The cell surface marker CD36 selectively identifies matured, mitochondria-rich hPSC-cardiomyocytes

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Dear Editor,

Human pluripotent stem cell (hPSC)-derived cardiomyocytes (CMs) are of significant translational value to in vitro studies of human cardiac development, drug and cardiotoxicity testing and cardiac disease modelling. Differentiation of hPSCs to CMs, however, yields mixed cultures of atrial-, ventricular-, and pacemaker-like cells as well as non-CMs in variable proportions. Strategies to enrich CMs from non-CMs and to generate ventricular versus atrial cells have been successful; however, enriched hPSC-CMs are developmentally immature and fail to recapitulate key functional traits that are fundamental to the (patho)physiology of adult CMs. Moreover, these strategies do not adequately address experimental variabilities caused by differences in the genomes and differentiation capabilities of diverse hPSC lines. One validated approach that overcomes issues of cell heterogeneity and experimental variability is immunophenotyping; however, accessible markers suitable for defining mature, live CMs are lacking. Although cell surface markers such as SIRPA(CD172a) and VCAM1(CD106) have been used to sort for hPSC-CMs, they do not distinguish between maturation states. Here, we report the identification of CD36 as a cell surface marker of cell heterogeneity and experimental variability is immunophenotyping.

To identify cell surface proteins informative of more mature CMs, we qualitatively profiled the surfaceome of cardiac Troponin T (TNNT2)-positive (>95%) human embryonic stem cell (hESC) (H7)-derived CMs (Supplementary information, Fig. S1) using mass spectrometry-based cell surface capture (CSC) technology. We identified 525 N-glycoproteins composed of membrane (85.5%), glycophosphatidylinositol-anchored (5.9%), and extracellular matrix (ECM) (5.0%) proteins. Cluster of differentiation (CD) molecules comprised 14% of the total glycoproteins identified (Supplementary information, Table S1). Comparisons with the Cell Surface Protein Atlas (CSPA) revealed 12 CD molecules with cardiac-prevalent expression (Supplementary information, Fig. S2). Among these, only CD36, a fatty acid translocase implicated in the uptake of long-chain fatty acids (FAs) in adult heart, was up-regulated in CMs as a function of differentiation time and differentially present on the surface of sub-populations of CMs (Fig. 1a, b, Supplementary information, Fig. S3). CD36 is present on the surface of multiple cell types including CMs, hematopoietic cells and adipocytes (Supplementary information, Fig. S2), but it is absent from undifferentiated hPSCs (Fig. 1b) and fibroblasts (not shown). Comparisons with public datasets show that CD36 transcripts increase in mouse and human hearts during in vivo development and are more abundant in ventricles than in atria from late embryonic mouse hearts (Supplementary information, Fig. S4).

Human PSC-CMs could be sorted into CD36hi or CD36lo cell populations with unique traits. Differentiation day (D) 45 CD36hi cells were enriched for α-actinin+ CMs, whereas CD36lo cells contained both α-actinin+ CMs and α-actinin− non-CMs (Supplementary information, Fig. S5). Sorting, after co-staining of CD172a (Supplementary information, Fig. S6) or pre-treatment with lactate, could eliminate most non-CMs. The percentages of CMs positive for CD36 at D45 ± 5 ranged from 34–78, 18–72, 30–81%, to 42–68% in differentiated cultures of H7 and H9 ESC lines and MD1- and JHU001-induced pluripotent stem cell (iPSC) lines, respectively (Supplementary information, Fig. S7). CMs derived from hPSC lines thus display a large degree of intra- and inter-line variability with respect to CD36. In atrial-like CMs induced with retinoic acid (RA), the fluorescent signal intensities for CD36hi CMs were lower than those observed from ventricular CMs derived from H7 or JHU001 lines (Supplementary information, Fig. S8).

Sorted CD36hi CMs (Supplementary information, Fig. S6 for gating) have traits characteristic of a more mature state. CD36hi CMs have similar morphology but on average a 10% greater mean surface area, compared to CD36lo CMs (Supplementary information, Fig. S9a, b). At D45, little to no cell proliferation could be demonstrated, and higher percentages of binucleate cells demonstrated, and higher percentages of binucleate cells were absent from undifferentiated hPSCs (Fig. 1b) and cardiac-prevalent expression (Supplementary information, Fig. S3). CD36hi CMs had a prolonged plateau phase, a decreased diastolic depolarization (DD) rate variability (i.e., arrhythmias) relative to CD36lo CMs. Electro-physiologically, all cells differentiated without RA, displayed ventricular-like action potentials (AP). CD36hi CMs had a prolonged plateau phase, a decreased diastolic depolarization (DD) slope and similar maximum diastolic potential (MDP) compared with CD36lo CMs (Fig. 1d, Supplementary information, S10b).

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amplitudes, reduced spontaneous firing frequencies, longer AP durations and a trend towards higher maximal upstroke velocities (Fig. 1e and Supplementary information, Fig. S10b). Monolayer cultures (500–600k) of CD36hi CMs could also be electrically captured and stimulated to contract over a broader range of pacing cycle lengths (500–1500 ms) than CD36mixed CMs (700–1500 ms). Consistent with this, we detected significantly increased mRNA levels of genes encoding subunits of the I K1 (KCNJ2: 2.69 ± 1.16 fold), Ica (CACNA1C: 3.30 ± 1.59 fold) and a trend towards increased Ikr (SCN5A: 3.27 ± 1.23 fold, p = 0.069). The gene encoding the Ikr subunit (HCN1: 0.29 ± 0.12 fold) was the main currently responsible for automaticity, was significantly reduced (Supplementary information, Fig. S11c). Independent assessments of H7-derived CMs from two laboratories confirmed that the average frequency of cytosolic Ca2+ release was significantly decreased (Fig. 1f, Supplementary information, Fig. S11a–c). The amplitude of Ca2+ transients was also increased, but the time-to-peak and half decay time were either unchanged or modestly increased in CD36hi CMs versus CD36lo CMs isolated from the same cultures (Fig. 1f, Supplementary information, Fig. S11c, d and movies S1 and S2). Genes important for Ca2+ handling, such as PLN and CASQ2, were significantly increased in CD36lo CMs by 1.83 ± 0.38 and 2.64 ± 0.63 fold, respectively (Supplementary information, Fig. S11e).

The major differences between CD36hi and CD36lo CMs were predicted by RNA-seq and gene ontology (GO) analyses to involve mitochondrial metabolism (Fig. 1g, Supplementary information, Tables S2 and 3). CD36hi CMs were characterized by an increased prevalence of mitochondrial and oxidative phosphorylation (OXPHOS)-associated genes (Fig. 1g, Supplementary information, Fig. S11a). Of the 67 OXPHOS transcripts detected by RNA-seq, 33 (49%) were significantly and positively correlated with CD36hi CMs (Supplementary information, Fig. S12b, Table S4). Mitochondrial proteins from Complexes II (SDHB) and III (UCRC2), and ACADVL were elevated in CD36hi CMs (Supplementary information, Fig. S12c, d). Transcripts from non-mitochondrial glycolysis associated genes (e.g., HK2 and ENO2) were largely unchanged and non-correlative. In contrast, CD36lo CMs showed significant increases in mRNA encoding cell adhesion and ECM proteins (Supplementary information, Tables S2 and 3). Metabolically, CD36hi CMs had increased mitochondrial function relative to CD36lo CMs. Mitochondrial mass was increased, and these organelles adopted a more elongated and well-aligned staining pattern (Fig. 1h, i). CD36hi CMs also had a >54% increase in mitochondrial membrane potential (Δψm) relative to CD36lo CMs (Fig. 1i). ATP production was enhanced, and the cellular uptake of a fluorescently labeled C16-palmitate analog was higher in CD36hi versus CD36lo CMs (Fig. 1k, l). When cultured in nutrient-deficient media lacking fatty acids, the Δψm were similar, but when supplemented with oleic acid, the Δψm in CD36lo CMs (43.0%) increased more significantly when compared with CD36lo CMs (22.9%) (Fig. 1m, Supplementary information, S13) consistent with improved β-oxidation. These phenotypic and metabolic traits of CD36lo CMs were maintained across diverse hPSC lines (Supplementary information, Fig. S14). Importantly, both hESC- and hiPSC-derived CD36hi CMs displayed a more metabolically mature phenotype with decreased experimental variability relative to CD36mixed cells in terms of Δψm, ATP production, fatty acid uptake and the %MLC2V* CMs (Supplementary information, Fig. S15). We investigated whether electrical stimulation, which promotes electrophysiological and structural maturation, or activation of the PPARα signaling pathway, which promotes a more advanced mitochondrial phenotype, could up-regulate CD36. Electrical stimulation failed to up-regulate CD36 or to significantly affect the mitochondrial content and Δψm (Supplementary information, Fig. S16a, b). Electrically stimulated CMs gated for high CD36 expression, however, exhibit a greater mitochondrial content and Δψm compared to either CD36mixed or CD36lo CMs (Supplementary information, Fig. S16c). Application of the PPARα agonist, WY-14643, significantly increased the proportion of cells with CD36 on the cell surface (Supplementary information, Fig. S16d). These results are consistent with publicly available data, which show that electrical stimulation does not increase the mRNA level of CD36, whereas application of fatty acids and HIF-1α increases (together with PPARα activation and postnatal factors) promote mitochondrial maturation and up-regulate CD36 mRNA levels (Supplementary information, Fig. S16e). Thus, CD36 is a marker of cardiac mitochondrial/metabolic maturation, and CD36 isolation can be combined with maturation protocols to immunofluorese and isolate CMs with enhanced mitochondrial function.

Mitochondria-rich CD36hi CMs showed enhanced sensitivity to oxidative stimuli compared to CD36mixed and CD36lo CMs. The viability of unsorted CMs was largely unaffected by 10–250μM hydrogen peroxide (H2O2) for treatment periods of 1 or 3 h. In contrast, treatment with 100 μM H2O2 for 30 min significantly reduced the viability of CD36hi, but not of CD36mixed and CD36lo CMs. CD36hi CMs also exhibited more damage in terms of a decline in Δψm, enhanced production of reactive oxygen species (ROS), and an increase in the proportion of cells with nuclear condensation (Supplementary information, Figs. S17–S19). Using a more complex hypoxia (H) and re-oxygenation (R) oxidative stress injury model, all CD36 subpopulations experienced a decrease in viability and demonstrated hypoxic damage (i.e., elevated ROS production and nuclear condensation, decreased Δψm), which was exacerbated by reoxygenation; but these changes and the production of lactate dehydrogenase (LDH) were significantly more pronounced in CD36lo CMs (Supplementary information, Fig. S20).
Doxorubicin (Dox), a commonly used anticancer drug, induces severe and irreversible cardiotoxicity through mitochondrial dysfunction and oxidative stress. Dox is toxic to both mixed and sorted CMs from hESCs and hiPSCs; however, CD36hi CMs are more sensitive to Dox-induced damage than either CD36lo or CD36mixed CMs (Fig. 1f, Supplementary information, Fig. S2f). Furthermore, dextrazoxane, the only FDA-approved drug that protects against doxorubicin-induced cardiotoxicity, is reported to be detrimental to conventionally-grown hPSC-CMs. Unexpectedly, CD36hi, but not CD36mixed or CD36lo CMs, responded positively to the protective effects of dextrazoxane (200 µM) (Fig. 1o). Indeed, pre-treatment with dextrazoxane significantly improved the viability and the ΔΨm of Dox-treated CD36hi CMs. Conversely, N-acetylcysteine, which is effective in mouse models of Dox-induced cardiotoxicity but unprotective in human clinical trials, did not have any significant impact on CD36hi CM viability except at non-physiological doses (10 mM). However, it did protect Dox-treated CD36lo CMs at a dose of 0.1 mM, consistent with results from in vitro animal experiments and in non-sorted hPSC-CMs (Fig. 1o).

In conclusion, we report the presence of a cell surface marker that can immuno-define more mature hPSC-CMs and reduce experimental variability. These properties make CD36hi CMs well suited for disease modelling and drug screening in general, and for studies of Dox-induced cardiotoxicity in particular. The CD36hi CM model is an improvement over existing animal models, which fail to eliminate false positives (e.g., N-acetylcysteine), and to existing hPSC-CM models, which fail to respond to authenticated cardioprotective agents (dextrazoxane). Since mitochondrial dysfunction contributes to a broad range of adult diseases (e.g., arrhythogenic right ventricular dysplasia/cardiomyopathy), injuries (e.g., ischemia/reperfusion) and cardiotoxicties (e.g., Dox), the study of HPSC-CD36 hi CM subpopulations will advance efforts to understand and promote cardiac mitochondrial maturation processes in vitro and provide the impetus for informed investigations of adult cardiac syndromes that involve mitochondrial dysfunction.

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AUTHOR CONTRIBUTIONS

E.N.P. and K.R.B. conceived and supervised the study. E.N.P., K.R.B., S.C.M.W., X.L., R.Z., S.E.W., Y.Y., P.Z., H.B., C.M.C., R.L.G. performed experiments and analyzed data with help from S.E.W., G.C.C., S.Y.T. and H.T.Y. B.Y. and J.S. contributed to the RNAseq data analysis. All the authors participated in data interpretation. E.N.P. and K.R.B. wrote the paper with editing support from S.E.W., and input from all authors.

ADDITIONAL INFORMATION

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