Ubiquitin-Dependent Proteolysis in Mammalian Spermatogenesis, Fertilization, and Sperm Quality Control: Killing Three Birds With One Stone

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ABSTRACT Ubiquitin and ubiquitin-like proteins control the degradation of substrates as diverse as cyclins, viral envelope proteins, plasma membrane receptors, and mRNAs. The ubiquitinated substrates are targeted towards the lysosomal or proteasomal degradation sites. The number and position of ubiquitin molecules bound to substrates' lysine residues and the number and position of ubiquitin molecules in polyubiquitin chains determine the astonishing substrate specificity of ubiquitin-mediated proteolysis. Ubiquitin is likely to be expressed in mammalian gametes and embryos at any given developmental step, but the information on ubiquitin dependence of gametogenesis and fertilization is sketchy. Ubiquitin ligases E1, E2, E3, and UBC4 are active in the testis. Ubiquitin and proteasomal subunits can be detected in the human sperm centrosome that undergoes dramatic reduction during spermatid elongation. Spermatid histones are ubiquitinated as they are being transiently replaced by transitional proteins and permanently by protamines. Ubiquitin tagging of the sperm mitochondrial membranes may serve as a death sentence for paternal mitochondria at fertilization, thus promoting the maternal inheritance of mitochondrial DNA (mtDNA) in mammals. The defective spermatozoa become surface-ubiquitinated during sperm descent down the epididymis. Finally, new evidence suggests the involvement of ubiquitin-proteasome pathway in the zona penetration by the acrosome-reacted spermatozoon. Such differential patterns of ubiquitination in the testis and epididymis, and inside the egg, may be necessary for reproductive success in humans and animals. Deciphering and eventually manipulating the ubiquitin-dependent proteolysis in the reproductive system could allow us to redirect the mode of mtDNA inheritance after cloning and ooplasmic transplantation, provide germ line therapy in some cases of male infertility, develop new contraceptives, manage polyspermia during in vitro fertilization, and establish objective markers for infertility diagnostics, semen evaluation, and prediction of future fertility. *Microsc. Res. Tech. 61:88–102, 2003.*

INTRODUCTION

Recent results from the human genome project indicate that fewer genes than originally thought are necessary to control the complexity of human and animal cells (Baltimore, 2001). Limited numbers of gene products available in a cell must therefore perform diversified functions wherein one molecule can exert control upon a multitude of cellular events. Ubiquitin, the cellular proteolytic marker peptide, is a prime example of such strategy, controlling events as diverse as cell cycle progression (Glotzer et al., 1991), protein degradation and recycling (Ciechanover et al., 1984), membrane receptor endocytosis (Strous and Govers, 1999), and even retroviral infection (Ott et al., 1998). This is achieved through the covalent binding of 76-amino acid, 8.5 kDa ubiquitin to the E-amino group on the substrate's Lys-residues. This reaction requires the hydrolysis of ATP and is catalyzed by a set of ubiquitin-carriers and ligases termed E1–E4 in mammals, or UBC-1 to UBC-5 in yeast (reviewed by Ciechanover, 1994; Hershko and Ciechanover, 1998).

The common patterns of ubiquitination include monoubiquitination, diubiquitination, and tetraubiquitination, all of them increasing the molecular weight of a substrate by an appropriate multiple of 8.5 kDa (Pickart, 1998). Often one ubiquitinated substrate can occur in a variety of ubiquitination orders, resulting in a typical ladder of bands, separated by 8.5 kDa spaces, or their multiples on Western blots. The purpose of the ligation of polyubiquitin chains is to deliver the ubiquitinated substrates to a cellular trash bin, a lysosome, an autophagosomal vacuole, or a 26 S proteasome. There the polyubiquitin chain is removed by ubiquitin C-terminal hydrolases and the substrate is hydrolyzed into small peptides and individual amino acids (Wilkinson and Hochstrasser, 1998).

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A quarter of a century after the initial characterization of the ubiquitin-dependent proteolytic pathway (Etlinger and Golberg, 1977; Goldstein et al., 1975), few reports exist on the role of ubiquitination in gametogenesis and fertilization (see recent reviews by Baarends et al., 1999; Bebington et al., 2001). Most work has emerged in the last decade and the present article is an attempt to review these data with emphasis on fertilization and sperm maturation during epididymal passage. Some yet to be published studies on ubiquitin-dependent proteolytic events in male and in the female reproductive system and embryogenesis, up to the point of fertilization, are also discussed.

ROLE OF UBIQUITINATION IN SUBSTRATE SPECIFICITY OF PROTEOLYSIS: STICKY LYSINES, MULTIUBIQUITIN CHAINS, AND UBIQUITIN LIGASES

Ubiquitination is likely one of the most versatile cellular regulatory mechanisms, controlling physiological and pathological events ranging from protein turnover (Ciechanover, 1994) to Alzheimer's disease and HIV (Cochran et al., 1991). While a number of enzymes and substrate-targeting sequences are involved in these events, ubiquitin, with its ability to bind covalently to Lys-residues of most known proteins, is a common denominator of these reactions. In addition to its affinity for Lys-residues of the substrate, ubiquitin has seven lysines of its own, at least five of which may participate in ubiquitin–ubiquitin conjugation. Ubiquitin molecules already attached to the substrate can therefore form dimers, tetramers, and a variety of polymers with additional, unconjugated ubiquitin molecules (Pickart, 1998). By a simple combinatoric, an almost infinite number of ubiquitination patterns can be achieved through such coupling. This, along with a variety of destruction boxes and N-end rules that determine protein life span, accounts for the unexpected substrate specificity of ubiquitin-protein conjugation (Laney and Hochstrasser, 1999; Varshavsky, 1997). In addition, there are a variety of ubiquitin carriers and conjugating enzymes that may contribute to a peculiar substrate specificity of ubiquitination. The respective roles of three common ubiquitin conjugating enzymes, designated E1, E2, and E3 in mammals, are to activate, carry/conjugate, and ligate ubiquitin to the substrates. A number of yeast analogs, Ubcs, and a novel mammalian ligase, E4, have been described recently (reviewed by Hershko and Ciechanover, 1998). Ubiquitin-related proteins with similar function in proteolysis include SUMO1, Rad23, and NEDD8 (reviewed by Tanaka et al., 1998; Jentsch and Pyrowolakis, 2000).

Monoubiquitination, i.e., the covalent ligation of a single ubiquitin molecule to a molecule of substrate, is sufficient to elicit substrate-proteolysis (e.g., Horak and Wolf, 2001). Similarly, ubiquitin conjugating enzymes E1 and E2, but not necessarily E3 and/or E4, are required for ubiquitination (Scheffler et al., 1998). While ubiquitin is the most conservative protein extrapolated from yeast to humans (Özkaynak et al., 1984), there is much variability in ubiquitin-protein and ubiquitin-ubiquitin conjugation, which is also thought to be responsible for the varied and limited specificity of anti-ubiquitin antibodies (Pickart, 1998). In most cases, these cross-react exclusively with certain orders of multiubiquitin chains and sometimes only recognize the unconjugated ubiquitin (reviewed by Pickart, 1998).

UBIQUITIN IN SPERMATOGENESIS: THE TESTIS

Ubiquitin (Fig. 1A–C,E), the ubiquitin conjugating enzymes E1, E2 (Fig. 1D), and UBC4 and the ubiquitin-recycling protein PGP 9.5 are highly expressed during gonad and germ cell differentiation by Sertoli cells, spermatogonia, spermatocytes, and spermatids (Agell and Mezquita, 1988; Baarends et al., 1999a; Bebington et al., 2001; Kon et al., 1999; Pusch et al., 1998; Rajapurohitam et al., 1999).

Dramatic reduction of cytoplasmic volume occurs during the haploid stage of spermatogenesis, i.e., spermatid elongation, when half of all spermatid mitochondria, along with most of the cytosol, are rejected in the form of a residual body destined for resorption by Sertoli cells of the testis (reviewed by Oko and Clermont, 1998). The ubiquitination of the spermatid mitochondria (Fig. 1E) is supported by the colocalization of ubiquitin with mitochondria in spermatids and mature sperm (Sutovsky et al., 1999b, 2000a). Expression of ubiquitin in the round spermatids and mature sperm was reported in roosters (Agell and Mezquita, 1988), bulls (Sutovsky et al., 2000a), men, and mice (Tipler et al., 1997). The ubiquitin-activating enzyme E1 (Kay et al., 1991) and ubiquitin carriers E2 (Wing and Jain, 1995; Sutovsky et al., 2000a) and UBC4 (Rajapurohitam et al., 1999; Wing et al., 1996) are active during spermatogenesis. Ubiquitin C-terminal hydrolase, PGP 9.5, which is responsible for the recycling of ubiquitin, is expressed in both testis (Kon et al., 1999) and the epididymis (Fraile et al., 1996; Santamaria et al., 1993). Ubiquitination is instrumental in the replacement of the spermatid's nuclear histones by transition proteins, followed by permanent substitution with protamines during spermatid elongation. Histone H2A (Baarends et al., 1999a,b) and histone H3 (Chen et al., 1998) are ubiquitinated and discarded in the cytoplasmic droplet prior to sperm release from seminiferous tubules. Accordingly, ubiquitin levels are decreased in ram elongated spermatids no earlier than after histone-protamine exchange (Loir et al., 1986). Nuclear pore complexes are also removed from the nuclear envelopes of elongating spermatids and hatched into their cytoplasmic lobe (Sutovsky et al., 1999c), where they could be subject to ubiquitination and proteolysis. A testicular homolog of human 26 S proteasome component, TPB-1, is present in the microtubules of the manchette and in the cytoplasmic lobe of the elongating rat spermatids, where it could participate in the proteolysis of the ubiquitinated proteins (Rivkin et al., 1997). At the genome level, targeting of the ubiquitin-conjugating DNA repair enzymes USP9Y and HR6B prevents normal spermatogenesis without affecting vital functions of the mutants (Baarends et al., 2000).

Centrosome reduction is yet another hallmark of spermatid elongation. A somatic cell-like spermatid centrosome is composed of two centrioles and a halo of pericentriolar, microtubule nucleating material. After fulfilling its role in generating the sperm axoneme, this centrosome is either completely removed (mouse, rat, hamster, probably most other rodents) or reduced to a

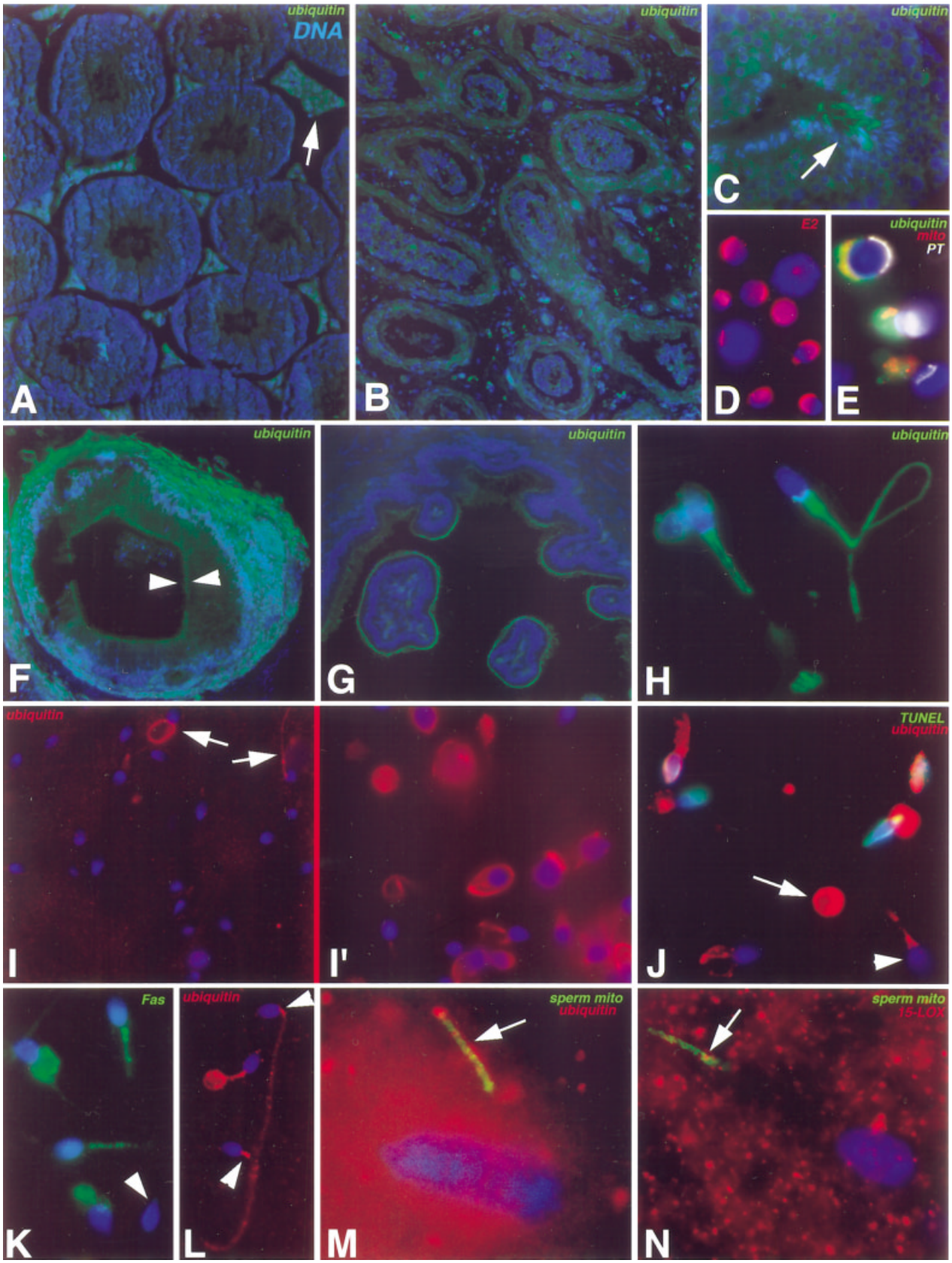
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Fig. 1.

single, inactive centriole (other mammals, including humans; reviewed by Sutovsky et al., 1999a). Ubiquitin immunoreactivity can be detected in the centrosomal part of the human and rhesus sperm tail when permeabilization is used in an immunocytochemistry protocol (Fig. 1L and unpubl. data). Proteasomal subunits were also detected inside or near the sperm centriole (Bialy et al., 2001; Mochida et al., 2000; Wojcik et al., 2000). These could be either an inactive carryover of dismantled proteasomes and ubiquitinated centrosomal proteins or a part of the proteolytic apparatus that could be activated and instrumental in the release of the sperm centriole at fertilization.

UBIQUITINATION DURING SPERM MATURATION AND STORAGE IN THE EPIDIDYMIS

After leaving the testis via testicular rete, spermatozoa are collected in the epididymis, where they undergo final maturation and storage. The mammalian epididymis is composed of three distinct compartments: caput, corpus, and cauda, each of them having a specific role in sperm maturation, sustenance, transport, and storage (reviewed by Bedford, 1979; Cooper, 1998). Numerous proteins, secreted in apocrine fashion by the epididymal epithelium, are implicated in sperm immobilization, stabilization of sperm perinuclear structure by disulfide bond-formation, and acquisition of fertilizing potential (reviewed by Kirchhoff, 1998). Epididymal protein secretion occurs by the detachment of prostasome-like particles and membrane vesicles from the apical surface protrusions of the epididymal epithelium into the lumen of epididymal tubules (Agrawal et al., 1988). This maturation and hardening step protects sperm from oxidative damage during their storage and after their release into the female genital tract. The sperm-associated droplets of residual cytoplasm carried over from the testis (Hermo et al., 1988; Fig. 2G) and a portion of the abnormal spermatozoa (Ramamohana et al., 1980; Roussel et al., 1967) are either liquefacted or phagocytosed by epididymal epithelial cells (EEC) during sperm descent down the epididymis.

Ubiquitin is present in human seminal plasma (Lippert et al., 1993) and the defective spermatozoa in both humans and animals become ubiquitinated during epididymal passage (Sutovsky et al., 2001a,b). Although the sex accessory glands could be a source of ubiquitin in seminal plasma, it is likely that most originates in the epididymis (Fig. 1F,G), a suggestion supported by the low levels of anti-ubiquitin cross-reactivity in the human and animal prostate, bulbourethral glans, seminal colliculus, and seminal vesicle (Sutovsky P., and Turner R., unpubl.).

It is not clear how the defective spermatozoa are recognized by the ubiquitination machinery and how they are disposed of. Paternal mitochondria are already tagged with ubiquitin in the testis (Sutovsky et al., 1999b, 2000a) and this nascent ubiquitin tag could be recognized by epididymal ubiquitination machinery in the abnormal sperm, where the disulfide bond cross-linking of mitochondrial membranes fails during epididymal passage. This is consistent with the accumulation of ubiquitin-cross-reactive substrates in the immotile sperm fraction, which contains most of the defective spermatozoa after gradient separation (Sutovsky et al., 2001a). Often, sperm mitochondria are the only structure with a ubiquitinated surface in defective bull sperm isolated from the upper epididymal compartment, caput epididymis (Sutovsky et al., 2001a; and unpubl. obs.). We have recently detected an unexpected, high molecular weight isoform of prohibitin, a highly conserved, 30 kDa protein of the inner mitochondrial membrane (Nuell et al., 1991), in bull sperm, where it appears to be ubiquitinated and masked by disulfide bond cross-linking of the sperm mitochondrial sheath (Sutovsky et al., 2000b). Similar to ubiquitin accumulation, an increase in prohibitin ubiquitination is evident in the immotile sperm fraction after gradient separation (Sutovsky et al., 2000b). Such a ubiquitinated isoform of prohibitin, or of another mitochondrial membrane component, could be

Fig. 1. Ubiquitin is expressed by the mammalian male reproductive system, germ cells, and embryos. **A–C:** Accumulation of ubiquitin (green) is seen in the stroma and Sertolli cells (**A**, arrow) and in the mitochondria of the fully differentiated testicular sperm (**C**, arrow) in the testis of a fertile mouse male, while the spermatogenesis is absent and ubiquitin accumulates predominantly inside the seminiferous tubules of a cancer patient who underwent chemotherapy (**B**). **D:** Ubiquitin conjugating enzyme E2 (red), a crucial component of ubiquitin-protein conjugation pathway, in the acrosomal cap and perinuclear theca of round and elongated rhesus monkey spermatids. **E:** Ubiquitin (green) colocalizes with mitochondria (red) in the cytoplasm of rhesus spermatids, as identified by the presence of perinuclear theca (white). Ubiquitin is inserted in the sperm mitochondrial membranes at this stage of spermatogenesis and carried over to mature sperm (Sutovsky et al., 2000a). **F:** Accumulation of ubiquitin (green) in the apical surface protrusions, microcillia (arrowheads) of the bovine epididymal epithelium, the site of apocrine secretion of epididymal sperm-binding proteins (Sutovsky et al., 2001b). **G,H:** Ubiquitin (green) in the apical microcillia lining the microvilli of horse epididymal lumen (**G**) and in the ejaculated horse sperm obtained during nonreproductive season (**H**). Seasonal changes in stallion fertility could be modulated by epididymal sperm ubiquitination. **I:** Comparison of ubiquitin levels (red) in the sperm of a fertile man (**I**) and of an infertility patient (**I′**). Spermatozoa with coiled tails (arrows) are seen in both fertile and infertile semen samples, but are more frequent in the later ones. Ubiquitin is a candidate semen quality marker in humans (Sutovsky et al., 2001a). **J:** Coincidence of ubiquitination (red) and DNA fragmentation (green; TUNEL assay kit) in the ejaculated sperm of an infertility patient. Arrow points to a ubiquitinated residual body, frequently seen in infertile semen samples. **K:** Localization of the apoptotic Fas-protein in the mitochondria of defective human sperm. Apoptotic markers are frequently seen in the ubiquitinated, defective human and animal sperm. **L:** Ubiquitin in the connecting piece and centriolar region of the permeabilized rhesus monkey sperm could be involved in the reduction of the male germ cell centrosome during spermatogenesis and/or participate in the release of the sperm proximal centriole into oocyte cytoplasm at fertilization. The center spermatozoon displays a coiled tail-defect. **M:** After a masking event during epididymal maturation, ubiquitin (red) becomes again detectable in the bull sperm mitochondria (arrow; green MitoTracker labeling) at the onset of the first embryonic cleavage. Note the accumulation of ubiquitin in the mitotic metaphase spindle. **N:** A protein cross-reactive with antibodies against 12/15-lipoxygenases (15-LOX; red), the enzymes implicated in the permeabilization of mitochondrial membranes during mitochondrial macroautophagy in differentiating reticulocytes, is abundant in bovine oocyte cytoplasm and appears to enter some of the sperm mitochondria (arrow; green) as they are degraded by the fertilized oocyte (see also Fig. 4). DNA in all panels was counterstained with DAPI (blue). Magnifications: **A** = 250×; **B** = 200×; **C** = 500×; **D** = 750×; **E** = 1,000×; **F** = 300×; **G** = 150×; **H** = 1,200×; **I** = 600×; **I′,J,K** = 750×; **L** = 600×; **M,N** = 1,500×.

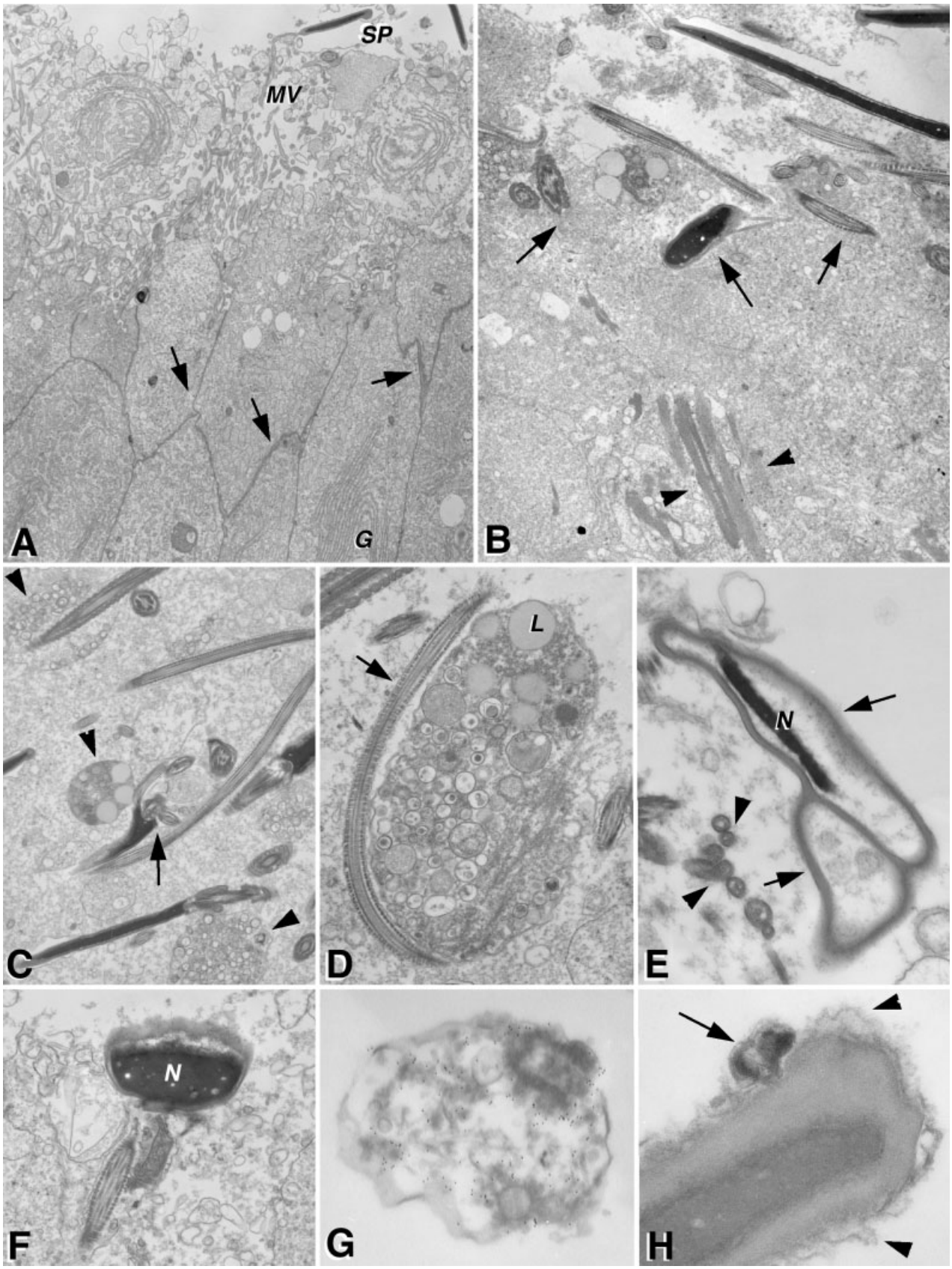

Fig. 2.

exposed by mitochondrial membrane rupture (Martinou et al., 1999) in the apoptotic sperm and rendered sensitive to further ubiquitination in the epididymis. The apoptotic Fas ligand localizes to the mitochondria of sperm from infertile men (Fig. 1K) and the TUNEL assay-detected DNA fragmentation coincides with the increased surface-ubiquitination in the epididymal and ejaculated sperm (Fig. 1J). An intriguing hypothesis has been proposed by Untergasser et al. (2001), suggesting that factors in seminal plasma may induce apoptosis in sensitized cells via the changes in mitochondrial potential and Bax/Blc-2 imbalance. While this mechanism would primarily target epithelial cells of the prostate and deferent ductuli, it could also induce apoptosis in the defective sperm. Overexpression of the ubiquitin-like protein FAT10 induced apoptosis in mouse fibroblasts (Raasi et al., 2001).

Epididymal protein secretion occurs by the way of the apocrine pathway, wherein secretory vesicles are detached from the apical surface of the epididymal epithelial cells (EEC) and sloughed into the lumen of epididymal tubules (Agrawal et al., 1988; Fouchecort et al., 2000; Yanagimachi et al., 1985). Accumulation of ubiquitin is seen in the microvilli, surface protrusions, and vesicles in bulls (Sutovsky et al., 2001b), men, rhesus monkeys, mice, and horses (Fig. 1F,G; Sutovsky P., and Turner R., unpubl. data). Ubiquitin can be pulse-chased in the cultured EEC and immunoprecipitated from the EEC-conditioned medium (Sutovsky et al., 2001b). Ubiquitin is present in epididymal vesicles and secretory granules that can be isolated from bovine epididymal fluid (Figs. 2F–H, 3F–G). Prostasome-like particles of similar size were isolated from bull epididymal fluid and shown to transfer epididymis-secreted protein p25 to the bull sperm plasma membrane (Frenette and Sullivan, 2001). Other major epididymal proteins such as the GPI-anchored sperm plasma membrane protein CD52/HE5 (Yeung et al., 1997b; Perry et al., 1992) and CD55 and CD59 (Kirchhoff and Hale, 1996) are inserted in the sperm plasma membrane during epididymal maturation. These or other epididymal sperm-surface proteins could be targeted for ubiquitination in defective sperm, or inversely, epididymal ubiquitination could prevent the anchorage of such proteins to the defective sperm plasma membrane. Anti-CD52 cross-reactivity is indeed reduced in asthenozoospermic and oligozoospermic patients (Yeung et al., 1997a).

Epididymal ubiquitination may intersect with apoptotic mechanisms operating in the testis (Sinha Hikim and Swerdloff, 1999), which may recognize the structural damage of sperm DNA and/or damaged sperm accessory structures. We found a high correlation between anti-ubiquitin cross-reactivity and TUNEL-labeling of fragmented sperm nuclear DNA, a common apoptotic marker, in bull and human sperm screened by flow cytometry and epifluorescence microscopy (Fig. 1L; Sutovsky et al., 2002). Components of an active apoptotic pathway, active caspase 3, fas-protein, and Fas-ligand (Fas-L), were found in mature sperm of mice (Weil et al., 1998) and men (Fig. 1K; and Sakkas et al., 1999). An elevated percentage of Fas ligand-positive sperm was found in the semen of infertile men (Sakkas et al., 1999). Annexin V, an early apoptotic indicator of the modification of plasma membrane during apoptosis, preferentially binds to the sperm from infertile patients (Oosterhuis et al., 2000). Ruffling and/or complete removal of the plasma membrane are frequently seen in defective epididymal and ejaculated sperm (Barth and Oko, 1989; Sutovsky et al., 2001a) and could make such spermatozoa prone to fusion with epididymal secretory vesicles. Similarly, the permeabilization or rearrangement of the sperm plasma membrane could expose the sperm-anchored ubiquitin conjugating enzyme E2 (Sutovsky et al., 2001a; Fig. 1D) and make it available for ubiquitin conjugation.

In our studies (Sutovsky et al., 2001a), the numbers of ubiquitinated spermatozoa seemed to decline progressively between caput and cauda epididymis. Other recent studies have documented the loss of defective spermatozoa during epididymal passage (Axner et al., 1999; Chenoweth et al., 2000; Ramamohana Rao et al., 1980). With respect to the disposal of the defective spermatozoa by epididymal epithelium (Figs. 2A–H, 3A,B), ubiquitination could facilitate sperm and cytoplasmic droplet degradation by clumping such cells together by its positively charged lysine residues and/or to trigger their internalization by a yet to be investigated membrane-docking event. While sperm phagocytosis in the epididymis is hard to document and has occasionally been disputed, some ultrastructural studies do support it (e.g., Goyal, 1982; Lopez Alvarez and Bustos Obregon, 1995). Alternatively, sperm liquefaction and intraluminal phagocytosis could lower the number of defective spermatozoa during epididymal passage (Barrat and Cohen, 1987; Flickinger, 1982). Solid evidence exists for the phagocytosis of the sperm cytoplasmic droplets by the clear cells of the caput-epididymal epithelium (Hermo et al., 1988; Temple-Smith, 1984). The defective spermatozoa are often clumped together in the epididymis and in the ejaculate (Sutovsky P., unpubl. obs.) and the preferential binding of the ubiquitin-coated microspheres (as opposed to the uncoated, or albumin-coated ones) to the surface of cultured epididymal cells was shown in bull EEC cultures (Sutovsky et al., 2001a; Fig. 3C–E). Other proteins such as clusterin (Ibrahim et al., 2000) and HEP64 (NagDas et al., 2000) are associated with

Fig. 2. Electron micrographs of the breakdown of defective spermatozoa in the bovine epididymis. **A:** Sperm (SP) are bound to surface stereocillia/microvilli (MV) in caput epididymis. Note the junctional complexes (arrows) separating the baso-lateral and apical cell compartments. **B:** Sperm heads and tails (arrows) intermingling with the apical cytoplasm of epididymal epithelial cells. Rootlets of the stereocillia (arrowheads) are seen in the apical cytoplasm. **C:** A defective epididymal spermatozoon (arrow) and the sloughed cytoplasmic droplets (arrowheads) in the lumen of a caput-epididymal tubule. **D:** A sperm tail with residual cytoplasmic droplet containing prominent lysosomes (L). **E:** Cauda-epididymal spermatozoon with a detaching perinuclear theca (arrow) and disintegrating mitochondrial sheath (arrowheads). **F:** A defective spermatozoon with deformed nucleus (N), engulfed by the cytoplasm of an epithelial cell in the cauda epididymis. **G:** Colloidal gold labeling of ubiquitin in a residual cytoplasmic droplet. The residual droplets are shed by the sperm and phagocytosed in the caput epididymis (Hermo et al., 1988) and appear to have a high content of ubiquitin. **H:** Binding of a prostasome-like particle to the ruffled plasma membrane of a defective epididymal spermatozoon. Epididymal secretory proteins are believed to be transferred from the epididymal epithelial cells to sperm in similar particles. Magnifications: **A** = 3,000×; **B** = 6,500×; **C** = 4,500×; **D** = 8,000×; **E** = 10,000×; **F** = 8,000×; **G** = 12,500×; **H** = 35,000×.

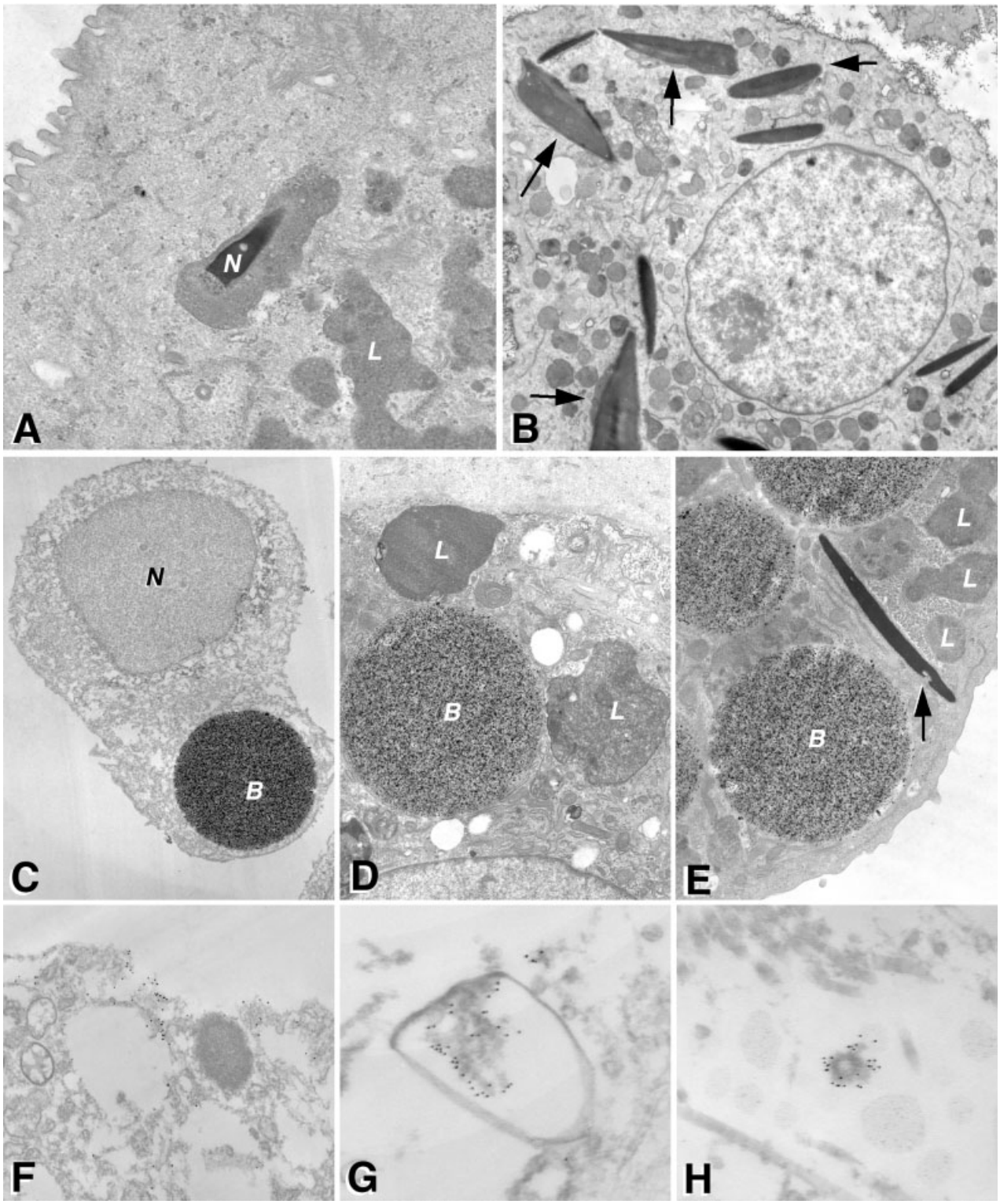

Fig. 3. Phagocytosis of the rhesus monkey sperm (**A,B**), and of the ubiquitin-coated magnetic microspheres (Dynabeads; **C–E**) by the in vitro-cultured bovine epididymal epithelial cells and the immunogold labeling of ubiquitin in such cultured cells (**F–G**). **A:** A rhesus sperm nucleus (N), completely engulfed by a large lysosome in the cytoplasm of a bovine epididymal cell. **B:** Multiple phagocytosed sperm (arrows) are seen in certain cell, presumed to be the clear cells, within epididymal epithelial plaques in vitro. **C:** A ubiquitin-coated Dynabead (B) occupies most of the cytoplasm of a cultured epididymal cell (N = nucleus). **D,E:** Lysosomes (L) and phagocytosed [bull] spermatozoon with a nuclear vacuole (arrow) in the cytoplasm of a cultured bovine epididymal epithelial cell. Coating of Dynabeads with purified bovine erythrocyte ubiquitin accelerated their binding to cultured epididymal cells in our previous studies (Sutovsky et al., 2001a). **F–H:** Accumulation of ubiquitin, as shown by colloidal gold labeling in the cortex of a cultured epididymal cell (**F**) and in the secretory vesicles (**G**) and granules (**H**) formed in vitro. Magnifications: **A,B** = 5,000×; **C** = 2,000×; **D,E** = 7,000×; **F** = 15,000×; **G,H** = 25,000×.

clumps of defective sperm and could be involved in their removal by the epididymal epithelium. It is of interest that the latter protein, HEP64, is only secreted by the lower epididymal compartments, corpus and cauda epididymis, in the hamster (NagDas et al., 2001), while ubiquitin is also produced by the caput epididymis (Sutovsky et al., 2001a). SpanX, a human testicular sperm protein (Westbrook et al., 2001), shows affinity for the nuclear vacuoles, considered to be a sperm head defect. Subunits of the 26 S proteasome (Bialy et al., 2001) and ubiquitin (Sutovsky P., unpubl. data) were also detected in the nuclear vacuoles of defective human sperm. Similar to the proteolysis of internalized plasma membrane receptors (Strous and Govers, 1999), the destruction of the defective sperm may occur by means of lysosomal proteolysis (Fig. 3A,D,E). Ubiquitin could be cleaved off from the phagocytosed sperms' surface by the ubiquitin C-terminal hydrolase, protein gene product, 9.5, abundant in epididymal tissue (Fraile et al., 1996; Santamaria et al., 1993).

Besides apoptotic signals, the pathways leading to the ubiquitination of defective spermatozoa could also recognize the misfolding or denaturation of sperm surface antigens. A specific motif within the amino acid sequence of the N-terminal domain, called the N-end rule, determines the half-life of most proteins and is subject to ubiquitination when the tertiary structure of such proteins is altered by the unveiling of their hydrophobic surfaces (Varshavsky, 1997). Ubiquitination is involved in a number of endocytotic events, including the endocytosis of membrane receptors and plasma membrane-anchored transporters (reviewed by Hershko and Ciechanover, 1998; Strous and Govers, 1999). While the concept of extracellular, sperm-surface ubiquitination may appear surprising at first, there may be other instances when ubiquitin is secreted in the extracellular space, exemplified by the presence of ubiquitin in the ovarian-follicular fluid (Einspanier et al., 1993).

FERTILIZATION, MITOCHONDRIAL INHERITANCE, AND EARLY EMBRYONIC DEVELOPMENT: A SHAKESPEARIAN TALE?

The consensus view prevails that the inheritance of mitochondria and mtDNA in mammals is predominantly maternal (Hutchinson et al., 1974; Giles et al., 1980). Sperm mitochondria, carrying paternal mtDNA, are destroyed in the egg cytoplasm shortly after fertilization (Fig. 4A–D; Sutovsky et al., 1996b). The reasons for enforcing maternal inheritance of mtDNA could be evolutionary (selection for fittest mtDNA genomes by a genetic bottleneck may operate during oogenesis but perhaps not in spermatogenesis) or developmental (an advantage of eliminating the mutation-prone sperm mtDNAs; reviewed by Ankel-Simon and Cummins, 1996). Ubiquitination of the sperm mitochondria after fertilization (Fig. 1M; Sutovsky et al., 1999) provides a feasible explanation for how the sperm mitochondria are targeted for degradation, while egg mitochondria are spared (Scheffler, 2001). The ubiquitin tag is acquired by the sperm mitochondria during spermatid differentiation in the testis and masked during disulfide-bond cross-linking of the sperm mitochondrial sheath in the epididymis. In immunocytochemical analysis of bull sperm, such hardening renders sperm mitochondrial ubiquitin undetectable to anti-ubiquitin antibodies, unless permeabilization or S-S reduction are included in the protocol (Sutovsky et al., 1999b, 2000a). After fertilization, the exposure of ubiquitin in the sperm mitochondria probably requires the relief of S-S cross-linking by the oocyte-produced reducing peptide, glutathione (GSH; Perreault et al., 1984; Sutovsky and Schatten, 1997) and glutathione synergists present in oocyte cytoplasm and/or in sperm (e.g., sperm thioredoxins; Miranda Vizuete et al., 2001). The exposed ubiquitin tag alone could be a sufficient signal for the degradation of the sperm mitochondria inside the egg cytoplasm, which can be prevented by the microinjection of anti-ubiquitin antibodies or by the treatment of fertilized eggs with lysosomal (Sutovsky et al., 2000a) or proteasomal (Sutovsky P., McCauley T.C., in prep.) inhibitors. Those findings inspired a comparison to Rosenkrantz and Guildenstern, Hamlet's ill-fated heroes delivering their own death sentence to the rulers of England (Travis, 2000). Prohibitin is a possible candidate for the ubiquitinated sperm mitochondrial substrate (Sutovsky et al., 2000a,b). Such a mechanism of mitochondrial inheritance may not be reserved to mammals: Prohibitin-family members were implicated in the control of mitochondrial inheritance in budding yeast (Berger et al., 1998) and the substitution of ubiquitin's Lys-63 residuum by arginine derailed the redistribution of mitochondria in the newly formed buds (Fisk and Yaffe, 1999).

A great deal can be learned about mitochondrial degradation, and perhaps extrapolated to fertilization, from the studies of organelle degradation by macroautophagy (reviewed by Klionsky and Emr, 2000). In the differentiating red blood cells, reticulocytes, all organelles, including the mitochondria, undergo autophagy. In addition to ubiquitin (Rappoport et al., 1985), a mitochondrial membrane-permeabilizing enzyme, 15-lipoxygenase (15-LOX), has been implicated in the degradation of reticulocyte mitochondria (van Leyen et al., 1998). The changes of the sperm mitochondria inside oocyte cytoplasm, i.e., swelling and loss of mitochondrial cristae (Fig. 4A–C), are reminiscent of macroautophagy, and our new data indeed show the presence of a protein cross-reactive with antibodies against 15-LOX and a related gene product, 12-LOX, in the oocyte cytoplasm (Sutovsky and van Leyen, unpubl. data; Fig. 1N).

Other events during fertilization may rely on proteolysis. Proteolysis may be necessary for the release of the sperm-borne, oocyte-activating factor (SOAF) during sperm head perinuclear theca removal at fertilization (Sutovsky et al., 1997). The dissolution of the sperm perinuclear theca in the oocyte cytoplasm can be inhibited by serine protease inhibitors (Perry et al., 2000), although this does not necessarily imply the participation of a ubiquitin system. The sperm centriole is released at fertilization from the compact mass of the sperm tail capitulum, which is restrained in the elaborate structure of the sperm tail connecting piece (Sutovsky et al., 1996a). Ubiquitin (Fig. 1L) and the 26 S proteasomal-subunits localize to the centriolar region of the sperm tail (Wojcik et al., 2000) and to the other parts of the sperm tail midpiece (Mochida et al., 2000), and may be instrumental in this process. Fol-

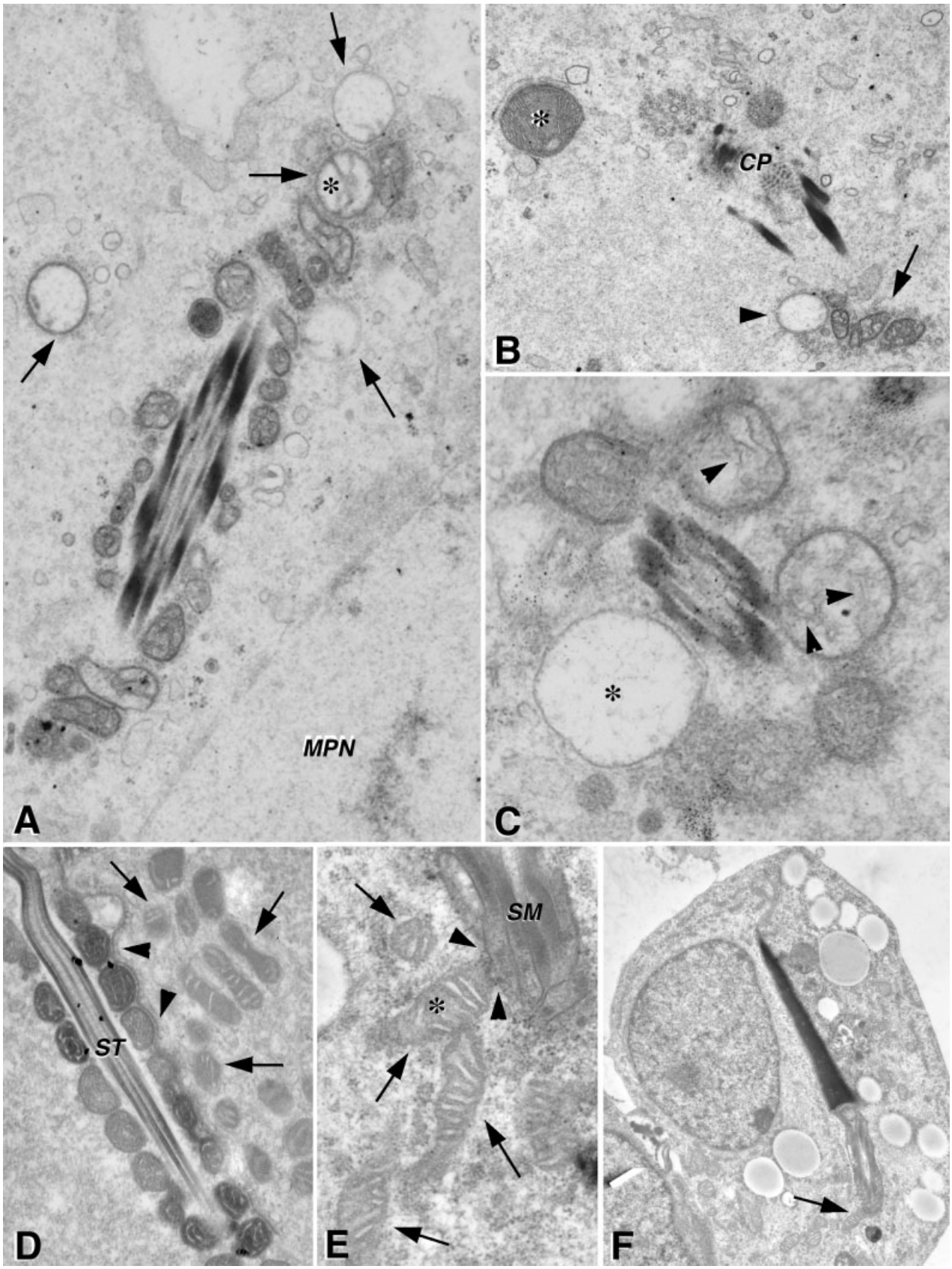

Fig. 4.

lowing the sperm aster formation, S-phase, and pronuclear apposition, the first embryonic mitosis is controlled by the ubiquitin-dependent degradation of cyclin B, a recurring component of the maturation promoting factor (Josefsberg et al., 2001). Accumulation of ubiquitin is also seen in the midbody of the second meiotic spindle in fertilized eggs and in the embryonic metaphase spindles (Sutovsky et al., 1999b, 2000a). The role of ubiquitination in fertilization and maternal–embryonic transition is further supported by the finding that ubiquitin-like proteins, ubiquitin ligases, and ubiquitin hydrolases are highly expressed in the mammalian zygote (Hwang et al., 2001).

Finally, new evidence that is just emerging implicates the proteasomal route for the degradation of ubiquitinated substrates in the process of acrosomal exocytosis and zona penetration.

Sawada et al. (2002a) showed that acrosomal exudate of the ascidian spermatozoa contains both ubiquitin and proteasomes and causes sequential ubiquitination and proteasomal degradation of the putative sperm receptor protein HrVC70 on the egg vitelline envelope. Consequently, these authors were able to block fertilization with anti-proteasomal antibodies. Further work shows that proteasomal inhibitors, small peptides binding to proteasome's catalytic subunit, block fertilization not only in ascidians (Sawada et al., 2002b) and sea urchins (Matsumara and Aketa, 1991), but also in mouse (Wang et al., 2002) and pig, where ubiquitin-cross-reactive substrates can be detected on the outer face of the zona pelluciada in both follicular and ovulated oocytes (Sutovsky, McCauley, and Day, in prep.). Proteasomal subunits were detected in the human (Bialy et al., 2001; Wojcik et al., 2000), ascidian (Sawada et al., 2002b), pig (Sutovsky, McCauley, and Day, in prep.), and sea urchin (Matsumara and Aketa, 1991) sperm. Component 2 and zeta chain of the proteasome are recognized by anti-sperm antibodies in patients diagnosed with autoimmune infertility (Bohring et al., 2001). These discoveries may completely change the current understanding of acrosome reaction and zona penetration, possibly providing new means for contraception in humans and management of polyspermy during in vitro fertilization in farm animals.

CLINICAL CONSIDERATIONS: INFERTILITY DIAGNOSTICS, VASECTOMY, CONTRACEPTION, IMMUNOSTERILITY, ART, AND HETEROPLASMY

Sperm morphology analysis by light microscopy provides useful, but not necessarily complete, information on sperm quality (Amann, 1989) and novel, objective markers of semen quality are sought to achieve a more accurate diagnostics of male infertility. Proteins with high affinity to defective sperm (e.g., annexin V, p64, Fas-ligand; see above) can be used as "negative" markers in infertility diagnostics. Other, "positive" markers such as p34H (Boue and Sullivan, 1996) and SP22 (Welch et al., 1998) may reveal some types of male infertility by the virtue of being absent from defective spermatozoa. Ubiquitin, however, may be a universal marker of sperm and semen abnormalities, as it recognizes an array of semen abnormalities including all variations of sperm head and tail defects, but also leukocytes, spermatids, cellular debris, and residual bodies present in the ejaculates of infertile men (Fig. 1I,I′; Sutovsky et al., 2001a). High ubiquitin levels are seen in the sperm of men suffering from some of the well-defined, presumably heritable spermatogenic defects. These include the dysplasia of the fibrous sheath, DFS, or stomp tail syndrome, and globozoospermia or round-headed sperm syndrome (Sutovsky et al., 2001b; Rawe et al., 2002; and unpubl. data). Similar to human globozoospermia, attributable to a heritable mutation of casein kinase II alpha gene (Xu et al., 1999), the males of *azh* mutant mice are infertile due to sperm head malformations (Meistrich et al., 1994) and display an increased ubiquitination by immunofluorescence. A shift in the MW of major ubiquitin-cross-reactive bands by Western blotting is also seen in *azh* mutants (Sutovsky and Moreno, unpubl. data).

Anti-sperm antibodies are produced by both men and women (reviewed by Bronson, 1999) and ubiquitin as a major surface antigen of defective spermatozoa could contribute to such immune response. Most anti-sperm antibodies recognize multiple bands in Western blots, making it difficult to determine which sperm antigens triggered the immune response. Such a pattern is consistent with multiple isoforms of polyubiquitinated substrates and a comparison of autoimmune sera with well-characterized anti-ubiquitin antibodies could yield some intriguing results. It is of interest to note high titers of anti-sperm immunoglobulins in patients with congenital blockage/absence of deferent ductuli and in vasectomized men (Primakoff et al., 1990), both conditions accompanied by the accumulation of defective sperm in the epididymis. In such men, the ubiquitination of defective spermatozoa could occur concomitantly with their accumulation resulting from the saturation of the proteolytic capacity of epididymal ep-

Fig. 4. Fate of the sperm mitochondria following in vitro fertilization of bovine oocytes. **A:** Disintegrating sperm mitochondrial sheath remains in the vicinity of the male pronucleus (MPN). Some of the sperm mitochondria became detached from the mitochondrial sheath and appear to be transformed into autophagosomal vacuole-like structures (arrows), one of them still containing residual cristae (asterisk). **B:** Sperm tail connecting piece (CP) with sperm centriole, detached sperm mitochondria (arrow), autophagosomal vacuole (arrowhead), and an intact oocyte mitochondrion (asterisk) with normal cristae at the pronuclear stage of the zygotic development. **C:** A cross-section of sperm tail midpiece with sperm mitochondria at various stages of swelling. Arrowheads point to the disintegrating mitochondrial cristae, asterisk shows a sperm mitochondrion that resembles an autophagosomal vacuole. **D:** Close contact between sperm tail (ST) mitochondria and oocyte mitochondria (arrows) after intracytoplasmic sperm injection in the rhesus monkey oocyte. Arrowheads point to the residual sperm plasma membrane, which could cause a delayed recognition and degradation of sperm mitochondria after this assisted reproductive procedure. **E,F:** Can sperm mitochondria fuse with other cells' mitochondria and eventually support homologous recombination of their respective mtDNAs? Fusion (arrowheads in **E**) between a sperm mitochondrion (SM) and a cumulus cell mitochondrion (asterisk in **E**; arrow in **F**), outlined by arrowheads in (**E**) has been observed when a bull spermatozoon penetrated a cell of bovine oocyte's cumulus oophorus. Cumulus cell mitochondria (arrows) display a distinct morphology with thick mitochondrial cristae. Some authors maintain that the leakage of paternal mtDNA and its recombination with oocyte-derived mtDNA occurs on occasion in the human and primate populations (discussed by Eyre-Walker, 2000). Magnifications: **A** = 20,000×; **B** = 15,000×; **C** = 25,000×; **D** = 12,000×; **E** = 30,000×; **F** = 5,000×.

ithelium. Even in fertile males, a substantial portion of ubiquitinated sperm is not removed during epididymal passage and can be found in the ejaculate (Sutovsky et al., 2001a,b). ICSI can efficiently treat such cases (Check et al., 2000), supporting the view that they are mainly caused by sperm surface antigen-induced antibodies (Shetty et al., 1999).

What is the significance of sperm ubiquitination in the epididymis? Besides clustering and removal of defective sperm (epididymal sperm quality control; Sutovsky et al., 2001b), it could serve to immobilize defective sperm and prevent them from competing for an egg. Once in the female genital tract, these spermatozoa could be targeted by resident leukocytes and trigger an immune response, preventing them from fertilizing an egg. If this is true, ubiquitin could provide an excellent target for either male or female immunocontraceptive. Is the increased output of defective sperm in infertile individuals compensated for by an overall increase in sperm production? In our studies, we found a slight, but statistically significant, increase in total sperm count in some men with increased sperm ubiquitin medians (Sutovsky et al., 2001a). A carefully controlled study comparing folate levels in seminal plasma of smokers and nonsmokers also reported higher sperm counts in the smokers (Wallock et al., 2001), whose fertility is presumably diminished. While the ubiquitination of defective sperm occurs mainly in the epididymis, the accumulation of ubiquitin in the testicular cells may also be indicative of pathological process. This is often seen in patients who underwent cancer treatments by chemotherapy or radiation (Fig. 1B) and in the age-related testicular tissue necrosis (not shown). This is perhaps a parallel to the accumulation of ubiquitin in the amyloid plaques in Alzheimer's disease (Cochran et al., 1991) and during apoptosis.

New assisted reproductive technologies (ART), including intracytoplasmic sperm injection (ICSI), round spermatid injection (ROSI), and oocyte cytoplasm donation/ooplasmic transplantation, were introduced in recent years to overcome infertility. Bypassing the sperm demembranation and sperm-oocyte fusion steps of natural fertilization, ICSI may cause delayed elimination of sperm accessory structures and the persistence of an intact plasma membrane on the surface of spermatozoa microinjected directly into oocyte cytoplasm (Sutovsky et al., 1996b). Such delay could hinder the degradation of the sperm mitochondria and facilitate the persistence of the sperm mtDNA, heteroplasmy, in the embryonic cytoplasm. So far, the completed studies of human ICSI embryos ruled out heteroplasmy (Danan et al., 1999; Houshmand et al., 1997). However, oocytes deficient in their ability to reduce/unmask the sperm mitochondrial capsule could be generated from superficially stimulated cycles and subsequently fail to expose the ubiquitin tag in the sperm mitochondrial membranes. If there is a specific window of opportunity for the degradation of sperm mitochondria after fertilization, this could be missed in some eggs compromised by ICSI and/or by in vitro maturation/culture. The paternal, sperm-derived mtDNA was indeed detected in the abnormal embryos conceived by conventional in vitro fertilization (St John et al., 1999). When somatic cell or other oocytes' mitochondria are introduced in the egg cytoplasm by microinjection (Shitara et al., 2000), cloning (e.g., Hiendleder et al., 1999) or ooplasmic transplantation (Barritt et al., 2001), a variable degree of heteroplasmy is observed in the resultant hybrid cells, or offspring, suggesting that only the sperm mitochondria are targeted for proteolysis.

EVOLUTIONARY CONSIDERATIONS: REPRODUCTIVE SEASONALITY, SPERM COMPETITION, mtDNA RECOMBINATION, AND SPECIES SPECIFICITY OF MITOCHONDRIAL INHERITANCE

In general, the sperm production is lowered only by a certain percentage in seasonally breeding animals such as horses and hamsters, while the fertilizing ability of such sperm is diminished almost completely (reviewed by Goodman, 1998). While the hormonal mechanism of daylight period-dependent male reproductive cycle is relatively well-defined along the hypothalamo-gonadal axis, the effects of sperm "hibernation" in the testis and epididymis are poorly understood. Reproductive seasonality could be executed by the ubiquitination of all sperm in the nonbreeding season. Our new data show the accumulation of ubiquitinated sperm in the epididymis of stallions probed during the nonreproductive season (Fig. 1H; Sutovsky and Turner, unpubl.).

Sperm competition, recently revisited by Birkhead (2000) in a delightfully entertaining and educational treatise, was believed to occur in female genital tract where "killer" and "blocker" sperm with coiled tails supposedly eradicate competing motile sperm (Baker and Bellis, 1988). Most of such "blockers" may in fact be the ubiquitinated, defective, and immotile sperm, in which the coiled tails are a common defect (Sutovsky et al., 2001b). Two decades prior to killer sperm hypothesis, the coiled tail, or "Dag defect," was described and named after a bull that suffered from this type of infertility (Bloom, 1966). After initial enthusiasm, the killer sperm hypothesis was dismissed by many andrologists, although it seems to persist in urban folklore. On the other hand, one could speculate that the "sticky" lysine-rich ubiquitin on the surface of defective sperm could bind motile sperm or trigger anti-sperm antibodies in the cervical fluid. Could the supernumerary, ubiquitinated, defective sperm of infertile patients agglutinate the remaining motile spermatozoa? Or, inversely, could the surface ubiquitination actually prevent such an effect and act as a natural defense against autoimmune infertility?

The carryover of paternal, sperm-derived mtDNA has been shown in interspecies hybrids of laboratory mice *Mus musculus* and a wild mouse, *Mus spretus* (Kaneda et al., 1995). Intact sperm mitochondrial sheaths with no signs of ubiquitin-cross-reactivity were detected in the hybrid, 8–16-cell embryos generated by the fertilization of domestic cow oocytes with the sperm of wild bull, gaur *(Bos gaurus;* Sutovsky et al., 2000a). If ubiquitination is indeed involved in the recognition and elimination of sperm mitochondria by oocyte cytoplasm, the species specificity of such a mechanism could be promoted by the interspecies differences in the order and pattern of sperm mitochondrial ubiquitination. This could explain how a highly conservative amino acid sequence of ubiquitin could be recognized by the lysosomal/proteasomal docking sites in the in-

traspecific, but not in the interspecific, crosses. Backcrossing, which seems to eliminate paternal mtDNA in the subsequent generations (Shitara et al., 1998), could then eliminate the conflict between mitochondrial genome encoding the foreign, paternal mtDNA, and the nuclear genome of the recipient oocyte, encoding the ubiquitination-prone mitochondrial membrane proteins. As discussed above, the interspecies differences in the amino acid sequence of participating ubiquitin ligases and carriers could contribute to this effect.

While it can be agreed that the sperm mitochondria are targeted for destruction after fertilization (Fig. 4A–D), opinions may vary as to whether the same holds true for sperm mtDNA. Some population studies defend the possibility of paternal–maternal mtDNA recombination after sperm mtDNA leakage (Awadalla et al., 1999; Eyre-Walker et al., 1999; Hagelberg, 1999a; Zhao et al., 2001) and it is yet to be demonstrated that the degradation of sperm mitochondrial proteins is parallel to the hydrolysis of sperm mtDNA. It should be considered that the sperm mitochondria can survive inside other cells (Manfredi et al., 1997). The mtDNA repair mechanism, no matter how simple, could in theory allow for recombination (Eyre-Walker, 2000), and some of the testicular ubiquitin-conjugating enzymes may also participate in DNA repair (Baarends et al., 2000). Our own observations also show that sperm mitochondria can occasionally fuse with the mitochondria of other cell types (Fig. 4E,F). On the other hand, there is little evidence that homologous recombination could occur between paternal and maternal mtDNAs (reviewed by Howell, 1997). At least one of the above studies (Hagelberg et al., 1999a), referring to a possible recombination event in human populations, was withdrawn (Hagelberg et al., 1999b), and others remain unsupported by a recent survey of the complete human mtDNA sequence (Ingman et al., 2000; Herrnstadt et al., 2002). It is of interest to note that the timing of sperm mitochondrion destruction in the mouse (one cell; Kaneda et al., 1995) and cattle (two to four cell; Sutovsky et al., 1996) roughly coincides with the maternal–embryonic transition of transcriptional control in these species (Memili and First, 1999; Schultz et al., 1999).

In summary, ubiquitin-dependent proteolysis plays a proven role in sperm cell differentiation inside the testicular seminiferous tubules and in the cell cycle control throughout spermatogenesis, oogenesis, fertilization, and embryonic development. In addition, ubiquitin emerges as a key player in epididymal sperm quality control and a regulator of mitochondrial inheritance. Further research on the respective roles of ubiquitination in the gametogenesis and fertilization may lead to advances in infertility diagnostics and treatment, immunocontraception, amelioration of farm animal reproduction, and animal cloning. This can be achieved by exploiting the ubiquitin-system to identify diagnostic markers, contraceptive targets, and means of controlling mitochondrial inheritance in the reconstructed, hybrid embryos. Stemming for the involvement of ubiquitin-proteasome pathway in the penetration of zona pellucida by the spermatozoon, new contraceptive targets and means for controlling polyspermia can be expected.

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NOTE ADDED IN PROOF

While this article was in press, an article documenting ubiquitin secretion in the rat epididymis was published (Hermo and Jacks. 2002. Mol Reprod Dev 63: 394–410). Transmission of paternal mtDNA, but not the mtDNA recombination, was documented in a male mitochondrial myopathy patient carrying a de novo deletion in the mitochondrial ND2 gene (Schwartz and Vissing. 2002. N Engl J Med 22:576–580). Seasonal sperm ubiquitination was documented in stallion (Sutovsky et al., Biol Reprod 68(2):688–698), and the contribution of the proteasomal pathway to the degradation of the paternal mitochondria was shown in the porcine zygote (Sutovsky et al., 2003. Biol Reprod 68(3): In press).

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