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REVIEW ARTICLE

Acrosome markers of human sperm

Chizuru Ito[1] · Kiyotaka Toshimori[1]

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Abstract Molecular biomarkers that can assess sperm acrosome status are very useful for evaluating sperm quality in the field of assisted reproductive technology. In this review, we introduce and discuss the localization and function of acrosomal proteins that have been well studied. Journal databases were searched using keywords, including "human acrosome", "localization", "fertilization-related protein", "acrosomal membrane", "acrosomal matrix", "acrosome reaction", "knockout mouse", and "acrosome marker".

Keywords Human acrosome · Fertilization-related protein · Acrosome marker · Acrosomal matrix · Acrosomal membrane · Acrosome reaction

Introduction

The acrosome is located at the anterior half of the sperm head (Fig. 1a, b). It is subdivided into the anterior acrosome (AA) and posterior acrosome (PA) or the equatorial segment (ES), which is the gamete fusion site. The AA is further divided into the apical region and the principal region. The posterior part of the head is called the postacrosomal region (PAR). The sperm head is covered with four types of membrane: the plasma membrane, the outer acrosomal membrane (OAM), the inner acrosomal membrane (IAM) and the nuclear envelope. The acrosome is enclosed by the OAM and IAM. The narrow space between the plasma membrane and the OAM is called the periacrosomal layer, and the space between the IAM and nuclear envelope is called the subacrosomal layer (perinuclear theca) where perinuclear substances are located (Toshimori and Eddy 2014).

The acrosome develops throughout spermiogenesis (Figs. 1c, d, 2). In the Golgi phase (Sa spermatids in human), many proacrosomal vesicles derived from the Golgi apparatus gather and fuse to form a single round acrosomal vesicle in the perinuclear space. In the cap phase (Sb spermatids), the acrosomal vesicle (cap) enlarges and spreads over the anterior half of the nucleus. In the elongation phase (Sc spermatids), which follows nuclear condensation, the acrosomal contents gradually condense into an electron-dense matrix, which is the acrosomal granule. In the maturation phase (Sd spermatids), the dense material in the acrosomal granule spreads over the entire acrosome. During acrosomal biogenesis, the acrosome accumulates many molecules, including molecules necessary for fertilization processes. These molecules are systematically transported and distributed to the proper site of the acrosome so that the following fertilization events proceed successfully (Russell et al. 1990; Toshimori 2009; Florman and Fissore 2014; Toshimori and Eddy 2014).

The acrosome is an indispensable organelle for the fertilization process, with the latter involving the acrosome reaction, binding of the zona pellucida (ZP) (Fig. 1e), penetration through the ZP, and sperm–egg membrane fusion. The acrosome contains hydrolytic enzymes associating with soluble components and detergent-resistant insoluble components. Highly soluble proteins are released during the acrosome reaction, whereas detergent-resistant insoluble proteins remain in association with the sperm head cytoskeleton even after the acrosome reaction (Hardy et al. 1991). Acrosomal proteins are individually released and exposed to the external environment according to a

✉ Chizuru Ito
chizuru@faculty.chiba-u.jp

[1] Department of Reproductive Biology and Medicine, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan

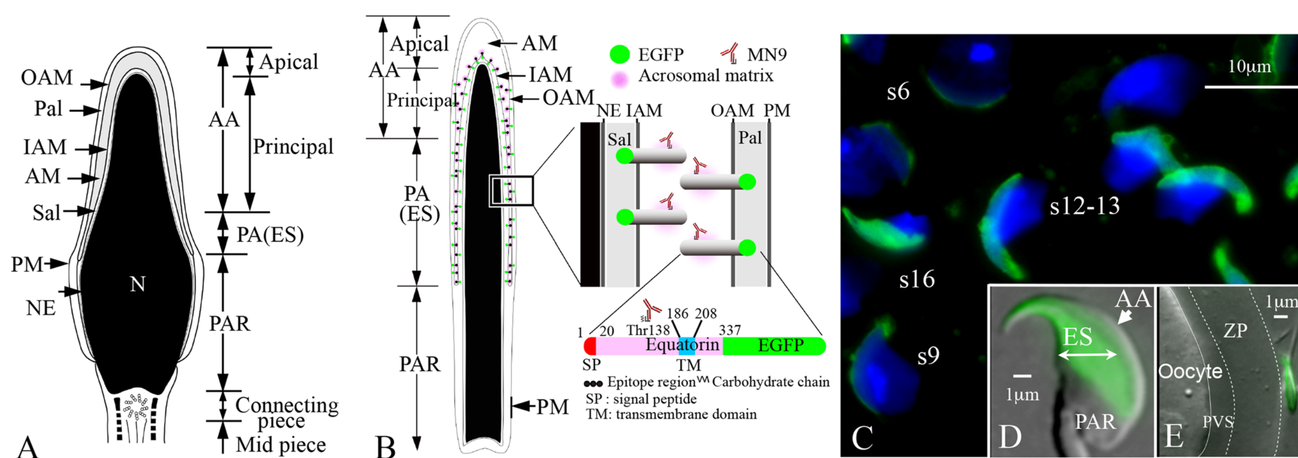

Fig. 1 Sperm head domain and subcellular localization of the acrosomal membrane protein equatorin (Eqtn). **a** Schematic drawing showing the localization and spatial relationship of the membrane system and cytoplasmic layers in the human sperm head. **b** Schematic drawing showing the subcellular localization of Eqtn in mature mouse sperm. Eqtn is localized throughout the entire inner acrosomal membrane (*IAM*) and the outer acrosomal membrane (*OAM*) of the principal segment and equatorial segment (*ES*). The equatorin antibody MN9 epitope is localized on the N-terminal side in the intra-acrosomal lumen, and EGFP is located on the C-terminal side in the periacrosomal layer (*Pal*) and subacrosomal layer (*Sal*). **c**–**e** Molecular structure of Eqtn fused to enhanced green fluorescent protein (*EGFP*). Expression of the Eqtn–EGFP complex in testicular spermatids (**c**), mature sperm (**d**), and sperm on the zona pellucida (*ZP*) (**e**). *AA* Anterior Acrosome, *AM* acrosomal matrix, *C* C-terminus, *IM* inner membrane, *N* nucleus, *NE* nuclear envelope, *PA* posterior acrosome, *PM* plasma membrane, *Sal* subacrosomal layer. Source of **b**, **c**: modified from Ito et al. (2015) with permission

time schedule determined by the location where they are organized, and then the proteins play their part in the fertilization process. Thus, the function of acrosomal molecules is strongly related to their localization in the acrosome. Acrosomal proteins localized on the OAM in the apical segment are, for example, thought to be associated with the initiation of the acrosome reaction (Yanagimachi 1994; Toshimori 2009; Toshimori and Eddy 2014), while the proteins that appear on the surface of the plasma membrane over the AA during the acrosome reaction are thought to be associated with binding to the ZP. During the acrosome reaction, many OAM proteins detach and disappear from the sperm head; to the contrary, other IAM proteins, one of which is equatorin, are transported into the oocyte and do not disappear during the acrosome reaction. These latter proteins are thought to function after they are transported into oocytes.

Knowledge of whether the sperm of interest possess key fertilization-related proteins and whether these proteins are properly distributed during the acrosome reaction will facilitate assessments of sperm quality. In this review, we discuss the localization and function of representative, well-studied acrosomal proteins.

Methods

For this review, we searched the relevant databases, including PubMed, Science Direct, and Wiley Online Library, using keywords, including “human acrosome”, “localization”, “fertilization-related protein”, “acrosomal membrane”, “acrosomal matrix”, “acrosome reaction”, “knockout mouse”, and “acrosome marker”. The most commonly used keywords were “human acrosome”, “localization”, “fertilization-related protein”, and varying combinations of these. Journal articles were included based on their quality and relevance. The reference list of selected journals was searched for relevant articles to include in this review. We then narrowed the search to focus primarily on human acrosomal proteins whose location and function have been well studied (see Table 1).

Matrix proteins of the anterior acrosome

Acrosin/proacrosin

Acrosin (Acr) is a serine proteinase located primarily in the acrosomal matrix and is initially synthesized as an enzymatically inactive zymogen, preproacrosin, which is then converted into proacrosin (Baba et al. 1989). Proacrosin and Acr have been purified from cauda epididymal and ejaculated sperm of various species, including humans (Polakoski and Parrish 1977; Siegel et al. 1986; Adekunle et al. 1987; Hardy et al. 1987). The subcellular localization of proacrosin is the apical segment and the principal segment of the AA (Westbrook-Case et al. 1994; Ferrer et al. 2012a, b). Acr was originally believed to be essential for sperm–ZP penetration and sperm–oocyte recognition and

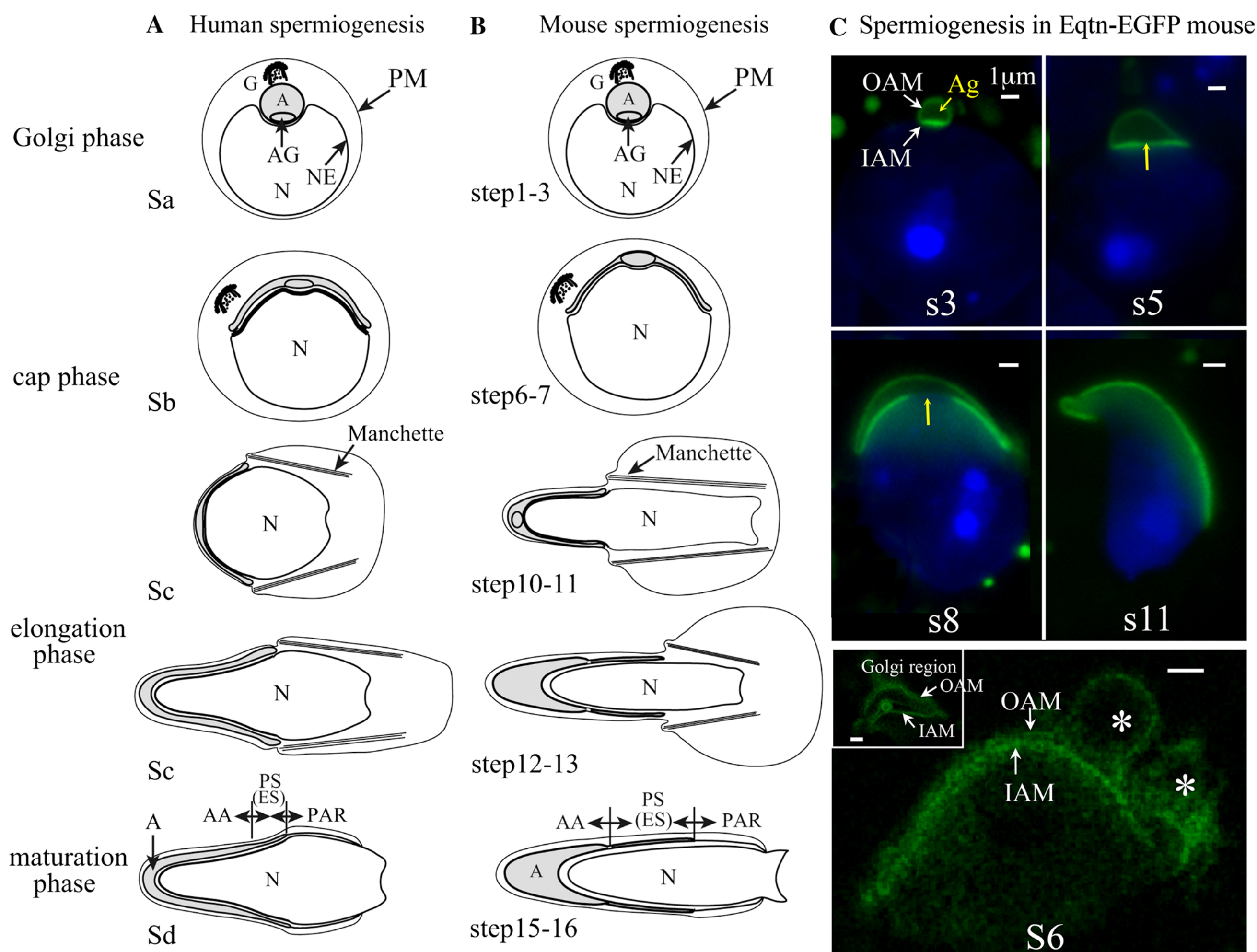

Fig. 2 Spermiogenesis in humans and mice. Schematic drawing of representative developing germ cells in humans (**a**) and mice (**b**), showing Sa, Sb, Sc, and Sd phase spermatids. **c** High- and super-resolution microscopy [simulated emission depletion (STED) nanoscopy] in a mouse Eqtn–EGFP transgenic developing acrosome: high-resolution microscopy [steps (*s*) *s3*, *s5*, *s8*, and *s11* spermatids) and super-resolution nanoscopy (STED CW; Leica Microsystems, Wetzlar, Germany) showing the region between the Golgi-derived components and the acrosome (*s6* spermatid). *A* Acrosome, *AG* acrosomal granule, *G* Golgi apparatus. Source of **a**, **b**: modified from Ito et al. (2010) with permission; source of **c**: modified from Ito et al. (2013) with permission

binding (Klemm et al. 1991), but $Acr^{-/-}$ male mice and mice carrying a deletion in the proline-rich region (PRR) of the proacrosin gene ($PRR^{-/-}$) are fertile (Baba et al. 1994; Adham et al. 1997; Nayernia et al. 2002). Detailed observation of fertilization using $Acr^{+/+}$ and $Acr^{-/-}$ sperm revealed that the loss of Acr results in delayed sperm penetration of the ZP at the early stage of fertilization in vitro (Baba et al. 1994; Adham et al. 1997). Subsequent studies revealed that Acr is involved in the dispersal of acrosomal components during the acrosome reaction (Yamagata et al. 1998; Honda et al. 2002). PRSS21 (a serine protease)/Acr-double deficient mice and SPAM1/Acr-double deficient male mice have also been reported to be fertile (Kawano et al. 2010; Zhou et al. 2012). However, a recent study showed that proacrosin is associated with the IAM and might function in sperm penetration of the ZP by cooperating with matrix metalloproteinase-2 (MMP2) (Ferrer et al. 2012b). A transgenic mouse line that expresses green fluorescent protein (GFP) in the acrosome (*Green-Acr* transgenic mouse) with an acrosin signal peptide and proacrosin peptide has been established and used to study exocytosis non-invasively. In the spermatogenesis of Green-Acr mice, green fluorescence is first detected in proacrosomal granules in step 2 spermatids and then in one large proacrosomal granule in step 3 spermatids. In mature sperm, GFP is mostly detected in the AA and weakly in the ES. Soon after the onset of the acrosome reaction induced by calcium ionophore A23187, GFP disappears from the reacted sperm (Fig. 3a; Nakanishi et al. 1999), followed by the appearance of intraacrosomal materials that have strong affinity to Alexa 594-conjugated peanut agglutinin (PNA) (Jin et al. 2011).

Table 1 Localization of representative acrosomal proteins in mature sperm and the phenotype of mice with the respective protein-encoding gene deleted

Acrosomal molecule	Presence in human sperm	Localization in sperm in nature					Phenotypes of mice with relevant protein-encoding gene deleted	
		Light microscopy level			Electronic microscopy (EM) level	References (EM)	Male	References
		Before AR		After AR	Before AR			
		Intact	Detergent-treated					
Intraacrosomal protein								
AA								
Acrosin/proacrosin	+	–	AA	–	Apical + principal	Ferrer et al. (2012a)	Fertile[d]	Baba et al. (1994); Adham et al. (1997); Nayernia et al. (2002)
ZP3R (SP56, AM67)	–	–	AA	–[a]	Apical	Foster et al. (1997)	Fertile	Muro et al. (2012)
MN7	+	–	AA	–	Apical	Oh-oka et al. (2001)	–	
p50 (AM50)	+	–	AA	–	Apical	Westbrook-Case et al. 1994	–	
Zonadhesin	+	–	AA	–	Apical	Bi et al. (2003)	Fertile	Tardif et al. 2010
AA + ES								
CRISP2 (TPX1)	+	–	AA + ES	ES	Apical + principal + ES	Hardy et al. (1991)	–	
SP10	+	–	AA + ES	ES	Principal + ES	Herr et al. (1990a, b)	–	
SLLP1 (SPACA3)	+	–	AA + ES	ES	Principal + ES	Mandal et al. (2003)	–	
ES								
SPESP1 (ESP1)	+	–	ES	ES	ES matrix associating with IAM + OAM	Wolkowicz et al. (2003)	Fertile	Fujihara et al. (2010)
Membrane protein								
AA + ES								
SPAM1 (PH20)	+	AA + ES + PAR	AA + ES	AA + ES	(before AR) (before + after AR) PM of entire head → IAM of entire head	Sabeur et al. (1997)	Fertile[d]	Baba et al. (2002)
CD46 (MCP)	+	–	AA + ES	AA + ES	IAM	Anderson et al. (1989)	Fertile	Inoue et al. (2003)

Table 1 continued

Acrosomal molecule	Presence in human sperm	Localization in sperm in nature					Phenotypes of mice with relevant protein-encoding gene deleted	
		Light microscopy level			Electronic microscopy (EM) level	References (EM)	Male	References
		Before AR		After AR	Before AR			
		Intact	Detergent-treated					
Equatorin (MN9)	+	–	AA + ES	AA + ES[b]	IAM: entire OAM:principal + ES	Toshimori et al. (1992) Yamatoya et al. (2009) Ito et al. (2013, 2015)	Fertile	Hao et al. (2014)
ADAM3 (cyritestin)	–	– or PM	AA + ES or ES	ES or –	IAM + OAM/?	Forsbach and Heinlein (1998)	Infertile	Shamsadin et al. (1999) Nishimura et al. (2001)
ZPBP1 (SP38, IAM38)	+	–	AA + ES	AA + ES	IAM: entire OAM: ES	Yu et al. (2006) Ferrer et al. (2012a)	Infertile (astheno-globozooospermia)	Lin et al. (2007)
Izumol (OBF13)	+	–	AA	AA + ES[c]	IAM: entire OAM: apical + principal	Satouh et al. (2012)[e]	Infertile	Inoue et al. (2005)
SPACA1 (SAMP32)	+	–	AA + ES	ES	IAM: entire OAM: ES	Hao et al. (2002) Ferrer et al. (2012a)	Infertile (globozoospermia)	Fujihara et al. (2012)
SAMP14 (SPACA4)	+	–	AA + ES	AA + ES	IAM + OAM + AM	Shetty et al. (2003)	–	

AA, Anterior acrosome; AM, acrosomal membrane; AR, acrosome reaction; ES, equatorial segment; IAM, inner acrosomal membrane; OAM, outer acrosomal membrane; PAR, postacrosomal region; PM, plasma membrane

[a] During capacitation, ZP3R is transiently translocated on the surface of the plasma membrane of the AA

[b] In acrosome-reacted sperm, some equatorin is translocated on the surface of the plasma membrane of the ES

[c] During the acrosome reaction, Izumol is transiently translocated on the surface of the plasma membrane of the ES and PAR

[d] SPAM1/ACR- and SPAM 1/PRSS21-deficient males are also fertile (Zhou et al. 2012)

[e] Light microsocopic level

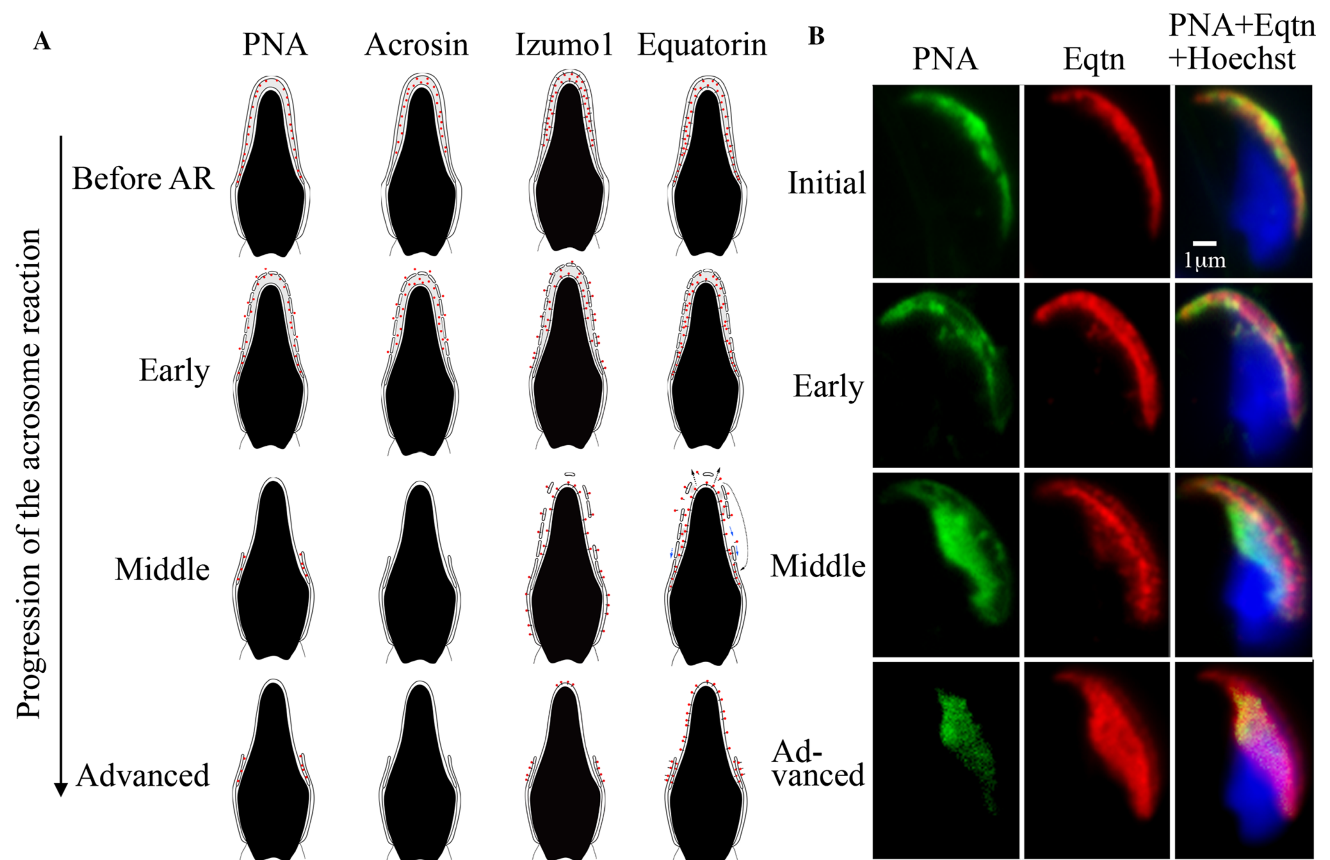

Fig. 3 The localization of peanut agglutinin (*PNA*) lectin, acrosin, Izumo1, and Eqtn during the acrosome reaction (*AR*). **a** Schematic drawing based on this review, showing the possible structural modification of the human sperm membrane system and the localization of the representative acrosome molecules during the acrosome reaction. **b** Anti-Eqtn antibody indirect immunofluorescence and PNA lectin fluorescence during the mouse acrosome reaction for comparison to the human acrosome reaction. Source of **a**: from this review; source of **b**: modified from Yoshida et al. (2010) with permission

Zona pellucida 3 receptor, sperm fertilization protein 56, and acrosomal matrix component 67

Zona pellucida 3 receptor (ZP3R) was initially identified as a 56-kDa mouse sperm surface protein with specific affinity for ZP3, one of the three ZP proteins (Bleil and Wassarman 1990; Cheng et al. 1994), but subsequent studies demonstrated that ZP3R is a component of the AM (Foster et al. 1997; Kim et al. 2001). Guinea pig acrosomal matrix component 67 (AM67) and rat sperm fertilization protein 56 (SP56) are the orthologs of mouse ZP3R (Foster et al. 1997; He et al. 2003); human ZP3R has not been reported to date. ZP3R is localized in the dorsal region of the apical segment (Foster et al. 1997; Kim et al. 2001). During the acrosome reaction, ZP3R is dissolved by proteases and released into the surrounding environment, where it remains transiently associated with the surface of the AA (Kim and Gerton 2003; Wassarman 2009) before finally disappearing from the completely reacted sperm. A recent study reported that ZP3R is not essential in sperm–ZP binding and/or the penetration of the ZP, showing that *ZP3R*$^{-/-}$ male mice were completely fertile and that there were no differences among *ZP3R*$^{+/+}$, *ZP3*$^{+/-}$, and *ZP3R*$^{-/-}$ mouse sperm based on the results of an in vitro fertilization assay, sperm–ZP binding assay, and analysis of acrosomal exocytosis (Muro et al. 2012).

Mn7

MN7 was initially identified as a 90-kDa mouse acrosomal protein (Tanii et al. 1994). This molecule is localized in the AA of rodents and humans and localized subcellularly to OAM/matrix-associated materials on the dorsal surface of the acrosomal apical segment (Yoshinaga et al. 1998). During spermatogenesis, MN7 is first detected in the proacrosomal granules of the early Golgi phase spermatids and, thereafter, in the cap and acrosomal phases, throughout the acrosome. including the acrosomal granule and especially in the head-cap portion. In mature sperm of rats, guinea pigs and humans, MN7 is detected on the acrosomal materials associated with the OAM and over the matrix at the AA (Tanii et al. 1994; Yoshinaga et al.

2000; Oh-Oka et al. 2001). MN7 is gradually released from the acrosome during the ZP-induced acrosome reaction, ultimately disappearing from completely reacted sperm (Saxena et al. 1999). An anti-MN7 antibody has been shown to inhibit the rate of fertilization of ZP-intact oocytes but not to affect the fertilization rate of ZP-free oocytes in mice, leading to the proposal that MN7 is associated with the penetration of sperm through the ZP in mice (Saxena et al. 1999).

Acrosomal pentraxin-like protein (protein 50, apexin)

Acrosomal pentraxin-like protein (AM50) [also known as protein 50 (P50) and appexin], was first identified as a 50-kDa acrosomal matrix protein localized to the ventral region of the apical segment (Noland et al. 1994; Reid and Blobel 1994; Westbrook-Case et al. 1994; Kim et al. 2001). Human neural pentraxin 2 (NPTX2) is the ortholog of AM50 (Hsu and Perin 1995). In spermatogenesis, AM50 is first detected in the matrix of proacrosomal granules and the acrosomal vesicle, then, in Golgi phase spermatids, in the acrosomal matrix and acrosomal granule. In spermatids at the acrosome phase, AM50 remains restricted to the apical segment, but it is later found in the ventral matrix of the apical segment in maturation phase spermatids (Westbrook-Case et al. 1995). During the course of capacitation and the ionophore-induced acrosomal reaction, AM50 is proteolytically processed (Westbrook-Case et al. 1994; Kim et al. 2011), then transiently exposed to the sperm surface (Kim et al. 2011); thereafter, on completely reacted sperm, it disappears completely.

Zonadhesin

Zonadhesin (ZAN) was originally identified as a porcine sperm membrane protein that bound in a species-specific manner to the ZP (Hardy and Garbers 1994, 1995). However, subsequent studies using immunoelectron microscopy revealed that ZAN is located in the acrosome—not in the plasma membrane (Bi et al. 2003; Olson et al. 2004). ZAN is a mosaic protein containing meprin/A5 antigen/mu receptor tyrosine phosphatase domains (MAM), mucin-like domains, and von Willebrand factor D domains (Hardy and Garbers 1995; Gao and Garbers 1998; Herlyn and Zischler 2008; Tardif et al. 2010; Tardif and Cormier 2011). It has been observed in many mammalian species, including mouse, human, rabbit, hamster, bull, primate, and horse (Gao et al. 1997; Gao and Garbers 1998; Lea et al. 2001; Olson et al. 2004; Herlyn and Zischler 2008; Tardif et al. 2010). In spermatogenesis, ZAN is first detected in the IAM and OAM in round spermatids and then in the OAM in elongating ones. During sperm transit in the epididymis, ZAN dissociates from the OAM and becomes incorporated into the acrosomal matrix throughout the dorsal and ventral regions of the AA (Bi et al. 2003; Olson et al. 2004). During capacitation, ZAN is transiently exposed at the sperm surface, then disappears from completely reacted sperm. A specific antibody against the D3p18 domain of mouse ZAN partially inhibits sperm adhesion to the ZP; however, ZAN-deficient ($Zan^{-/-}$) male mice are fertile, although the $Zan^{-/-}$ sperm decreases the species specificity of ZP adhesion (Tardif et al. 2010).

Matrix proteins of the anterior acrosome and equatorial segment

Cysteine-rich secretory protein 2 (testis-specific protein 1)

Cysteine-rich secretory protein 2 (CRISP2) [also known as testis-specific protein 1 (TPX1)] is a cysteine-rich secretory protein expressed specifically in male haploid germ cells in the testis. CRISP2 is an intra-acrosomal protein localized to the apical and principal segments of the AA and ES (Hardy et al. 1991). After capacitation and the acrosome reaction, CRISP2 remains associated with the ES in humans and mice (Busso et al. 2005, 2007); it is released from the acrosome during the acrosome reaction and reassociates at the ES (Nimlamool et al. 2013). CRISP2 is also present on the surface of spermatogenic cells and mediates the specific adhesion of germ cells to Sertoli cells (Maeda et al. 1998, 1999). In rat sperm, CRISP2 is also found in the outer dense fibers, the longitudinal columns of the fibrous sheath, and the connecting piece (O'Bryan et al. 1998, 2001). Gibbs et al. (2006) proposed that CRISP2 is a regulator of Ca^{2+} influx through the ryanodine receptors during capacitation. Anti-CRISP2 antibodies inhibit the sperm–oolemma interaction without interfering with ZP penetration in humans and mice (Busso et al. 2007).

Sperm protein-10

Sperm protein 10 (SP-10) is an intra-acrosome protein that was first identified in human sperm (Herr et al. 1990a) and has subsequently been detected in baboon, macaque, and porcine sperm (Herr et al. 1990b). SP-10 cDNAs have been cloned and sequenced in humans (Wright et al. 1993), macaques, and baboons (Freemerman et al. 1993); a mouse SP-10 homolog has also been identified (Liu et al. 1992; Reddi et al. 1995). In spermatogenesis, SP-10 is first detected in the acrosomal vesicle of Golgi phase spermatids. In the cap phase, SP-10 is detected throughout the acrosomal vesicle, including the acrosomal granule.

Thereafter, SP-10 is concentrated in the acrosomal matrix at the site of the acrosomal granule and within the acrosomal matrix associated with the acrosomal membranes. As the spermatids elongate and the nuclei condense, the localization of SP-10 shifts from the acrosomal matrix to an association with the IAM and OAM (Kurth et al. 1991). In mature ejaculated sperm, SP-10 is located on the IAM and OAM adjacent to the acrosomal matrix, especially within the principal segment and posterior bulb of the ES of the human sperm acrosome (Herr et al. 1990a; Foster et al. 1994). During acrosomal swelling followed by the formation of hybrid vesicles of the OAM with the plasma membrane, SP-10 is observed on electron-dense acrosomal matrix material throughout the AA. After the acrosome reaction, SP-10 is detected on the IAM in the ES and on the surface of hybrid vesicles (Foster et al. 1994). Human SP-10 on the equatorial region of acrosome-reacted sperm is considered to be associated with sperm–oolemma binding in a β_1 integrin-independent manner, based on the results of an inhibition assay using a monoclonal antibody against SP-10 (Hamatani et al. 2000). SP-10–GFP transgenic mice were established in 2002 (Reddi et al. 2002).

Sperm lysozyme-like protein 1 (sperm acrosome membrane-associated protein 3, sperm protein reactive with antisperm antibodies)

Sperm lysozyme-like protein 1 (SLLP1) [also known as sperm acrosome membrane-associated protein 3 (SPACA3) and sperm protein reactive with antisperm antibodies (SPRASA)] was identified as a non-bacteriolytic c (chicken or conventional type) lysozyme-like protein in the acrosome of human sperm and is localized in the acrosomal matrix, including the principal segment and ES (Mandal et al. 2003). In capacitated human sperm, SLLP1 is observed on the luminal face of both the IAM and OAM (Mandal et al. 2003). Imunofluorescence studies using an anti-mouse SLLP1 antibody revealed that mouse SLLP1 is localized mainly to the AA before the acrosome reaction and is retained in the ES following capacitation and the acrosome reaction (Herrero et al. 2005). Recombinant SLLP1 and antibodies against SLLP1 have inhibitory effects on in vitro fertilization with both cumulus-intact and zona-free oocytes (Herrero et al. 2005). An oocyte-specific membrane metalloproteinase, SAS1B (sperm acrosomal SLLP1 binding), was recently identified as a SLLP1 binding partner (Sachdev et al. 2012). SAS1B is localized on the microvillar oolemma of MII oocytes and SAS1B protein or an antibody against SAS1B significantly inhibits fertilization, suggesting that SLLP1 and SAS1B are involved in fertilization (Sachdev et al. 2012).

Matrix proteins of the equatorial segment

Sperm equatorial segment protein 1 (equatorial segment protein)

Sperm equatorial segment protein 1 (SPESP-1) [also referred to as equatorial segment protein (ESP)] was cloned and characterized as a protein localized to the ES of ejaculated human sperm (Wolkowicz et al. 2003). In spermatogenesis, SPESP-1 is first detected in Golgi-derived acrosomal vesicles of the early round spermatids and then in the peripheral region of the acrosome, with the exception of the acrosomal granule in cap-phase spermatids. In elongating spermatids and mature sperm, SPESP-1 is located in the acrosomal matrix in the ES. In capacitated acrosome-reacted sperm and sperm tightly bound to the oolemma of eggs, human SPESP-1 persists in the ES (Wolkowicz et al. 2003, 2008). Antisera raised against recombinant SPESP-1 inhibits the binding and fusion of human sperm to hamster eggs (Wolkowicz et al. 2008). Testicular SPESP-1 (77 and 67 kDa, respectively) is highly glycosylated, but is deglycosylated and proteolyzed just before spermiation and during the capacitation and acrosome reaction; thus, epididymal SPESP-1 is 47 and 43 kDa, respectively (Suryavathi et al. 2015). SPESP-1-deficient mice are fertile, although *Spesp1*$^{+/-}$ and *Spesp1*$^{-/-}$ sperm have a lower fusing ability than that of wild-type sperm (Fujihara et al. 2010). Moreover, a deficiency in SPESP1 causes embrittlement of the ES and increases the expression of equatorin (Fujihara et al. 2010).

Acrosomal membrane proteins of the anterior acrosome and the equatorial segment

Sperm adhesion molecule 1 (PH-20 hyaluronidase)

Sperm adhesion molecule 1 (SPAM1) (also known as PH-20 hyaluronidase) is a glycosylphosphatidylinositol-anchored sperm hyaluronidase found in humans, guinea pigs, mice, macaques, and bulls (Primakoff et al. 1985; Phelps et al. 1988; Overstreet et al. 1995; Thaler and Cardullo 1995; Sabeur et al. 1997; Morin et al. 2005). In acrosome-intact sperm, SPAM1 is localized on the plasma membrane over the entire head and on the IAM. In human sperm, during the acrosome reaction SPAM1 is observed on the IAM and on hybrid vesicles derived from the fusion of the plasma membrane and the OAM; after the acrosome reaction SPAM1 is observed on the IAM of the AA and on the plasma membrane of the ES (Overstreet et al. 1995; Sabeur et al. 1997). The function of SPAM1 in fertilization

was originally thought to enable acrosome-intact sperm to pass through the layer of cumulus cells and reach the ZP. However, *Spam1*$^{-/-}$ male mice are fertile, although sperm lacking SPAM1 have a reduced ability to disperse cumulus cells for fertilization (Baba et al. 2002). SPAM1/Acr- and SPAM1/PRSS21-double deficient male mice are also fertile (Zhou et al. 2012). SPAM1 is also detected in the epididymis, accessory organs, and female genital tracts. The molecular weight of SPAM1 in the extratesticular organ and female genital tracts is the same as that of the testicular form (64–68 kDa) (Zhang and Martin-DeLeon 2003; Zhang et al. 2004), with the exception of bulls, where the former is shorter than the latter (Morin et al. 2010). SPAM1 in the extratesticular organ and female genital tracts is transferred to the sperm surface during its residence in the male and female genital tracts (Griffiths et al. 2008a). The binding of epididymal or uterine SPAM1 during in vitro capacitation significantly increases cumulus penetration, and the acquisition of uterine SPAM1 significantly increases hyaluronic acid-binding ability, which enhances the induction of the acrosome reaction (Griffiths et al. 2008b).

CD46 complement regulatory protein (membrane cofactor protein)

CD46 complement regulatory protein (CD46) [also known as membrane cofactor protein (MCP)] was first described as a cell-surface complement regulatory protein that facilitates enzymatic cleavage of complement component C3b. CD46 is a type 1 membrane glycoprotein (Post et al. 1991) which acts as a multitasking molecule, such as being a receptor for several species of bacteria and viruses (Dörig et al. 1993; Okada et al. 1995; Greenstone et al. 2002) and a regulator of T cell-mediated immunity (Marie et al. 2002; Kemper et al. 2003). Although CD46 exists as multiple isomeric forms and is widely distributed in humans (Post et al. 1991), the distribution of CD46 protein in mice and rats is restricted to the testis (Inoue et al. 2003; Mizuno et al. 2004). The expression of CD46 on mature sperm is restricted to the IAM before and after the acrosome reaction (Anderson et al. 1989; Fénichel et al. 1990). In spermatogenesis, CD46 is first detected in the acrosome of late, round spermatids, and the expression increases in intensity through spermatid development (Mizuno et al. 2005). Treatment with an anti-CD46 antibody significantly decreases the ability of human sperm to facilitate hamster egg penetration (Anderson et al. 1989), but CD46-deficient male mice are fertile (Inoue et al. 2003). However, CD46 is thought to play some role in regulating the sperm acrosome reaction because *CD46*$^{-/-}$ sperm show increased spontaneous acrosome reactions and because the average number of pups born from *CD46*$^{-/-}$ males is significantly greater than that from *CD46*$^{+/+}$ males (Inoue et al. 2003).

Equatorin/MN9 antigen

Equatorin (EQTN) [also known as acrosome formation-associated factor (Afaf)] is a widely distributed acrosomal protein in mammalian sperm, including human sperm (Toshimori et al. 1992, 1998). Mouse equatorin (Eqtn) is a highly glycosylated type 1 transmembrane protein with an N-terminus facing the acrosomal lumen on both the OAM and IAM (Yamatoya et al. 2009). In spermatogenesis, Eqtn is first detected on the acrosomal membranes in step 2–3 spermatids and then in the peripheral region of the acrosome, with the exception of the acrosomal granule, in cap-phase spermatids. In elongating spermatids and mature sperm, Eqtn is located primarily in the ES and partly in the AA (Figs. 1c, 2c). A detailed study of sperm from *Eqtn–EGFP* transgenic mice by super-resolution and immunoelectron microscopy revealed that Eqtn is located on the IAM of the entire acrosome, the OAM of the principal segment of the AA, and the ES and that it is associated with the structure made of acrosomal matrices and acrosomal membranes, which we have termed the complex of the IAM and associated acrosomal matrix (CIAMAM) and the complex of the OAM and associated acrosomal matrix (COAMAM), respectively (Fig. 1b; Ito et al. 2013, 2015). The anti-Eqtn antibody MN9 inhibits the release of cortical granules without inhibiting sperm–ZP binding or sperm–egg binding, suggesting the possibility that Eqtn is involved in fusion with the oolemma or in activation of the oocyte (Toshimori et al. 1998; Yoshinaga et al. 2001). During the acrosome reaction, a certain amount of Eqtn is translocated onto the plasma membrane covering the ES, while the majority remains on the IAM until the male pronucleus is formed (Fig. 3a; Manandhar and Toshimori 2001). Yoshida et al. (2010) monitored the staining pattern of MN9 immunofluorescence and compared it with that of *Arachis hypogaea* agglutinin (PNA–fluorescein isothiocyanate). Based on their results, the authors presented a progressive model of the acrosome reaction that was classified into four stages (initial, early, advanced and final). These authors reported that as the acrosome reaction progressed from the initial to the early stage, Eqtn spread from the peripheral region of the AA toward the center of the ES; then, during the advanced stage, it spread gradually over the entire region of the ES; in the final stage, it was present as at the ES (Fig. 3b; Yoshida et al. 2010). A combination analysis using immunoelectron microscopy and high-resolution fluorescence microscopy also showed that EQTN is a good acrosome membrane marker for the spatio-temporal behavior of acrosomal membrane proteins (Toshimori 2011). Hao et al. (2014) reported that *Eqtn*$^{-/-}$

male mice are subfertile due to a disorder of the acrosome reaction; however, their conclusion seems to be debatable. Our detailed studies on *Eqtn*$^{-/-}$ mice will be published in the near future.

A disintegrin and metalloprotease 3 (cyritestin)

A disintegrin and metalloprotease 3 (ADAM3) (also known as cyritestin) is a member of the ADAM (a disintegrin and metalloprotease) family. The localization of ADAM3 is controversial. It has been reported to be an acrosomal transmembrane protein that becomes distributed over the entire sperm surface, especially in the PAR, after interaction with the ZP and a successful acrosome reaction (Linder et al. 1995; Forsbach and Heinlein 1998), a sperm surface protein restricted to the equatorial region before and after the acrosome reaction (Yuan et al. 1997), or a sperm surface protein located on the AA and ES that disappears from the sperm head during A23187-induced acrosomal reaction (Kim et al. 2004). *ADAM3*$^{-/-}$ male mice are infertile, but the function of ADAM3 has long been controversial. Analyses of *ADAM3*$^{-/-}$ sperm, immunoblotting and immunohistochemistry of wild-type sperm, and inhibition assays using active site peptides or antibodies against ADAM3 have shown that ADAM3 is involved in sperm–egg adhesion and fusion, acrosome formation, or the sperm–ZP interaction (Yuan et al. 1997; Forsbach and Heinlein 1998; Shamsadin et al. 1999; Nishimura et al. 2001; Kim et al. 2004). However, sperm lacking ADAM3 are reported to have a deficiency in the migration from the uterus into the oviduct, but not in zona binding or in the membrane fusion between the sperm and egg (Yamaguchi et al. 2009; Tokuhiro et al. 2012). The localization of ADAM3 to the sperm surface is reported to require PDILT and TEX101 presentation (Tokuhiro et al. 2012; Fujihara et al. 2014). Human cyritestin genes (CYRN1 and CYRN2) are non-functional (Grzmil et al. 2001).

Zona pellucida-binding protein 1 [sp38, sperm inner acrosomal membrane protein)

Zona pellucida-binding protein 1 (ZPBP1) [also known as sp38 and sperm inner acrosomal membrane protein (IAM38)] was identified and purified from a detergent extract of porcine epididymal sperm; the human and mouse orthologs have also been identified (Mori et al. 1993, 1995). ZPBP1 binds to the 90-kDa family of ZP glycoprotein in a calcium-dependent manner, and proacrosin inhibits the binding of sp38 to ZP, suggesting that sp38 competes with proacrosin for binding to the ZP during the early steps of fertilization (Mori et al. 1993, 1995). Subsequent studies of the bovine ortholog IAM38 and mouse IAM38 revealed that ZPBP1/IAM38 is located on the IAM and its peripheral coat, the IAM coat, of the entire acrosome, and on the OAM of the ES; in addition, IAM38 is retained on the IAM surface after the acrosome reaction and after zona penetration (Yu et al. 2006; Ferrer et al. 2012a). During spermiogenesis, IAM38 is first detected in the proacrosomal granules in the medullary region of the Golgi apparatus in round spermatids and then is concentrated in the acrosomal granule in early, round spermatids. In the cap phase, IAM38 migrates from the acrosomal granule to the acrosomal membrane and finally locates on the IAM of the AA and on both the IAM and OAM of the ES (Yu et al. 2006, 2009). The absence of ZPBP1 prevents proper acrosome compaction, resulting in acrosome fragmentation and disruption of the Sertoli–spermatid junctions. *ZPBP1*$^{-/-}$ male mice are sterile with abnormal round-headed sperm (globozoospermia) that have no forward motility (Lin et al. 2007). A mutation in the human *ZPBP1* gene has been reported to be associated with abnormal sperm head morphology in infertile men (Yatsenko et al. 2012).

Izumo1

Izumo1 is the only candidate sperm–egg fusion protein in sperm that has been probed using a gene knockout method. Izumo1 was first identified as a sperm antigen against the OBF13 antibody, which inhibits mouse sperm–egg fusion (Okabe et al. 1988; Inoue et al. 2005). Izumo1 is a type I membrane glycoprotein with one immunoglobulin-like domain and a putative *N*-glycoside link motif. It is concealed inside the acrosome and is not detectable on the surface of mature intact sperm. At a very early stage of the acrosome reaction, Izumo1 becomes detectable in the acrosomal cap and then on the entire surface of the head, including the AA and PA and PAR (Fig. 3a). Because the redistribution of Izumo1 is blocked by an inhibitor of actin polymerization, the polymerization of actin is thought to be critical to the distribution mechanism (Sosnik et al. 2009). A testis-specific serine kinase, Tssk6, has been reported to be involved in the redistribution of Izumo1 through regulation of the polymerization of actin after the acrosome reaction (Sosnik et al. 2009). Nishimura et al. (2011) reported that TMEM190, a small transmembrane protein containing a trefoil domain, co-localizes with mouse Izumo1 both before and after the acrosome reaction. *Izumo1*$^{-/-}$ male mice are infertile due to a failure in sperm–egg membrane fusion, although the sperm does undergo the acrosome reaction, can penetrate the ZP, and does accumulate in the perivitelline space of the eggs. Intracytoplasmic sperm injection using *Izumo1*$^{-/-}$ sperm allowed *Izumo1*$^{-/-}$ male mice to have offspring. Human sperm have Izumo1, and an anti-human Izumo1 antibody inhibits the binding of human sperm with zona-free hamster

oocytes (Inoue et al. 2005). A study of *Izumo1-mCherry-Acr3-EGFP* (*Red-IZUMO1: Green-Ac*) double transgenic mice found that Izumo1 is localized in both the OAM and IAM in the AA—not in the ES—in acrosome-intact sperm (Satouh et al. 2012). Inoue et al. (2013) recently reported that Izumo1 generates sperm–egg adhesion, and these authors considered a site in the N-terminal region of Izumo1 to be essential for binding the oolemma. Juno, a glycosylphosphatidylinositol-anchored protein previously called folate receptor 4, was reported to be the egg receptor for Izumo1 (Bianchi et al. 2014) and a partner of egg Cd9 (Chalbi et al. 2014).

Sperm acrosome associated 1 (sperm acrosomal membrane-associated protein 32)

Sperm acrosome associated 1 (SPACA1) [also known as sperm acrosomal membrane-associated protein 32 (SAMP32)] was identified and purified from the detergent extract of human ejaculated sperm (Hao et al. 2002). During spermatogenesis, SPACA1 is detected in the acrosome at all stages of spermatid development, but it is not found prior to acrosome formation. In mature sperm, SPACA1 is localized on the IAM of the entire acrosome and on the OAM of the ES, and it remains on the IAM in capacitated and acrosomal-reacted sperm (Hao et al. 2002; Ferrer et al. 2012a). Because antibodies against the recombinant protein inhibit the binding and fusion of capacitated human sperm with zona-free hamster eggs, it was originally believed that SPACA1 played a role in sperm binding and fusion with the oolemma. A strong reaction of recombinant SPACA1 antigen with serum from an anti-sperm antibody-positive infertile man also suggested that SPACA1 might be an antigen that causes immunoinfertility. However, *Spaca* $1^{-/-}$ mice are infertile due to their mature sperm having a round head without an acrosome, indicating that the SPACA1 protein has an important role in shaping the sperm head (Fujihara et al. 2012).

Sperm acrosomal membrane-associated protein 14 (sperm acrosome associated 4)

Sperm acrosomal membrane-associated protein 14 (SAMP14) [also known as sperm acrosome associated 4 (SPACA4)] is a member of the Ly-6 and urokinase plasminogen activator receptor family. In noncapacitated human sperm, SAMP14 is primarily associated with the OAM and IAM and partly found in the acrosomal matrix. SAMP14 is retained on the IAM after the acrosome reaction. Rat anti-recombinant SAMP14 serum blocks the binding and fusion of human sperm to zona-free hamster eggs in a dose-dependent manner (Shetty et al. 2003).

Others

Although many other acrosomal enzymes are known to be present in the acrosome, we will not discuss them here for two primary reasons: (1) many of them disappear at the very early stage of the acrosome reaction; (2) they cannot function as biomarkers for the long life of the acrosome. These "other" enzymatic molecules are localized in the mammalian acrosome are (1) glycohydrolases, including α-L-fucosidase, α-D-galactosidase, β-D-galactosidase, β-D-glucuronidase, β-*N*-hexosaminidase, β-*N*-acetylglucosaminidase, α-D-mannosidase, β-D-mannosidase, neuraminidase, and Aryl sulfatases A, B and C (Brandon et al. 1997; Tulsiani et al. 1998); (2) proteinases, including dipeptidyl peptidase II, acrolysin, cathepsins, and metalloproteases. MMP2 has recently been reported to be associated with the IAM and play a number of roles in sperm penetration of the ZP in cooperation with acrosin (Ferrer et al. 2012b); further studies are expected. The acrosome also contains esterases, sulfatases, phosphatases, and phospholipases. These enzymes have been described in detail in a book chapter (Zaneveld and De Jonge 1991). Some other molecules, such as VAMP/synaptobrevin and acrogranin, are also not discussed here because their exact localization is not clear (Anakwe and Gerton 1990; Ramalho-Santos et al. 2002).

Conclusion

Acrosome biogenesis is closely associated with nuclear formation (Toshimori and Ito 2003; Kierszenbaum and Tres 2004; Toshimori 2009; Toshimori and Eddy 2014). Acrosome malformation is often accompanied with nuclear deformity, which is observed in human teratozoospermic sperm and mouse gene-knockout infertile sperm. Therefore, visualization of the acrosome can provide valuable information on sperm nuclear integrity. Fluorescent-labeled lectins, such as PNA, *Pisum sativum* (PSA), and *Canavalia ensiformis* (Con A), can often be used to show the morphology of the acrosome. However, lectins only interact with a specific carbohydrate and thus do not provide information on the molecules that actually function during the fertilization process.

Many studies using fertilization-related gene knockout mice have revealed that a defect in a single acrosomal protein—with the exception of Izumo1—does not functionally cause male infertility, suggesting that several other proteins could be involved in each step of the fertilization process. Knowledge of the distribution of fertilization-related proteins is very helpful for the precise assessment of sperm quality.

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Compliance with ethical standards

Conflict of interest None.

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