicz et al. 2003). A two-exon gene on the q22 locus of chromosome 15 encodes ESP. ESP is glycosylated strongly on the spots at 34kDa (pI 5.0) and 36kDa (pI 5.1) in the sperm proteome. Immunoelectron microscopy reveals that the ES becomes visible as a defined, discrete domain within the acrosomal

vesicle as early as in the Golgi phase. ESP expression is confined to an electron-lucent subdomain of the condensing acrosomal matrix in Golgi-phase spermatids and persists in a similar electron-lucent subdomain in cap-phase spermatids. It is subsequently localized at electron-dense regions of the ES and the expanded equatorial bulb in elongating spermatids and mature spermatozoa. The functions of ESP remain unclear.

5.6 Failure of Acrosome Biogenesis

Normal acrosome biogenesis is a prerequisite for normal sperm-head formation. Failure of acrosome biogenesis results in impaired formation and shaping of the sperm head. This has been typically demonstrated in studies on *Azh/azh* (Mochida et al. 1999), *Hrb* (Kang-Decker et al. 2001), and *GOPC* (Yao et al. 2002) gene mutations.

Azh/azh: The *azh* mutation is an autosomal recessive mutation. Individuals homozygous for this mutation produce sperm with 100% aberrantly shaped heads but without any other prominent somatic changes (Cole et al. 1988; Hugenholtz and Bruce 1983).

The sperm-tail architectures of *azh/azh* mutant mice are similar to those of the wild-type mice, but the head architectures differ markedly in terms of the point of attachment. The wild-type sperm nuclei are usually flat, whereas the mutant nuclei exhibit tapered cylindrical structures toward the posterior region. The manchette, a microtubular structure, differs between the wild-type and mutant sperm in terms of its size and configuration. At the midstage of spermiogenesis, the manchette microtubules of the wild-type spermatids extend outward at a 45° angle from the long axis of the flattened head, while those of the mutant spermatids form tapered cylindrical structures around the long axis of the caudal region of the nucleus. Thus, the sperm-head shape of *azh/azh* mutants is strongly affected by manchette formation.

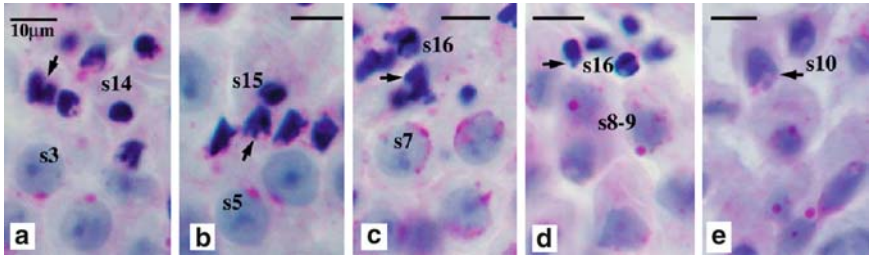

Fig. 5.5 Light micrograph showing abnormal acrosome formation in the spermatogenesis of *GOPC*-deficient (−/−) mouse. PAS staining. **a–e** Developing spermatids (s) at stage III, V, VII, VIII–IX, respectively. Compare this image to that of normal acrosomal biogenesis, shown in Fig. **5.1**. Modified from a photograph shown by Ito et al. (2004)

Hrb and *GOPC*: *Herb*- and *GOPC*-deficient mice exhibit a condition resembling globozoospermia in humans; these spermatozoa have round heads lacking acrosomes (Kang-Decker et al. 2001; Yao et al. 2002). This indicates the existence of an important relationship between acrosome formation and nucleus formation during spermiogenesis. The primary defect exhibited by these mutants is that the Golgi-derived proacrosomal vesicles fail to fuse, eventually resulting in the absence of acrosomes (Figs. 5.5 and 5.6) (Ito et al. 2004). Consequently, acrosomal materials are not transported into the acrosome, and the spermatozoa produced are incapable of performing the acrosome reaction and penetrating the zona pellucida. In addition, in *GOPC*$^{-/-}$ mutant spermatozoa, the organization of the tail cytoskeletal components (ODFs and FSs) fails, and the excess cytoplasm remains accumulated around the nucleus (Suzuki-Toyota et al. 2007).

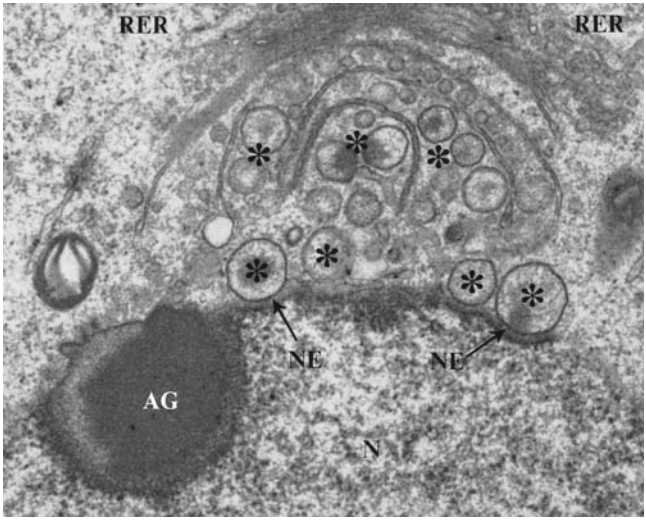

Fig. 5.6 TEM image of abnormal acrosome formation found in *GOPC*-deficient (−/−) male mouse. Many transport vesicles or proacrosomal vesicles (*asterisk*) derived from the Golgi apparatus cannot fuse each other and appear to attach to the nuclear envelope

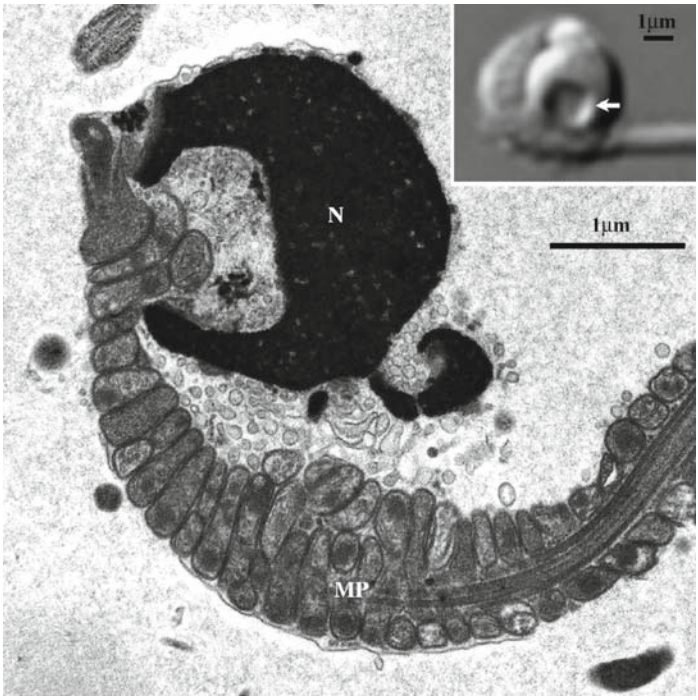

Fig. 5.7 TEM image of a round-headed spermatozoon lacking acrosome. *Inset:* DIC image corresponding to the TEM image shown in Figure 5.7. A large vacuole (*arrow*) is seen in the head

Chapter 6
Dynamics of the Perinuclear Theca

6.1
Biogenesis During Spermatogenesis

The perinuclear theca contains many molecules such as MN13 (Manandhar and Toshimori 2003; Toshimori et al. 1991), PERF15 (Oko and Morales 1994), multiple-binding proteins (capping proteins α3 and β3) (von Bülow et al. 1997), actin-related protein (Arp)-T1 and Arp-T2 (Heid et al. 2002), the calmodulin-binding peptide (CaMBP) (Lécuyer et al. 2000), calicin (expressed in the postacrosomal calyx) (Leclerc and Goupil 2000), phospholipase C (PLC) zeta (Saunders et al. 2002; Yoda et al. 2004), SubH2B (Aul and Oko 2002), cysteine-rich PT1 (CYPT1) (Hansen et al. 2006), and postacrosomal sheath WW domain-binding protein (PAWP) (Wu et al. 2007). Ultrastructural observations reveal that electron-dense materials gradually accumulate in the nascent PAR of elongating spermatids as precursor substances for the biogenesis of the perinuclear theca (Bellvé et al. 1992; Kann et al. 1991; Longo and Cook 1991). This accumulation occurs actively in late elongating spermatids and gradually results in the formation of the paracrystalline sheet or the PAS.

Interestingly, the cytoskeletal components of the paracrystalline sheet and PAS are not formed either in the round-headed spermatozoa produced by *GOPC*$^{-/-}$ mutant mice (Ito et al. 2004) or in the spermatozoa produced by humans with globozoospermia (Escalier 1990). These lines of evidence suggest that substances in the perinuclear theca regulate the shaping of the sperm head by keeping the developing nucleus bound to the acrosome and plasma membrane. Similar findings have been reported previously (Oko and Maravei 1995; Oko and Morales 1994).

The perinuclear theca also contains molecules such as capping proteins α3 and b3 (von Bülow et al. 1997), calicin (Leclerc and Goupil 2000), and actin-related protein (Arp)-T1 and Arp-T2 (Heid et al. 2002), which have an affinity for actin. It is important to investigate the roles of these molecules in fertilization.

6.2 Functions of Perinuclear Theca-Associating Molecules

In addition to their role in sperm-head shaping, the substance(s) in the perinuclear theca play an important role in egg activation, since they contain various structural and functional molecules required for egg activation (Kimura et al. 1998; Longo et al. 1987; Manandhar and Toshimori 2003; Yanagimachi 1994). The egg activation-related substances are presumed to be present in the acrosomal and/or postacrosomal layers, since egg activation is induced immediately (i.e., within 1–3 min) after sperm–egg fusion, during which time no prominent structural changes are induced. Egg activation is triggered by a dramatic increase in the intracellular Ca^{2+} concentration. These topics are discussed later.

Chapter 7
Dynamics of the Sperm Nucleus

Sperm DNA and the associated nuclear matrix are completely enclosed within the nuclear envelope and are not directly exposed to the cytoplasm. In contrast, the maternal genome is not enclosed within a nuclear envelope and is directly exposed to the cytoplasm. Despite being protected by a rigid enclosure, sperm chromatin DNA strands are likely to be damaged during the long process of spermatogenesis, in which apoptosis also causes DNA fragmentation. Further, sperm DNA fragmentation is also induced during the long journey of the sperm to reach the oocyte (Aitken and Krausz 2001). Such DNA fragmentation may lead to poor fertilization and embryonic development, reduced rates of implantation and successful pregnancy, and recurrent pregnancy losses (Chohan et al. 2006).

During spermatogenesis, the sperm chromatin is essentially remodeled, and it condenses. The genomic DNA is transformed from a histone-associated nucleosome to a protamine-rich toroid (doughnut-like) structure (Fig. 7.1). Although these doughnut-like structures are highly resistant to external agents such as environmental toxins, genetic mutagens, oxidative stress, and smoking, the nuclear DNA is still considerably susceptible to oxidative stress (Aitken and Baker 2004; Bennetts and Aitken 2005; Lewis and Aitken 2005). Chromatin modification during the epididymal maturation of the sperm is important to ensure that the nucleus (genome) develops resistance to such external agents. During the entry of the sperm into the oocyte, the nuclear envelope and nuclear matrix proteins are gradually degraded by the action of the reducing agent glutathione, which is abundant in the ooplasm.

It is interesting for researchers to evaluate the status of sperm DNA and to investigate the factors that induce DNA damage. In this regard, I describe the basic findings on the sperm nucleus and then proceed to discuss recently developed techniques for evaluating the degree of DNA damage in sperm.

7.1
Formation and Maturation of the Nucleus

The formation of the sperm nucleus is intimately related to the processes of spermhead shaping and acrosome formation, as discussed earlier. As the golgi-derived proacrosomal vesicles gradually adhere to the nuclear envelope, the nuclear lamina

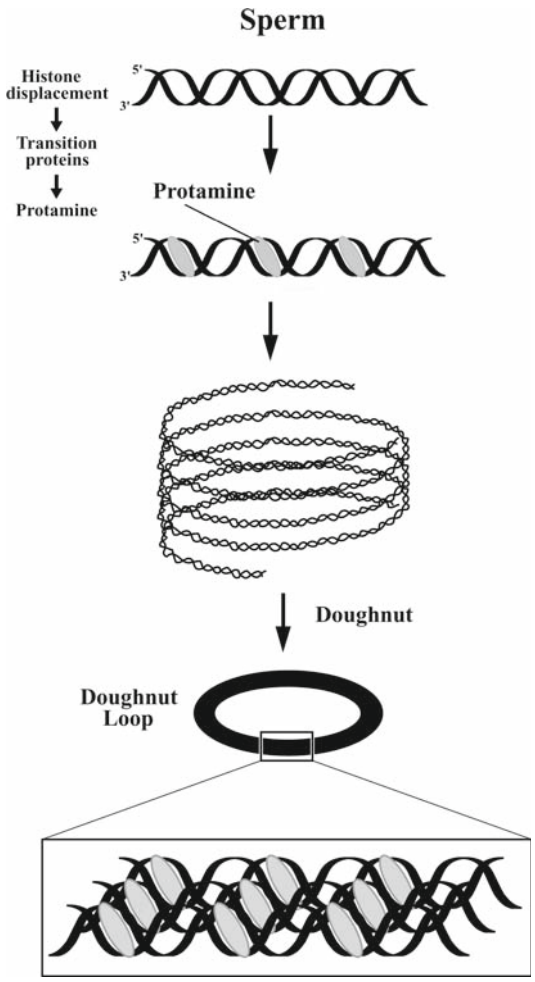

Fig. 7.1 Scheme of sperm DNA strands, doughnut loop (toroid), and their structural relationship. Redrawn based on the scheme presented by Braun (2001)

begins to resemble the thickening region of the inner nuclear envelope that faces the developing acrosome (Figs. 3.4 and 3.5). This event continues up to the stage of elongating spermatids or the mid- to late stage of spermatid elongation. Eventually, the mature sperm nucleus occupies almost the whole of the sperm head, extending anteriorly to the proximal-most region of the head and attaching posteriorly to the basal plate.

In rodents, the sperm nucleus is highly condensed and, in general, a few or no vacuolar structures are found in the nuclear matrix. In contrast, human spermatozoa, including those found in the semen of healthy individuals, contain many vacuoles, which are occasionally in contact with the nucleus. The vacuoles vary in

size and number among individuals. It is possible that the number and size of vacuoles reflect the condition of nuclear maturity/immaturity. In fact, large vacuoles exhibiting membranous structures and containing dense amorphous substances can occasionally be observed in mature spermatozoa. The membranous structures appear to be derived from the nuclear envelope and/or plasma membrane (Fig. 7.2). Similar abnormal findings are found in mice carrying deletions for genes such as *GOPC* (Sukardi et al. 2001), the mouse germ cell-less gene (*mGCL*) (Maekawa et al. 2004), and casein kinase-2α (*CK2α*) (Escalier et al. 2003). These nuclear vacuoles contain the excess cytoplasmic components that are eliminated by the Sertoli cells during spermatogenesis under normal conditions. In this context, morphologically abnormal spermatozoa exhibiting unusually large amounts of cytoplasmic components are considered to be immature in terms of the nuclear-matrix integrity. This finding raises the possibility that DNA strands may be readily fragmented when exposed to the oxidative stress induced by the presence of

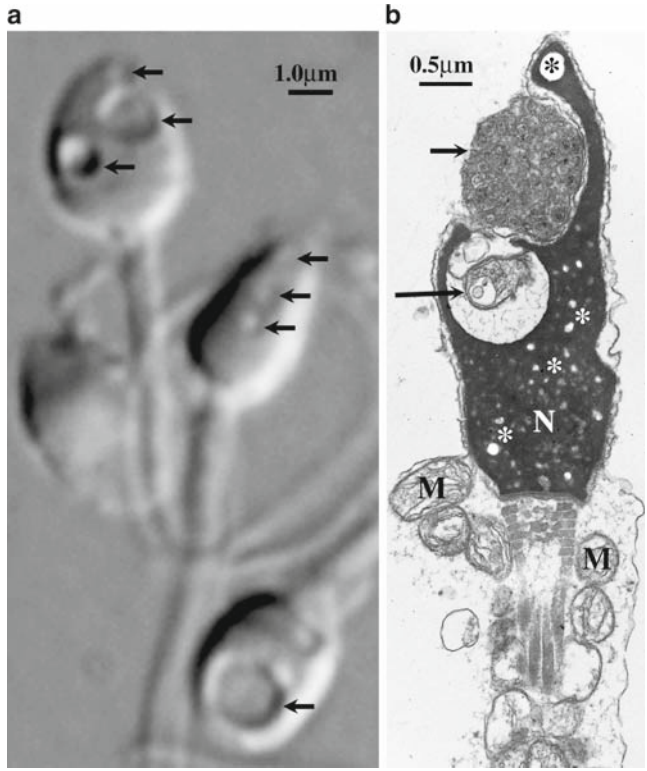

Fig. 7.2a–b Light micrograph and TEM images of the head vacuoles. In general, the head vacuoles are recognized as the nuclear vacuoles by electron microscopy. **a** DIC image to show various-sized head vacuoles (*arrows*). **b** TEM image. Lead and uranyl acetate double-staining. Note that the two large vacuoles differ in content; one large vacuole has membraneous structures (*long arrows*), and the other has amorphous substances (*arrows*). There are many small vacuolous structures without any structures inside (*asterisk*)

cytoplasmic components in excess. Thus, it is important to establish a technique for evaluating the nuclear integrity as well as the sperm quality.

Next, I will elaborate on the failure of nucleus formation: sperm-nucleus formation is easily impaired, and this occasionally results in abnormalities in the sperm-head shape.

7.2 Abnormal Nucleus Formation in Gene-KO Mice

The formation of the sperm nucleus is typically impaired in *Gcl*– (Kimura et al. 2003; Maekawa et al. 2004) and CK2a-KO mice (Escalier et al. 2003).

Gcl: The product of the germ cell-less (*gcl*) gene (GCL) is a component of the germ plasm and an essential factor for the formation of germ cells in drosophila embryos (Jongens et al. 1992, 1994). Mouse gcl-1 (*mgcl-1*), a mouse homolog of *gcl* (de la Luna et al. 1999; Kimura et al. 1999; Leatherman et al. 2000), functions as a repressor against the transcriptional activity of E2F-DP. GCL is localized on the nuclear envelope. E2F is a transcription factor that plays a crucial role in orchestrating the progression of the early cell cycle (Lavia and Jansen-Dürr 1999). DP is an E2F dimerization partner. The DP-interacting protein (DIP) interacts with the DP component of E2F. mGCL-1 binds to lamina-associated polypeptide (LAP)2β (Nili et al. 2001), which is an integral membrane protein expressed on the nuclear envelope and is involved in maintaining the chromatin and nuclear architecture (Gerace and Foisner 2004). GCL is colocalized with LAP2β on the nuclear envelope of mammalian cells (Nili et al. 2001). mGCL-1 binds to emerin, another integral nuclear protein, the deficiency of which causes Emery–Dreifuss muscular dystrophy (Holaska et al. 2003). Thus, mGCL-1 is localized on the nuclear lamina and is directly or indirectly involved in important nuclear activities such as DNA replication and transcription, nuclear and chromatin organization, cell-cycle regulation, and cell development and differentiation (Mattout-Drubezki and Gruenbaum 2003).

When the *mgc1-1* gene is deleted (Kimura et al. 2003), the mutant individual develops teratozoospermia, exhibiting spermatozoa with multiple heads (Maekawa et al. 2004). The nuclei of *mgc1*-null spermatozoa are frequently invaginated, and microtubules are occasionally found in the intruded cytoplasm. The nuclear envelope is abnormal and exhibits myelin-like structures. Both the posterior ring and PAS develop in the spermatozoa of *mgc1*$^{-/-}$ mice, although a part of the PAS is occasionally found to be detached from the nucleus. Deformities are also evident as acrosomal abnormalities and the presence of redundant cytoplasm around the sperm head. Further, incomplete chromatin condensation is frequently observed.

CK2α: CK-2 is a protein kinase casein kinase II with a cyclic-AMP and calcium-independent serine–threonine kinase that is composed of two catalytic subunits (alpha and alpha′). CK-2 uses both ATP and GTP as phosphate donors and specifically targets serine/threonine residues in the vicinity of acidic amino acids.

The active CK-2 homolog is a heterotetrameric protein comprising catalytic (α αvδ α′) and regulatory (β) subunits. The CK2α′ subunit is encoded by the *Csnk2α2* gene and is expressed in developing spermatozoa throughout spermatogenesis (Xu et al. 1999).

Csnk2α2-null male mice are infertile. Their spermatids exhibit a variety of anomalies such as protrusion of the outer nuclear envelope and dilation of the nuclear envelope at the PAR. The number of spermatozoa undergoing epididymal maturation in these mutants is less than 30% of that in the wild-type individuals, and the mutant spermatozoa exhibit abnormally shaped heads, with the acrosomes detached from the nucleus. The acrosomal substances are invaginated into the nucleus or into the abnormally detached nucleoplasm (Escalier et al. 2003).

7.3 DNA Fragmentation

The sperm chromosome is composed of DNA strands and nuclear proteins. The DNA strands are readily damaged not only by aberrations in the chromatin-packing mechanism (Gorczyca et al. 1993; Sailer et al. 1998) but also by defective apoptosis during spermatogenesis (Sakkas et al. 1999, 2002). Further, sperm DNA can also be damaged by reactive oxygen species (ROS) that are produced in sperm per se and oxidative stress in ejaculates (Aitken and DeLuliis 2007; Aitken and Krausz 2001; Aitken et al. 1998; Irvine et al. 2000; Kodama et al. 1997; Lopez-Fernandez et al. 1998; Moustafa et al. 2004; Sun et al. 1997). DNA fragmentation can be visualized in individual spermatozoa by the use of appropriate DNA-fragmentation assays, as described below.

7.3.1 DNA Damage in Germ Cells and Mature Spermatozoa

During spermatogenesis, the somatic histones of germ cells are transformed into transition proteins and subsequently into arginine-rich protamines (protamines 1 and 2 in humans and mice) in developing spermatids. The transition begins in elongating spermatids and is completed in elongated spermatids. Deletion of the genes encoding protamines 1 and 2 causes haploinsufficiency in germ cells and impairs spermatogenesis, resulting in infertility. In particular, when the gene for protamine 2 is deleted, the sperm morphology is severely altered, and the sperm produced exhibit head deformities (Cho et al. 2001).

Apoptosis is induced in germ cells during spermatogenesis, and defective apoptosis causes DNA fragmentation (Aitken and DeLuliis 2007; Aitken and Krausz 2001). During epididymal maturation, the sulfhydryl (S–H) residues in the nuclear proteins of mature sperm form disulfide bonds (S–S) (Calvin and Bedford 1971). Thus, the sperm DNA is protected from oxidative stress. However, spermatozoa themselves produce hydrogen peroxidases, which, if released into the cytoplasm

in excess, are likely to cause DNA fragmentation. Fragmentation of sperm DNA is also induced during sperm storage in the male reproductive tract, especially in males suffering from inflammation of the reproductive tract. Further, sperm may also undergo DNA fragmentation in the female reproductive tract, during their transit to the oocyte, or even in the artificial incubation medium used for in vitro fertilization (IVF). Thus, sperm DNA strands are readily damaged by oxidative stress, and severe damage to sperm DNA is presumed to cause fertilization failure, abnormal embryonic development, spontaneous abortion, or cancer during childhood (Aitken and DeLuliis 2007; Aitken et al. 2006; Lewis and Aitken 2005).

7.3.2 Evaluation of DNA Fragmentation

Through evaluation of sperm DNA fragmentation, it could be possible to predict the degree of chromatin aberration or disintegration. This information is especially useful for clinical examinations performed prior to IVF and ICSI. DNA fragmentation can be evaluated or visualized using DNA-fragmentation assays such as the acridine orange assay (Kosower et al. 1992), deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Sun et al. 1997), alkaline gel electrophoresis (Sawyer et al. 2003), quantitative PCR (QnPCR) for nuclear and mitochondrial DNA to determine ladder-like patterns of base pairs (Sawyer et al. 2003), single-cell gel electrophoresis (SCGE) or the comet assay (McKelvey-Martin et al. 1993; Singh and Stephens 1998), the sperm chromatin structure assay (SCSA) (Evenson et al. 2002, 1999), and the sperm chromatin dispersion (SCD) test (Fernandez et al. 2003). Of these tools, the acridine orange assay does not appear to accurately reflect DNA fragmentation (Chohan et al. 2006).

The rationale of the SCD test is based on the principle that abnormal spermatozoa with fragmented DNA fail to produce the characteristic big halo of DNA loops that is generally produced by normal spermatozoa with intact DNA strands.

However, it is important to note that there has been no evidence proving that the sperm-head morphology reflects the status of DNA integrity (i.e., the sperm quality) or the ability of the sperm to perform functions such as zona pellucida binding, the acrosome reaction, zona pellucida penetration, gamete membrane fusion, and egg activation. Nevertheless, it is possible that the morphology of the sperm head and tail may reflect the sperm functions. Thus, it is important to establish assessment methods for accurately determining the sperm quality with regard to all aspects, including the degree of DNA fragmentation. In this regard, an interesting report has recently been published that presents the outcomes of pregnancy and normal birth after ICSI performed using electrophoretically isolated spermatozoa (Ainsworth et al. 2007).

One possible method by which the sperm quality can be evaluated is by the establishment of an assay to be performed in combination with the sperm morphology assay, using high-resolution microscopy and the predictive test, such as the SCD (sperm chromatin dispersion) test—an assay for DNA fragmentation. In this

context, it is particularly important to evaluate how the morphological characteristics of sperm, such as the number of sperm, their size, and the vacuole content of the heads (in many cases, vacuoles are present within the nucleus) are related to the degree of DNA fragmentation. This is because almost all (usually more than 95%) human spermatozoa, including those of healthy unmarried young men, contain heterogeneous vacuoles varying in number, size, and content (Fig. 7.2).

The system of intracytoplasmic morphologically selected sperm injection (IMSI) that has recently been applied by clinicians will be also useful for these evaluations; however, this assay should be carefully validated to determine its applicability for the selection of good-quality spermatozoa.

To predict the sperm quality in terms of aspects other than nuclear integrity, analyses can be performed using specific probes for sperm-head components that are intrinsically related to the fertilization process. To determine the acrosome status, antiacrosome probes such as antibodies against the acrosome lectins PNA and Con-A and MN9 (antiEquatorin antibody) can be used. Antibodies against postacrosomal molecules such as MN13 are also useful for evaluating the status of the PAR (perinuclear theca) or egg activation ability.

Epigenesis: Epigenesis or genomic imprinting by events such as DNA methylation also occurs in germ cells. Epigenesis does not cause evident structural changes, but it can seriously affect sperm development for normal embryogenesis. Although epigenesis has considerable physiological significance, it is not discussed in this book due to the paucity of evidence regarding sperm events occurring during this phenomenon.

Chapter 8
Dynamics of the Membrane System

Proteins in the sperm plasma membrane are modified when the spermatozoa pass through the extracellular matrix surrounding the cumulus cells. Thus far, I have investigated the IgSF proteins basigin, CE9, and MC31 in this regard (Fig. 8.1).

8.1
Modifications in Sperm Plasma-Membrane Proteins

Mouse basigin (expressed in mice with the Ok blood group) (Igakura et al. 1998; Kuno et al. 1998; Miyauchi et al. 1990) is homologous to the human extracellular matrix metalloproteinase inducer (EMMPRIN), HT-7, CD147, 5A11, M6 (leukocye-activation antigen), 5F7, tumor cell-derived collagenase factor (TCSF), gp42, neurothelin, and the MRC OX-47 T-cell-activation antigen. The rat homolog of basigin is CE9 (de la Luna et al. 1999; Petruszak et al. 1991), which is identical to MC31 (Toshimori et al. 1992a; Wakayama et al. 2000). Mouse *bsg* is localized at locus 42.2 on chromosome 10, and human *EMMPRIN* is localized on chromosome 19p13.3.

Molecular structure of BSG and CE9/MC31: Basigin and CE9 (MC31) are predicted to be single-pass type-1 glycoproteins that comprise 269 amino acids and contain three potential N-glycosylation sites. Basigin is composed of 269 amino acids, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting analyses have revealed its molecular size to range from 35 to 60 kDa. The extracellular domain is composed of 187 amino acids and contains two Ig-related domains. The basigin-encoding gene *BSG* contains seven exons with a total length of 1.8 kb. Its molecular structure, as predicted on the basis of genetic analysis, is shown in Fig. 8.1.

The N-terminal amino acids 1–21 form a prodomain that is possibly involved in cell-membrane trafficking. The mature forms of basigin and CE9 (MC31) possess a prodomain at the N-terminal region and two Ig-like domains (C2 and V loops) containing three putative glycosylation sites and at least one potential phosphorylation site at a serine/threonine residue. The membrane-spanning domain is conserved in most species, including humans, and is connected to the intracytoplasmic domain at the C terminus. The mature form of basigin undergoes homooligomer formation (dimerization) at the cell surface in a *cis*-dependent manner; the N-terminal Ig-like domain is presumed to be essential for this oligomerization. In the testis, basigin

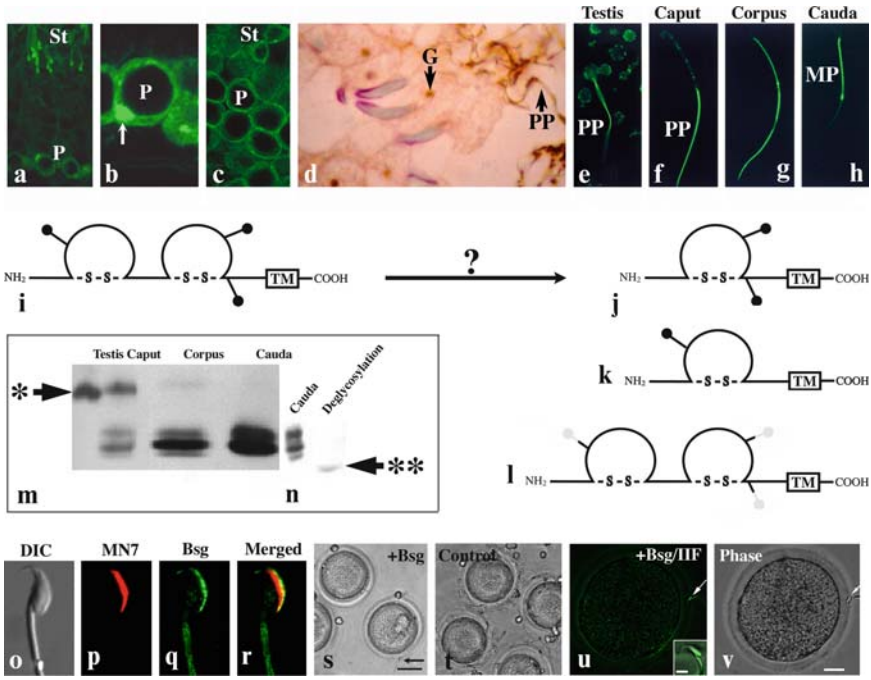

Fig. 8.1a–v Modification of IgSF protein MC31 (**a–h**) and basigin (**i–v**) on the sperm plasma membrane. **a–c**, **e–h** and **u** IIF. **d** Indirect immunoperoxidase staining. **i–l** Scheme showing possible changes of MC31 and the basigin protein structure based on gene analysis and epididymal maturation leading to fertilization. **m**, **n** Immunoblot. **a–d** Expression during spermatogenesis and **e–h** post-testicular modification during epididymal maturation. **i–l** Hypothesis for the processing that occurs on proteineous region (**i–j**) and on the carbohydrate region during the acrosome reaction (**k–l**). **o–r** Basigin is expressed on the anterior acrosome and tail region in intact spermatozoa. **s–t** Inhibition of sperm binding to zona pellucida in the presence of antibasigin antibody (**s**). **t** Control in the absence of antibasigin antibody. **u–v** Basigin is present on the head region (*arrows*). *Inset* of **u**: Binding to zona pellucida, corresponding to Fig. 8.1s

expressed on the surface of germ cells is considered to bind to Sertoli cells via heterophilic interactions or to germ cells per se via homophilic interactions.

Expression of basigin and CE9 (MC31) during spermiogenesis: The expression patterns of basigin (Miyauchi et al. 1990), CE9 (de la Luna et al. 1999; Petruszak et al. 1991), and MC31 (Wakayama et al. 2000) in the testis are essentially identical, as has been determined by in situ hybridization and immunohistochemical staining.

MC31 (CE9) and basigin are first expressed in early spermatocytes prior to meiosis I, after which they are integrated into the sperm plasma membrane for their subsequent interactions with the oocyte. Basigin is ubiquitously expressed in a variety of tissues such as those of the testis, uterus, brain, and retina. The anti-CE9 antibody recognizes various types of cells (de la Luna et al. 1999; Petruszak et al. 1991), while the anti-MC31 antibody recognizes only rat germ cells (Toshimori et al. 1992b); this difference is presumably due to the differences in the antigenic

epitopes expressed by the cells (i.e., due to the species- and tissue-specific expression of carbohydrate moieties).

Deletion of the bsg gene: Male *bsg*-null mice are infertile. This etiology is observed because sperm maturation is arrested at meiosis I. On the other hand, the *bsg*-deficient homozygous female mice are infertile due to the failure of embryonic implantation within the uterus (Igakura et al. 1998). The detailed mechanisms underlying these phenomena remain to be clarified.

Modification of basigin and MC31/CE9 in the epididymis: Basigin and MC31 (CE9) expressed on the sperm surface undergo postmeiotic maturation in the epididymis. The immunostaining pattern of these molecules in the sperm tail changes spatiotemporally, from the distal region (principal segment) to the proximal region (midpiece), as spermatozoa move into the cauda epididymidis (Fig. 8.1). These changes in the immunostaining patterns of these molecules are correlated with a reduction in their molecular sizes: they are approximately 45 kDa in size when expressed on testicular germ cells and on spermatozoa in the caput epididymidis, 35 kDa when expressed in spermatozoa in the corpus epididymidis, and approximately 25 kDa when expressed in spermatozoa in the cauda epididymidis. A possible explanation for this reduction in the molecular sizes may be that the external carbohydrate moieties and/or peptides near the N-terminal loop domain (C_2) are modified or cleaved (Fig. 8.1). If this is true, hydrolytic enzymes present in the epididymal fluid or on the sperm surface must be involved in this event.

Possible roles of basigin and MC31 in the fertilization process: During sperm–egg interaction, the expression of basigin and MC31 can be detected on the sperm head (Fig. 8.1) (Huang and Yanagimachi 1985; Saxena and Toshimori 2004). An affinity-purified antibasigin antibody, if used to test the effects of basigin on sperm–egg interaction, is found to suppress sperm–zona binding. During sperm–egg interaction, basigin is expressed on the head and proximal region of the tail of capacitated spermatozoa. After the acrosome reaction, basigin undergoes deglycosylation with a reduction in its molecular size from 25 to 20 kDa, and its expression tends to disappear from the tail region (Fig. 8.1). Thus deglycosylation is likely to be involved in this event, which is related to sperm–egg interaction.

Since basigin is an EMMPRIN (or a collagenase-stimulating factor) in humans, sperm basigin is likely to play a role in the fertilization process.

8.2 Zona Pellucida Binding and the Acrosome Reaction

Prior to the binding of the sperm to the zona pellucida, the sperm plasma membrane is modified to enable this interaction. This event specifically involves the periacrosomal plasma membrane and not only enables the spermatozoa to recognize the zona pellucida but facilitates the primary binding of the spermatozoa to the zona pellucida. The required modifications occur during sperm capacitation but before the binding of the sperm to the zona pellucida. Presumably, fully capacitated spermatozoa express specific molecules that are expected to be present on the periacrosomal plasma membrane.

8.3 Primary Binding of Sperm to the Zona Pellucida

Binding of spermatozoa to the zona pellucida is inevitable for the entry of the spermatozoa into the perivitelline space. The binding is considered to occur in two steps: primary binding and secondary binding.

Primary binding is a temporal event and is not adequate to support sperm penetration into the zona pellucida; that is, spermatozoa are readily detached from the zona pellucida during this step. The acrosome reaction is considered to begin only after the primary binding, as has been typically shown in studies on mice (Bleil and Wassarman 1990; Wassarman 1990). However, the findings of studies on several other animals such as *Suncus* spp. (Kaneko et al. 2001) and guinea pigs (Huang et al. 1981) indicate that the acrosome reaction is completed or at least initiated before the sperm reaches the zona pellucida. Thus, it is unclear as to precisely when and how the acrosome reaction occurs in spermatozoa during fertilization in mammals, including humans.

Under natural conditions in mammals, the primary binding is a highly species-specific event, depending on the relevant carbohydrates associated with the zona pellucida molecule ZP2 (Bleil and Wassarman 1990; Wassarman 1990).

Novel molecular models of sperm–egg binding have recently been presented on the basis of the findings in mice (Clark and Dell 2006). One such model presents the protein–protein interactions occurring at the initial stage of sperm–zona pellucida binding. Another is a mixed model wherein approximately 75–80% of the sperm–zona binding events are shown to be carbohydrate dependent, while the rest are shown to occur via protein–protein interactions. In fact, several proteins are known to be involved in the sperm functions of zona pellucida recognition, binding, and adhesion, as has been elucidated in studies on animal models with deletions of genes such as *apolipoprotein B* (Huang et al. 1996), *Calmegin* (Ikawa et al. 2001), *ACE* (Hageman et al. 1998), and *Cyritestin* or *ADAM3* (Nishimura et al. 2007, 2001; Shamsadin et al. 1999). Although the molecular mechanisms underlying these protein–protein interactions and carbohydrate-mediated events that govern sperm–zona binding remain to be clarified, it is possible that the signal transduction leading to the acrosome reaction may be carbohydrate independent (Clark and Dell 2006).

8.4 The Acrosome Reaction

Based on the evidence accumulated thus far, a model of the signal-transduction cascade that follows the acrosome reaction has been proposed (Fig. 8.2). The mechanisms involved in this signal cascade are not described in detail here, but I will later discuss in greater detail the membrane-associating molecules.

The acrosome reaction must be completed before the entry of the sperm into the zona pellucida. The entry of extrinsic calcium into the sperm is required to trigger the cascade of events involved in the acrosome reaction. During the acrosome reaction, many hybrid microvesicles are formed via fusion of the overlying

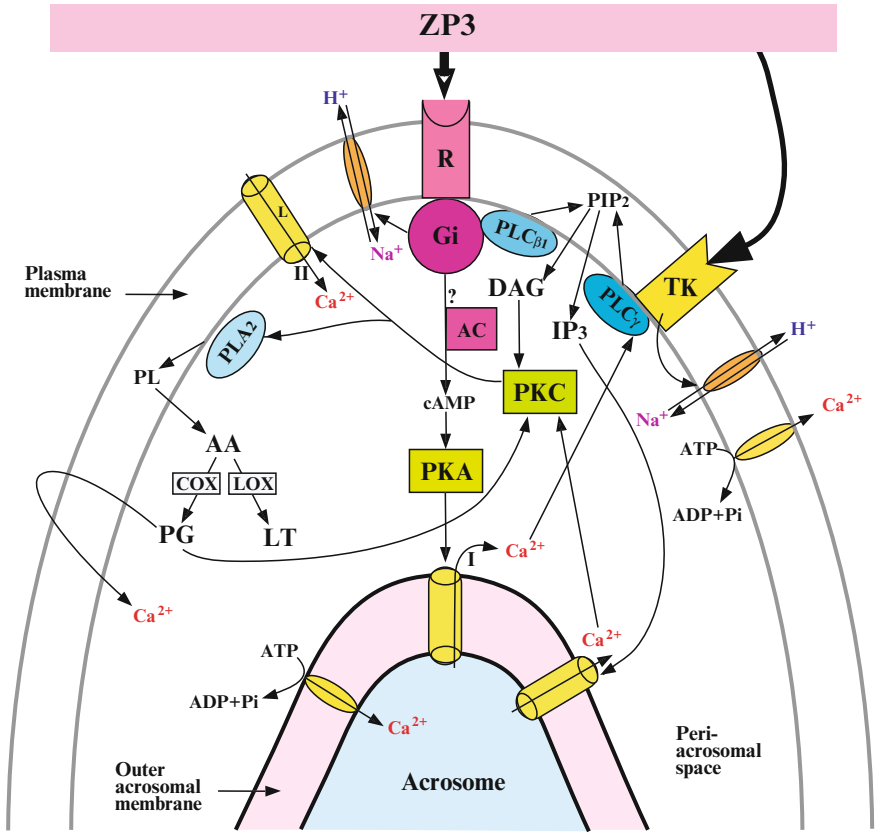

Fig. 8.2 Scheme showing the possible interactions including signal cascades among the activities involved during acrosome reaction. Zona pellucida glycoprotein 3 (ZP3) binds to at least two different receptors on sperm plasma membrane. One (R) is a G_i-coupled receptor that activates phopholipase C (PLCβ1). The other (TK) is a tyrosine kinase receptor coupled to PLCγ. Binding to receptor (R) would regulate adenylate cyclase (AC), leading to the elevation of cAMP and protein kinase (PKA) activation. The PKA activates a voltage-dependent Ca^{2+} channel in the outer acrosomal membrane, which releases Ca^{2+} from the interior of the acrosome to the cytosol. This is the first (relatively small) rise in $[Ca^{2+}]_I$ (I) which leads to activation of the PLCγ. The products of phosphatidyl-inositol biphosphate (PIP_2) hydrolysis by PLC diacylglycerol (DAG) and inosotol triphosphate (IP_3) will lead to protein kinase C (PKC) translocation to the plasma membrane and its activation. PKC opens a voltage-dependent Ca channel (L) in the plasma membrane, leading to the second (II; higher) increase in $[Ca^{2+}]_I$. The G_i or TK can also activate a Na^+/H^+ exchanger, leading to alkalization of the cytosol. PKC also activates phospholipase A2 (PLA_2) to generate arachidonic acid (AA) from membrane phospholipids. AA will be converted to prostaglandins (PG) and leukotriens (LT) by the enzymes cyclooxygenase (COX) and lipoxygenase (LOX) respectively. The increase in $[Ca^{2+}]_I$ and pH will lead to membrane fusion and acrosomal exocytosis. *ADP*, adenosine diphosphate; *ATP*, adenosine triphosphate. The original drawing and its legend were presented by Breitbert and Spungin (1997)

periacrosomal plasma membrane and the outer acrosomal membrane. A calcium-binding protein is found to be expressed in the outer acrosomal membrane of spermatozoa in rams (Sukardi et al. 2001).

After the acrosome reaction, the peri-ES plasma membrane partially loses cholesterol, and this alters the distribution of intramembranous particles and the composition of membrane molecules (Suzuki and Yanagimachi 1989). In this context, the emergence of a cholesterol-free area in the equatorial plasma membrane may possibly reflect raft formation, an event that is a prerequisite for fusion of the sperm and egg membranes. The raft is considered to be composed of the membrane subdomains that are rich in sterols and sphingolipids. Candidate molecules and substances that have previously been identified as raft components of the sperm plasma membrane are listed in Table 8.1 (Sleight et al. 2005). Interestingly,

Table 8.1 Detergent-resistant membrane proteins (candidates of raft-relating molecules) identified by MS/MS, and their respective genes and NCBI accession numbers. Modified from Sleight et al. (2005)

Detergent-resistant membrane protein	Gene (mouse/human)	NCBI
Hexokinase I (HK1)	*Hk1/HK1*	AAH72628
Mouse keratin complex 2 (KRT2)	*Krt1/KRT1*	NP 032499
Izumo	*Izumo1/IZUMO1*	BAD91011
Similar to PH-20/Hya15 (4933439A12RIK)	*Hyal5/ND*	BAC55071
Mouse sperm albumin (ALBI)	*ND/ND*	CAA09617
Seminal vesicle secretion 5 (SVS5)	*Svs5/ND*	NP 033327
Similar to CGI-49 (C330023F11RIK)	*Sccpdh/SCCPDH*	NP 848768
PH-20/Sperm adhesion molecule (SPAM1)	*Spam1/SPAM1*	AAP49832
Testis serineprotease 1 (TESP1)	*Tesp1/ND*	NP 033381
Testis-specific TES101RP (TEX101)	*Tex101/TEX101*	NP 064365
GLUT3/Solute carrier family 2 (SLC2A3)	*Slc2a3/SLC2A3*	NP 035531
L-Lactate dehydrogenase A-like (LDHAL6B)	*Ldhal6b/LDHAL6/*	NP 780558
SCP/TPX-1/CRISP-LIKE #1 (4921505011RIK)	*Crisp2/CRISP2*	NP 080499
Testis serine protease 2 (TESP2)	*Tessp2/TESSP2*	BAB78735
Vacuolar ATPase D (ATP6V0D1)	*Atp6v0d1/ATP6V0D1*	NP 038505
Carbonic anhydrase IV (CAR4)	*Car4/CA4*	NP 031633
Pantophysin isoform 1 (SYP1)	*Syp/SYP*	NP 038663
Polycythemia rubra vera-like #1	*ND/ND*	NP 877586
Polycythemia rubra vera-like #2	*ND/ND*	XP 355991
Basigin (BSG)	*Bsg/BSG*	NP 033898
Serine protease-like #1	*Gm1019/ESSPL*	BAB63919
SCP/TPX-1/CRISP-like #2	*(Crisp2/CRISP2)*	BAB24280
Ig Light chain (IGK-V8)	*Igh-3/ND*	AAB59659
Ig Gamma 2b (IGH-3)	*Crisp1/ND*	NP 033768
Cystein-rich secretory protein 1 (CRISP1)	*Mup1/ND*	NP 112465
Major urinary protein 1 (MUP1)	*Svs7/ND*	NP 064660
Caltrin/seminal vesicle secretion 7 (SVS7)	*Svs7/ND*	NP 064660

ND not detected

the membrane fusion associated with the acrosome reaction involves the periacrosomal membrane, which is relatively rich in chole sterol, and the outer acrosomal membrane, which is deficient in cholesterol. In contrast, the fusion between the sperm and egg membranes involves the equatorial plasma membrane, wherein cholesterol is focally depleted, and the oolemma, which is rich in cholesterol. It can be presumed that the fusion mechanisms that operate in the acrosome reaction and in gamete membrane fusion differ. Recently, SNARE-family proteins have been suggested to be involved in the acrosome reaction.

SNARE: SNARE-family proteins are expressed in the acrosome. v-SNARE is associated with the outer acrosomal membrane, while the target t-SNARE is associated with the plasma membrane. Interactions between v-SNARE and t-SNARE require Rab proteins and a complex of two proteins: NSF and SNAP. Rab proteins are members of a small family of GTPases related to Ras proteins, while the NSF–SNAP complex is recruited via interactions between Rab proteins and the v-SNARE/t-SNARE complex. Fusogenic molecules such as VAMP, syntaxin (Katafuchi et al. 2000; Ramalho-Santos et al. 2000; Tsai et al. 2007), synaptotagmin, and rab3A (Iida et al. 1999; Ward et al. 1999) are reported to be involved in the exocytosis event of the acrosome reaction. A schematic representation of the role played by SNARE proteins in membrane fusion is shown in Fig. 8.3.

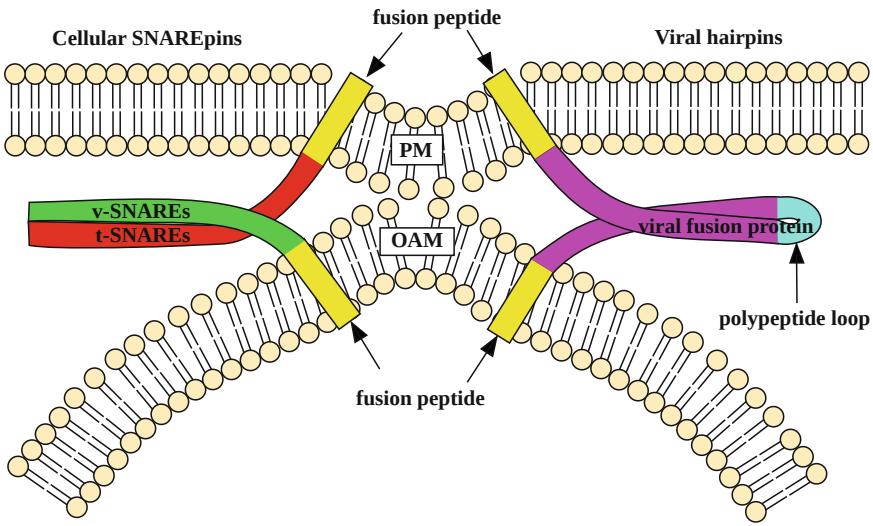

Fig. 8.3 Scheme showing a hypothesis for the acrosome reaction mediated by SNARE proteins, compared to viral fusion proteins. Modified based on the original drawing presented by Weber et al. (1998)

8.5 Rapid and Slow Release of Acrosomal Molecules

The acrosomal matrix interacts with the outer acrosomal membrane, forming matrix–membrane complexes, and these complexes in turn interact with substances in the zona pellucida (Olson et al. 2003). During these interactions, soluble hydrolytic enzymes are readily and rapidly released. In contrast, insoluble structural molecules appear to be released slowly.

Acrosomal molecules are apparently associated with hybrid microvesicles formed during the acrosome reaction, as has been shown in the case of hamsters (Fig. 8.4). Some of these complexes reach the surface of the equatorial plasma membrane, and this presumably triggers the modification of preexisting but dormant molecules on the equatorial plasma membrane (Fig. 8.5). Several possible mechanisms have been proposed for the membrane modifications that occur during the acrosome reaction, and these are schematically presented in Fig. 8.6.

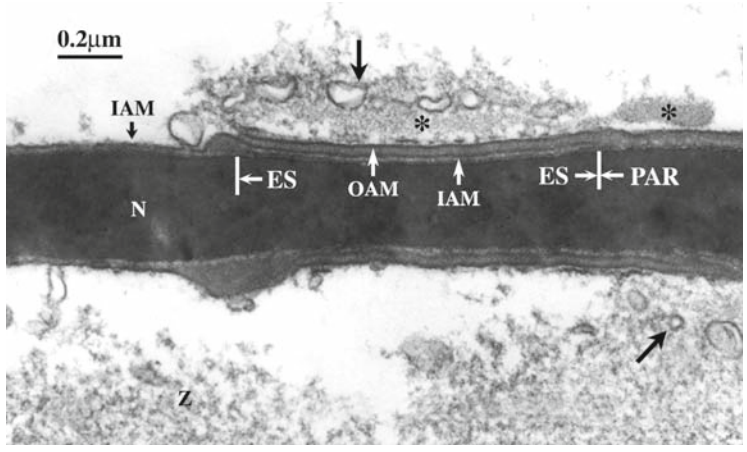

Fig. 8.4 TEM image showing the hybrid vesicles (*arrows*) that are formed by membrane fusion between periacrosomal plasma membrane and the outer acrosomal membrane. Amorphous substances (*asterisk*) are found associating with the hybrid vesicles. Samples were recovered from hamster fertilization in vivo

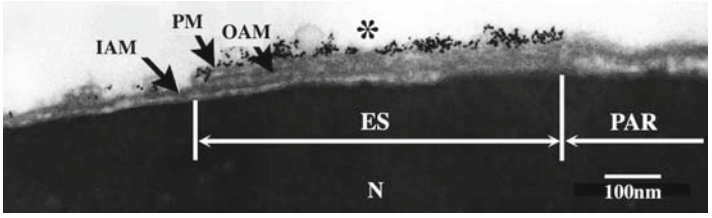

Fig. 8.5 TEM image of immunogold (IGS) showing the relocation of Equatorin (MN9 antigen) during the acrosome reaction. Immunogold particles are found on the plasma membrane over the equatorial segment. Equatorin is present within the acrosome before the acrosome reaction starts (Toshimori et al. 1992a)

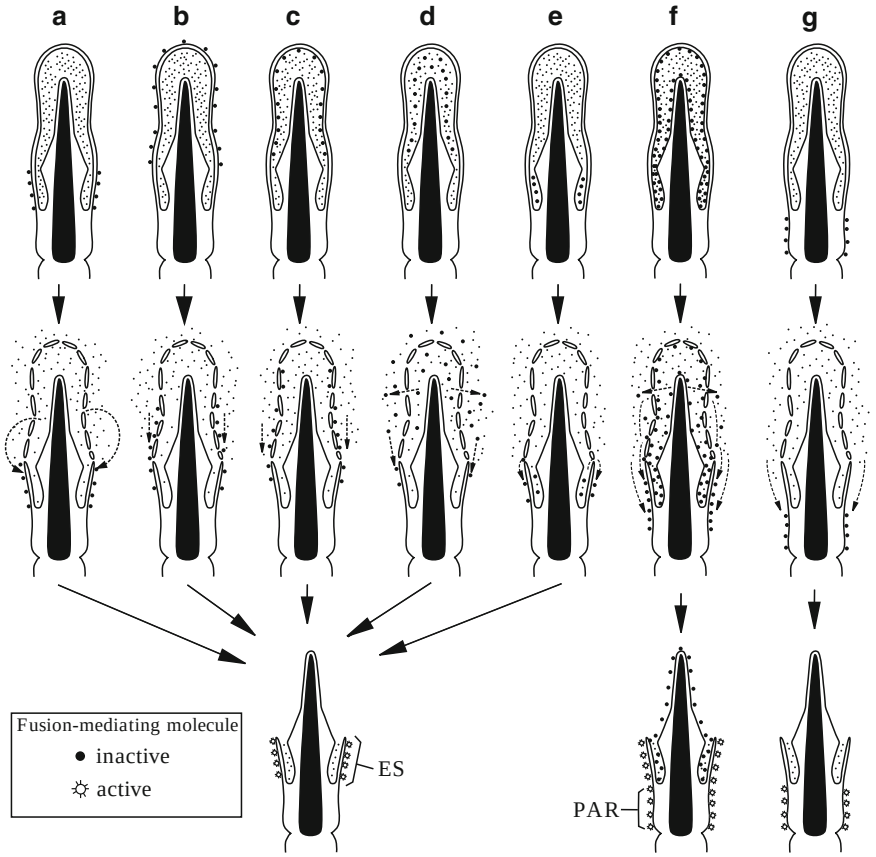

Fig. 8.6a–g Scheme showing possible routes for the relocation of acrosomal substances during the acrosome reaction. **a** Putative fusion proteins in/on the periequatorial segment plasma membrane are in a dormant phase before the acrosome reaction starts. Acrosomal enzymes such as acrosin activate the proteins during the acrosome reaction (Takano et al. 1993). **b** Fusion-mediating molecules migrate from the periacrosomal plasma membrane to the periequatorial segment plasma membrane (Rochwerger and Cuasnicu 1992). **c** Fusion-mediating molecules migrate from the outer acrosomal membrane to the periequatorial segment plasma membrane (Allen and Green 1994). **d** Part of the acrosin released from the acrosome binds to the periequatorial segment plasma membrane. **e** Some molecules within the equatorial segment migrate to the periequatorial segment plasma membrane to mediate sperm–oolemma fusion (Toshimori et al. 1992). **f** Fusion-mediating molecules relocate from the acrosomal membrane (IAM and OAM) to the periequatorial segment plasma membrane; this type is the current hypothesis of the author (unpublished data which will be published separately). **g** Fusion-mediating molecules on/in the peripostacrosomal plasma membrane are activated after the acrosome reaction (Blobel et al. 1992). Modified from the original drawing by Yanagimachi (2003)

8.6 Secondary Binding to the Zona Pellucida and the Candidate Molecules Involved

Secondary binding between the sperm and zona pellucida is strong and always follows the acrosome reaction (Bleil et al. 1988). Therefore, acrosome-reacted spermatozoa are expected to express adhesion molecules and/or appropriate receptor molecules on the inner acrosomal membrane and/or the equatorial plasma membrane. If this is true, specific molecules may be present on the inner acrosomal membrane itself and/or among the acrosomal matrix substances (Foster et al. 1997). Secondary binding facilitates the digestion of the zona pellucida. Candidate molecules involved in secondary binding are proacrosin/acrosin (Jones 1991; Töpfer-Petersen and Henschen 1988a; Urch and Patel 1991), zonadhesin (Hardy and Garbers 1995), sperm adhesion molecule 1 (SPAM1) or PH-20 (Cowan et al. 1991; Hunnicutt et al. 1996; Yudin et al. 1999), PH-30 (Blobel et al. 1992), SP17 (Kong et al. 1995; Richardson et al. 1994), MC41 (Tanii et al. 2001), sperm acrosomal membrane-associated protein 32 (SAMP32) (Hao et al. 2002), SAMP14 (Shetty et al. 2003), 26S proteasome (Sutovsky et al. 2004), IAM 38 (Yu et al. 2000) or Sp38 (Mori et al. 1995), the MN9 antigen or Equatorin (Toshimori et al. 1998, 1992a), Izumo (Inoue et al. 2005), and Afaf (Li et al. 2006). Of these molecules, several proteins of particular interest are further described.

IAM38 or Sp38: IAM38 is a 38-kDa polypeptide expressed at the peripheral region of the inner acrosomal membrane coat (IAMC), as shown in Fig. 8.7 (Yu et al. 2006). It is also reported to be associated with the underlying perforatorium. IAM38 is identical to Sp38, which has been identified in pigs in a previous study (Mori et al. 1995). The nucleotide and amino acid sequences of this protein are highly conserved (90%) in boars, bulls, humans, and murid rodents. Treatment with the anti IAM38 antibody can block in vitro fertilization in porcine systems, and this suggests the involvement of IAM38 in secondary binding between sperm and the zona pellucida.

Zonadhesin: Zonadhesin binds to the zona pellucida of mammalian oocytes in a species-specific manner that is distinct in the cases of mice (Gao and Garbers 1998), pigs (Hardy and Garbers 1994), rabbits (Lea et al. 2001), and humans (Gao et al. 1997; Glockner et al. 1998).

The precursor of pig zonadhesin is a 267-kDa mosaic protein (Fig. 8.8). It exhibits the topology of a type I membrane protein and has a large extracellular region comprising a unique N-terminal domain containing a meprin/A5 antigen/mu receptor tyrosine phosphatase, a mucin-like domain, and five tandem domains that lie proximal to the membrane and are homologous to the pre-pro-von Willebrand (VWD) factor. The polypeptide composition of zonadhesin expressed in mature spermatozoa differs drastically among species. Sperm-specific surface proteins contain multiple domains resembling those of cell adhesion molecules. The zonadhesin precursor is predicted to possess a transmembrane segment, localized

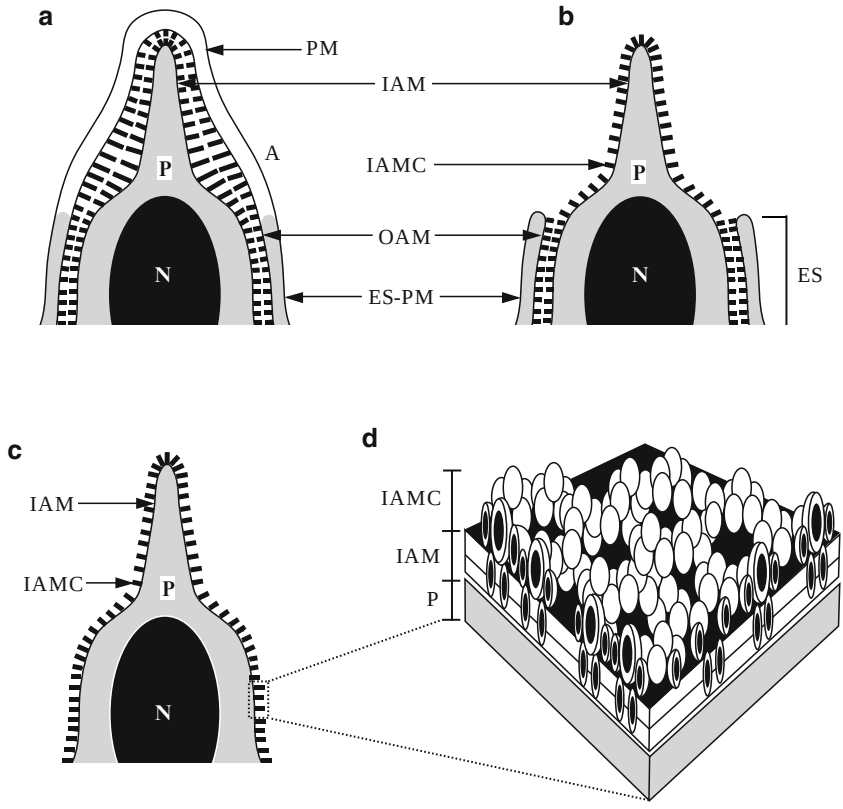

Fig. 8.7 Scheme showing the features of the acrosomal membranes and the inner acrosomal matrix complex (IAMC). **a** Intact Sperm (before acrosome reaction). **b** Acrosome-reacted sperm. **c** and **d** Hypothetical structure of IAMC. Modified from the drawing by Yu et al. (2006)

on the anterior head of the sperm. Interestingly, however, the N-terminal and mucin-like domains are absent from the zonadhesin molecule that binds to the zona pellucida. The precursor molecule is processed into an intermediate molecule and then into the mature zonadhesin molecule; these molecules exhibit M(r) values of 300 kDa (p300), 105 kDa (p105), and 45 kDa (p45), respectively. The latter two molecules are N-glycosylated, while p300 is strongly O-glycosylated, with glycosyl groups spanning the meprin/A5 antigen/mu receptor tyrosine phosphatase and mucin-like domain (Bi et al. 2003). The zonadhesin precursor contains 19 potential N-glycosylation sites in its MAM and VWD domains, and the putative mucin domain includes numerous potential O-glycosylation sites. Thus, zonadhesin is processed (proteolysed) and undergoes deglycosylation during sperm maturation and/or capacitation. Thus, glycosylation and deglycosylation are important events that affect the roles of the acrosomal matrix and membrane-bound molecules.

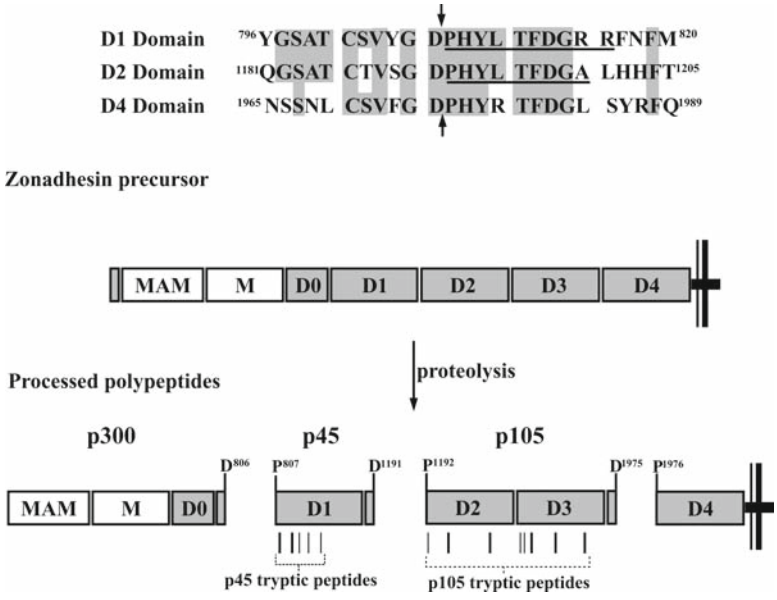

Fig. 8.8 Scheme of zonadhesin molecule. Redrawn based on the scheme by Bi et al. (2003)

In terms of protein trafficking into the acrosomal matrix, immunoelectron microscopy reveals that zonadhesin is uniquely transported to its destination within the acrosome during spermiogenesis and sperm maturation in the epididymis (Olson et al. 2004). In round spermatids, zonadhesin is specifically localized on the acrosomal membrane and is evenly distributed between the outer- and inner-membrane domains. It is subsequently removed from the inner acrosomal membrane and redistributed to the apical and principal segments of the outer acrosomal membrane and to the posterior acrosome. In addition, during sperm maturation in the epididymis, zonadhesin is detached from the outer acrosomal membrane for its incorporation into the acrosomal matrix.

Since zonadhesin undergoes these post-testicular processing and modification events within the fine structural domains of the acrosome, it is presumed to be involved in the step of secondary binding.

In addition, recent evidence indicates that a mutation in the zonadhesin-like domain of α-tectorin, which is a major component of the noncollagenous matrix of the tectorial membrane in the organ of Corti, is associated with autosomal dominant nonsyndromatic hearing loss (Alloisio et al. 1999). Interestingly, severe midfrequency hearing loss is induced in the families with missense mutations (C1619S) within the zona pellucida-binding domain of α-tectorin. The details of how this finding is related to fertilization remain unclear.

SPAM1 or PH-20: SPAM1 was formerly known as PH-20 (Cowan et al. 1991; Hunnicutt et al. 1996). PH-20, which was initially reported to be a 64/53-kDa

protein, is translocated from the plasma membrane to the surface of the inner acrosomal membrane in the sperm of guinea pigs (Cowan et al. 1991). However, PH-20 is now known to be a GPI-anchored protein of 56–64 kDa, localized on both the plasma membrane and inner acrosomal membrane of sperm in guinea pigs, mice, cynomolgus monkeys, and humans (Primakoff et al. 1985; Sabeur et al. 1997; Zheng et al. 2001). PH-20 exhibits hyaluronidase activity and is implicated in the sperm penetration of the cumulus oophorus. Monoclonal and polyclonal antibodies against PH-20 are able to inhibit the adhesion of guinea-pig spermatozoa to the zona pellucida. However, PH-20 is not related to the activity of enzymes involved in sperm–egg interactions, since *PH-20*-deficient male mice are still fertile (Baba et al. 2002). Thus, PH-20 may not be essential for sperm penetration through the cumulus cell layer, although the dispersal of the cumulus cells is delayed in its absence. However, the immunization of female guinea pigs with purified PH-20 was found to induce 100% infertility; this suggests the importance of this molecule in the fertilization process. SPAM1 has recently been reviewed (Martin-DeLeon 2006).

PH-30: PH-30 was formerly known as fertilin and was initially suggested to be involved in sperm–egg fusion (Blobel et al. 1992). The *Adam2* gene encodes ADAM2 that forms the ADAM1/ADAM2 heterodimer (i.e., fertilin). Male mice carrying a deletion for the *Adam2* gene are infertile. However, the infertility is not found to originate at the point of sperm–egg fusion but rather at the point of sperm–zona pellucida interaction (Cho et al. 1998).

SAMP14: Sperm acrosomal membrane-associated protein 14 (SAMP14) is a newly identified putative GPI-anchored receptor of the Ly-6/urokinase-type plasminogen activator receptor (uPAR) superfamily expressed in human spermatozoa (Shetty et al. 2003). uPAR binds to the urokinase plasminogen activator via the N-terminus, and this region is conserved in SAMP14. In addition, uPAR functions to generate proteolytic activity that is restricted to the plasma membrane and is involved in cell–cell and cell–matrix interactions through its participation in signal transduction (Ossowski and Aguirre-Ghiso 2000). Treatment with the anti SAMP14 antibody significantly inhibits not only the sperm–zona pellucida binding but also sperm–egg fusion under a zona pellucida-free condition in hamsters; this suggests that SAMP14 plays a role in one or more events leading to fertilization. SAMP14 is retained on the inner acrosomal membrane after the acrosome reaction; this suggests that it functions as an orphan-class acrosomal membrane receptor, which has an undefined ligand, and participates in proteolysis or events involved in the adhesion of proteins to the acrosomal membrane.

SAMP32: SAMP32 is 32-kDa protein with a predicted pI of 4.57. It contains a putative transmembrane domain at its C-terminus and is phosphorylated at serine 256 in vivo (Hao et al. 2002). SAMP32 is associated with the inner acrosomal membrane, as has been determined by immunoelectron microscopy performed using a specific antibody raised against recombinant SAMP32. This antibody also recognizes the ES of human spermatozoa and inhibits sperm–egg membrane fusion, as has been determined using a human sperm–hamster zona pellucida-free egg penetration assay (Hao et al. 2002).

Other molecules: MC41 is a soluble protein with a high salt content. It binds to mouse ZP2 via other high-salt soluble proteins; this suggests its involvement in the secondary binding of the sperm to the zona pellucida (Tanii et al. 2001). *26S proteasome* is a multisubunit acrosomal protease that is retained on the inner acrosomal membrane. It degrades egg coat-expressed substrate proteins that are tagged with ubiquitin. During fertilization in pigs, 26S proteasome expressed on sperm selectively degrades a ubiquitinated glycoprotein on the zona pellucida (Yi et al. 2007). This indicates that one or more of the zona pellucida proteins are ubiquitinated and that proteasomes associating with the inner acrosomal membrane are exposed after acrosomal exocytosis. Sperm penetration of the zona pellucida is presumed to be involved in the deubiquitination of zona pellucida proteins. At this stage, several proteasomal subunits are phosphorylated. Further, the sperm recognition of the polyubiquitin chain is likely to be related to the sperm acrosomal proteasome. Thus, the 26S proteasome is implicated in the process of sperm penetration of the zona pellucida (Sutovsky et al. 2004; Yi et al. 2007).

8.7 Priming for Gamete Membrane Fusion and the Candidate Molecules Involved

The fertilizing spermatozoon that has entered the perivitelline space should recognize the molecules necessary for its adhesion to or fusion with the egg plasma membrane. Such molecules are presumably present on the egg microvilli, since the sperm plasma membrane first encounters the microvillar plasma membrane, as discussed below. On the other hand, the sperm-surface molecules involved in adhesion and fusion are presumably present on the equatorial plasma membrane, as described above.

Initial priming step: As an initial step, the fluidity of the sperm membrane is altered during capacitation or incubation in a culture medium containing albumin. Cholesterol absorption by extracellular albumin reduces the cholesterol content of the sperm plasma membrane, as has been shown in a study on rats (Davis 1980). In fact, evaluation of this event using the FRR technique reveals that the cholesterol content in the equatorial plasma membrane of the acrosome decreases, as described above. Cholesterol efflux occurring in this manner during capacitation could alter the biophysical properties of the sperm plasma membrane, presumably by altering the lipid constituents of the raft (Nixon et al. 2007). If this is true, the dissociation of these lipid constituents could be involved in the initiation of a signaling cascade (pathway) that leads to the acrosome reaction or even the events occurring after the acrosome reaction, possibly during the priming of the plasma membrane for gamete membrane fusion.

An interesting finding relating to the raft event is that acrosomal molecules are translocated from the acrosome to the sperm surface across the equatorial plasma membrane, as described below (Fig. 8.5). This relocation is thought to be an essential

event for sperm to prime the equatorial plasma membrane, biochemically modifying its properties.

Equatorin (MN9 antigen): Equatorin is an MN9 antigenic protein with a molecular weight of 40–50 kDa in mice (Toshimori et al. 1992a) and 50–60 kDa in humans (unpublished data). As mentioned above (Fig. 8.5), Equatorin is localized throughout the acrosome in intact spermatozoa before the acrosome reaction and is therefore not recognized by the antiEquatorin antibody MN9 (Manandhar and Toshimori 2003). However, its expression becomes more distinct at the ES after the acrosome reaction. Therefore, the MN9 antigenic molecule is designated as "Equatorin." The biochemical nature of this molecule has recently been clarified, the gene encoding it has been identified, and a novel antiEquatorin antibody EQ_{70-83} has been developed (manuscript in preparation).

During the acrosome reaction, equatorin is found to be associated with the surface of hybrid vesicles, which comprise segments of the periacrosomal plasma membrane and the outer acrosomal membrane. On the basis of an antibody-mediated inhibition assay, it has been presumed that the translocated or newly exposed Equatorin eventually participates in gamete membrane fusion (Toshimori et al. 1998). Other lines of evidence obtained recently show that the egg-derived fusion molecule CD9 is translocated to the head of the fertilizing sperm (Barraud-Lange et al. 2007); this translocation appears to be exosome mediated, similar to that observed in immune cells. In fact, many microvesicles and amorphous substances on the sperm surface penetrate the perivitelline space (Fig. 8.9) (Toshimori et al. 1998). Thus, further priming of sperm-surface proteins could be intrinsically induced by a cascade event that follows the acrosome reaction. Nevertheless, this

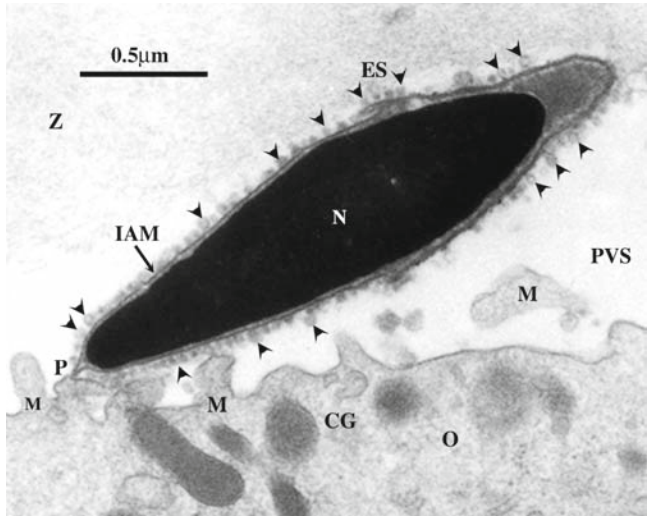

Fig. 8.9 TEM image showing microvesicles and amorphous substances around a sperm head that had reached perivitelline space (PVS)

does not rule out the possibility that the sperm plasma membrane is extrinsically affected by the oocyte factor(s).

IZUMO: IZUMO (Inoue et al. 2005), which was formerly known as the OBF13 antigen (Okabe et al. 1987), contains an extracellular Ig-like domain that has one putative asparagine-linked glycosylation site at amino acid residue 204. Mouse IZUMO has been identified as a testis/sperm-specific 56.4-kDa antigenic molecule of the OBF antibody. Immunoblot analysis indicates that IZUMO expressed in human sperm is a 37.2-kDa protein. When spermatozoa derived from *Izumo*$^{-/-}$ mice are used to inseminate eggs derived from *Izumo*$^{+/+}$ mice, the spermatozoa bind to the egg plasma membrane, but sperm–egg fusion does not occur; this finding reveals that IZUMO is involved in sperm–egg membrane fusion. IZUMO expression cannot be detected on the surface of newly formed spermatozoa but is observed over the entire posterior region of the sperm head after the acrosome reaction, as determined by immunofluorescence analysis. Thus, IZUMO is probably translocated from the acrosome to the surface of the plasma membrane during the acrosome reaction.

DE/CRISP-1: The epididymal protein DE (Cameo and Blaquier 1976) has been analyzed extensively. It is a 32-kDa protein, with carbohydrates comprising 10% of its structure, and is synthesized by the proximal epididymis in an androgen-dependent manner. The secreted DE binds to the sperm surface during epididymal maturation. DE is a member of the family of cysteine-rich secreting proteins (CRISPs), which consists of secreted proteins that have molecular weights of 20–30 kDa and are characterized by the presence of conserved cysteine residues. DE exhibits sequence homology (84%) with the murine epididymal protein AEG-1/CRISP-1 (Haendler et al. 1993) and with human CRISP-1 (Krätzschmar et al. 1996).

DE/CRISP-1 is localized on the dorsal region (anterior acrosome) of the periacrosomal plasma membrane and is relocated to the equatorial plasma membrane (Rochwerger and Cuasnicu 1992). Some DE/CRISP-1 molecules (30% of the total number expressed) are strongly associated with the membrane surface and function as integral proteins involved in gamete membrane fusion (Cohen et al. 2000), but the ligand molecule on the egg surface is not identified. CRISP-family members contain a plant pathogenesis-related domain (PR-1) and a cysteine-rich domain (CRD), connected via a short hinge region. The egg-binding site is located in the Signature 2 region of the PR-1 domain. Most DE/CRISP-1 molecules (70%) are presumed to exhibit ion-channel regulatory activity in the CRD domain; these molecules function as decapacitating factors and are released from the head surface during capacitation because of their weak association with the membrane.

8.8 Gamete Membrane Fusion

Here, I present electron micrographs showing the changes induced in the sperm head immediately after sperm–egg fusion in vivo in mice and hamsters (Figs. 8.10–8.11). At the time of sperm–egg fusion, the structure of the sperm-head

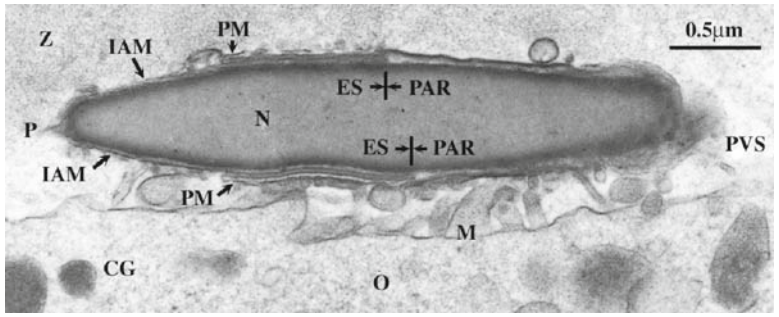

Fig. 8.10 TEM images of spermatozoa during gamete membrane fusion and entry into the ooplasm. At sperm–egg fusion. Note the many microvilli approaching the plasma membrane at the equatorial segment and postacrosomal region

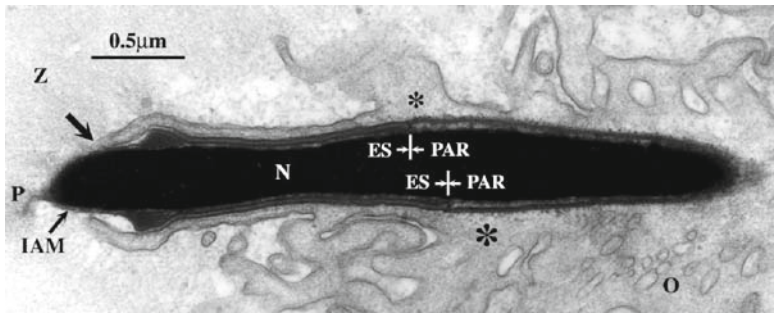

Fig. 8.11 TEM images of spermatozoa during gamete membrane fusion and entry into the ooplasm. At sperm entry. Note the ooplasmic feature around the border of the equatorial segment and postacrosomal region (*asterisk*). Also note that the anterior tip of the equatorial segment protrudes anteriorly (*arrows*)

domain involved in the fusion and in egg activation is of particular significance. On the basis of data obtained in studies on mice and hamsters (Bedford et al. 1979; Oura and Toshimori 1990; Toshimori 1982; Toshimori and Oura 1993; Yanagimachi and Noda 1970), the initial point of fusion is considered to be the ES, presumably including the proximal-most part of the PAR (Figs. 8.10–8.12); a schematic diagram of this region is presented in Fig. 8.13. During the short time required for sperm–egg fusion, structural changes are only observed on the sperm plasma membrane or in the microvillar structures formed around the ES and the proximal part of the PAR. This information is especially important for understanding the structure–function relationship of the sperm components. From a structural viewpoint, it is unclear as to how the sperm plasma membrane interacts with the microvillar plasma membrane, since projections that are thicker than the width of the microvilli are formed at the fusion site. After the sperm plasma membrane interacts with the microvillar plasma membrane, these thick projections may be

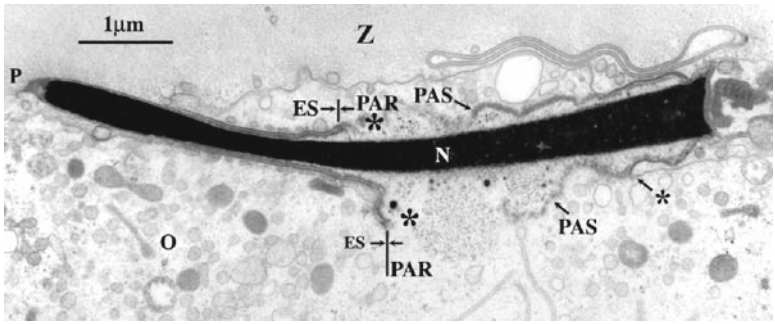

Fig. 8.12 TEM image of spermatozoon during gamete membrane fusion and entry into the ooplasm. At sperm nuclear decondensation. Note that decondensation occurs first at the proximal region of the postacrosomal region (*asterisk*). The acrosomal membrane system is relatively intact at this stage

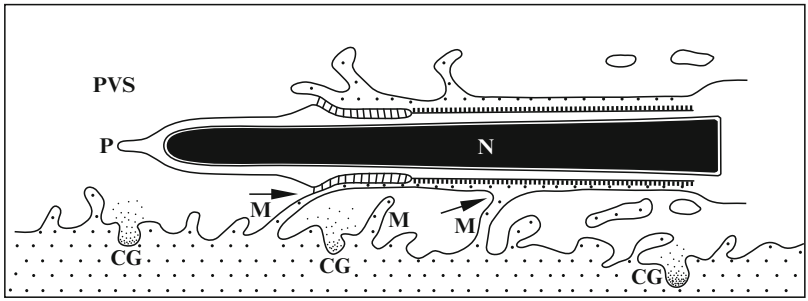

Fig. 8.13 Scheme showing the very early stage of sperm–egg fusion and a possible event, oocyte cytoplasmic flow. *Arrows* indicate the sperm–egg fusion points at which the ooplam enters into sperm cytoplasm

formed because of the cytoplasmic flow from the oocyte (ooplasmic pressure) into the sperm cytoplasm (i.e., the periacrosomal and peri-PAS layers). Further, the mechanism by which actin filaments function to elongate these projections remains unclear. From a functional viewpoint, the structural degradation is found to be intimately related to the release of egg activation-related molecules from the sperm cytoplasm, as discussed in the next topic.

Chapter 9
Dynamics of Egg (Oocyte) Activation

Fluctuations in the intracellular calcium concentration ($[Ca^{+2}_i]$) are a prerequisite for egg activation. The initial increase in $[Ca^{+2}_i]$is mainly due to Ca^{2+} release from the ER into the cytoplasm. The Ca^{2+} wave begins at the site of sperm–egg fusion, and this signal is then propagated throughout the cytoplasm. The repetitive increase in $[Ca^{+2}_i]$is termed "Ca^{2+} oscillation" and is maintained up to the time of pronucleus formation (Jones et al. 1995; Marangos et al. 2003).

Two egg-activation pathways have been proposed (Fig. 9.1). The first pathway is via the sperm (cytoplasmic) factor; in this case, the factor(s) can be directly inserted into the ooplasm. Among these factors, sperm-derived PLCzeta (ζ) is currently a candidate molecule. The other pathway is via the sperm receptor; in this case, the receptor is expected to be present on the oolemma.

9.1
Sperm (Cytoplasmic) Factor and Candidate Molecules Involved in Egg Activation

Factors involved in oocyte activation were formerly known as sperm-borne oocyte-activating factors (SOAFs). SOAFs exist in two forms: the heat-sensitive form SOAF-1 and the heat-stable form SOAF-2 (Perry et al. 2000). When an SOAF is introduced into the oocyte, Ca^{2+} oscillation is activated. This factor can then be recovered from the cytosolic component of the perinuclear theca; this suggests that the SOAF is rather cytosolic in nature.

The finding that Ca^{2+} oscillation is completely inhibited in the presence of a monoclonal antibody against type-1 IP3R/CaC gives rise to the possibility of IP3-induced Ca^{2+} release (IICR) (Miyazaki et al. 1993; Miyazaki et al. 1992). IP3 is produced by PLC-catalyzed hydrolysis of phosphatidyl-4,5-inositol (PIP2) expressed on the membrane, and this process is referred to as the PI pathway.

It is important to note that egg activation can start quickly, within 1–3 min after sperm–egg fusion (Lawrence et al. 1997), and that the signal originating at the site of sperm–egg fusion is multiplied in the ooplasm; this increases the concentration of the Ca^{2+} ion, which mediates Ca^{2+} oscillation. Similarly, it has been reported that the sperm factor(s) is released into the ooplasm between 15 min and 60 min

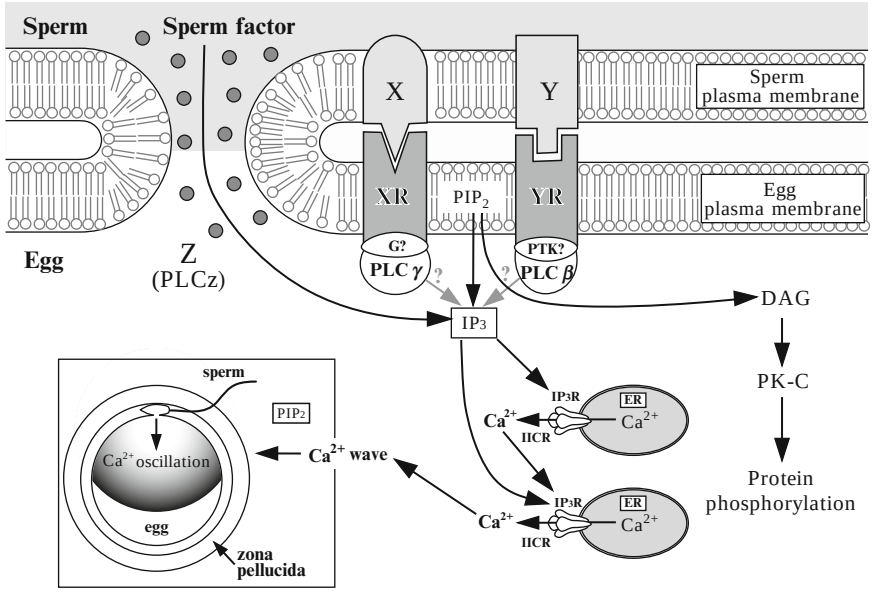

Fig. 9.1 Scheme of sperm–egg signaling that leads to Ca^{2+} release from the ER through IP_3R and forms a Ca^{2+} wave in mammalian eggs (adopted from Miyazaki and Ito 2006). When gamete cytoplasmic continuity is established, sperm factors such as PLCzeta (PLCz) are capable of directly entering ooplasm from the sperm–egg membrane fusion site. Thus, upon entry, the PLCzeta induces IP^3-induced Ca^{2+} release (IICR) through the IP_3R on the intra-ooplasmic Ca^{2+} storage reservoir, smooth endoplasmic reticulum. The released Ca^{2+} is supposed to eventually induce Ca^{2+} waves and Ca^{2+} oscillations. There is also a possibility that the released Ca^{2+} induces Ca^{2+} release (CICR) through the ryanodine receptor (RyR)/Ca^{2+} channel system synergically in a cascade fashion, eventually causing Ca^{2+} oscillations, which are present in some mammals. However, the Ca^{2+} oscillations can be induced in hamsters lacking RyR (Miyazaki et al. (1993). IP_3 is a hydrolytic product of membrane phosphatidylinositol 4,5-biphosphate (PIP_2) via phospholipase C (PLC) in the PI pathway

after the entry of the sperm into the oocyte and the establishment of Ca^{2+} oscillation (Knott et al. 2003). Thus, Ca^{2+} oscillation is always initiated after sperm–egg fusion. Sperm–egg continuity is required for the cytosolic factor(s) to directly enter the ooplasm. This further indicates that the cytosolic factor(s) is localized in the sperm-head cytoplasm, in the ES or PAR near or in the periacrosomal layer. In particular, the extrusion of perinuclear theca substances is required for the initiation of Ca^{2+} oscillation, regardless of the cell-cycle stage in which the sperm is arrested. It has been reported that the sperm factor(s) is not a structural component of the perinuclear theca but is anchored to it (Knott et al. 2003). This report is in accordance with the findings of previous studies (Fujimoto et al. 2004; Kimura et al. 1998; Perry et al. 2000; Sutovsky et al. 2003).

Recent evidence indicates that the perinuclear theca substances remain within spermatozoa even after lysolecithin treatment (Morozumi et al. 2006); lysolecithin

is produced by the hydrolysis of a membrane phospholipase under natural conditions. This evidence supports the notion that the oocyte activation factor(s) is present as a component of the perinuclear theca. Thus far, PLC zeta-KO mice have not been generated for further analysis.

In light of these findings, the sperm-factor theory is more likely to be accurate than the sperm-receptor theory in the present clinical settings.

PLCzeta: PLCzeta, a 74-kDa protein, is expressed in spermatozoa. It was originally considered a candidate Ca^{2+} oscillation-inducing protein (COPI), identical to the sperm (cytosolic) factor. PLCzeta is now known as the smallest PLC isozyme identified thus far (Fig. 9.2). Similar to other PLC isozymes, it contains four EF-hand domains at its N-terminus, X and Y catalytic domains that can cleave PIP2, and a C2 domain at its C-terminus; however, it lacks an N-terminal pleckstrin homology (PH) domain that binds to PI (Saunders et al. 2002). Further, PLC zeta has homologous domains that resemble those of PLCbeta, gamma, and delta, except for the PH domain. Short PLCzeta, a splicing variant of PLCzeta, contains only one EF-hand domain at its N-terminus and is expressed in the mouse testis.

It has been reported that if PLCzeta RNA is injected into the oocyte, Ca^{2+} oscillation occurs in the fertilized egg, and the zygote develops normally into the blastocyst. The amount of PLCzeta expressed in the oocyte is estimated to be 20–50 $\times$ 50^{-15} g, which is almost identical to the amount that has been estimated for a spermatozoon by performing comparative densitometry analyses with synthetic PLC zeta as a control. Absorption of the sperm extract by the anti-PLCzeta antibody suppresses the Ca^{2+} oscillation activity.

Miyazaki's group injected PLCzeta RNA bound to the fluorescent protein Venus into mature oocytes and found that PLCzeta–Venus expression was detected in the oocyte, initially 40–50 min after the injection, and then it increased linearly up to 3 h after the injection (Yoda et al. 2004). In addition, the RNA injection induced Ca^{2+}

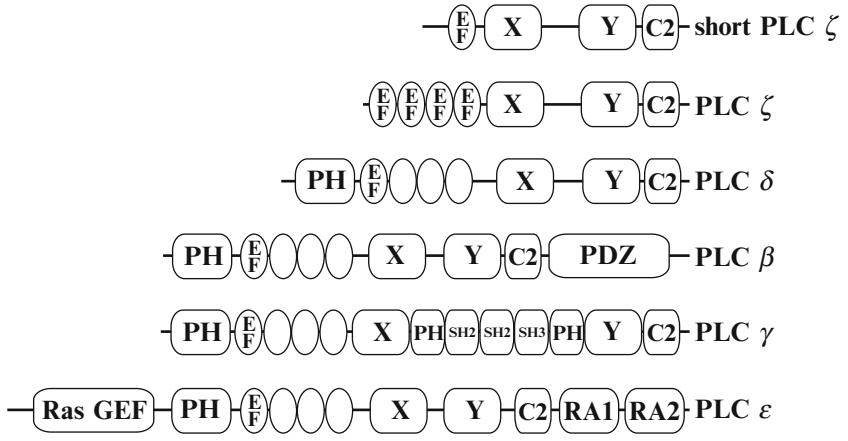

Fig. 9.2 Scheme showing the family of PLC molecules. Adopted from the drawing by Saunders et al. (2002)

oscillations similar to those observed under IVF conditions. The EF-hand domain located at the N-terminus of PLCzeta is reported to be essential for Ca^{2+} oscillations (Kouchi et al. 2005). Structural and functional analyses of PLCzeta have revealed that its EF-hand domain, which usually contains a Ca^{2+}-binding site, is important for its enzymatic activity (Kouchi et al. 2005; Yoda et al. 2004). Thus, since PLCzeta expressed in sperm exhibits adequate activity for the initiation and maintenance of Ca^{2+} oscillations after the entry of the sperm into the oocyte, PLCzeta is most probably involved in egg activation.

The precise localization of PLCzeta in spermatozoa is unknown. According to a previous report (Fujimoto et al. 2004), PLCzeta is expressed in the sperm head and its localization is similar to that of MN13, which is localized in the PAR of the peri-PAS layer. This suggests that PLCzeta may be one of the perinuclear theca materials expressed in the peri-PAS layer. However, PLCzeta-KO mice have not yet been generated for analysis.

Tr-kit: Tr-kit, which is a truncated product of *c-kit*, is expressed in germ cells at the postmeiotic stage and is accumulated in mature spermatozoa, where it is localized in the midpiece and PAR (Sette et al. 1998). Tr-kit is 30-kDa cytoplasmic protein containing only the second box of the split kinase domain (catalytic site of phosphotransferase) and the C-terminal tail of the full-length c-kit receptor. Tr-kit may interact with an unknown tyrosine kinase that is present in the ooplasm and may function as a scaffold protein for the recruitment of signal-transduction proteins required for egg activation. Tr-kit-induced egg activation is likely to be mediated by the activation of the gamma (γ) isoform of PLC (PLCγ1) (Rossi et al. 2003; Sette et al. 2002). When the split-catalytic domain of PLCγ1 becomes active, the consequent PIP2 hydrolysis triggers inositol triphosphate (IP3)- and Ca^{2+}-mediated events required for egg activation.

MN13: As mentioned earlier, the submicroscopic localization of MN13 is restricted to the top of the periodic striations just beneath the plasma membrane covering the postacrosomal sheath (**Fig. 2.3**). MN13 is involved in egg activation, as has been revealed by an inhibition assay performed using the anti MN13 antibody (Manandhar and Toshimori 2003). Although the biochemical nature of MN13 remains to be clarified, the anti-MN13 antibody is currently considered a good probe for studying PAS-related events.

Calmodulin: Calmodulin is localized in the PAS (Kann et al. 1991).

9.2 Sperm Receptor

The receptor/G protein/PI pathway or the receptor/protein tyrosine kinase/PI pathway is known to operate in various types of somatic cells. Although cell-surface proteins appear to mediate sperm–egg binding, no evidence obtained thus far has proved the association between these proteins and intercellular signal transduction (Runft et al. 2002).

Candidate sperm ligands and sperm receptors on the ooplasm are shown in Fig. 9.1. Of these molecules, integrin alpha-6 beta-1 is a putative receptor for fertilin beta and is localized on the egg surface. Although previous studies have investigated alpha-6 beta-1 (Almeida et al. 1995) and the sperm ligand fertilin (Cho et al. 1998), none of the knockout male mice generated for these genes developed infertility; this finding indicates that these molecules are not essential for sperm–egg fusion (Talbot et al. 2003). In addition, no reports published thus far have discussed Ca^{2+} oscillation in relation to these molecules.

GPI-anchored molecules are expressed on the egg surface. Of these, tetraspanin CD9 is essential for gamete fusion (Kaji et al. 2000; Le Naour et al. 2000; Miyado et al. 2000). Further, CD81 expressed on the egg surface probably facilitates the process of gamete membrane fusion, since treatment with the anti-CD81 antibody is found to moderately inhibit sperm binding and fusion.

Since the enzymatic activity of PLCzeta in spermatozoa is suppressed before the entry of the sperm components into the ooplasm, it is important to determine the precise localization of PLCzeta in the sperm cytoplasm and its function.

9.3 After Egg Activation

After egg activation, all of the components of the fertilizing spermatozoon enter the ooplasm. The fertilizing spermatozoon then provides a centriole that gives rise to a microtubule-organizing center (sperm aster), which is essential for normal embryonic development in nonrodent mammals (Sathananthan et al. 1996; Sathananthan et al. 1997; Sun and Schatten 2007; Sutovsky and Schatten 2000). However, in rodent mammals such as mice, hamsters, rats, and guinea pigs, the fertilizing spermatozoa do not provide the centriole for microtubule formation; the microtubules are nucleated in the ooplasm, which exhibits multiple microtubule-organizing centers. Thus, events involving the sperm centriole are of significance after egg activation, but further discussion of these events is beyond the scope of this book.

Chapter 10
Status of Sperm-Head Components During Normal Fertilization, IVF, ICSI, and Round Spermatid Injection

The differences in the behavior of the sperm-head components during sperm entry into the oocyte in the case of normal (natural) fertilization, IVF, ICSI, and round spermatid injection (ROSI) are listed in Table 10.1.

ICSI bypasses the normal events (sperm selection) described above that occur during fertilization. The spermatozoa do not undergo normal modifications and events for the maturation of the plasma membrane, acrosomal membrane, and internal acrosomal and cytoplasmic materials, including the perinuclear theca and DNA. This artificial shortcut bypasses natural sperm-selection processes that routinely occur in the male and female reproductive tracts during natural fertilization. It is also important to note that the process of natural selection against DNA-damaged spermatozoa that occurs during fertilization is not yet clearly understood. Further, it remains unclear as to how healthy and functional spermatozoa with that can successfully trigger egg activation are selected. In this context, as mentioned above, it is important to develop a method for assessing qualities of sperm such as their ability to perform the acrosome reaction and to trigger egg activation, their membrane function, and the stability of their DNA or degree of DNA fragmentation.

Table 10.1 Sperm-head components that enter into ooplasm under various conditions; normal (natural) fertilization, IVF, ICSI and ROSI

Type of entry (carrier of paternal genome)	Sperm components					
	PM	OAM	IAM	PT	Nucleus	Centrosome
Normal (natural) fertilization (ejaculated sperm)	–	–	+ (phagocytosis)	+ (after fusion)	+ (decondensation)	+
IVF (ejaculated sperm)	–	–	+ (phagocytosis)	+	+ (decondensation)	+
ICSI (ejaculated sperm)	+*	+*	+*	+*	+*	+*
TESE–ICSI (ejaculated sperm)	+*	+*	+*	+*	+*	+*
TESE–ICSI (elongated spermatid)	+*	+*	+*	+*	+*	+*
ROSI (round spermatid)	+*	+*	+*	+*	+*	+*

+ enter; – does not enter; +* entry is compulsory

Chapter 11
Conclusion

Spermatozoa perform the function of carrying the paternal genome to the resting oocyte or the maternal genome. The activation of the resting oocyte is followed by fertilization, which leads to the embryonic development of the new individual (next generation). The factors involved in egg activation are expressed on the sperm head. The mammalian sperm head is formed by a highly programmed process and is further modified morphologically and functionally in the male and female reproductive tracts, after spermiation. These modifications are imperative for successful fusion between the sperm and the oocyte at the time of fertilization (i.e., when the genomes meet). Functionally primed mature spermatozoa that have undergone the necessary modifications are able to safely carry the paternal genome to the maternal genome. Each process involves dynamic modifications for the maturation of the sperm components, and these processes are governed by a large number of molecules. Genes control the expression of these molecules, and this expression is affected by both intrinsic and extrinsic factors. The paternal genome is constantly exposed to harmful environments, since spermatozoa are required to travel through sequentially changing microenvironments until the moment of gamete membrane fusion. To overcome obstacles presented by these changing environments, the sperm head and tail develop elaborate structures consisting of functional proteins, carbohydrates and lipids during spermatogenesis, and these structures are continuously modified and mature until fertilization/embryonic development.

Over the past two decades, powerful genetic, biophysical, biochemical, and molecular biology tools have been developed and have substantially improved our understanding of sperm biology. Since numerous molecules and genes involved in these processes have been identified thus far and are still under investigation, they cannot all be cited in this book. In the future, many studies undertaken worldwide will discover new molecules and features and will provide many invaluable insights into sperm biology, from spermatogenesis to egg activation. The progress of research in this manner will be useful for practical application in mammals, including humans, especially for clinical patients with infertility.

I hope that the information provided in this book will not only improve the understanding of sperm biology but also aid the development of innovative

approaches for infertility programs such as IVF and ICSI, including TESE–ICSI and TESE–IMSI, for the clinical treatment of infertility in humans and animals.

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