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Exogenous paternal mitochondria rescue hybrid incompatibility and the destiny of exogenous mitochondria

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ABSTRACT

The mitochondria of most organisms follow strict maternal inheritance, and the mechanism of elimination of paternal mitochondria is unclear. Our previous studies showed that the paternal mtDNA presented in the embryo of hybrid *Megalobrama Amblycephala* (BSB,♀) × *Carassius auratus* red var(RCC,♂) (BR) and its reciprocal hybrid (RB), but its expression was quiescent. However, the microinjected mitochondria persisted in the embryo and its mtDNA expressed throughout embryonic development. In addition, the chromosome number of RCC (2n = 100) is much larger than that of BSB (2n = 48). All BR embryos were severely abnormal and ultimately died, while the RB embryos could survive and grow up into adult. It implied that the nuclear-cytoplasm incompatibility may be an important cause of BR abnormality. In this study, exogenous parental mitochondria were microinjected into BR embryos, and it was found that paternal mitochondrial DNA persisted and expressed throughout embryonic development. Meanwhile, the study also found that microinjection of isolated paternal (RCC) mitochondria into BR embryos significantly improved the degree of early embryonic abnormality with some fry swimming normally, comparing to the control embryos. However, injection of maternal (BSB) mitochondria did not improve the development of BR embryos. We safely concluded exogenous mitochondria escape the mechanism of elimination and its DNA persist and express throughout embryonic development. In addition, the expression of paternal mitochondrial genes largely reduces the cyto-nuclear conflict, so that the early BR embryos exhibit less degree of abnormality and enhanced activity.

1. Introduction

Mitochondria are one of the most important organelles in eukaryotic cells. In the past few decades, the study on mitochondria mainly focused on the molecular evolution and phylogeny of mtDNA [1,2]. Recently, the scientific questions on mitochondria have been increasingly discussed. Many studies focused on the structure and function of mitochondria as an organelle and its gene regulation, the production and regulation of biochemical energy, the relationship between mitochondrial dysfunction and disease, apoptosis and lifespan [3–7]. Mitochondria produce energy through oxidative phosphorylation and are widely involved in various cellular life activities, including nucleic acid biosynthesis, cell division and ion metabolism [8]. The process of oxidative phosphorylation is controlled by both nuclear and mitochondrial genes. And many other functional activities of mitochondria, including mtDNA replication and transcription, are also regulated by nuclear genes [8,9]. Therefore, the coordination of nuclear genes and mitochondrial genes is very important for the basic life activities. The disharmony between nuclear and mitochondrial genes is called nuclear-cytoplasm incompatibility, which is common in hybrid species and leads to disorders of many life activities [10–14].

Interspecific hybridization plays a very important role in the evolution of higher animals [15–17]. The interspecific hybridization incompatibility causing reproductive isolation is a common phenomenon driving speciation. As we all know, the divergence of intra-species usually results from selection pressures, and the emergence of new species leads to interspecific hybridization incompatibility. Surprisingly, the differentiation within many species is not driven by the selection pressures of adaptation to the ecological environment, but the result of gene mutations or inter-gene conflicts [15,18]. The incompatibility between

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nuclear genes and cytoplasmic genes (usually mitochondrial genes) is one of the most important reasons for interspecific hybridization incompatibility [14,19–21]. The conflict between nuclear and cytoplasmic genes in hybrids is mainly due to the different inheritance patterns of mitochondrial genes and nuclear genes. Mitochondria are strict maternal inheritance, while nuclear genes inherit from both parents. Aerobic respiration depends on the cooperation between mitochondrial and nuclear genes, so genetic variation on either of them would increase the pressure on the other for adaptation [20,22]. The inefficient repair system of mitochondrial genes would increase the mutation rate [23]. Because maternal inheritance and lack of recombination of mtDNA leads to the failure of elimination of minor harmful mutations, so harmful mutations in the mitochondrial genome accumulate. In order to adapt to these minor mutations in mtDNA, nuclear genes may have compensatory mutations for repairing interaction of nuclear genes and cytoplasmic genes [14,24]. Interspecific hybrid species inherit parental genes and many unique genes emerge through recombination and mutation [25,26]. And new species would appear, only when the interaction of heterogeneous nuclear and cytoplasmic genes reaches a new balance. If the interaction fails to reach a new balance, it will cause reproductive barriers to hybrid species [27]. Hybridization between different species often generates hybrids with various degrees of nuclear and cytoplasm incompatibility, resulting in infertility, lethality and phenotypic abnormality in their offspring [28–30]. In recent years, a lot of researches on nuclear-cytoplasmic incompatibility have been carried out, but lacking of direct experimental evidences.

Mitochondrial microinjection is an effective approach to study the dynamic changes of mitochondria and metabolic diseases caused by inactivation and mutation of mitochondrial function [31,32]. Meanwhile, mitochondria from different tissues were introduced into the embryo by microinjection to study the changes of mitochondria and their effects in embryo [33]. Microinjection of isolated mitochondria into the fertilized eggs also has a significant effect on embryonic development [34].

Our previous studies showed that paternal mitochondria gradually decreased along with embryonic development and ultimately disappeared after hatching, and the expression of paternal mitochondrial genes was not detected throughout embryonic development [35]. In addition, our experiment on exogenous mitochondria microinjection found that mtDNA of exogenous mitochondria persisted in all stages of embryonic development, and its mitochondrial genes expressed [36]. *Megalobrama Amblycephala* (BSB) ($2n = 48$) and *Carassius auratus* red var. (RCC) ($2n = 100$) belong to the family of Cyprinidae with significant difference of chromosome number, but respectively belong to different subfamilies of Cultrinae and Cyprininae. In this study, through the hybridization of BSB and RCC, it was found that the offspring of BSB (♀) × RCC (♂) (BR) were all abnormal and finally died after a few days of hatching, but the reciprocal offspring of RCC (♀) × BSB (♂) (RB) survived, which provided a good experimental model for us to study nuclear-cytoplasm incompatibility.

2. Materials and methods

2.1. Experimental animals

All animal work conducted here adhered to the relevant Guidelines for the Care and Use of Laboratory Animals of the National Advisory Committee for Laboratory Animal Research in China and approved by the Animal Care Committee of Hunan Normal University (Permit Number: 4237). All the experimental fish were collected from the National Education Ministry Breeding Center of Polyploidy Fish, Hunan Normal University. The 2-year-old RCC and BSB with well-developed gonads were selected as breeding parents at breeding season (From April to June), We conducted reciprocal cross experiments using RCC and BSB as breeders to obtain embryo from different embryonic stages of RB and BR. All the fish dissected in the experiment were anesthetized with 2-phenoxyethanol before dissection and operated according to the relevant regulations of experimental animals. Embryos and tissues were stored at −80 °C for DNA and RNA extraction.

2.2. Mitochondria extraction

Mitochondria were extracted using the mitochondrial Extraction Kit (purchased from ScienceCell research laboratories Cat. No.8268). Firstly, 100 mg of adult organs (heart, muscle, liver and testis) was rinsed in precooled PBS at 4 °C for 3 times. And then 1 ml 1x mitochondrial isolation buffer A was added to homogenize the tissue in a glass homogenizer. The homogenates were moved to 1.5 ml EP tube and centrifuged at 4 °C, 1000 g for 5min. And the supernatants were centrifuged at 4 °C, 10000 g for 20min. After removing the supernatants, 1 ml 1x mitochondrial isolation buffer A was added, and precipitates were re-suspended with lightly pipetting followed by centrifuging at 4 °C, 10000 g for 20min. Finally, after removing the supernatants, 100 μl mitochondrial isolation buffer B was added to re-suspend precipitates containing mitochondria. The activity of extracted mitochondria was examined in time, and these isolated mitochondria were used immediately or stored at −80 °C for later mitochondria microinjection.

2.3. Measurement of isolated mitochondria protein concentration and activity

BSA (Cat. No. b9001s) purchased from NEW ENGLISH Biolabs was used to prepare 6 standard samples with different protein concentrations (2000 μg/ml, 1000 μg/ml, 500 μg/ml, 250 μg/ml, 125 μg/ml, 0 μg/ml). And then the concentration of standard samples was measured, using BCA protein quantitative Kit (product No.: p001b-1), to generate standard curve for evaluating protein concentration from isolated mitochondria. Then protein concentration of mitochondria samples was estimated based on the standard curve.

Mitochondria were diluted with buffer B to a concentration of 0.1 μg/ml protein. Finally, Cytochrome C oxidase activity was measured using Cytochrome Oxidase Measurement Kit (GMS10014.2.v.A) according manufacture's instructions.

2.4. Mitochondria microinjection and observation

Embryos at 1–2 cell stage were injected with approximately 1 nl of Buffer B or mitochondrial preparation at 0.01 μg/μl protein using a FemtoJet (Eppendorf, Germany). Experimental embryos and control embryos were incubated in Petri dishes in water at 22 °C. More details about mitochondria microinjection were depicted in our previous study [36]. The survival and phenotype of embryos throughout the whole embryonic development were observed and photographed with the help of Leica MZFIII stereo microscope.

2.5. DNA and RNA extraction

The zona pellucida and spermatozoa attached to or within the perivitelline space were removed by washing with acidified Tyrode's solution prior to the DNA and RNA extraction. DNA was extracted from a group of frozen embryos ($n > 20$) using the TaKaRa Mini BEST Universal Genomic DNA Extraction Kit (TaKaRa, Japan) as specification described. RNA was extracted using the E.Z.N.A. Total RNA Kit II (OMEGA).

2.6. Species-specific PCR

Specific primers of *cytb* for BSB and RCC mtDNA were previously [35] developed for identifying the existence of mtDNA from each species in the embryo or adult organs. Using these species-specific primers, the presence of BSB and RCC mtDNA and their *cytb* genes expression were examined by PCR. Genomic PCR and rt-PCR were conducted with 0.25 units of Ex Taq, 1 μl of 10 × PCR Buffer, 100 μM dNTP mixture, 1 μl of 50

ng/μl genomic DNA, 0.1 μM of each primer in a total volume of 25 μl. The conditions of PCR were denatured at 94 °C for 30s, annealed at 56 °C for 30s and extended at 72 °C for 30s for 35 cycles. At last, PCR products were run on 1.5 agarose gels.

3. Experimental results

3.1. Comparison of mitochondrial activity in different tissues

Mitochondria from four organs, including heart, liver, muscle and testis of BSB were extracted. Subsequently, its protein concentration and activity were examined. The result showed that there were significant differences between mitochondrial number and activity in different organs within the same individual (Table 1). The testis possessed the largest number of mitochondria with the highest protein concentration, whereas the isolated mitochondria from heart showed a highest activity in vitro (Table 1). This data was consistent with our isolated mitochondria data from various organs of RCC, where isolated mitochondria from heart exhibited the highest activity [36].

3.2. The behavior of mtDNA in BR embryos

Mitochondria isolated from heart were injected into 1–2 cell stage of BR embryos and the dynamics of its mtDNA was monitored using species-specific primers of BSB and RCC (Fig. 1A) throughout embryonic development. As expected, it was found that maternal (BSB) mtDNA was stably present throughout embryonic development. However, paternal (RCC) mtDNA persisted throughout embryonic development, and not gradually disappeared with embryonic development (Fig. 1B, upper panel). It was necessary to mention that our previous research showed that sperm mtDNA presented in early embryo and gradually decreased along embryonic development in the hybrids BR and RB [35]. In addition, our previous study on the behavior of exogenous mitochondria during embryonic development showed that microinjection-delivered mtDNA persistently presented in the whole embryonic stages, even after birth [36]. These results implied that microinjection-delivered mitochondria were persistently existed throughout embryonic development of BR.

Mitochondrial gene (*cytb*) expression was examined throughout embryogenesis of BR with mitochondria injection. Reasonably, the maternal *cytb* gene was expressed in the whole embryogenesis of mitochondria injected BR (Fig. 1B, below panel). Interestingly, paternal (RCC) cytb gene was also detected throughout embryogenesis (Fig. 1B, below panel). Sperm mtDNA presented in embryos of the BR and RB hybrids, its RNA was quiescent throughout embryogenesis [35]. Whereas, RNA of microinjection-delivered mtDNA was expressed accompanying the presence of exogenous mtDNA. It indicated that RNA, encoded by microinjection-delivered mtDNA, expressed throughout hybrid BR embryogenesis.

3.3. BR embryos improved after mitochondria injection

Isolated paternal (RCC) mitochondria were introduced to 1–2 cells BR

Table 1
Comparison of the activity of isolated mitochondria from different organs of blunt-snout bream.

	Heart	Liver	Muscle	testis
A	0.10 ± 0.21	0.12 ± 0.16	0.11 ± 0.19	0.13 ± 0.09
B	4.87 ± 1.03	6.53 ± 0.86	1.66 ± 0.43	**8.56** ± 2.12
C	**7.56** ± 1.98	1.19 ± 0.25	2.55 ± 0.36	1.06 ± 0.37

A: Initial tissue weight (g).
B: Total protein concentration of isolated mitochondria (μg/μl).
C: Enzyme activity of isolated mitochondria with 0.1 g/ml protein concentration (μM/mg/min).

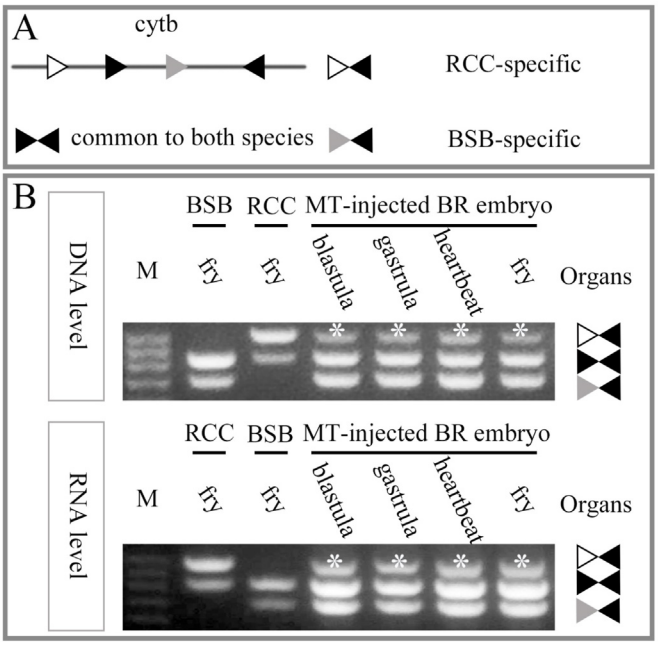

Fig. 1. The dynamics of exogenous mtDNA behavior during embryonic development. (A) Scheme of PCR primers for *cytb*, showing primers specific to goldfish (open ar) or blunt-snout bream (grey ar) and common to both species (black ars). (B) Our previous studies showed paternal mtDNA was present and gradually reduced in developing embryos, and it disappeared around hatching. In this study, paternal mtDNA and mtRNA were both clearly persistent throughout embryogenesis after microinjection with paternal organ mitochondria. Asterisks depict paternal mtDNA from goldfish, respectively. DNA and RNA were isolated from 20 pooled embryos at each stage from parental species and injected BR and analyzed by PCR at representative stages. PCR and gels were run under the same conditions. The upper panel is genomic DNA PCR analysis, and the below panel is RT-PCR analysis.

embryo with the controls of untreated embryos and embryos injected with isolated maternal (BSB) mitochondria and mitochondrial isolation buffer B. The survival rate of untreated embryos was obviously higher than that of injection embryos from blastula to fry. However, the survival rate between embryos injected with mitochondria and those injected with control was not significant difference during blastula stage to fry (Table 2). It implied that microinjection operation, to some extent, caused damage to embryonic development. It was found that the abnormality rate of embryos injected with paternal (RCC) mitochondria (Table 2, Fig. 2B) was significantly lower than that of embryos injected with maternal (BSB) mitochondria, untreated embryos and embryos injected with buffer B (Table 2, Fig. 2A). Some BR fry, after microinjection with paternal mitochondria, exhibited normal morphology and

Table 2
Statistics of BR embryo with mitochondria (MT) injection and control sets.

Injection category		RCC MT	BSB MT	Untreated	Buffer
Total no. of zygote		2351	2213	2563	1688
Blastula	Survival	2227	2052	2555	1531
		(94.7%)	(92.7%)	(99.7%)	(90.7%)
Gastrula	Survival	1211	1262	1889	963
		(51.5%)	(57.0%)	(73.7%)	(57.1%)
Heart-beat	Survival	820	903	1329	548
		(34.9%)	(40.8%)	(51.9%)	(32.5%)
Fry	Survival	565	592	**1067**	481
		(24.0%)	(26.8%)	**(41.6%)**	(28.5%)
	Abnormality	**497**	592	1067	481
		(88.0%)	(100%)	(100%)	(100%)
The longest survival days after hatching		~20	~20	~20	~20
No. of adult fishes		0	0	0	0

swam normally (Fig. 2C and B). However, with the development, these fry gradually became abnormal. All fish including paternal-mitochondria injected and controls showed severely abnormal with organs exposure to environment, and finally died around 20 days after hatching (Table 2, Fig. 2D and E).

4. Discussion

Mitochondria play an important role in embryonic development [37]. Mitochondrial dysfunction is usually caused by exposure to certain environmental factors or genetic abnormalities including mitochondrial and nuclear DNA, which can result in various diseases [38]. Mitochondrial microinjection technique can facilitate animal models establishment for the study of mitochondrial-related diseases. Previously, mitochondria from *Mus spretus* liver were introduced into fertilized eggs of *Mus musculus domesticus* to obtain an animal model for studying mitochondrial-related diseases [31,32,39]. Exogenous mitochondria may have a certain effect on embryonic development. Previously reported that injection of somatic cell-derived mitochondria into fertilized eggs led to the remodeling of embryonic development [34]. Meanwhile, introduction of exogenous mitochondria can enhance embryonic development, but it possibly raises potential risks, such as mitochondrial heteroplasmy, nuclear-mitochondrial interaction and epigenetic aspects [40]. Our previous research showed that injection-delivered mitochondria had little adverse impact on embryogenesis [36]. In this study, we observed expression of injection-delivered mtDNA throughout BR embryogenesis, and the abnormality of BR was significantly improved. It indicated that introduced parental mitochondria had positive influence on embryonic development of BR.

Before microinjection of isolated mitochondria into the embryo, the most important thing is to determine whether the isolated mitochondria are active or not. The activity of mitochondria isolated from heart was the highest comparing to mitochondria isolated from other organs of RCC [36]. In this study, we also measured the activity of mitochondria from different organs of BSB, and found that the activity of mitochondria isolated from heart was also the highest in comparison to mitochondria isolated from other organs. Mitochondria are strictly maternal inheritance, but the number and activity of mitochondria are various in different organs. It reflected that mitochondria from different organs evolve to different number and activity along embryonic development and cell differentiation to meet the requirement of different energy demands of different tissues [41]. In addition, the isolated mitochondria with activity will ensure them functional in the receptor embryos.

Our previous studies showed that, the paternal mitochondria gradually decreased and disappeared with development, while the mitochondrial gene expression was quiescent throughout the entire process of embryonic development of both RB and BR [35]. In addition, injection-delivered mtDNA was stably persisted and its RNA expressed accompanying its presence throughout embryogenesis [36]. Sperm mitochondria inside fertilized cow and monkey eggs are tagged by the recycling marker protein ubiquitin [42]. In this study, it was found that paternal mtDNA, including fertilization-delivered and injection-delivered, persisted and its RNA expressed all over the whole BR embryogenesis. It implied that injection-delivered mitochondria were functional in the embryo. These primary studies implied that paternal mitochondria were immediately recognized by some certain factors from eggs around fertilization pore, resulting in paternal mitochondria dysfunction. However, the injection-delivered mitochondria entered eggs after fertilization and escaped factors' capture. Mitochondria from different delivery strategies may have different destines throughout embryogenesis. Here, we proposed possible destines for these mitochondria based on our primary data, whereas it requires further experimental evidence to confirm (Fig. 3). The possible destinies for different mitochondria are as below: before sperm mitochondria entering into eggs, egg mitochondria were marked as maternal mitochondria; paternal mitochondria markers converged around fertilization pore, and all sperm mitochondria would be labelled when entering eggs; once paternal mitochondria were marked, some digestion factors surrounded mitochondria resulting in mitochondria dysfunction; exogenous mitochondria introduced into fertilized eggs and escaped being marked as paternal or maternal mitochondria. Hence, the destiny of different mitochondria is totally different among the three sources of mitochondria. Paternal mitochondria are rapidly marked and functionally inactivated, and its mtDNA is eliminated gradually. Exogenous mitochondria escaped elimination without replication function, but its mitochondrial RNA express throughout embryogenesis. Only the marked maternal mitochondria are fully functional. Previous studies showed that

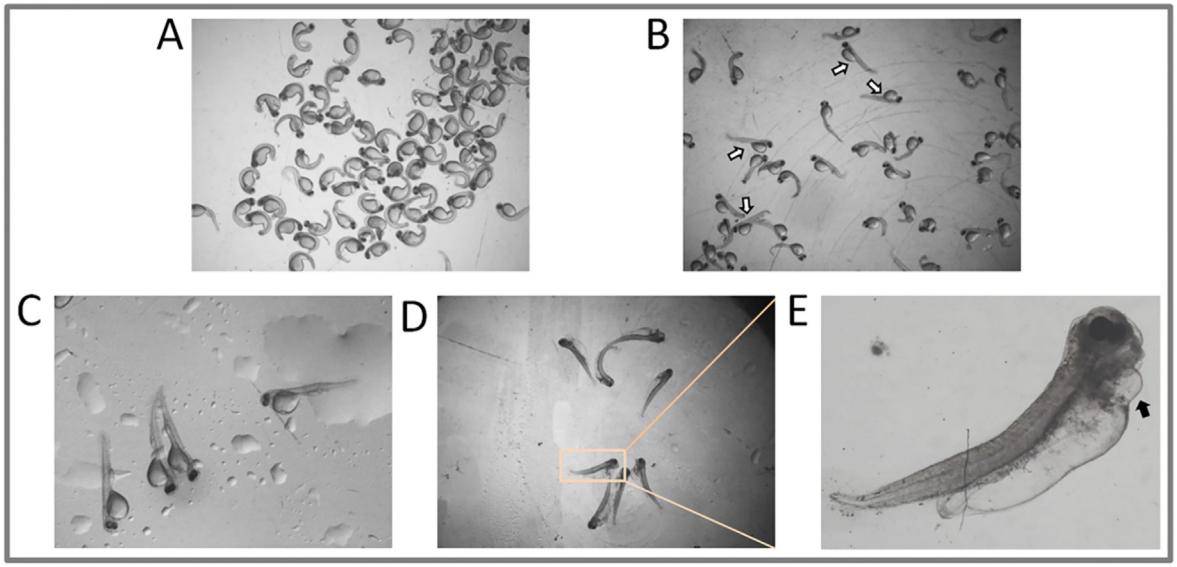

Fig. 2. The abnormality of BR embryos improved after microinjection of isolated paternal mitochondria (A) BR embryos exhibited 100% abnormality in all control groups including untreated, buffer injected, and isolated maternal (BSB) mitochondria injected group. (B) The abnormality of BR fry was significantly improved after microinjection of isolated paternal mitochondria with the presence of normal morphological fry. (C) The normal morphological fry with microinjection of isolated paternal mitochondria. (D) The normal morphological fry with microinjection of isolated paternal mitochondria exhibited abnormal with tail twisted at 7 days after hatching. (E) The magnification of abnormal fry of 7 days after hatching showed these fry were with organs naked. And all fry including treated and untreated groups died around 20 days after hatching.

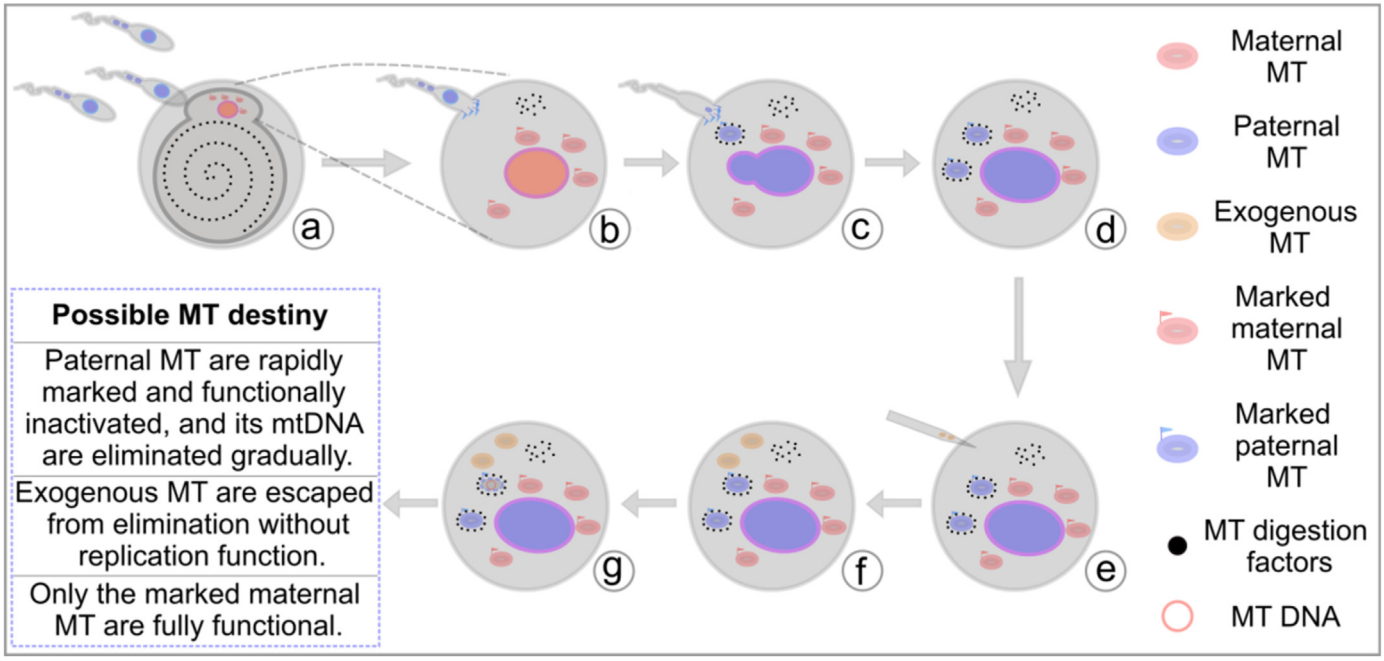

Fig. 3. The possible destinies of mitochondria from different delivery strategies during embryogenesis. The possible destinies of mitochondria that enter into the eggs in different ways are different. When sperm move onto egg fertilization pore (a), sperm mitochondrial marker factors are rapidly assembled around the fertilization pore (b), sperm mitochondria that enter into the egg will be marked by these factors (c), and the digestive factors in the egg will quickly surround the marked sperm mitochondria (d), inactivating mitochondria function. However, the paternal mitochondrial DNA was not eliminated in time and remained in the cells (g), so that the paternal mitochondrial DNA was detected in early embryos, but not expressed. Isolated mitochondria were injected at 1-cell embryos (e). These mitochondria escape from being marked, so their function is not inactivated (f). However, they have no replicative function and are therefore gradually diluted during embryonic development. Maternal mitochondria may be marked prior to fertilization, thus retaining intact mitochondrial activity (g).

autophagy is involved in the elimination of sperm mitochondria and mtDNA in early embryos in *Caenorhabditis elegans* [43]. In contrast, autophagy is not involved in the degradation of sperm mitochondria after fertilization in mice [44]. Therefore, more researches in the future should be conducted to better elucidate mechanism of elimination of paternal and exogenous mitochondria.

Hybridization between two distant species often generates offspring with sterility, lethality and/or phenotypic abnormality, which may be caused by the incompatibility between the different genomes of the two species [45]. The incompatibility between genomes includes the incompatibility between paternal nuclear genome and maternal mitochondrial genome, and the incompatibility between two parent nuclear genomes. The interaction between nuclear genomes from the two species is complex involving numerous genes interacting with each other. Mitochondria are involved in many important life activities, such as energy production, metabolism, apoptosis, etc [46]. And more than 100 functional proteins exist in mitochondria, among which only a dozen are encoded by mtDNA. Therefore, the effective interaction of nuclear genes and mitochondrial genes is an important guarantee for the normal life activities.

In recent years, the nuclear-cytoplasm incompatibility in hybrid species has also raised extensive interest among scientists. Hsin-YiLee et al. hybridized two closely related yeasts, *S. bayanus* and *S. cerevisiae*, and found that *S. bayanus aep2*, a mitochondrial protein encoded by nuclear genome, was incompatible with *S. cerevisiae* mitochondria resulting in sterility in hybrid offspring and driving speciation [11]. In the hybridization experiment of *Nasonia giraulti* and *N. vitripennis*, it was found that the nuclear-cytoplasm incompatibility caused increased mortality of F2 hybrids [12]. In this study, RCC and BSB belong to two different subfamilies, and their chromosome numbers are largely different with 100 in RCC and 48 in BSB. The embryos of hybrid BR were of 100% abnormality and eventually died. Through mitochondria microinjection, paternal mitochondrial genes expressed throughout BR embryogenesis. These paternal mitochondrial genes would interact with paternal nuclear genes without conflicts to relieve the incompatibility between maternal mitochondrial genes and paternal nuclear genes in hybrid BR. Meanwhile, the abnormality of hybrid BR was significantly improved by introducing paternal mitochondria, implying the nuclear-cytoplasm incompatibility had an important impact on BR embryonic development. However, all BR hybrids, including paternal mitochondria injected offspring, eventually died of organ exposure, indicating that the injection-delivered paternal mitochondria exerted temporary influence on individual development. The cause of BR death should be ascribed to multiple unknown factors, and to reveal these unknown reasons, combination of a variety of approaches would be helpful in future exploration and research.

5. Conclusion

Our study revealed that microinjected paternal mitochondria persisted and were functional throughout embryogenesis. Comparing to microinjection with maternal organ mitochondria, the abnormality of BR hybrids was significantly improved by injection of paternal organ mitochondria. However, the fate of BR hybrids did not change through paternal mitochondria injection. Our results confirmed that nuclear-cytoplasm incompatibility did have a great effect on the abnormality and lethality of BR hybrids, but the causes of their death are complicated.

Authors' contributions

Shaojun Liu conceived the project, designed the experiments and acquired funding. Ming Wen, Liangyue Peng, Qilin Li, Xinjiang Hu, Yuling Zhao conducted investigation. QinBo Qin, Min Tao, Chun Zhang, Kaikun Luo, Rurong Zhao, Shi Wang, Fangzhou Hu, Qingfeng Liu, Chenchen Tang helped in collecting samples and doing investigation. Shaojun Liu, Ming Wen, Yuxin Zhang, Siyu Wang, and Qian Li wrote and modified this manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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