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Metabolic substrate shift in human induced pluripotent stem cells during cardiac differentiation: Functional assessment using *in vitro* radionuclide uptake assay

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ABSTRACT

Background: Recent developments in cellular reprogramming technology enable the production of virtually unlimited numbers of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM). Although hiPSC-CM share various characteristic hallmarks with endogenous cardiomyocytes, it remains a question as to what extent metabolic characteristics are equivalent to mature mammalian cardiomyocytes. Here we set out to functionally characterize the metabolic status of hiPSC-CM *in vitro* by employing a radionuclide tracer uptake assay.

Material and methods: Cardiac differentiation of hiPSC was induced using a combination of well-orchestrated extrinsic stimuli such as WNT activation (by CHIR99021) and BMP signalling followed by WNT inhibition and lactate based cardiomyocyte enrichment. For characterization of metabolic substrates, dual tracer uptake studies were performed with ^{18}F 2 fluoro 2 deoxy D glucose (^{18}F-FDG) and ^{125}I β methyl iodophenyl pentadecanoic acid (^{125}I-BMIPP) as transport markers of glucose and fatty acids, respectively.

Results: After cardiac differentiation of hiPSCs, *in vitro* tracer uptake assays confirmed metabolic substrate shift from glucose to fatty acids that was comparable to those observed in native isolated human cardiomyocytes. Immunostaining further confirmed expression of fatty acid transport and binding proteins on hiPSC-CM.

Conclusions: During *in vitro* cardiac maturation, we observed a metabolic shift to fatty acids, which are known as a main energy source of mammalian hearts, suggesting hi-PSC-CM as a potential functional phenotype to investigate alteration of cardiac metabolism in cardiac diseases. Results also highlight the use of available clinical nuclear medicine tracers as functional assays in stem cell research for improved generation of autologous differentiated cells for numerous biomedical applications.

1. Introduction

Human induced pluripotent stem cells (hiPSC) have been shown to represent a virtually unlimited source of spontaneously contracting cardiomyocytes by applying appropriate differentiation protocols [1]. These pluripotent stem cell technologies promise improved therapies of cardiovascular diseases such as patient-specific cardiac repair without immune rejection [2], more robust disease modelling, scalable and standardized drug discovery screenings, as well as stratified or personalized toxicology studies. A bottleneck of induced cardiomyocyte technology is the difficulty in establishing standardized differentiation strategies for homogeneous and fully maturated cardiomyocytes [3, 4]. The cardiac differentiation process is known to be very delicate and needs to be tightly orchestrated by the sequential activation of different signalling pathways resulting in variable cellular maturity with different protocols [5, 6]. Recent protocol improvements achieved by BMP and

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WNT signalling pathway control include enrichment processes to obtain pure and matured cardiomyocyte populations [7]. However, the development of appropriate functional assays to functionally characterize these differentiated cells remains a challenge.

Molecular imaging using radionuclide tracers is a clinically routinely available technology for the visualization of key molecular events in cardiovascular disease [8–10]. Radiolabelled energy substrate analogues such as ^{125}I β methyl iodophenyl pentadecanoic acid (^{125}I-BMIPP) (fatty acid transport marker) [8] and ^{18}F 2 fluoro 2 deoxy D glucose (^{18}F-FDG) (glucose transport marker) [9] are well-established for the clinical diagnosis of ischemic heart diseases. We hypothesized that these well-characterized radionuclide tracers in molecular imaging can be used to assess the functional characteristics of hiPSC-derived cardiomyocytes (hiPSC-CM) and as such provide a novel tool for functional validation of hiPSC-CM *in vitro*. In this study, we harnessed the radionuclide technology in order to test to what extent hiPSC-CM are metabolically equivalent to mature mammalian cardiomyocytes.

2. Material and methods

2.1. Radiopharmaceuticals

^{18}F-FDG was supplied from the in-house University Hospital of Würzburg cyclotron unit. ^{125}I-BMIPP was kindly gifted from Nihon Medi-Physics Co., Ltd., Japan. Radiochemical purity was >95% for both radiolabeled compounds.

2.2. Reprogramming to hiPSC (AF-, K-, and FS-hiPSC)

hiPSC from human adult skin fibroblasts (AF-hiPSC) were obtained by transgene-free technology as previously described [11], after obtaining informed consent and ethical clearance by the ethics committee of the University of Würzburg, Germany (ethical report number 96/11). Briefly, human fibroblasts obtained from a skin punch biopsy were infected with the human STEMCCA Cre-Excisable constitutive polycistronic (OKSM) lentiviral vector and were seeded on irradiated mouse embryonic fibroblasts in a reprogramming medium. After 3 weeks, iPSC-like colonies were picked, expanded on hESC-qualified Matrigel™ (Corning, USA) - coated dishes in mTeSR™1 media (STEMCELL Technologies, Germany) and characterized for the pluripotency markers Oct4 and SSEA-4. Additionally, hiPSCs obtained from human keratinocytes (K-hiPSC, kindly gifted by Dr. Stefan Liebau, University of Tübingen, Germany, [12]) and foreskin fibroblasts derived hiPSC (FS-hiPSC) generated from normal human dermal fibroblasts (NHDF; PromoCell, Germany) were used in this study.

2.3. Cardiac differentiation of hiPSCs

hiPSC (AF-, K- and FS-hiPSC, each cell line from one donor) were differentiated as previously described with minor changes [13]. Respectively, 10 times of cardiac differentiations with AF-hiPSC and 3 times of cardiac differentiations with both K- and FS-hiPSC were performed. For each cardiac differentiation, we carried out n = 3–9 technical measurement replicates (in total, n = 12–30). Briefly, hiPSC were maintained on hESC-qualified Matrigel™-coated plates in mTeSR™ 1 medium until they reached 80% confluency (37 °C, 5% CO_2). Cardiac differentiation was induced by BMP4 (25 ng/ml) (Thermo Fisher Scientific, USA) and CHIR99021 (5 μM) (Axon Medchem, The Netherlands) in RPMI1640 medium (Thermo Fisher Scientific) containing B27 (Thermo Fisher Scientific), β-Mercaptoethanol (100 μM) (Thermo Fisher Scientific) and L-Ascorbic acid (50 μg/ml) (Sigma-Aldrich Inc., USA) as basal medium A. After 24 h, the medium was replaced with the same basal medium A containing CHIR99021 (5 μM) for another 24 h. Afterwards, cells were kept in basal medium B, media similar to media A but containing B27 without insulin (Thermo Fisher Scientific), for 24 h, then the medium was replaced with basal medium B containing the WNT inhibitor IWR1 (10 μM) (Sigma-Aldrich Inc.) for 5 to 7 days followed by further cultivation in basal medium A. Subsequently, cells were kept in basal medium A for 4 to 5 days followed by replacement with cardiac enrichment medium (RPMI1640 without glucose (Thermo Fisher Scientific) + 4 mM sodium L-lactate (Sigma-Aldrich Inc.)). Cells were kept in cardiac enrichment medium for 4 to 5 days. After this enrichment phase, the medium was switched back to basal medium A. Native adult human cardiomyocytes (Adult hCM) were isolated from left atrial appendage biopsy specimens belonging to patients which underwent heart surgery as described [14, 15] with minor modifications after obtaining informed consent and ethical approval by the local ethics committee from the University Hospital Würzburg, Germany (ethical report number 182/10) and kindly gifted from Department Tissue Engineering and Regenerative Medicine, University of Würzburg, Germany.

2.4. Dual-tracer uptake assay for the assessment of functional energy substrates transport

Glucose and fatty acids uptake were assessed with ^{18}F-FDG and ^{125}I-BMIPP as previously reported [8, 9] with a minor modification. In brief, cells (1.0–2.0 × 10^6 cells/well in 12-well plate) were washed with Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich Inc.). DPBS containing 3.0–5.0 × 10^{-1} MBq/mL ^{18}F-FDG and 4.0 × 10^{-2} MBq/mL ^{125}I-BMIPP was added to each well. After 30 min incubation (37 °C, 5% CO_2), the medium was removed and ^{18}F-FDG as well as ^{125}I-BMIPP uptake into the cells were immediately terminated by applying 1 ml of ice-cold DPBS. The cells were washed once with 1 ml of

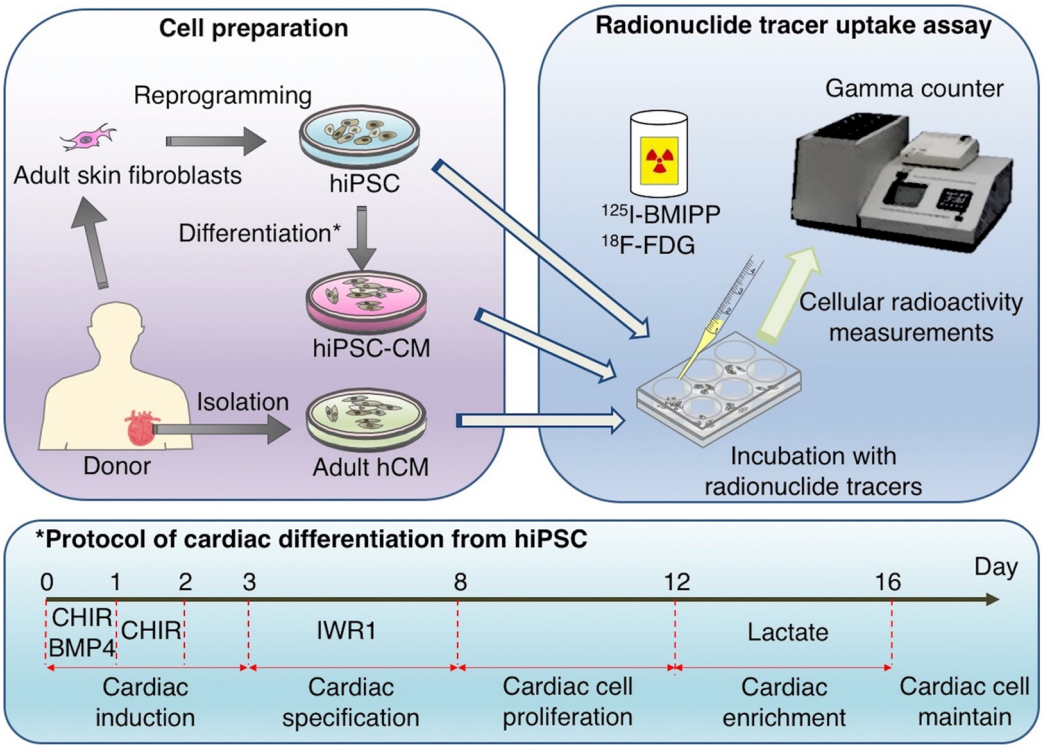

Fig. 1. Schematic presentation of the cell preparation and radionuclide tracer uptake assay.

ice-cold DPBS and then solubilized with 0.1 M NaOH for 1 h. The radioactivity of each lysate was measured by a γ-counter (FH 412; Frieseke & Höpfner, Germany) (Fig. 1). A tracer uptake ratio of ^{125}I-BMIPP radioactivity divided by ^{18}F-FDG radioactivity was calculated.

2.5. Immunostaining

Immunostaining was performed using antibodies for octamer binding transcription factor 4 (Oct4) (1:100; sc-5279, Santacruz Biotechnology, USA), cardiac Troponin T (cTnT) (1:100; ab-8295, Abcam), α-actinin (1:400; A7811, Sigma-Aldrich Inc.), Connexin 43 (Cx43) (1:1000; ab-11370, Abcam), long-chain fatty acid transport protein 6 (SLC27A6) (1:50; 600-401-EP0, Rockland Immunochemicals Inc., USA), cardiac fatty acid binding protein (FABP3) (1:50; TA590642-OR, BioCat GmbH, Germany) and cluster of differentiation 36 (CD36) (1:100; ab-17044, Abcam) as primary antibodies. Briefly, cells were fixed and incubated with the primary antibody at 4 °C overnight followed by incubation with the secondary antibodies at room temperature for 1 h. After nuclear staining with DAPI (1 μg/ml; 10236276001, Sigma-Aldrich Inc.), micrographs were taken with an Oxion3070 microscope (Euromex Microscopen BV, Netherlands).

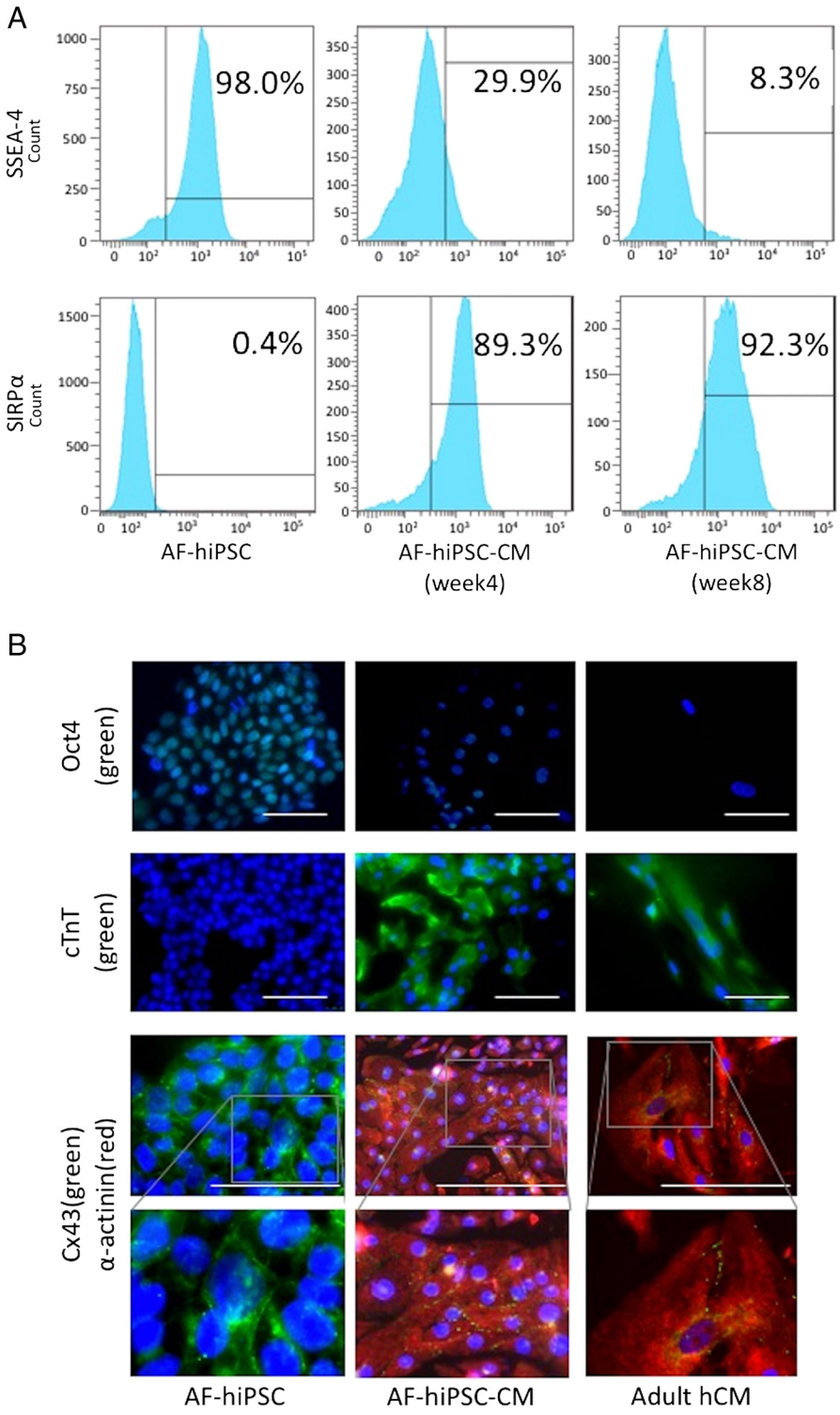

Fig. 2. Results of FACS analysis for pluripotency marker SSEA-4 and cardiomyocyte marker SIRPα (A). Representative immunostaining images for pluripotency marker (Oct4) and cardiomyocyte markers (cTnT, α-actinin) as well as gap junctional marker (Cx43) (B). Scale bars:100 μm, AF-hiPSC: human adult skin fibroblast-derived human induced pluripotent stem cells. AF-hiPSC-CM: AF-hiPSC derived cardiomyocytes, Adult hCM: isolated native adult human cardiomyocytes.

2.6. Flow cytometry

To detect cell surface expression of signal regulatory protein α (SIRPα) and stage-specific embryonic antigen-4 (SSEA-4), 1×10^6 trypsinized cells were incubated with SIRPα antibodies (1:11; 130-099-783, Miltenyi Biotec Inc.) or SSEA-4 antibodies (1:11; 130-098-34, Miltenyi Biotec Inc.) for 15 min at 4 °C, respectively, and measured for fluorescence-activated cell sorting (FACS) analysis. The analysis was performed using FlowJo software (FlowJo LLC).

2.7. Statistics

Results are given as mean ± SD. The two-tailed paired Student's *t*-test was used to compare differences between two dependent groups and the two-tailed independent Student's *t*-test between independent groups. Multiple group comparisons were performed using analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant. Statistical analysis was done with StatMate III (ATMS Co., Ltd.).

3. Results

hiPSC were successfully differentiated into cardiomyocytes using a well-established protocol [13] in all three tested hiPSC lines. Cardiac differentiation after maturation and enrichment process was confirmed by the disappearance of Oct4 positivity and appearance of α-actinin, cTnT and Cx43 positive cells as assessed by immunofluorescence staining. FACS analysis further confirmed the differentiation with an increased number of SIRPα positive cells (>90%) and a decreased number of SSEA-4 positive cells (<10%) after cardiac differentiation (week 8) (Fig. 2).

Dual-tracer uptake analysis with ^{18}F-FDG and ^{125}I-BMIPP was performed at different stages of cardiac differentiation of hiPSC. Tracer uptake ratios of ^{125}I-BMIPP/^{18}F-FDG began to increase at week 2 after cardiac specification and proliferation with WNT inhibitor IWR1, further increased with cardiac enrichment process using lactate, and plateaued

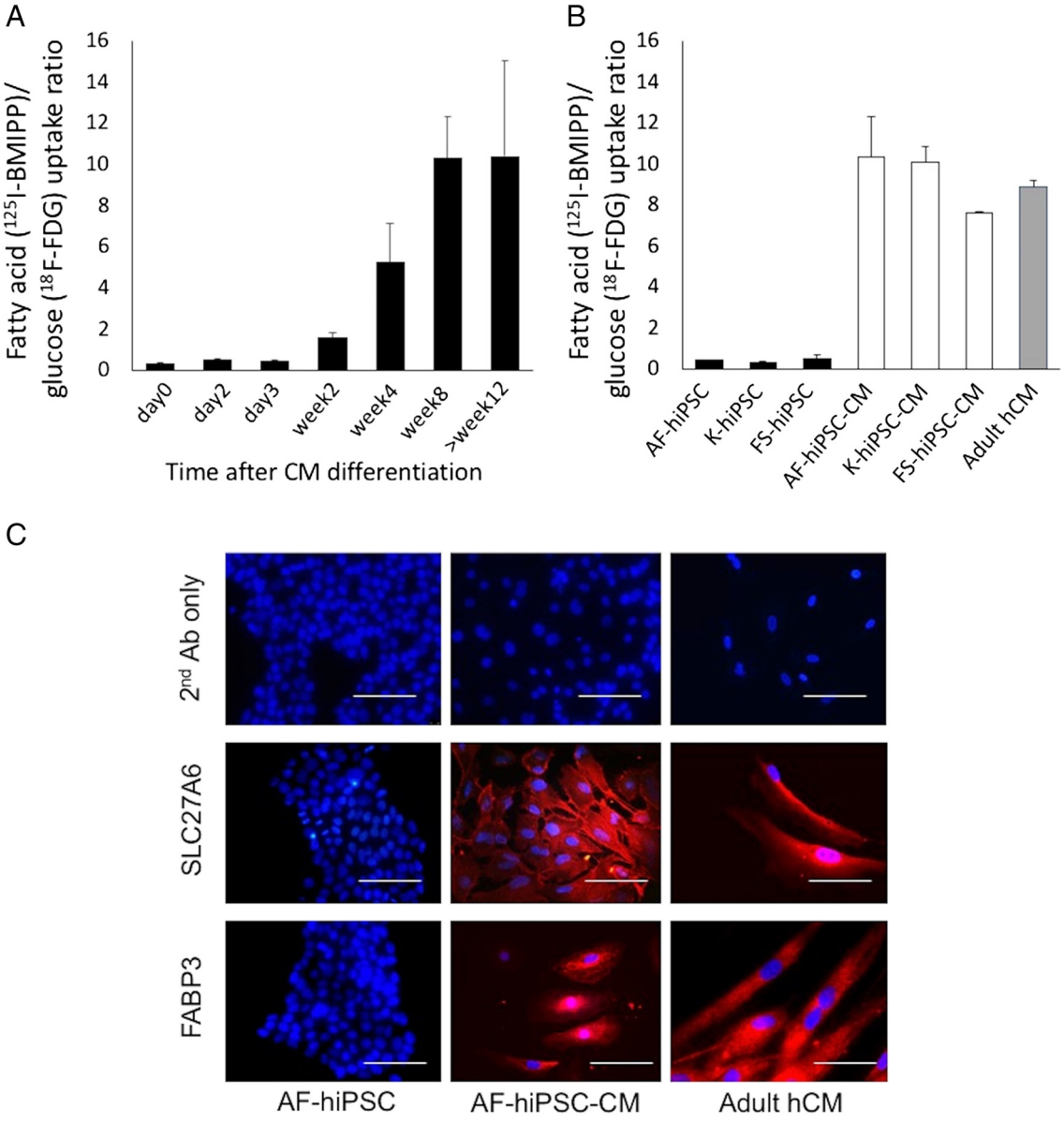

Fig. 3. Results of dual-tracer uptake studies with ^{18}F 2 fluoro 2 deoxy D glucose (^{18}F-FDG) and ^{125}I-β-methyl-iodophenyl-pentadecanoic acid (^{125}I-BMIPP) as a functional marker of glucose and fatty acid transport, respectively. A metabolic substrate shift from glucose to fatty acids is observed during cardiac differentiation of hiPSC (n = 3–9 per uptake measurement replicates) (A). A metabolic substrate shift during cardiac differentiation was confirmed in three different hiPSC lines (n = 3–9 per uptake measurement replicates) (B). Expression of fatty acid transport protein (SLC27A6) and fatty acid binding protein (FABP3) is confirmed by immunostaining in hiPSC-derived cardiomyocytes as well as Adult hCM. (C). Scale bars: 100 μm, K-hiPSC: human keratinocytes-derived induced pluripotent stem cells, FS-hiPSC: human foreskin fibroblasts-derived induced pluripotent stem cells, K-hiPSC-CM: K-hiPSC derived cardiomyocytes, FS-hiPSC-CM: FS-hiPSC derived cardiomyocytes, 2nd Ab only: only the secondary antibodies are used as a negative control.

after maturation at week 8 (Fig. 3A). The increased ratio of ^{125}I-BMIPP/^{18}F-FDG uptake was observed in three different hiPSC lines after cardiac differentiation and was also found in isolated native Adult hCM (Fig. 3B). The results of the *in vitro* tracer uptake study indicated a shift of metabolic substrate usage from glucose to fatty acids after cardiac differentiation. Immunofluorescence staining of hiPSC-CM and Adult hCM confirmed strong positivity for fatty acid transporter (SLC27A6) and fatty acid binding protein (FABP3) while hiPSC showed no expression of both markers (Fig. 3C, Supplemental Figs. 1 and 2). Additionally, increased expression of cluster of differentiation 36 (CD36) during cardiac differentiation of hiPSC was also confirmed by immunostaining (Supplemental Fig. 3).

4. Discussion

The present study is the first to describe functional properties of an energy substrate shift of hiPSC after cardiac differentiation. We elaborated a tracer uptake assay for the assessment of energy substrate transport activity of hiPSC and differentiated cardiomyocytes. Decreased ^{18}F-FDG (radiolabelled glucose analog) uptake along with increased ^{125}I-BMIPP (radiolabelled fatty acid analog) uptake confirmed an energy substrate shift from glucose to fatty acids consistent with cardiac differentiation. The metabolic shift is one of the unique characteristics of *in vivo* mammalian cardiomyocytes that primarily use of fatty acids (>70%) as an energy source (Fig. 4). Immunofluorescence staining confirmed the cellular expression of fatty acid transport and binding proteins on hiPSC-CM supporting the results of tracer uptake assays.

Cardiac differentiation of hiPSC is increasingly recognized in cardiovascular research as a platform for drug discovery, toxicology studies, disease modelling and comprehension of a development of the heart [16]. Many different protocols for cardiac differentiation from pluripotent stem cells have been reported [12]. In this study, we employed a novel, robust strategy to obtain cardiomyocytes from hiPSC lines using state-of-the-art reprogramming technology of well-orchestrated extrinsic stimuli including BMP4 and CHIR followed by WNT inhibition (*via* XAV939 or IWR1) and enrichment of cardiomyocytes by supplying lactate as energy source [7]. The differentiation process can be divided into four phases, namely cardiovascular induction (day 0–3), cardiac specification (day 4–7), cardiac cell proliferation (day 8–12) and cardiomyocyte enrichment (day 13–16) [13]. The results of immunostaining for cardiomyocyte markers (α-actinin and cTnT) and gap junctional marker (Cx43) and FACS analysis for SIRPα indicate successful induction of cardiomyocytes from hiPSC (>90%) after the enrichment phase, a finding that has well consisted with our previous reports [13]. Tracer assays demonstrated metabolic substrate shift (fatty acids uptake > glucose uptake) already beginning in the cardiac cell proliferation phase. The uptake ratio increased during cardiomyocyte enrichment process and ultimately reached approximately a ^{125}I-BMIPP (fatty acid analogue) uptake 10 times higher than ^{18}F-FDG (glucose analogue) uptake. The strongly positive immunostaining for fatty acid transport protein and fatty acid binding protein nicely corresponded to the functional properties of hiPSC-CM as assessed by the tracer assays performed.

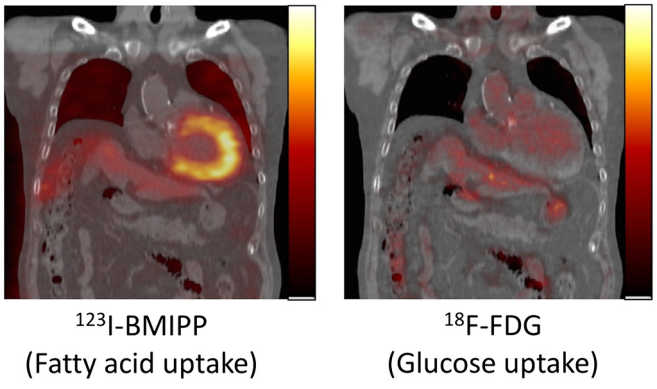

Fig. 4. *In vivo* chest coronal images of radionuclide metabolic imaging in a healthy subject under fasting conditions. Increased cardiac ^{123}I-BMIPP uptake (fatty acids uptake marker) (arrow) as compared to ^{18}F-FDG uptake (glucose uptake marker) is identified.

Moreover, we confirmed a time-dependent increase of fatty acid uptake during cardiac differentiation of hiPSC that is considered as a reference of isolated Adult hCM. It should be noted that, due to our technical limitations, the hCM used in this study were isolated from left atrial appendage. Since our cardiac differentiation protocol would rather result in ventricular like cells, there might be some differences of metabolic status between atrial and ventricular hCM.

In general, a mammalian cardiac function is considered to be maintained by overall oxidative metabolism to generate ATP using different energy substrates. Switching between one to another depends on environmental factors, and loss of plasticity in substrate preference is known to play an important role in pathological conditions. Radionuclide imaging approaches for myocardial substrates including fatty acids (*e.g.* ^{123}I-BMIPP) and glucose (*e.g.* ^{18}F-FDG) have been demonstrated to contribute to understanding the relationship between altered myocardial metabolism and abnormal cardiac conditions [17, 18]. Physiologically, under fasting conditions, fatty acid oxidation is the predominant energy source in the human heart (Fig. 4). Decreased fatty acids metabolism and increased glucose utilization is observed in pathologic conditions such as ischemic myocardium and heart failure, while a lack of glucose utilization is found in diabetic cardiomyopathy. However, still many important issues remain unsolved including the question whether the alteration of energy substrate represents a maladaptive process that can be potentially used as a therapeutic target or an adaptive process [19]. Present results including the combination of metabolic tracer uptake studies and recent technology of hiPSC-CM suggest potential novel implications of metabolic tracer assays as a marker for cardiac functional differentiation as well as further insights into the role of metabolism in cardiac diseases.

In the present *in vitro* cell uptake assay, we employed the clinically well-established radionuclide imaging tracers ^{123}I-BMIPP and ^{18}F-FDG. ^{123}I-BMIPP is the most frequently used single photon emission computed tomography (SPECT) tracer for cardiac fatty acid metabolism imaging [20]. After being specifically taken up into cells *via* CD36 fatty acid transport proteins, ^{123}I-BMIPP is resistant to beta-oxidation because of the methyl group at the beta-position. This results in prolonged cellular tracer retention rendering ^{123}I-BMIPP as a suitable marker of fatty acid transport. On the other hand, ^{18}F-FDG is the most widely used positron emission tomography (PET) tracer for oncological, cardiac and neurological studies. ^{18}F-FDG is transported into cells *via* glucose transporters and subsequently phosphorylated to ^{18}F-FDG-6-phosphate by hexokinase. ^{18}F-FDG-6-phosphate is not a substrate for further glycolytic pathways and – as a charged molecule- retained in the cells. Therefore, cellular ^{18}F-FDG uptake is considered as a reflection of glucose transport and hexokinase activity [21]. To be noted, we conducted dual tracer uptake assays to minimize the variation of the experimental setting between the two tracers. Cells were simultaneously incubated with both ^{125}I-BMIPP and ^{18}F-FDG. Due to differences in gamma ray energy spectra (35 keV and 511 keV, respectively) and radioactive half-lives (59.43 day and 109.8 min, respectively), the respective amount of each radiotracer contained in the cells could be accurately measured.

Thus, our study demonstrates that hiPSC-CM metabolically represent that of native human cardiomyocytes *in vivo* and *in vitro*. As a result, hiPSC-CM harbour a yet unidentified potential for modelling of cardiac energy metabolism. Furthermore, tracer uptake assays might develop into robust and reliable standard validation tests for cardiac differentiation protocol optimization and preclinical quality control.

5. Human and animal rights

All procedures performed in this study involving human participants or human tissues were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from participants included in the study.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2018.06.089.

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Declaration of interest

None.

Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

Disclosure

All authors have approved the final article. Author contributions were as follows:

Study supervision: NN, RAW, YU, CL, MSJ, KF, FE, TH. Conception and design: NN, RAW, YU, CL, KF, MSJ, KF, FE, TH. Development of methodology: NN, RAW, YU, KG, CL, KF, FE, TH. Acquisition of data: NN, RAW, CL, YU, FE, TH. Analysis and interpretation of data: NN, RAW, YU, KG, MSJ, KF, FE, TH. Writing, review and/or revision of the manuscript: all authors. Administrative, technical, or material support: NN, RAW, YU, KG, CL, MSJ, KF, FE, TH.

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