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Review:

Cell models and drug discovery for mitochondrial diseases*

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Abstract: Mitochondrion is a semi-autonomous organelle, important for cell energy metabolism, apoptosis, the production of reactive oxygen species (ROS), and Ca^{2+} homeostasis. Mitochondrial DNA (mtDNA) mutation is one of the primary factors in mitochondrial disorders. Though much progress has been made, there remain many difficulties in constructing cell models for mitochondrial diseases. This seriously restricts studies related to targeted drug discovery and the mechanism and therapy for such diseases. Here we summarize the characteristics of patient-specific immortalized lymphoblastoid cells, fibroblastoid cells, cytoplasmic hybrid (cybrid) cell lines, and induced pluripotent stem cells (iPSCs)-derived differentiation cells in the study of mitochondrial disorders, as well as offering discussion of roles and advances of these cell models, particularly in the screening of drugs.

Key words: Mitochondrial diseases; Mitochondrial DNA; Cell model; Drug discovery
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1 Introduction

In many mitochondrial diseases, mitochondrial dysfunction triggers profound clinical manifestations, especially those relating to defects in oxidative phosphorylation. Incidences of mitochondrial diseases are conservatively estimated to occur in one in 6500 people (Yan and Guan, 2008). Primary mutations in the mitochondrial DNA (mtDNA) could disrupt mitochondrial function and contribute to mitochondrial diseases. These mainly affect tissues and organs that have high energy requirements, including those of the heart, brain, and skeletal muscle (Wallace, 2013). Mitochondria are semi-autonomous organelles, which play central roles in cell energy production, regulation of apoptosis, generation of reactive oxygen species (ROS), and calcium homeostasis. Mitochondrial functions are cooperatively controlled by nuclear DNA (nDNA) and mtDNA. While nDNA encodes approximately 1500 mitochondrial proteins, mtDNA encodes only 13 polypeptide components of the electron transport chain complexes (ETCs), as well as the 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) in the mitochondrion. Since the mtDNA mutation and deletion were first reported to be associated with mitochondrial diseases in 1988 (Holt et al., 1988; Wallace et al., 1988), hundreds of variants in mtDNA related to mitochondrial diseases have now been identified.

Drug discovery is the preliminary process of detecting and studying the pharmacological activity of potential therapeutic compounds. Traditionally, drug discovery is based on the search for target

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molecules, which possibly represents the primary focus of the pharmaceutical industry. However, identifying disease-related drug targets is rarely a straight forward or simple process. One of the reasons is the existence of compensation mechanisms and feedback pathways in the complex cellular environment, which often causes the targeting and therapeutic efficacy of drugs to be greatly reduced, even when the drug has been previously demonstrated to be effective at the molecular level in vitro (Inak et al., 2017). In this way, attempts at target-based drug discovery not only cost time and money, but also have less success. Currently, phenotypic screening has regained momentum and is receiving extensive attention. The discovery of phenotypic drugs initially requires the identification of disease-specific features. Although cellular or animal models of nuclear gene mutation-associated diseases can be generated by techniques such as gene knockout, knockdown, or editing, there are still many difficulties in constructing cell or animal models for mitochondrial diseases. Every mammalian cell contains hundreds or even thousands of mitochondria with lipid bilayer membranes. Each mitochondrion contains multiple mtDNA copies. Specific and efficient genetic operating systems have not yet been established for site-directed mutagenesis or modification of mtDNA (Wallace and Fan, 2009; Farrar et al., 2013). Currently, somatic cell nuclear transfer (SCNT) technology has been shown to be capable of replacing mtDNA of fibroblasts from Leigh syndrome patients harboring the m.8993T>C mutation (Ma et al., 2015). The pathogenic mitochondrial mutations of the oocyte have been exchanged using mitochondrial replacement therapy (Kang E et al., 2016), and "three-parent babies" have been born based on mitochondrial donation linked with in vitro fertilization (IVF) (Reardon, 2016). Nevertheless, for mitochondrial replacement therapy, ethical and practical questions remain. The possibility that phenotypic changes may result due to the mismatch between nDNA and mtDNA is of concern and overall, the whole process is still hampered by technical difficulties and high costs (Inak et al., 2017). Up to the present, peripheral blood lymphocytes or fibroblasts from patients, and their derived cytoplasmic hybrid (cybrid) cells have been the main focus for conventional strategies of the study of mitochondrial diseases (Wu et al., 2016). However, in recent years, patient-specific induced pluripotent stem cells (iPSCs) reprogrammed from somatic cells and iPSC-derived differentiation cells have been widely recognized as providing new opportunities for novel cell models of disease (Yamanaka, 2007).

Here we summarize the generation and characteristics of patient-specific immortalized lymphoblastoid cells, fibroblastoid cells, cybrid cells, and iPSC-derived cells in mitochondrial disorders. We assess the roles and advances of these cell models in the screening of mitochondrial drugs, and in the light of these, analyze and discuss the existing problems and potential developmental directions.

2 Lymphoblastoid cells and fibroblasts for mitochondrial drug discovery

Immortalized lymphoblastoid cell lines are produced from the transformation of peripheral blood B lymphocytes by the Epstein Barr virus (EBV) (Amoli et al., 2008). There are several advantages of using immortalized lymphoblastoid cells as a drug screening model for mitochondrial diseases. Firstly, large quantities of lymphocytes are available and can be easily obtained from human peripheral blood. Secondly, lymphocytes can be immortalized effectively to proliferate indefinitely (Sun et al., 2016). Finally, cells carrying mtDNA mutations can perpetually maintain their biological properties at a cellular level. A large proportion of the mitochondrial drugs that have been approved or are currently being developed are small molecule compounds. The efficiency and accuracy of mitochondrial drug discovery can be improved greatly using such cell models of disease. To evaluate the use of patient-derived lymphoblastoid cell lines for small molecule drug discovery in mitochondrial disease, Chin et al. (2018) constructed five immortalized lymphoblastoid cell lines harboring mtDNA point mutations. Idebenone, an analog of coenzyme Q (CoQ), increased the basal respiration of Leber's hereditary optic neuropathy (LHON) lymphoblastoid cell lines harboring the m.3460G>A variant in *MT-ND1* or the m.11778G>A variant in *MT-ND4*, but not in Leigh disease cells harboring the m.8993T>G variant in *MT-ATP6* (Chin et al., 2018). GNE-7915, a leucine-rich repeat kinase 2 (LRRK2) inhibitor, could repair the mtDNA damage

caused by the LRRK2 p.G2019S mutation associated with Parkinson's disease (Howlett et al., 2017). These results indicate that patient-specific immortalized lymphoblastoid cells carrying mtDNA variants can exhibit different sensitivities to the same drug. This reveals the potential value of lymphoblastoid as a mitochondrial drug screening cell model.

Fibroblasts are much enriched and easily obtained during a muscle biopsy. Fibroblast cultures are highly proliferative and provide a renewable source of cells in vitro. Patient's fibroblasts harboring an mtDNA variant may provide a suitable platform for the search of small molecules for the treatment of mitochondrial disease (Saada, 2011). The deficiency of the mitochondrial ETC complex I (nicotinamide adenine dinucleotide (NADH) ubiquinone oxidoreductase) is the most common form of oxidative phosphorylation (OXPHOS) defect. It has no known effective cure and results in decreased ATP, increased ROS, and imbalanced NAD^+/NADH ratio due to NADH accumulation (Pfeffer et al., 2013). Mitochondria-targeted antioxidants, succinate or short chain quinones, could bypass the defective complex I and directly transfer electrons to the next complexes. This is the main treatment strategy of complex I defects (Pfeffer et al., 2013; Koopman et al., 2016). Saada (2011) evaluated the effectiveness of the drug by measuring the ATP content of a fibroblast cell line with complex I deficiency. However, in clinical trials, different responses to the same drug have been found from different patients, suggesting that it is necessary to test the therapeutic effect of drugs on individual patients, such that patient-specific cell models may be necessary in order to deliver personalized therapy. The m.3243A>G (*MT-TL1*) variant is one of the major pathogenic mutations in mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS). In this condition, a decrease in mitochondrial membrane potential and mitochondrial respiratory chain enzyme activity was found in fibroblasts, together with the activation of autophagy. All of these abnormalities were significantly restored after CoQ treatment (Cotán et al., 2011). Antioxidants such as *N*-acetylcysteine (NAC) and dihydrolipoic acid have been applied to treat patients with neuropathy, ataxia, and retinitis pigmentosa (NARP) fibroblasts harboring the m.8993T>G variant. In this treatment, mitochondrial function was recovered with a notable increase in mitochondrial oxygen consumption rate and ATP synthesis (Mattiazzi et al., 2004). Overall, patient-derived fibroblasts are suitable cell models for drug discovery. More information about immortalized lymphocytes and fibroblasts as models of drug discovery is available in Table 1.

3 Cytoplasmic hybrid cells for mitochondrial drug discovery

Mitochondrial function is controlled by both nuclear and mitochondrial genes. Immortalized lymphocytes or fibroblasts harboring the same pathogenic mtDNA variants derived from the same pedigree may exhibit different mitochondrial function resulting from their different nuclear genes. In order to eliminate the interference of this nuclear background, the nucleus was removed from patient-derived cells such as immortalized lymphocytes or fibroblasts, and the cytoplasts were fused with ρ^0 cells lacking mtDNA. After screening, cybrid cell lines with a consistent nuclear background were constructed. This provided an important and effective cell model for mitochondrial disorders reflecting the effects of mtDNA mutations on mitochondrial function (Wilkins et al., 2014).

Table 1 Lymphoblastoid cells and fibroblasts for mitochondrial drug discovery

Cell model	Disease	Variant site	Drug	Reference
Lymphoblastoid cells	LHON	m.3460G>A	Idebenone	Chin et al., 2018
	LHON	m.11778G>A	Idebenone	Chin et al., 2018
Fibroblastoid cells	MELAS	m.3243A>G	CoQ_{10}	Cotán et al., 2011
	MELAS	m.3243A>G	Riboflavin or CoQ_{10}	Garrido-Maraver et al., 2012
	NARP and MILS	m.8993T>G	*N*-acetylcysteine; dihydrolipoic acid	Mattiazzi et al., 2004
	MERRF	m.8344A>G	CoQ_{10}	de la Mata et al., 2012

LHON: Leber's hereditary optic neuropathy; MELAS: mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; NARP: neuropathy, ataxia, and retinitis pigmentosa; MILS: maternally inherited Leigh syndrome; MERRF: myoclonic epilepsy with ragged red fibers; CoQ: coenzyme Q

This cybrid cell model is of great value for mitochondrial drug discovery. Barrow et al. (2016) designed and developed a high-throughput in-cell enzyme-linked immunoassay based on the mitochondrial cell model and identified the I-BET 525762A, a bromodomain inhibitor, from 10015 small molecule compound libraries. This could lead to the dramatic accumulation of the complex IV subunit COX5A expression levels, and protect the cybrids with the oxidative respiratory chain complex I mutation (m.3796A>G, found in adult onset dystonia) from cell death. Since cells are forced to produce ATP by oxidative phosphorylation in media with galactose, they found that m.3796A>G mutant cybrids died within 72 h while I-BET 525762A-treated cells could still survive. After performing genome-wide editing clustered regularly interspaced short palindromic repeat (CRISPR) screening, the researchers confirmed that I-BET 525762A could remodel the mitochondrial proteome and increase the expression and activity of OXPHOS protein complexes, which rescued from the bioenergetic defects and cell death caused by complex I variants (Barrow et al., 2016). NARP and maternally inherited Leigh syndrome (MILS), which are both mitochondrial disorders, could be attributed to the m.8993T>G mutation. Fibroblasts from patients with NARP tend to undergo apoptosis and abnormal OXPHOS with decreased ATP synthesis, owing to a large amount of generated free radicals. Mattiazzi et al. (2004) found that antioxidants such as NAC and dihydrolipoic acid (DHLPA) could rescue mitochondrial functions, including aerobic respiration and ATP synthesis in transferring mitochondria cells with the m.8993T>G variant. Cybrid cells carrying the same mutation also present CoQ_{10} deficiency and elevated autophagy activity. All these abnormal phenotypes were ameliorated after CoQ treatment. This was in accordance with m.3243A>G variant fibroblasts (Cotán et al., 2011). β-Lapachone (β-lap) is a substrate of NAD(P)H:quinone oxidoreductase 1 (NQO1), which can increase significantly energy production, reduce ROS and lactate generation, and restore mitochondrial content in MELAS cybrid cells (Jeong et al., 2014). In general, patient-derived mitochondrial cybrids are promising cell models in drug screening and may be applied equally to other mitochondrial diseases. More information about cybrid cells as models of mitochondrial disease drug screening is shown in Table 2.

4 iPSCs and iPSC-derived cells for mitochondrial drug discovery

The phenotypes that result from mtDNA mutations often exhibit tissue specificity where lymphocytes or fibroblasts, as common models, have significant differences in morphology, structure, and function from the cells of affected tissues. Whether such models can reflect the real functions and characteristics of the such tissues remains an area of debate. iPSCs are a class of cells reprogrammed from terminally differentiated cells. They are identical to embryonic stem cells in self-renewal and multi-directional differentiation potential. iPSCs have attracted great attention as a new disease cell model due to their abundant cell sources, unlimited powers of proliferation, and ability to differentiate into various functional cells. During the process of reprogramming and directed differentiation, disease-specific iPSCs derived from somatic cells of patients retain the disease-causing genes.

Table 2 Cytoplasmic hybrid cells for mitochondrial drug discovery

Disease	Variant site	Drug	Reference
MELAS	m.3243A>G	CoQ_{10}	Cotán et al., 2011
	m.3243A>G	Riboflavin or CoQ_{10}	Garrido-Maraver et al., 2012
	m.3243A>G	β-Lapachone	Jeong et al., 2014
MERRF	m.8344A>G	CoQ_{10}	de la Mata et al., 2012
Adult onset dystonia	m.3796A>G	I-BET 525762A	Barrow et al., 2016
NARP and MILS	m.8993T>G	*N*-acetylcysteine or dihydrolipoic acid	Mattiazzi et al., 2004
NARP	m.8993T>G	Sodium pyrithione	Aiyar et al., 2014
	m.8993T>G	Chlorhexidine or oleic acid	Couplan et al., 2011

MELAS: mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; MERRF: myoclonic epilepsy with ragged red fibers; NARP: neuropathy, ataxia, and retinitis pigmentosa; MILS: maternally inherited Leigh syndrome; CoQ: coenzyme Q

Recently, we generated patient-specific iPSCs and iPSC-derived cardiomyocytes (iPSC-CMs) from a maternally inherited hypertrophic cardiomyopathy (HCM) family who carry the m.2336T>C variant in the mitochondrial 16S rRNA gene (*MT-RNR2*) (Li et al., 2018). Results showed that m.2336T>C variant led to enlarged cell size, increased action potential delay after depolarizations (DADs), and prolonged action potential duration (APD), all of which were similar to that observed in HCM cardiomyocytes. Functional research on such cells has shown that the ATP/ADP ratio and mitochondrial membrane potential ($\Delta\psi_m$) are also reduced, thereby elevating the intracellular Ca^{2+} concentration and decreasing in L-type Ca^{2+} current (I_{CaL}) (Li et al., 2018). Our findings therefore provide an ideal model for both the study of the pathogenesis of maternally inherited HCM and for drug discovery. Several other patient-specific iPSCs carrying mtDNA mutations and iPSC-derived cell models have also been generated. These include a 2.5-kb deletion of mtDNA associated with Pearson bone marrow pancreatic syndrome (PS) (Cherry et al., 2013), the m.3243A>G variant in mitochondrial causing MELAS (Ma et al., 2015) or diabetes (Fujikura et al., 2012; Matsubara et al., 2018), the m.8344A>G variant correlated with myoclonic epilepsy with ragged red fibers (MERRF) syndrome (Chou et al., 2016, 2018), the m.11778G>A variant implicated in LHON (Lu et al., 2018; Wu et al., 2018), and the m.1555A>G variant related to sensorineural hearing loss (SNHL) (Hsu et al., 2017).

The iPSC-derived cells from patients were able to model the specific related pathology (Ebert et al., 2009; Kang JF et al., 2016), which could be rescued after drug therapy. Lorenz et al. (2017) generated iPSCs and their differentiated neural progenitor cells (NPCs) carrying the m.9185T>C variant in MT-ATP6 related to Leigh syndrome. Using high-throughput screening, it was found that avanafil as a phosphodiesterase type 5 (PDE5) inhibitor could dramatically improve the mitochondrial membrane potential and adjust mitochondrial calcium homeostasis, which in turn rescued the function of patient's NPCs and differentiated neurons (Lorenz et al., 2017). mtDNA depletion syndrome 3 (MTDPS3) is a kind of genetic disorder characterized by mtDNA depletion and comprised ATP production, while deoxyguanosine kinase (DGUOK) deficiency is among the most common causes of hepatic mtDNA depletion syndrome (Cámara et al., 2014). $DGUOK^{-/-}$ iPSCs were generated using CRISPR/CRISPR-associated protein 9 (CAS9) and iPSC-derived hepatocyte-like cells to identify drugs that could elevate ATP synthesis in DGUOK-deficient hepatocytes by phenotypic screening. It was found that NAD treatment resulted in an improvement in intracellular NAD^+ levels, promoting Sirtuin 1 (SIRT1)-mediated peroxisome proliferator-activated receptor-γ coactivator-1-α (PGC-1α) activation. Addition of NAD also increased the levels of messenger RNAs (mRNAs) of fatty acid oxidation (FAO) and tricarboxylic acid (TCA) cycling, indicating a boost in fatty acid and glucose metabolism, which further strengthened mitochondrial activity and ATP synthesis (Jing et al., 2018).

iPSCs, along with iPSC-derived cells, still have a long way to go for drug discovery. Differentiating iPSCs into specific cells not only takes time, but also tends to lead to heteroplasmy when cells differentiated from different batches of iPSCs are compared (Cherry et al., 2013). In addition, iPSC-derived cells as a drug screening model have certain limitations. Since human beings represent an integrated and complex system, different types of cells can interact with each other. Even though iPSCs can differentiate into specific cells, it is difficult to totally imitate the pathophysiological condition of the human body (Cámara et al., 2014). Improving the differentiation efficiency of iPSCs and studying how to effectively simulate complex human physiological states are key areas for future research.

5 Conclusions

It should also be noted that there are some limitations of the above cell models of mitochondrial diseases for drug screening. Mitochondrial disorders usually affect high energy demand tissues. Lymphoblasts and fibroblasts are not considered as high energy demand. Cybrid cells disrupt the patient-specific interaction between nDNA and mtDNA, as all nDNA-encoded mitochondrial proteins in cybrids come from ρ^0 cells (Inak et al., 2017). Human iPSC-derived cells exhibit high phenotypic immaturity and variability, which presents some differences compared with adult terminally differentiated cells (Boulting et al., 2011;

Sala et al., 2017). On the other hand, with the continuous improvement of high throughput drug screening technology, intelligent information collection and analysis, and a small molecule compound library and natural product library, it will be possible to miniaturize, scale, automate, and intellectualize phenotype drug screening. Appropriate cell models thus contribute to providing insights into the pathogenesis of mitochondrial disorders with mtDNA mutations, and shortening the period of drug discovery for mitochondrial diseases.

Contributors

Qing-feng YAN conceived and designed the review. Shuang-yi HU, Qian-qian ZHUANG, Yue QIU, and Xu-fen ZHU collected the references and wrote the manuscript. Xu-fen ZHU and Qing-feng YAN revised the manuscript. All authors read and approved the final manuscript.

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Compliance with ethics guidelines

Shuang-yi HU, Qian-qian ZHUANG, Yue QIU, Xu-fen ZHU, and Qing-feng YAN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：线粒体疾病细胞模型及其药物筛选

概　要：线粒体是一种半自主性细胞器，不仅是细胞能量代谢的重要场所，而且与细胞凋亡、活性氧（ROS）产生和 Ca^{2+}稳态等密切相关。线粒体DNA（mtDNA）突变是线粒体疾病发生的重要原因。当前，线粒体疾病细胞模型构建仍存在许多困难，严重制约线粒体疾病的致病机制、靶向药物筛选和临床治疗等研究。本文将重点介绍线粒体疾病细胞模型的构建和特征，包括患者特异性永生化淋巴细胞模型、成纤维细胞模型及其衍生的转线粒体细胞模型和诱导多能干细胞及其定向分化细胞模型，以及这些细胞模型在线粒体药物筛选中的作用和研究进展。

关键词：线粒体疾病；线粒体 DNA；细胞模型；药物筛选