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Fatty acid metabolism driven mitochondrial bioenergetics promotes advanced developmental phenotypes in human induced pluripotent stem cell derived cardiomyocytes

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ABSTRACT

Background: Preferential utilization of fatty acids for ATP production represents an advanced metabolic phenotype in developing cardiomyocytes. We investigated whether this phenotype could be attained in human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) and assessed its influence on mitochondrial morphology, bioenergetics, respiratory capacity and ultra-structural architecture.

Methods and results: Whole-cell proteome analysis of day 14 and day 30-CMs maintained in glucose media revealed a positive influence of extended culture on mitochondria-related processes that primed the day 30-CMs for fatty acid metabolism. Supplementing the day 30-CMs with palmitate/oleate (fatty acids) significantly enhanced mitochondrial remodeling, oxygen consumption rates and ATP production. Metabolomic analysis upon fatty acid supplementation revealed a β-oxidation fueled ATP elevation that coincided with presence of junctional complexes, intercalated discs, t-tubule-like structures and adult isoform of cardiac troponin T. In contrast, glucose-maintained day 30-CMs continued to harbor underdeveloped ultra-structural architecture and more subdued bioenergetics, constrained by suboptimal mitochondria development.

Conclusion: The advanced metabolic phenotype of preferential fatty acid utilization was attained in hiPSC-CMs, whereby fatty acid driven β-oxidation sustained cardiac bioenergetics and respiratory capacity resulting in ultra-structural and functional characteristics similar to those of developmentally advanced cardiomyocytes. Better understanding of mitochondrial bioenergetics and ultra-structural adaptation associated with fatty acid metabolism has important implications in the study of cardiac physiology that are associated with late-onset mitochondrial and metabolic adaptations.

1. Introduction

Cardiomyocytes harbor the largest number of mitochondria, among all cell types, generating >95% of ATP consumed by the adult human heart. These ATPs are utilized for critical cellular functions including growth, contraction, calcium homeostasis, signaling and survival [1]. Adaptative changes to the mitochondrial bioenergetic machinery is crucial in meeting increased metabolic demands of developing cardiomyocytes. It is not surprising, therefore, that dysregulation in

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mitochondrial function leads to various cardiomyopathies and heart failure [2,3]. Cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CMs) offer a valuable platform to study cellular pathophysiological processes [4,5]. We have previously identified key signaling pathways governing formation of cardiomyocytes from hiPSCs [6,7]. While mitochondrial biogenesis has been reported to be important for cardiomyocyte derivation [8], information regarding their involvement in metabolic remodeling and ultra-structural development post-cardiomyocyte formation remains relatively undefined.

Maintenance of mitochondrial morphology through fusion and fission events together with cytoplasmic motility are key in remodeling mitochondrial networks necessary for intra-cellular distribution of ATP in adult cardiomyocytes [9]. During these events, adaptive modification of mitochondrial morphology through interplay between fusion proteins such as optic atrophy 1 (OPA1) and mitofusins (MFN1, MFN2) as well as fission proteins like dynamin-related protein 1 (DRP1) and fission protein 1 (FIS1) is continuously observed to sustain energy flux associated with supply and demand of cellular activities. Fetal cardiomyocytes are populated with mostly fragmented mitochondrial networks [10] and rely majorly on glucose as their main energy substrate [11]. In contrast, as cardiomyocytes undergo terminal differentiation, and adapt to postnatal life, metabolic remodeling towards β-oxidation occurs with fatty acids becoming the dominant energy substrate. This metabolic shift results in increased mitochondrial bioenergetic capacity [1], a change which is necessary for maintaining the high levels of ATP.

In the current study, as evidence of bona fide metabolic remodeling, we show that by day 30 of differentiation, hiPSC-CMs adapt to metabolizing fatty acids as the primary energy substrate, resulting in increased mitochondrial bioenergetics and respiratory capacity coinciding with ultra-structural organization of junctional complexes of fascia adherens, gap junctions and desmosomes supporting intercalated discs/t-tubule-like structures, consistent with developmentally advanced cardiomyocytes. Although glucose-maintained day 30-CMs showed improved mitochondrial functionality in comparison to day 14-CMs, their bioenergetics, respiratory capacity and ultra-structural architecture remain subdued when compared to those of the day 30-CMs supplemented with fatty acids. We conclude that attaining the advanced metabolic phenotype of preferential fatty acid utilization is crucial for sustaining cardiac bioenergetics required for augmenting ultra-structural architecture in hiPSC-CMs.

2. Methods

2.1. hiPSC maintenance and cardiac differentiation

Normal dermal fibroblast-derived hiPSC line (CL-1) [12] and BJ fibroblast-derived-mRNA reprogramed hiPSC line (CL-2) [13] were maintained and differentiated into cardiomyocytes using a previously described embryoid body (EB) based protocol [14].

2.2. Western blot and whole-cell proteome analysis

Western blots and whole-cell proteome analysis were performed as described previously [6,15]. Antibodies used in the study are listed in Supplementary Table 1. Peptides obtained from day 30-CMs were compared against day 14-CMs and those with a 20%-fold change (up-regulated and down-regulated) (nonparametric *t*-test; $p < 0.05$) were analyzed using DAVID Bioinformatics Resources 6.8. The proteome assay was performed in triplicate and the principal component analysis (PCA) was performed using Clustvis [16].

2.3. Mitochondrial functional assays

Cardiac clusters were dissociated into single cells and stained with either JC-1 dye (10 μg/mL; Thermo Fisher Scientific) or TMRM (0.25 μM; Thermo Fisher Scientific) for 15 min at 37 °C. For mitochondrial complex inhibition studies, JC-1 stained cells were treated with rotenone, thenoyltrifluoroacetone and antimycin A (Sigma-Aldrich), respectively. Changes in JC-1 fluorescence intensity were measured using SpectraMax M3 (Molecular Devices, CA, USA). TMRM-stained cells were analyzed on BD FACSAria II (BD Biosciences, CA, USA). A total of 10,000 gated events were evaluated for each time-point and data analysis was performed using FlowJo software. Hexokinase Colorimetric Assay Kit (Sigma-Aldrich), L-Carnitine Assay Kit (Sigma-Aldrich) and ATP Determination Kit (Thermo Fisher Scientific) were used in this study. Cardiac clusters were lysed in respective buffers and assayed as per manufacturer's instructions.

2.4. MitoTracker and immunostaining

Cardiac clusters were dissociated into single cells and stained with MitoTracker (0.1 μM; Thermo Fisher Scientific) for 15 min at 37 °C. For immunostaining, cells were fixed with 4% PFA, permeabilized with 0.3% Triton X-100, blocked with 5% BSA and stained with primary antibodies overnight (Supplementary Table 1). Cells were washed, probed with respective fluorophore-conjugated secondary antibodies and counterstained with DAPI the following day. Stained cells were examined under Zeiss LSM710 NLO multi-photon confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). For sarcomere length measurements, cells were stained with sarcomeric α-actinin (Sigma-Aldrich) and the distance between sarcomeres was measured by drawing a perpendicular line across adjacent *Z*-discs using ImageJ. The profile of stained Z-discs was plotted and the distance between the maximum intensity of each neighboring Z-disc was tabulated as sarcomere length.

2.5. Mitochondrial respiration assay

Cardiac clusters were dissociated into single cells and seeded on a Seahorse 24-well XF Cell Culture Microplate (Agilent Technologies, CA, USA) at approximately 1×10^5 cells/well. Prior to initiation of the assay, cardiomyocyte maintenance media was replaced with XF Media (with/without fatty acids). During measurements of OCR, oligomycin (2.5 μM), FCCP (1 μM) and antimycin A/rotenone (2.5 μM) was injected into the system. Non-mitochondrial OCR values (average values post antimycin A/rotenone treatment) were deducted from basal and maximal OCR values. Basal OCR was the average values taken from the start of the experiment until addition of oligomycin. Maximal OCR was the average values taken from addition of FCCP until treatment with antimycin A/rotenone. Respiratory reserve capacity was the difference in values between maximal and basal OCR.

2.6. Cardiac troponin T isoform analysis

RNA from cardiac clusters was converted to cDNA using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). Using Platinum PCR SuperMix (Thermo Fisher Scientific) and *TNNT2* primers listed in Supplementary Table 2, cDNA templates were cycled as follows: 2 min at 94 °C, followed by 35 cycles of 30s at 94 °C, 30s at 60 °C and 30s at 72 °C. Electrophoresis of amplified products was performed on a 3% agarose gel, run at 100 V for 50 min.

2.7. Statistical analysis

Data with normal Gaussian distribution were analyzed by standard parametric tests. Data with non-Gaussian distribution were analyzed by nonparametric *t*-test and Kruskal-Wallis test was applied for multi-group comparison followed by Dunn's post-hoc test. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Extended culture influences mitochondrial function and network expansion

We previously reported the use of an embryoid body (EB)-based cardiac differentiation protocol which yielded vigorously beating cardiac clusters by day 14 with high purity of hiPSC-CMs (approximately 95% $NKX2–5^+$ cardiac committed cells with up to 85% positive for cTnT; Supplementary Fig. 1A) [12,14]. To study subsequent bioenergetic, metabolic and developmental advancements, our day 14 hiPSC-CMs were further maintained as cardiac clusters until day 30 in standard glucose medium.

A whole-cell proteome profiling revealed 654 down-regulated and 844 up-regulated proteins (Supplementary Table 3) when day 14-CMs were maintained until day 30. Among the most enriched biological processes (Supplementary Table 4) and cellular compartments (Supplementary Table 5), protein families located in the extracellular matrix, cytoplasm and nucleus which regulate cell adhesion, transcription and translation activities were down-regulated in the day 30-CMs, while families belonging to the mitochondria and sarcomere compartments which regulate energy production (including that of fatty acid β-oxidation) and contractile function, were up-regulated (Fig. 1A and Supplementary Fig. 1B–C), signifying a major switch from basic cellular functions towards specific metabolic and bioenergetic development.

Upon further evaluation of the enriched proteins in mitochondrial-related processes, family members involved in the formation of

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