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Complex Regulation of Mitochondrial Function During Cardiac Development

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Abnormal heart development is a key feature contributing to the cardiac defects of congenital heart diseases, which are the most common birth defects in humans, occurring in 1% to 5% of newborns.[1–4] Revealing the cellular, genetic, and molecular mechanisms that govern normal heart development will provide key information necessary for the development of effective clinical applications to prevent or treat congenital heart diseases.

The heart is the first organ formed in mammals and is essential for embryo survival.[5–10] Cardiogenesis involves a series of complicated and precisely controlled processes (Figure 1). During gastrulation in mice, the cardiac progenitor cells enter the primitive streak and assimilate to constitute the primary heart field in the anterior splanchnic mesoderm at embryonic day 6.5 (E6.5). Cardiogenic mesodermal cells then coalesce to form a cardiac crescent at E7.5.[11,12] These cells move toward the ventral midline and form the linear heart tube at E8.0. At this stage, another group of cardiac precursor cells from the second heart field join the primitive heart tube from the pharyngeal mesoderm and dorsal mesocardium.[10–16] Shortly thereafter, the linear heart undergoes rightward looping to bring the future inflow chambers (atria) to the top of the outflow chambers (ventricles). At E9.5, a heart with 4 primordial chambers is formed, and cardiac precursor cells from the second heart field continue to join the hearts from both arterial and venous poles until E11.5.[10–16] Cells derived from the primary heart field give rise to the left ventricle and parts of the atria, whereas cells derived from the second heart field generate the right ventricle, outflow tract (OFT) and main parts of the atria.[10–16] From E11.5 to E18.5, embryonic hearts undergo complicated maturation and remodeling processes including myocardial wall trabeculation and compaction, septation between chambers, septation of the OFT, valvulogenesis, formation of coronary vessels, and formation of the conduction system. In mice, nearly all cardiomyocytes withdraw from the cell cycle and undergo maturation during the first 2 weeks after birth.[17–19] In contrast to the hyperplastic growth observed in embryos, postnatal cardiac growth primarily relies on hypertrophic growth.[17–19] The major processes of cardiogenesis are highly conserved between rodent and human; therefore, mice and rats serve as power model systems to study human heart development.[20]

Accumulated evidence suggests that mitochondria are critical for embryonic heart development in mammals, in addition to their well-established roles in postnatal hearts. Impaired mitochondrial activities in embryonic hearts may lead to severe cardiomyopathy and embryonic/neonatal lethality in animal models and human patients.[21–33] This review will summarize recent knowledge regarding the regulatory roles of mitochondria during embryonic and early postnatal heart development in mammals.

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Mitochondrial Structure and Function

Mitochondria arose ≈2 billion years ago through engulfment of α-proteobacteria by precursors of modern eukaryotic cells and have evolved to become a vital organelle with multiple activities in eukaryotes.[34–38] Mitochondria are composed of 2 lipid bilayer membranes (ie, the relatively permeable outer membrane and the much less permeable, highly folded inner membrane) and 2 aqueous spaces (ie, the intermembrane space and the matrix)[39,40] (Figure 2). The outer membrane serves as the boundary between the cytoplasm and mitochondria. It contains multiple receptors to mediate communication between mitochondria and other organelles.[41,42] The inner membrane is folded into cristae protruding into the matrix and

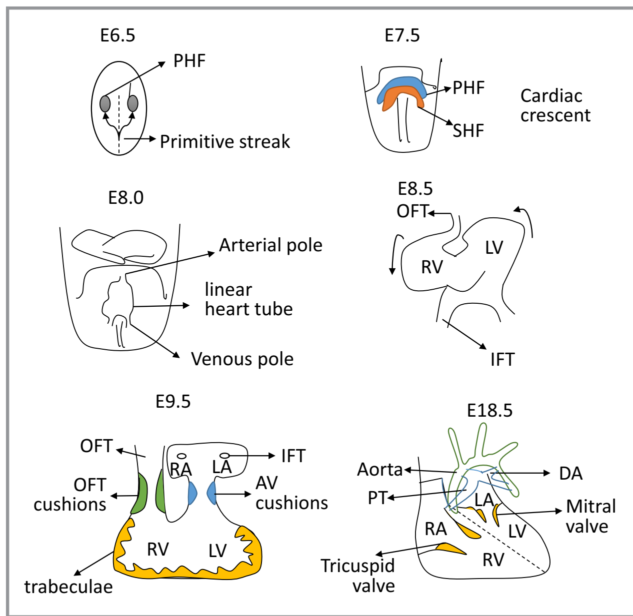

Figure 1. Cardiogenesis in mice. AV indicates atrioventricular; DA, ductus arteriosus; E, embryonic day; IFT, inflow tract; LA, left atrium; LV, left ventricle; OFT, outflow tract; PHF, primary heart field; PT, pulmonary trunk; RA, right atrium; RV, right ventricle; SHF, second heart field.

thus has a much larger surface area than the outer membrane.[38] The oxidative phosphorylation (OXPHOS) complexes, complexes I to V, are located on the inner membrane and mediate electron transportation to generate ATP.[43,44] The mitochondrial permeability transition pore (mPTP) contains both inner and outer membrane components, and its exact biomolecular configuration has not been finally determined.[45] Under normal conditions, this pore is closed to maintain proper membrane integrity. When open, it allows passage of molecules <1.5 kDa.[46] The intermembrane space between the inner and outer membranes contains cytochrome c, which is essential for transporting electrons and inducing apoptosis.[47,48] The mitochondrial matrix is the space inside the inner membrane. It contains the mitochondrial DNA (mtDNA), ribosomes, enzymes, and ions and is the location of the tricarboxylic acid cycle.[49,50]

At the cellular level, mitochondria form dynamic networks with morphologies varying with cell types,[51] for example, a highly ordered lattice in adult cardiac cells and a more irregular tubular arrangement in neonatal cardiomyocytes.[52–54] Functionally, mitochondria play a pivotal role in regulating cellular functions (eg, energy production) and signaling molecules (eg, reactive oxygen species [ROS] and Ca^{2+}).[55,56]

In adult cardiomyocytes, mitochondria are the major cellular powerhouse and produce >95% of the cell's energy in the form of ATP. Once the substrates (eg, glucose and fatty acids) enter the cell, they are sequentially oxidized to produce acetyl coenzyme A, which drives the tricarboxylic acid cycle to produce the reducing equivalents NADH (Nicotinamide Adenine

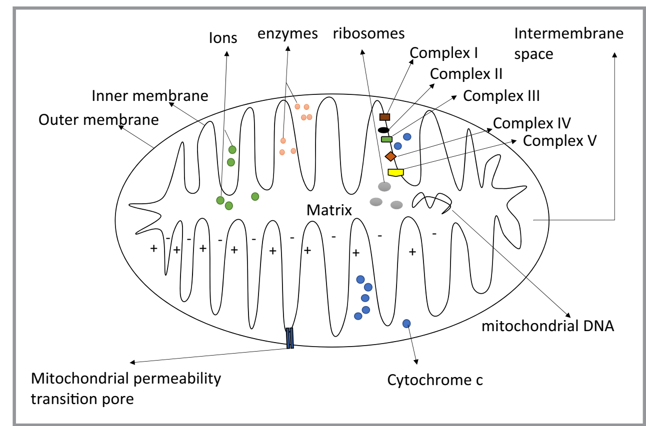

Figure 2. Structure of mitochondria.

Dinucleotide-Hydrogen) and $FADH_2$ (Flavin Adenine Dinucleotide-2 Hydrogen) in the mitochondrial matrix. NADH and $FADH_2$ are oxidized by NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II), respectively, in the inner membrane, driving the electron transport chain (ETC; complex I–IV) to establish the proton-motive force. In the last step of OXPHOS, the energy derived from the proton-motive force is utilized by F_1F_O ATP synthase (complex V) to convert ADP and inorganic phosphate to ATP.

In addition to energy production, mitochondria are the major site of ROS production.[57–60] In vitro studies have shown that up to 2% of electrons flowing through the ETC are partially reduced to form superoxide anion (O_2^-),[61] likely at complexes I, II, and III.[57,58] Superoxide is rapidly dismutated by superoxide dismutase (eg, MnSOD (manganese superoxide dismutase) and CuZnSOD (copper-zinc superoxide dismutase)) to form H_2O_2 (hydrogen peroxide), which is further reduced to H_2O by various antioxidant enzymes such as catalase, glutathione peroxidase, and peroxiredoxins.[62–67] As natural byproducts of oxygen metabolism, ROS signaling molecules regulate a variety of oxygen-sensing machineries, including gene expression.[68–70] However, excessive ROSs are toxic and can oxidize large molecules such as proteins, DNA, and lipids, which can lead to oxidative damage.[57,64,71] In addition, oxidative stress can promote the pathological opening of the mPTP, causing mitochondrial swelling, membrane rupture, and cell death.[72–75] ROS overload also can deplete the intracellular redox pool,[76] impairing cellular Ca^{2+} handling,[77] ion channel activity, and ROS-mediated redox signaling pathways.[78–81]

In cardiomyocytes, Ca^{2+} homeostasis is maintained by various Ca^{2+} channels and exchangers in the sarcolemma and subcellular membranes, including the L-type Ca^{2+} channel, the Na^+/Ca^{2+} exchanger, RyR (ryanodine receptor), and SERCA (sarcoplasmic reticulum Ca^{2+} ATPase). Recent studies suggest that Ca^{2+} dynamics also may be shaped by mitochondria,[82–84] either directly by inner membrane Ca^{2+} transport or indirectly by ROS-mediated signaling pathways. The mitochondrial Ca^{2+}

uniporter is the primary route for Ca^{2+} entry into the mitochondrial matrix,[85] and RyR1 has been proposed as an alternative Ca^{2+} uptake mechanism.[86] Evidence suggests that mitochondrial Ca^{2+} uptake channels are located at the proximate side of sarcoplasmic reticulum Ca^{2+} release sites (ie, RyRs),[87,88] facilitating rapid mitochondrial Ca^{2+} uptake and ATP production in response to increased workload. The extrusion of Ca^{2+} from the mitochondria is mediated by a mitochondrial Na^{+}/Ca^{2+} exchanger,[89] which is much slower compared with the mitochondrial Ca^{2+} uniporter. The transient mPTP opening also may be associated with mitochondrial Ca^{2+} release.[90] Under pathological conditions, mitochondria can affect Ca^{2+} handling by releasing ROS close to redox-sensitive ion transporters involved in cytoplasmic Ca^{2+} handling, such as the L-type Ca^{2+} channel,[91] RyRs,[80,92] and SERCA.[93] Thus, mitochondria play a multifactorial role in regulating Ca^{2+} homeostasis and signaling in cardiomyocytes.[94]

Mitochondria in mature cardiomyocytes are different from those found in other tissue and cell types. Mitochondria are significantly more abundant in hearts than in skeletal muscle, brain, kidney, and liver.[95] Consistent with these observations, the ratio of mtDNA to nuclear DNA in hearts is also significantly higher than in other tissues.[96] Transmission electron microscopy reveals that mitochondria in striated muscles (skeletal and cardiac muscles) appear to be located within membranous compartments along the myofiber.[95] In contrast, mitochondria are more perinuclear in liver cells and more dispersed throughout the cytosol in brain. In addition to the differences in abundance and morphology, mitochondria from different tissues can utilize different fuel to generate ATP. For example, cardiac mitochondria primarily use fatty acid as fuel, whereas most other organs use glucose as the major energy substrate.[96]

Maturation of Cardiac Mitochondria in Mammalian Embryos

In this section we summarize the process of mitochondrial maturation at different stages of mammalian heart development. Little is known regarding mitochondria in cardiac precursor cells. Between E6.5 and E8.5 in mice, the whole embryo is under low-oxygen conditions; therefore, aerobic glycolysis presumably serves as the primary source of ATP for cardiac precursor cells.[97,98] At E8.5 in mouse embryos or E10 in rat embryos, some mitochondria can be observed in cardiomyocytes through transmission electron microscopy; however, these mitochondria are considered "very immature" (class 1; Figure 3).[24,32,99] They contain few cristae and no expanded matrix. At E9.5 in mice and E11 in rats, the number of mitochondria in cardiomyocytes is increased and cristae can be clearly observed within mitochondria.[24,32,99] At this stage most mitochondria are still Class 1. At E10.5 in mice and E12 in rats, the number of cristae in cardiac mitochondria is increased compared with earlier stages. Some mitochondria contain tubular cristae, which form connections to the periphery. The matrix in these mitochondria is slightly expanded. This type of mitochondria is considered "immature" (class 2).[24,32,99] In class 1 and 2 mitochondria, ETC activity and

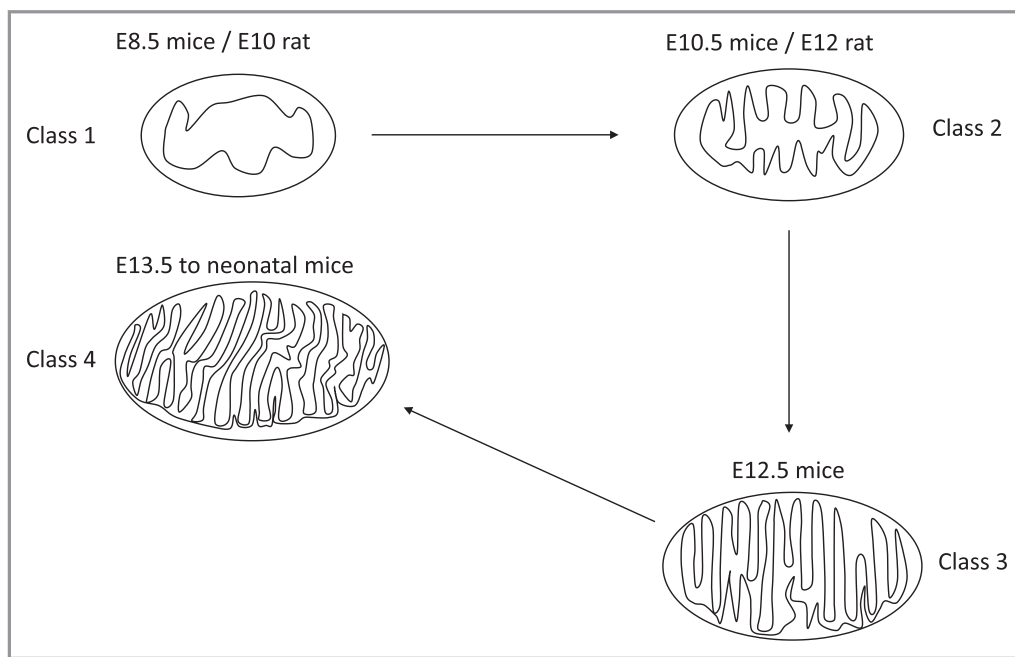

Figure 3. Maturation of mitochondria during mouse heart development. E indicates, embryonic day.

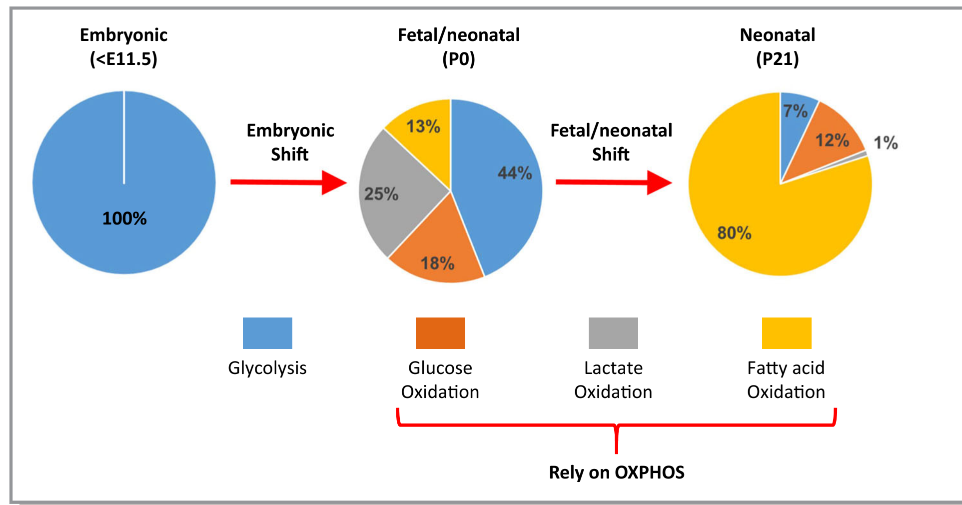

Figure 4. Embryonic and fetal/neonatal metabolic shift.[101] E indicates embryonic day; OXPHOS, oxidative phosphorylation.

ATP generation are not coupled, although OXPHOS complexes can be detected in the inner membrane of mitochondria.[24,32,99] Cardiomyocytes primarily acquire ATP through anaerobic glycolysis (Figure 4). At E11.5 in mouse embryonic hearts, OXPHOS complexes begin to assemble into highly efficient respirasomes (supercomplexes) and ETC activity and ATP generation are coupled.[100] Initiation of ETC activity coincides with the activation of complex I,[100] suggesting that complex I activation is critical for initiation of electron transportation along the OXPHOS complexes. Unfortunately, no ultrastructural examination of mitochondria at this stage has been published. In rodent hearts corresponding to E12.5 and E13.5 of mouse embryos, most cardiac mitochondria become "almost mature" (class 3), with structures resembling the mature mitochondria (class 4) observed in postnatal and adult heart tissues.[32,99] Class 3 mitochondria contain many tubular cristae that are connected with the periphery.[32,99] Their matrices are highly compacted with occasional "voids" observed in transmission electron microscopy images. More respirasomes can be observed on the inner membranes of mitochondria. By E13.5 the mitochondria are functionally matured and their mPTPs are closed.[32,99] Electron transportation and OXPHOS activities in E13.5 cardiac mitochondria are indistinguishable from those in adult mitochondria.[32,99] At this stage, embryonic cardiomyocytes acquire ATP through both anaerobic glycolysis and OXPHOS.[101,102] By the end of gestation in prenatal and newborn hearts, ≈44% of total ATP is generated through anaerobic glycolysis; production of the remaining ATP relies on OXPHOS (Figure 4).[101,103]

It is a common misconception that aerobic metabolism in hearts becomes critical only after birth. The metabolic shift from anaerobic glycolysis to aerobic metabolism already occurs during the mid- to late-gestation stages in mammalian hearts. By the end of gestation, the generation of >50% of ATP relies on OXPHOS in prenatal hearts (Figure 4).[101,103] This embryonic metabolic shift, originally proposed by Baker and Ebert,[21] can serve at least 2 major purposes. First, the normal assembly of respiratory complexes in embryonic hearts is necessary to prepare hearts to undergo the fetal to neonatal metabolic shift after birth. Second, OXPHOS provides >50% of ATP in fetal hearts to support their pumping activity.[21,98,101,104] Defective OXPHOS in embryonic hearts leads to severe heart defects and embryonic or neonatal lethality in mouse models and human patients.[21–31] This "embryonic" metabolic shift remains poorly studied in the literature.

Mitochondrial Biogenic Surge During the Fetal/Neonatal Metabolic Shift and Potential Roles of Mitochondria in Regulating Neonatal Cardiomyocyte Maturation

After birth, the cardiac environment shifts from hypoxia in embryos (10–30 mm Hg oxygen) to normoxia (80–100 mm Hg oxygen).[98,105] Neonatal hearts undergo the well-defined fetal/neonatal metabolic shift through which the primary source of ATP generation transitions from glycolysis to fatty acid β-oxidation.[98,101,105] By postnatal day 21 (P21), nearly 80% of ATP is generated through fatty acid β-oxidation in cardiomyocytes (Figure 4).[98,101] Compared with glycolysis, fatty acid β-oxidation is much more efficient with respect to ATP generation; therefore, the fetal/neonatal metabolic shift is essential for cardiomyocytes to fulfill the increased cardiac workload during this period.[98,101] Accompanying this metabolic shift, the mitochondrial biogenic surge occurs in cardiomyocytes soon after birth.[106] For example, the mtDNA copy number increases by ≈1.5-fold at P1 and by ≈5.0-fold at P21 compared with fetal hearts in rats.[107]

The essential functions of mitochondria in neonatal and infant hearts can be clearly demonstrated through studies of *Tfam* (transcription factor A, mitochondrial) in mice. TFAM is a DNA-binding protein that is required for replication and transcription of the mitochondrial genome.[108–110] Inactivation of *Tfam* in muscle cells at the perinatal stage uses *MCK* (muscle creatine kinase)–*Cre* reduced expression of TFAM in cardiac and skeletal muscle cells at E18.5, as determined from Western analysis.[111] The steady-state level of mitochondrial transcripts in these mutant hearts between P20 and P30 was ≈30% of the control level, suggesting that the function of TFAM was efficiently inactivated at this stage.[111] Deletion of *Tfam* by *MCK-Cre* caused deficiency in the respiratory chain, blockage of atrioventricular heart conduction, dilated cardiomyopathy, and animal lethality between P15 and P35.[111] Knocking out *Tfam* using another muscle Cre line, *MCK-Nls-Cre*, reduced mtDNA to ≈25% of the control level at P14 and allowed mutant animals to survive 10 to 12 weeks after birth.[112] In these mutant hearts, respiration was severely impaired and a fetal gene expression program was activated between 4 and 9 weeks after birth.[112] Reactivation of the fetal gene expression program is a well-recognized phenotype of cardiac diseases.[113,114] The studies described, in which *Tfam* was deleted, clearly show that mitochondria are required for normal cardiac postnatal development. Whether cardiomyocyte maturation was impaired in these mutant mice was not reported.[111,112]

A classical study[115] published a decade ago strongly supported the idea that mitochondrial biogenesis and the perinatal biogenic surge are required for postnatal cardiomyocyte maturation. PGC-1α (PPARG [peroxisome proliferator-activated receptor γ] coactivator 1α; encoded by *Ppargc1a* [PPARG coactivator 1α]) and its homolog PGC-1β (encoded by *Ppargc1b*) play a central role in regulating mitochondrial biogenesis and maturation.[24,115–117] To understand the role of *Ppargc1a* and *Ppargc1b* during heart development, *Ppargc1b* was specifically inactivated in fetal hearts using *Myh6* (*Myosin heavy chain 6)-Cre* on the *Ppargc1a*-deficient (*Ppargc1a*$^{-/-}$) background (*Ppargc1a*$^{-/-}$;*Ppargc1b*$^{loxp/loxp}$;*Myh6-Cre*).[115] At P0.5, expression of *Ppargc1b* mRNA in *Ppargc1a*$^{-/-}$;*Ppargc1b*$^{loxp/loxp}$;*Myh6* mice was reduced to <5% of the control level.[115] Double inactivation of *Ppargc1a* and *Ppargc1b* in mouse fetal hearts decreased mitochondrial volume density and arrested mitochondrial biogenesis and maturation at the perinatal stage.[115] Double-knockout (DKO) animals survived to P0 and died during the first week after birth because of heart failure. Heart size and activity in DKO animals was significantly decreased compared with controls. Molecular examination showed that the expression of fetal cardiac genes (including *Nppa* (natriuretic peptide A) and *Nppb* (natriuretic peptide B)) remained high, whereas the expression of the adult sarcomeric isoform, *Myh6*, was reduced within neonatal DKO hearts.[115] These data collectively suggest that blocking mitochondrial biogenesis at the perinatal stage impairs cardiomyocyte maturation.

The conclusion from the study described above is challenged by a recent study in which *Tfam* was specifically inactivated in neonatal hearts using an adeno-associated viral system, AAV9-*cTnt-Cre* (Cre driven by the cardiac Troponin T promoter in the AAV9 vector).[31] Mice at P0 were treated with either high- or low-dose AAV9-*cTnt-Cre* virus, resulting in ≈55% and ≈30% Cre-mediated recombination in cardiomyocytes, respectively. Four weeks after injection with low-dose AAV9-*cTnt-Cre*, expression of TFAM was undetectable in isolated cardiomyocytes.[31] Mutant mice displayed mitochondrial dysfunction leading to dilated cardiomyopathy.[31] However, cardiomyocyte maturation did not appear to be impaired, as judged from the development of transverse tubules, sarcomere organization, physiological hypertrophy of cardiomyocytes, and switching of sarcomeric isoform expression.[31] Consequently, the authors concluded that normal mitochondrial function is not essential for postnatal cardiomyocyte maturation.

It remains unclear how to reconcile these opposing opinions. Other than the simple explanation that the difference is due to the different mouse genetic backgrounds used by the 2 groups, we propose 6 possible explanations. First, most *Ppargc1a* and *Ppargc1b* DKO mice die within the first week after birth, and the cardiomyocyte maturation defect was evaluated by examining the expression of 3 marker genes (*Nppa*, *Nppb*, and *Myh6*) at P0.5.[115] The early postnatal lethality of these mice precludes the detailed examination of cardiomyocyte maturation using multiple complementary experiments at later stages, as performed by another group.[31] It is thus unclear to what degree the cardiomyocytes are immature and whether maturation is completely blocked or simply delayed at the neonatal stage in *Ppargc1a* and *Ppargc1b* DKO mice. A second, alternative possibility is that PGC-1α and PGC-1β have broader activities in mitochondrial biogenesis through regulating nuclear encoded mitochondrial genes,[24] whereas *Tfam* only regulates mtDNA-encoded genes.[108–110] This could explain why the phenotype caused by DKO of *Ppargc1a* and *Ppargc1b* is stronger than knocking out *Tfam*. Third, PGC-1α and PGC-1β may exert certain unidentified mitochondrial-unrelated activities to promote cardiomyocyte maturation. Therefore, the observed defect in cardiomyocyte maturation is not directly caused by mitochondrial dysfunction in the DKO hearts. Fourth, in the *Tfam* knockout study, the AAV9-*Cre* virus was injected into P0 *Tfam*$^{loxp/loxp}$ mice. Considerable time is required to allow the cardiomyocytes to express CRE, to knockout *Tfam*, and to deplete the expressed *Tfam* mRNA and protein. It is possible that neonatal cardiomyocytes have already begun the maturation program before the expression of *Tfam* is efficiently

inactivated in these cells and that once the program is initiated, it no longer relies on mitochondrial biogenesis. Fifth, related to the fourth explanation, *Tfam* was inactivated using AAV9-*Cre* virus,[31] whereas *Ppargc1b* was inactivated using the *Myh6-Cre* transgenic line.[115] A high dose of virus led ≈55% of cardiomyocytes to express the reporter; however, the efficiency of inactivation of *Tfam* expression was not examined directly.[115] Thus, we cannot exclude the possibility that the failure to observe the maturation defect in AAV9-*Cre; Tfam*$^{loxp/loxp}$ mice is due to incomplete deletion of *Tfam*. The sixth possibility would be that the embryonic metabolic shift is required for the postnatal heart to undergo the neonatal metabolic shift and maturation. In this scenario, inactivation of *Ppargc1a* and *Ppargc1b* in embryonic hearts impairs the embryonic metabolic shift, which in turn causes maturation defects in postnatal hearts. In contrast, AAV9-*Cre*–mediated deletion of *Tfam* occurred postnatally, and the embryonic metabolic shift was not affected in these mice. Future studies are warranted to solve this important issue regarding the relationship between mitochondrial functions and postnatal heart maturation.

Role of ROS in Heart Development

ROS is a physiological byproduct of ETC electron flow, and its production can be increased or uncontrolled if ETC electron flow is compromised.[57–60] In addition, ROS can be generated through cell membrane–bound NADPH oxidase complexes.[118] Recent studies have indicated that ROS may act as signaling molecules.[119] Using embryonic stem cells as a model system, a high level of ROS increased the percentage of beating cardiomyocytes in embryoid bodies.[118] ROS may stimulate cardiomyocyte differentiation through multiple signaling pathways including JNK, ERK1/2, p38, Ca^{2+} and BMP.[118,120] However, a role for ROS in cardiac precursor cells has not been demonstrated through in vivo genetic studies.

ROS levels remain high in mouse E9.5 cardiomyocytes and decrease as embryos age.[32,118,121] The reduction in ROS levels in later stage embryos stimulates cardiomyocyte maturation, based on studies of mPTP.[32,121] Located on the mitochondrial inner membrane, mPTP is closed in matured cardiomyocytes under physiological conditions. In mouse embryonic hearts, mPTP is open until E9.5 and closes between E9.5 and E13.5.[32] Closure of mPTP not only increases the mitochondrial membrane potential ($\Delta\psi$m) to promote OXPHOS (aerobic respiration) but also decreases ROS levels in embryonic cardiomyocytes.[32] The forced closure of mPTP using a pharmacological reagent (cyclosporin A) or deletion of *Ppid* (peptidylprolyl isomerase D; *CyP-D* [cyclophilin D]), which is required for mPTP opening, led to prematuration of both mitochondria and cardiomyocytes at E9.5.[32] Treatment of E9.5 cardiomyocytes with an antioxidant (Trolox, Hoffmann-La Roche Inc) stimulated, and treatment with a stable oxidant (tertiary butyl hydroperoxide) inhibited, cardiomyocyte differentiation, regardless of whether mPTP was open or closed.[32] Collectively, these data support the idea that closure of mPTP acts upstream of redox signaling and reduces ROS levels in embryonic cardiomyocytes, which stimulates their maturation.[32]

In addition to regulating cardiomyocyte differentiation and maturation, ROS may regulate cardiomyocyte proliferation. The treatment of cardiomyocytes derived from embryonic stem cells or mouse neonatal hearts with 100 nmol/L H_2O_2 significantly enhanced their proliferation.[120,122] H_2O_2 treatment increased nuclear localization of cyclin D1, reduced the expression of $p27^{Kip1}$ (a negative cell cycle regulator), and enhanced phosphorylation of retinoblastoma in cultured myocardial cells.[120]

Abnormally high ROS levels due to mitochondrial dysfunction in embryonic hearts may lead to severe inborn cardiomyopathy. For example, embryonic heart inactivation of *Tfam* led to mitochondrial dysfunction, elevated ROS products, reduced cardiomyocyte proliferation, and embryonic lethality.[31] Furthermore, inhibition of ROS or the DNA damage response pathway using MitoTEMPO (Santa Cruz Biotechnology) or MK-1755 (a WEE1 [WEE1 G2 checkpoint kinase] kinase inhibitor) rescued the cell proliferation defect observed in cultured fetal cardiomyocytes in which *Tfam* was deleted.[31]

Taken together, ROSs exert complex activities during cardiogenesis, and their activities are stage dependent. Increased ROS levels stimulate cardiomyocyte differentiation from precursor cells. At later stages, reduction of ROS levels through closure of mPTP is required for normal maturation of cardiomyocytes. In addition, ROS can promote cardiomyocyte proliferation, according to evidence from in vitro cell culture analysis. Mitochondrial dysfunction in fetal hearts elevates ROS species, which triggers the DNA damage pathway to block cardiomyocyte proliferation.

Role of Apoptosis in OFT Remodeling

In addition to their role as the cellular powerhouse, it is well established that mitochondria play key roles in controlling cell apoptosis (programmed cell death).[123–125] In contrast to necrosis, which is caused by acute cellular injury, apoptosis is a precisely controlled process that regulates multiple processes during embryonic development.[126–128] In apoptotic cells, the death signal activates the proapoptotic protein BAX (BCL-2–associated X protein) and BAK (BCL2-antagonist/killer 1), which then associate with the mitochondrial outer membrane to form pores.[123–125] The pore alters the outer membrane potential and releases cytochrome c from the intermembrane space to the mitochondrial cytosol, where it interacts with APAF-1 (apoptotic protease activating factor 1)

to form the apoptosome.[123–125] Apoptosomes activate the caspase cascade to induce cell death.[123–125] In addition to cytochrome c, mitochondria may also release SMAC (second mitochondrial-derived activator of caspases) to activate caspases.[124,125,129]

During heart development, apoptosis serves as the driving force for OFT shortening and remodeling. Watanabe et al initially reported that, in chicken embryos, OFT shortening and rotation occurs through cardiomyocyte apoptosis in the proximal OFT region.[130] Pharmacologically blocking apoptosis in *ex ovo* chicken cultures resulted in an abnormally long infundibulum. In some embryo cultures, the OFT failed to rotate, leading to the double-outlet-right-ventricle defect.[131] The function of cardiomyocyte apoptosis in the OFT appears to be evolutionarily conserved from chicken to mammals. In mouse embryonic hearts, apoptosis in the proximal OFT region can be observed initially at E12.5, peaks at E13.5 to E14.5, and declines thereafter.[132] The stages of apoptosis in the OFT region correlate well with the shortening period of the OFT.[132] In addition to the OFT region, apoptosis can be detected in the ventricle and endocardial cells, suggesting that it may also be involved in ventricular morphogenesis.[131]

Considering the well-established role of mitochondria in apoptosis, we speculate that apoptosis during OFT remodeling is mitochondria dependent; however, we cannot exclude the involvement of mitochondria-independent mechanisms. Further studies are required to reveal the signal that initiates cell death in the OFT and to understand exactly which apoptotic pathway is used to regulate OFT remodeling.

Regulation of Heart Development by Mitochondrial Fission and Fusion

Mitochondria are highly dynamic organelles with constantly changing morphologies in response to altered inter- and intracellular environments.[133–136] The mitochondrial tubular network is regulated by fusion and fission. Reducing or blocking mitochondrial fusion (or overfission) leads to the fragmentation of mitochondria and the loss of mtDNA, whereas reducing or blocking fission (or overfusion) results in enlargement of mitochondria and overly interconnected tubules.[38,137–139]

Regulators of mitochondrial fission and fusion are GTPases and belong to the dynamin family.[136,140] MFN1 (mitofusin 1) and MFN2 are the GTPases that act on the mitochondrial outer membrane to promote fusion. Systematic knockout and rescue experiments have revealed that MFN1 and MFN2 possess partially overlapping functions in regulating mitochondrial morphology.[141,142] To reveal their potential functions during heart development, *Mfn1* and *Mfn2* were simultaneously inactivated in embryonic hearts using the *Nkx2.5-Cre* line,[143,144] which inactivates target genes in the early cardiac crescent at E7.5.[145] *Mfn1/Mfn2* cardiac DKO mice die between E9.5 and E15.5. Double-mutant hearts displayed severe hypocellular defects in their myocardial wall at E13.5.[143] Expression of multiple cardiac differentiation markers was impaired by deletion of the 2 genes at E9.5. Further mechanistic studies using embryonic stem cells as the model system suggested that blocking mitochondrial fusion decreases the capacity of Ca^{2+} to enter mitochondria and increases the Ca^{2+} concentration in the cytoplasm. Consequently, calcineurin activity is upregulated, which increases Notch signaling and impairs cardiomyocyte differentiation.[143] The studies on double deletion of *Mfn1* and *Mfn2* using *Nkx2.5-Cre* thus provided definitive evidence to support the essential role of mitochondrial fusion during cardiomyocyte differentiation.[143,144]

Cardiac functions of *Mfn1* and *Mfn2* have also been examined using another Cre line, *Myh6-Cre*, which inactivates target genes at midgestation.[146] The transcripts of both genes were efficiently reduced at E15.5 in DKO mice; however, no cardiac phenotype was observed in these mice from E15.5 to P0, suggesting that mitochondrial fusion is not essential for heart development from midgestation to birth.[146] Starting from P7, many DKO mice start to display dilated cardiomyopathy, and they all die before P16, supporting the essential role of mitochondrial fusion in postnatal cardiac development. Therefore, the results from *Nkx2.5-Cre* and *Myh6-Cre* lines have revealed that mitochondrial fusion is critical for heart development at different stages.

The DRP1 (Dynamin Related Protein 1) GTPase acts on the outer membrane of mitochondria to promote fission.[38,137,138,147] The potential role of *Drp1* during mouse heart development has been studied using 2 different Cre drivers to knockout *Drp1* in embryonic hearts. Inactivation of *Drp1* using the *Myh6-Cre* line reduced the expression of DRP1 to 50% of the control level at P1, and the activity of the left ventricle in mutants was reduced at this stage according to echocardiographic analyses.[148] However, it is unclear whether the reduced activity of the left ventricle in mutant hearts began during the fetal stage. At P7, the mutant animals displayed multiple cardiac defects, including reduced heart rate, abnormal patterns of electrocardiography and reduced left ventricle contraction. The sizes of mitochondria were abnormally enlarged in cardiomyocytes with *Drp1* deleted, and OXPHOS was also impaired.[148] All mutant mice died between P9 and P11. In another study, *Drp1* was inactivated by a muscle cell Cre line, *MCK-Cre.*[149] Reduced expression of DRP1 was not observed until P1 in mutant hearts. Mice with *Drp1* inactivated using *MCK-Cre* died between P7 and P10, which was slightly earlier than observed with mice in which the *Myh6-Cre* line was used. Mutant mice at P7 showed dilated cardiomyopathy, disorganized myofibrils, impaired mitochondrial respiration,

and reduced hypertrophic growth of postnatal hearts.[149] Collectively, the 2 complementary studies support the essential role of mitochondrial fission in regulating early postnatal heart development and function. Whether mitochondrial fission also regulates embryonic heart development, as mitochondrial fusion does, will need to be addressed using Cre lines acting at earlier stages in embryonic hearts.

It should be noted that in addition to the canonical activities, MFN1, MFN2, and DRP1 also exhibit noncanonical functions.[150] MFN1/2 tethers mitochondria to the endoplasmic reticulum or the sarcoplasmic reticulum to form contact sites, which are important for mitochondrial Ca^{2+} uptake and bioenergetics.[151–153] In addition, MFN2 is involved in mitophagy, which will be discussed next. The noncanonical activities of DRP1 include regulating mPTP opening, respiration, mitophagy, and cell death.[154–156] Therefore, the phenotypes observed in *Mfn1, Mfn2*, and *Drp1* knockout hearts are likely caused by the combined defects of multiple aspects of mitochondrial activities rather than solely by impaired mitochondrial fusion or fission.

Regulation of the Perinatal Metabolic Shift by Mitophagy in Mouse Hearts

Mitophagy refers to the selective degradation of mitochondria by autophagy, a process that can be induced through both Parkin-dependent and Parkin-independent pathways.[157,158] A previous elegant study provided strong evidence to support the critical role of Parkin-dependent mitophagy in regulating the perinatal metabolic shift in mouse hearts.[159] The MFN2 T111A/S442A (MFN2 AA) mutation inhibits mitochondrial Parkin localization and thus blocks Parkin-dependent mitophagy. However, this mutation does not affect mitochondrial fusion or Parkin-independent mitophagy induced by starvation.[159] Ectopic expression of MFN2 AA in neonatal cardiomyocytes led to cardiac dilation, impaired contraction, pulmonary congestion, and eventually heart failure.[159] All mutant animals died 7 to 8 weeks after birth. Conversely, ectopic expression of a comparable level of wild-type MFN2 in neonatal hearts did not result in any overt defects. MFN2 AA mitochondria retained fetal mitochondrial morphology, failed to undergo maturation, and exhibited impaired functionality.[159] Furthermore, high-throughput RNA-sequencing examination showed that perinatal cardiac expression of MFN2 AA blocked metabolic gene reprogramming in postnatal hearts,[159] suggesting that Parkin-dependent mitophagy is required for perinatal mitochondrial maturation at both the morphological and gene expression levels. A mismatch between mitochondrial programming and the substrate availability was proposed as the major cause for the juvenile cardiomyopathy observed in mice with cardiac ectopic expression of MFN2 AA.[159]

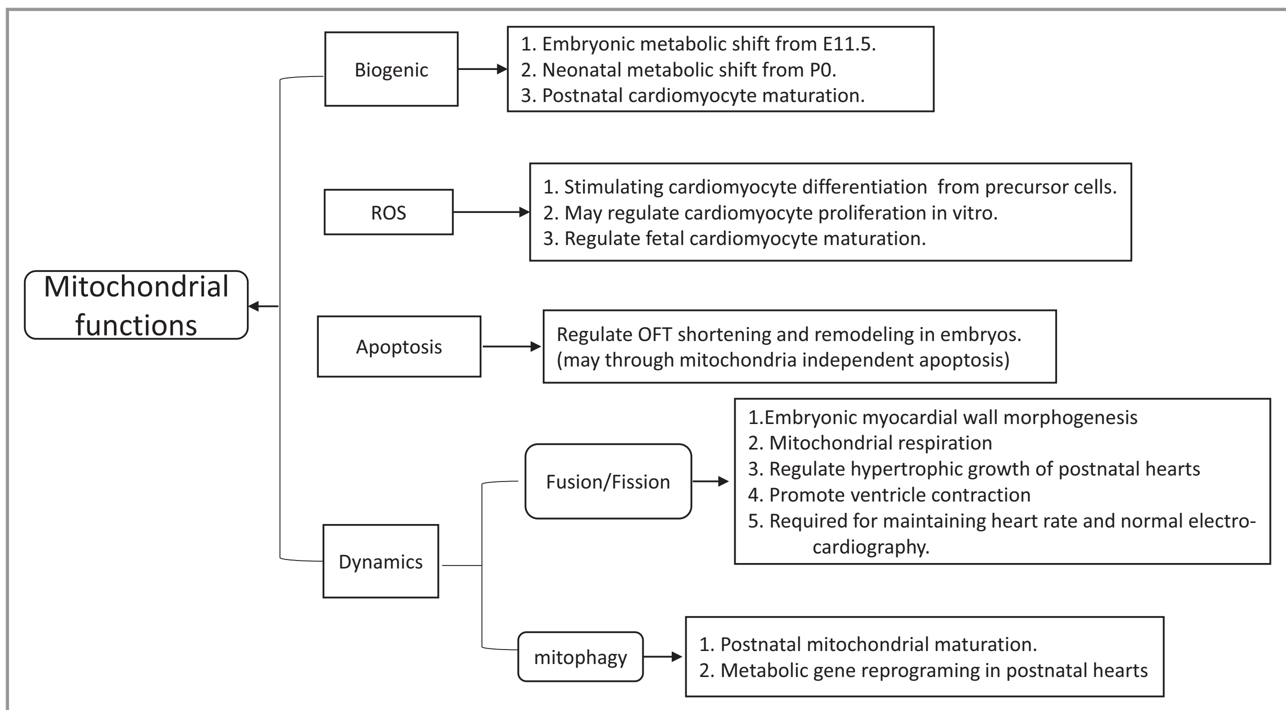

Figure 5. Summary of mitochondrial functions during cardiogenesis. E indicates, embryonic day; OFT, outflow tract; P, postnatal day; ROS, reactive oxygen species.

Table. Functions of Genes Important for Mitochondrial Morphology/Activity During Cardiogenesis

Names of Gene	Genetic Manipulation Methods	Defects in Mutants
Drp1	1. *Myh6-Cre*, inactivation of target genes in fetal hearts 2. *MCK-Cre*, perinatal inactivation of target genes in cardiac and skeleton muscle cells	Lethality between P9 and P11. Reduced heart rate, abnormal patterns of electrocardiography, and reduced left ventricle activity at P7. Enlarged mitochondria in cardiomyocytes. Impaired OXPHOS.[148] Lethality between P7 and P10. Dilated cardiomyopathy, disorganized myofibrils, impaired mitochondrial respiration, and reduced hypertrophic growth at P7.[149]
Mfn1 and *Mfn2*	1. *Nkx2.5-Cre*, cardiac precursor cells 2. *Myh6-Cre*, inactivation of target genes in fetal hearts	Lethality between E9.5 and E15.5. Severe hypocellular defects in the myocardial wall at E13.5.[143] Lethality before P16. Dilated cardiomyopathy at P7.[146]
Mfn2 (T111A/S442A)	Transgenic expression of *Mfn2(T111A/S442A)* in neonatal hearts, only affects mitophagy	Lethality 7 to 8 wks after birth. Impaired mitophagy. Cardiac dilation, impaired contraction, pulmonary congestion, and eventually heart failure.[159]
Ppargc1a and *Ppargc1b*	*Myh6-Cre* inactivation of *Ppargc1b* on the *Ppargc1a*$^{-/-}$ background (*Ppargc1a*$^{-/-}$; *Ppargc1b*$^{loxp/loxp}$; *Myh6-Cre*), inactivation of target genes in fetal hearts	Lethality during the first week after birth. Heart failure, reduced heart size and activities. Impaired neonatal cardiomyocyte maturation.[115]
Tfam	1. *MCK-Cre*, perinatal inactivation of target genes in cardiac and skeleton muscle cells 2. *MCK-Nls-Cre*, perinatal inactivation of target genes in cardiac and skeleton muscle cells. CRE activity weaker than *MCK-Cre* 3. AAV9-*cTnt-Cre*, neonatal injection	Deficiency in the respiratory chain, blockage of atrioventricular heart conduction, dilated cardiomyopathy and animal lethality between P15 and P35.[111] Survive to 10 to 12 weeks after birth. Deficiency in respiration. Reactivation of a fetal gene expression program between 4 and 9 weeks after birth.[112] Dilated cardiomyopathy. No defect in postnatal cardiomyocyte maturation.[31]

Conclusion

Accumulated evidence has established that mitochondria not only serve as the cellular powerhouse enabling the heart to beat but also play a critical role in regulating embryonic and neonatal heart development in mammals (Figure 5). In Table, we summarize the roles of genes important for mitochondrial morphology and function during mammalian cardiogenesis that are discussed in this review. Revealing the role of mitochondria in heart development and the underlying mechanisms will provide crucial clues to the development of novel clinical applications aimed at treating cardiomyopathies caused by mitochondrial dysfunction in infants.

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