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Metabolic switching in pluripotent stem cells reorganizes energy metabolism and subcellular organelles

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Abstract

Metabolic studies of human pluripotent stem cells (hPSCs) have focused on how the cells produce energy through the catabolic pathway. The less-studied anabolic pathway, by which hPSCs expend energy in the form of adenosine triphosphate (ATP), is not yet fully understood. Compared to fully differentiated somatic cells, hPSCs undergo significant changes not only in their gene expression but also in their production and/or expenditure of ATP. Here, we investigate how hPSCs tightly control their energy homeostasis by studying the main energy-consuming process, mRNA translation. In addition, change of subcellular organelles regarding energy homeostasis has been investigated. Lysosomes are organelles that play an important role in the elimination of unnecessary cellular materials by digestion and in the recycling system of the cell. We have found that hPSCs control their lysosome numbers in part by regulating lysosomal gene/protein expression. Thus, because the levels of mRNA translation rate are lower in hPSCs than in somatic cells, not only the global translational machinery but also the lysosomal recycling machinery is suppressed in hPSCs. Overall, the results of our study suggest that hPSCs reprogram

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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gene expression and signaling to regulate energy-consuming processes and energy-controlling organelles.

Graphical Abstract

Keywords

Pluripotent stem cell; Mitochondrial; Lysosome; Translation

INTRODUCTION

Pluripotent stem cells (PSCs) have two major characteristics that distinguish them from other types of cells. The first of these characteristics is the capability for “long-term self-renewal,” which enables PSCs to replicate by division. The second characteristic is the “potency of differentiation,” which enables PSCs to make all types of cells under the appropriate environmental conditions. Recently published data show that not only cancer cells but also human PSCs, which include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), prefer to generate ATP in the cytoplasm through glycolysis instead of through oxidative phosphorylation (OXPHOS) in the mitochondria (a phenomenon known as the Warburg effect) [1–7]. Although switching from the more efficient and common OXPHOS system to glycolysis is a hallmark of hPSCs, the reason for this switching is unclear. We have previously reported that downregulation of SIRT2, a NAD-dependent deacetylase, is a molecular signature of human PSCs (hPSCs), distinguishing them from fully differentiated somatic cells. SIRT2 critically regulates the metabolic switch from mitochondrial OXPHOS to glycolysis by targeting glycolytic enzymes to induce the metabolic switch [3]. As a result of this switch, the overall production of cellular energy in the form of ATP is significantly reduced in hPSCs. Thus, hPSCs fine-

tune cellular processes requiring energy expenditure, which includes anabolic metabolism, the process by which macromolecules such as proteins, lipids, and carbohydrates are generated in cells. In addition, compared with somatic cells, hPSCs have large nuclei and scanty cytoplasm, which implies a disconnect between transcription and translation in these cells [8–10]. As a result of the changes in energy generation and energy requirements in hPSCs, many cellular organelles exhibit altered size and function [11]. We examined the changes in two such organelles: mitochondria and lysosomes.

Mitochondria are essential organelles in eukaryotic cells for energy homeostasis, generating 90% of the ATP needed for cellular function. In addition to generating ATP, mitochondria play a crucial role in the regulation of programmed cell death, or apoptosis, which is mediated by cell signaling [12, 13]. The major metabolites and their intermediates are generated in the mitochondrial matrix through four known biochemical pathways: the tricarboxylic acid (TCA) cycle, the urea cycle, OXPHOS, and fatty acid oxidation [12]. It has been reported that mitochondrial numbers, morphology, activity and mtDNA integrity are changed during reprogramming of hPSC, suggesting reorganization of cellular energy and nutrient metabolism in stem cells are required [14].

Lysosomes are commonly referred to as recycling centers; they contain several hydrolytic enzymes that break down macromolecules for recycling and maintain a highly acidic environment. Lysosomes are also regarded as one of the central organelles for energy metabolism. Lysosomes are involved in degradation and recycling of macromolecules and cellular organelles to provide small molecules, nutrients, and energy. Disruption of lysosomal homeostasis leads to dysregulated degradation and recycling of macromolecules such as proteins. In addition, Lysosomes also play an important role in autophagy which is a precise mechanism to remove unnecessary cellular compartments [15]. Therefore, mutations in lysosomal enzymes result in various diseases, such as neurodegenerative disorders (i.e., Parkinson disease, Huntington disease, and Alzheimer disease), cancer, and ageing-related diseases [16, 17]. However, not much is known about the lysosomes in hPSCs.

In this study, we investigated the metabolic switch in hPSCs that affects energy homeostasis. We also determined how hPSCs organize energy generation and energy expenditure by investigating major energy control centers such as mitochondria, lysosomes, and mRNA translation, which is one of most energy consuming processes in cells. Our findings also show that mitochondrial and lysosomal development is incomplete in hPSCs due to metabolic switch.

MATERIALS AND METHODS

Cell Culture

Human iPSCs were generated by infecting fibroblasts (BJ and PCS201 cells, purchased from ATCC) with a Sendai virus vector encoding four reprogramming factors (L-Myc, Oct4, Sox2, and Klf4) (Invitrogen, Carlsbad, CA). Embryonic stem cell (ES)-like colonies were formed after 3 weeks of viral infection or transfection with an episomal vector, and the observed ES-like colonies were manually picked and transferred to mouse feeder cells (mouse embryonic fibroblasts [MEFs]) to generate iPSC lines. The iPSCs were maintained on irradiated MEFs

(GlobalStem). The iPSCs were maintained in ES medium (Dulbecco's modified Minimal Essential Medium (DMEM; Invitrogen) supplemented with 2mM L-glutamine (Invitrogen), 1mM β -mercaptoethanol, 1 $\times$ nonessential amino acids (NEAA; Invitrogen), 20% knockout serum replacement (KOSR; Invitrogen), 100 U/mL penicillin, 100 μ g/mL streptomycin (Invitrogen), and 10 ng/mL basic fibroblast growth factor (bFGF; Invitrogen). Until the iPSC lines were established, iPSC colonies were manually picked. The established iPSC lines were maintained in mTeSR Human Embryonic Stem Cell Culture Medium (STEMCELL Technologies, Vancouver, BC, Canada) with Geltrex™ LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix (Invitrogen).

Fibroblasts were grown in a normoxic condition (5% CO₂ and 21% O₂), whereas iPSCs were cultivated under hypoxic conditions (5% CO₂ and 5% O₂).

Coomassie Staining and Liquid Chromatography Mass Spectrometry

After the iPSC colonies were collected, the cells were washed with PBS for proteomics analysis based on a previously reported protocol [18]. Briefly, proteins were extracted and subjected to concentration analysis by BCA assay (Pierce, Rockford, IL) and short SDS gel staining [19] using bovine serum albumin (BSA) as a standard. Samples were then digested by trypsin and labeled with Tandem Mass Tag (TMT) 11-plex for two-dimensional liquid chromatography and tandem mass spectrometry (LC/LC-MS/MS) analysis. Mass spectrometry data were processed and analyzed by JUMP software suite for protein identification [20] and quantification [21].

Western Blot Analysis

Protein samples (100 μ g) were electrophoresed on 12% SDS-PAGE gels and transferred to Immobilon-NC membranes (Bio-Rad, Richmond, CA). For Western blot assays, each membrane was blocked for 3–5 h in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% (w/v) dry skim-milk powder and then incubated overnight at 4 °C with the primary antibody. The membrane was washed three times with TBS containing 0.05% Tween-20 (TBST) and then incubated for 2 h with the appropriate secondary antibody (Pierce, Rockford, IL). After washing the membrane twice with TBST and once with TBS, the bound antibody was imaged with the Li-COR system. Antibodies to TFEB, LAMP1, LIPA, GAPDH, 4EBP, phospho-4EBP, AKT and phospho-AKT were purchased from Cell Signaling Technology (Danvers, MA). The antibody to puromycin was purchased from Kerafast (Boston, MA).

Immunofluorescence

For immunofluorescence assays, iPSCs were fixed immediately with fixing solution (2% paraformaldehyde, 100mM KCl, 200mM sucrose, 1mM EGTA, 1mM MgCl₂, 10mM PIPES, pH 6.8) for 10 min, washed with PBS for 10 min, and then treated with permeabilization buffer (0.2% Triton X-100, 100mM KCl, 200mM sucrose, 1mM EGTA, 1mM MgCl₂, 10mM PIPES, pH 6.8) for 10 min. The cells were then washed three times with PBS and incubated for 15 min with blocking solution containing 3% BSA in PBS. The cells were then washed a further three times with PBS and incubated overnight with an anti-Oct4 antibody (Invitrogen) in blocking solution. Next day, the cells were again washed three

times with PBS and incubated with the secondary antibody for 2 hrs, a goat anti-mouse IgG conjugated with Alexa Fluor® 488 (green) fluorescent dye (Molecular Probes, Eugene, OR) in blocking solution. The cells were then washed with PBS, and Hoechst dye was added to stain the nuclei before the cells were examined with a confocal laser scanning microscope (Olympus, Melville, NY).

Mitochondrial Activity Measurement

Mitochondrial activity was measured using a Seahorse XF24 Extracellular Flux Analyzer (Agilent, Santa Clara, CA). The Seahorse XF24 Extracellular Flux Analyzer allowed the simultaneous quantification of the mitochondrial respiration rate (the oxygen consumption rate [OCR]). The serial injection of oligomycin, FCCP, and Rotenone/AntimycinA which were mitochondrial inhibitors (1µM oligomycin, and 0.5µM Rotenone/AntimycinA) and mitochondrial OXPHOS uncoupler (1µM FCCP) were used in succession to monitor the mitochondrial activity in fibroblast and iPSCs. Fibroblasts and iPSCs were incubated under normoxic conditions (5% CO₂ and 21% O₂) during the assay.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cells by using a Direct-zol RNA Purification Kit (Zymo Research, Irvine, CA), and cDNA was synthesized with the ThermoScript™ RT-PCR System (Invitrogen). Primers and a TaqMan probe to determine viral integration were purchased from Invitrogen.

ATP Determination Assays

Cellular ATP concentrations were measured with an ATP Determination Kit (Molecular Probes, Carlsbad, CA). Cells (fibroblasts, human iPSCs, and human ESs) were washed three times with PBS and boiled for 5 min in water to lyse them. Cell lysates were collected by centrifugation (14000 rpm) for 15 min at 4 °C. To measure the ATP, chemiluminescent detection was performed using firefly luciferase and luciferin, with the signal being measured by a SpectraMax Microplate Reader (Molecular Devices, San Jose, CA). The protein concentration of the cell lysates was determined by BCA assay (Bio-Rad), and the result in RLU (relative luminescent units) was normalized to the protein concentration.

Transmission Electron Microscope

Samples were fixed overnight with 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7, then post fixed for 1.5 h in 2% osmium tetroxide in 0.1M cacodylate buffer with 0.3% potassium ferrocyanide. After being rinsed in the same buffer, the tissue was stained with 4% aqueous uranyl acetate and dehydrated through a graded ethanol series to propylene oxide. It was then infiltrated through a propylene oxide:epon series, ending with 100% epon overnight. This routine processing was performed on a Leica EM TP Tissue Processor. Next day, the tissue was embedded in fresh epon and polymerized at 70 °C overnight. Semithin (0.5-µm) sections were stained with toluidine blue for light microscope examination. Ultrathin (80-nm) sections were imaged with an FEI Tecnai 200Kv FEG Electron Microscope with an ATM XR41 2K Digital Camera.

ATAC-seq

ATAC-seq libraries for 50,000 cells per sample were constructed in accordance with the published protocol [22]. Libraries were paired-end 100bp sequenced using an Illumina HiSeq 2500 System. The adaptor sequences were trimmed by skewer [23] and then mapped and aligned to hg19 by using BWA (version 0.7.1). ATAC-seq peaks were called using MACS2 [24] with the following parameters: macs2 callpeak --nomodel --shift -100 --extsize 200.

RNA-seq

Total RNA was extracted using the RNeasy Plus Micro Kit (Qiagen). cDNA libraries were constructed using an Illumina TruSeq Stranded mRNA Kit with poly-A selection. Libraries were paired-end 100bp sequenced using an Illumina HiSeq 2500 System. The sequencing reads were aligned to human cDNA from ensembl.org by using Kallisto (version 0.43.0) [25] with the default settings. Differentially expressed genes were called using the Sleuth R package [26].

Flow Analysis for TMRE Dye Staining

To determine the mitochondrial membrane potential (Ψ_m), the potentiometric, cell-permeable fluorescent probe TMRE (tetramethylrhodamine ethyl ester) was used in accordance with the protocol described previously [27].

Translation Assays

To quantify nascent protein synthesis in fibroblasts and iPSC cells, Click-iT Cell Reaction Buffer Kits were purchased from Invitrogen and experiments were performed according to the protocol described previously [28]. Fibroblasts and iPSCs were plated on 15 μ -Slide 8 well (ibidi GmbH, Germany) chamber slide and nascent mRNA translation was measured using Click-iT protein synthesis assay kit under normoxic conditions (5% CO₂ and 21% O₂).

Mitochondrial DNA Quantitative Analysis

To determine the level of mitochondrial DNA in fibroblasts and iPSCs, total DNA was extracted with a Quick-DNA™ Plus Kit (Zymo Research). The DNA was quantified with a Qubit Fluorometer (Invitrogen), and qPCR with MT-ND2 primers (ACCATCTTTGCAGGCACACT, GCTTCTGTGGAACGAGGGTT) was used to quantitate the mitochondrial DNA. B2M primers (TGCTGTCTCCATGTTTGATGTATCT, TCTCTGCTCCCCACCTCTAAGT) were used for normalization.

RESULTS AND DISCUSSION

Mitochondrial Function is Suppressed in Pluripotent Stem Cells

Wild-type male and female iPSC lines were generated by introducing four reprogramming factors (L-Myc, Oct4, Sox2, and Klf4) into human fibroblasts using a Sendai virus vector (known as a non-integration method). Each clone was tested for pluripotency, viral integration, and normal karyotype before being used in further experiments. In brief,

immunofluorescence assays for canonical pluripotency markers (Oct4, SSEA3, and SSEA4) confirmed the pluripotency of the established iPSC lines. Clearance of Sendai virus was confirmed by qPCR using a Sendai virus-specific TaqMan FAM-labeled probe and primers. A VIC-labeled probe for GAPDH was used as an endogenous control. G-band karyotyping was performed to show normal karyotypes (Supplement Fig. 1A, and Supplement Fig. 1B).

The Seahorse XF Cell Mito Stress Test is the gold standard for analyzing mitochondrial function by directly measuring the oxygen consumption rate (OCR). Mitochondrial respiration was significantly lower in iPSCs (blue circle) than in the original fibroblasts (red circle) (Supplement Fig. 2A). In addition, the metabolic switch in hPSCs was demonstrated by the Seahorse XF Cell Energy Phenotype Test. In this analysis, we provided compounds which were necessary to measure metabolic phenotypes and potential of cell and mitochondrial stressors (oligomycin and FCCP) were injected into cells simultaneously to check energy usage and demand to response to mitochondrial stressors. The iPSCs showed a preference for glycolysis in the baseline phenotype (Fig. 1A, red open square (fibroblasts) vs. blue open square (iPSCs)) and a modest increase in glycolysis to meet energy challenge (blue open square to blue solid squares). In contrast, fibroblasts showed an increase in both OXPHOS and glycolysis response to mitochondrial stressors (red open square to red solid squares). Next, we labeled active mitochondria with TMRE and measured the mitochondrial membrane potential, which is closely linked to ATP generation in mitochondria. FCCP, a well-known ionophore uncoupler of OXPHOS, eliminated the mitochondrial membrane potential in fibroblasts (Supplement Fig. 2B). However, FCCP treatment did not change the TMRE intensity significantly in iPSCs (Supplement Fig. 2C), which implied that the mitochondrial membrane potential was lower in iPSCs. Overall, TMRE labeling was less intense in iPSCs than in fibroblasts, which suggests that mitochondrial function is less active in iPSCs (Supplement Fig. 2D). Several types of stem cells exist during development. In pre-implantation embryos, PSCs are referred to as “naïve state” pluripotent stem cell which is from inner cell mass of early blastocyst, and “naïve state” pluripotent stem cell become “primed state” pluripotent stem cell during post-implantation development [29]. Both derivation of human ESC and reprogrammed iPSC from somatic cells belong to “primed state” pluripotent stem cells. Recently published data claimed that “naïve state” pluripotent stem cells still have active mitochondrial function compared “primed state” pluripotent stem cells [30–32].

Mitochondrial function could be suppressed as a result of changes in the number and/or morphology of the mitochondria. Therefore, we investigated whether the mitochondrial number and morphology were altered in iPSCs. There was no significant difference in the number of mitochondria in iPSCs as determined by both electron microscopy and confocal microscopy of cells stained with MitoTracker (Fig. 1B and 1C). In addition, the overall levels of mitochondrial proteins in iPSCs were not significantly different from those in fibroblasts (Supplement Fig. 3, Supplement Table1). However, iPSC mitochondria showed altered morphology, when compared to fibroblast mitochondria, which implied that there was a malfunction in the iPSC mitochondria with regard to OXPHOS. Mitochondrial dynamics play a critical role in mitochondrial function [33], and the remodeling of mitochondria formation by developing cristae has also been linked to mitochondrial function [34]. This indicates that a structural difference in the iPSC mitochondria lead to an

inability to use the OXPHOS metabolic pathway for ATP production. Our data support the hypothesis that incomplete mitochondrial development would influence the shift from OXPHOS to glycolysis in iPSCs, whereas the quantity of mitochondria is not a factor in this phenomenon.

Next, we investigated whether the copy number of the mitochondrial DNA (mtDNA) was linked to mitochondrial function. Even though previous studies found that the copy number of mtDNA did not always correlate closely with the mitochondrial contents in a physiologic setting [35, 36], depleted levels of mtDNA have been reported in ESs and in neural progenitor cells [14, 37, 38]. In this study, we compared the mtDNA levels in iPSCs and in the original fibroblasts. As a control for the mtDNA level, we used rho0 cells (an mtDNA-obliterated osteosarcoma cell line). The level of mtDNA was reduced significantly in iPSCs (Supplement Fig. 4A) and the expression of 13 mtDNA-encoded genes was slightly decreased in iPSCs, as quantified by RNA-seq (Supplement Fig. 4B). Taken together, these findings indicate that iPSCs contain mitochondria that are morphologically different and functionally less active by comparison with those in the original fibroblasts, which leads to insufficient energy being generated and the metabolic switch in iPSCs.

Protein Synthesis is Reduced in Pluripotent Stem Cells

The intracellular ATP levels were significantly lower in iPSCs than in the original fibroblasts as a result of the less active mitochondrial function in iPSCs (Fig. 2A). Changes in energy production altered the energy expenditure in these cells. The translation of functional proteins in the cytoplasm is a major cause of energy consumption in cells, and we investigated its link to metabolic switching in iPSCs. To examine the overall translation rate in iPSCs, we applied several different puromycin-incorporation assays. First, we employed the SUnSET (SURface SENSing of Translation) technique to detect changes in mRNA translation by detecting puromycin-labeled peptides in the cells with an anti-puromycin antibody [39]. Translation in iPSCs was significantly reduced, as shown by the lower density of Western blot bands, which is an indicator of reduced puromycin incorporation and protein synthesis (Fig. 2B). In fibroblasts treated with cycloheximide, an inhibitor of protein synthesis, there was a complete block of puromycin incorporation, demonstrating the specificity of the assay (Supplement Fig. 5). We next used a puromycin analog, O-propargyl-puromycin (OPP), to confirm further the reduction in mRNA translation. OPP is incorporated efficiently into newly synthesized proteins, enabling their quantitative analysis with flow cytometry. iPSCs showed reduced OPP incorporation, confirming that the rate of translation in those cells was lower than that in the original fibroblasts (Fig. 2C). Next, we measured OPP incorporation at the single-cell level. The level of OPP incorporation in each cell was determined by confocal microscopy, and the signal intensity was normalized with a cytoplasm marker (HCS CellMask stain) and nucleus marker (DAPI) (Fig. 2D). Each cell was represented by the segmented color as a single cell, and the level of signal density was determined by Imaris, a confocal microscope image-analysis software (Supplement Fig. 6). Taken together, these all results confirmed that mRNA translation was significantly reduced in iPSCs.

As translation is the most energy-consuming process in the cell, it requires tight, systematic regulation. Mechanistic target of rapamycin (mTOR) is one of the well-known signaling pathways involved in translation. Because the iPSCs were cultivated under hypoxic conditions (5% CO₂ and 5% O₂), whereas the fibroblasts were grown in culture in a normoxic (5% CO₂ and 21% O₂) incubator, we tested fibroblasts under both hypoxic and normoxic culture conditions to check the activation of the downstream target of mTOR. The levels of pan 4EBP (total) and phosphorylated forms of 4EBP proteins were similar, regardless of the culture conditions used in fibroblast (Supplement Fig. 7). However, relatively less phosphorylation due to significantly upregulated 4EBP expression was observed in iPSCs (Fig. 2E and 2F), suggesting that the mTOR signaling pathway was inactivated in iPSCs. In addition, the upstream regulator of mTOR signaling, AKT expression was downregulated in iPSCs, suggesting that total AKT activity is low in iPSCs and that overall mTOR signaling inhibition plays a role in regulating translation in these cells (Fig. 2E). Downregulation of AKT and upregulation of 4EBP was observed in both male- and female-derived iPSCs, which suggests that a sex-independent universal translational mechanism operates in hPSCs.

Comparative Proteomics Profiling Reveals Change of Subcellular Organelles in Pluripotent Stem Cells

We performed comparative proteomics profiling with 11-plex TMT-based quantitative mass spectrometry, which enabled the identification and quantitation of proteins in fibroblasts and iPSCs. The experimental design is shown in Supplement Fig. 8. Among the top 10% proteins that were most variable between fibroblasts and iPSCs, most showed significantly reduced levels in iPSCs (Fig. 3A, blue). Because a discrepancy between transcription and translation had been previously reported [40], we performed RNA-seq and quantitative proteomics (TMT-MS) to identify gene and protein expression changes in iPSCs, respectively. By comparing the levels of transcripts (determined by RNA-seq) and proteins (determined by TMT-MS), we could identify correlations between changes in transcription and protein synthesis during reprogramming. Proteomic profiling using the TMT-MS assay resulted in the identification and quantification of proteins encoded by 9,891 genes (Supplement Table3 and Fig. 3B). Corresponding transcripts for 8,433 of 9,891 total proteins were also detected by RNA-seq (Supplement Table2 and Fig. 3B). Among the 8,433 transcript/protein pairs, we identified 3,295 differentially expressed proteins (fold change $> \pm 2$, FDR < 0.01 , calculated by a moderated t-test in limma software [41] and 5,258 differentially expressed mRNAs (fold change $> \pm 2$, FDR < 0.01) in iPSCs (Fig. 3C). There were 1,941 overlapping differentially expressed genes/proteins; 858 gene products were upregulated and 1,060 gene products were downregulated at both the mRNA and protein levels in iPSCs. In addition, enrichment analysis using KEGG pathways showed that those upregulated and downregulated genes were involved in DNA replication and lysosomes, respectively (Fig. 3D). DNMT3a and 3b play a crucial role in gene regulation in pluripotent stem cells by modifying chromatin, as reported previously [42]. Our data from the present study showed upregulation of DNMT3a and 3b in iPSCs (Supplement Fig. 9). Similarly, Gene Ontology (GO) and pathway enrichment analyses showed that most of the proteins upregulated in iPSCs (Fig. 3E) were involved in DNA replication, whereas downregulated proteins (Fig. 3F) were involved in organelle components such as lysosomes. Taken together,

global changes in transcription and translation in iPSCs alter not only cellular metabolism but also subcellular organelles. Surprisingly, most lysosomal proteins were significantly downregulated in iPSCs, as compared to the original fibroblasts (Fig. 3G).

Lysosomes Do Not Fully Develop in Pluripotent Stem Cells

In iPSCs, overall translation is suppressed, and our analyses of transcription (by RNA-seq) and protein synthesis (by TMT-MS assay) pointed to a reduction in the number of lysosomes (Supplement Table4). To confirm this finding, we applied both electron microscopy and confocal microscopy of cells stained with LysoTracker to visualize lysosomes. Typical lysosomal structures were observed in fibroblasts by both techniques (Fig. 4A). However, lysosomes were not observed in iPSCs (Fig. 4B). Lysosomal acid lipase (LIPA) is a well-known lysosomal protein found in all lysosomal compartments in the cell. Our analyses of transcriptome (by RNA-seq) and proteomics (by TMT-MS assay) showed a reduction in LIPA at both the transcript and protein levels in iPSCs (Fig. 4C). The assay for transposase-accessible chromatin using sequencing (ATAC-seq) identifies chromatin accessibility, which corresponds to transcriptional regulation in cells. ATAC-seq showed that the chromatin in the promoter region of the *LIPA* gene in fibroblasts had an open structure to enhance transcription, whereas the chromatin in the same region in iPSCs had a closed structure, which supports the hypothesis that *LIPA* transcription is inactive in iPSCs (Fig. 4C). In addition, other master regulators of lysosomal biogenesis, such as TFEB (Fig. 4D) and LAMP1 (Fig. 4E) showed significant reductions in gene/protein expression in iPSCs. TFEB has been known as essential transcription factor in the regulation of expression of lots of lysosomal protein, lysosomal hydrolases, and autophagy mediated genes [43], whereas LAMP1 play a crucial role in lysosome integrity and function of lysosome formation [44, 45]. ATAC-seq for *TFEB* and *LAMP1* showed that the chromatin in the promoter region of the *TFEB* and *LAMP1* genes in fibroblasts had an open structure to enhance transcription, whereas the chromatin in the same region in iPSCs had a closed structure, which supports the hypothesis that *TFEB* and *LAMP1* transcriptions were inactive in iPSCs (Fig. 4D and 4E). Whole-cell lysates were isolated and subjected to Western blot analysis with anti-LIPA, TFEB, and LAMP1 antibodies to validate the protein levels in fibroblasts and iPSCs. LIPA, TFEB and LAMP1 expression were significantly down-regulated in iPSCs compared to fibroblasts (Fig. 4F). Lysosomes play an important role in digestive system to remove unnecessary cellular materials and recycling system in the cell. Lysosomes are also critically responsible for autophagy which is a precise process of degradation and recycling of cellular components. Microtubule-associated protein 1A/1B-light chain 3A (LC3A) and ubiquitin binding protein p62 (p62/SQSTM1) have been reported essential marker of lysosome mediated autophagy processing in the cells. Our analyses of the transcriptome and proteomics showed a reduction in LC3 and p62 in iPSCs (Supplement Fig. 10A and 10B, respectively). Taken together, our data suggest that iPSCs do not fully develop lysosomes because there might be unnecessary to make them for recycling macromolecules.

A hallmark of hPSCs is the metabolic switch from OXPHOS to glycolysis. Glucose is a fundamental nutrient for generating cellular energy. In most mammalian cells, glucose is converted into two molecules of pyruvate, and two pyruvate molecules are transported into the mitochondria and fed into the TCA cycle and OXPHOS to generate 34 ATP molecules;

in contrast, glycolysis produces only two ATP molecules. However, OXPHOS requires oxygen and produces reactive oxygen species (ROS), which can be a notorious mutagen, as a by-product. A mutation in hPSCs could be detrimental during their development, so it is necessary to minimize the occurrence of mutagenic events in hPSCs. Therefore, hPSCs generate energy by bypassing OXPHOS and mitochondrial function. Although the fundamental mechanisms of glycolysis and the OXPHOS pathways have been investigated, it is not fully understood how the balance between oxidative and glycolytic metabolism in hPSCs is regulated. Through the present study, we have provided further mechanistic insights into the metabolic switch that occurs in both the anabolic (i.e. translation) and catabolic (i.e. lysosome) pathways in hPSCs. In conclusion, our study suggests that metabolic switch in hPSCs reprograms energy homeostasis system by regulating mitochondria, lysosomes, and mRNA translation that are associated with energy generation and expenditure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHT

Not only energy generation but also energy usage is significantly reduced in human pluripotent stem cells (hPSCs) due to metabolic switch.

The mRNA translation which is one of the most energy consuming process in the cells is reduced in hPSCs.

The metabolic changes are associated with changes in the expression of lysosomal protein and lysosome integrity to be distinguished from other types of cells.

Figure 1.

(A) The Seahorse XF Cell Energy Phenotype Kit was used to measure the metabolic switch in iPSCs. iPSCs (blue, n=10) showed a modest stress response toward the bottom right of the graph, which implied an increase in glycolysis to meet the energy challenge. Fibroblasts (red, n=10) responded to stress by changing their energy phenotype toward the top right of the graph, which is indicative of a combination of increased mitochondrial respiration and glycolysis. (B) Images of mitochondria in fibroblasts were acquired by electron microscopy (left) and by confocal microscopy with MitoTracker being used to

visualize the mitochondria (right). The black and white scale bars represented 400 nm and 10 μm , respectively. (C) Images of mitochondria in iPSCs were acquired by electron microscopy (left) and by confocal microscopy with MitoTracker being used to visualize the mitochondria (right). The black and white scale bars represented 400 nm and 10 μm , respectively.

Figure 2.

(A) Intracellular ATP levels were determined in fibroblasts and iPSCs. The result in RLU (relative luminescent units) was normalized to the total protein concentration. The data were shown as the mean $\pm$ SD of triplicate experiments. The statistical analysis was performed using GraphPad Prism. The results for fibroblasts and iPSCs were compared using an unpaired Student's *t*-test ($P < 0.05$) (B) A puromycin incorporation assay was performed to measure the newly synthesized proteins. Cell lysates were subjected to Western blot analysis with an anti-puromycin antibody. Probing with an anti-GAPDH antibody was employed to

ensure equal loading amounts. **(C)** The nascent protein synthesis rate was measured by an OPP incorporation assay and detected by flow cytometry. **(D)** The nascent protein synthesis rate at the single-cell level was measured by OPP incorporation, using confocal microscopy. The signal intensity after normalization with a cytoplasm marker (HCS CellMask stain) was determined by Imaris, a confocal microscope image-analysis software. Each dot represents a single cell for this analysis. The data were shown as the mean $\pm$ SD. The statistical analysis was performed using GraphPad Prism. Statistical comparisons among the different groups were performed using 1way ANOVA (***P* value <0.01 compared to fibroblasts). **(E)** Whole-cell lysates were isolated and Western blotting analyses were performed to determine the protein levels in fibroblasts and iPSCs. **(F)** The data were shown as the mean $\pm$ SD of triplicate experiments. The band density was determined by Image Studio Lite software (Li-COR).

(A) The 11-plex TMT mass spectrometry enabled the identification and quantitation of proteins in fibroblasts and iPSCs. The top 10% most variable proteins are shown. **(B)** Proteins identified by the TMT-MS assay (9,891 genes). Of the 9,891 proteins, corresponding transcripts for 8,433 were detected by RNA-seq. **(C)** Among those 8,433 proteins, 3,295 were differentially expressed in fibroblasts and iPSCs (fold change >2, FDR < 0.01) and 5,258 transcripts were differentially expressed (fold change >2, FDR < 0.01). **(D)** KEGG pathway enrichment analysis with differentially expressed upregulated

and downregulated genes (**E, F**) Top enriched GO terms and KEGG pathways in terms of significantly upregulated (E, red) and downregulated (F, blue) proteins in iPSCs, as compared to fibroblasts. (**G**) Lysosomal proteins were significantly downregulated in iPSCs, as compared to original fibroblasts, as determined by the TMT-MS assay.

Figure 4.

(A) Images of fibroblasts acquired by electron microscopy (left) and by confocal microscopy with LysoTracker dye being used to visualize lysosomes (right). The black and white scale bars represented 600 nm and 10 μ m, respectively. (B) Images of iPSCs acquired by electron microscopy (left) and by confocal microscope with LysoTracker being used to visualize lysosomes (right). The black and white scale bars represented 600 nm and 10 μ m, respectively. (C) Expression of LIPA. The *LIPA* transcript level was measured by RNA-seq. LIPA protein expression was measured by the TMT-MS assay. The chromatin

accessibility of the *LIPA* locus was determined by ATAC-seq. **(D)** Expression of *TFEB*. The *TFEB* transcript level was measured by RNA-seq, and *TFEB* protein expression was measured by the TMT-MS assay. **(E)** Expression of *LAMP1*. The *LAMP1* transcript level was measured by RNA-seq, and *LAMP1* protein expression was measured by the TMT-MS assay. **(F)** Whole-cell lysates were isolated and subjected to Western blot analysis with anti-*LIPA*, *TFEB*, and *LAMP1* antibodies to validate the protein levels in fibroblasts and iPSCs. Western blot probing with anti-GAPDH antibody was employed to ensure equal loading amount.