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MINI-REVIEW

Regulation of metastasis; mitochondrial DNA mutations have appeared on stage

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Abstract It has been controversial whether mtDNA mutations are responsible for tumorigenesis and for the process to develop metastases. To clarify this issue, we established trans-mitochondrial cybrids with mtDNA exchanged between mouse tumor cells that possess high and low metastatic potential. The results revealed that the G13997A mutation in the ND6 gene of mtDNA from highly metastatic tumor cells reversibly controlled development of metastases by overproduction of reactive oxygen species (ROS). The transmitochondrial model mice possessing G13997A mtDNA showed symptoms of impaired glucose tolerability, suggesting that ROS generated mtDNA mutations can regulate not only metastatic potential, but also age-associated disorders such as diabetes. We also identified other mtDNA mutations that affect metastatic potential but the mechanisms are independent of ROS production. The mtDNA-mediated reversible control of metastasis and age-associated disorders are novel functions of mtDNA, and suggests that ROS scavengers may be therapeutically effective to suppress these phenotypes.

Keywords mtDNA · Metastasis · Reactive oxygen species (ROS) · Transmitochondrial cybrids · Deabetes

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Introduction

Mitochondria are multi-functional organelles, which play pivotal roles in not only ATP generation, but also the apoptotic pathway, maintenance of Ca^{2+} homeostasis in cells, biosynthetic pathway of lipids or steroids, and many other biological pathways. Mitochondrial dysfunction itself has been observed in various disorders such as mitochondrial diseases, diabetes, neurodegenerative diseases, and cancers, suggesting that normal mitochondrial function is indispensable for our life. However, because mitochondrial function is regulated by both mitochondrial DNA (mtDNA)-encoded genes and nuclear DNA-encoded genes, both mtDNA and nuclear DNA mutations can induce mitochondrial dysfunction. Because no technologies to manipulate mtDNA artificially have been established so far, it has been difficult to confirm whether certain mtDNA mutations are the cause of the mitochondrial dysfunction observed in the diseases listed above. Moreover, even if the mtDNA mutations are confirmed as pathogenic mutations that affect the mitochondrial respiratory functions, it is still possible that real causes of the diseases are nuclear DNA mutations, not the mtDNA mutations. Due to these difficulties, direct evidence that has been reported and confirming the relationship between mtDNA mutations and

certain disease phenotypes is very limited. Our laboratory has approached this issue using transmitochondrial technology, which has enabled us to exchange the mtDNA between 2 different cell lines completely and reciprocally. Using this technology, novel mtDNA functions that are involved in regulation of metastatic potential and age-associated phenotypes have been uncovered.

"Mitochondrial theory of cancer" and contrary evidences

Mitochondria of tumor cells have been reported to differ functionally and morphologically from those of normal cells (Pedersen 1978). Moreover, many chemical carcinogens have been shown to bind preferentially to mtDNA rather than to nuclear DNA (Allen and Coombs 1980; Backer and Weinstein 1980). Although there has been no direct evidence for the creation of mtDNA mutations by carcinogens, and for their contribution to tumor development in mammalian cells, recent studies showed high frequencies of homoplasmic mutations in mtDNA of tumors rather than in mtDNA of normal tissues of the same patients (Polyak et al. 1998; Fliss et al. 2000). Many subsequent studies supported preferential accumulation of mutated mtDNAs in tumor cells (Penta et al. 2001; Taylor and Turnbull 2005; Czarnecka et al. 2006; Gallardo et al. 2006). Therefore, mtDNA was considered to be the major cellular target of chemical carcinogens, and the resultant creation of mutations in mtDNA could be responsible for the oncogenic transformation of normal cells and their ability to develop tumors (Shay and Werbin 1987). This theory is called "the mitochondrial theory of cancer".

However, subsequent studies revealed that most of the mutations found in tumor mtDNA are synonymous mutations, which do not affect the amino acid sequences, or polymorphic mutations that have been reported already in healthy control samples (Meierhofer et al. 2006; Sanchez-Cespedes et al. 2001). Therefore, these mutations can be considered to be not harmful for mitochondrial functions. Moreover, it is well known that mtDNAs are a maternally inherited genome (Kaneda et al. 1995; Shitara et al. 1998). If certain mtDNA mutations could trigger tumorigenesis, maternal inherited cancers should exist. Nevertheless, no cancers have been proven to be maternally inherited so far. These contrary pieces of evidences suggest that mtDNA mutations found in cancer cells frequently are not the cause of tumorigenesis, but the results of rapid and repeated cell divisions.

We previously tried to provide experimental evidences to answer the question, whether or notmtDNA mutations can induce tumorigenesis. We generated tumorigenic transformant from non-tumorigenic mouse embryonic fibroblast cells through chemical carcinogen treatment, then exchanged their mtDNA completely with each other: one possessed nuclear DNA from normal fibroblast but mtDNA from transformant, and the other possessed nuclear DNA from transformant but mtDNA from normal fibroblast. Because only the latter showed tumorigenicity, not mtDNA but nuclear DNA is responsible for the tumorigenesis at least in this cell line (Akimoto et al 2005). We have obtained similar results using human cell lines (Hayashi et al. 1986; Hayashi et al. 1992). Although we cannot assert that all of the causes for tumorigenesis are due to only nuclear DNA from these experiments, our results are one of the convincing contrary evidences for the mitochondrial theory of cancer.

From "the mitochondrial theory of cancer" to "the mitochondrial theory of tumor metastasis"

Then, is mtDNA unrelated with any process of tumor development and progression? Of course, it is still possible that mtDNA mutations are involved in processes other than tumorigenesis, such as in the malignant progression of tumor cells to develop metastatic potential. Recent studies have demonstrated that the dysfunction of the TCA cycle caused by mutations in nuclear DNA controls tumor phenotypes by the induction of a pseudo-hypoxic pathway under normoxic conditions (Baysal et al. 2000; Gottlieb and Tomlinson 2005). Thus, the abnormality of mitochondrial function caused by mtDNA mutations also possibly affects tumor phenotypes. However, there has been no direct evidence of the involvement of mtDNA mutations in malignant progression or in the regulation of the pseudo-hypoxic pathway, because of the difficulty of excluding possible involvement of nuclear DNA mutations in these processes (Augenlicht and Heerdt 2001). To overcome this difficulty, we applied our unique technique that enables the exchange of mtDNA between different cell lines, to test the possibility (Ishikawa et al. 2008a).

The cell lines we used were C57BL/6 (B6) mouse-derived low metastatic Lewis lung carcinoma cell line P29 and A11, and the high metastatic subline of P29. Although both P29 and A11 cell lines share the same nuclear background, A11 shows high metastatic potential while P29 does not, and their difference of metastatic potential can be distinguished clearly by counting the number of lung metastatic nodules after inoculating them into the tail vein of B6 mice (Takenaga et al. 1997; Takasu et al. 1999). First, we compared the activity of each respiratory chain complex of mitochondria, and found that highly metastatic A11 cells show complex I defects (Ishikawa et al. 2008a).

However, as mentioned above, because not only mtDNA is responsible for the respiratory function of mitochondria, there is a possibility that nuclear DNA mutations but not mtDNA mutations are involved in these complex I defects.

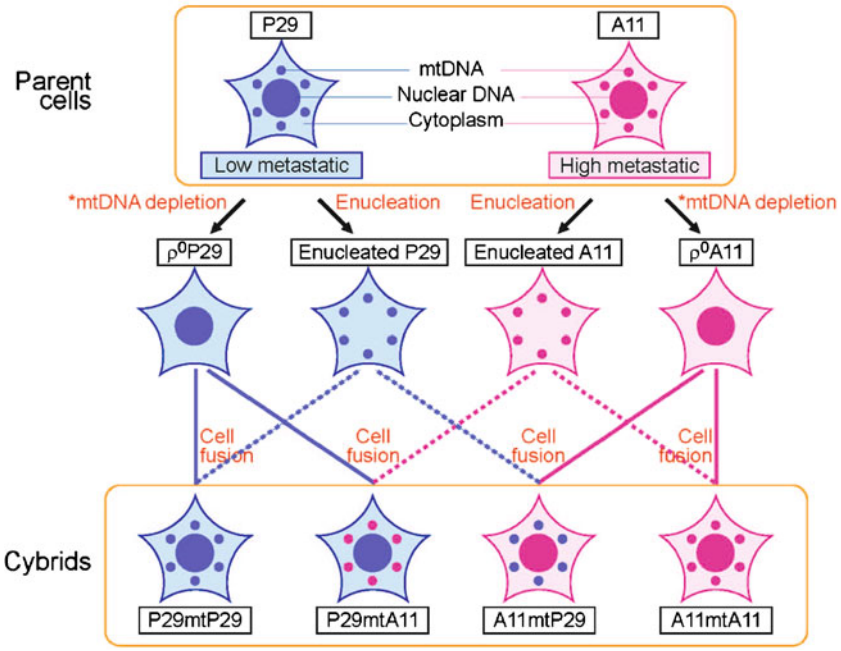

Fig. 1 Scheme for the isolation of cybrids with completely exchanged mtDNA. *, Parental P29 and A11 cells were treated with ditercalinium (Inoue et al. 1997) for isolation of ρ^0P29 and ρ^0A11 cells, which have no mtDNA, and then the G418-resistance plasmid was introduced into the ρ^0P29 and ρ^0A11 cells to permit isolation of ρ^0 cells resistant to G418. High metastatic potential and complex I defects are transferred with the transfer of mtDNA from the A11 cells, and low metastatic potential and normal complex I activity are transferred with the transfer of mtDNA from the P29 cells

To clarify which genome is responsible for complex I defects and high metastatic potential of A11, we performed complete and reciprocal exchange of mtDNA between A11 and P29 (Fig. 1). As mtDNA recipient cells, we isolated mtDNA-less cells called ρ^0 cells, from A11 and P29 cell lines respectively. We also obtained cytoplasts of A11 and P29 as mtDNA donors. Fusing the ρ^0 cells and cytoplasts, we established 4 cytoplasmic hybrid (cybrid) cells that possess nuclear DNA from P29 cells and mtDNA from A11 (P29mtA11), nuclear DNA from A11 and mtDNA from P29 (A11mtP29), both nuclear DNA and mtDNA from P29 (P29mt29), and nuclear DNA and mtDNA from A11 (A11mtA11), respectively.

Evaluation of metastatic potential of each cybrid cells revealed that cybrid cells possess high metastatic A11-derived mtDNA and show high metastatic potential irrespective of whether their nuclear DNAs came from P29 or A11. And vice versa, cybrid cells with mtDNA derived from P29 showed low metastatic potential even if their nuclear DNAs came from A11 cells. Moreover, cybrid cells possess mtDNA derived from A11 (P29mtA11 and A11mtA11) showed complex I defects, while complex I activity of cybrid cells possess mtDNA from P29 (P29mtP29 and A11mtP29) were normal. Thus, both phenotypes of metastatic potential and complex I defects were co-transferred with mtDNA between P29 and A11 cells, and these results mean that mtDNA, not nuclear DNA, is responsible for high metastatic potential and complex I defects observed in A11 cells. These are the first experimental results that revealed mtDNA can regulate metastatic potential (Ishikawa et al. 2008a).

The cause of mtDNA-mediated metastasis

Next we compared mtDNA sequences between P29 and A11 cells, and found that mtDNA from A11 cells has a G to A point mutation at n.p. 13,997 (G13997A). We concluded that this G13997A mutation is the cause of complex I defects and high metastatic potential of A11 cells from the following reasons:

1. This mutation is the only difference found in A11 mtDNA compared to P29 mtDNA.
2. This mutation causes amino acid substitution in the ND6 protein, one of the sub-units of complex I.
3. The amino acid sequences of this mutation site in ND6 is highly conserved among not only vertebrates but also arthropods, suggesting that this site is important for ND6 functions, and this mutation can be harmful.

Since hundreds to thousands of copies of mtDNA are contained in one cell, and the mutation rate of mtDNA is higher than nuclear DNA, probably we could have found more mutations than G13997A if we had surveyed the sequences of all of the mtDNA copies in A11 cells. However, mitochondrial respiratory functions are protected from potentially harmful mutations but exist at a low rate through “mitochondrial complementation”. It is known that mitochondrial functions are kept at normal levels even if cells contain small numbers of harmful mtDNA mutations, because the majority of normal mtDNA complements the functions. Thus, mutations that exist at a low rate cannot be the cause of the complex I defects and metastatic potential observed in A11 cells. To confirm the mutation rate of G13997A mutation

in A11 cells, we amplified the mtDNA fragment including this mutation site by PCR and compared RFLP (restriction fragment length polymorphism). The results showed that almost all of the mtDNA in A11 cells are G13997A mutants, while mtDNA in P29 cells are wild type, suggesting that G13997A mutation in A11 mtDNA is the cause of complex I defects and high metastatic potential (Ishikawa et al. 2008a).

The mechanisms of mtDNA-mediated metastasis

The next question is about the mechanisms of mtDNA-mediated metastasis. How does the G13997A mutation induce high metastatic potential? To metastasize, cancer cells have to survive under severe conditions such as hypoxia which leads cells to apoptosis, during which they transfer from primary lesion to other organs passing through blood vessels. In fact, it has been revealed that the highly metastatic parent cell A11 shows up-regulation of some metastasis-related genes such as HIF1-α (hypoxia inducible factor-1α), VEGF (vascular endotherial growth factor), and MCL-1 (myeloid cell leukemia-1) (Takasu et al. 1999; Koshikawa et al. 2003; Koshikawa et al. 2006). Then we compared gene expression levels of these metastasis-related genes involved in the process to acquire the resistance to hypoxia or apoptosis, and found that protein expression levels of HIF1-α, VEGF, and MCL-1 were higher in P29mtA11 and A11mtA11 than P29mtP29 and A11mtP29 (Ishikawa et al. 2008a; Koshikawa et al. 2009). HIF1-α is one of the master regulators to adopt hypoxic condition, VEGF is an essential factor for angiogenesis, and its expression levels are controlled by HIF1-α. MCL-1 is an anti-apoptotic factor under hypoxia. Thus, higher expression levels of these genes enable tumor cells to acquire the resistance and survive under the severe conditions, and induce metastasis effectively.

Then, expression levels of these metastasis-related genes are somehow thought to be regulated by the G13997A mutation through some pathways. As the pathway, we focused on ROS (reactive oxygen species). Mitochondrion, the place for oxidative phosphorylation, is the major source of ROS because electrons are transmitted continuously within the inner membrane of mitochondria, and leaked electrons change into ROS easily. Especially complex I as well as complex III is known as a main origin of ROS (Wallace 1999), and thus complex I defects caused by the G13997A mutation possibly induce ROS overproduction. As expected, ROS production was increased in cybrids with A11-derived mtDNA (Ishikawa et al. 2008a).

Next we tested whether the overproduced ROS is the cause of up-regulation of metastasis-related genes. Once cybrids possessing A11-derived mtDNA were treated with NAC (*N*-acetyl cysteine), a well-established antioxidant, the expression levels of MCL-1 were decreased, and metastatic potential was reduced simultaneously. Our results suggest that ROS overproduced by complex I defects induced by the G13997A mutation causes reversible gene expression changes that possibly lead to high metastatic potential (Fig. 2) (Koshikawa et al. 2009). ROS overproduction can be a kind of mutagen for nuclear DNA and sometimes causes somatic mutations that possibly change phenotypes of the tumor cells. These somatic mutations and resultant phenotypes should be irreversible, but our observation revealed that the G13997A mutation-mediated metastasis is reversible. Therefore, for these types of reversible metastasis, antioxidants such as NAC seem to be effective in preventing or treating them (Ishikawa et al. 2008a).

mtDNA-mediated metstasis – recent updates

Although our results showed that ROS overproduction induced by complex I defects is the trigger of high metastatic potential, the other pathways may be involved simultaneously in the process of acquiring metastatic potential. For instance, to adapt to dysfunction of oxidative phosphorylation, generally anaerobic glycolysis is enhanced even under the normoxic condition (called aerobic glycolysis, also known as the Warburg's effect), and lactate is overproduced as a byproduct.

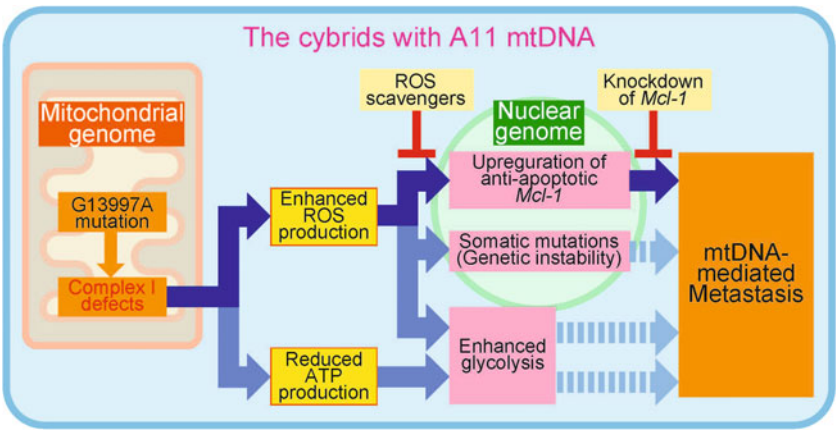

Fig. 2 Reversible control of metastasis by ROS-generating mtDNA mutations. A scheme we proposed as a mechanism of mtDNA-mediated metastasis. Because NAC treatment succeeded in preventing metastasis of cybrid cells possessing A11 mtDNA, this metastasis can be considered as reversible

Because aerobic glycolysis seems to increase resistance of cells under hypoxic condition due to their low dependence on oxygen, cells with enhanced glycolysis may possess high metastatic potential. Since the A11 cells and their cybrids we used show complex I defects, the effect of aerobic glycolysis also may be involved in metastatic potential.

To address this issue, we used cell lines possessing mtDNAs with a large deletion mutation (lacking 4,696 bp, named ΔmtDNA) and lacking mtDNA completely (called ρ^0 cells). Both cells with ΔmtDNA and ρ^0 cells show respiration defects and accelerated glycolysis but ROS production levels are comparable with cells with wild type mtDNA. We established trans-mitochondrial cybrid cells sharing the same nuclear background with P29 but possessing ΔmtDNA (P29mtΔ) and compared its metastatic potential among P29mtP29, P29mtA11, and ρ^0P29 cells. Only P29mtA11, showing complex I defects with slightly enhanced glycolysis and ROS overproduction, has high metastatic potential, while P29mtΔ and ρ^0P29, showing respiration defects and enhanced glycolysis but without ROS overproduction, did not possess metastatic potential. These results indicate that respiration defects and enhanced glycolysis *per se* are not involved in metastatic process in our experimental system (Ishikawa et al. 2008b).

On the other hand, our recent study revealed ROS-independent but mtDNA mutation-mediated metastasis using human breast cancer cell lines. A breast cancer cell line, MDA-MB-231, is malignant and possesses high metastatic potential. We identified 2 pathogenic point mutations C12084T and A13966G, that change amino acid sequences of ND4 and ND5, respectively, in MDA-MB-231 mtDNA. We isolated 231mt231 (both nuclear DNA and mtDNA are derived from MDA-MB-231) and 231mtFt (nuclear DNA from MDA-MB-231 but possesses fetal fibroblast-derived normal mtDNA), and compared their characters. Only 231mt231 showed complex I defects and high metastatic potential, but both 231mt231 and 231mtFt showed ROS overproduction. These results suggest that mtDNA derived from MDA-MB-231, but not mtDNA from fetus, is metastasis-inducible, but the mechanisms to acquire the metastatic potential seem to be ROS-independent (Imanishi et al. 2011). The mechanisms of MDA-MB-231-derived mtDNA-mediated metastasis remains to be solved, but this study revealed that not all of the metastasis induced by mtDNA mutation is ROS-dependent, and mechanisms of mtDNA-mediated metastasis can be varied depend on cell types or other factors.

An animal model with metastasis-inducible mtDNA mutation

It is known that pathogenic mtDNA mutations that induce significant respiration defects can be causes of mitochondrial diseases (Wallace 1999). Several mtDNA mutations that cause mitochondrial diseases have been identified, but it has been difficult to understand the relationship between specific mtDNA mutations and specific phenotypes because nuclear backgrounds are varied with patients and establishment of a mitochondrial disease animal model is not easy due to the lack of mtDNA manipulation technologies. In 2000, we generated the first mitochondrial disease model mice which possess ΔmtDNA applying trans-mitochondrial techniques (Inoue et al. 2000), and using this model, we have revealed several important biological functions that mtDNA mutations are involved in, such as, the process of spermatogenesis (Nakada et al. 2006), and the differentiation process of hematopoietic stem cells (Inoue et al. 2010).

The G13997A mutation we identified in A11 mtDNA also induces significant complex I defects, and was expected to be the cause of some phenotypes when introduced into mouse model. Then we introduced A11 cells-derived G13997A mutant mtDNA into mouse ES cells, and injected them into embryos to obtain mouse models possessing the mutant mtDNA (Yokota et al. 2010). The resultant mouse model showed complex I defects in several organs and symptoms of lactic acidosis, but did not show other severe disease phenotypes when they were young (Yokota et al. 2010). However, after aging, they showed phenotypes of impaired glucose tolerability in all mice tested and lymphoma in higher frequency compared to wild type mice (Hashizume et al. 2012). The transformed mouse embryonic fibroblasts (MEF) derived from this model showed high metastatic potential, but the transformation rate of MEF was not enhanced by the G13997A mutation (Hashizume et al. 2012). Highly frequent lymphomas observed in this model may seem to be a discrepancy because the G13997A mutation itself is not tumorigenic. However since tumors observed in this model were restricted in only lymphomas throughout their lifespans, and their nuclear donor (B6 mice) tend to develop lymphoma naturally, frequent lymphoma of this model may be observed only with a B6-strain nuclear background. Meanwhile, impaired glucose tolerability was observed in all of the model mice, and this phenotype could be rescued by treating them with antioxidant (Hashizume et al. 2012). Because diabetes is one of the disease symptoms of mitochondrial diseases, this model can be the mitochondrial disease model reflecting clinical phenotypes.

Conclusions

Several reports suggest that mtDNA mutations can cause tumorigenesis, but our findings that some mtDNA mutations can mediate metastasis, rather than tumor formation, reveal novel functions of mtDNA. It seems that there are several

mechanisms to explain the metastasis mediated by mtDNA mutations: ROS-dependent and ROS-independent pathways. Moreover our transmitochondrial model mice uncovered that ROS generating metastasis-inducible mtDNA mutation can lead to symptoms of diabetes also, implicating that treatment with antioxidant is possibly effective not only for inhibition of metastasis, but also for suppression of diabetes.

Although our research has partly resolved the wide-varied functions of mitochondria or mtDNA, many undefined functions or effects still remain to be solved. A deeper understanding of mitochondrial function will probably provide us a novel approach to treat diseases or symptoms that are difficult to treat currently.

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