Chapter 11

MAMMALIAN ADAMS WITH TESTIS-SPECIFIC OR -PREDOMINANT EXPRESSION

Testicular ADAMs

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Abstract: A family of multidomain membrane proteins, the ADAM family (a disintegrin and metalloprotease), comprises at least 32 members. Based on an ADAM phylogenetic tree, mammalian ADAMs with testis-specific or -predominant expression are divided into two major groups: ADAMs 1, 4, 6, 20, 21, 24, 25, 26, 29, 30 and 34 (the first group), and ADAMs 2, 3, 5, 27 and 32 (the second group). All of the mammalian, testicular ADAMs predicted as active metalloproteases (ADAMs 1, 20, 21, 24, 25, 26, 30 and 34) belong to the first phylogenetic group and are unique in several aspects. All of these ADAM genes lack introns in their coding sequences and many of them are present as multiple copies in the mouse genome, resulting in total of 11 functional genes (ADAMs 1a, 1b, 21, 24, 25a, 25b, 25c, 26a, 26b, 30 and 34) predicted to encode active proteases. These genes are transcribed by both somatic and germ cells with higher expression level in post-meiotic germ cells in the testis. The ADAM 1 protein expressed in testicular germ cells is complexed with ADAM 2 to form a heterodimer and processed during spermatogenesis. Mouse knockout studies indicate that ADAM 1a/2 heterodimer in testicular germ cells is implicated in the regulation or localization of sperm proteins involved in sperm progression in the female reproductive tract, sperm penetration into the cumulus cell layer and sperm-egg zona pellucida binding, thus playing a central role in fertilization. ADAM 24 is a potential sperm protease implicated in sperm function during sperm maturation or fertilization. A number of the 11 mouse ADAMs do not have orthologues in human and, even if they exist, some orthologues are pseudogenes in human. As a result, only 3 human ADAMs (ADAMs 20, 21 and 30) are functional genes encoding potential metalloproteases. Uncovering the in vivo functions of the testicular ADAM proteases present in both species should provide insights into the mammalian reproductive system involving protease-mediated events.

Key words: Disintegrin; fertilization; metalloprotease; orthologue; sperm; testis

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1. INTRODUCTION

ADAMs are a family of transmembrane proteins that contain A Disintegrin And Metalloprotease domain. The ADAM family members have been discovered in a variety of tissues and species, and the family currently has at least 32 members. The ADAM family proteins share conserved multidomain structure: pro-, metalloprotease, disintegrin, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic domains (Fig. 1). They show sequence homology with soluble snake venom peptides and proteins that contain one or more of pro-, metalloprotease, disintegrin, and cysteine-rich domains. A primordial ADAM gene, having arisen by the assembly of the separate domains, may have been amplified to generate new genes. The ADAM proteins have at least two potential functions. They function as cell adhesion molecules. In many snake venom disintegrin peptides, the active site of the molecules is defined by a tripeptide sequence motif RGD. The RGD-containing disintegrins bind to platelet integrin gpIIb/IIIa and prevent fibrinogen binding and platelet aggregation. In the disintegrin domains of the ADAM proteins, the RGD sequence is replaced by other amino acids. The predicted binding partners of the ADAM proteins are integrins, but non-integrin adhesion partners could bind to particular ADAMs. The ADAM proteins with a consensus sequence for an active metalloprotease site potentially have protease activity. The consensus sequence to be an active protease is HEXGHXXGXXHD in which the histidines bind zinc and the glutamic acid is the catalytic residue. ADAMs with the consensus sequence contain a conserved, free cysteine residue responsible for the cysteine-switch mechanism in the prodomain. In addition, these ADAM proteins contain a 'methionine turn' downstream to the catalytic site and thus belong to the 'metzincin' class together with the ADAM-TS proteins and matrix metalloproteases. The ADAM proteins having cell adhesion activity or protease activity have been found to be active in various biological processes (reviewed in Wolfsberg *et al.* 1995a; Blobel 1997; Black and White 1998; Primakoff and Myles 2000; White 2003).

Of the 32 known ADAMs, 16 are expressed exclusively or predominantly in the mammalian testis. The 16 ADAMs are divided phylogenetically into two major groups: ADAMs 1, 4, 6, 20, 21, 24, 25, 26, 29, 30 and 34 (the first group); and ADAMs 2, 3, 5, 27 and 32 (the second group) (Fig. 1). All of the mammalian, testicular ADAMs predicted as active metalloproteases belong to the first phylogenetic group. They are ADAMs 1, 20, 21, 24, 25, 26, 30 and 34 (Fig. 1). This chapter focuses mainly on ADAM 1, one of the best-studied ADAMs, and also the other testicular

ADAMs of which functions are largely unknown, discussing their genomic nature, protein characteristics and roles in reproduction.

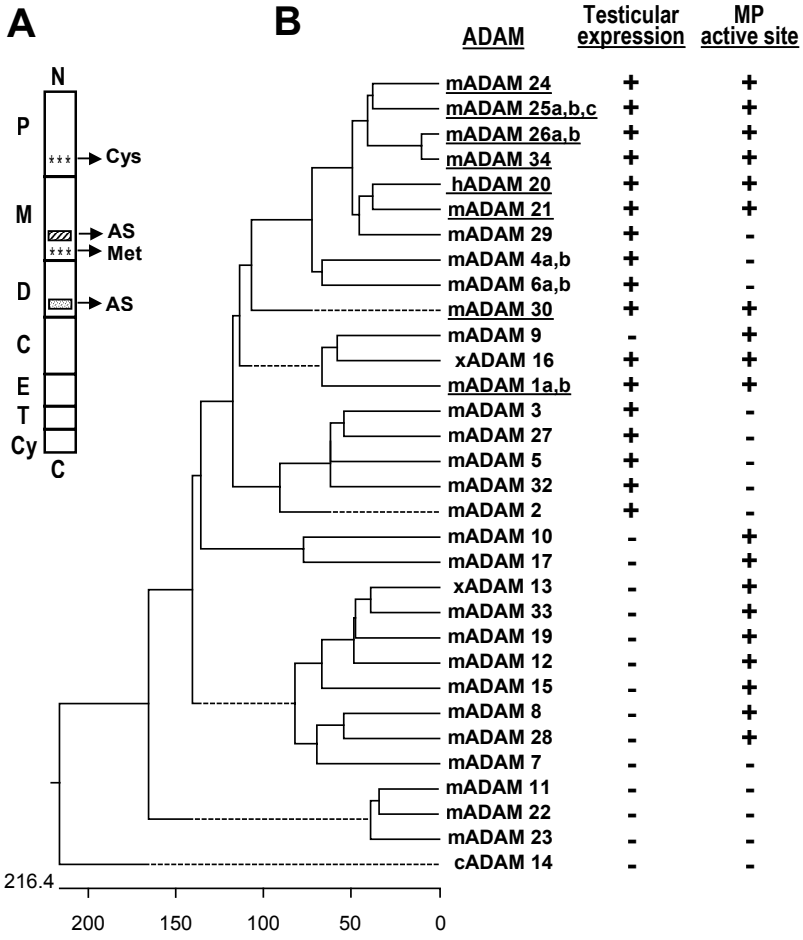

Figure 1. ADAM domain structure (A) and the phylogenetic tree of ADAM family members (B). Residues responsible for cysteine-switch (Cys), active sites (AS) and methionine-turn (Met) are indicated at the domain structure. Domain abbreviations are as follows: P, prodomain; M, metalloprotease; D, disintegrin; C, cysteine-rich; E, EGF-like; T, transmembrane; Cy, cytoplasmic. The length of the branch in the phylogenetic tree represents the level of divergence between ADAMs. Mammalian ADAM members with testis-specific or -predominant gene expression, and the active metalloprotease (MP) site are underlined. GenBank accession numbers for the sequences are AB048844 (ADAM 1a), AB048843 (ADAM 1b), NM_009618 (ADAM 2), X64227 (ADAM 3), AY158688 (ADAM 4a), BK001466 (ADAM 4b), U22059 (ADAM 5), AY158689 (ADAM 6a), BK001467 (ADAM 6b), NM_007402 (ADAM 7), NM_007403 (ADAM 8), U41765 (ADAM 9), NM_007399

(ADAM 10), AB009676 (ADAM 11), NM_007400 (ADAM 12), U66003 (ADAM 13), U68185 (ADAM 14), AF006196 (ADAM 15), U78185 (ADAM 16), AB021709 (ADAM 17), AF019887 (ADAM 19), AF158643 (ADAM 20), AF251559 (ADAM 21), AB009674 (ADAM 22), AB009673 (ADAM 23), AF167402 (ADAM 24), AF167403 (ADAM 25a), BN000121 (ADAM 25b), BN000119 (ADAM 25c), AF167404 (ADAM 26a), BK001468 (ADAM 26b), AF167405 (ADAM 27), AF153350 (ADAM 28), AY190759 (ADAM 29), AY190760 (ADAM 30), AF513715 (ADAM 32), AF472524 (ADAM 33) and AF373288 (ADAM 34). Species abbreviations: m, *Mus musculus*; h, *Homo sapiens*; x, *Xenopus lavies*; c, *Caenorhabditis elegans*.

2. ADAM 1

2.1 Organization and Expression of the ADAM 1 Gene

ADAM 1 was one of the first-identified and best-studied ADAMs. Complementary DNA for ADAM 1 was first cloned in guinea pig (Blobel *et al.* 1992; Wolfsberg *et al.* 1993) and subsequently, ADAM 1 cDNA sequences from a number of mammalian species have been reported, including mouse, rat, rabbit, bull and monkey (Perry *et al.* 1995; Wolfsberg *et al.* 1995b; Hardy and Holland 1996; McLaughlin *et al.* 1997; Waters and White 1997; Nishimura *et al.* 2002).

One of the notable features of the ADAM 1 gene is the presence of two copies for the gene in a range of mammalian species. In mouse, two genes for ADAM 1 were identified, encoding different isoforms named ADAM 1a (791 residues) and ADAM 1b (806 residues). Both genes for ADAM 1 were mapped to one site of a single chromosome in mouse (distal region of chromosome 5). Consistently, a genome database search revealed that the two genes are adjacent in the mouse chromosome, suggesting that the primordial ADAM 1 gene has undergone duplication during evolution (Cho *et al.* 1996, 1997; Nishimura *et al.* 2002). The amino acid sequences of mouse ADAM 1a and ADAM 1b are highly homologous (99%) to each other in the N-terminal region such as the pro- and metalloprotease domains. The metalloprotease active site sequence is the same between the two isoforms: HELGHNLGIQHD. By contrast, the two isoforms share a low degree of identity (37%) in the C-terminal region including the disintegrin and cysteine-rich domains (Nishimura *et al.* 2002). Similarly, cDNAs for two ADAM 1 isoforms, fertilin αI and fertilin αII, were identified in monkey (*Macaca fascicularis*), encoding proteins of 905 and 825 residues, respectively. The two monkey isoforms share extensive identity, but differ significantly towards their N- and C-termini (Perry *et al.* 1995). A study with a range of additional primate species revealed the presence of two copies for the ADAM 1 gene in monkey species such as baboon and tamarin (Jury *et*

al. 1998). On the contrary, higher primates such as orang-utan, gorilla and human were found to have only a single gene for ADAM 1. Nucleotide sequence comparison showed that the 5' end of the ADAM 1 gene in these three species is highly homologous to the 5' end of monkey fertilin αI (but not fertilin αII), the central region shows a high degree of identity to the central region common to both monkey isoforms and the 3' end of the genes are significantly homologous to the 3' region of monkey fertilin αII (but not fertilin αI). This suggests that the ADAM 1 gene in orang-utan, gorilla and human has been derived by genetic recombination between the two ADAM 1 genes maintained presumably from mouse to monkeys during evolution. This recombination event may have occurred during the divergence of monkeys and great apes.

Another distinctive feature of the ADAM 1 gene is that all the mammalian ADAM 1 genes lack introns, and the gorilla and human genes are inactive (Jury *et al.* 1997, 1998; Nishimura *et al.* 2002). This suggests that the ADAM 1 gene may have arisen through retrotransposition of spliced mRNA transcribed from a primordial ADAM gene into the mammalian genome, resulting in the loss of introns. The gorilla and human ADAM 1 genes contain a number of nucleotide insertions and deletions in the coding regions, disrupting the reading frames. This suggests that the processed, intronless ADAM 1 genes may have accumulated mutations in the mammalian lineage over time, due to the absence of selective pressure during evolution.

The ADAM 1 gene is expressed exclusively or predominantly in the mammalian testis. Northern blot analyses demonstrated the testis-specific expression of the mouse and guinea pig ADAM 1 genes, whereas RT-PCR analyses, a more sensitive assay, showed that the mouse and rat ADAM 1 genes are expressed abundantly in the testis and at low levels in other tissues (Wolfsberg *et al.* 1993, 1995b; Frayne *et al.* 1997; Nishimura *et al.* 2002). When a certain gene is found to be expressed in the testis, it is of particular interest whether the gene is expressed in germ cells or somatic cells in the testis. Male germ cell development occurs in seminiferous tubules containing a mixture of spermatogenic cells and somatic cells such as Sertoli cells. In the first round of spermatogenesis in prepubertal animals, stem cells proliferate and differentiate gradually to yield the sequence of spermatogonia, spermatocytes, spermatids and sperm. This process takes about 35 days in the mouse with three different phases such as mitotic (11 days), meiotic (10 days) and postmeiotic (14 days) phases (Eddy 1998). If a particular gene is expressed in germ cells during spermatogenesis, a transcript for that gene will appear in the testis at a certain post-partum time point corresponding to a specific stage of spermatogenesis. Northern blot

analysis using prepubertal and mature mouse testes showed that the ADAM 1a and ADAM 1b genes are first expressed from days 24 and 20, respectively, suggesting germ cell-specific expression of the genes (Nishimura *et al.* 2002). By contrast, separate studies using PCR assay demonstrated that the rat ADAM 1 gene is expressed at all stages of development. This suggests that the rat ADAM 1 gene is not exclusively expressed in germ cells (Fryne *et al.* 1997; McLaughlin *et al.* 1997). Considering a difference between Northern blot and PCR assay in sensitivity and quantification, it is likely that the ADAM 1 gene is transcribed by both somatic and germ cells in the testis with higher level expression in germ cells. Alternatively, the expression of the ADAM 1 gene may be differentially regulated between mouse and rat testes.

2.2 Biochemical and Cellular Properties of ADAM 1

ADAM 1 (also known as PH30 α or fertilin α), an integral membrane glycoprotein, was identified originally in guinea pig sperm on which the protein is dimerized with the ADAM 2 protein (PH30 β or fertilin β) (Primakoff *et al.* 1987; Blobel *et al.* 1990). Although ADAM 2, unlike ADAM 1, does not have the correct amino acid sequence at the position of the metalloprotease active site to be a functional protease, it is closely related with ADAM 1 in protein interaction and function (reviewed in Myles 1993; Wolfsberg *et al.* 1995a; Snell and White 1996; Wolfsberg and White 1996; Myles and Primakoff 1997; Wassarman 1999; Blobel 2000; Primakoff and Myles 2000, 2002; Evans 2001; Talbot *et al.* 2003). A comprehensive, biochemical analysis using testicular cells, testicular sperm and epididymal sperm from guinea pig revealed the complicated processing pattern of ADAM 1/2 heterodimeric complex during sperm development and maturation. The guinea pig ADAM 1 and ADAM 2 proteins are synthesized as precursors in spermatogenic cells and undergo subsequent proteolytic cleavages during spermatogenesis (ADAM 1) and during passage through the epididymis (ADAM 2), a highly convoluted tubule in which sperm migrate from the testis to the vas deferens (Blobel *et al.* 1990). A further analysis of guinea pig ADAM 1 showed that only the processed form of ADAM 1, but not the ADAM 1 precursor, can be cell-surface biotinylated and some processed ADAM 1 is sensitive to endoglycosidase H. The proteolytic cleavage site was found to be next to four arginine residues (RRRR) between the metalloprotease domain and the disintegrin domain. This tetrabasic sequence is consistent with a consensus cleavage site, RX(K/R)R, as a target for a pro-protein convertase such as furin. These data suggest that ADAM 1 is processed by a pro-protein convertase in the

secretory pathway of testicular cells before emerging on the cell surface (Lum and Blobel 1997). The proteolytic processing of the ADAM 2 precursor also removes the pro- and metalloprotease domains, leaving an N-terminal disintegrin domain on the processed form. The ADAM 1/2 complex is distributed throughout the testicular sperm head but becomes concentrated in the posterior head region simultaneously with ADAM 2 proteolytic processing during epididymal maturation of the sperm (Blobel *et al.* 1990; Phelps *et al.* 1990; Hunnicutt *et al.* 1997; Lum and Blobel 1997).

Similar ADAM 1 features with additional information were found in other mammalian species. Monkey fertilin αI, one of the ADAM 1 isoforms, is processed during spermatogenesis in testicular cells (Frayne *et al.* 1998). In bovine sperm, ADAM 1 and ADAM 2 are complexed to form a heterodimer and this complex appears to exist as a higher-order oligomer of the basic heterodimer (Waters and White 1997). As described above (section 2.1), there are two intronless mouse genes encoding different ADAM 1 isoforms, ADAM 1a and ADAM 1b (Nishimura *et al.* 2002). Both ADAM 1 isoforms were found to be present in testicular germ cells (Kim *et al.* 2003). The ADAM 1a precursor (100 kDa) is partially converted into a processed form (48 kDa) in round and elongating spermatids. Unlike ADAM 1 in other species, mouse ADAM 1a is missing in sperm from the epididymis and vas deferens. Both the ADAM 1a precursor and the processed form of ADAM 1a are sensitive to endoglycosidase H, suggesting that they reside in the endoplasmic reticulum (ER). ADAM 1a does not contain the motifs required for protein retention in the ER. Thus it is unknown how the ADAM 1a proteins are retained within the ER. The ADAM 1a precursor forms a heterodimeric complex with the ADAM 2 precursor in spermatogenic cells. On the other hand, ADAM 1b is made as a precursor (120 kDa) in pachytene spermatocytes, converted to an intermediate form (63 kDa) during spermiogenesis and further processed to a mature form (60 kDa) during the sperm transit in the epididymis. Both of the precursor and mature forms of ADAM 1b are capable of forming heterodimers with the ADAM 2 precursor and processed ADAM2, respectively. Most of the ADAM 1b precursor is associated with ADAM 2 in testicular cells, but only a part of the processed form of ADAM 1b contributes to the complex formation with ADAM 2 in mature sperm. Both of the heterodimeric and monomeric forms of ADAM 1b are localized on the cell surface of mature sperm. Taken together, the two ADAM 1 isoforms are distinguishable from each other in processing pattern and subcellular localization (Kim *et al.* 2003). Mouse ADAM 1b, but not ADAM 1a, is similar to guinea pig, bovine and monkey ADAM 1. Despite the existence of the two functional genes for ADAM 1 in several mammalian species lower than great apes (see section 2.1) besides mouse, it is unknown

whether the ADAM 1a protein exists in these species. On the contrary, the human ADAM 1 gene is nonfunctional. It is possible that an ADAM 1a-like protein may be present and active in human. Resolution of these issues, requiring further investigation, is important because knockout studies imply that each of the two mouse ADAM 1 isoforms has a differential function in reproduction (see below; section 2.3).

ADAM 1 contains the pro-protein convertase consensus sequence between the metalloprotease domain and the disintegrin domain, directing removal of the pro- and metalloprotease domains from the membrane-anchored protein. This presents an apparent contrast to other catalytic ADAM members of which activation is dependent upon removal of the prodomain, leaving the N-terminal metalloprotease domain on the membrane-anchored, processed form (Blobel 2000). To answer if prodomain removal of ADAM 1 occurs and whether this processing event is necessary for protease activity will require further investigation.

2.3 Functions of ADAM 1 in Fertilization

Mammalian fertilization is a multi-step process. After sperm enter the uterus, they migrate into the oviduct where sperm fertilize eggs. The utero-tubal junction, a barrier for sperm ascent to the ampulla, permits migration of only a small fraction of uterine sperm into the oviduct (Yanagimachi, 1994). When sperm reach the egg, they first encounter a mass of cells, called the cumulus oophorus, that surrounds the egg. Sperm swim between these cells, dissolving the extracellular matrix rich in hyaluronic acid. When sperm reach the egg extracellular coat, the zona pellucida (ZP), they recognize it and bind to it. Subsequently, sperm penetrate the ZP and come to lie in the perivitelline space. Finally, the sperm bind to the egg plasma membrane and then fuse with it.

Mouse knockouts of ADAM 1 and related ADAMs revealed direct or indirect involvement of the ADAMs in the fertilization process. Recently, mice were produced that are homozygous nulls for ADAM 1a (Nishimura *et al.* 2004). The mutant male mice are infertile because of the severely impaired ability of sperm to migrate from the uterus into the oviduct. ADAM 1a-deficient sperm are also impaired in the dispersal of cumulus cells and binding to the egg ZP. The analysis of protein expression phenotype revealed that ADAM 1b and ADAM 2 are normally present in both testicular germ cells and sperm from ADAM 1a-deficient mice. However, the loss of ADAM 1a resulted in the severe reduction of ADAM 3 in epididymal sperm (Table 1). ADAM 3, also known as cyritestin, is a testicular ADAM member similar to ADAM 2 in structure, processing and subcellular localization: it is predicted as an inactive protease, processed

during sperm maturation and present on mature sperm (Yuan *et al.* 1997). Mice lacking ADAM 2 were also produced and the mutant males were found to be infertile (Cho *et al.* 1998). Like ADAM 1a knockout, sperm from ADAM 2-deficient mice are defective in migration from the uterus into the oviduct and adhesion to the egg ZP. However, the protein expression phenotype of ADAM 2-deficient mice is dissimilar to that of ADAM 1a-deficient mice. In ADAM 2-deficient testicular germ cells, the amounts of ADAM 1a and ADAM 3 are normal, while the level of ADAM 1b is significantly reduced. Furthermore, ADAM 2-deficient sperm completely lack ADAM 1b and barely contain ADAM 3 (Table 1) (Cho *et al.* 1998, 2000; Nishimura *et al.* 2004). Finally, male mice with a deletion in the ADAM 3 gene were reported to be also infertile (Shamsadin *et al.* 1999; Nishimura *et al.* 2001). Comparing to ADAM 1a- and ADAM 2-deficient sperm, ADAM 3 knockout resulted in similar, but more restricted, effects on fertilization. ADAM 3 null sperm are deficient in adhesion to the egg ZP, but show normal transport from the uterus into the oviduct. The levels of ADAM 1a, ADAM 1b and ADAM 2 are unchanged in testicular germ cells and mature sperm from ADAM 3-deficient mice (Table 1) (Nishimura *et al.* 2004).

Table 1. Protein expression phenotype in ADAM knockouts.

Knockout	Cell stage*	Expression level or integrity of ADAM proteins**					
		1a	1b	2	1a/2	1b/2	3
ADAM 1a	TGC	-	+	+	-	+	+
	ES	-	+	+	-	+	-
ADAM 2	TGC	+	-	-	-	-	+
	ES	-	-	-	-	-	-
ADAM 3	TGC	+	+	+	+	+	-
	ES	-	+	+	+	+	-

*Cell stage: TGC, testicular germ cell; ES, epididymal sperm.

**The normal expression level, and the absence or severely reduced expression level of ADAMs are indicated as + and -, respectively. 1a/2 or 1b/2 indicates ADAM 1/ADAM2 heterodimer. Lack of ADAM 1a and ADAM 1a/ADAM 2 in epididymal sperm is not due to gene knockouts since ADAM 1a is missing in wild-type sperm as described in section 2.2.

The intriguing feature of the ADAM knockouts is the intricate relationships between the protein expression phenotypes and the fertilization phenotypes (Table 1). Both of ADAM 1a- and ADAM 2-decificent sperm, but not ADAM 3 null sperm, are defective in migration from the uterus to the oviduct. A common protein expression phenotype between ADAM 1a and ADAM 2 knockouts is the absence of ADAM 1a/2 heterodimer and ADAM 3 in testicular germ cells and epididymal sperm, respectively (Table 1). Because ADAM 3 null sperm are normal in progression into the oviduct,

ADAM 1a/2 heterodimer, but not ADAM 3, is important for sperm migration. Considering lack of ADAM 1a/2 complex in wild-type sperm, this complex is indirectly involved in sperm transport from the uterus into the oviduct. During spermatogenesis, the ADAM 1a metalloprotease, of which activity presumably requires interaction with ADAM 2, might process and thus regulate sperm molecules directly involved in sperm transport (Fig. 2).

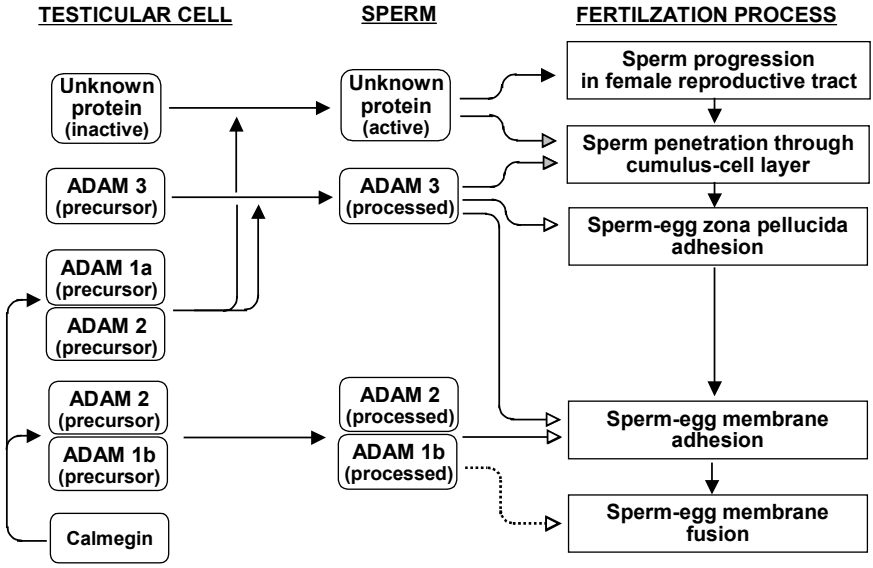

Figure 2. Schematic diagram showing the relationships between ADAMs and fertilization. Calmegin is necessary for ADAM 1/2 heterodimerization. ADAM 1a/2 regulates the activity or localization of sperm proteins including ADAM 3. An unknown sperm protein(s) regulated by ADAM 1a/2 is directly involved in sperm transport from the uterus to the oviduct. Sperm penetration through the layer of cumulus cells is partially dependent on the unknown protein and/or ADAM 3 (shaded arrowhead). The fertilization steps of sperm-egg zona pellucida binding involving ADAM 3, and sperm-egg plasma membrane binding involving ADAM 3, ADAM 2 or ADAM 1b are not physiologically essential for successful fertilization (open arrowhead) as found for the mutant sperm showing normal fertilization in the subsequent steps. ADAM 1b is not an absolute requirement for sperm-egg plasma membrane fusion (dotted arrow).

All ADAM 1a-, ADAM 2- and ADAM 3-deficient sperm are defective in binding to the egg ZP and all commonly lack ADAM 3 in mature sperm (Table 1). This suggests that the formation of the complex between ADAM1a and ADAM 2 in testicular germ cells is responsible for the appearance of ADAM 3 on the sperm surface, which plays a critical role in

sperm-egg ZP binding (Fig. 2). Despite the impaired ability to bind the ZP, ADAM 1a-deficient sperm lacking ADAM 3 are capable of fertilizing cumulus-intact, ZP-intact eggs normally (Nishimura *et al.* 2004). This indicates that the step of sperm-egg ZP adhesion involving ADAM 3 is not a prerequisite for successful fertilization.

In vitro sperm-egg plasma membrane interaction analysis and amino acid sequence analysis suggested that ADAM 1 plays roles in sperm-egg plasma membrane adhesion and fusion. It was found that a recombinant protein and a peptide mimetic corresponding to the mouse ADAM 1a disintegrin domain inhibit sperm adhesion to the plasma membrane of ZP-free eggs (Evans *et al.* 1998; Wong *et al.* 2001). Because ADAM 1a is absent from mature sperm, however, assessment of ADAM 1a in sperm-egg binding is physiologically irrelevant. By contrast, mouse ADAM 1b is present on the surface of mature sperm and contains the RGD sequence, the integrin-binding motif, in the cysteine-rich domain (Nishimura et al. 2002; Kim et al. 2003). Consistent with this, ADAM 2 knockout sperm lacking ADAM 1b are defective in adhesion to the plasma membrane of ZP-free eggs (Table 1) (Cho *et al.* 1998). It should be noted that ADAM 2 and ADAM 3 also play a role in sperm-egg membrane binding because they are present on sperm surface and sperm lacking either or both proteins are deficient in adhesion to the egg membrane (Cho *et al.* 1998; Nishimura *et al.* 2001). The adhesion role of ADAM 2 and ADAM 3 is also supported by a number of *in vitro* studies in which peptide mimetics of the ADAM 2 and ADAM 3 disintegrin domains inhibit sperm-egg membrane binding (Myles *et al.* 1994; Almeida *et al.* 1995; Evans *et al.* 1995; Yuan *et al.* 1997). However, sperm lacking ADAM 1b, ADAM 2 or ADAM 3 were found to have little defect in fusion with the egg plasma membrane. Thus the adhesion step involving ADAM 1b, ADAM 2 or ADAM 3 is not an essential, physiological process that leads to fusion (Fig. 2).

A previously proposed model for the mechanism of gamete fusion suggests that a hydrophobic fusion peptide present in the ADAM 1 cysteine-rich domain promotes sperm-egg fusion (Blobel *et al.* 1992; Wolfsberg *et al.* 1995a; Snell and White 1996; Waters and White; 1997). A number of investigators have searched for fusion-promoting properties of peptides corresponding to the putative fusion peptide of ADAM 1 (Muga *et al.* 1994; Niidome *et al.* 1997; Martin *et al.* 1998; Wolfe *et al.* 1999). Fusion of mouse sperm lacking ADAM 2 with the egg plasma membrane occurs at about 50% of the wild-type rate (Cho *et al.* 1998). Because ADAM 1b is absent from the mutant sperm which can fuse, this indicates that the ADAM 1b fusion peptide is not an absolute requirement for sperm-egg membrane fusion (Fig. 2) (Cho *et al.* 2001; Nishimura *et al.* 2004).

The ADAM knockouts provide an insight into the molecular basis of mammalian fertilization. Figure 2 summarizes the relationships between the ADAM proteins and the fertilization process. Each of the two ADAM 1 isoforms interacts with ADAM 2 to form a heterodimer and differentially participates in fertilization. Spermatogenesis-specific chaperone calmegin was reported to be required for ADAM 1/2 heterodimerization (Ikawa *et al.* 1997; 2001). ADAM 1a is implicated in the regulation of sperm proteins, including ADAM 3, that function in sperm progression from the uterus into the oviduct. Identification of target proteins for ADAM 1a/2 complex should provide more information about the molecular network of fertilization.

3. ADAMS 20, 21, 24, 25, 26, 30 AND 34

3.1 Molecular and Genomic Nature of ADAMs

According to the ADAM phylogenetic tree (Fig. 1), the mammalian ADAMs with testis-specific or -predominant gene expression are divided into two major groups: ADAMs 1, 4, 6, 20, 21, 24, 25, 26, 29, 30 and 34 (group I); and ADAMs 2, 3, 5, 27 and 32 (group II). The first-group ADAMs can be further divided phylogenetically into two subgroups: ADAM 1 (group I-1) and the rest of the ADAMs (group I-2) (Fig. 1 and Table 2). Among the members in the group I-2, ADAMs with the metalloprotease active site are ADAMs 20, 21, 24 (testase 1), 25 (testase 2), 26 (testase 3), 30 and 34 (testase 4). Full-length cDNAs for these ADAMs were cloned from mouse and/or human (Hooft van Huijsduijnen 1998; Cerretti *et al.* 1999; Brachvogel *et al.* 2002; Poindexter *et al.* 1999; Zhu *et al.* 1999; Liu and Smith 2000; Choi *et al.* 2004). All of these ADAMs possess the consensus zinc-binding and catalytic sites HEXGHXXGXXHD and the 'methionine turn' downstream (Table 2). It is noteworthy that the group-I-2 ADAMs without the correct amino acid sequence to be a functional protease (especially ADAMs 4 and 29) show high similarity to the consensus sequence at the position of the metalloprotease active site, presenting a contrast to the group-II ADAMs which also do not have the consensus sequence (Table 2). It is likely that the metalloprotease active site arose in a common ancestor and subsequently, maintained or lost in the lineages of the group-I-2 ADAMs. Thus the testicular ADAMs with protease activity appear to be closely related by evolution (Cerretti *et al.* 1999; Choi *et al.* 2004). In addition to the conserved zinc-dependent metalloprotease active site, the prodomain of all of the group-I-2 ADAMs having the consensus sequence to be a functional protease contains an unpaired cysteine involved in the inhibition of the metalloprotease activity by interacting with zinc (Table 2).

Convertase-induced cleavage of the prodomain, removing the free cysteine, is expected to activate the metalloprotease activity. Unlike other ADAM proteases that have the consensus target sequence for a furin-like convertase, the group-I-2 ADAMs predicted as active proteases do not have this cleavage site (Table 2), suggesting a different processing mechanism (see section 3.2).

Table 2. Comparison of metalloprotease characteristics among mammalian, testicular ADAMs.

Phylo-genetic Group	ADAM*	Prediction of active protease	Free cys.	Target site for furin	Active site sequence**	Met-turn
I-1	1	+	+	+	**HE**L**GH**NL**G**IQ**HD**	+
I-2	20	+	+	-	**HE**L**GH**NL**G**MQ**HD**	+
	21	+	+	-	**HE**L**GH**TF**G**MK**HD**	+
	24	+	+	-	**HE**I**GH**NL**G**MS**HD**	+
	25	+	+	-	**HE**M**GH**NL**G**ME**HD**	+
	26	+	+	-	**HE**M**GH**NF**G**MK**HD**	+
	30	+	+	-	**HE**L**GH**CV**G**MI**HD**	+
	34	+	+	-	**HE**M**GH**NL**G**MM**HD**	+
	4	-	+	-	**H**AV**GH**LLDVS**HD**	+
	6	-	-	-	NRGVRSL**G**LK**HD**	+
	29	-	-	-	**H**HL**GH**NL**G**MK**HD**	+
II	2	-	-	-	QLLSLSM**G**LAY**D**	+
	3	-	-	-	QLL**G**INL**G**LAY**D**	+
	5	-	-	-	QLLSIGM**G**LTY**D**	+
	27	-	-	-	QLI**G**LHI**G**LTY**D**	+
	32	-	-	-	QML**G**LSL**G**ISY**D**	+
				Consensus	**HE**X**GH**XX**G**XX**HD**	

*Except for ADAM 21 (human), all of the listed ADAMs are from mouse. In some ADAMs having isoforms, only the first isoforms (25a, 26a, 4a, 6a and 1a) are listed. The second or third isoform is similar to the first isoform in the characteristics of each ADAM.
** Amino acids identical to the conserved residues in the consensus sequence are bolded.

In genomic organization, ADAMs in the phylogenetic group I-2, containing most of testicular members predicted as active proteases, are similar to ADAM 1, but strikingly different from the group-II ADAMs without protease activity. First, all of the group-I-2 ADAM genes lack introns that interrupt protein-coding exons (Poindexter *et al.* 1999; Choi *et al*. 2004). In PCR analysis using genomic DNA and testis cDNA with primers amplifying the complete coding sequence of each mouse ADAM, PCR products with the identical size were generated in each ADAM (Choi *et al*. 2004). Similarly, genome database searches revealed lack of introns in the open reading frames in all of the ADAMs belonging to the group I-2. This presents a great contrast to the group-II ADAMs containing multiple

(19-20) introns interrupting the protein-coding exons (Cho *et al*. 1997; Choi *et al*. 2003). Second, many of the intronless ADAMs are present as multicopy genes in the mouse genome and these gene copies are clustered in the same chromosome (Table 3) (Bolcun *et al*. 2003; Choi *et al*. 2004). Southern blot analysis and genome database searches demonstrated that more than one gene for ADAM 4 (2 copies), ADAM 6 (2 copies), ADAM 25 (3 copies) and ADAM 26 (2 copies) exist in the mouse genome. Two clustered genes (ADAM 4a and ADAM 4b) for mouse ADAM 4 with 90% nucleotide sequence identity are present in the middle region of mouse chromosome 12. Two ADAM 6 genes (ADAM 6a and ADAM 6b) with 95% nucleotide sequence identity are adjacent to each other in the proximal region of mouse chromosome 12. For ADAM 25, three gene copies (ADAM 25a, ADAM 25b and ADAM 25c) with 80-94% nucleotide sequence homology among the copies are present as a cluster in the mid-proximal region of mouse chromosome 8. At a close distance from the ADAM 25 genes on chromosome 8, there exist two genes (ADAM 26a and ADAM 26b) for ADAM 26, having 93% nucleotide sequence homology. In contrast with these ADAM genes, none of the ADAMs belonging to the phylogenetic group II was found to have more than one gene in the mouse genome. Third, the pattern of correspondence between the mouse and human ADAM genes is unusual in more than half of the ADAMs and some human orthologues are pseudogenes (Table 3) (Poindexter *et al.* 1999; Choi *et al*. 2004). Orthology with 1:1 relationship in gene numbers was found in ADAMs 4, 24, 29 and 30 but not in ADAMs 6, 20, 21, 25, 26 and 34. Whereas there are two genes for ADAM 6 in mouse, only a single homologous gene for ADAM 6 is present in the human genome. For ADAM 21, the gene exists as a single copy in the mouse genome, whereas it is present as two copies in the human genome. The most unusual orthology relationship was found for ADAMs 20, 25, 26 and 34. There is no mouse gene for human ADAM 20. The human genome does not contain genes corresponding to the genes for mouse ADAM25 (3 copies), ADAM26 (2 copies) and ADAM34 (1 copy). Like the mouse ADAM genes, all the human ADAM genes do not have introns. The intriguing feature of the human intronless ADAM genes is that many of them (ADAMs 4a, 4b, 6, 21b and 24) are pseudogenes with frameshift or stop codons. As described above (section 2.1), the ADAM 1 gene is a single-copy pseudogene in the human genome but two functional copies for the gene are present in the mouse genome. It was suggested that the ADAM 1 gene in human is derived by genetic recombination between the two ADAM 1 genes present in some primates closely related to human. Although such type of evolutionary selection might explain the relationship of the intronless ADAM genes (group I-2) between mouse and human, a comprehensive,

comparative analysis of these ADAM genes from a wide range of rodents and primates would be needed to validate the hypothesis.

Table 3. Genomic characteristics of ADAMs belonging to the phylogenetic group I-2

ADAM	Mouse				Human			
	Gene	Chr*	AA**	F***	Gene	Chr	AA**	F***
ADAM 4	4a	12 (D2)	763	+	4a	14q24	300	-
	4b	12 (D2)	751	+	4b	14q24	375	-
ADAM 6	6a	12 (F2)	754	+	6	14q32	610	-
	6b	12 (F2)	726	+	-			
ADAM 20	-				20	14q24	726	+
ADAM 21	21	12 (D3)	729	+	21a	14q24	722	+
	-				21b	14q24	132	-
ADAM 24	24	8 (B1)	761	+	24	8p22	3	-
ADAM 25	25a	8 (B1)	760	+	-			
	25b	8 (B1)	756	+	-			
	25c	8 (B1)	760	+	-			
ADAM 26	26a	8 (B1)	697	+	-			
	26b	8 (B1)	699	+	-			
ADAM 29	29	8 (B3)	763	+	29	4q34	820	+
ADAM 30	30	3 (F3)	732	+	30	1p12	790	+
ADAM 34	34	8 (B1)	714	+	-			

*Chromosomal location of the genes. Letters and numbers in parenthesis indicate subregions in chromosomes.

**Number of amino acids (AA).

***Functionality (F) of the genes based on the integrity of open reading frames. Functional genes and pseudogenes are indicated by + and -, respectively.

The phylogenetic ADAM group I-2 enriched with the active proteases is also unique in gene expression pattern. Northern blot analysis showed that the mouse ADAM 24, 25 and 26 genes are expressed predominantly in postmeiotic germ cells (Zhu *et al*. 1999). RT-PCR analysis revealed that all the ADAM genes belonging to the group I-2 are expressed in mouse testis from postnatal day 6 to adult (Choi *et al*. 2004). Since the majority of cells in the testis at day 6 are somatic cells and the proportion of somatic cells decreases as spermatogenesis proceeds, this indicates that the mouse ADAM genes are expressed by both somatic and germ cells. Furthermore, all of the mouse ADAM genes were found to be transcribed in a Sertoli cell line (Choi *et al.* 2004). Thus it is likely that the group-I-2 ADAM genes are transcribed by both somatic and germ cells with higher expression level in postmeiotic germ cells in the testis. The gene expression pattern of these ADAMs is similar to that of ADAM 1 but not other ADAMs belonging to the separate phylogenetic group (group II) showing a germ-cell specific expression pattern (Wolfsberg *et al*. 1995b; Zhu *et al*. 1999; Choi *et al*. 2003). These

suggest a potential relationship between the function of the ADAMs with protease activity and the process of somatic cell -germ cell interaction during spermatogenesis.

3.2 Properties of the ADAM 24 protein

To date, among the testicular ADAMs belonging to the phylogenetic group I-2 and having the metalloprotease active site, only ADAM 24 (testase 1) was investigated at the protein level (Zhu *et al*. 2001). Immunoblot analysis revealed that ADAM 24 is synthesized as a precursor (108 kDa) in testicular germ cells and sperm. The protein is partially converted to a processed form (88 kDa) in caput sperm and the processing is complete in corpus sperm, suggesting that the prodomain of ADAM 24 is removed during sperm passage through the caput epididymis. The ADAM 24 precursor and processed form reside on the plasma membrane of testicular and epididymal sperm, respectively. The processing of ADAM 24 is unique in several aspects. First, unlike ADAM 1 whose proteolytic processing removes the pro- and metalloprotease domains, only the prodomain is cleaved in ADAM 24. Second, ADAM 24 does not contain the consensus cleavage site recognized by the furin-type pro-protein convertase (see sections 2.2 and 3.1) known to be responsible for removal of the prodomain in a number of ADAM proteases. Third, the processing of ADAM 24 occurs on the plasma membrane, presenting an apparent contrast to other ADAM proteases of which processing takes place in the secretory pathway before emerging on the cell surface. Thus these indicate the existence of the differential, molecular mechanism underlying the processing of ADAM 24 and insure further investigation of cellular source (sperm or epididymis) and identity of a processing enzyme.

Considering ADAM 24 is a plasma membrane-anchored sperm protease, it is tempting to hypothesize that the protein plays a direct role in the fertilization process requiring protease activity, such as sperm passage through the cumulus cell layer, sperm penetration into the egg ZP and sperm-egg plasma membrane fusion. There exist a number of testicular ADAMs predicted as active proteases (ADAMs 20, 21, 25, 26, 30 and 34) in the phylogenetic group I-2 containing ADAM 24. These ADAMs are highly similar to ADAM 24 in gene structure, gene expression pattern and metalloprotease characteristics based on amino acid sequence analysis (Table 2). While these ADAMs need extensive investigation that concerns processing and function, one can speculate on the dynamic participation of these ADAM proteases in the process of sperm maturation and fertilization.

4. CONCLUSION

A number of testicular ADAMs predicted as active proteases have been discovered in various mammalian species. The unique features of these ADAM genes are that all of them belong to the same phylogenetic group and lack introns. Furthermore, many of these ADAM genes are present as multicopy genes in the mouse genome and a number of these mouse genes do not have human orthologues. Even if human orthologues exist, some of them are pseudogenes in human. Noteworthy in relation to this is a global difference between the mouse and human genomes. Comparative genomic analysis revealed that over 90% of the two genomes are syntenic. Nevertheless, each genome is evolving in a non-uniform manner, showing substantial variation across the genome. The proportion of mouse genes with a single identifiable orthologue in the human genome is about 80%. The remainder are genes lacking a strict 1:1 relationship due to differential expansion in at least one of the two genomes. It was found that most genes expanded in the mouse lineage have common features. They are present as a family and clustered in the mouse genome, suggesting that they have been generated by local gene duplication. The significant proportion of these genes seems to be involved in reproduction (Waterson *et al*. 2002). Similarly, the global view of human and mouse proteases revealed that the mouse degradome is more complex and many of genes expanded in the mouse genome correspond to proteases that are involved in reproductive functions (Puente *et al*. 2003). There exist 11 testicular ADAM proteases in mouse (ADAMs 1a, 1b, 21, 24, 25a, 25b, 25c, 26a, 26b, 30 and 34), whereas only 3 ADAM genes (ADAMs 20, 21 and 30) with testicular expression are predicted to encode functional proteases in human. The studies of mouse ADAMs indicate that ADAM 1a is central to the fertilization process by regulating sperm proteins responsible for fertility and ADAM 24 is potentially involved in the various steps of fertilization. These ADAMs may be related to rodent-specific aspects of fertilization. It is possible that the human testicular ADAM proteases have similar functions in fertilization. In this regard, function studies on the ADAM proteases present in both species (ADAMs 21 and 30) should provide more information about the molecular mechanisms underlying mammalian fertilization.

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