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Mitochondrial metabolism transition cooperates with nuclear reprogramming during induced pluripotent stem cell generation

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ABSTRACT

Induced pluripotent stem cells (iPSCs) hold great clinical potential for regenerative medicine. Much work has been done to investigate the mechanisms of their generation, focusing on the cell nucleus. However, the roles of specific organelles and in particular mitochondria in the potential mechanisms of nuclear reprogramming remain unclear. In this study, we sought to determine the role of mitochondrial metabolism transition in nuclear reprogramming. We found that the mitochondrial cristae had remodeled in iPSCs. The efficiency of iPSC generation was significantly reduced by down-regulation of mitochondrial inner membrane protein (IMMT), which regulates the morphology of mitochondrial cristae. Moreover, cells with the oxidative phosphorylation (OXPHOS) advantage had higher reprogramming efficiency than normal cells and the glycolysis intermediate lactic acid enhanced the efficiency of iPSCs generation. Our results show that the remodeling of mitochondrial cristae couples with the generation of iPSCs, suggesting mitochondrial metabolism transition plays an important role in nuclear reprogramming.

1. Introduction

Induced pluripotent stem cells (iPSCs) are generated from murine embryonic fibroblasts by introducing four transcript factors, Oct4, Sox2, Klf4 and c-Myc [1]. For the potential clinical application of iPSCs in the future, much work has been done to dissect the mechanisms of nuclear reprogramming focusing on cell nucleus [2–6]. However, little work has been done to study the role of mitochondria in the generation of iPSCs. Our work is trying to reveal a new mechanism of nuclear reprogramming.

Mitochondrion is regarded as an important organelle in the cell. Emerging evidence suggests that mitochondrial metabolism is involved in the differentiation of embryonic stem cells. In addition, the status of mitochondrial metabolism modulates the capability of pluripotency of embryonic stem cells [7]. iPSCs have the similar mitochondrial metabolism features to that of embryonic stem cells and these cells mainly depend on glycolysis as their preferred source of energy production regardless of oxygen availability [8]. Moreover, mitochondrial metabolism has also been demonstrated to affect the efficiency of iPSC generation. The study by Sheng Ding implicates that mitochondrial metabolism conversion from oxidative phosphorylation (OXPHOS) to glycolysis possibly enhances nuclear reprogramming [9]. In addition, Andre Terzic reports that iPSCs induced by four transcript factors (Sox2, Klf4, Oct4 and c-Myc) have few immature mitochondrial cristae and glycolysis facilitates nuclear reprogramming [10]. Although the effects of mitochondrial metabolism transition on nuclear reprogramming have been illustrated by these two studies, the relationship between mitochondrial metabolism transition and nuclear reprogramming needs further clarification.

Herein, our results demonstrate that mitochondrial crista remodeling couples with iPSC generation. The ultrastructure of mitochondria of iPSCs is similar to that of embryonic stem cells. Our results also show that cells with OXPHOS advantage will have higher reprogramming efficiency than normal cells and facilitating glycolysis enhances the efficiency of iPSC generation. Our study will extend our understanding of the potential mechanisms of nuclear reprogramming and will assist in the development of future clinical application of iPSCs.

2. Materials and methods

2.1. Cell culture

Mouse embryonic fibroblasts (MEFs) were derived from OG2 mice. OG2 MEFs carried a transgenic Oct4 promoter driving GFP expression [11]. MEFs or human adult fibroblasts (donated from

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adults in our lab) were cultured in DMEM (Hyclone) medium containing 10% FBS (PAA), 100× NEAA (Invitrogen) and 100× GlutaMAX (Invitrogen). Plat-E cells (purchased from ATCC) were cultured in DMEM (Hyclone) medium containing 10% FBS (PAA), 100× NEAA (Invitrogen) and 100× GlutaMAX (Invitrogen) for retrovirus packaging. Retrovirus packaging was performed according to standard protocol [12]. OG2 MEFs were infected with retrovirus encoding Oct4, Sox2, Klf-4 with or without c-Myc. 24 h after the second infection, the medium was changed to embryonic stem cell culture medium. Embryonic stem cell culture medium was DMEM (Hyclone) containing 15% FBS (GIBCO), 100× NEAA, 100× GlutaMAX, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 100× penicillin/streptomycin (Invitrogen) and 1000× leukemia inhibitory factor (LIF) (Sigma). The cells of mouse embryonic stem cell line R1 (purchased from ATCC) were plated onto 1% gelatin-coated plates and maintained in a feeder-free, serum-free N2B27 medium [13]. Human iPSCs were plated onto matrigel-coated plates and maintained in a feeder-free, serum-free mTesR™1 medium (Stem Cell). All cells used in the experiments were cultured in cell incubator containing 5% CO2 at 37 °C. For 5 mM L-lactate treatment, L-lactate was steadily added into embryonic stem cell culture medium until the GFP positive clones were counted after 15 days or 16 days.

2.2. Plasmids cloning

pMXs based vector encoding mouse Oct4, Sox2, Klf4 and c-Myc purchased from Addgene. MCT1, MCT2 and MCT4 used in the experiments were cloned into pMXs-Flag vector (Addgene) . IMMT shRNA was cloned into pSuper-retro-puro vector (Addgene). The target sequence was inserted into BglII and HindIII site. The target sequence of IMMT was supplied in Table 1.

2.3. Western blotting

Cell lysates were processed according to standard protocols. The primary antibody anti-IMMT was produced in rabbit (Abcam). The secondary antibody was goat anti-rabbit with HRP (Sigma). We used ECL (GE healthcare) to detect the target protein.

2.4. Flow cytometer sorting

For cell sorting, we used tetramethyl rhodamine methyl ester (TMRM; Invitrogen) to dye OG2 MEFs at the final concentration of 25 nM for 15 min. Cells were sorted by flow cytometer according to high $\Delta\Psi m$.

2.5. Quantification the efficiency of iPSC generation

We used OG2 MEFs to study the efficiency of iPSC generation. MEFs were infected with retrovirus based vectors encoding mouse Oct4, Sox2, Klf4 in the presence or absence of c-Myc. After twice retrovirus infection, the culture medium was changed to embryonic stem cell culture medium. We calculated the reprogramming efficiency by counting the number of GFP positive colonies under fluorescence microscope after 15 days or 16 days.

Table 1
Quantitative real time PCR primers and shRNA target sequence.

IMMT qPCR primer forward	TGAAATGCGGACCCAACT
IMMT qPCR primer reversed	TTGACTGCGACGACGAAA
Actin qPCR primer forward	TGCTAGGAGCCAGAGCAGTA
Actin qPCR primer reversed	AGTGTGACGTTGACATCCGT
IMMT shRNA target sequence	GCTGGCAAACTCTCTACTGAT

2.6. Transmission electron microscope (TEM)

Cells were washed by phosphate buffer solution (PBS) twice and were then fixed by 1% glutaraldehyde and 4% formaldehyde (Sigma). The ultrastructure of mitochondria was examined by TEM.

2.7. RT-PCR and quantitative real time PCR

Cells were washed by PBS twice and were then lysed by Trizol reagent (Invitrogen). Total RNA was extracted according to standard protocol (Takara RNA extraction kit). The cDNA synthesis was performed with RT-PCR Kit (Takara RT kit). The mRNA level of target gene was quantified by real time PCR-SYBR Green kit (Takara) and mouse actin was used as internal control. All quantitative real time PCR primers were supplied in Table 1.

3. Results

3.1. Mitochondrial cristae have remodeled after nuclear reprogramming

To characterize the features of mitochondria of iPSCs, we compared the ultrastructure of mitochondria in several cell types. These cell types including mouse embryonic fibroblasts, mouse embryonic stem cell line R1, human adult fibroblasts, mouse and human iPSCs. As shown in Fig. 1, we observed that somatic cells have more mature mitochondrial cristae. However, iPSCs have few immature mitochondrial cristae. The ultrastructure of mitochondria of iPSCs is similar to that of embryonic stem cells. Our results indicate that mitochondrial cristae have remodeled after nuclear reprogramming.

3.2. The effect of mitochondrial inner membrane protein (IMMT) on nuclear reprogramming

To better understand the role of mitochondrial cristae in nuclear reprogramming, we turned our attention to the mitochondrial cristae regulating protein IMMT. IMMT localizes in mitochondrial inner membrane. It has two expressed isoforms [14]. The previous study demonstrates that IMMT controls mitochondrial cristae morphology [15]. Emerging study suggests that IMMT also maintains the integrity and stability of mitochondrial inner membrane [16]. As shown in Fig. 2A, IMMT is markedly up-regulated during nuclear reprogramming. We selected the effective short hairpin RNA (shRNA) expression construct to knock down IMMT (Fig. 2B). Down-regulation of IMMT suppresses nuclear reprogramming (Fig. 2C). These results indicate that IMMT is involved in the generation of iPSCs.

3.3. Mitochondrial metabolism transition enhances the efficiency of iPSC generation

In an effort to explain the possibility that mitochondrial cristae have remodeled after nuclear reprogramming in order to adapt to mitochondrial metabolism transition, we examined the status of mitochondrial metabolism at the early stage of generation of iPSCs. Mitochondrial membrane potential ($\Delta\Psi m$) is considered as an indirect indicator of cell oxygen consumption rates. It is reported that mitochondrial membrane potential couples with the status of intrinsic mitochondrial metabolism [7]. In our study, we used the mitochondrial membrane potential specific probe TMRM to dye mouse embryonic fibroblasts for cell sorting. The sorted cell population with high $\Delta\Psi m$ is only 3–5% in the whole populations of mouse embryonic fibroblasts (Fig. 3A). When we used these cells with high $\Delta\Psi m$ to reprogram, we found that these cells with high

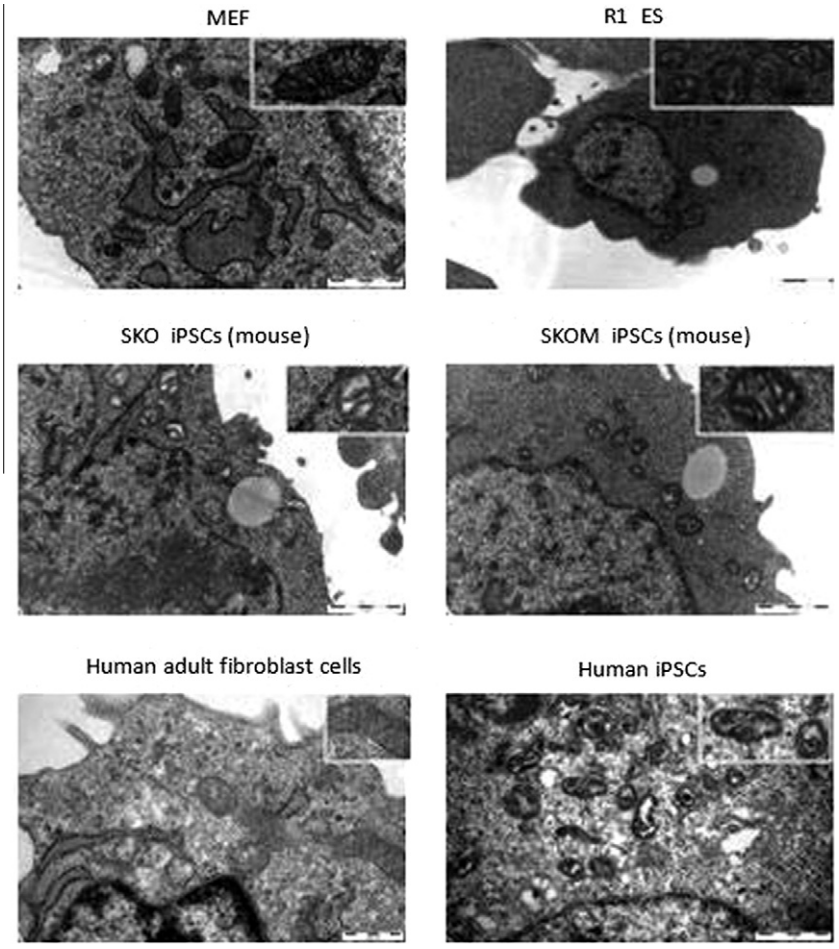

Fig. 1. The ultrastructure of mitochondrial cristae in the mouse and human fibroblast cells, mouse embryonic stem cell line R1, mouse and human iPSCs induced by Oct4, Sox2, Klf4 in the presence or absence of c-Myc.

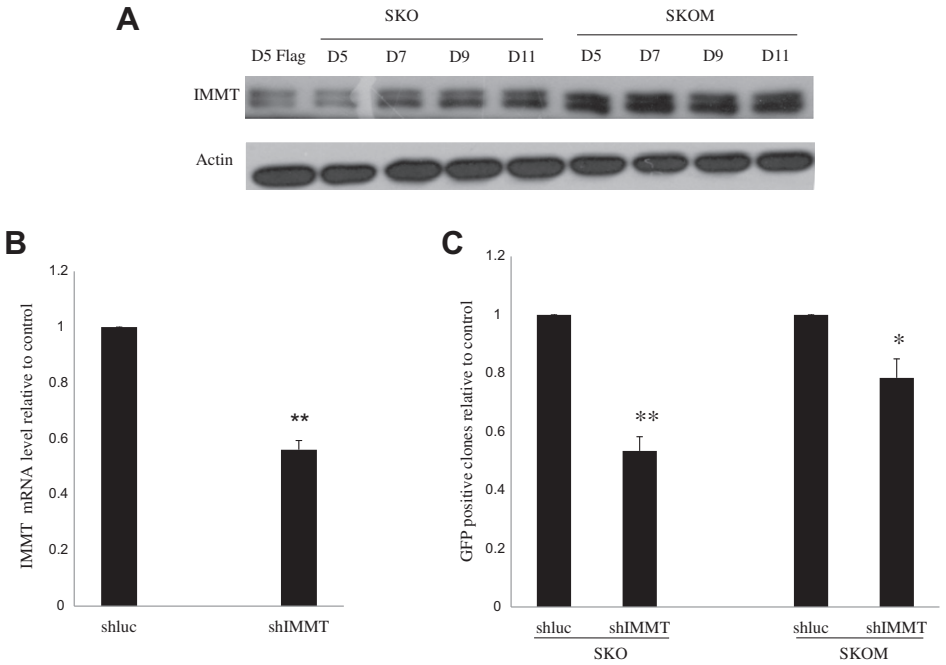

Fig. 2. The effect of silencing IMMT on nuclear reprogramming. (A) IMMT expression was detected by Western blotting. (B, C) The effect of silencing IMMT on the efficiency of iPSCs generation. We used TTEST to analyze the data. When $p < 0.05$, it was represented as "$*$", when $p < 0.01$, it was represented as "$**$", and when $p < 0.001$, it was represented as "$***$".

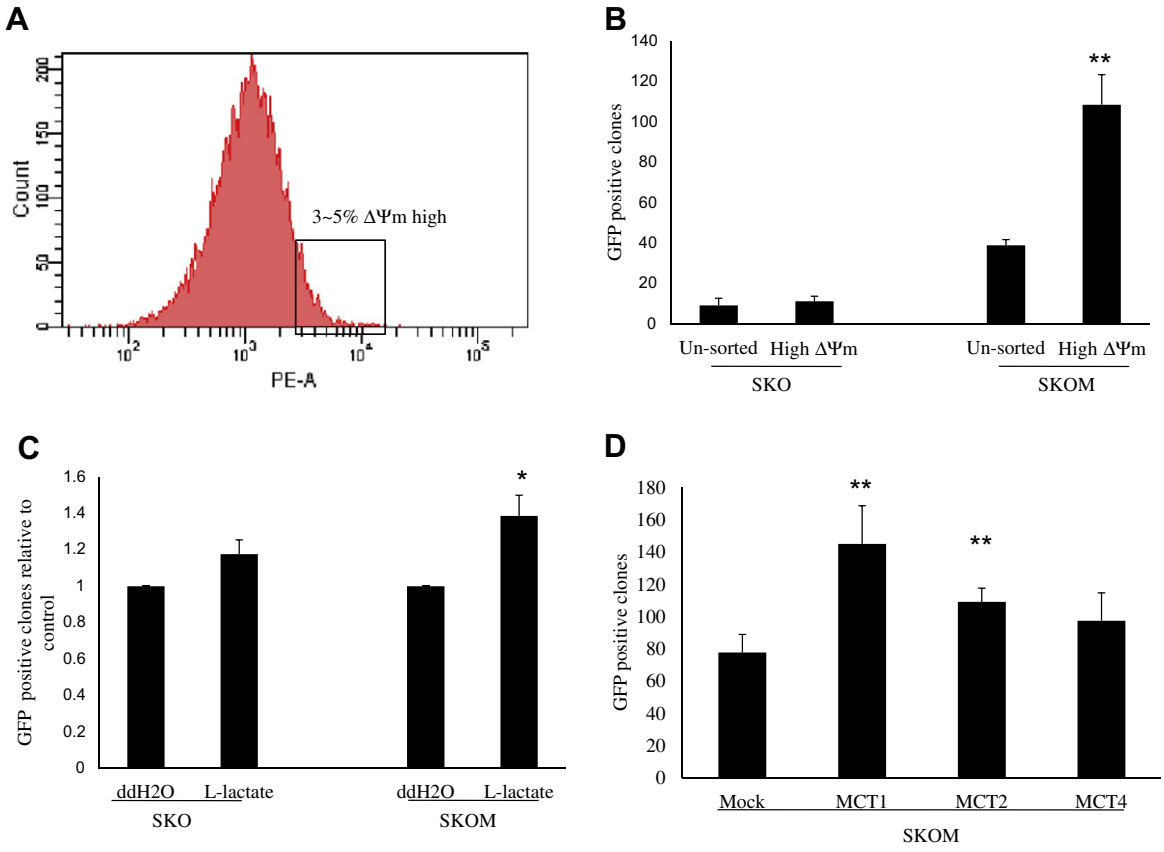

Fig. 3. OXPHOS is necessary for nuclear reprogramming. (A) Sorting MEFs with high $\Delta\Psi m$. (B) Cells with high $\Delta\Psi m$ were used to generate iPSCs. (C, D) Addition of L-lactate or overexpression of lactic acid transporter genes significantly enhances the efficiency of iPSCs generation. The 5 mM L-lactate was steadily added into mouse embryonic stem cells culture medium.

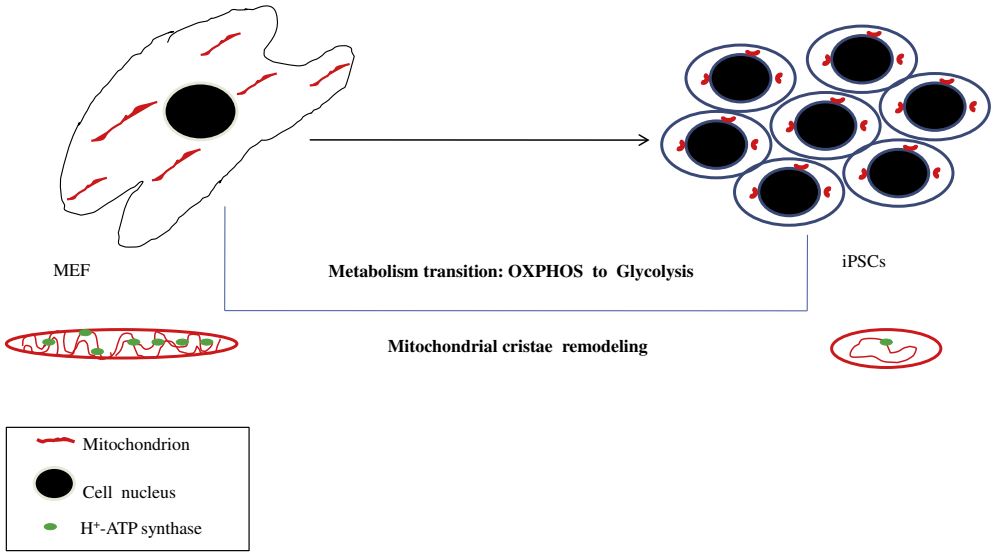

Fig. 4. Model for role of mitochondrial metabolism transition in the generation of iPSCs.

$\Delta\Psi m$ tended to be more effectively reprogrammed to iPSCs (Fig. 3B). These results demonstrate that cells with OXPHOS advantage have higher reprogramming efficiency than normal cells.

To characterize further the role of mitochondrial metabolism transition in nuclear reprogramming, we asked whether facilitating glycolysis might improve the efficiency of iPSC generation. When we added the glycolysis intermediate L-lactate into the culture medium during nuclear reprogramming, we found that addition of L-lactate significantly enhanced the generation of iPSCs (Fig. 3C). MCT1, MCT2 and MCT4 have been reported that they have important function in lactic acid transportation [17–19]. As shown in Fig. 3D, nuclear reprogramming efficiency is significantly enhanced by overexpression of the lactic acid transporter proteins MCT1, MCT2 and MCT4 in order to promote lactic acid uptake. Our

results are in accord with previous reports that glycolysis facilitates nuclear reprogramming [10].

4. Discussion

These findings demonstrate that OXPHOS advantage facilitates nuclear reprogramming. Mitochondrial cristae supply sites for H^+-ATP synthases. H^+-ATP synthases are important for ATP production in OXPHOS. iPSCs have few immature mitochondrial cristae compared with somatic cells. These cells possibly depend on a different bioenergetics. Although previous study has demonstrated that iPSCs use glycolysis as its energy resource, our study shows that OXPHOS is necessary for nuclear reprogramming. Somatic cells prefer OXPHOS for their ATP production compared to iPSCs [8,20]. Hence, more mature mitochondrial cristae are likely required for nuclear reprogramming at the early stage. Suppressing IMMT possibly results in few immature mitochondrial cristae and restrains OXPHOS to produce ATP. Cell proliferation is inhibited because of lacking of energy. Eventually, silencing IMMT suppresses nuclear reprogramming. This explanation is in accord with previous reports that high cell proliferation is essential for nuclear reprogramming [3,21,22]. Moreover, previous study suggests that glycolysis facilitates nuclear reprogramming [20].

Our study shows that the efficiency of iPSCs generation is enhanced by addition of glycolysis intermediate L-lactate or overexpression of lactic acid transportation proteins in order to facilitate glycolysis. Our results support the view that glycolysis is also necessary for nuclear reprogramming. However, when we reprogrammed cells into iPSCs by three transcript factors (SKO), cells with OXPHOS advantage or cells with glycolysis advantage did not have a higher reprogramming efficiency. It can account for this phenotype that nuclear reprogramming induced by three transcript factors (SKO) or by four transcript factors (SKOM) likely depends on different pathway.

In summary, our results support a model that the remodeling of mitochondrial cristae of iPSCs is likely to adapt to mitochondrial metabolism transition from OXPHOS to glycolysis. OXPHOS is necessary for nuclear reprogramming at the early stage. Glycolysis might play an important role in the generation of iPSCs at the later stage. Mitochondrial metabolism transition may interact with nuclear reprogramming during the generation of iPSCs (Fig. 4).

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