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Metabolic Maturation of Human Pluripotent Stem Cell Derived Cardiomyocytes by Inhibition of HIF1α and LDHA

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Abstract

Rationale: Human Pluripotent Stem Cell-Derived Cardiomyocytes (hPSC-CMs) are a readily available, robustly reproducible and physiologically appropriate human cell source for cardiac disease modeling, drug discovery, and toxicity screenings in vitro. However, unlike adult myocardial cells in vivo, hPSC-CMs cultured in vitro maintain an immature metabolic phenotype where majority of ATP is produced through aerobic glycolysis instead of oxidative phosphorylation in the mitochondria. Little is known about the underlying signaling pathways controlling hPSC-CMs' metabolic and functional maturation.

Objective: To define the molecular pathways controlling CMs' metabolic pathway selections and improve CM metabolic and functional maturation.

Methods and Results: We cultured hPSC-CMs in different media compositions including glucose-containing media, glucose-containing media supplemented with fatty acids, and glucose-free media with fatty acids as the primary carbon source. We found that CMs cultured in the presence of glucose utilized primarily aerobic glycolysis and aberrantly upregulated hypoxia-inducible factor 1-alpha (HIF1α) and its downstream target lactate dehydrogenase A (LDHA). Conversely, glucose deprivation promoted oxidative phosphorylation and repressed HIF1α. Small molecule inhibition of HIF1α or LDHA resulted in a switch from aerobic glycolysis to oxidative phosphorylation. Likewise, siRNA inhibition of HIF1α stimulated oxidative phosphorylation while inhibiting aerobic glycolysis. This metabolic shift was accompanied by an increase in

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DISCLOSURES

None.

mitochondrial content and cellular ATP levels. Furthermore, functional gene expressions, sarcomere length and contractility were improved by HIF1α/LDHA inhibition.

Conclusions: We show that under standard culture conditions, the HIF1α-LDHA axis is aberrantly upregulated in hPSC-CMs, preventing their metabolic maturation. Chemical or siRNA inhibition of this pathway results in an appropriate metabolic shift from aerobic glycolysis to oxidative phosphorylation. This in turn improves metabolic and functional maturation of hPSC-CMs. These findings provide key insight into molecular control of hPSC-CMs' metabolism and may be used to generate more physiologically mature CMs for drug screening, disease modeling and therapeutic purposes.

Keywords

Cardiomyocyte; metabolism; maturation; HIF1α; LDHA; stem cell; glucose glycolysis; cell signaling; contractility; calcium transients; Basic Science Research; Cell Signaling/Signal Transduction; Contractile Function; Metabolism; Stem Cells

INTRODUCTION

Human Pluripotent Stem Cell-Derived Cardiomyocytes (hPSC-CMs) have been widely used for cardiac disease modeling[1–3], drug discovery[4, 5] and cardiac toxicity screenings[6–8] in vitro. Unlike primary human CMs which have limited availability and are difficult to maintain in culture, hPSC-CMs can be easily generated and cultured in vitro with unlimited quantities. They have also been shown to exhibit similar electrical activities and contractile capabilities compared to primary CMs[9, 10]. However, hPSC-CMs exhibit metabolically and functionally immature phenotypes when cultured in traditional culture media[11–13]. They mainly utilize glycolysis for energy production in vitro as opposed to the predominantly oxidative metabolism observed in normal adult hearts[14, 15].

To assess maturation of hPSC-CMs, biochemical, structural, and biophysical parameters are commonly used in the field including measurements of gene expression, cell shape and size, sarcomeric lengths and organizations, conduction velocity, mitochondria-to-cell volume ratio, resting membrane potential, calcium transient kinetics and cellular ATP levels, and force of contractions[10, 11, 16–20]. Many promising approaches have been proposed to enhance hPSC-CMs' maturation including biophysical, biochemical and bioelectrical stimulations[10]. Ribeiro et. al. for example showed improved CM shortening velocities, action potential amplitudes, improved sarcomeric organizations and lower resting membrane potentials by culturing hPSC-CMs in commercial media optimized for cardiomyocyte maturation[21]. By combining 3-dimentional, aligned cardiac tissues with electrical stimulations, Nunes et. al. demonstrated improvements in CM ultrastructural organization and conduction velocities[18]. Recently, Kadota et. al. showed improved maturation of hPSC-CMs transplanted in rat hearts[22]. While most studies focused on characterizing CMs' morphological, electrical and contractile phenotypes, the role of energy substrate and metabolic pathway utilizations on CM maturation remains relatively less well studied. Several signaling molecules have been identified as important factors in promoting maturation of hPSC-CMs including AMPK/mTORC1 pathway[23], thyroid hormone[24, 25] and microRNAs[20, 26]. In a recent publication, Correia et al. showed that by depriving glucose

and supplementing fatty acids in the culture media, hPSC-CMs are metabolically and functionally more mature[15]. However, the regulatory pathways that controls hPSC-CMs' metabolic maturation remains to be elucidated.

During heart development, a major metabolic switch from glycolytic to oxidative energy production occurs shortly after birth[27]. In the fetal heart, most of ATP is produced by glycolytic breakdown of glucose whereas the majority of ATP synthesis in the adult heart comes from fatty acid β oxidation in the mitochondria[27]. Since tremendous amount of energy is required constantly for proper heart function, it is essential for mature cardiomyocytes to utilize oxidative phosphorylation (OXPHOS) as a robust and effective pathway for ATP synthesis. Therefore, the metabolic switch to oxidative energy production is a hallmark of cardiac metabolic maturation.

Hypoxia-inducible factor 1-alpha (HIF1α) has been identified as a primary transcriptional factor and master regulator of cellular hypoxia responses in multiple organ systems in the body[28, 29]. Under physiological conditions, HIF1α is targeted by E3 ubiquitin ligase Von Hippel–Lindau tumor suppressor (VHL) and degraded rapidly by the ubiquitin protease pathway. A decrease in cellular oxygen levels stabilizes HIF1α and causes HIF1α proteins to accumulate in the nucleus[30, 31]. An extensive body of literature has shown that HIF1α acts as a central regulator of metabolism in a variety of cell types and promotes glycolytic pathways and suppresses oxidative energy production in the mitochondria[32]. The adaptive activation of HIF1α in hypoxic tumor promotes cancer cell survivor and growth by activating a large number of downstream transcripts regulating cellular metabolism and proliferation [32–35]. Okamoto et al. have recently shown that HIF1α reduces cellular oxygen consumption and mitochondria electron transport chain activities[36]. HIF1α activation has also been shown to upregulate glycolytic enzymes including lactate dehydrogenase A (LDHA), which is required for the conversion of pyruvate to lactate[37, 38]. Since LDHA plays a critical role in cancer metabolism, targeting LDHA has been proposed as a potential therapeutic strategy for inhibiting tumorigenesis[39, 40] and a number of small molecule inhibitors of LDHA activity have been generated and validated.

In the heart, HIF1α signaling has been shown to control metabolic signaling during cardiac development and maturation in vivo[41–43]. HIF1α signaling has also been shown to be required for CM proliferation during the mid-gestation stage. Furthermore, cardiac specific HIF1α deletion results in severe alterations in cardiac calcium flux, contractility and energy availability. Early ablation of HIF1α has been shown to produce severe cardiac defects and embryonic lethality[44]. Recently, Menendez-Montes et. al, showed that HIF1α signaling is essential in controlling the in vivo embryonic metabolic switch from glycolysis to oxidative phosphorylation, which is essential for cardiac maturation in vivo[43]. Given the central role of HIF1α in regulating metabolic substrate selection and the abnormal glucose utilization in hPSC-CMs, we hypothesized that the HIF1α-LDHA signaling pathway plays an essential role in regulating metabolic and functional maturation of cultured CMs.

Accordingly, the aim of this study is to investigate the underlying signaling mechanism controlling CMs' energy substrate selection and metabolic maturation of hPSC-CMs. To address this, we investigated the metabolic and physiological consequences of culturing

hPSC-CMs in three different media compositions: glucose media (GLM), glucose media supplemented with fatty acids (GFAM) and fatty acids only media deprived of glucose (FAM). Metabolic and functional characterizations of CMs cultured in these three media reveals that glucose rich medium promotes glycolytic metabolism, even in the presence of fatty acids whereas media containing fatty acids as the only energy source allowed for normal physiological metabolic substrate utilization. We then show that HIF1α-LDHA axis is aberrantly upregulated in CMs cultured on glucose containing media. Small molecule inhibition of the HIF1α-LDHA pathway results in enhanced metabolic and functional maturation of hPSC-CMs. Collectively our results highlight the importance of controlling the metabolic maturation of stem cell derived CMs in order to generate viable stem cell models of human cardiovascular disease in vitro.

METHODS

The authors declare that all supporting data are available within the article [and its online supplementary files].

Human pluripotent stem cell culture and differentiation.

The pluripotent stem cells used in this study include **HUES9** (NIH Human Embryonic Stem Cell Registry Number 0022, generated by HSCI iPS Core at Harvard University), **H7** (WA07, NIH Human Embryonic Stem Cell Registry Number 0067, generated by WiCell Research Institute) and **iPSC** (BJ RiPS-D - Healthy Donor Control (Male), generated by HSCI iPS Core at Harvard University). We characterized the pluripotency of these stem cells and found no significant difference between several pluripotency markers (Online Figure I).

The schematic of Figure 1A illustrates the hPSC culture, differentiation and dissociation timeline. hPSC-CM differentiation methods were adapted from previously published manuscripts[6, 10, 15, 50].

The three media compositions used are as follows: GLM contains RPMI Medium with GlutaMAX Supplement (Thermo Fisher Scientific, MA) plus Gem21 NeuroPlex Serum-Free Supplement (Gemini Bio-Products, CA). GFAM contains GLM plus 50 μmol/L of Palmitic Acid (Sigma, MO) and 100 μmol/L Oleic Acid. FAM contains RPMI Medium with GlutaMAX Supplement without glucose (Thermo Fisher Scientific, MA) plus 50 μmol/L of Palmitic Acid (Sigma, MO) and 100 μmol/L Oleic Acid[15, 45]. To prepare the fatty acids, stock solutions of palmitic acid and oleic acid were made by dissolving fatty acid powder in DMSO at 37ºC followed by vortex mixing and aseptic filtration. The stock solutions were prepared fresh for each new media. CMs were cultured in different media compositions with or without small molecule treatments for 7 days prior to experiments. Media was refreshed at day 4 after re-plating with the appropriate substrates and small molecules.

Animal procedures and hexokinase activity assay.

C57BL/6 mouse were used in this study. Hearts from embryonic day 15, postnatal day 2 and adult mice were harvested in lysis buffer and homogenized using TissueLyser LT (Qiagen, Germany). Mice studies were approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. Hexokinase Activity Assays were performed using a

commercial assay kit (BioVision, CA) according to manufacture suggested protocol. Hexokinase converts glucose into glucose-6-phosphate, which is oxidized to form NADH. The resulting NADH then reduces a colorless probe into a colorimetric product indicative of the hexokinase enzyme activity levels.

Adult CMs were isolated using direct enzyme injection protocol[46]. Briefly, C57BL/6 mice were anaesthetized and cut open below the diaphragm to expose the heart. Following injection of 7ml EDTA buffer to the right ventricle, the aorta was clamped by forceps and the heart was removed. Multiple rounds of injections to the apex of the heart were performed with the following: 10ml EDTA buffer, 3ml of perfusion buffer and 30ml of collagenase buffer. The hearts were then pulled apart using forceps and filtered through 100um strainers to remove large tissue debris. After three rounds of gravity settling, the adult cardiomyocytes were re-suspended in culture media.

Statistical analysis.

Statistical analysis was performed using GraphPad Prism software (La Jolla, CA). The sample size for each group is shown in the figure legends. Results for single cell assays were collected from at least three sets of independent experiments. Experimental sample sizes, normality test results, statistical testing and post hoc corrections used are detailed in Online table II. Statistically significance was defined as $p < 0.05$.

RESULTS

hPSC-CMs cultured in GLM and GFAM lacks metabolic and functional maturation.

Prior reports have suggested that the mammalian embryonic heart depends on glycolysis for ATP production whereas the adult heart switches to primarily oxidative phosphorylation to produce energy. To verify these findings and establish a baseline control for hPSC-CMs, we followed the changes in glycolysis rates of at different stages of heart development and maturation. We measured hexokinase enzyme activity levels of heart samples from embryonic day 15, postnatal day 2 and adult mice using colorimetric commercial assay kit. As expected, we observed significantly higher hexokinase activities in immature embryonic and postnatal hearts compared to adult hearts (Fig. 1B). We then used the same hexokinase assay to compare the glycolysis rates of hPSC-CMs cultured in glucose containing media (GLM, GFAM) and media with fatty acids as the only energy source (FAM). hPSC-CMs cultured in FAM had significantly lower hexokinase activities compared to CMs cultured in GLM and GFAM (Fig. 1C). No difference in hexokinase protein levels was observed among the three groups (Online Figure V). Glycolysis results in the conversion of glucose into pyruvate, which is subsequently reduced into lactate. To further validate that the increase in hexokinase activity is due to an increase in glycolysis, we compared the total cellular lactate levels in CMs cultured under different growth conditions using a commercial lactate assay kit. As shown in Figure 1D, CMs cultured in GLM and GFAM had significantly higher lactate levels compared to CMs cultured in FAM. These results show that hPSC-CMs cultured in the glucose containing media GLM and GFAM have a high glycolytic rate that is similar to embryonic and postnatal hearts. In contrast, CMs cultured in FAM media where

fatty acids are the only energy source had increased oxidative phosphorylation rates and reduced glycolysis rates as would be expected during myocardial maturation.

We then assessed rates of oxidative metabolism in hPSC-CMs cultured in GLM, GFAM and FAM using the Seahorse Live-cell Metabolic Assay. CMs cultured in GLM and GFAM had significantly lower oxygen consumption rates (OCR, Fig. 1E) compared to CMs cultured in FAM. We then performed mitochondria stress test by sequentially adding oligomycin, FCCP, and antimycin to CMs cultured in all three media. CMs cultured in FAM had a significant increase in OCR with FCCP treatment compared to CMs cultured in GLM and GFAM (Fig. 1F). This response indicates a much larger spare capacity for CMs cultured in FAM compared to GLM and GFAM. To assess ATP production, we measured CM ATP levels using the CellTiter-Glo assay. CMs cultured in GLM and GFAM had significantly lower levels of ATP per cell compared to CMs cultured in FAM (Fig. 1G). These results support our findings that hPSC-CMs cultured in GLM and GFAM are metabolically reliant on glycolysis rather than oxidative phosphorylation and furthermore that they generate less ATP compared to CMs cultured in FAM.

Since oxidative phosphorylation is a mitochondrial process, we then compared the number of mitochondria among hPSC-CMs cultured in GLM, GFAM and FAM using mitotracker deep red staining and quantitative flow cytometry. We observed that CMs cultured in GLM and GFAM have significantly lower median mitotracker intensities compared to that of FAM (Fig. 1H-I). We also observed a higher sarcomere length (Fig. 1J) and peak contractile force generation (Fig. 1K) in CMs cultured in FAM compared to CMs cultured in GLM and GFAM. These results taken together show that CMs cultured in the presence of glucose resemble immature embryonic and neonatal cardiomyocytes with respect to their energy substrate utilization, active metabolic pathways, and mitochondrial content. In contrast, CMs cultured in the presence of fatty acids as the primary energy source more closely resemble adult CMs, consistent with previous reports[15].

Aberrant activation of HIF1α and LDHA pathway in cultured CMs.—HIF1α is a central regulator of metabolism and promotes glycolysis at the expense of oxidative phosphorylation[29, 37, 38, 41, 43]. To test a potential role for this pathway in controlling the observed inappropriate energy substrate selection in glucose cultured CMs, we performed immunofluorescent staining of HIF1α in hPSC-CMs cultured in GLM, GFAM and FAM. We found that CMs cultured in the presence of glucose had high levels of HIF1α in the nucleus and low levels in the cytoplasm (Fig. 2A). This high nuclear to cytoplasmic ratio of HIF1α protein (Online Figure IV.A) and localizations are consistent with the activation of the HIF1α pathway. In contrast, HIF1α was primarily localized to the cytoplasm in cells cultured in FAM (Fig. 2B). We then quantified HIF1α protein expressions using western blot. HIF1α was expressed at significantly higher levels in hPSC-CMs cultured in GLM and GFAM compared to FAM (Fig. 2C). We then measured the mRNA levels of direct and indirect downstream targets of activated HIF1α. Among these transcripts, we found that lactate dehydrogenase A (LDHA) has a significantly higher expression in hPSC-CMs cultured in the presence of glucose (GLM and GFAM) compared to those cultured in FAM without glucose (Fig. 2D). Other transcripts involved in cellular metabolism were not significantly different among the three groups (Online Figure IV.B-C).

Activated HIF1α is a transcriptional regulator that has been previously shown to act in a positive feedback loop to promote its own transcriptional activation[48–50]. We therefore reasoned that repression of HIF1α activity might result in a normalization of its levels at the mRNA and protein level. To test this, we then evaluated the effect of HIF1α inhibition by treatment with CTM, a small molecule inhibitor of HIF1α's transcriptional activity (Fig. 2C). Inhibition of HIF1α activity normalized its protein expression level. Furthermore, inhibition of HIF1α with CTM also significantly reduced LDHA mRNA levels in GLM and GFAM cultured CMs (Fig. 2D). These results demonstrate an aberrant activation of HIF1α in hPSC-CMs cultured in glucose rich media and raises the possibility that repression of this pathway may result in improved metabolic function of CMs cultured in vitro.

Inhibition of HIF1α and LDHA improves CMs' metabolic maturation.—To assess the metabolic impact of inhibiting the HIF1α-LDHA regulatory axis, we treated hPSC-CMs cultured in GLM, GFAM and FAM with GSKA (a selective and potent LDHA inhibitor) or CTM. We first compared hexokinase enzyme activity levels of CMs treated with GSKA, CTM, or carrier control and showed that both GSKA and CTM treatments significantly reduced hexokinase enzyme activity levels in CMs cultured in GLM and GFAM (Fig. 3A). Similarly, total cellular lactate levels were also decreased with this treatment (Fig. 3B). We then compared the cellular ATP levels of CMs treated with GSKA or CTM. As shown in Figure 3C and Online Figure VI.A, hESC-CM and hiPSC-CM inhibition of this pathway resulted in an increase of cellular ATP production in cells cultured in GLM and GFAM consistent with the shift from aerobic glycolysis to oxidative phosphorylation. Next, we quantified the impact of GSKA, CTM or GSKA+CTM treatment on cellular respiration and oxidative metabolism using the Seahorse Analyzer and found that CMs treated with GSKA had significantly increased OCRs (Fig. 3D) and spare capacities (Fig. 3E). These results demonstrate that inhibition of the HIF1α or LDHA inhibits aerobic glycolysis and restores normal CM dependence on oxidative phosphorylation. Metabolic pathway utilizations of CMs cultured in FAM, GLM and GFAM treated with GSKA or CTM are illustrated in scheme 1. Collectively, these results demonstrate that small molecular inhibition of HIF1α-LDHA promotes CMs' metabolic maturation with reduced glycolysis, increased oxidative metabolism and higher cellular ATP productions

Inhibition of HIF1α and LDHA enhances CMs' structural and functional maturation.—To assess the impact of HIF1α-LDHA inhibition on CMs' structural maturation, we quantified the sarcomere lengths of CMs cultured in GLM, GFAM and FAM treated with GSKA, CTM or control. CMs cultured with fatty acids as the only energy source (FAM) had significantly longer sarcomere length compared to cells cultured in the presence of glucose (Fig. 4A). Likewise, significant improvements in sarcomere lengths were observed in CMs treated with GSKA or CTM (Fig. 4A). We then investigated the impact of metabolic substrate availability and HIF1α-LDHA regulation on mitochondrial content. As shown in Figure 4B, we found a significant increase in mitochondria DNA to genomic DNA ratio with CTM or GSKA treatments in CMs cultured in GFAM. We also found a similar increase in median mitotracker staining intensities with CTM or GSKA treatments in CMs cultured in GFAM (Fig. 4C-D). Likewise, TEM imaging quantification showed a number of mitochondria per cell normalized to cytoplasmic area. We observed a

significant increase in mitochondria number in CMs cultured in GFAM with CTM compared to control (Fig. 4E and Online Figure IX). No significant changes in myofibril content were observed in these experiments. We subsequently examined the effect of HIF1α-LDHA inhibition by treatment with GSKA or CTM on structural and functional gene expressions. We analyzed several key cardiac functional genes and observed a significant increase in the expression levels of gap junction protein alpha 1 (*GJA1*), myosin heavy chain 6 (*MYH6*), beta-myosin heavy chain (*MYH7*), potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 (*HCN4*), and sodium voltage-gated channel alpha subunit 5 (*SCN5A*) when CMs were treated with GSKA or CTM (Fig. 4F).

We then quantified the impact of HIF1α-LDHA inhibition on CMs' calcium transient kinetics and contractility. We show significant increases in peak calcium amplitude (Fig. 5A-B), maximum upstroke and decay velocities (Fig. 5C-D), and decreases in time to 50% upstroke and decay (Fig. 5E-F) in CMs cultured in GFAM and treated with GSKA or CTM compared to FAM. We also observed significant increases in peak contractile force generations (Fig. 5G), maximum shortening velocities (Fig. 5H) and maximum relengthening velocities (Fig. 5I) in CMs cultured in GFAM and treated with GSKA or CTM compared to FAM. Measurements of peak contractile forces of HUES9-CMs (Online Figure VI.B) and H7-CMs (Online Figure VI.C) also showed significant increases in GSKA or CTM treated CMs cultured in GLM and GFAM. Subsequently, we performed whole-cell current-patch clamp recordings of cardiac action potentials in hiPSC-CMs cultured in GFAM plus CTM and control. We found that CTM treated CMs showed a significantly more negative resting membrane potential (−75±1 mV, N=6 cells) compared to control group (−70±1 mV, N=6 cells, Fig. 5J-K) consistent with the finding HIF1α-LDHA inhibition promotes CMs' structural and functional maturation.

To confirm that our findings were generalizable, we replicated key experiments with different pluripotent stem cell lines. We used H7-ESC-CMs for cellular metabolic and functional studies. We observed similar improvements in cellular hexokinase activities, ATP per cell, sarcomere lengths, cell size and median mitotracker intensities with HIF1α-LDHA inhibition (Online Figure VII and 10A). We also assessed the long-term effect of HIF1α-LDHA inhibition in hiPSC-CMs. Similar improvements in mitochondria DNA to genomic DNA ratio, median mitotracker intensity, sarcomere lengths and ATP per cell were observed with 30-day inhibition of HIF1α-LDHA in GFAM cultured CMs (Online Figure VIII). We also used siRNA inhibition of HIF1α as an alternative approach to promote hPSC-CMs' metabolic and functional maturation (Fig. 6). In hiPSC-CMs cultured in GFAM treated with HIF1α siRNA, we found similar decrease in hexokinase activities, increases in mitochondria DNA to genomic DNA ratio, improvements in cellular ATP levels and percentage fractional shortenings from controls compared to CTM small molecular approach (Fig. 6).

DISCUSSION

hPSC-CMs have been proposed as a promising cell source for drug development, human disease modeling, and cardiac toxicity screening in vitro[1, 2, 8]. Compared to human adult cardiomyocytes, hPSC-CMs are relatively immature both morphologically and functionally, lacking normal levels of binucleation, well-developed sarcomeres, fast electrical

conductivity and oxidative metabolism[10]. To address these limitations, numerous studies have aimed to enhance hPSC-CMs' maturation using genetic, chemical, and biomechanical approaches[16–18]. These studies have achieved varying degrees of success in terms of improving hPSC-CMs' morphological, electrical and contractile maturation. In this study we focus on cellular metabolism as a critical rubric for maturation state of CMs. We have previously shown that culturing hPSC-CMs in fatty acids media in the absence glucose enhanced CM maturation. An important caveat of this approach, however, is the cytotoxicity that resulted from the accumulation of lipid intermediates and leading to cellular dysfunction and death[15]. This lipotoxicity occurred only after 10 days of in vitro culture and could be abrogated by the addition of galactose to the culture media[15]. Of important note however, both glucose and fatty acids are available as potential energy sources for CMs in vivo. We therefore sought to investigate the mechanism underlying metabolic substrate selection of hPSC-CMs cultured in the presence of both glucose and fatty acids and to determine how these metabolic pathways controlled myocardial maturation. In so doing we CMs cultured in FAM for 7-days as positive controls, a time point when meaningful cellular maturation could be observed but prior to the development of lipotoxicity. Accordingly, our study advances the mechanistic understanding of metabolic substrate utilization and at the same time establishes a novel methodology of CM metabolic maturation that is compatible with physiological growth conditions.

A number of studies have demonstrated that the embryonic and neonatal mammalian heart depends on aerobic glycolysis as the principal energy production pathway[27]. As a result of a postnatal metabolic shift in CM substrate utilization, the majority of the energy required for CMs' contractile function in the adult heart is produced through β-oxidation of fatty acids in the mitochondria[27]. Hexokinase is a key enzyme in glycolysis that converts glucose into glucose-6-phosphate and its activity levels have been used as a surrogate for glycolysis rates[51–53]. Similarly, lactate, a byproduct of glycolysis in mammalian cells, can be used as an indicator for the level of glycolysis[27]. Transitioning hPSC-CMs from glucose containing to glucose free conditions forces an analogous shift in substrate utilization due to the lack of availability of the substrate of glycolysis[15]. In the native heart, although both glucose and fatty acids are available as energy sources, adult CMs utilizes primarily fatty acids for energy production. In contrast, hPSC-CMs cultured in the presence of both glucose and fatty acids preferentially utilized glucose as the principle energy source. HIF1α has been identified as a master transcriptional regulator of cellular metabolism[28, 38] and its expression is crucial for early fetal cardiac development[54, 55]. Recent studies have further suggested that activation of the HIF1α pathway is required for the maintenance of a myocardial proliferative state during early embryonic development[43]. We therefore hypothesized that aberrant activation of this pathway may contribute to the persistence of an immature metabolic phenotype in hPSC-CMs. Activated HIF1α is localized to the nucleus whereas inactive HIF1α remains in the cytoplasm where it is rapidly turned over[28, 30, 31]. Of note, we show that HIF1α is aberrantly activated in hPSC-CMs cultured in the presence of glucose and inactivated when cells were grown with fatty acids as the only carbon source. Inhibition of HIF1α activity further resulted in a decrease in HIF1α nuclear localization, consistent with prior reports showing that a positive feedback loop coordinates HIF1α activity with its protein localization and transcriptional expression[48–50]. LDHA is a key target of HIF1α. Its

expression is required for the conversion of pyruvate to lactate and the restoration of the cellular NAD^+ storage required for glycolysis. We show that LDHA is significantly upregulated in hPSC-CMs cultured in the presence of glucose, consistent with the dependence of these cells on glycolytic pathways for energy production. Inhibition of HIF1α activity with CTM resulted in reduced LDHA gene expression, confirming that LDHA is a downstream target of HIF1α in hPSC-CMs. Likewise, small molecule inhibition of LDHA activity suppresses aerobic glycolysis and facilitates oxidative phosphorylation in hPSC-CMs.

The expression and activity of HIF1α in hPSC-CMs cultured in the presence of glucose is similar to its reported expression during early fetal development. Moreover, deletion of VHL during midgestation stage of cardiac development has been shown to stabilize HIF1α proteins and prevent metabolic shift from glycolytic to oxidative pathway resulting in impaired cardiac maturation and function[43]. Aerobic glycolysis has been previously shown to be activated in oncogenesis [32, 34, 40] and inhibiting HIF1α-LDHA signaling in cancer cells decreases glycolysis and lactate fermentation rates while reactivating oxidative phosphorylation[39, 40]. Here, we propose to improve cultured hPSC-CMs' metabolic and functional maturation by shifting its metabolism using small molecule HIF1α-LDHA inhibitors. Indeed, increases in OCR, mitochondria spare capacities and ATP productions as well as decreases in cellular hexokinase activity and lactate levels were observed in hPSC-CMs cultured in GLM and GFAM with inhibition of HIF1α/LDHA by CTM/GSKA. Critically, shifting CM metabolism to oxidative pathways promotes cellular maturation including sarcomere lengths, mitochondrial number, functional gene expressions, calcium transient kinetics and contractile force generation. Furthermore, peak contractile force generated by single hPSC-CMs treated with CTM/GSKA reached similar levels of adult murine CMs reported in the literature[56, 57]. These results indicate that inhibiting HIF1α-LDHA signaling is an effective strategy for improving hPSC-CMs' metabolic and functional maturation. We did observe an increase in the expression of *HCN4*, which has been previously shown to inversely correlate with CM maturation. Jaconi and colleagues showed that *HCN4* is highly expressed in ESC and day 30 ESC-derived CMs but dramatically reduces at day 60 of in vitro culture. Interestingly, HCN4 expression is restored by day 110 of culture and at that point is no statistically difference with its expression level for day 30. Since the role of *HCN4* during CM maturation in vivo and in vitro is complex and difficult to infer electrical maturation state of CMs, we performed whole-cell current-patch clamp experiments. We observed significantly more negative MDP in CTM treated CMs, which supports our conclusions that inhibiting HIF1α promotes hPSC-CMs' electrical maturation.

The findings of this study highlight the importance of bioenergetics and energy substrate utilization in the functional maturation of human pluripotent stem cell-derived CMs. Aberrant activation of the HIF1α/LDHA signaling pathway leads to a metabolic shift toward aerobic glycolysis resulting in decreased number of mitochondria, lower cellular ATP levels, impaired calcium handling, and diminished contractility. Correcting hPSC-CMs' metabolic pathway selection and ensuring proper mitochondria function resulted in an increased number of mitochondria, higher cellular ATP levels, improved calcium handling, and contractility. Therefore, this approach may be important in developing more clinically relevant in vitro cardiac disease model and drug-screening platforms using hPSC-CMs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms:

FAM	Fatty Acids Media
GLM	Glucose Media
GFAM	Glucose and Fatty Acids Media

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NOVELTY AND SIGNIFICANCE

What Is Known?

- Human Pluripotent Stem Cell-derived Cardiac myocytes (hPSC-CMs) are a ready source for in vitro human cardiac disease modeling, drug discovery, and toxicity screening.
- Cultured hPSC-CMs maintain an immature metabolic phenotype in which the majority of ATP is produced through aerobic glycolysis instead of oxidative phosphorylation.
- Glucose deprivation results in a metabolic shift from glycolysis to oxidative phosphorylation and improves functional maturation of hPSC-CM; however, the pathways that regulate metabolic maturation of these cells remain poorly understood.

What New Information Does This Article Contribute?

- CMs cultured in the presence of glucose aberrantly upregulate hypoxia-inducible factor 1-alpha (HIF1α) and its downstream target - lactate dehydrogenase A (LDHA), two factors that are known to promote aerobic glycolysis.
- Glucose deprivation results in the inhibition of HIF1α and LDHA activity, and repression of aerobic glycolysis.
- Small molecule or siRNA inhibition of HIF1α or small molecule inhibition of LDHA results in a metabolic switch from aerobic glycolysis to oxidative phosphorylation, a more mature metabolic phenotype.
- The metabolic maturation promoted by HIF1α inhibition improves functional maturation of hPSC-CMs.

hPSC-CMs have found wide spread use in cardiac disease modeling, drug discovery, and toxicity screenings in vitro. Mature adult myocardial cells rely on fatty acid oxidation for energy production. In contrast, hPSC-CMs cultured in vitro maintain an immature metabolic phenotype, where a majority of ATP is produced through aerobic glycolysis, not oxidative phosphorylation. Herein we show that hPSC-CMs cultured in the presence of glucose aberrantly upregulate HIF1α and its downstream target LDHA. Whereas, glucose deprivation promotes oxidative phosphorylation and represses HIF1α activity. Small molecule inhibition of HIF1α or LDHA triggers a switch from aerobic glycolysis to oxidative phosphorylation as does siRNA inhibition of HIF1α. This metabolic maturation is accompanied by an increase in mitochondrial content, increased cellular ATP levels, improved functional gene expression, increased sarcomere length, and improved contractility. These findings provide key insights into the regulation of metabolic and functional maturation of HPSC-CMs and may be used to generate more physiologically mature CMs for drug screening, disease modeling, and therapeutic applications.

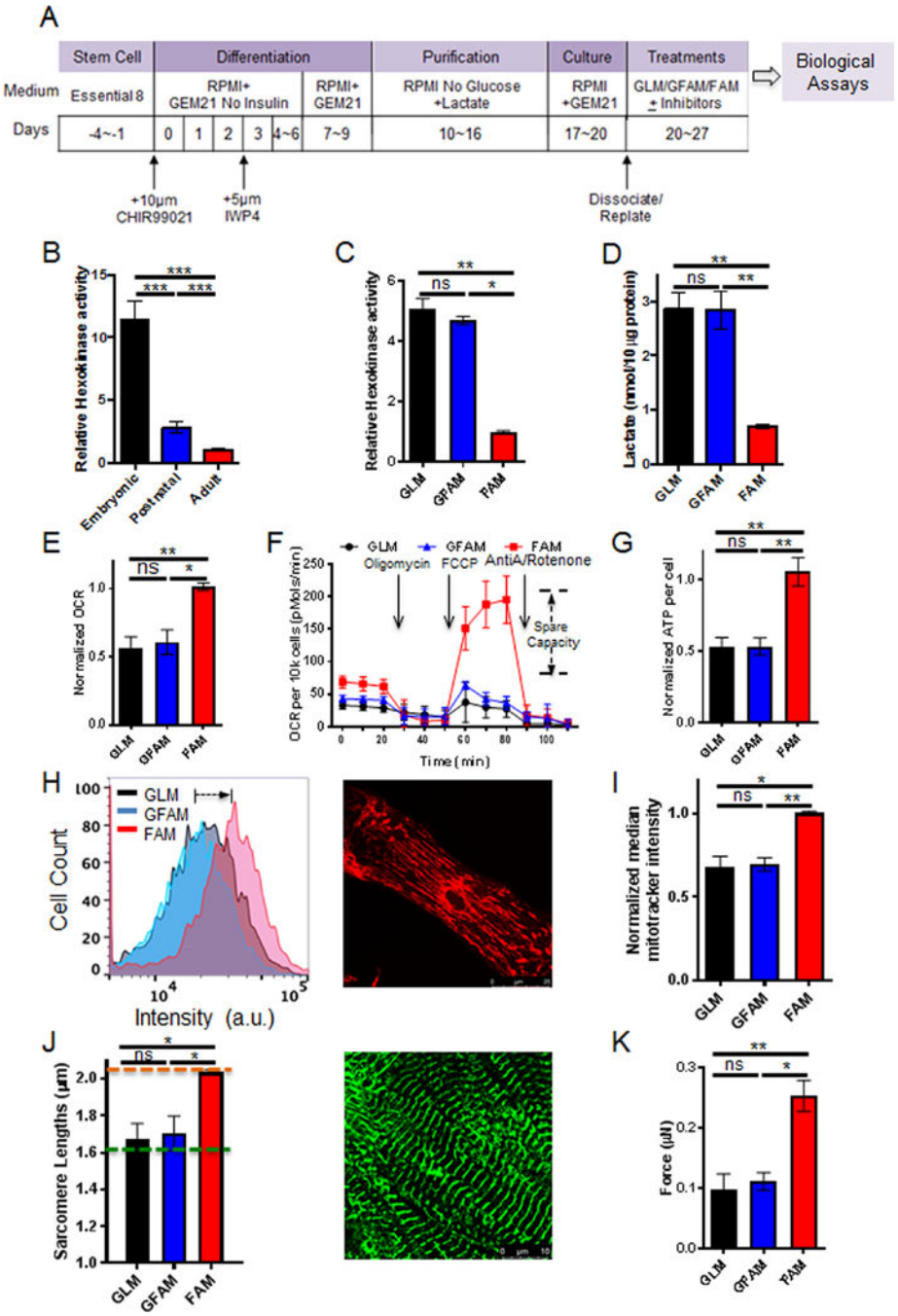

Figure 1. hPSC-CMs cultured in glucose media lack metabolic and functional maturity.

A) Schematic of experimental timeline including stem cell culture, cardiomyocyte differentiation, purification, culture and treatments. Different media used at different days of the timeline indicated. **B)** Relative hexokinase enzyme activity in heart tissue samples from embryonic (n=7 embryos), neonatal (n=10 mice), and adult mice (n=9 mice), **C)** Relative hexokinase enzyme activity in hPSC-CMs cultured in GLM, GFAM and FAM (n=1 iPSC line and 2 ESC lines), **D)** Lactate measurements of hPSC-CMs cultured in GLM, GFAM and FAM (n=1 iPSC line and 2 ESC lines), **E)** Baseline OCR measurements of hPSC-CMs

cultured in GLM, GFAM and FAM normalized to cell number (n=1 iPSC line and 2 ESC lines), **F)** Mitochondria respiration rates of hPSC-CMs cultured in GLM, GFAM and FAM per 30,000 cells (n=1 iPSC line and 2 ESC lines), **G)** ATP measurements of hPSC-CMs cultured in GLM, GFAM and FAM (n=1 iPSC line and 2 ESC lines), **H)** Histogram of hPSC-CMs cultured in GLM, GFAM and FAM with mitotracker staining analyzed by FACS with a representative mitotracker staining; 10,000 events recorded per group, **I)** Mean mitotracker staining intensities of hPSC-CMs cultured in GLM, GFAM and FAM (n=1 iPSC line and 2 ESC lines), **J)** Sarcomere lengths measurements of hPSC-CMs cultured in GLM, GFAM and FAM (n=1 iPSC line and 2 ESC lines) with a representative α-actinin staining, green and orange lines represent hPSC-CMs' and adult CMs' sarcomere lengths reported in the literature, respectively[10, 19, 22], **K)** Contractile force generated by single hPSC-CMs cultured in GLM, GFAM and FAM (n=1 iPSC line and 2 ESC lines).

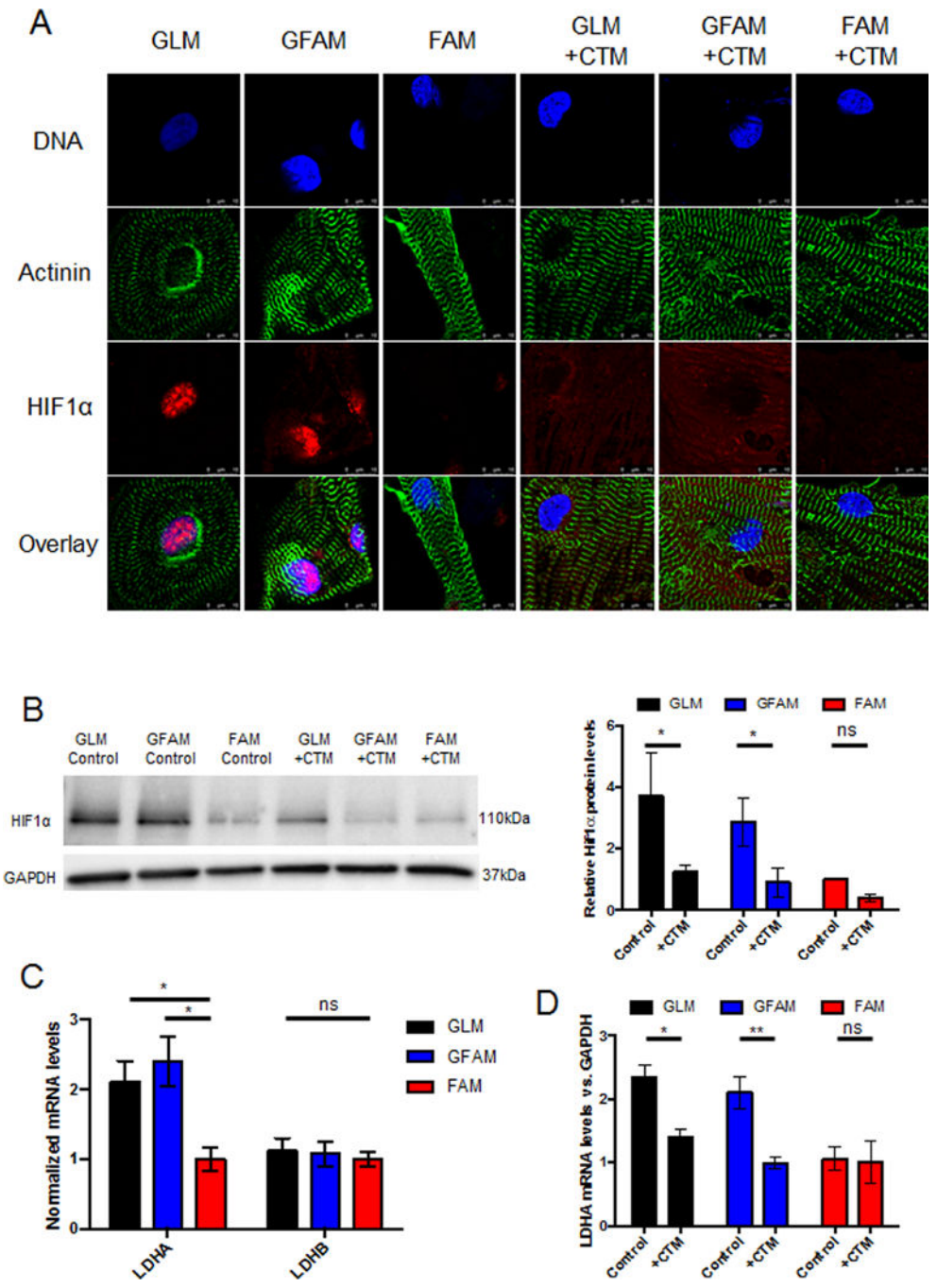

Figure 2. Aberrant activation of HIF1α-LDHA pathway in hPSC-CMs cultured in glucose media.

A) Immunofluorescent staining of DAPi (blue), α-Actinin (green), and HIF1α (red) in hPSC-CMs cultured in GLM, GFAM and FAM, **B)** Western blot (left) and quantifications (right) of HIF1α protein expressions in hPSC-CMs cultured in GLM, GFAM and FAM treated with CTM (n=3 independent experiments using HUES9-ESC-CMs), **C)** mRNA levels of LDHA and LDHB in hPSC-CMs cultured in GLM, GFAM and FAM (n=3 independent experiments using HUES9-ESC-CMs), **D)** mRNA levels of LDHA in hPSC-

CMs cultured in GLM, GFAM and FAM treated with CTM (n=3 independent experiments using HUES9-ESC-CMs).

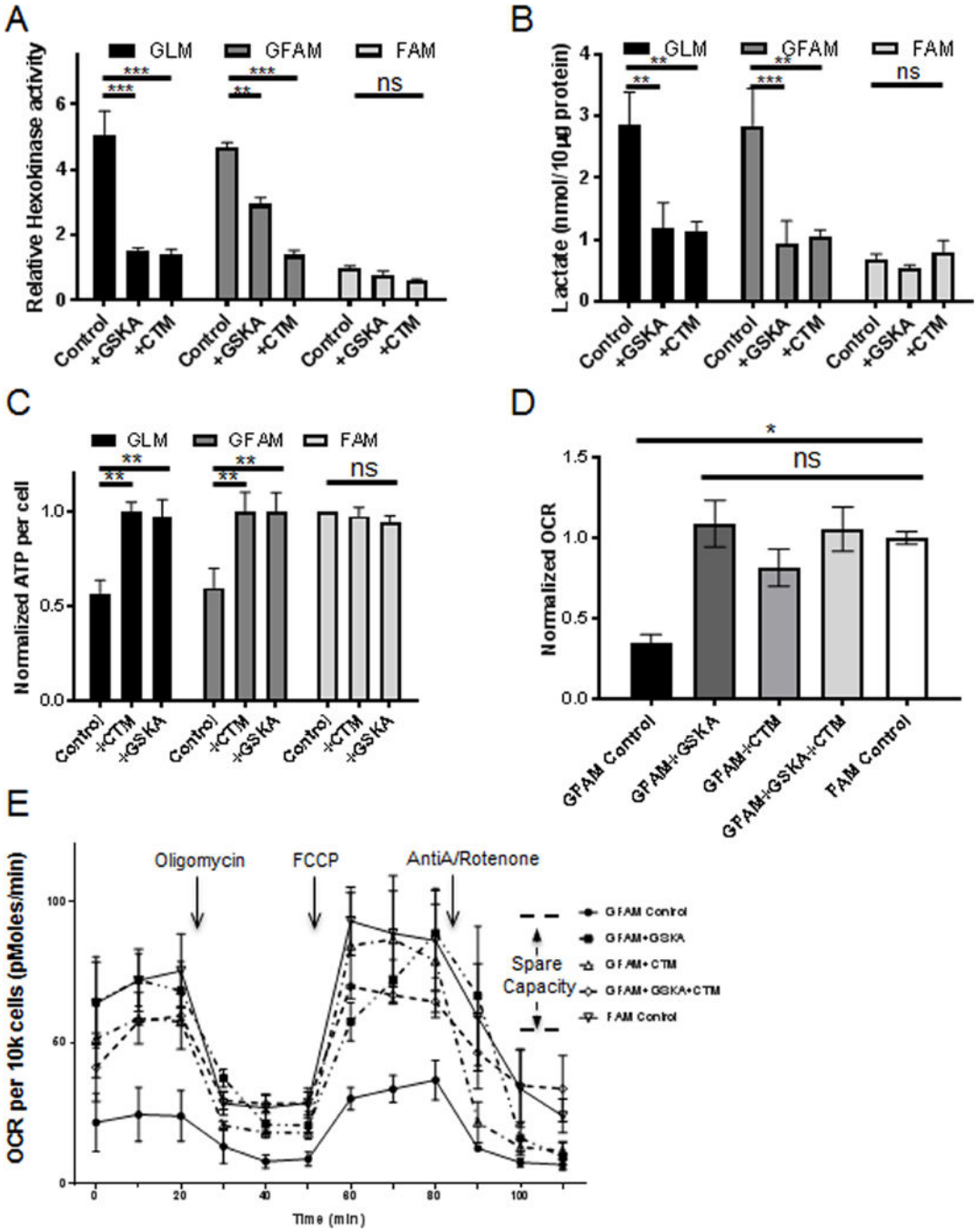

Figure 3. Inhibition of HIF1α and LDHA improves hPSC-CMs metabolic maturation.

A) Relative hexokinase enzyme activity of hPSC-CMs cultured in GLM, GFAM and FAM treated with CTM or GSKA (n=1 iPSC line and 2 ESC lines), **B)** Normalized lactate measurements of hPSC-CMs cultured in GLM, GFAM and FAM treated with CTM or GSKA (n=1 iPSC line and 2 ESC lines), **C)** Normalized ATP measurements of hPSC-CMs cultured in GLM, GFAM and FAM treated with CTM or GSKA (n=1 iPSC line and 2 ESC lines), **D)** Normalized baseline OCR measurements of hPSC-CMs cultured in GFAM, GFAM treated with GSKA, CTM, GSKA+CTM, and FAM (n=1 iPSC line and 2 ESC lines),

E) Mitochondria respiration rates of hPSC-CMs cultured in GFAM, GFAM treated with GSKA, CTM, GSKA+CTM, and FAM (n=1 iPSC line and 2 ESC lines).

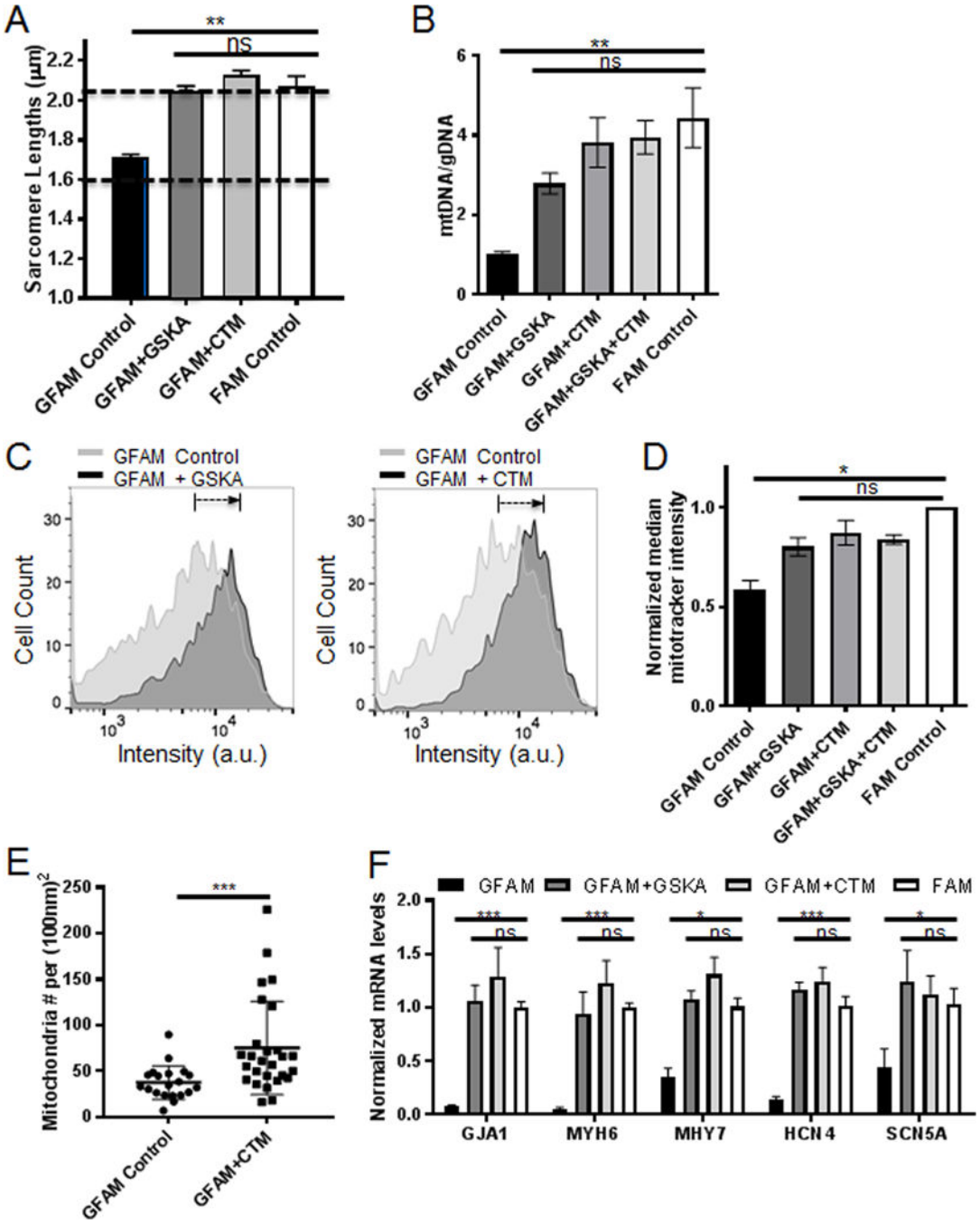

Figure 4. Inhibition of HIF1α and LDHA improves hPSC-CMs structural maturation.

A) Sarcomere lengths measurements of hPSC-CMs cultured in GLM, GFAM and FAM treated with CTM or GSKA, top and bottom dotted lines represent hPSC-CMs' and adult CMs' sarcomere lengths reported in the literature, respectively; (n=1 iPSC line and 2 ESC lines), **B)** Mitochondria DNA to genomic DNA ratio of hPSC-CMs cultured in GFAM treated with CTM, GSKA, or both and FAM (n=1 iPSC line and 2 ESC lines), **C)** Histogram of hPSC-CMs cultured in GFAM treated with CTM or GSKA with mitotracker staining, **D)** Quantification of median mitotracker staining intensities of hPSC-CMs cultured in GFAM

treated with GSKA, CTM, GSKA+CTM and FAM (n=1 iPSC line and 2 ESC lines), **E)** Numbers of mitochondria per $(100nm)^2$ cytoplasmic area for each cell measured by transmission electron microscopy in hiPSC-CMs cultured in control GFAM or GFAM +CTM, (n=20 cells for control GFAM and n=27 GFAM+CTM analyzed, Mann–Whitney test were used for statistical analysis), **F)** mRNA levels of selected transcripts involved in CM function and maturation in hiPSC-CMs cultured in GFAM, GFAM treated with GSKA or CTM, and FAM (n=3 independent experiments). All statistical analysis were performed comparing to GFAM controls.

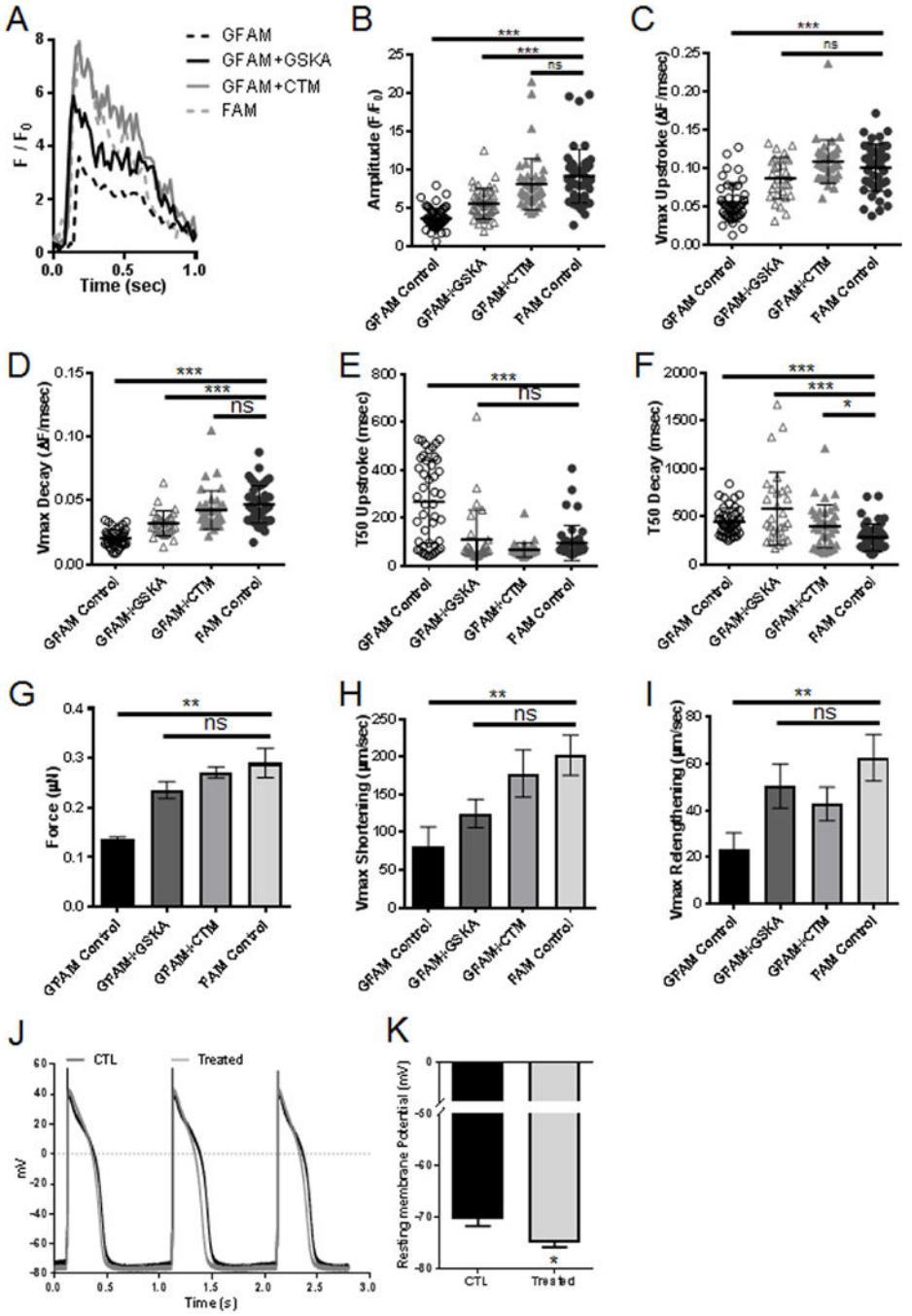

Figure 5. Inhibition of HIF1α and LDHA improves HUES9-hPSC-CMs functional maturation.

A) Representative calcium transient of hPSC-CMs cultured in GFAM, GFAM treated with CTM or GSKA and FAM, **B)** Maximum calcium transient amplitude, **C)** Maximum calcium transient upstroke velocities, **D)** Maximum calcium transient decay velocities, **E)** Time to 50% upstroke calcium transient amplitude, and **F)** Time to 50% decay calcium transient amplitude of hPSC-CMs cultured in GFAM, GFAM treated with CTM or GSKA and FAM (n=30–50 cells per group, from 3 independent experiments). **G)** Contractile force, **H)** Maximum shortening velocities, and **I)** Maximum relengthening velocities of hPSC-CMs

cultured in GFAM, GFAM treated with CTM or GSKA and FAM (n=10–14 cells per group, from 3 independent experiments). **J)** Representative action potential traces and **K)** Resting membrane potentials of hPSC-CMs cultured in control GFAM or GFAM+CTM measured by patch clamp, N=6 cells for each group. All statistic analysis were performed comparing to GFAM controls.

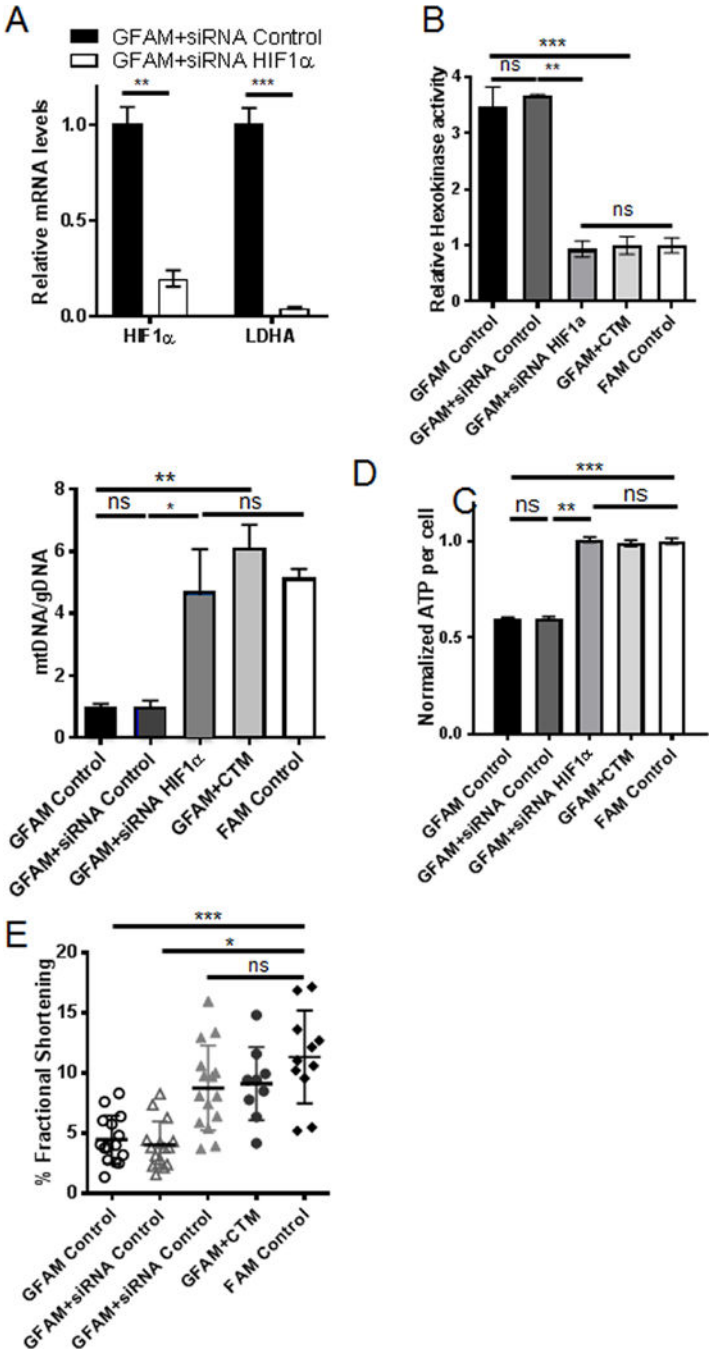

Figure 6. siRNA knock-down of HIF1α Improves hiPSC-CM maturation similar to HIF1α small molecule inhibition.

A) Relative mRNA levels of HIF1α and LDHA of hiPSC-CMs cultured in GFAM transfected with HIF1α siRNA compared to control siRNA after 96 hours (n=3 independent experiments). Measurements of **B)** relative hexokinase activities, **C)** ratio of mitochondrial DNA and genomic DNA, **D)** normalized cellular ATP levels, and **E)** fractional shortenings of hiPSC-CMs cultured in GFAM treated with HIF1α siRNA or CTM compared to control conditions and FAM cultured hiPSC-CMs (n=3 independent experiments).