Chapter II

Stage-specific enhanced expression of mitochondrial fusion and fission factors during spermatogenesis in rat testis
The mitochondrial fusion factors mitofusins 1 and 2 (Mfn1 and Mfn2) and the fission factor dynamin-related protein 1 (Drp1) were found to be highly expressed in the pubertal and adult rat testis by Northern blot analysis. Immunohistochemistry using specific antisera to Mfn2 and Drp1 revealed a pronounced expression of the fusion and fission factors in the round and elongating spermatids in the seminiferous tubules, suggesting that at precise steps of spermiogenesis (i.e. steps 8–12), spermatid mitochondria are rapidly homogenized by frequent fusion and division. Although physiological relevance of this phenomenon remains to be clarified, a role is proposed for it as an effective means of achieving complete and homogeneous ubiquitination of mitochondria, which has recently been demonstrated to be a mechanism for the elimination of paternal mitochondria during fertilization, based on the fact that the timing of expression of Mfn2 and Drp1 coincides well with that reported for a spermatid-specific ubiquitin-conjugating enzyme.
INTRODUCTION

Mitochondria are multitasking organelles essential for normal eukaryotic cell function. They exist in multiple copies and play central roles not only in the oxidative energy metabolism by producing most of the cell’s energy currency ATP (39) but also in apoptosis by orchestrating death signals triggered from both inside and outside the cell (40, 41). Mitochondria are organelles bounded by two membranes. Despite this structural complexity, they display an amazing plasticity of morphology and distribution. Their number, shape, and distribution are altered to meet the specialized energy requirements of a cell during cellular growth and differentiation or in response to external stimuli. For example, in muscle fibers, mitochondria are organized into regular columns stacked between myofibrils (42), and in many other cell types, a dynamic tubular network of mitochondrial membranes is seen with projections that constantly move, break, and reseal (43, 44). Through this frequent fusion and fission, mitochondria exchange their components including mitochondrial DNA and matrix proteins and maintain their homogeneity as a virtually single dynamic unit (4, 9--11). The molecular mechanisms underlying the mitochondrial fission and fusion processes are just now beginning to be uncovered by genetic and biochemical approaches (12--18).

Recently, Hales and Fuller (3) have identified a molecule mediating mitochondrial fusion through the analysis of a *Drosophila* mutant defective in sperm development. The molecule, termed Fzo, is a mitochondrial outer membrane protein with both its large N-terminal GTPase domain and short C-terminal ends exposed to the cytoplasm (21--23). Further insights into the function of Fzo have emerged from studies on the yeast homologue Fzo1p (19--25) and mammalian homologues mitofusins 1 and 2 (Mfn1 and Mfn2) (1, 2, 27--31). Loss of Fzo1p function causes the mitochondrial network to rapidly fragment and no fusion of mitochondria occurs when this protein is defective or absent. Fzo1p has been suggested to be a component of multiprotein fusion machinery composed of at least two other proteins, Mgm1p and Ugo1p (22--25), and its levels appear to be regulated by Mdm30, an F-box-containing ubiquitin ligase-like protein (26). An essentially identical functional role has been
Mitochondrial fusion has been shown to be essential for embryonic development by gene targeting. Mice deficient in either Mfn1 or Mfn2 died in midgestation and the embryonic fibroblasts established from the knockout mice displayed fragmented mitochondria (2).

Molecules required for mitochondrial fission have also been identified in yeast and mammalian cells and shown to act antagonistically with the fusion factors mentioned above (1, 19, 20, 45--48). One of the key components of the fission machinery is a novel dynamin-related protein (Drp1) that was originally thought to function in membrane traffic but later shown to control mitochondrial fission without affecting vesicular trafficking and to assemble on the outer mitochondrial membrane at sites that mark actual mitochondrial division events (49--51). Drp1 is therefore considered to wrap around the constriction points of dividing mitochondria like dynamin assembling into collars around the base of clathrin-coated pits. Drp1 is also responsible for apoptotic fragmentation of mitochondria (5, 52).

Sperm mitochondria are unique in being selectively tagged for extinction. When microinjected into eggs, mitochondria from sperm are completely eliminated whereas those from liver cells are not (53, 54), suggesting a mechanism that selectively recognizes and eliminates sperm mitochondria, which ensures strictly maternal inheritance of mitochondria and mitochondrial DNA in mammals. Recently, Sutovsky et al. (55--57) have shown that prohibitin, a 30–32-kDa membrane protein of sperm mitochondria is ubiquitinated during spermatogenesis and suggested that the ubiquitination may represent a mechanism for the elimination of paternal mitochondria during fertilization. Consistent with this observation, a ubiquitin-conjugating enzyme whose expression is restricted to round and elongating spermatids (steps 8--11) has been identified (55, 58, 59). In addition to the high level expression of enzymes for ubiquitination, the following mechanism appears to be necessary for ensuring an appropriate level of ubiquitination of individual mitochondria: a mechanism that stimulates fusion and fission of mitochondria, and thereby assures homogeneous ubiquitination by mixing their components. Here I report that the latter mechanism is operating by showing stage-specific marked enhancement in expression levels of the fusion and fission factors, Mfn2 and Drp1, respectively, in rat spermatids.
While trying to identify, by differential display, the genes whose products are involved in the fusion and formation of multinucleated osteoclasts, I identified a clone that was highly expressed in differentiated osteoclast-like cells (OCLs) but only at trace levels in precursor cells. Sequence analysis revealed that the clone represents a mouse homologue of *Drosophila* Fzo (3), yeast Fzo1 (21, 22), and human Mfn2 (27). Northern blot analysis using RNA preparations from various rat tissues indicated that Mfn2 is most highly expressed in the testis and its mRNA levels could easily be detected using total RNA preparations. I therefore decided to determine its cellular location by immunohistochemistry, which revealed developmental stage-specific and greatly enhanced expression of Mfn2 in spermatids suggesting, as mentioned above, the necessity of intensive homogenization of mitochondria at a certain stage of spermatogenesis, and provided supporting evidence for this suggestion by analyzing the expression pattern of the counterpart of Mfn2, i.e. Drp1.
RESULTS

Tissue distribution of mitofusins

Tissue distributions of Mfn1 and Mfn2 were determined using total RNA preparations from rat tissues by Northern analysis (Fig. 11, A and B). Relatively high levels of the Mfn1 and Mfn2 messages were found in the testis; moderate levels in the heart, brain, and kidney; and low but significant levels in the other tissues examined. The size of Mfn2 mRNA (~5 kb) is slightly larger than that of Mfn1 mRNA (~4 kb). This difference is consistent with the difference in the sizes of their translation products (757 versus 703 amino acid residues). Intensities of the Mfn1 and Mfn2 signals were almost identical indicating that both mitofusins are expressed in an approximately equal ratio in the rat testis.

Age-dependent expression of Mfn1 and Mfn2 mRNA in rat testis but not in epididymis

I extended Northern analysis to RNA preparations of testis from rats of different ages and to that of epididymis. The Mfn1 and Mfn2 transcript levels were found to be markedly elevated in testis of pubertal (4 week-old) and adult (7 week-old) rats, suggesting that pronounced expression of both mitofusins occurs at the onset of puberty (Fig. 12, A and B). In the epididymis of adult rats, however, only very weak signals were detected (Fig. 12, A and B). The low level expression in the epididymis that stores sperm indicates that the enhanced expression of mitofusins occurs either in sperm transiently at particular stages of their development or in other cells including the Sertoli cell and Leydig cell. As shown later, immunohistochemistry revealed that the former is the case.

Stage-specific enhanced expression of mitofusins in developing spermatids revealed by immunohistochemistry

As mentioned above, strong hybridization signals of mitofusins were detected in the testis by Northern blot analysis without the necessity of enriching poly(A)$^+$ RNA, suggesting high level expression of mitofusins and hence the potential usefulness of the
FIG. 11. Tissue distribution of rat Mfn1 (A), Mfn2 (B), and Drp1 (C). Blots with 20 μg of total RNA from the indicated rat tissues were hybridized with radiolabeled Mfn1, Mfn2, Drp1, and β-actin cDNA probes and processed for autoradiography. In C, testis sample shows two bands of Drp1 mRNA (~3 and ~4 kb), which has been demonstrated to be splice variants that differ only in the 3'-noncoding region (68). The ratio of the short (~3 kb) and long (~4 kb) messages varied considerably among individual rats (Fig. 3C).
FIG. 12. Expression of Mfn1, Mfn2, and Drp1 during testis and epididymis development. Blots with 20 µg of total RNA from rat testis and epididymis at the indicated developmental stages were hybridized with radiolabeled Mfn1, Mfn2, Drp1, and β-actin cDNA probes and processed for autoradiography. As mentioned in Fig. 13 legend, two forms of Drp1 mRNA are present in the rat testis that arise from alternative splicing (68). Their relative abundance varied greatly among the individual rats; in the third lane of panel C is shown a typical example of a rat testis expressing mainly the short form of Drp1 mRNA.

immunohistochemical approach for determining the cell types that express mitofusins. I therefore performed immunohistochemistry using rat testis sections.

I first raised antisera against an N-terminal fragment and a C-terminal fragment of mouse Mfn2, purified them by affinity chromatography, and termed anti-Mfn2-N and anti-Mfn2-C, respectively. The antisera recognized rat Mfn2 as well as mouse Mfn2. Their specificities were confirmed by Western blot analysis using extracts of COS7 cells expressing exogenous mouse Mfn2 (Fig. 13A). Both antisera gave essentially identical results but anti-Mfn2-C was much superior to anti-Mfn2-N in the sensitivity. For staining mitochondria, I used a commercially available monoclonal antibody against cytochrome oxidase subunit I (COX I), a mitochondrial inner membrane protein.

As shown in Fig. 14A, immunostaining of Mfn2 was found inside a subset of the seminiferous tubules. Some seminiferous tubules were not stained at all probably because of the asynchronous nature of their development; it is known that each seminiferous tubule displays particular collections of germ cells and stages of germ cell development and therefore exhibits a distinct staining pattern for the developmental stage-specific antigens depending on the stage of spermatogenesis. Close examination at higher magnification indicated that the staining appeared to be restricted to mitochondria of step 8–12 spermatids in the tubules at stages VIII-XII (Fig. 15; left
FIG. 13. Immunoblot analysis of COS-7 cells transfected with Mfn2 and Drp1. 
A, Total cell lysates from COS-7 cells mock-transfected (left) and transfected with Mfn2-pcDNA3 expression vector (right) were resolved on a 10% polyacrylamide gel. Blots were incubated with purified anti-Mfn2-C antibody and detected with alkaline phosphatase-conjugated secondary antibody. 
B, Total cell lysates from COS-7 cells, mock-transfected (left) and transfected with Drp1-pcDNA3 (right), were resolved on a 10% polyacrylamide gel. Blots were incubated with purified anti-Drp1 antibody and detected with alkaline phosphatase-conjugated secondary antibody.

Mitochondrial staining, as revealed by merged images of the anti-Mfn2-C immunofluorescence (green) and of anti-COX I immunofluorescence (red), is consistent with the fact that Mfn2 is an integral mitochondrial outer membrane protein. Spermatogonia, spermatocytes, Sertoli cells, and intertubular Leydig cells were not labeled above the basal level.

Similar stage-specific enhanced expression of Drp1, a fission GTPase

The above results on mitofusins indicate that highly frequent mitochondrial fusion is essential for the developing round and elongating spermatids. To see whether the fusion alone or a combination of both fusion and fission is necessary at these developmental steps, I cloned the mitochondrial fission factor Drp1 cDNA (accession number AB079133), raised antiserum against its recombinant protein (Fig. 13B), and performed Northern analysis and immunohistochemistry. Drp1 is very similar to
mitofusins in the tissue distribution with testis being the highest (Fig. 11C), in enhanced testicular expression after puberty (Fig. 12C), and in developmental stage-dependent enhanced expression in spermatids (Figs. 14C and 15). The marked enhancement of Drp1 expression, however, seemed to occur slightly later than that of Mfn2 (step 9 versus step 8; Fig. 15; right panels). The Drp1 immunofluorescence was observed as punctate structures associated with mitochondria in the step 9–12 spermatids (Fig. 15, K–N). At late steps of spermiogenesis (steps 13–16), the majority of Drp1 appeared to be present in the residual bodies (Fig. 15, O and P).

**Fig. 14. Immunofluorescence staining of Mfn2 and Drp1 in rat testis.** Sections (5 μm) of adult rat testis were reacted with affinity-purified anti-Mfn2-C antibody (A) and anti-Drp1 antibody (C) and their corresponding preimmune sera (B and D), and visualized with Alexa 488-labeled goat anti-rabbit IgG (green). Mitochondria were stained using mouse monoclonal anti-COX I antibody and TRITC-labeled goat anti-mouse IgG (red). Nuclei were visualized using Hoechst 33342 (blue). Bars, 50 μm.
FIG. 15. Immunofluorescence staining of Mfn2 and Drp1 in the various steps of the rat spermatogenesis. Sections (5 µm) of adult rat testis were reacted with affinity-purified anti-Mfn2-C (A–H), anti-Drp1 (I–P) antibodies and visualized with Alexa 488-labeled goat anti-rabbit IgG (green). Mitochondria were stained using mouse monoclonal anti-COX I antibody and TRITC-labeled goat anti-mouse IgG (red). Nuclei were visualized using Hoechst 33342 (blue). Bars, 10 µm.
DISCUSSION

Mitochondria undergo regulated fusion and division in many cell types during cellular division and differentiation. These events are essential for maintaining the shape and function of mitochondria in coordination with cellular energy demands and for normal mitochondrial inheritance (43). Stimulated by the recent identification of the molecules involved, the molecular mechanisms controlling the mitochondrial fusion and division are rapidly becoming better understood (12--18). The molecules identified include, as fusion factors, *Drosophila* Fzo (3), yeast Fzo1p (21, 22), yeast Ugo1p (37), yeast Mgm1p (60), and mammalian mitofusins Mfn1 and Mfn2 of the outer mitochondrial membrane (27, 29, 31) and OPA1 of the intermembrane space (6--8, 61, 62); and, as fission factors, dynamin-related protein (termed Dnm1p in yeast and Drp1, DLP1, DVLP, or Dymple in mammals) (49--51, 63--68), mitochondrial integral membrane protein Fis1/Mdv2 (46, 48, 69), and its association molecule Mdv1/Fis2/Gag3 (45, 46, 48). Interactions among these molecules that form fusion or fission machinery are currently being studied intensively (17, 24, 25, 69, 70). In relation to these fusion and fission events, the joint research group of Tsukuba and Tokyo has provided, by using mitochondrial transplantation technology (71), direct evidence for the extensive and continuous exchange of mtDNA between mitochondria and suggested that mammalian mitochondria should be considered as a single dynamic cellular unit (4, 9). In the present study, I found unexpectedly high level expression of Mfn2 and Drp1 in the round and elongating spermatids (Fig. 15). This high level coexpression strongly suggests that at precise steps of spermiogenesis, the frequency of spermatid mitochondrial fusion and fission should be greatly increased for certain purposes. One possibility is that such accelerated fusion and fission is a mechanism for achieving homogenous distribution of mtDNA into individual mitochondria, but this seems not to be the case because the inheritance of mtDNA is strictly maternal (72). Another possibility is achieving complete homogenization, among individual mitochondria, of their membrane components that are tagged with ubiquitin for selective extinction of paternal mitochondria. The ubiquitin tagging of sperm mitochondria for selective elimination has recently been demonstrated (55, 56). Furthermore, a testis-specific isoform of the ubiquitin-conjugating enzyme (UBC or E2)
FIG. 16. Schematic presentation of stage-dependent expression of mitochondrial fusion and fission proteins (Mfn2 and Drp1) during spermatogenesis in the rat testis. Spermatid development in the rat testis that is well delineated morphologically into steps is schematically shown to indicate the timing of induction of Mfn2 and Drp1 together with that of ubiquitin-conjugating enzyme (E2). For the physiological significance of the induction (arrow), see the text. Red dots, mitochondria; blue and black area, nucleus.

has been identified and shown to be induced at puberty in the round and elongating haploid spermatids (55, 58, 59); this cell-type specific and developmentally regulated expression pattern is very similar to that of Mfn2 and Drp1 reported here. To attain ubiquitination of all mitochondria over a threshold level within a limited period of the particular stage of spermiogenesis, their homogenization by fusion and division seems to be necessary as a security measures in addition to increasing the E2 enzyme. The fusion and fission factors studied here therefore likely participate in the tagging process by facilitating, for example, homogenous ubiquitination (Fig. 16).

Although mitochondria constantly fuse and divide even in the ordinary somatic cells in culture, the expression levels of the fusion and fission factors are usually relatively low in such cells, making it quite difficult to determine their subcellular localization. To circumvent this difficulty, green fluorescent protein (GFP)-tagged constructs of the fusion and fission factors have been used in most localization studies.
and there are only two reports describing immunohistochemical visualization of endogenous Drp1 in COS7 cells (50) and HeLa cells (52). My observation of the endogenous Drp1 in patches on spermatid mitochondria (e.g. Fig. 15K) may therefore serve to strongly support the mode of interaction of Drp1 (or Dnm1 in yeast) with mitochondria, emerged from the previous localization studies, that Drp1/Dnm1 assembles on the mitochondrial outer membrane in punctate structures where mitochondrial division occurs.