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Title: *In vitro model of ischemic heart failure using human induced pluripotent stem cell-derived cardiomyocytes*

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Conflict of Interest Statement: Jeffery Creech and Andre Monteiro da Rocha are consultants to CARTOX, Inc.; Todd J. Herron is co-founder of and has financial interest in CARTOX, Inc. CARTOX, Inc. is a Michigan company focused on the development of novel hiPSC-CM based assays for cardiotoxicity screening. T.J.H is also a consultant to StemBioSys, Inc.

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Abstract
Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) have been used extensively to model inherited heart diseases, but hiPSC-CM models of ischemic heart disease are lacking. Here our objective was to generate an hiPSC-CM model of ischemic heart disease. To this end, hiPSCs were differentiated to functional hiPSC-CMs and then purified using either a simulated ischemia media or by using magnetic antibody based purification targeting the non-myocyte population for depletion from the cell population. Flow cytometry analysis confirmed that each purification approach generated hiPSC-CM cultures of >94% cTnT+ cells. Following purification hiPSC-CMs were re-plated as confluent syncytial monolayers for electrophysiologically phenotype analysis and protein expression by Western blotting. Metabolic selected hiPSC-CM monolayers’ phenotype recapitulated many of the functional and structural hallmarks of ischemic cardiomyocytes, including: elevated diastolic calcium, diminished calcium transient amplitude, prolonged action potential duration, depolarized resting membrane potential, hypersensitivity to chemotherapy induced cardiotoxicity, depolarized mitochondrial membrane potential, depressed SERCA2a expression, reduced maximal oxygen consumption rate and abnormal response to β1-adrenergic receptor stimulation. These findings indicate that metabolic selection of hiPSC-CMs generates cell populations with phenotype similar to what is well known to occur in the setting of ischemic heart failure, and thus provides a novel opportunity for study of human ischemic heart disease.

Introduction
The use of human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) makes it possible now to generate a virtually unlimited supply of human cardiomyocytes that can be used for research purposes. (1, 2) hiPSC-CMs have been utilized to model many monogenic cardiac diseases such as long QT syndrome (3, 4), hypertrophic cardiomyopathy (5), catecholaminergic polymorphic ventricular tachycardia (CPVT) (6) and Timothy syndrome (7). On the other hand, there are few reports on the generation of hiPSC-CM models of acquired heart disease such as ischemic heart disease and failure. Ischemic heart failure is a significant health problem that affects over 5 million patients in the US and currently the only cure is cardiac transplantation. The development of new and effective therapies to treat ischemic heart failure will be hastened by the availability of an in vitro hiPSC-CM model of ischemic cardiomyopathy.

Importantly the in vitro hiPSC-CM ischemic heart failure model should recapitulate the molecular and functional hallmarks that are well known to occur in patients and research models of heart failure. For example, contractile function of the individual cardiomyocytes is depressed in human heart failure and this has been confirmed in numerous animal models of ischemic heart failure. (8) Molecular and genetic modification of key contractile and calcium handling proteins underlie the poor contractile function of the failing heart and cardiomyocytes. Specifically, heart failure is associated with reduced SERCA2a gene expression and reduced phospholamban (PLN) phosphorylation. (9, 10) These proteins directly regulate the amount of calcium available to trigger contraction and therefore represent molecular targets for heart failure therapy. SERCA2a gene overexpression in the heart rapidly hastens the removal of cytosolic calcium during diastole, thus alleviating diastolic dysfunction. In fact, SERCA2a gene therapy was shown in vitro to improve the contractile function of human cardiomyocytes isolated from patients afflicted with heart failure and is being tested as a new therapy for heart failure patients. (11-13) Thus, an in vitro model of human ischemic heart failure should present with reduced contraction, reduced calcium flux amplitudes, reduced SERCA2a protein expression, prolonged calcium transient duration, prolonged action
potential duration and reduced PLN phosphorylation. Additionally, the functional phenotype should be rescued using established SERCA2a gene therapy approaches known to improve cardiomyocyte contractile function. Further, the ischemic heart failure in vitro model should recapitulate elevated sensitivity to cardiotoxic therapies such as doxorubicin chemotherapy. Our goal here was to demonstrate these features of human heart failure phenotypes using hiPSC-CMs in vitro.

Currently, the most widely utilized approach for purification of hiPSC-CMs relies on marked metabolic differences in glucose and lactate metabolism between cardiomyocytes and non-cardiomyocytes.(14) Tohyama et al(14) cultured pluripotent stem cell derivative cells with glucose-depleted culture medium supplemented with abundant lactate and found that only cardiomyocytes survived. This method of hiPSC-CM purification has been adopted by many laboratories and is a highly efficient method for large scale purification.(15, 16) However, this same media composition has been utilized historically to simulate ischemia pre conditioning in cultured adult cardiomyocytes.(17) Also, similar glucose free solution has been referred to as “injury solution” before to produce localized ischemic injury to cardiac monolayers.(18, 19) Here we hypothesized that this simulated ischemia media (0 glucose, abundant lactate) generates purified hiPSC-CMs with an ischemic heart failure-like phenotype. To test this hypothesis, we compared the phenotype of metabolic stress purified hiPSC-CMs with the phenotype of hiPSC-CMs purified using a commercially available magnetic activated cell sorting approach (MACS, Miltenyi Biotec). The MACS approach and subsequent enrichment of the hiPSC-CMs was recently described and validated for use in cardiotoxicity proarrhythmia assays.(20, 21)

Figure 1A outlines the general cardiac directed differentiation and purification approaches used here. All functional and structural phenotype analysis was performed on syncytial hiPSC-CM monolayers. The electrophysiological dysfunction (calcium transient alternans, red traces of figure 1C) apparent during metabolic selection is depicted in figure 1C and provided us rationale for further study.
**Results**

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) were enriched for preparation of syncytial monolayers with a metabolic selection protocol or with magnetic activated cell sorting (MACS) targeting non-cardiomyocytes for immunodepletion (Figure 1A). Two separate established hiPSC lines were used here: 1) 19-9-11 hiPSC line (WiCell, iPS DF19-9-11 T.H)(22), and 2) BJ-hiPSC line (generated in house using mRNA reprogramming).(23, 24)

Cardiomyocyte enrichment was successful with both methods across the cell lines used, and the two methods yielded similar percentages of cardiomyocytes (figure 2A, metabolic selection=97.03±2.9% purity, n=4 and MACS selection=95.2±3.0% cTnT+ cells, n=4). Cardiomyocytes selected with each of the two different methods formed functional syncytial cardiac monolayers with cell-cell propagation of electrical impulses as described before(21, 23-26); nevertheless, there were several phenotypic differences between cardiomyocytes obtained by the two methods such as heterogeneous expression and mislocalization of connexin 43 (Figure 2B), decrease in sarcomere length and organization (Figure 2C), and the appearance of actin stress fibers in metabolic stress selected hiPSC-CMs (Figure 2D). Additionally, functional phenotyping of cardiomyocytes indicated changes in important functional characteristics of cardiomyocytes like intracellular calcium handling and electrophysiology.

**Metabolic selection alters intracellular calcium handling and blunts β-adrenergic response of cardiomyocytes**

Figure 1C shows that metabolic selection induces calcium transient alternans in hiPSC-CM monolayers prior to re-plating; whereas calcium transient alternans were not observed in non-selected hiPSC-CM monolayers. Next, hiPSC-CM monolayers selected with the two purification methods were loaded with calcium sensitive dye (Fura2AM) for quantification of intracellular calcium
transients and displayed divergent functional phenotypes. The frequency of spontaneous intracellular calcium activations was higher in syncytial monolayers of cardiomyocytes prepared after metabolic selection (0.52±0.07Hz, n=9 vs. MACS=0.27±0.05Hz, n=7; *significant difference t-test, P<0.001; figure 3A) in comparison to those prepared after MACS sorting. Furthermore, metabolic selected hiPSC-CM monolayers had higher diastolic cytosolic calcium concentrations in comparison to MACS enriched cardiomyocytes (0.04±0.008 fura ratio units, n=15 vs. MACS=0.01±0.004, n=13; t test, P<0.001; figure 3A). Calcium transient amplitude was significantly greater in the MACS enriched hiPSC-CM monolayers compared to metabolic selected monolayers (MACS=0.14±0.05fura ratio units, n=13 vs. metabolic selection=0.09±0.03fura ratio units, n=15; t test, P=0.014; figure 3A).

Next, we sought to determine the molecular mechanism to account for the differences in calcium flux observed between metabolic and MACS based purification approaches. Western blotting was employed to determine the relative expression levels of SERCA2a - the primary calcium pump of cardiomyocytes responsible for cytosolic calcium sequestration into the sarcoplasmic reticulum. Impaired intracellular calcium handling in metabolic selected cardiomyocytes was mechanistically associated with significantly lower expression of SERCA2a protein (0.94±0.21au, n=6 vs. 2.31±0.56au, n=6; significant difference t-test, P=0.002 Figure 3B) in comparison to MACS selected cardiomyocytes. Furthermore, in figure 3C, imaging intracellular calcium flux (with fura-2) after isoproterenol treatment demonstrated that MACS selected cardiomyocytes present expected chronotropic and inotropic responses to β-adrenergic stimulation, as both spontaneous frequency and calcium transient amplitude significantly increased after isoproterenol treatment (from 0.27±0.05Hz to 0.5±0.1Hz, n=3 and amplitude increased from 0.09±0.06 Fura2 ratio units to 0.15±0.10 fura ratio units, n=3). On the other hand, in figure 3C, β-adrenergic stimulation of cardiomyocytes purified with metabolic selection did not induce an inotropic effect, as spontaneous frequency significantly increased (from 0.5±0.08 up to 1.0±0.4Hz, n=3 red traces) without concomitant increase in
intracellular calcium amplitude (pre isoproterenol=0.08±0.05 Fura2 ratio units and post isoproterenol amplitude=0.06±0.06 Fura2 ratio units, n=3).

To further explore molecular mechanisms at play, we examined the phosphorylation state of two key cardiomyocyte proteins following isoproterenol (500nmol·L⁻¹) stimulation of β1 adrenergic receptors: namely cTnI and phospholamban. Cardiomyocytes harvested from syncytial monolayers prepared after metabolic selection had higher base-line cTnI phosphorylation in comparison to MACS sorted cardiomyocytes, but presented lower cTnI phosphorylation after isoproterenol treatment (figure 3D). Investigation of phospholamban phosphorylation levels by Western blotting provided complementary mechanistic insight on metabolic selected cardiomyocytes intracellular calcium dysregulation. Untreated metabolic selected hiPSC-CM monolayers presented lower levels of phosphorylated phospholamban in comparison to MACS selected cardiomyocytes (figure 3E). After isoproterenol treatment, MACS selected cardiomyocytes had a ~15-fold increase in phosphorylated phospholamban whilst metabolic selected cardiomyocytes did not have any detectable increase in phosphorylated phospholamban.

**Metabolic stress selection impacts hiPSC-CM electrophysiology**

Following hiPSC-CM purification using MACS or metabolic stress selection, cells were replated as electrically and mechanically confluent monolayers for electrophysiological analysis as described before.(20, 21, 27) First electrophysiology of hiPSC-CM monolayers was investigated using sharp microelectrode recordings or optical mapping with a voltage sensitive dye. Figure 4A shows original action potential recordings and quantification of Maximal Diastolic Potential (MDP) and Action Potential Amplitude (APA). MDP was less negative (depolarized) in metabolic selected monolayers compared to MACS purified hiPSC-CM monolayers (MACS= -71.4±4.84mV; n=11 vs. metabolic stress hiPSC-CMs=-59.9±7.59mV, n=9 *denotes significance, unpaired t-test, P<0.001). This baseline membrane potential difference translated to greater frequency of spontaneous activations in metabolic stress selected monolayers (MACS=0.41±0.16Hz; n=24 monolayers vs. metabolic stress hiPSC-CM).
selection=0.97±0.29Hz; n=24 *significant difference unpaired t-test, P<0.001) and lower action potential amplitude (Figure 4B). To precisely investigate action potential duration, hiPSC-CM syncytial monolayers were paced at different frequencies during optical mapping with voltage sensitive dye (Figure 4C). Action potential duration at 80% of repolarization (APD80) was significantly longer in metabolic stress selected hiPSC-CM monolayers in comparison to MACS selected monolayers at 1.0, 1.5, 2.0 and 2.5Hz pacing frequencies (Figure 4C). Finally, metabolic stress selected and MACS selected hiPSC-CM syncytial monolayers were submitted to an arrhythmia test consisting of abrupt decrease in pacing from 2.5Hz to 1.0Hz while recording optical action potentials. All syncytia prepared with MACS selected cardiomyocytes were able to follow the decrease in pacing frequency with 1:1 capture; however, seventy-two percent of metabolic stress selected hiPSC-CM monolayers displayed early afterdepolarizations (Figure 4D).

**Effect of hiPSC-CM purification approach on mitochondrial membrane potential (Δψm)**

Mitochondria have been implicated in several human diseases, including cardiac disease and are sensitive to energy substrates.(28) Next, we determined the impact of hiPSC-CM purification approach on mitochondrial membrane potential (Δψm) using the carbocyanine compound, JC-1. Figure 5A shows confocal images of hiPSC-CM monolayers made from metabolic stress selected or MACS selected CMs loaded with JC-1. It is apparent in this image that there is a greater amount of red staining in the MACS sorted cardiomyocytes relative to metabolic stress selected hiPSC-CMs. Indeed, quantification of the ratio of red/green fluorescence shows that metabolic selected hiPSC-CMs have a lower ratio and thus have depolarized Δψm (0.57±0.06 au, n=5 vs. MACS=0.65±0.04, n=4; *significant difference-unpaired t-test, P<0.001). Δψm depolarization is indicated by the reduction of the red to green fluorescence intensity ratio and this difference persisted even following treatment with the mitochondrial uncoupler, FCCP (metabolic selected hiPSC-CM=0.21±0.01 au, n=5 vs. MACS=0.34±0.06, n=4). Further complementary analysis of mitochondrial membrane potential was done using MitoTracker Red CMXRos dye (Figure 5B). MitoTracker Red CMXRos dye accumulates
in the mitochondria and its accumulation is dependent on mitochondrial membrane potential. (29)

Figure 5B shows greater MitoTracker Red dye accumulation in MACS purified hiPSC-CMs, indicating
more hyperpolarized, active mitochondria compared to CMs purified using metabolic stress selection.

**Metabolic selection increases susceptibility of cardiomyocytes to doxorubicin induced cardiototoxicity (DOX-TOX)**

Doxorubicin (Adriamycin) is a chemotherapy drug with well-documented cardiotoxicity referred to as DOX-TOX. DOX-TOX has been linked to direct effects on mitochondrial function and intracellular calcium flux. We hypothesized that metabolic selected hiPSC-CMs are more sensitive to DOX-TOX owing to the baseline differences in mitochondrial membrane potential, electrophysiology and intracellular calcium flux identified in figures 3-5. To test this, metabolic stress and MACS selected cardiomyocyte monolayers were challenged with doxorubicin concentrations ranging from 100 to 1000nM. First, cell survival was assessed in cardiomyocyte monolayers using annexin V staining (figure 6A). AnnexinV live cell staining demonstrated similar DOX-TOX of metabolic stress selected and MACS selected cardiomyocytes to doses equal to or below 750nM of doxorubicin. However, the number of cells undergoing apoptosis after exposure to 1000nM of doxorubicin was ~3X higher in metabolic stress selected cardiomyocyte syncytial monolayers compared to MACS selected monolayers (169.4±75.1 AnnexinV+cells/mm², n=4 vs. 51.8±5.1 AnnexinV+cells/mm², n=4; mean±sem, t-test P<0.001; figure 6A).

Optical mapping with calcium sensitive dye (rhod2AM, figure 6B&C) was performed after chronic exposure (48h) to doxorubicin and percentage of monolayers with arrhythmias was calculated for each group. Arrhythmia phenotypes included early after depolarization, extra calcium release and tachycardia. Doxorubicin concentration of 250nM or below did not induce arrhythmias in monolayers from either group. 500nM or greater concentrations of doxorubicin induced arrhythmias and significantly higher arrhythmia burden in metabolic stress selected cardiomyocytes syncytia than in MACS selected syncytial monolayers (Figure 6B&C). Arrhythmia burden reached 100% in metabolic
stress selected hiPSC-CM syncytial monolayers at 750nM of doxorubicin treatment with absolute
predominance of EADs (Figure 6B&C) and severe tachyarrhythmia at 1000nM of doxorubicin.

**SERCA2a over expression corrected calcium transient duration prolongation associated with heart failure**

Finally, we tested the effectiveness of SERCA2a gene therapy to improve the function of the
metabolic stress selected hiPSC-CMs. Recombinant adenovirus (Vector Biolabs) designed to deliver
SERCA2a gene and mCherry promoter was applied to MACS sorted and metabolic stress treated
monolayers (100 moi, multiplicity of infection). mCherry expression indicated robust expression
throughout the monolayers (figure 7A) and Western Blotting confirmed elevated SERCA2a
expression levels (figure 7A). Functional analysis measuring intracellular calcium flux (fluo-4)
indicated spontaneous arrhythmias in the metabolic stress media treated cells and prolonged calcium
transient duration 50 (CaTD50). The quantification in Figure 7C indicates correction of the CaTD50
with SERCA2a gene therapy to control levels.

**Metabolic stress media affects the electrophysiological phenotypes of MACS Purified hiPSC-
CMs & Antibiotic Resistance Purified hiPSC-CMs**

Next we tested the effect of the CDML3 metabolic selection media on hiPSC-CMs that were
purified using the MACS approach. This enabled testing the effect of the metabolic stress condition of
0 glucose and abundant lactate on hiPSC-CM function without interference of non-cardiomyocytes
death. Here we used the same hiPSC line (19-9-11), differentiated to cardiomyocytes as outlined in
figure 1 and purified hiPSC-CMs using MACS selection. hiPSC-CMs were re-plated into two separate
96 well plates: 1 plate was cultured in maintenance media (RPMI/B27+insulin) and the other plate
was cultured in CDML3 media, each for 10 days prior to electrophysiological recordings. Figure 8
outlines the effects of CDML3 media on hiPSC-CM electrophysiological function, assessed using
voltage sensitive dye (fluovolt). Figure 8A shows representative action potential traces from hiPSC-
CMs cultured in media containing glucose (RPMI+B27). Figure 8B and C show examples of spontaneous arrhythmias recorded in hiPSC-CMs that were cultured in zero glucose/abundant lactate media (CDML3) for 10 days following MACS sorting and re-plating. Importantly, all recordings were made in the same physiological salt solution (HBSS) following the media treatments—indicating that the effects of each media persist following media changes. Quantification of action potential duration and action potential triangulation are presented in figure 8D-F. APD30 was significantly prolonged in hiPSC-CM monolayers cultured in CDML3 (309.7± 29.0 ms, n=95 vs. 284.7± 27.8, n=94; unpaired t-test, P<0.0001). APD90 was also significantly prolonged in hiPSC-CM monolayers cultured in CDML3 media (1,266.7±499.9 ms, n=95 vs. 823.2± 366.1 ms, n=94; unpaired t-test, P<0.0001). Action potential triangulation is a unitless parameter calculated by the equation: 

$$AP\ Triangulation = \frac{(APD90 - APD30)}{APD90}.$$ 

Increased AP Triangulation occurs when the APD is prolonged and is used as an index to describe the potential to cause arrhythmias. hiPSC-CM monolayers cultured for 10 days in CDML3 media had greater AP triangulation values (0.72±0.12 au, n=95 vs. 0.60±0.13 au, n=94; unpaired t-test, P<0.0001). Increased AP triangulation increases the odds for observing spontaneous arrhythmias in these monolayers cultured in zero glucose and abundant lactate (CDML3). Indeed, figure 8G shows that the increased APD values and increased AP triangulation manifested as greater number of spontaneous arrhythmias occurring in the CDML3 treated group (22/95 well with arrhythmias vs. 00/94 wells with arrhythmias).

To rigorously test the effect of CDML3 media on a separate hiPSC-CM source, high purity commercially available cardiomyocytes (iCell cardiomyocytes\textsuperscript{2}) were cultured in CDML3 media for 10 days. Action potential duration (APD50%) was significantly increased by metabolic stress induced with CDML3 (APD50%:499.24± 9.3ms, n=24; p=0.01) in comparison to RPMI media (APD50%:464.9±9.91ms, n=24, Figure 8H). Intracellular calcium transient duration (CaTD50%) was significantly increased in syncytia treated with CDML3 (596.77±9.48 ms, n=24) in comparison to syncytia cultured in control media (536.82±8.89 ms, n=24; p<0.0001, Figure 8I). Further, in two
separate 96 well plates where one full plate was maintained in CDML3 media and the other plate in RPMI media, we found reduced intracellular calcium amplitude induced by the CDML3 media treatment (RPMI B27=0.0866±0.0013 a.u., n=96 and CDML3=0.0369±0.0012 a.u., n=96, p<0.0001, Figure 8J). These data collectively support the hypothesis that media containing zero glucose and abundant lactate can be used to simulate cardiac injury and create an arrhythmogenic substrate in syncytia of commercially available hiPSC-CMs.

At last, in another set of experiments, MACS purified hiPSC-CMs were cultured in CDML3 or RPMI media prior to bioenergetics assessment with Seahorse XFe96 analyzer (19-9-11 hiPSC-CMs 7 days of CDML3 exposure). Oxygen consumption rate was affected by 7 days of exposure of MACS separated cardiomyocytes to CDML3 media. Mitochondrial oxygen consumption rate (OCR) was significantly lower in cardiomyocytes after 7 days of exposure to metabolic stress, although there are no differences in ATP-driven and non-mitochondrial OCR (Figure 8K-M). Bioenergetics analysis showed significant reduction of maximal respiration [RPMI B27=34.14±3.58 (pmoles/min)/mg of protein and CDML3=25.34±1.9 (pmoles/min)/mg of protein, p=0.02 Figure 8L] and decrease in respiratory reserve capacity (RPMI B27=23.04±3.25 (pmoles/min)/mg of protein and CDML3=14.56±1.05 (pmoles/min)/mg of protein, p=0.006, Figure 8M) in cardiomyocytes cultured under metabolic stress conditions. Seahorse XFe96 analyzer data concatenate with mitochondrial membrane potential data described in the previous section.

**Discussion**

To date the most widely used method for hiPSC-CM purification relies on metabolism differences between cardiomyocytes and non-cardiomyocytes. The widely used hiPSC-CM purification media contains 0 glucose and abundant lactate; a media composition that has been used historically to simulate ischemia in native adult cardiomyocytes *in vitro.* (17-19) Here we tested the hypothesis that this metabolic mediated hiPSC-CM selection subjects the CMs to a metabolic stress that induces an ischemic-like phenotype. To this end, we completed a direct comparison of the
functional and structural phenotypes of hiPSC-CMs purified using either metabolic selection media (0 glucose, abundant lactate) or magnetic activated cell sorting (MACS) using commercially available reagents (figure 1). A robust feature of this approach is that we were able to differentiate a batch of hiPSCs to hiPSC-CMs and then utilize either purification approach on the same batch of cells. Thus, the comparisons are tightly time-matched.

We report distinct structural and functional phenotypes induced by each hiPSC-CM purification approach. hiPSC-CM selected by means of alteration of energetic substrate presented a reduction in membrane bound connexin43 in contrast to hiPSC-CMs that were not submitted to metabolic stress (Figure 2B). Changes in connexin43 expression are not pathognomonic of cardiac ischemia; nevertheless, it has been long known from studies using animal models of ischemia/hypoxia and post-mortem human specimens that ischemia induces alterations in connexin43 expression, localization and phosphorylation, impacts on cardiac function and produce a substrate for reentrant arrhythmias (30-35).

Cardiomyocytes exposed to 0 glucose and abundant lactate had sarcomere shortening in comparison to hiPSC-CMs selected with MACS (Figure 2C), similarly to the decrease in sarcomere length demonstrated a long ago in in vitro and animal models of myocardial infarction, hypoxia and hypoxia with glycolytic blockade(36-38). Similar sarcomere disorganization was apparent with cTnT staining in a separate hiPSC cell line (supplemental figure 1). Furthermore, staining with phalloidin revealed the abundant presence of non-striated F-actin fibers amongst sarcomeres (also known as stress fibers) in metabolic stress selected cells (Figure 2D); whilst MACs purified hiPSC-CMs presented striated pattern of phalloidin staining characteristic of sarcomeres. Stress fibers are present in different cell types and have important roles in cell adhesion and migration. In cardiomyocytes, stress fibers participate in early stages of sarcomere morphogenesis (39) and they can be observed in dedifferentiated cardiomyocytes adjacent to immobilized areas of the heart (40). Whether the presence of stress fibers in cardiomyocytes submitted to metabolic stress is indicative of
dedifferentiation or immature phenotype is out of the scope of this manuscript; nevertheless, cardiomyocytes purified with glucose deprivation and high availability of lactate present nuclear expression of Ki67 and retain proliferation potential (supplemental figure 2)(14); and suggest the hypothesis that metabolic selection delays hiPSC-CM maturation. Although we have not further explored the expression of genes associated to maturation of cardiomyocytes, Ordono et al. (41) has shown that exposure of hiPSC-CMs to 6mM of lactate for 3 days is able to induce upregulation of genes associated to dedifferentiation and proliferation.

Functionally, metabolic stress selected hiPSC-CM monolayers showed electrophysiological and intracellular calcium handling properties reminiscent of cardiomyocytes isolated from ischemic failing hearts. Figure 3 summarizes the differences in hiPSC-CM calcium handling between the two purification approaches. Metabolic selected syncytial monolayers had higher diastolic calcium concentrations, reduced calcium transient amplitudes and reduced SERCA2a expression levels at baseline relative to MACS sorted hiPSC-CMs (figure 3). These are all features reminiscent of the phenotypes well documented for heart failure cardiomyocytes.

The expression and phosphorylation status of calcium handling proteins regulate cardiac function and are implicated as causal for poor function of the failing heart. SERCA2a expression, for example, is significantly reduced in animal models of heart failure and this molecular defect contributes to diastolic dysfunction and poor contraction of the failing heart.(10) In fact, increasing SERCA2a expression levels has shown to be effective to restore cardiac function in animal models of heart failure and clinical trials have been conducted to deliver the SERCA2a gene to patients with heart failure.(42) Here we have discovered that metabolic stress selection of hiPSC-CMs produces cardiomyocytes with reduced SERCA2a expression compared to hiPSC-CMs purified using MACS approach (figure 3B). Furthermore, we used SERCA2a gene therapy approach in vitro to restore the function and arrhythmia phenotypes of metabolic stress selected hiPSC-CM monolayers (figure 7).
Another calcium handling protein consistently implicated in contributing to heart failure phenotypes is phospholamban.\(^{43}\) It is well established that phospholamban is phosphorylated upon myocardial β-adrenergic stimulation and this contributes to positive inotropic and lusitropic effects in healthy hearts. In cases of heart failure, however, phosphorylation of phospholamban is reduced and this is attributed to dysfunctional adrenergic signaling.\(^{9, 43, 44}\) Here we have found that metabolic selection generated hiPSC-CMs in which phospholamban phosphorylation did not occur upon isoproterenol treatment (figure 3E). MACS selected hiPSC-CM monolayers, however showed significant phosphorylation of phospholamban upon isoproterenol stimulation (figure 3E). This suggests a dysfunctional or underdeveloped β-adrenergic signaling pathway in metabolic stress selected hiPSC-CMs. These molecular differences most likely underlie the disparate functional responses to isoproterenol presented in figure 3C. MACS selected hiPSC-CM monolayers responded to isoproterenol with positive chronotropic and inotropic responses. On the other hand, metabolic selected hiPSC-CM monolayers only showed a positive chronotropic effect of isoproterenol treatment with no significant positive inotropic effect. cTnl phosphorylation, however, was induced by isoproterenol treatment in both groups. These molecular and cellular differences of intracellular calcium flux in hiPSC-CMs purified by distinct methods resemble the extensive comparisons that have been made historically between native cardiomyocytes isolated from healthy and failing hearts.

Key electrophysiological parameters including MDP, APD and rate adaptation were also impacted by purification approach. Figure 4 outlines the key electrophysiological differences that we observed between metabolic selected hiPSC-CMs and MACS selected hiPSC-CMs. Microelectrode recordings in figure 4A show differences in maximal diastolic potential (MDP) and action potential amplitude (APA) between hiPSC-CMs purified by each method. MDP was significantly depolarized in metabolic selected hiPSC-CMs syncytial monolayers compared to MACS selected hiPSC-CMs. Depolarization of MDP and smaller APA may be attributed to elevated diastolic calcium concentrations observed in metabolic selected hiPSC-CMs relative to MACS selected hiPSC-CM.
monolayers. Depolarization of MDP also contributes to the differences of baseline spontaneous contraction rate between the groups (figure 4B)-depolarized MDP sets the membrane potential closer to threshold for firing an action potential. Action potential duration (APD) was significantly prolonged in metabolic selected hiPSC-CM monolayers even over a range of electrical pacing frequencies (figure 4C). APD prolongation is a feature well known to occur in heart failure, contributes to QT prolongation of the EKG and increases heart failure patients’ risk for suffering a fatal arrhythmia. (45)

Arrhythmia testing by rapid reduction of pacing frequency from 2.5 to 0.5Hz revealed significantly greater incidence of EADs and extra contractions in metabolic selected hiPSC-CM monolayers relative to MACS selected monolayers (figure 4D). The electrophysiological differences between hiPSC-CM monolayers purified by each method outlined in figure 1 resemble the well-established differences between healthy and heart failure cardiomyocytes. (45)

Furthermore, we have observed changes in mitochondrial function that recapitulate bioenergetic features of cardiomyocytes submitted to ischemia (Figure 5 and Figure 8K-M); namely, an overall reduction of mitochondrial membrane potential and function. This was measured using fluorescent mitochondrial membrane potential sensitive dyes and with a respirometry assay performed with a Seahorse XFe96 analyzer. Figure 8K-M show the bioenergetic differences between hiPSC-CM monolayers that were maintained in either CDML3 (metabolic selection media) or glucose containing RPMI media for seven days. Metabolic selection media produced hiPSC-CM monolayers with reduced maximal oxygen consumption rate and respiratory reserve capacity. The reduction of maximal oxygen consumption rate is similar to clinical data showing that human cardiomyocytes isolated from the left atrial appendage from patients suffering of chronic myocardial ischemia have loss of maximal oxygen consumption rate(46). Inherent mitochondrial functional differences also underlie the enhanced sensitivity to DOX-TOX observed in metabolic selected hiPSC-CMs (figure 6). In combination with elevated calcium concentrations in metabolic stress selected hiPSC-CMs, the mitochondrial dysfunction combines to increase sensitivity to DOX-TOX. Metabolic selected hiPSC-
CMs showed increased apoptosis after doxorubicin treatment compared to MACS sorted hiPSC-CMs (figure 6B). Also, DOX-TOX indexed by arrhythmia occurrence was more apparent in metabolic stress selected hiPSC-CMs relative to MACS sorted hiPSC-CM monolayers (figure 6C&D). This indicates that the purification approach should be carefully considered when using hiPSC-CMs to predict patient specific sensitivity to chemotherapy induced cardiotoxicity.

The approach of metabolically starving non-myocytes in order to enrich cardiomyocyte populations derived from stem cells has been employed for over seven years (14) and has proven to be an effective approach. The metabolic starvation technique is attractive owing to its ease of use, applicability to bulk processing and extremely low cost. Here we have used the same metabolic starvation approach with subtle modification for comparison to a novel extracellular receptor-magnetic bead based approach that is commercially available (Miltenyi Biotec). Compared to the MACS based purification, metabolic selection generates cardiomyocytes with a heart failure like molecular and functional phenotype. This provides a novel acquired heart failure model system for in vitro testing and research. There are some slight differences in protocols that require discussion. The first reports of using metabolic starvation used 8 days of treatment for purification of cells, and later publications using this method applied longer durations up to 10 days (16). Figure 1 shows that we used 10 days of metabolic media selection to obtain hiPSC-CMs for our comparisons, except for oxygen consumption rate experiments that were performed after 7 days of continuous exposure to CDM3L. Although we demonstrated that 7 days of treatment with CDM3L affects energy metabolism of cardiomyocytes, we cannot state that shorter exposures to CDM3L such as used by Sharma et al. (47) induce an ischemic phenotype and this should be subjected to further investigation. During the differentiation protocol, previous reports began the metabolic media purification treatment at day 10, however we have initiated the metabolic selection at day 14. The timing of introducing metabolic selection and the duration of exposure may impact on cellular phenotypes. Despite the subtle protocol differences, the rigorous experimental design using time matched controls in each
differentiation experiment for each purification approach here enabled the comparisons between hiPSC-CM purification approaches.

**Conclusion**

Heart failure affects approximately 6.5 million Americans(48), the majority of these heart failure patients suffer ischemic heart failure which generally occurs secondary to coronary heart disease. Current heart failure treatments are palliative in nature and cardiac transplantation is possible, but inadequate to address a significant portion of the population. A major obstacle to development of new and effective heart failure treatments has been the lack of viable human cardiac tissue and myocytes available for research. Our results indicate that human heart failure molecular and functional phenotypes can be modeled *in vitro* using metabolic selection of hiPSC-CMs. The use of acquired heart failure *in vitro* models using human cardiac syncytial monolayers offers advantages over the use of animal models and provides a testing platform for development of new heart failure therapies. The use of syncytial monolayers of human cardiomyocytes can only be accomplished using pluripotent stem cell derived cardiac myocytes (PSC-CMs) and here we have validated that these monolayers are sensitive to metabolic stress and can recapitulate many molecular and functional features well known to occur in cardiomyocytes obtained from failing human hearts.

**Methods**

**hiPSC maintenance.** Vector free hiPSCs were maintained on matrigel coated 6 well plates using Xeno-free media (iPS Brew, Miltenyi Biotec) essentially as described before.(20, 49) Two vector free control cell lines were utilized to determine efficiency of cardiomyocyte purification approaches (19-9-11 and BJ iPSCs). The 19-9-11 hiPSC line was obtained from WiCell and the BJ iPSC line was generated from commercially available human dermal fibroblasts in the laboratory using mRNA
reprogramming. hiPSCs were maintained as colonies and passaged once every week. Pluripotent stem cell use was approved by the University of Michigan HPSCRO Committee.

**Cardiac Directed Differentiation and hiPSC-CM purification.** Figure 1A outlines the cardiac directed differentiation and purification protocols. We utilized the highly efficient small molecule approach relying on temporal modulation of the Wnt signaling pathway. (50) On day 14 cardiac differentiation cultures were switched to metabolic stress selection media (CDM3L, 0 glucose, 5mM sodium DL-lactate) or maintained in cardiomyocyte maintenance media (RPMI/B27+insulin). Phase contrast images of the differentiated cardiomyocytes in CDM3L or RPMI maintenance media are shown in figure 1B and indicate significant non-myocyte cell death in CDM3L media as expected based on previous reports. (16) Figure 1C data shows time matched spontaneous calcium transient recordings of hiPSC-CM monolayers either with no selection or on day 10 of metabolic stress selection process prior to replating. The non-myocyte cell death creates holes in the monolayer that, in addition to the high lactate concentrations, contribute to the observed dysfunction. For metabolic selection of hiPSC-CMs cell cultures were kept in CDM3L for 10 days and on day 24 purified hiPSC-CMs were trypsinized and re-plated as confluent monolayers for phenotype analysis. This is a slight deviation from the original reports of Tohyama et al, which used 6 to 8 days of metabolic stress selection with CDM3L to purify stem cell derived cardiomyocytes to 93% and 98% respectively. (14, 51, 52) There is however, a precedence for our use of 10 days of CDM3L metabolic stress selection media in the work of Burridge et al. (16) In 2014 Burridge et al(16) reported that 6-10 days of glucose deprivation using CDM3L was optimal to purify cardiomyocytes from 85% to >95% purity. In fact, 10 days of selection was found to purify hiPSC-CMs to a greater extent than shorter time periods (6 days). For MACS hiPSC-CM purification, differentiation plates were kept in normal maintenance media and processed for cardiomyocyte purification on day 24 of the protocol (Miltenyi Biotec, PSC-derived cardiomyocyte isolation kit, human). The MACS purification approach uses antibody to target a non-myocyte extracellular epitope to magnetize the non-myocytes, which are trapped in a magnetic
field while non-labelled cardiomyocytes flow through the magnetic field and are collected; similarly to
the alternative approach to the use of SIRPA antibodies for positive selection of cardiomyocytes
suggested by Dubois et al (53). In each group the media was changed every other day. Flow
cytometry was performed to assess the relative purity of cardiomyocyte cultures purified with the two
methods by probing cTnT expression (Anti-cardiac Troponin T-APC; Miltenyi Biotec 130-120-403).
On the day of MACS purification hiPSC-CMs were re-plated in parallel to the metabolic selected
hiPSC-CMs for phenotype comparison. Plating procedures have been described recently in
detail.(20, 26, 49) In each condition, phenotype analysis to study the structure and function of the
hiPSC-CM monolayers was done 7 days after re plating. This approach enabled phenotype analysis
of the same batch of hiPSC-CMs that were time-matched, but purified by distinct methods.
Experiments were done to determine the effectiveness of an established gene therapy reported to
reverse heart failure phenotypes. This data is in figure 8. Ad-mCherry-hATP2A2 was obtained from
Vector Biolabs (Malvern, PA), stored at -80C and applied to hiPSC-CM monolayers plated in 96 well
plates using MOI=100 (MOI=Multiplicity of Infection, 50,000 hiPSC-CMs per well). mCherry
expression was detected using live cell time lapse imaging (Incucyte Zoom, Essen Bioscience, Ann
Arbor, MI) and expression was robust by 48h following gene transfer. Following optical mapping of
intracellular calcium flux using fluo4AM (10μM), monolayers were collected/solubilized in SDS sample
buffer for Western blot detection of SERCA2a protein.

**Cardiac monolayer optical mapping and electrophysiology.** hiPSC-CM monolayer
electrophysiology was measured using optical mapping or micro electrode recording as previously
described.(20, 26, 49) Action potential properties were measured using the voltage sensitive dye,
fluovolt (ThermoFisher) or by microelectrode recording. Action potential duration (APD80) restitution
was quantified using field stimulation of monolayers (20V, 5ms duration, various Hz). Arrhythmia
screening was done using a dynamic stimulation frequency assay as previously described.(54)
Briefly, monolayers were paced at 2.5Hz and action potential responses to immediate slowing of pacing frequency (1.0Hz) were quantified.

Calcium transients were recorded using either rhod2AM, fluo-4AM or the more quantitative calcium dye-Fura 2AM. For fura2AM measurements, monolayers were re-plated on to glass bottom dishes for microscopic imaging using an IonOptix system with alternating excitation between 340nm and 380nm ultraviolet light with emission recordings in the green spectrum (515nm). Fura2 measurements are independent of cellular motion and amount of dye loaded and thus reflect intracellular calcium concentration changes. Fura2 measurements are expressed as ratio units. In each purification approach, intracellular calcium response to β-adrenergic receptor stimulation (500nM isoproterenol) was quantified using Fura2 loaded monolayers. Rhod2 loaded monolayers (10µM) were used to determine sensitivity to doxorubicin induced arrhythmias in large monolayers.

**Control Experiments using Commercially Available Purified hiPSC-CMs.** In control experiments we tested the hypothesis that CDML3 metabolic stress media also induces electrophysiological abnormalities in commercially available human iPSC-CMs. To test the effect of CDML3 metabolic stress media on hiPSC-CMs from another source, we applied this media to commercially available human cardiomyocytes (iCell2; Cellular Dynamics International, Madison, WI) that are purified by mechanisms other than those used in this study. These cells were handled, plated and used for optical mapping as outlined before. Results of these experiments are presented in supplemental figure 5. First we generated a 96 well plate of these cells, using 50,000 cells per well to form monolayers. One half of the plate was cultured in RPMI (glucose containing) media and the other half of the plate was cultured in CDML3 (zero glucose, high lactate) media. After 10 days in culture, the plate was used for functional analysis with half of the plate being used for action potential measurements (Fluovolt) and the other half being used for calcium transient analysis (fluo-4AM). Next, to determine the effect of CDML3 media on calcium transient amplitudes two additional 96 well
plates of iCell² cardiomyocytes were generated. In one plate, all 96 wells were cultured in RPMI media for 10 days while the other plate was maintained in CDML3 media for the same 10 days. On day 10, cells were loaded with rhod2AM (10µM for 15min) and spontaneous calcium flux was recorded using the LED based optical mapping system.

**Mitochondrial membrane potential measurements.** Mitochondrial membrane potential ($\Delta \psi_m$) was determined using the carbocyanine compound JC-1, the optimal dye to use for quantification of changes of $\psi$ in cardiomyocytes.(55, 56) JC-1 dye exhibits potential dependent accumulation in mitochondria, indicated by a shift in fluorescence emission from green to red. As a result, mitochondrial depolarization is indicated by a decrease of the red/green fluorescence intensity ratio. JC-1 dye was loaded into cardiomyocytes (2µM, 30 min) in the incubator at 37°C. Red and green emission was recorded in live cells using a Nikon A1R confocal microscope with the appropriate laser and detection wavelengths. FCCP (50 µM, carbonilcyanide p-trifluoromethoxyphenylhydrazone), a potent uncoupler of mitochondrial oxidative phosphorylation, was applied to validate the JC-1 measurements.

**Western blotting analysis.** Protein expression was analyzed by Western blotting essentially as previously described.(20, 49) For each purification approach SERCA2a protein expression was determined using a monoclonal antibody (ThermoFisher, MA3-919; 1:300). Phospho-specific antibodies were used to determine phosphorylation levels of cardiac troponin I (cTnI) (Cell Signaling Technology, 4004S; 1:100) and phospholamban (PLB) (Millipore, 07-052; 1:150) with or without isoproterenol treatment (500nM).

**Immunofluorescence.** Monolayer structure was observed by immunofluorescence as previously described.¹⁴ For each purification approach, sarcomeric $\alpha$-actinin (Sigma, A7811; 1:300) and connexin 43 (Sigma, C6219; 1:100) protein expression were visualized using monoclonal antibodies. Cell nuclei were also labeled using DAPI (1:1000). $\alpha$-actinin spacing was measured using fluorescence intensity-space plots as shown in figure 2C. Confocal images were collected using a
Nikon A1R confocal microscope (60X objective) from five separate monolayers generated using each purification method. Average sarcomere spacing was quantified from at least seven intensity-space plots drawn perpendicular to separate sarcomere patterns for each image.

**Apoptosis Assay.** The extent of hiPSC-CM apoptosis immediately following each purification approach was quantified using Annexin V Green reagent (Essen Bioscience, Cat. No. 4642). Monolayers resulting from each purification method were plated on PDMS and incubated with annexin V (ThermoFisher, A13201) and doses of doxorubicin ranging from 0nM to 1000nM for 48 hours. Cells were then imaged using the Incucyte Zoom time lapse microscope (Essen Biosciences, Ann Arbor, MI) and annexin V fluorescence analyzed with its software.

**Author Contributions**

Experimental design was by all authors. Cell culture experiments including stem cell maintenance, cardiac differentiation, purification and replating were done by JD, AC, JC, AMR, and KC. Microelectrode recordings of action potentials were done by DPB and EJV. Optical mapping of action potentials and calcium transients were done by JD, TJH, JC and AMR. Mitochondrial membrane potentials were measured by JD, TJH and AMR. AL, AMR and NM executed bioenergetic analysis using the SeaHorse. All authors contributed to writing and editing the manuscript.

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References


Figure 1. **hiPSC-CM differentiation protocol and purification approaches.** **A**, Timeline of small molecule based cardiac directed differentiation and purification approaches. **B**, Phase contrast images showing the impact of metabolic selection on monolayer confluence (19-1-11 hiPSC line, day 24). **C**, hiPSC-CM spontaneous calcium transient recordings in 19-9-11 hiPSC-CMs without any selection (black, n=6 monolayers) and with metabolic selection (red, n=5 monolayers). Spontaneous calcium transient alternans were induced by metabolic selection media (red traces and bars). * denotes significant difference of amplitude between even and odd numbered beats. Quantification in panel C shows the relative amplitude of each beat following beat #1-normalized to beat #1 amplitude. Without selection, the amplitude did not vary beat to beat (beat #2=0.97±0.02, beat #3=0.98±0.01; n=6). However, in lactate selection media (CDML3) the beat amplitude varied on a beat to beat basis (beat#2=0.84±0.11, beat #3=0.99±0.01; n=6, paired t-test, P<0.05), indicating calcium transient alternans.
Figure 2. hiPSC-CMs generated using two distinct purification approaches. 

A, Each purification approach is equally effective to enrich hiPSC-CM population analyzed by flow cytometry with cTnT labelling. 

B, Connexin43 (Cx43) mislocalization was apparent in metabolic stress selected monolayers. 

C, Metabolic stress selected hiPSC-CMs had shorter sarcomere length (MACS=1.85±0.17µm; n=35 vs. metabolic stress selection=1.77±0.13µm; n=40, *denotes difference, unpaired t-test, P=0.009. Sarcomere length was quantified using the repeating fluorescence pattern of the α-actinin staining. 

D, Phalloidin staining was used to examine the actin cytoskeleton. Stress fibers rather than sarcomeres were identified in the metabolic stress selected hiPSC-CMs.
Figure 3. hiPSC-CM purification approach impacts on cardiomyocyte intracellular calcium flux. 

A, hiPSC-CM Monolayer Calcium Transients: Metabolic stress selection significantly alters baseline calcium flux of hiPSC-CMs. B, SERCA2a expression levels were reduced in metabolic stress selected hiPSC-CMs. C, MACS purified cardiomyocytes (black traces, symbols) responded as expected to isoproterenol; metabolic stress selected hiPSC-CMs however only responded with positive chronotropy. D, cTnI phosphorylation state was elevated at baseline in metabolic stress selected hiPSC-CMs (0.19±0.05, n=3 vs. 0.03±0.02au, n=3). cTnI phosphorylation increased following isoproterenol stimulation (metabolic stress=0.75±0.29, n=3 and MACS=0.99±0.30au, n=3). E, PLN phosphorylation levels (PLN-P) were similarly low at baseline in each group, and only in MACS sorted hiPSC-CMs was PLN-P detected at significant levels following isoproterenol treatment (metabolic stress=0.05±0.06au, n=3 vs. MACS=0.77±0.35au, n=3; *P=0.02).
Figure 4. hiPSC-CM purification approach impacts electrophysiology phenotypes. A, Microelectrode recordings show Maximal Diastolic Potential (MDP) was depolarized in metabolic stress selected hiPSC-CMs Action potential amplitude (APA) was greater in MACS selected hiPSC-CM monolayers (MACS=103.5±12.5mV; n=11 vs. metabolic stress selection=81.5±17.9mV; n=9*denotes significant difference unpaired t-test, P<0.001). B, Metabolic selected monolayers had significantly greater spontaneous beating frequency. C, Metabolic stress selection monolayers had significantly longer APD80 than MACS selected monolayers over a range of pacing frequencies (1Hz: metabolic=357.12±6.85ms; 1.5Hz: metabolic=312.24±7.03ms; 2.0Hz: metabolic=279.38±6.21ms; 2.5Hz metabolic=250.29±4.20ms; n=9-12 monolayers) vs. (1Hz: MACS= 309.56±6.85ms; 1.5Hz: MACS= 279.16±3.83ms; 2.0Hz: MACS= 248.60±6.31ms; 2.5Hz: MACS=216.93±4.20ms; n=12 monolayers, mean±sem). D, Arrhythmia testing was done by abrupt slowing of pacing from 2.5 to 1.0Hz with continuous recording. MACS purified monolayers could follow the pacing change with 1:1 capture, however 72.2% of metabolic selected monolayers displayed arrhythmias.
Figure 5. **Metabolic selection induces mitochondrial dysfunction in hiPSC-CM monolayers.**

A, JC-1 staining indicates more polarized mitochondria in MACS purified monolayers. Numeric values are in the main text.

B, Mitotracker Red CMX Ros staining supports the JC-1 results. Mitotracker signal, MACS=1369.11±355.66 au vs. metabolic stress selection=635.45±302.12, * t test P<0.001.
Figure 6. Metabolic stress selected hiPSC-CM monolayers have increased sensitivity to chemotherapy induced cardiotoxicity (DOX-TOX). A, Annexin V staining indicates greater extent of apoptosis in metabolic purified hiPSC-CMs treated with 1000nM Doxorubicin (DOX). B, Calcium transient optical mapping using rhod2 example traces show greater sensitivity of electrophysiological function of metabolic stress selection hiPSC-CMs (red traces). C, Metabolic stress selected hiPSC-CM monolayers show arrhythmias in response to DOX-TOX with greater sensitivity than MACS purified hiPSC-CM monolayers.
Figure 7. **SERCA2a calcium pump gene therapy reverses hiPSC-CM heart failure phenotype.**

A, Viral transduction was verified by mCherry live cell expression in hiPSC-CM monolayers. Further, SERCA2a protein expression was determined by Western Blotting. Quantification indicates that SERCA2a protein expression was reduced in metabolic stress treated hiPSC-CMs at baseline (MACS=2.11±0.22, n=3 vs Metabolic stress=1.27±0.06, n=3). AdSERCA2a treatment restored SERCA2a levels to control levels (Metabolic stress + Ad SERCA2a=2.15±0.17, n=3; *ANOVA P<0.05). B, Calcium transient measurements indicate that metabolic stress selection media prolongs the cardiac action potential and spontaneous arrhythmias were observed in the metabolic stress selection purification approach. hiPSC-CM monolayers treated with AdSERCA2a did not develop arrhythmias. C, Calcium transient duration 50 (CaTD50) was prolonged in metabolic stress media treated cells at baseline and was corrected to control values with AdSERCA2a gene therapy (CaTD50s: MACS baseline=475.5 ±18.1 ms; metabolic stress baseline=577.37 ±17.7 ms; MACS + AdSERCA2a=396.6 ±11.2 ms; Metabolic stress + Ad SERCA2a=493.67 ±19.9 ms; ANOVA *P=0.01, **P=0.002, ns=not significant).
Figure 8. CDML3 metabolic selection media induces arrhythmia phenotypes in MACS purified 19-9-11 hiPSC-CM monolayers and antibiotic resistance purified hiPSC-CMs. A, Action potential traces (fluovolt) of hiPSC-CMs maintained in RPMI+B27 maintenance media. B&C, CDML3 induced arrhythmia phenotypes observed using voltage sensitive dye. D, APD30, E, APD50 and F AP triangulation are greater in CDML3 treated hiPSC-CMs. RPMI+ B27, APD30= 284.7±27.8 ms; CDML3 APD30=309.7±29.0 ms; * unpaired t-tests, P<0.001, n=94 and n=95. RPMI APD90=823.2±366.1 ms; CDML3 APD90=1266.7 ± 499.9 ms; *unpaired t-test, P<0.001. RPMI Triangulation=0.60±0.13; CDML3 Triangulation=0.72±0.12; *unpaired t-test, P<0.001. G, zero of 94 wells showed spontaneous arrhythmias in RPMI+B27 monolayers, while 24/95 monolayers presented with spontaneous arrhythmias in the CDML3 treated group. H-J electrophysiology and calcium flux of iCell² Cardiomyocytes is affected by maintenance in CDML3 media. APD50 and CaTD50 are prolonged in the CDML3 media treated monolayers. Calcium transient amplitude of CDML3 media treated monolayers plated in 96 well plates is reduced compared to RPMI-B27 maintenance media. K, Seahorse measurement of oxygen consumption rate shows reduced maximal OCR (L) and reduced reserve respiratory capacity in CDML3 treated hiPSC-CM monolayers.