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Techniques for investigating mitochondrial gene expression

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ABSTRACT

The mitochondrial genome encodes 13 proteins that are components of the oxidative phosphorylation system (OXPHOS), suggesting that precise regulation of these genes is crucial for maintaining OXPHOS functions, including ATP production, calcium buffering, cell signaling, ROS production, and apoptosis. Furthermore, heteroplasmy or mis-regulation of gene expression in mitochondria frequently is associated with human mitochondrial diseases. Thus, various approaches have been developed to investigate the roles of genes encoded by the mitochondrial genome. In this review, we will discuss a wide range of techniques available for investigating the mitochondrial genome, mitochondrial transcription, and mitochondrial translation, which provide a useful guide to understanding mitochondrial gene expression.

Mitochondria in mammals have their own genome, which encodes 2 mitochondrial rRNAs (mt-rRNAs), 22 mitochondrial tRNAs (mt-tRNAs), and 13 proteins in the oxidative phosphorylation system (OXPHOS) (1, 2). Each heavy- and light-strand mitochondrial DNA

(mt-DNA) is transcribed to polycistronic transcripts followed by sequential maturation in the mitochondrial RNA granule (3). There are 11 mRNAs with 13 reading frames that are then translated by mitochondrial ribosomes. These proteins make up components of OXPHOS, which are composed of nuclear-encoded and mitochondria-encoded subunits (4). Thus, proper assembly of OXPHOS requires precise coordination of mitochondrial and cytoplasmic translation; however, mechanisms underlying their communication are not well understood.

There are several unique features of mitochondria that need to be considered for investigating gene expression in mitochondria. First, since a mitochondrion is an individually compartmentalized organelle, a method that engineers RNAs or proteins to be transported into mitochondrion is necessary. Importing proteins into mitochondria has been achieved by inserting a mitochondrial targeting sequence (MTS) into desired proteins (5, 6). However, targeting RNA into mitochondria is still challenging because of our poor understanding of the RNA delivery mechanism; hence small RNA-mediated knockdown or chimeric mRNA-based tools have limited success. Second, a mitochondrion is a highly dynamic organelle that constantly undergoes fusion and fission and changes position (7, 8). Therefore, imaging analysis of mitochondria dynamics still requires more sophisticated tools. Third, mitochondria have independent gene expression machinery. (9, 10). The mitochondrial ribosome consists of 28S and 39S subunits, and thus is smaller than cytoplasmic ribosome, which contains 40S and 60S subunits (11), indicating that factors involved in mitochondrial translation are different from those for cytoplasm translation.

Mitochondrial gene expression changes according to cellular environments (10), suggesting that mitochondrial translation is regulated by cellular activity to maintain cellular homeostasis or to meet cellular demands. Thus, it is desirable to examine mitochondrial translation in various types of cells. Here, we review biochemical and imaging tools that are available for studying mitochondrial DNAs, mitochondrial RNAs, and mitochondrial gene expression.

Mitochondrial DNA

A mitochondrion contains 2 to 10 copies of mitochondrial DNA (mt-DNA), which is a 16.5 KB circular structure containing a D-loop (12). The total number of mitochondrial DNAs in a single cell is usually about 10^3 to 10^4 (13), but fertilized cells or cells with diseases, such as cancer, cardiovascular diseases, and neurological disorders, have altered mt-DNA copy numbers (14-17). Quantification of an mt-DNA copy number is a simple experiment, which involves DNA extraction and quantitative PCR (13). However, how different mt-DNA copy numbers alter cellular phenotypes is not fully understood. Furthermore, whether the number of mt-DNA copy numbers directly affects the mechanism of mitochondrial transcription and translation is not clear.

Editing the mitochondrial genome

For mitochondrial genome editing, restriction endonucleases that recognize a mutated mitochondrial DNA sequence have been successfully targeted to mitochondria by the insertion of the MTS (18). Once inside the mitochondria, restriction endonucleases remove only mutated mitochondrial DNA from mitochondrial heteroplasmy. However, since mutated sequences that can be recognized by restriction endonucleases are rare, the use of this method is limited. Other genome editing techniques, such as zinc-finger nucleases (ZFNs) (19), transcription activator-like effector nuclease (TALENs) (20), and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system (21), have been developed to replace mutated genes found in human diseases (22). Accordingly, mitochondrial DNA editing tools have been developed by delivering the respective editing machinery to mitochondria (Fig. 1A): mt-ZFN (23, 24), mt-TALENs (25), and mt-CRISPR/Cas9 system (26). ZFN is a synthetic nuclease constructed

by adding a zinc-finger DNA-binding domain to a DNA-cleavage domain of a restriction enzyme, Fok1, which can recognize three nucleotides (19), and mt-ZFN contains both MTS and a nuclear export sequence that efficiently localizes mt-ZFN in mitochondria (23, 27). Studies have shown that mt-ZFN selectively removes mt-DNA containing a large deletion that causes human mitochondrial diseases, as well as restored biochemical defects in models of mitochondrial diseases (27). It also reduced heteroplasmy by eliminating mutant mt-DNA, which rescued defects in a cardiac disease mouse model (28).

On the other hand, TALEN is based on transcription activator-like elements, which can recognize a single nucleotide instead of the triplet nucleotides recognized by ZFN. In the same way, transcription activator-like elements are conjugated with nuclease, Fok1, and are imported into mitochondria by adding an MTS and UTR sequence from *ATP5B* and *SOD2*. Mt-TALEN has been used to cleave mutated mt-DNAs found in Leber's hereditary optic neuropathy (LHON), dystonia, myoclonic epilepsy with ragged red fibers, and MELAS/Leigh syndrome in osteosarcoma cells (25, 29). Since the mt-ZFN and mt-TALEN editing systems require different types of DNA-binding proteins to remove various mutated mt-DNAs, it is still challenging to obtain the right combination of zinc-finger proteins or transcription activator-like effectors that correspond to each nucleotide.

The mt-CRISPR using mitochondria-targeted Cas9 and guide RNA (gRNA) has also been used to manipulate the mitochondrial genome (26). However, it is still controversial whether gRNA is properly translocated into mitochondria, since RNAs are difficult to import into mitochondria without specific sequences that form a stem loop structure. Nonetheless, if the CRISPR/Cas9 system efficiently works in mitochondria, it would be a useful tool for adding or deleting specific sequences.

Another method for manipulating mt-DNA is by applying a mitochondrial targeted adeno-associated virus. A modified virus capsid protein VP2 containing wild-type *ND4* was delivered into mitochondria having a mutation in *ND4* and successfully rescued the defective

phenotypes shown in a cell-culture model of LHON (30). However, a mitochondria-targeted adeno-associated virus has a size limit of ~5 kb, which restricts its use in treating mitochondrial genome mutations. Although current editing tools still have limitations, improved genome-editing machinery in the future will surely be successful at treating mitochondrial diseases caused by mutations in the mitochondrial genome.

Visualization of mitochondrial DNA

The location of mt-DNA provides clues to how mitochondrial gene expression is regulated during different conditions (31, 32). Endogenous mitochondrial DNA in the intact cell can be visualized by incorporating ethidium bromide (EtBr), SYBR dye, and thymine analogues, such as bromodeoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU), to DNA (Fig. 1B) (33-38). EtBr staining is a general method used to visualize DNA. However, EtBr enlarges mitochondrial nucleoids and inhibits mt-DNA replication (39), which makes EtBr usage more suitable for other purposes. For example, EtBr has been used to deplete mt-DNA and to investigate the effects of reduced mt-DNA on mitochondrial functions (40). SYBR dye labels mitochondrial nucleoids without affecting cell viability, and thus can also be used to establish time-lapse images (34, 35). Both BrdU and Edu detect nascent mt-DNA generated by mitochondrial-genome replication (36-38). However, BrdU incorporation requires a stringent DNA denaturation step, which renders low reproducibility (35). Thus, quantification analysis using BrdU incorporation becomes less reliable. On the other hand, Edu incorporation does not require the denaturation step, but is less sensitive than is BrdU (35). Hence, a combination of BrdU and Edu incorporation has been used to label nascent mt-DNAs (41). To visualize the endogenous mt-DNA, FISH with specific probe sets for target genes is used to detect the location and expression level of mt-DNA (Fig. 1C) (42). Various cellular events and disease conditions can induce heteroplasmy (22). Using mt-DNA FISH containing a mixture of 60 unique mt-DNA specific probes, the spatiotemporal regulation of

the mt-DNA level in primordial germ cells was clearly observed (43). Furthermore, the authors discovered that changes in the mt-DNA copy number and mitochondrial distribution alter germline development in *D. melanogaster* (43). In addition to detecting circular mt-DNA, mt-DNA FISH can be used to detect a small portion of mt-DNAs in nuclear DNAs (44). Relocation of mt-DNA into the nucleus, or nuclear mitochondrial DNA (NUMT), was discovered to occur during tumorigenesis by using mt-FIBER FISH (44). Also, there is another method called the mitochondrial transcription and replication imaging protocol (mTRIP) that combines with DNA-FISH, RNA-FISH, and immunofluorescence of mitochondrial protein to simultaneously visualize mt-DNA, mt-RNA, and proteins (45). However, all the FISH experiments have a limitation of temporal resolution because fixed samples are used.

Mitochondrial transcription factor A (TFAM) is an mt-DNA binding protein regulating mitochondrial transcription and mt-DNA compaction (46); so fluorescent protein-tagged TFAM is widely used to analyze mt-DNA dynamics in live cells (Fig. 1D). Using TFAM, it was discovered that mt-DNA is released from mitochondria during apoptosis to activate the innate immune pathway (47), and that ER-mitochondria tethering regulates mitochondrial fission and mt-DNA replication (32). However, the use of TFAM requires careful interpretation, since TFAM overexpression increases the mt-DNA copy number and upregulates mitochondrial transcription (48, 49).

Mitochondrial RNA

RNA import into mitochondria

When two strands of the mitochondrial genome are transcribed, two polycistronic RNA transcripts containing all mitochondrial genes are generated (12), indicating that extensive RNA processing is important for gene expression. For this reason, normal sequencing does

not yield insights into mechanism of RNA processing in mitochondria. To overcome this limitation, a method of preparing circularization of RNA prior to sequencing was developed, to identify and characterize various intermediate processing products in mitochondria (50). Furthermore, RNA import into mitochondria plays key roles in regulation of mitochondrial DNA replication, transcription, and translation, and only a few factors involved in these processes are known (51, 52). One of them is polynucleotide phosphorylase (PNPASE), which transports RNAs containing a specific stem-loop structure (Fig. 1E) (52). RNase P, mitochondrial ribosomal protein, and 5S rRNAs are targets of PNPASE-mediated mitochondrial importing (52). Using the targeted mitochondrial RNA import, phenotypes of human mitochondrial DNA disease models were partially rescued (53), suggesting that mitochondrial RNA import may provide a new opportunity to treat various human diseases containing mutations in the mitochondrial genome.

microRNAs in mitochondria

Nuclear-encoded microRNAs (miRNAs) are transported into mitochondria to regulate expression of mitochondrial-encoded DNA (54). The miRNA binds to an RNA-induced silencing complex (RISC) that helps it to target specific mRNAs (55). The argonaute protein family is the essential component of RISC in the cytoplasm, but argonaute 2 (Ago2) is present in mitochondria (56). Ago2 is discovered in mitochondria as a mitochondrial tRNA^Met associated protein (57). Small RNA sequencing and microarray profiling revealed 13-20 miRNAs in mitochondria (54); miR-181c-5p and miR-146a-5p may regulate 19 potential targets in mitochondria (*12S RNA, 16S RNA, ND1, ND2, ND4, ND5, ND6, CO1, CO2, CO3, ATP6, ATP8, Cytb, tRNA^Ala, tRNA^Glu, tRNA^Gly, tRNA^Ser(UCN), tRNA^Ser(AGY), and D-loop*) (58). Since miR-378 targets mitochondrial *ATP6*, miR-378 overexpression leads to accumulation of miR-378 in mitochondria and reduces the level of ATP6 (59). miR-181c targets mt-*CO1* and its overexpression leads to heart failure *in vivo* (60). miR-1, a muscle-specific miRNA in

C2C12 cell, improves *CO1, ND1, CO3*, and *ATP8* translation (61). The mechanism by which miRNAs are transported into the mitochondrial matrix, however, is not known. It is plausible that a complex of Ago2 and miRNA is delivered by vesicle uptake into mitochondria (54), or that pre-miRNAs are translocated by a PNPASE-mediated mechanism before being processed by an Ago2 protein (Fig. 1E) (54). It is also possible that miRNA biogenesis occurs in mitochondria, since pre-mRNAs are found in the mitochondrial matrix (62). Interestingly, miR-1974, miR-1977, and miR-1978 in humans are suggested to be encoded by mt-DNA (56).

Visualization of mitochondrial RNA

FISH can be used to visualize mitochondrial RNAs on fixed samples but not living cells (63). However, a genetically encoded fluorescent probe that can visualize the location and dynamics of mt-RNAs in living cells has been developed (64). Two identical RNA recognition motif domains of human PUMILIO1 protein (PUM-HD) are modified differently to recognize 16 different nucleotide sequences in tandem (65), followed by tagging with different split fragments of fluorescent protein (Fig. 1F) (64). These probes are then located in mitochondria using a mitochondrial targeting sequence. Mitochondrial RNA of *NADH dehydrogenase 6* is successfully visualized, and the dynamics of *ND6* mRNA in the oxidative condition is analyzed (64) using this tool. Although single-molecule imaging of cytoplasmic translation is achieved by combining SunTag and MS2 stem loops (66-68) based on chimeric RNAs (66-68), it is still challenging to apply the same technique to mitochondrial study, because of difficulties in importing engineered mitochondrial mRNAs into mitochondria.

Mitochondrial protein

Labeling proteins synthesized in mitochondria

To directly study mitochondrial translation, methods were developed to monitor the levels of 13 proteins encoded by mt-DNA. Pulse-chase labeling of a mitochondrial translation product can be used to investigate the efficiency of mitochondrial translation under different conditions (69-71). Like labeling cytoplasmic translation products, radioactive isotopes, such as [^{35}S]-methionine or a [^{35}S]-methionine/cysteine mixture, can label nascent proteins in mitochondria (Fig. 1G) (63, 70-74). To exclude contamination of cytoplasmic translation products, a cytosolic translation inhibitor, such as emetine or anisomycin, should be added to methionine or methionine/cysteine-free media before labeling mitochondrial translation products (63, 70-74). Mitochondrial translation inhibitors, such as actinonin, chloramphenicol, or erythromycin, are also needed for a negative control (63, 70-74). SDS-PAGE followed by autoradiography can measure the efficiency of mitochondrial translation (63, 70-74). Furthermore, mitochondrial initiation factor 2 and 3 are discovered as an activator of mitochondrial translation initiation by using the labeling method (75-77).

In situ imaging of mitochondrial translation

A nonradioactive method that detects newly synthesized proteins in mitochondria has been developed (78). L-homopropagylglycine (HPG), a methionine analog that contains an alkyne moiety, can be incorporated into an actively translating peptide in mitochondria, which can be visualized by a click-reaction between the alkyne moiety of HPG and a fluorescent azide or biotin azide (Fig. 1G). Only nascent proteins from mitochondria are detected if cycloheximide, a cytoplasmic translation inhibitor, is treated (78). Using this *in situ* imaging technique, Estell et al. observed that mitochondrial translation has a poor correlation with the mitochondrial DNA amount, and that mitochondrial translation is not decreased during mitosis (78).

Summary

Energy production in cells is strongly correlated with the efficiency of the OXPHOS system, and OXPHOS dysfunction is frequently found in various human mitochondrial diseases (79). Since all mitochondrial protein products become components of OXPHOS, it is crucial to precisely regulate mitochondrial gene expression. In the near future, we expect that development of the CRISPR/Cas 9 system together with tools that regulate RNA will rescue dysfunctional mitochondrial gene expression, which will provide intervention therapy for human diseases caused by defective mitochondria.

Acknowledgements

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Fig. 1. An overview of biochemical and imaging tools for investigating mitochondrial

gene expression. Mitochondrial DNA (A) mt-ZFN and mt-TALEN use a zinc-finger DNA-binding domain and transcription activator-like elements, respectively, and each links to a DNA-cleavage domain of the restriction enzyme, Fok1. (B) EtBr or SyBr binds to double-stranded mt-DNA, whereas BrdU and EdU can incorporate into mt-DNA during mt-DNA replication, which allows intact mt-DNA to be visualized in live cells. (C) An mt-DNA specific sequence can be detected by using a FISH probe. (D) GFP-tagged TFAM is also used to visualize mt-DNA in live cells. **Mitochondrial RNA** (E) Three possible mechanisms of RNA transport into mitochondrial matrix are suggested: (1) PNPASE transports RNA of *RNase P*, RNAs of *mitochondrial ribosomal proteins*, and *5S rRNAs* by recognizing the stem-loop structure of their RNAs; (2) miRNA can be delivered to mitochondria by Ago2; (3) PNPASE also translocates pre-miRNA into mitochondria. (F) RNA recognition motif domains of human PUMILIO1 are tagged with split fragments of EGFP. When the two split EGFPs are reassembled by binding to ND6 mRNA, EGFP signals appear and the dynamics of ND6 mRNA can be visualized. **Mitochondrial protein** (G) Mitochondria-specific metabolic labeling can be achieved by using isotope or non-canonical amino acids while inhibiting cytoplasmic translation. Isotope-labeled mitochondrial proteins are separated by SDS-PAGE. Azide-conjugated non-canonical amino acids are detected by biotin or a fluorescent dye through click reaction.
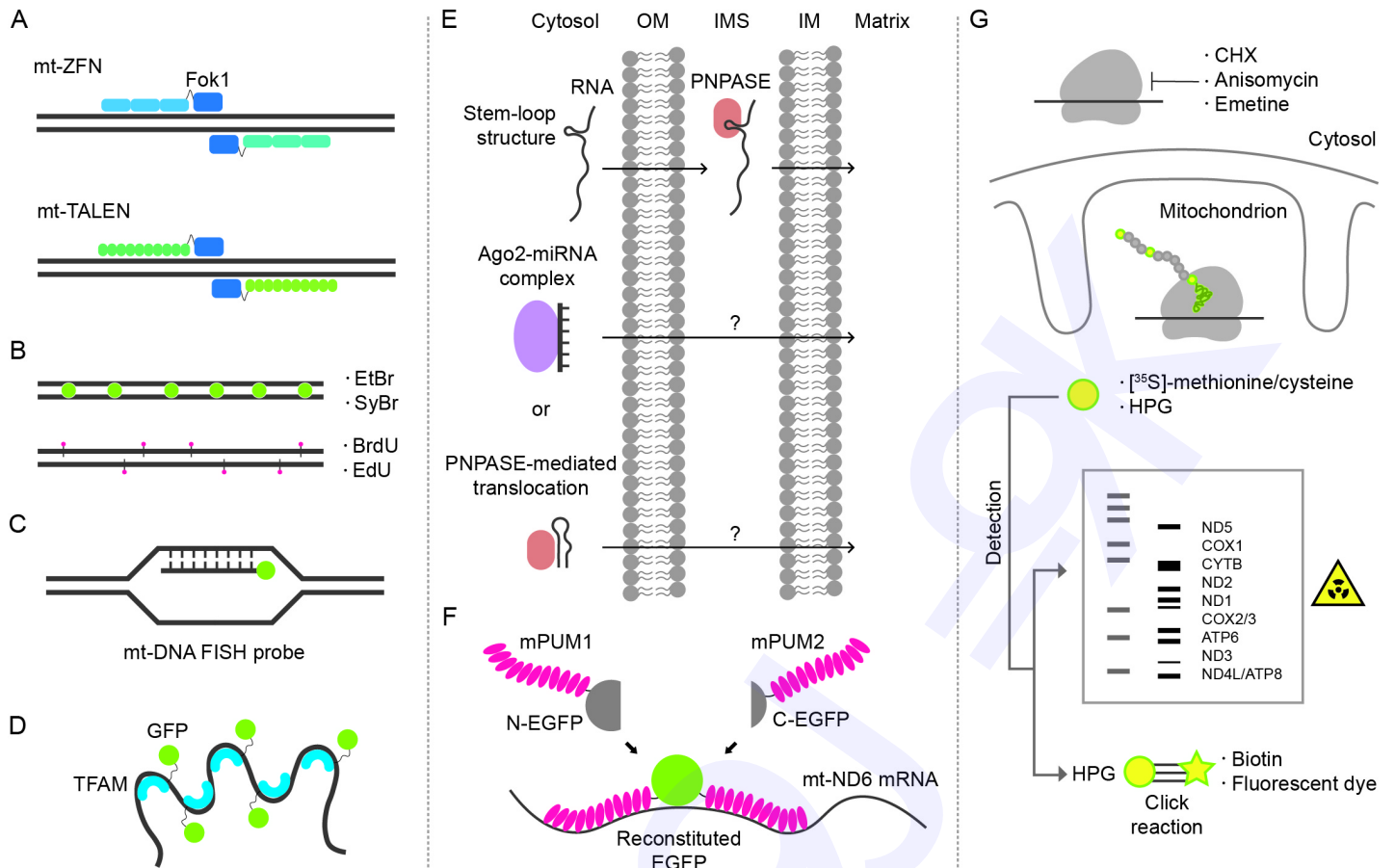
Mitochondrial DNA

A

mt-ZFN
Fok1

mt-TALEN

B
· EtBr
· SyBr

· BrdU
· EdU

C
mt-DNA FISH probe

D
GFP
TFAM

Mitochondrial RNA

E
Cytosol OM IMS IM Matrix

RNA PNPASE
Stem-loop
structure

Ago2-miRNA
complex
?
or

PNPASE-mediated
translocation
?

F
mPUM1 mPUM2

N-EGFP C-EGFP

mt-ND6 mRNA
Reconstituted
EGFP

Mitochondrial protein

G
· CHX
· Anisomycin
· Emetine

Cytosol

Mitochondrion

· [^{35}S]-methionine/cysteine
· HPG

Detection

ND5
COX1
CYTB
ND2
ND1
COX2/3
ATP6
ND3
ND4L/ATP8

HPG
· Biotin
· Fluorescent dye
Click
reaction

Fig. 1.