

Is the mitochondrial cloud the selection machinery for preferentially transmitting wild-type mtDNA between generations? Rewinding Müller's ratchet efficiently

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Abstract In animal mitochondrial DNA inheritance, it remains largely unclear where the mitochondrial genetic bottleneck localizes and how it works in rewinding Müller's ratchet. In a variety of different animals germ plasm mRNAs typically aggregate along with numerous mitochondria to form the mitochondrial cloud (MC) during oogenesis. The MC has been found to serve as messenger transport organizer for germ plasm mRNAs. Germ plasm RNAs in MC will specifically distribute to the primordial germ cells of the future embryo. It has been proposed that the MC might be the site where selected mitochondria accumulate for specific transmission to grandchildren but this idea received relatively little attention and the criterion by which mitochondria are selected remains unknown. Our recent results in zebrafish provided further evidence for selective mitochondria accumulation in the MC by showing that mitochondria with high-inner membrane potential tend to be recruited preferentially into the MC, and these mitochondria are transported along with germ plasm to the cortex of the vegetal pole. By analyzing the composition, behavior and functions of the MC, and in reviewing related literature, we found strong support for the proposition that the MC corresponds to the position and function of the mitochondrial genetic bottleneck.

Keywords Mitochondrial cloud · Oogenesis · Mitochondrial transmission · Wild-type mtDNA

Abbreviations

MC Mitochondrial cloud
mtDNA Mitochondrial DNA
PGCs Primordial germ cells
METRO Messenger transport organizer

Introduction

Müller's 'ratchet' anticipates that organisms reproducing asexually with high mutation rates will accumulate irreversible deleterious mutations over generations (Felsenstein 1974). In time, these mutations will initially compromise the function of individual cells, then the whole organism, and eventually the species (Chinnery et al. 2000). Animal and human mtDNAs are asexually transmitted from mother to offspring; the mtDNA suffers from high mutation rates and accumulates deleterious mutations in oocytes as well as in somatic cells during the life span of an organism (Chen et al. 1995; Nagley and Wei 1998; Jansen and Burton 2004). Despite solid data that almost all metazoan animals transmit mitochondria to offspring only through their female germlines, we still know little about the stage and the mechanism(s) by which deleterious mutant mtDNAs might be eliminated in the female germline. Based on our review of related literature, analysis of composition, behavior and functions of the MC, and additional supporting evidence from our recent experiments, we found strong support for Spradling and colleagues' idea (Pepling and Spradling 1998; Cox and Spradling 2003) concluding that the MC not only organizes and transports germ plasm, but also functions as the efficient selection machinery for

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transmission of wild-type mtDNA to offspring in animals; the MC may well correspond to the mitochondrial genetic bottleneck.

The ‘bottleneck’ hypothesis

It is generally believed that there is a mitochondrial genetic bottleneck during female germline development, which can lead to rapid segregation of different mitochondrial genotypes within a few generations (Hauswirth and Laipis 1982; Ashley et al. 1989; Koehler et al. 1991). But based on different observations and experimental evidence, different authors proposed that the bottleneck exists at different sites and works through different mechanisms. The assumed stages include the earliest PGCs where the germ cell number is small and there are less than ten mitochondria in each PGC (Jansen and de Boer 1998); during expansion of the oogonial population (Jenuth et al. 1996); during postnatal folliculogenesis, during which a clone of mtDNA replicates fast and populates the developing oocyte, diluting out pre-existing mtDNA (Wai et al. 2008); during oocyte maturation, during which there is about a 100-fold increase of mtDNA, the amplification may use a limited number of template mtDNA molecules and yield one predominating genotype in the mature oocyte (Hauswirth and Laipis 1982; Marchington et al. 1998); or during early embryonic development cells that form the embryonic inner cell mass rather than extraembryonic tissues may receive very different ratios of heteroplasmic mtDNAs (Laipis et al. 1988).

Aimed at identifying the exact process by which different types of mtDNA are transmitted between human and animal generations, different transmission modes have been investigated in various human pedigrees with various mtDNA mutations (Chinnery 2002; Yu-Wai-Man et al. 2009), and different heteroplasmic animal models have been created and analyzed. Animal models include neutral mtDNA mutations, neutral and pathogenic mtDNA mutations, and mouse mtDNA common deletions (Hauswirth and Laipis 1982; Jenuth et al. 1996; Meirelles and Smith 1997; Inoue et al. 2000; Toivonen et al. 2001; Fan et al. 2008). Studying heteroplasmy in the mouse, Jenuth et al. (1996) found that in sharp contrast to results obtained in primary and mature oocytes, the coefficient of mtDNA genotype variation in PGCs was very small which indicates that the mtDNA genotype is more uniform in early PGCs than in oocytes. Recent studies in mice suggest that each of the earliest discernable PGC contains more than 200 mtDNA (Cao et al. 2007; Cree et al. 2008). Such a large number cannot explain convincingly the rapid mtDNA genotype segregation observed in different animals through pure random genetic drift (Olivo et al. 1983; Ashley et al. 1989; Koehler et al. 1991). It appears that the reduction of

mtDNA copy number in early PGCs does not correspond well to the bottleneck.

The expansion of PGCs before colonizing the gonad is thought to account for the increase in mtDNA genotypic variance observed among mature oocytes from heteroplasmic mothers (Jenuth et al. 1996), and expansion of the oogonial population may distribute different mtDNA genotypes to different germ cells by a series of asymmetric mitoses. In *Drosophila* and mouse, germ cell death proportionally occurs in each germline cyst (see below). It is difficult to understand that deleterious mutations are always proportionally distributed in each germline cyst. In other animals, such as *Xenopus*, there is no colossal purge of germ cells during oogenesis (Kloc et al. 2007). These data do not support the oogonial expansion bottleneck hypothesis.

Jenuth et al. (1996) compared the variance in the frequency of mtDNA genotypes between primary oocytes from heteroplasmic mice (postnatal day 2–3) and mature oocytes from adult mice, and observed similar variation coefficients in these two populations. They again obtained immature oocytes from one ovary of postnatal day 2 mouse and mature oocytes from the other ovary of the same mouse after transplantation and allowed them to mature in the ovarian capsule of a nude mouse. The results revealed that the mtDNA variation was virtually identical in primary and mature oocytes derived from the same animal. They compared the distribution of mtDNA genotypes in mature oocytes and F1 offspring from the same founder females and observed no significant difference in the mtDNA genotype proportions. These data show that from primary oocytes to mature oocytes and to the offspring there is no mtDNA segregation.

Another disagreement regarding the bottleneck hypothesis is the question on how deleterious mtDNA mutations are eliminated in the female germline. Jansen and de Boer (1998) suggested that following the mitochondrial bottleneck in PGCs, female germ cells propagate into more than 6 million at the time of five-month pregnancy. Then, a colossal purge of germ cells takes place to purge defective mitochondrial genomes as well as those that fail to sustain adequate nuclear genomic functions. Pepling and Spradling (2001) found that during mouse oogenesis, 16-cell cysts are formed but in each cyst about one-third of the total number of germ cells will develop into primordial follicles. The other germ cells in the cyst will die. This means that if there is no asymmetrical distribution of high-functional mitochondria, or selective transfer of functionally different mitochondria between germ cells within each cyst, colossal purge (about two-thirds) of the germ cells will hardly play any role in eliminating defective mitochondrial genomes since all the germ cells in a cyst are derived from the same cystoblast (or ancestor) through four consecutive mitoses;

they would contain quite similar mtDNA genotypes. Similar to mouse oogenesis, in human embryonic ovaries, also about two-thirds of germ cells die from the time of 5-month pregnancy (more than 6 million germ cells) to the prenatal stage (about 2 million germ cells); whether there is a definite proportion of germ cell elimination in each germline cyst or similar germ cell group is not known. In *Drosophila*, 16-cell cysts are also formed and only one of the 16 germ cells will develop into an oocyte. All others will become nurse cells and undergo apoptosis later. A selective transfer of a subgroup of mitochondria with some specific feature from nurse cells to a specific position of the oocyte does occur (see below). The colossal purge of germ cells in humans, mice and *Drosophila* may play an important role in eliminating deleterious mtDNAs if wild-type mtDNA or mtDNA with advantageous characteristics has been selectively transported into the destined oocytes before the purge takes place. In *Xenopus* and perhaps in all anuran amphibians, all germ cells in each germline cyst develop into primary oocytes (Kloc et al. 2007); without colossal purge of germ cells, deleterious mtDNAs are also eliminated through some other processes between generations of these animals. The MC formation process in *Xenopus* and fish is different from that in mammals and in *Drosophila*; the process of eliminating deleterious mtDNA mutations may also be different among these species (see below).

Is the MC the competitive selection machinery in the female germline to ensure the wild-type or fittest mtDNA being transmitted to offspring?

Mitochondrial cloud and its significance

Mitochondrial cloud or Balbiani body has been shown in early oogenesis in many animals including Actinia, spider, insects, fish, amphibian, avian and mammals (Kloc et al. 2004; Pepling et al. 2007). MC has been extensively studied in *Xenopus*, and many authors emphasized its function in germ plasm RNA organization and translocation from the nucleus to the vegetal pole of the early oocyte; the MC was even called messenger transport organizer METRO (Kloc et al. 1996, 2004). In the early oocyte, such as the pre-stage I *Xenopus* and zebrafish oocyte, mitochondria aggregate into small clusters surrounding the nucleus (Kloc et al. 1996; Zhang et al. 2008). These structures were named nuage/germ plasm/polar plasm/oosome by different authors (Kloc et al. 2004). Kloc et al. (1996) referred to these structures as pre-mitochondrial clouds (there are usually 2–4 of them in the early *Xenopus* oocyte). Germ plasm RNAs become increasingly more abundant within one of the pre-mitochondrial clouds, and eventually only one pre-mitochondrial cloud develops into the MC (Kloc et al.

1996). More than ten germline-determining RNAs, such as *Xlsirts*, *Xwnt-11*, and *Xcat2* RNAs, have been found within the *Xenopus* MC; in the cloud they are transmitted to the vegetal pole of the oocyte (Kloc and Etkin 1998; Kosaka et al. 2007).

There are specific proteins in the MC, such as RNA-binding protein Hermes, XPAT and XPIX1, and Trailer Hitch (Kloc et al. 2007; Pepling et al. 2007; Hames et al. 2008); elongation factor 1 alpha (EF-1 alpha) was shown to be concentrated in the MC in *Xenopus* (Viel et al. 1990). Milton (a mitochondria-specific adaptor protein) was shown to be necessary for mitochondria transport from nurse cells to the oocyte and for Balbiani body (or MC) formation in *Drosophila* (Cox and Spradling 2006). Products of many conservative genes (such as *vasa*, and *mago nashi*) have been found both in invertebrate and vertebrate MC or Balbiani bodies (Saffman and Lasko 1999; Matova and Cooley 2001).

In the MC of many vertebrate and invertebrate animals, densely glomerate mitochondria are always assembled within the MC; ATP supply alone may not be a satisfactory answer for such density of mitochondrial aggregation in this area. Large and small mitochondrial rRNAs (mtlrRNA and mtsrRNA) have been determined in the MC but outside mitochondria in *Drosophila* (Iida and Kobayashi 1998; Amikura et al. 2005), and *Xenopus* (Kashikawa et al. 2001). It was also shown that injection of mitochondrial rRNA into ultraviolet-irradiated *Drosophila* embryos restored the pole cell-forming ability (Kobayashi and Okada 1989). Besides ATP supply, mitochondria in the MC are likely to play other important roles, and the MC may play an important role in mitochondrial selection and inheritance.

The possible role of MC in active selection of high-functional mitochondria for PGCs of the next generation

The role of the MC in transmitting mitochondria and other organelles to the cortex of the vegetal pole in *Xenopus* (Chang et al. 2004; Kloc et al. 2007), fish (Zhang et al. 2008), and quail (D'Herde et al. 1995) has been observed. It has been noted that mitochondria in the MC have specific characteristics compared to mitochondria in other areas of the ooplasm (D'Herde et al. 1995; Cox and Spradling 2003, 2006; Kloc et al. 2007; Zhang et al. 2008). It has also been noted that mitochondria in the MC might be specifically transmitted to the PGCs of the future embryo (D'Herde et al. 1995; Cox and Spradling 2003, 2006; Kloc et al. 2004). Fewer investigators, primarily Cox and Spradling (2006), have addressed its possible role in selecting high-functional organelles for distribution to the PGCs of the next generation. The function of the MC in selecting high-functional organelles and transmitting them to PGCs of the

future embryo may be as important as transmission and distribution of germ plasm RNAs.

In stage I zebrafish oocytes (Zhang et al. 2008) and *Xenopus* oocytes (Kloc et al. 2007), the MC contains a large number of mitochondria. Mitochondria inside and outside the MC are interchangeable. After treatment with oxidative phosphorylation uncoupler, FCCP [carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone], the inner membrane potential is lost or becomes very low in most mitochondria; the few mitochondria left with comparatively high-inner membrane potential, which can assemble 'J aggregations' and emit red fluorescence after staining with JC-1, are eventually recruited into the MC (Zhang et al. 2008). These data suggest that some molecules or components may be present that selectively attract high-inner membrane potential mitochondria into the MC; these molecules or components may act as the machinery for selecting high-functional mitochondria into the germ plasm and distribute them to PGCs of the future embryo. If there is a large number of high-functional (high-inner membrane potential) mitochondria in the cytoplasm, they would compete for attachment to these molecules or components, and mitochondria with the highest inner membrane potential would have better probabilities to attach to these molecules (components) and to be preferentially transmitted into PGCs of the future embryo. Highest inner membrane potential indicates mitochondria containing wild-type or normal functional mtDNA. The process by which mitochondria with high-inner membrane potential compete for attachment to the molecules (components) in the MC would be an efficient selection mechanism for mtDNA segregation during germ cell development. Mitochondria with similar mtDNA function would be stochastically recruited into the MC and transported into PGCs of the next generation; mtDNA with a defective impact on mitochondrial function would be excluded from passing into PGCs of the future embryo.

Different distribution patterns of low- and high-inner membrane potential mitochondria in human and animal oocytes have been reported (Sun et al. 2001; Van Blerkom et al. 2003; Van Blerkom 2008; Zhang et al. 2008). But direct motility tracking of high-inner membrane potential mitochondria into the MC in developing oocytes is still lacking. We found that mitochondria in the MC always show high-inner membrane potential; after treatment with FCCP almost no high-inner membrane potential mitochondria were left in the cell, a few mitochondria showing comparatively high-inner membrane potential were all recruited into the MC (Zhang et al. 2008). This still cannot totally exclude the possibility that mitochondria with normal inner membrane potential randomly aggregate into the MC, and after reaching the MC, their inner membrane potential increases by stimulation of MC component(s). Further

study is needed to determine whether high-inner membrane potential or other specific characteristics are the specific criteria for selection of mitochondria into the MC.

Selection processes are different in different animals

Almost all studied animals form a MC (or Balbiani body) in the early oocyte stages but the process of MC formation differs in different animals. *Drosophila* forms a typical germ cell cyst during oogenesis. Each germline stem cell divides into a new stem cell and a cystoblast. The cystoblast consecutively divides four times with incomplete cytokinesis to generate an interconnected 16-cell cyst surrounded by somatic cells. Only one of the 16 germline cells will develop into an oocyte while all others will become nurse cells (Cox and Spradling 2003). Transport of cytoplasm from all nurse cells to the oocyte occurs through the ring canals in two phases: an early slow phase during which specific mitochondria and molecules are transported into the oocyte, and a late rapid phase (or dumping) during which the nurse cells empty almost all their remaining cytoplasm into the oocyte (Buszczak and Cooley 2000; Fig. 1a1, a2). A specific subpopulation of mitochondria in nurse cells moves along the fusome and enters the oocyte in the early (slow) phase and coalesces into the MC (or Balbiani body; Fig. 1a1). The remaining mitochondria in nurse cells are blocked from entering the oocyte and will enter the oocyte during a late (dumping) phase, and they will not coalesce into the MC (Fig. 1a2). A specific feature sets the early subpopulation of mitochondria apart from the others, but the nature of this specific feature is still unknown (Cox and Spradling 2003); it may be related to the inner membrane potential. Cox and Spradling (2006) found that the *Drosophila* oocyte acquires the majority of mitochondria by competitive bidirectional transport along microtubules, and proposed that the genomes in Balbiani body-associated mitochondria will be preferentially inherited in the second generation of offspring.

Unlike *Drosophila*, all 16 germ cells in the *Xenopus* cyst develop into functional oocytes. Although materials exchange among cystocytes through ring canals (Kloc et al. 2004), functional mitochondria may not be specifically selected for one or a few oocytes. In *Xenopus* and zebrafish oocytes, the MC may be formed by competitive selection of high-inner membrane potential mitochondria into the germ plasm area (Fig. 1b). Specific molecules (or components) in the germ plasm area may preferentially attract high-inner membrane potential mitochondria to attach to them as described above. In this case, all mitochondria in the MC are recruited from the same oocyte. *Xenopus* and zebrafish oocytes containing significantly more mitochondria compared to those of *Drosophila* and mammals might represent an advantage for such selection.

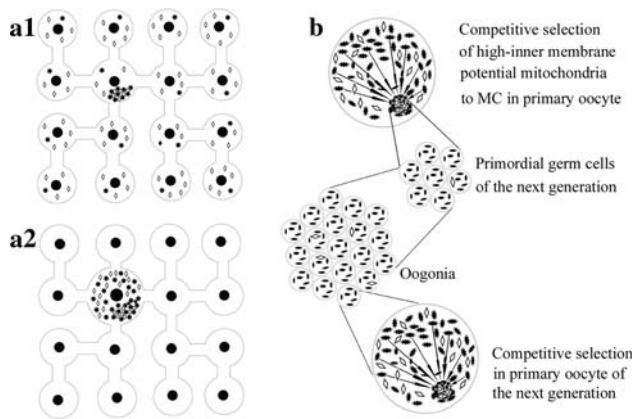


Fig. 1 Schematic diagram of the two typical processes (**a1** and **a2** in *Drosophila*; **b** in *Xenopus*) by which mitochondria with high-inner membrane potential (*asterisks*) are recruited into the mitochondrial cloud (MC) together with germ plasm (*circles*). **a1** During the germline cyst stage of *Drosophila* oogenesis, in the slow transporting phase a subpopulation of mitochondria with high-inner membrane potential together with pole plasm (or germ plasm) are transported from the 15 nurse cells to the destined oocyte and coalesce into the MC, while mitochondria with low-inner membrane potential (*rhombuses*) remain in the nurse cells. **a2** Following the slow phase there is a late rapid phase during which nurse cells empty almost all their remaining cytoplasm into the oocyte (Buszczak and Cooley 2000), but mitochondria entering the oocyte in the later rapid phase will not coalesce into the MC (Saffman and Lasko 1999; Matova and Cooley 2001). **b** In early primary oocyte of *Xenopus*, a subpopulation of mitochondria with high-inner membrane potential move into the MC through competition. During early embryonic development, these mitochondria become distributed into primordial germ cells (PGCs). From these PGCs many oogonia are produced, and oogonia will eventually develop into primary oocytes. During the stages from PGCs to mature oocytes mutant mitochondria with low-inner membrane potential may occur and accumulate again. Through competition, another subpopulation of mitochondria with high-inner membrane potential will move into the MC in primary oocyte of the next generation

During mouse oogenesis, 16-cell cysts are also formed, but in each cyst about one-third of the total number of germ cells will develop into primordial follicles. The other germ cells in the cyst will die (Pepling and Spradling 2001). Mitochondrial transport takes place through ring canals between cystocytes. Shortly after germline cyst breakdown, mitochondrial aggregations (precursors of the MC) appear in the oocytes of primordial follicles (Pepling and Spradling 2001). Compared to the MC in *Xenopus* oocytes, the MC in mouse oocytes is smaller, contains fewer mitochondria, and persists for a shorter period of time. Pepling and Spradling (2001) proposed that cysts may ensure that oocytes destined to form primordial follicles acquire populations of high-functional mitochondria, but the details of the selection machinery remain unclear. Whether high-functional mitochondria are selectively transported from the perished cells to the oocytes destined to form primordial follicles remains unknown.

In birds, the high energy demands of flight must place a stronger selection pressure on mitochondrial genes than

that occurring in flightless animals (Lane 2008). D'Herde et al. (1995) showed that in pre-lampbrush stage quail oocytes the perinuclear MC is bulky and composed of numerous mitochondria. As oocytes develop into lampbrush chromosome stages, the MC initially disperses homogeneously but then segregates into 2 populations: (1) a population localized in the cortical layer of the vegetal pole; and (2) clusters of mitochondria distributed geometrically around the germinal vesicle in the animal pole. The authors suggested that the perinuclear group of mitochondria will be distributed into the somatic cells of the future embryo while the original subcortical group near the vegetal pole will be localized in the PGCs. This suggests that a specific group of mitochondria in the MC will be assigned to PGCs of the future embryo.

In different taxa of animals, MCs are different in size, duration, and mitochondrial number. Animals like *Xenopus* and fish producing large numbers of progeny need to select more high-functional mitochondria for PGCs of the next generation; the MCs in these animals are usually large and contain significantly more mitochondria. Other animals like mammals producing small numbers of progeny need to select fewer high-functional mitochondria for PGCs; the MCs in these animals are usually smaller and contain fewer mitochondria. This may explain the differences of MCs in different animals.

Regarding the high attrition rate of oocytes from the time of primary follicle formation which usually takes place during mid-pregnancy in humans and mammals to the time of mature oocyte ovulation, or to menopause, we speculate that this process would be another safeguard to eliminate the deleterious mtDNA mutations accumulated after MC selection. As it usually takes several years or even dozens of years from the time of primary follicle formation to mature oocyte ovulation, deleterious mtDNA mutations would occur and accumulate in many oocytes during such a long period of time. In amphibians and fish, it has been reported that there is no colossal purge or attrition of germ cells but the low survival rate of their embryos and young offspring may correspond to the elimination of deleterious mtDNA mutations that occurred to some extent after MC selection.

Why do some pathogenic mtDNA mutations transmit from mother to offspring?

Mitochondrial inheritance is perhaps most extensively investigated in humans but the mechanism underlying the inheritance process is still uncertain. Chinnery and colleagues (Chinnery et al. 2000) identified a large number of transmissions of six most common pathogenic mtDNA point mutations between offspring and their corresponding mothers, and found that three mutations significantly increased the mutant levels in offspring; two other muta-

tions also increased their mutant levels in offspring but did not reach the statistical significance level. It is frequently seen that specific pathogenic point mutations, such as T8993G, tend to rapidly increase their levels in offspring (Carelli et al. 2002; Wong et al. 2002). There are various published examples of children with high pathogenic mutant levels being born to mothers with low mutant levels, but few examples of the reverse exist (Chinnery et al. 2000). Each pathogenic mtDNA mutant type may have its own causes and transmission rule. For example, both A3243G and T8933G tend to be amplified in offspring, but A3243G mutation level decreases in blood with age and varies in different tissues; T8933G mutation level does not change in blood with age and does not vary in different tissues. It appears that they pass the female germline and accumulate in germ cells or somatic cells through different mechanisms. The detailed mechanisms of transmission of some pathogenic mtDNA mutations from mother to offspring appear to be complex pathological processes, and not simply a random genetic drift.

Furthermore, mtDNA deletions are rarely, if ever, transmitted from clinically affected mothers to their offspring (Zeviani and Di Donato 2004). Similar to pathogenic point mutations, low levels of mtDNA deletions may have no obvious effect on the cell or organism. Why are low levels of mtDNA deletions rarely transmitted from mothers to their offspring? It implies an active selection mechanism that exists in the female germline. Contrary to deletions, partial duplications are transmittable under certain conditions both in human and in animals (Poulton and Holt 1994; Ballinger et al. 1992; Martin Negrier et al. 1998; Jacobs 2000). Partially duplicated mtDNA may contain all the normal mitochondrial genes, and the mitochondrial function may not be affected for a limited period of time. During this time, the selection machinery may assess this mitochondrion as a normal one and recruit it into the MC which will result in partially duplicated mtDNA transmission to offspring.

Conclusion

The fact that animal mtDNAs are not susceptible to Müller's 'ratchet' implies that an active restricting mechanism exists in the female germline to exclude deleterious mtDNA mutations from passing to offspring. This mechanism may be called 'mitochondrial genetic bottleneck', but its position and molecular criteria remain unclear. The MC forms in early oocytes of all animals studied so far and functions to specifically transmit germ plasm RNAs to PGCs of the future embryo. A specific group of mitochondria is always recruited into the MC and transported along with germ plasm RNAs to the cortex of the vegetal pole of the oocyte. Mitochondria in the MC display high-inner

membrane potential, which may be a criterion for the oocyte to recruit high-functional mitochondria to the MC and specifically distribute them to the PGCs of the next generation along with germ plasm RNAs. There are typically fewer copies of mtDNA in each mitochondrion in the germ cells compared with that in somatic cells; high-inner membrane potential or high-function of the mitochondrion represents integrity of its mtDNA. All evidence discussed in this review show that the MC appears to be an active and efficient selection machinery to guarantee wild-type mtDNA transmission to offspring. It corresponds well to the position and function of the mitochondrial genetic bottleneck. Further studies may identify molecule(s) or component(s) in the MC specifically attracting mitochondria with high-inner membrane potential or other high-functional criteria, which may result in significant advances to artificially select high-quality mitochondria for therapeutic use in fields such as rescue of oocytes containing high-level of mutant mtDNA or low-numbers of mtDNA.

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