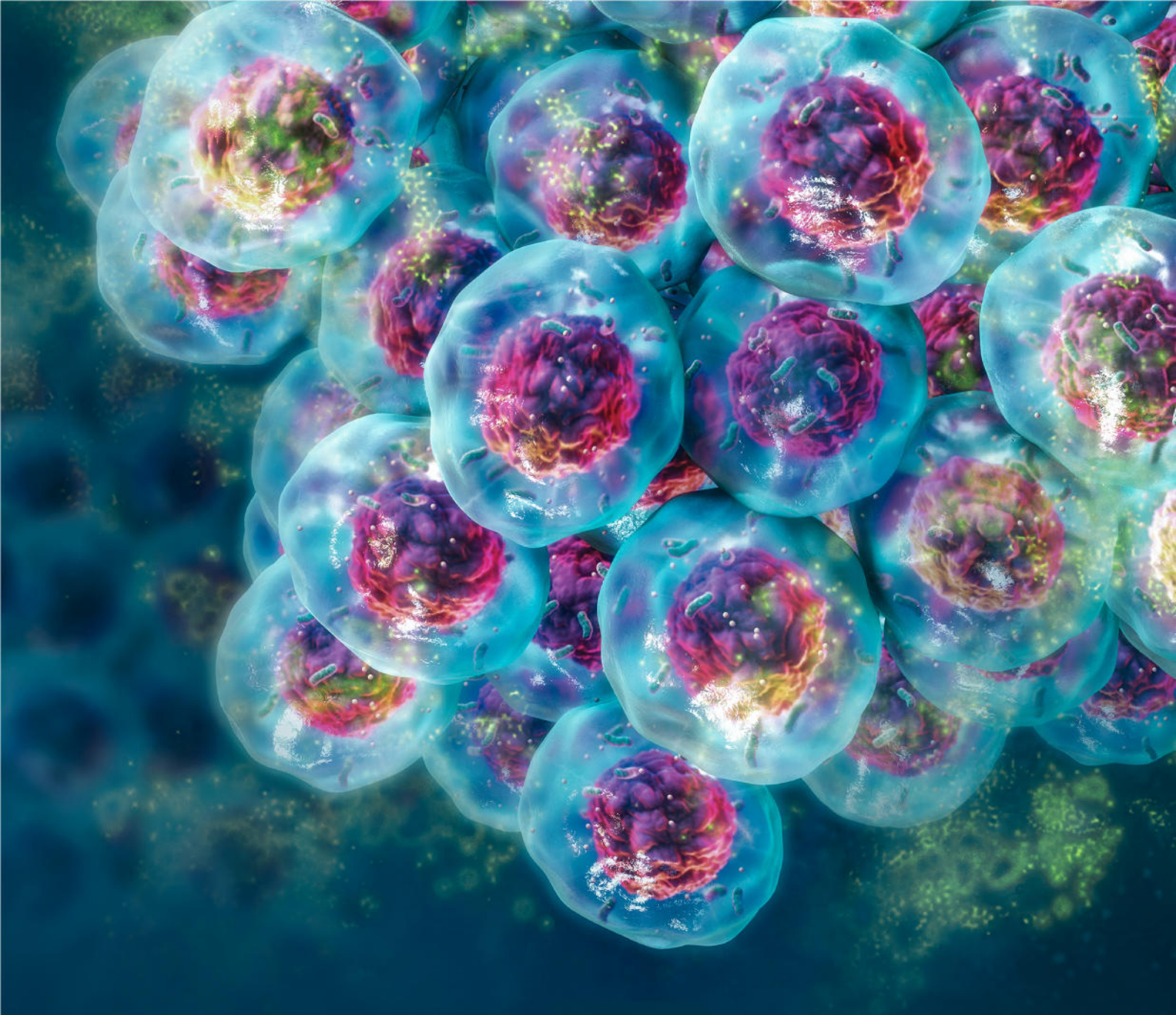




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Epigenetic Priming of Human Pluripotent Stem Cell-Derived Cardiac Progenitor Cells Accelerates Cardiomyocyte Maturation

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Key Words. Cardiac progenitor cells • Cardiomyocyte maturation • Epigenetics • Notch signaling • Human pluripotent stem cells

ABSTRACT

Human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) exhibit a fetal phenotype that limits *in vitro* and therapeutic applications. Strategies to promote cardiomyocyte maturation have focused interventions on differentiated hPSC-CMs, but this study tests priming of early cardiac progenitor cells (CPCs) with polyinosinic-polycytidylic acid (pIC) to accelerate cardiomyocyte maturation. CPCs were differentiated from hPSCs using a monolayer differentiation protocol with defined small molecule Wnt temporal modulation, and pIC was added during the formation of early CPCs. pIC priming did not alter the expression of cell surface markers for CPCs (>80% KDR+/PDGFRα+), expression of common cardiac transcription factors, or final purity of differentiated hPSC-CMs (~90%). However, CPC differentiation in basal medium revealed that pIC priming resulted in hPSC-CMs with enhanced maturity manifested by increased cell size, greater contractility, faster electrical upstrokes, increased oxidative metabolism, and more mature sarcomeric structure and composition. To investigate the mechanisms of CPC priming, RNAseq revealed that cardiac progenitor-stage pIC modulated early Notch signaling and cardiomyogenic transcriptional programs. Chromatin immunoprecipitation of CPCs showed that pIC treatment increased deposition of the H3K9ac activating epigenetic mark at core promoters of cardiac myofilament genes and the Notch ligand, *JAG1*. Inhibition of Notch signaling blocked the effects of pIC on differentiation and cardiomyocyte maturation. Furthermore, primed CPCs showed more robust formation of hPSC-CMs grafts when transplanted to the NSGW mouse kidney capsule. Overall, epigenetic modulation of CPCs with pIC accelerates cardiomyocyte maturation enabling basic research applications and potential therapeutic uses. *STEM CELLS* 2019;37:910–923

SIGNIFICANCE STATEMENT

The results of this study show that epigenetic priming of human pluripotent stem cell-derived cardiomyocytes with polyinosinic-polycytidylic acid accelerates differentiation and cardiomyocyte maturation *in vitro* and following heterotopic transplantation *in vivo*. Epigenetic and transcriptional profiling of primed cardiac progenitor cells reveals increased histone acetylation and earlier transcription of Notch and cardiomyogenic genes. The present study unmasks pathways regulating the time course of cardiac progenitor cell differentiation and cardiomyocyte maturation, providing a novel strategy to rapidly induce cardiomyocyte maturation for research and clinical applications.

INTRODUCTION

Human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) are powerful tools for disease modeling, drug development, basic research, and possible therapies. However, hPSC-CMs exhibit developmental immaturity that limits these applications. Therefore, intense efforts to promote maturation of hPSC-CM have been undertaken

including changing media to favor oxidative metabolism [1], manipulating action potentials by electrical pacing or Kir2.1 ion channel overexpression [2,3], creating three-dimensional tissue constructs [4], and extending cell culture over more than 3 months [5]. Despite progress in enhancing maturity, these interventions are often time-consuming, cumbersome, or promote only certain aspects of maturation (Table 1).

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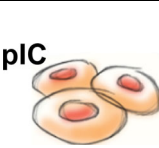
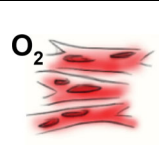
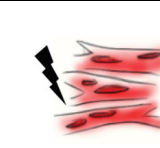
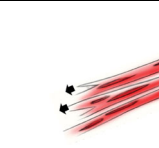
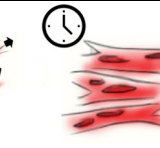
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Table 1. Methods to enhance hPSC-CM maturation

						
Method	Priming	Biochemical	Metabolic	Electrical	Mechanical	Temporal
Stage	hPSC-CPCs	hPSC-CMs	hPSC-CMs	hPSC-CMs	hPSC-CMs	hPSC-CMs
Approach	Early epigenetic modulation	Activate maturation signals	Induce switch to FFA oxidation	Electrical pacing; hyperpolarization	Morphologic, force constraints	Prolonged cell culture
Reagents	pIC	Let-7 miRNA, T3, IGF-1, dex.	IBMX, insulin, rosiglitazone, indomethacin, dex.	Pacing, coculture, overexpress CSQ/Kir2.1	Collagen, hydrogel, PDMS, Matrigel, PEG/PCL	Basal medium
Equipment	Standard	Standard	Standard	Pacing device; 3D tissue; gene modification	Engineered materials	Standard
Speed	+++	++	++	++/+++	++	+
Economy	+++	++	++	+/++	++	+
Scalable	+++	+++	+++	+/++	+/++	+++
References	This study	[6–8]	[1]	[2, 3], [9–13]	[4], [13–20]	[5], [21], [22, 23]

Abbreviations: 3D, three dimensional; dex., dexamethasone; FFA, free fatty acid; hPSC-CMs, human pluripotent stem cell-derived cardiomyocytes; hPSC-CPCs, human pluripotent stem cell-derived cardiac progenitor cells; IBMX, isobutylmethylxanthine; IGF-1, insulin-like growth factor 1; PCL, polycaprolactone; PDMS, polydimethylsiloxane; PEG, polyethylene glycol.

Although environmental cues clearly modulate cardiomyocyte development, there is evidence that cell-intrinsic processes also regulate the time course of maturation. For example, studies comparing mouse PSC and hPSC differentiation demonstrate species-specific differences in the kinetics of differentiation both in vitro and during in vivo teratoma formation suggesting a cell autonomous developmental clock [24]. Consistent with an intrinsic developmental clock, distant cardiomyocytes found in pulmonary and azygous veins show maturational changes in troponin and myosin isoforms that are synchronous in time with cardiomyocytes of the heart proper, although spatially remote and subject to dramatically different hemodynamics and cell signaling [25]. These observations suggest that both environmental and intrinsic processes control cardiac cellular maturation. Although the mechanisms controlling intrinsic pathways of maturation are largely unknown, epigenetic factors are likely important [26, 27].

We therefore hypothesized that appropriate priming of hPSC-derived cardiac progenitor cells (CPCs) can alter their epigenetic status and accelerate cardiomyocyte differentiation and maturation. For this purpose, the double-stranded RNA and innate immunity activator, polyinosinic-polycytidylic acid (pIC), was investigated. pIC has previously been found to accelerate the reprogramming of skin fibroblasts to induced pluripotent stem cells (iPSCs) by augmenting activating epigenetic marks at the transcription factor loci responsible for the observed lineage changes [28]. Other agents that promote epigenetic changes have also been associated with accelerated cell type transitions in reprogramming [29], and pIC has also shown efficacy in enhancing reprogramming of fibroblasts to cells of the cardiovascular lineage such as cardiomyocytes and endothelial cells [30,31]. Like reprogramming, the differentiation of hPSCs to the cardiomyocyte lineage involves a progression of epigenetic transitions [32]; so, we hypothesized that pIC could aid the transitions needed for hPSC-CM differentiation and maturation.

Here, we demonstrate that pIC treatment of CPCs accelerates maturation of hPSC-CMs broadly assessed by structural, electrical, and metabolic assays. Primed CPCs have epigenetic changes in myofilament and Notch signaling-related genes, and inhibition of Notch signaling blocks the effect of pIC on maturation. Heterotopic transplantation of primed CPCs to the immunodeficient mouse

leads to more robust cardiomyocyte differentiation. Thus, a simple biopolymer intervention at the CPC stage can substantially accelerate the differentiation and maturation of hPSC-CMs, which can improve in vitro applications and potentially therapeutic approaches.

METHODS

Methods are described in the Supporting Information Materials and Methods section, available online.

RESULTS

pIC Priming of CPCs Accelerates hPSC-CM Maturation

To test the effect of pIC on CPCs, we used a defined, small molecule hPSC cardiac differentiation protocol based on sequential activation of the Wnt pathway by GSK3 β inhibition followed by Wnt inhibition (GiWi; Fig. 1A) [33]. pIC was added on days 3–5 of the GiWi protocol during CPC formation, and we first assessed whether pIC impacted the formation of CPCs identified as the cell population colabeled by kinase insert domain receptor (KDR) and platelet-derived growth factor Receptor alpha (PDGFR α) [34]. Approximately 80% of day 5 cells in the presence or absence of pIC express the KDR⁺/PDGFR α ⁺ immunophenotype (Supporting Information Fig. S1a). pIC treatment does not alter the expression of key transcription factors present in CPCs including *MESP1*, *GATA4*, *TBX5*, *ISL1*, and *NKX2-5* (Supporting Information Fig. S1a). Because prior studies used pIC treatment to promote lineage reprogramming, we also assessed if pIC treatment of CPCs altered the composition of the differentiated progeny. Flow cytometry for cardiac troponin T (cTnT) revealed that cells differentiated from pIC-treated and -untreated CPCs using the GiWi protocol were primarily hPSC-CMs (80%–95%) for both hES and hiPS cell lines (Supporting Information Fig. S1b). Thy1⁺ cells accounted for the majority of noncardiomyocytes differentiated from the CPCs and were primarily a fibroblast population supported by colabeling with other markers of cardiac fibroblasts such as Wilm's tumor 1 (WT1), matrix metalloproteinase 1 (MMP1), and fibroblast-specific protein 1 (FSP1) (Supporting

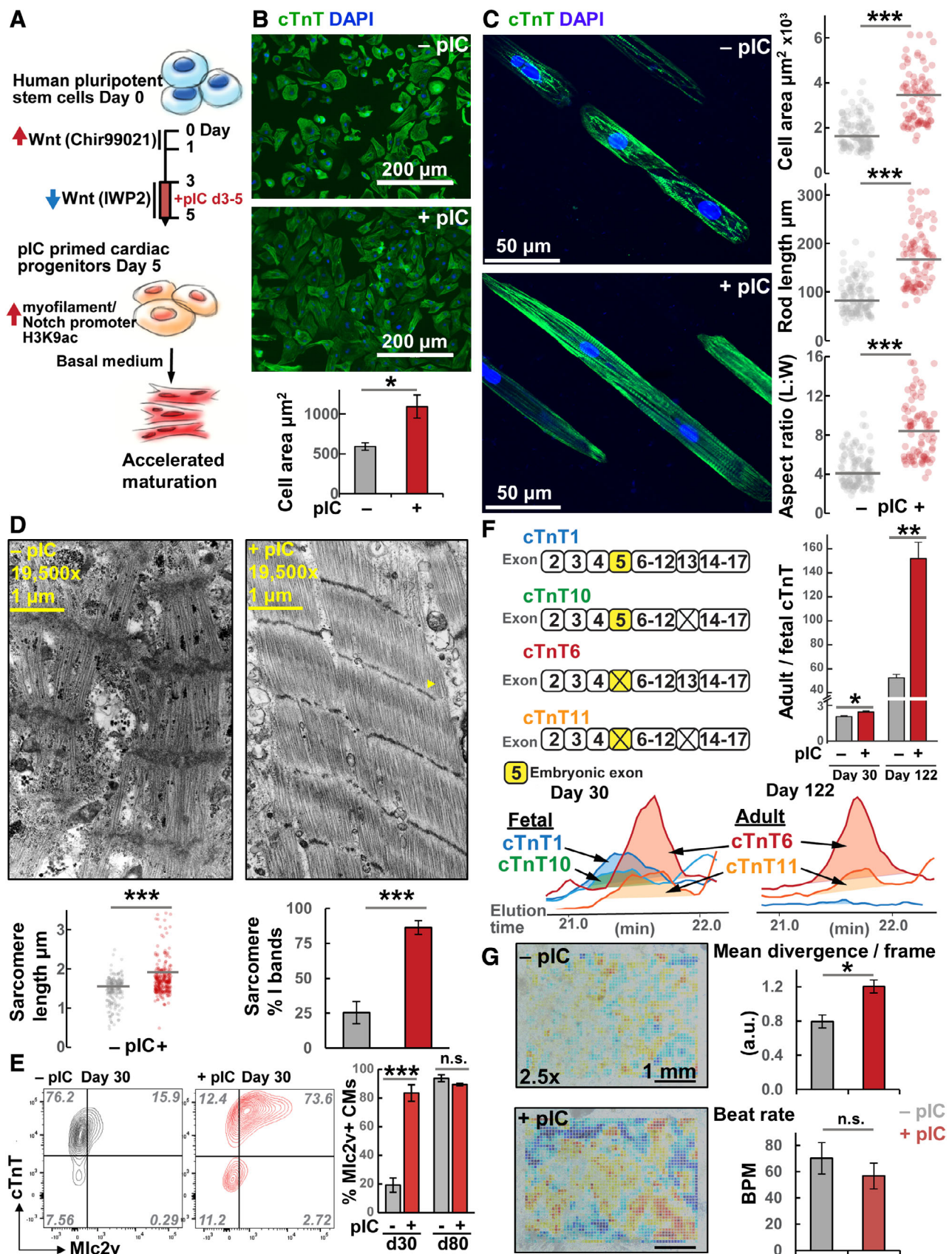


Figure 1. (legend appears on next page.)

Information Fig. S1c) [35]. Endothelial and smooth muscle cells were rare based on CD31 and smooth muscle-myosin heavy chain (SM-MHC) labeling (Supporting Information Fig. S1d). We detected no evidence for chamber-specific lineage changes in the mostly ventricular-fated GiWi cardiomyocytes as assessed by gene expression and immunostaining of atrial (*HEY1* and *COUPTFII*) and ventricular (*IRX4*, *HAND1*, and *HAND2*) cardiomyocyte markers (Supporting Information Fig. S1e, S1f), action potential shape quantified by APD30/80 (Supporting Information Fig. S1g), or ventricular-specific myosin light chain 2v (Mlc2v) flow cytometry at day 80 (Fig. 1E). Thus, pIC treatment did not alter the formation of CPCs or their differentiation potential.

We then assessed if pIC treatment of CPCs impacted the maturation of the differentiated hPSC-CMs. Compared with adult cardiomyocytes, hPSC-CMs are developmentally immature exhibiting smaller size, less organized sarcomeres, expression of fetal cardiac myofilament isoforms, decreased oxidative metabolism, smaller amplitude action potentials, and reduced contractility [21, 25, 33, 36–39]. We began by characterizing the morphological and structural properties of hPSC-CMs differentiated from pIC-treated or -untreated CPCs. Accordingly, we found pIC treatment of CPCs resulted in day 30 hPSC-CMs that were twice as large (Fig. 1B); however, the larger hPSC-CMs following pIC treatment still lacked adult rod-like morphology when cultured in this standard 2D tissue culture format. We therefore conducted cell size and morphology measurements on hPSC-CMs from treated and untreated CPCs that were dissociated and replated on 20- μ m-wide lanes of Matrigel patterned on a soft polydimethylsiloxane (PDMS) substrate to mimic the stiffness found in the native heart ($\sim$ 10 kPa) to promote hPSC-CM structural organization [40,41]. Rod-like hPSC-CMs emerged in this environment from pIC-treated CPCs that were again approximately twice as large and had nearly double the L:W aspect ratio ($\sim$ 8:1 vs. 4:1) of hPSC-CMs from untreated CPCs (Fig. 1C; Supporting Information Fig. S2). We found that lane patterning was a necessary feature for rod formation in this assay, wherein plating hPSC-CMs on unpatterned, soft PDMS did not result in rod formation (Supporting Information Fig. S2). Ultrastructural characterization by electron microscopy showed longer, more regular sarcomeres-containing I-bands in hPSC-CMs from pIC-treated CPCs, which are features of sarcomeres found in more mature cardiomyocytes (Fig. 1D) [21]. The myofilament protein Mlc2v gradually increases in expression over 80 days as the ventricular-fated cardiomyocytes of the GiWi protocol mature, such that few early hPSC-CMs have detectable Mlc2v by flow cytometry [33,42]. However, pIC treatment of CPCs markedly increased the percentage of cTnT⁺ hPSC-CMs that produced

Mlc2v at day 30 compared with day 80 when more than 90% of hPSC-CMs were Mlc2v⁺ in both groups (Fig. 1E). This shows accelerated maturation of Mlc2v expression in hPSC-CMs from pIC-treated CPCs, and we next investigated markers which change at a later stage in hPSC-CM maturation. The myofilament protein cTnT is expressed throughout development, but it undergoes developmental changes in protein spliceoforms based on the presence or absence of embryonic exon 5 [43], leading to initial expression of fetal isoforms 1 and 10 and subsequently adult isoforms 6 and 11 (Uniprot numbering). We performed top-down mass spectrometry to measure these isoforms of cTnT in hPSC-CMs and found that pIC treatment of CPCs increased the ratio of adult to fetal isoforms in day 30 hPSC-CMs. Notably, during prolonged culture to day 122, the adult to fetal ratio and the relative difference between hPSC-CMs from pIC treated and untreated CPCs increased dramatically (Fig. 1F). This suggests that although Mlc2v expression normalized at later-stage culture, myofilaments with slower maturation timelines were affected greater than 100 days after the day 3–5 pIC treatment and showcases the ability of top-down mass spectrometry for cTnT isoforms specifically to distinguish the maturation status of late-stage hPSC-CMs. Together these data indicate that pIC treatment of CPCs accelerates cardiomyocyte structural maturation based on hPSC-CM size, morphology, ultrastructure, and myofilament expression patterns.

To determine if the changes in maturation of structural properties translate into increased contractility of hPSC-CMs, we performed video analysis of contraction divergence patterns as previously described [44]. Beat divergence is derived from a local cross-correlation (or “optical flow”) analysis of image sequences, and has been shown to distinguish active contraction from passive motion in mixed cultures [44]. We found that hPSC-CMs from pIC-treated CPCs had increased average divergence (Fig. 1G; Supporting Information Fig. SV1), as well as increases in other parameters of sheet contractility (for extended data see Supporting Information Fig. S3) [44, 45]. Although some of these methods may be sensitive to the rate of beating, we found no significant difference between the spontaneous beat frequency in hPSC-CMs from pIC-treated and -untreated CPCs (Fig. 1G). We then assessed for changes in other functional properties of hPSC-CMs. As cardiomyocytes mature, they exhibit faster action potential upstroke velocity and increased oxidative metabolism [37,46]. We recorded spontaneous optical action potentials from day 30 hPSC-CMs using the voltage-sensitive dye RH237. The optical upstroke velocity in hPSC-CMs from pIC-treated CPCs was significantly faster (Fig. 2A; Supporting Information Fig. S4a), quantified as decreased rise time (the time required to change from 10% to 90% depolarization voltage

Figure 1. Increased structural maturation in human pluripotent stem cell (hPSC)-derived CMs from pIC primed cardiac progenitor cells (CPCs). **(A):** Overview: Differentiation of hPSCs using a small molecule, biphasic Wnt modulation protocol with pIC treatment to generate primed CPCs that exhibit accelerated differentiation and maturation. **(B):** Cell size analysis of day 30 human induced PSC (hiPSC)-CMs quantified by the area of cTnT immunofluorescence in standard two-dimensional culture, $n = 4$ passages approximately >50 cells. **(C):** Cell morphology analysis of cTnT immunolabeled hiPSC-CMs dissociated after day 30 and cultured for 1 week on 20- μ m-wide Matrigel patterned lanes on polydimethylsiloxane substrate. **(D):** Transmission electron micrographs of human ESC (hESC)-CMs showing I bands (yellow arrow). Individual sarcomere length measurements across all images and percentages of I banded sarcomeres in individual images quantified with means illustrated. **(E):** Flow cytometry plots for day 30 and day 80 hiPSC-CMs costained with cTnT and Mlc2v with average data, $n = 3$. **(F):** Top-down mass spectrometry for cTnT isoforms area-under-the-curve ratio of adult to fetal isoforms for day 30 and day 122 hESC-CMs, $n = 3$. **(G):** Representative day 30 contractility heatmaps and average data calculated from divergence analysis of hiPSC-CM monolayers, $n = 7$. Red indicates regions of higher divergence, and blue negative divergence (convergence). t test *, $p < .05$; **, $p < .01$; ***, $p < .001$; hiPSC line DF19-9-11, hESC line H9-TNNT2-GFP. Abbreviations: CM, cardiomyocytes; cTnT, cardiac troponin T; pIC, polyinosinic-polycytidylic acid.

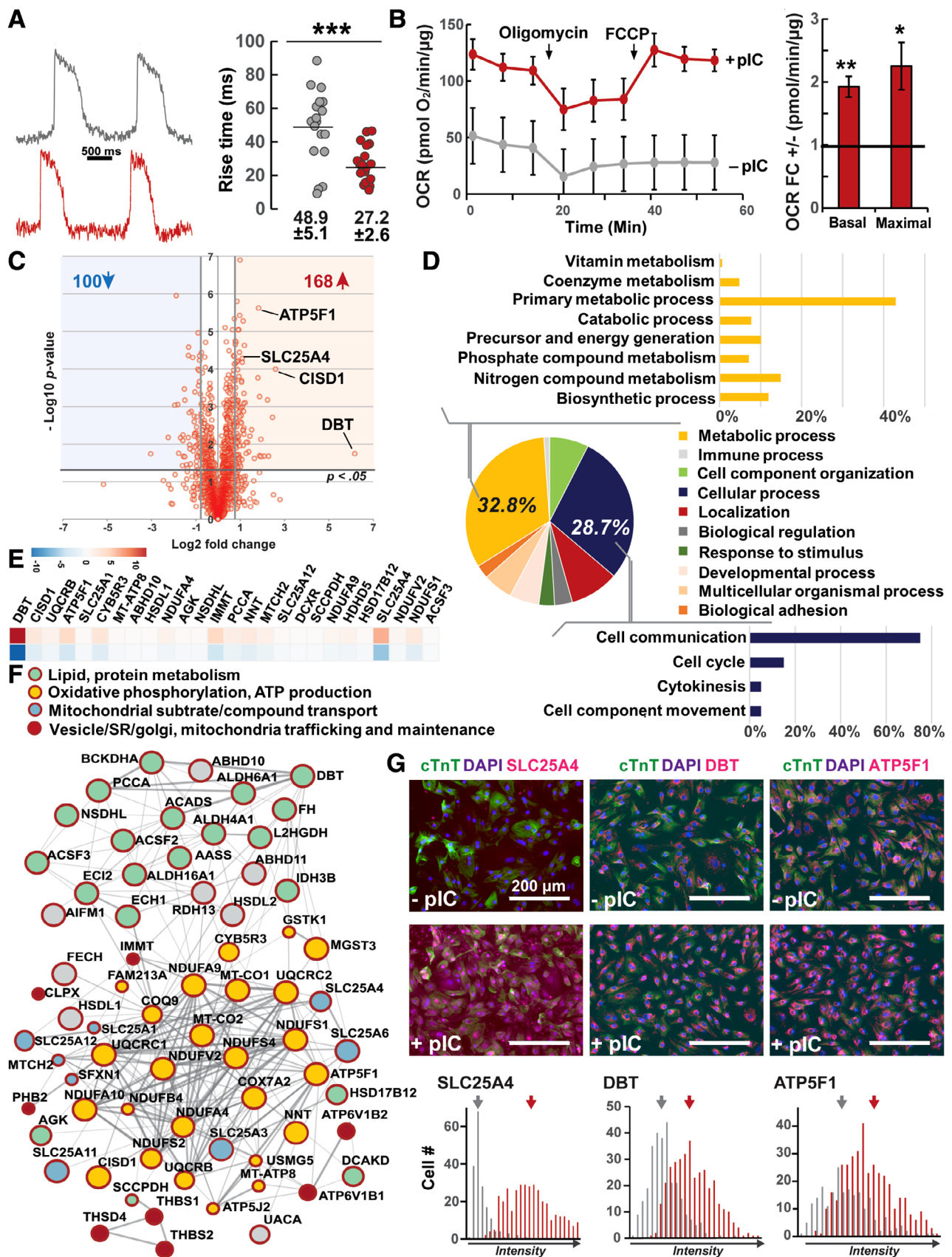


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amplitude) [47]. There was no difference in the spontaneous rate or other parameters measured for the spontaneous action potentials, except pIC treatment did decrease APD80 (Supporting Information Fig. S4a). To examine the metabolism of hPSC-CMs, we lactate-purified [48] hPSC-CMs (>95% cTnT⁺) from both pIC-treated and -untreated CPCs and used the Seahorse bioanalyzer to measure basal and maximal oxygen consumption in hPSC-CMs. hPSC-CMs from treated CPCs had approximately two-fold greater basal and maximal oxygen consumption rates (Fig. 2B). These studies suggest an increase in maturation of functional properties of the hPSC-CMs differentiated from pIC-treated CPCs.

To provide an unbiased evaluation of changes in protein expression in the hPSC-CMs generated by treated and untreated CPCs, we performed global label-free quantitative bottom-up proteomics on day 30 hPSC-CMs [49]. We detected approximately 2,600 proteins (Supporting Information Fig. S4b) and found that overall, 168 proteins were significantly upregulated and 100 downregulated in hPSC-CMs from treated relative to untreated CPCs ($p < .05$ and >50% change in expression; Fig. 2C). The upregulated proteins were mainly involved in metabolic and cell communication processes (Fig. 2D). Inspection of upregulated metabolic proteins suggested predominant roles in oxidative phosphorylation, mitochondrial transport, and the metabolism of amino acids and lipids (Fig. 2E, 2F; Supporting Information Fig. S4c and Table S1). This was consistent with our functional data showing increased oxygen consumption rates. By quantitative immunostaining, we verified increased expression of four of the top upregulated proteins in the dataset involved in oxidative and lipid metabolism: branched chain amino acid dehydrogenase DBT, NADP-dependent steroid dehydrogenase-like NSDHL, ATP synthase subunit ATP5F1, and the cytoplasmic mitochondrial DNA-stabilizing ATP transporter SLC25A4 (Fig. 2G; Supporting Information Fig. S4d). SLC25A4 protein in particular was almost absent from untreated CPC-CMs and is a known mediator of mitochondrial function, where its loss in humans results in the mitochondrial myopathy MTDPS type 12 [50]. In contrast, downregulated proteins were often involved in cytoskeletal organization (Myl6, Talin, Transgrelin, and collagens 18a1, 3a1, and 1a2; Supporting Information Fig. S4c). We found proteins involved in ribosomal biogenesis or mRNA synthesis and translation in both upregulated and downregulated protein sets; however, we noticed mitochondrial ribosomal complex proteins MRPL1, MRPL44, and MRPS22 only among upregulated proteins in hPSC-CMs from pIC-treated CPCs.

Through the combination of this proteomics data with the functional and structural data above, we conclude that there is increased maturation broadly assessed in structural, metabolic, and electrical properties of cardiomyocytes from pIC-treated CPCs, which we refer to hereafter as primed CPCs.

pIC Epigenetically Primes CPC Notch and Cardiomyogenic Transcriptional Programs

We hypothesized that the effect of pIC on CPCs to promote hPSC-CM maturation involves modulation of cardiac developmental signaling networks and early cardiomyogenic transcriptional programs. To discover these networks, we performed RNA sequencing of day 5 pIC primed and untreated CPCs to evaluate gene expression.

Among pIC-upregulated genes, we found enrichment in gene ontology (GO) terms associated with cardiac development, such as insulin signaling pathways, and we also noted upregulation of multiple genes in Notch signaling (Supporting Information Table S2). We first validated this by targeted Notch pathway quantitative polymerase chain reaction (qPCR) array, which demonstrated 48/65 Notch genes in this panel to be upregulated in primed CPCs (Fig. 3B). The top-enriched gene in RNA sequencing was the Notch pathway ligand *JAG1* (Fig. 3A; Supporting Information Table S2), and qPCR validation confirmed upregulation of *JAG1* and other Notch-related genes such as *HEY1*, *DLL3*, and *ATOH1* and the retinoic acid signaling enzyme *ALDH1A2* (which requires Notch signaling for its expression in the heart [51] and is also known to be necessary for early ventricular maturation during formation of the chambered heart in vivo) [52–54]. Jag1 protein expression has not been previously reported during heart development prior to the early heart tube myocardium [55]. To determine if Jag1 is expressed in developing CPCs in vivo in analogy to our findings during in vitro differentiation, we performed immunostaining in cardiac crescent-stage E8.25/4-somite mouse embryos (Fig. 3D). Intense Jag1 immunolabeling was present in the CPCs of the cardiac crescent and the neighboring cells of the endoderm as well as in the yolk sac. *JAG1* and *NOTCH2* were the most abundant Notch ligand/receptor pair in the CPCs in RNAseq (Supporting Information Fig. S5a), so we investigated Jag1 and Notch2 protein surface localization on the hPSC-derived CPCs by flow cytometry. The majority of both primed and untreated CPCs coexpressed Jag1 and Notch2, but pIC treatment increased the percentage of Jag1⁺/Notch2⁺ cells (Fig. 3E). A pIC-induced increase in Jag1 expression was also confirmed by quantitative, IR Western blotting (Fig. 3F). These data suggest that Jag1 and Notch2 function together in Notch signaling in CPCs, which is consistent with human genetic studies in which loss of function mutations in either *JAG1* or *NOTCH2* result in cardiomyopathy in patients with Alagille syndrome [56].

GO analysis of pIC downregulated genes showed pathways in cancer, pluripotency, and transforming growth factor beta (TGFβ) signaling in the top four downregulated GO terms in Kyoto Encyclopedia of Genes and Genomes pathways analysis (Supporting Information Table S2), and we confirmed many of these genes by qPCR

Figure 2. Functional and proteomic analysis of maturation in human pluripotent stem cell-derived cardiomyocytes, effect of pIC treatment of cardiac progenitor cells (CPCs). **(A):** Optical upstroke velocity with the voltage-sensitive dye RH237 (quantified by time required to traverse 10% to 90% action potential amplitude, rise time) for day 30 human ESC-derived cardiomyocytes (hESC-CMs), individual cells plotted from four cell preparations. **(B):** Seahorse quantification of OCR normalized to total protein fold change of hESC-CMs from primed to untreated CPCs, $n = 4$. **(C):** Global quantitative bottom up proteomic analysis of upregulated and downregulated proteins as volcano plot of hESC-CMs from primed CPCs compared with no treatment. **(D):** Ontology analysis revealed the majority of the upregulated proteins were involved in primary metabolic processes and cell communication. **(E):** Heatmap of the top 34 upregulated proteins involved in metabolic processes and mitochondrial function. **(F):** Protein–protein interaction network of upregulated metabolic proteins, predominantly involved in oxidative phosphorylation and ATP production (yellow), as well as lipid/amino acid metabolism (green). Proteins important for mitochondrial substrate/compound transport (blue) and the crosstalk of mitochondrial and SR/Golgi compartments (red) were also upregulated. For full interactome see Supporting Information Figure S4c. **(G):** Immunostaining and quantification of mean fluorescence of three top upregulated metabolic proteins. Means labeled. For Unmerged images see Supporting Information Figure S4d. t test *, $p < .05$; **, $p < .01$; ***, $p < .001$; hESC line H9-TNNT2-GFP. Abbreviations: cTnT, cardiac troponin T; OCR, oxygen consumption rate; pIC, polyinosinic-polycytidylic acid.

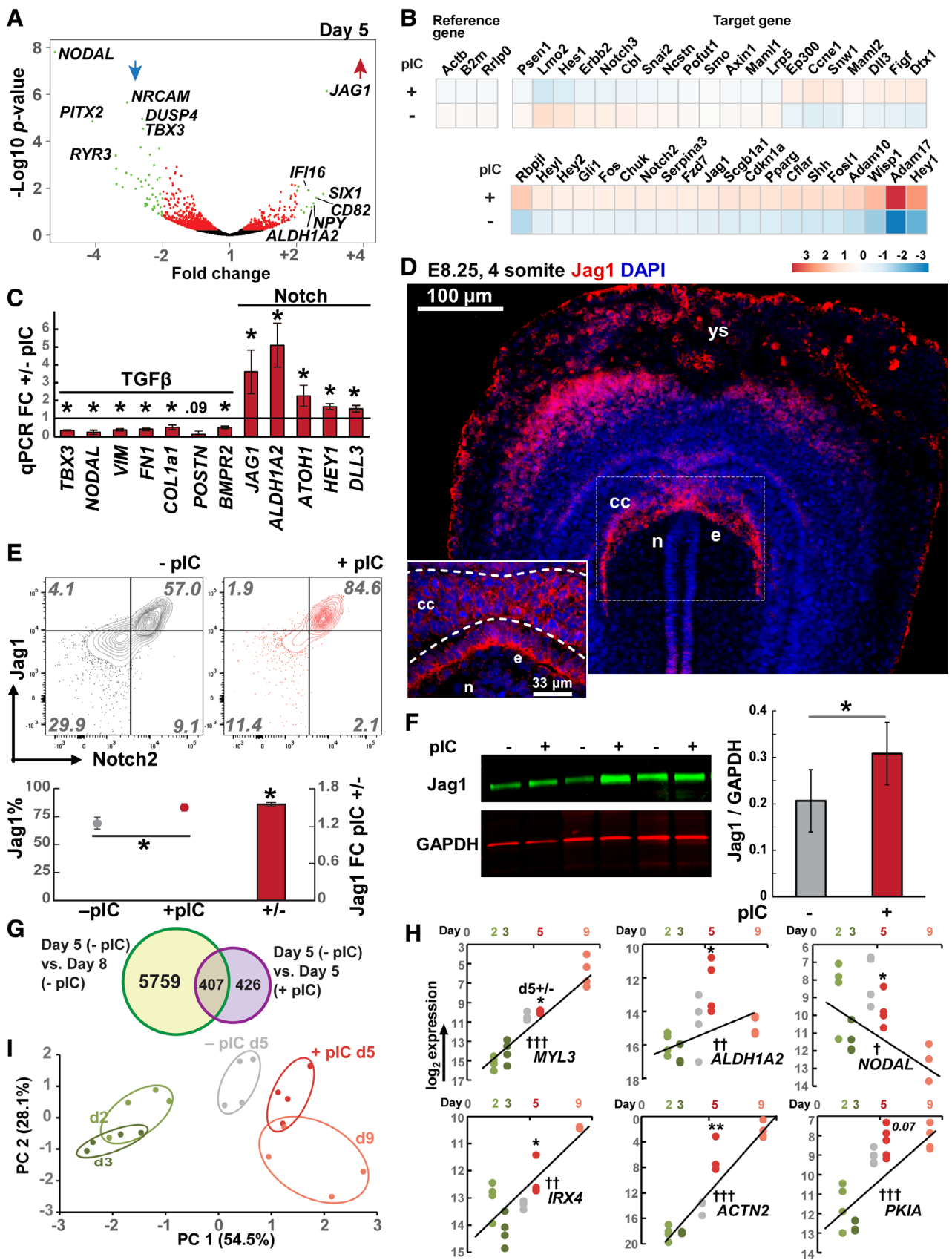


Figure 3. (legend appears on next page.)

(Fig. 3A, 3C). We found that the downregulated TGF β -related genes were largely classical epithelial-to-mesenchymal transition (EMT) markers such as *FN1* and *VIM*, or found in the GO analysis among “networks involved in pluripotency signaling” (#4 downregulated GO term; Supporting Information Table S2). Two of the top downregulated genes, *TBX3* and *NODAL*, are established necessary factors for pluripotency [57, 58]. TGF β also mediates the EMT of primitive streak-like mesendoderm formation in the initial phase of cardiac differentiation protocols [33, 59]. Thus, we interpreted pIC's downregulation of TGF β pathways as consistent with inhibiting residual expression of genes of pluripotency and early EMT/mesendoderm.

We hypothesized that pIC causes a change in gene expression in CPCs consistent with an acceleration of the differentiation process. We therefore sought to compare the transcriptional patterns of pIC primed and untreated CPCs to changes in gene expression occurring in early cardiac differentiation. We used a publicly available RNAseq dataset evaluating the time course of hPSC-CMs differentiation during the GiWi protocol (PCBC synapse ID 25822579). We analyzed early days in this series (2, 3, 5, and 8) to detect leading indicator genes first differentially expressed in the earliest day 8 cardiomyocytes. Consistent with the initial formation of terminally differentiated cardiomyocytes, the day 8 reference dataset exhibited the greatest differences compared with days 2, 3, and 5 (Supporting Information Fig. S5b). Five thousand three hundred seventy five genes were differentially expressed between day 5 and day 8 in the reference RNA sequencing of the GiWi protocol (Supporting Information Table S3). In our RNAseq of day 5 pIC primed and untreated CPCs, pIC affected a much smaller set of just 833 genes (out of ~10,500 genes detected; Fig. 3G). Nonetheless, there were significant overlaps in these data sets, with 49% of genes differentially expressed following pIC treatment overlapping the differentially expressed genes between day 5 and day 8 of the GiWi protocol. Gene ontology analysis shows that these overlap genes were predominately involved in cardiac processes and development (Supporting Information Fig. S5c and Table S3). We then focused on validating several of these differentially expressed genes by qPCR on day 5 pIC primed and untreated CPCs, as well as time points before and after the progenitor stage (days 2, 3, and 9 of GiWi). We found increased expression of a subset of the genes discovered in our time course RNAseq analysis that have been previously discovered as early-upregulated cardiomyogenic factors in other large-scale transcriptomic and epigenetic studies—*ACTN2*, *MYL3*, *ALDH1A2*, *IRX4*, and *PKIA* [26, 27]. These genes increased in GiWi

cultures between precardiac (days 2 and 3) to postcardiac (day 9) progenitor stages in a log-linear fashion and increased in pIC primed day 5 CPCs compared with untreated CPCs (Fig. 3H). Oppositely, we found that *NODAL* (as described above) decreased from days 2–3 to day 9 and was decreased in pIC primed CPCs in the direction of differentiation. When these markers were combined into principal component analysis (PCA), the statistically largest component of analysis, PC1 accounted for the majority of the variability in gene expression and convincingly separated earlier (days 2, 3) and later (day 9) GiWi samples (Fig. 3I). PCA showed that pIC primed CPCs were most similar to the untreated day 5 CPCs, but had greater similarity to the later day 9 samples in this subset of early cardiomyocyte genes than did untreated CPCs.

Given that the Notch pathway was upregulated in primed CPCs (Fig. 3B, 3C), we tested the effects of inhibiting Notch signaling with the gamma-secretase inhibitor N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester (DAPT). Although *JAG1* and *ALDH1A2* were significantly enriched in day 5 CPCs, a time course experiment demonstrated that progenitor stage treatment with pIC from days 3 to 5 also had prolonged effects on the cells, with RNA levels of *JAG1* and *ALDH1A2* remaining upregulated until day 9–11 (Fig. 4A). We therefore assessed the maturation of hPSC-CMs from day 3 to 5 pIC-untreated CPCs in the presence of DAPT inhibition. We first assessed the morphology of hPSC-CMs in the PDMS rod-formation assay and found that DAPT specifically blocked the increased cell area of hPSC-CMs from primed CPCs but did not decrease the size of hPSC-CMs from untreated CPCs (Fig. 4B). This effect on primed CPCs was present with Notch inhibition with DAPT from days 3 to 7 and intensified when DAPT included the early cardiomyocyte window (days 3–16). Treatment from days 3 to 16 with DAPT also blocked other markers of maturation including the increase in optical upstroke velocity in cardiomyocytes from primed CPCs (decreased rise time; Fig. 4C) and the protein isoform change toward adult cTnT 6 and 11 (Fig. 4D). Conversely, we found that Notch inhibition from days 3 to 7 and days 3 to 16 blocked the increase in contractility of hPSC-CMs from primed CPCs to a similar extent (Fig. 4E). The role of Notch signaling in promoting maturation of hPSC-CMs is consistent with previous reports in vivo in which *Jag1* deletion driven by the cardiomyocyte-specific *Tnnt2* promoter inhibits ventricular maturation [60].

To uncover the upstream mechanisms for the transcriptional changes induced by pIC, we profiled the epigenetic status of

Figure 3. Transcriptomics of primed progenitors reveals Notch and cardiomyogenic transcriptional program enrichment. **(A):** RNAseq volcano plot of day 5 pIC primed versus untreated human induced pluripotent stem cell-cardiac progenitor cells (hiPSC-CPCs). Significantly altered genes with fold change $\geq \pm 2$ in green, $\geq \pm 1$ in red. **(B):** Notch pathway real-time quantitative polymerase chain reaction (RT-qPCR) array showing upregulation in 48 of 65 Notch genes in pIC primed CPCs. Reference genes shown in left with no changes between cell groups. **(C):** RT-qPCR validation of RNAseq candidates in the TGF β and Notch pathways, $n = 4$. **(D):** E8.25/4-somite stage mouse embryo transverse section immunolabeled for Jag1. ys, yolk sac; cc, cardiac crescent; n, notochord; e, endoderm. **(E):** Flow cytometry of day 5 CPCs for Jag1 and Notch 2 showing pIC treatment increased the number of Jag1 positive cells and median fluorescence signal-to-noise. **(F):** Licor near-IR Western blot of CPC Jag1 with quantitation normalized to GAPDH, $n = 3$. **(G):** Heatmap of top differentially expressed genes from RNAseq data comparing the earliest cardiomyocytes (day 8) and CPCs (day 5) in the GSK3 β inhibition followed by Wnt inhibition protocol. Venn diagram illustrates overlap of a subset of these genes with those differentially expressed in RNAseq comparing days 3–5 $\pm$ pIC. **(H):** RT-qPCR validation of overlap genes from **(D)** and comparison of expression levels between day 5 $\pm$ pIC with pre-progenitor (days 2 and 3) and postprogenitor (day 9) time points. Regression computed from days 2, 3, and 9 with analysis of variance test for slope: $\dagger$, $p < .05$; $\dagger\dagger$, $p < .01$; $\dagger\dagger\dagger$, $p < .001$. **(I):** PC analysis of CPC priming genes and cell samples in **(H)**. t test *, $p < .05$; **, $p < .01$; ***, $p < .001$; hiPSC line DF19-9-11. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PC, principal component; pIC, polyinosinic-polycytidylic acid.

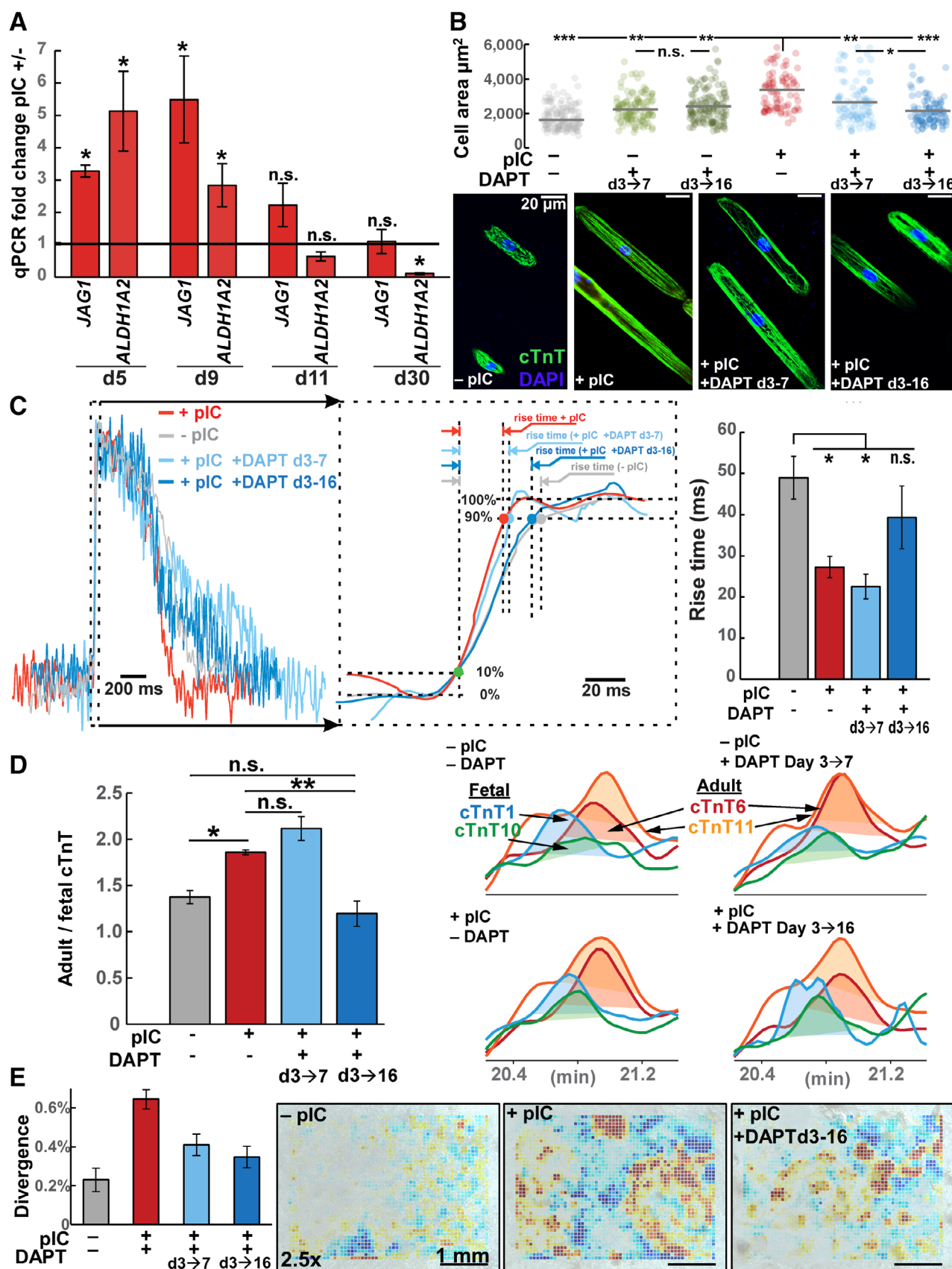


Figure 4. (legend appears on next page.)

both primed and untreated day 5 CPCs. Previous investigation demonstrated that pIC-mediated augmentation of fibroblast reprogramming is associated with decreased expression of repressive epigenetic modifiers, including type I and type II histone deacetylases, and this led to increased activating marks at the loci of pluripotency factors [28]. We examined the expression of type I and II histone deacetylases in CPCs differentiated from multiple hPSC lines. qPCR demonstrated that primed CPCs had universally lower levels of type II histone deacetylases (*HDAC4-7*) but unchanged to upregulated levels of type I *HDAC1-3* (Fig. 5A). Type I HDACs are ubiquitous enzymes generally present in all cell types, but the heart is the only organ to express all type II HDACs 4-7 and changes in type II HDACs alone or in combination cause cardiac-specific phenotypes [61,62]. We viewed predominant type II HDAC modulation in primed CPCs compared with the mixed type I and II repression of pIC treatment in fibroblasts, as a distinct cardiac progenitor response suggesting that epigenetic activation in CPCs could involve the loci of the early cardiomyogenic and Notch transcriptional programs. In ChIP-qPCR of day 5 primed and untreated CPCs, we found a significant pattern of increased deposition of the activating histone acetylation mark H3K9ac near the transcriptional start sites of both *JAG1* and multiple cardiac myofilament genes: *TNNT2*, *TNNI3*, *MYL2*, *MYL7*, *MYH6*, *MYH7*, *ACTC1*, and *ACTN2* (Fig. 5B). These changes with pIC treatment were specific for H3K9ac and not found in the H3K4me3 activating mark. There were also no changes in the repressive epigenetic modification H3K27me3 or H3K9ac deposition at a negative control intergenic region. Promoters with codeposition of both H3K27me3 and H3K4me3 have previously been described in stem cells and progenitors as epigenetically “poised,” [63] and we found greatest co-occupancy of these marks for *ACTN2*, which also saw the greatest relative increase in expression with pIC in day 5 CPC real time-qPCR (Fig. 3H; Supporting Information S1b). This suggests that poised genes may be expressed earlier in primed CPCs. These data and the pattern of increased H3K9ac across multiple cardiac myofilaments and *JAG1* suggest greater epigenetic priming for differentiation along the cardiac lineage, and we hypothesized that it would lead to faster onset of myofilament transcription and cell contraction. A time course experiment of myofilament gene expression revealed that day 9 cells from primed CPCs had increased expression of all cardiac myofilaments studied compared with cells from untreated CPCs and faster onset of contractions in the differentiated cells by day 9 (Fig. 5C). By day 30, the relative increases induced by pIC remained only for *MYH6*, *TNNI3*, and *ACTC1*, myofilament proteins with greater adult than fetal expression or force production. These results suggested that

epigenetic priming causes earlier activation of the cardiomyogenic transcriptional program and accelerated differentiation.

Given the impact of pIC on CPC epigenetics and acceleration of differentiation in the in vitro analyses, we sought to determine if pIC priming promoted the differentiation of CPCs and accelerated cardiomyocyte maturation following in vivo delivery. We therefore heterotopically grafted both primed and untreated day 5 CPCs into the kidney capsules of immunodeficient NSGW mice [64], and after 30 days, the human grafts were identified by anti-human mitochondrial antibody. We observed approximately fourfold more cardiac actin and sarcomeric myosin heavy chain-expressing hPSC-CMs in grafts from primed CPCs, and treatment of the CPCs with the Notch inhibitor, DAPT, specifically reduced the number of differentiated hPSC-CMs (Fig. 5D, 5E). The grafts from primed CPCs exhibited hPSC-CMs with elongated morphology and aligned myofilaments in contrast to the more immature appearing circular clusters of hPSC-CMs from untreated CPCs. We cannot exclude that longer in vivo follow-up of animals would equalize certain of these differences, but the finding of accelerated differentiation of primed CPCs is consistent with the previously reported ability of later stage, committed CPCs to more readily differentiate into hPSC-CMs in the kidney capsule in contrast to early stage CPCs [65]. In addition, these results from the heterotopic kidney capsule transplant model agree with the observed in vitro effects of pIC and suggest that this strategy could have relevance for cell therapy applications.

DISCUSSION

Here, we describe the striking effects of pIC on hPSC-derived CPCs to accelerate differentiation and maturation of hPSC-CMs. Treatment of CPCs with pIC promotes H3K9ac deposition at the loci of Notch and cardiomyocyte genes, which leads to accelerated transcription of cardiac myofilaments and earlier onset of electromechanical contractions of the differentiated hPSC-CMs. Furthermore, pIC priming accelerates the overall maturation process of hPSC-CMs as broadly assessed by transcriptional, structural, electrical, and metabolic assays. We find that Notch signaling has an essential role in mediating this accelerated maturation, which is consistent with the observation that loss of function mutations in pIC's top upregulated gene, *JAG1*, are a known cause of immature ventricular development and cardiomyopathy in humans [56]. The effect of pIC priming of CPCs is also evident following heterotopic transplantation to the kidney capsule of NSG mice with formation of larger and more mature cardiac grafts.

Figure 4. Notch inhibition blocks acceleration of human pluripotent stem cell-derived cardiomyocyte maturation induced by pIC treatment of cardiac progenitor cells (CPCs). **(A):** real-time-qPCR expression of top Notch pathway-associated genes from Figure 3C, *JAG1* and *ALDH1A2*, at the progenitor stage (day 5), earliest cardiomyocytes (days 9 and 11), and later (day 30) using human induced pluripotent stem cell (hiPSC) line DF19-9-11, $n = 3-4$. **(B):** Confocal microscopy with morphology analysis cTnT-immunolabeled hiPSC-derived cardiomyocytes (CMs) from cells treated with indicated conditions, dissociated and cultured for 1 week on 30- μ m-wide Matrigel patterned lanes on polydimethylsiloxane substrate, $n = 6$. Analysis of variance (ANOVA) $p < .001$. **(C):** Optical upstroke velocity measured using the voltage-sensitive dye RH237 (quantified by time required to traverse 10% to 90% action potential amplitude, rise time) from d30 hiPSC-CMs differentiated from primed and untreated CPCs, in the presence or absence of the Notch inhibitor DAPT at times indicated, $n = 17$ individual cells from four passages. ANOVA $p < .001$. **(D):** Top-down mass spectrometry for cTnT isoforms, area-under-the-curve ratio of adult to fetal isoforms for day 30 hiPSC-CMs from primed and untreated CPCs, in the presence or absence of the Notch inhibitor DAPT, $n = 3$. ANOVA $p < .001$. **(E):** Video contractility analysis of hiPSC-CM monolayers from cells treated with indicated conditions. t test or post hoc Holm *, $p < .05$; **, $p < .01$; ***, $p < .001$; hiPSC line, DF19-9-11. Abbreviations: cTnT, cardiac troponin T; pIC, polyinosinic-polycytidylic acid; qPCR, quantitative polymerase chain reaction.

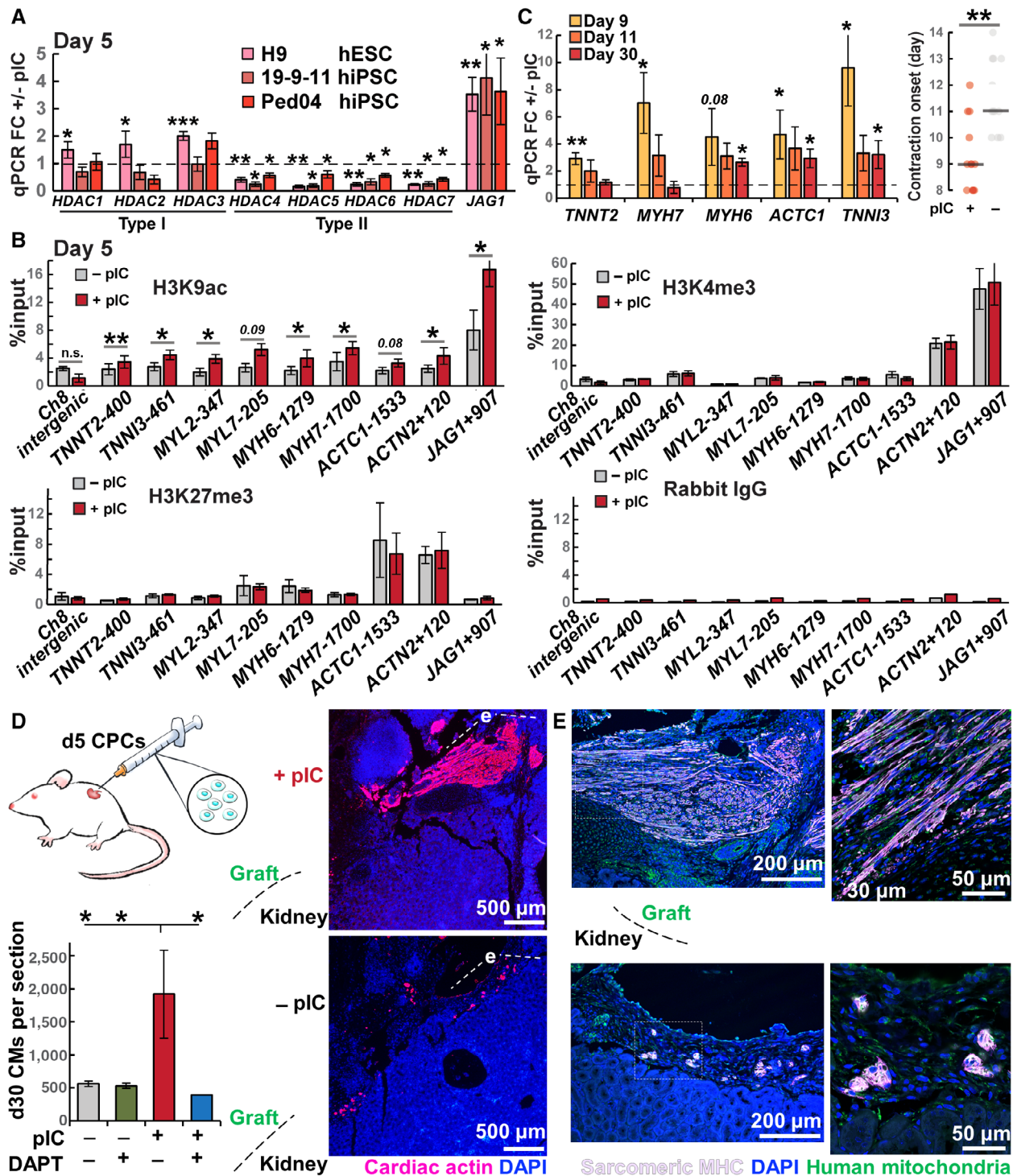


Figure 5. Epigenetic and functional cardiac priming of CPCs by pIC. **(A):** Expression of type I and type II HDACs in pIC primed versus untreated CPCs from three hPSC lines measured by real-time (RT)-qPCR, $n = 3-4$. *JAG1* was upregulated and type II HDACs downregulated in all three lines, confirming consistency of day 5 priming. **(B):** ChIP-qPCR of cardiac myofilament genes, *JAG1*, and chromosome 8 intergenic negative control as raw percent input binding to activating marks H3K9ac and H3K4me3, repressive mark H3K27me3, or rabbit IgG negative control, in hiPSC line 19-9-11, $n = 4$. **(C):** RT-qPCR of myofilament genes for days 9, 11, and 30 cardiomyocytes, fold change +/- pIC-treated CPCs and day of onset of contractions and in hiPSC line 19-9-11, $n = 3$. **(D):** Cardiac actin (AC1-20.4.2) immunostaining of NSGW mouse kidney xenografts day 30, posttransplantation of primed and untreated CPCs from hiPSC line 19-9-11, $n = 3-4$. ANOVA $p < .05$. **(E):** Sarcomeric myosin heavy chain (MF20) immunostaining of areas in **(D)** showing overlap with anti-human mitochondria (113-1) in confocal microscopy. t Test or post hoc Holm *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: CPCs, cardiac progenitor cells; hESC, human ESC; hiPSC, human induced pluripotent stem cell; pIC, polyinosinic-polycytidylic acid; qPCR, quantitative polymerase chain reaction.

Previous studies aiming to advance the maturation of hPSC-CMs have focused interventions on differentiated cardiomyocytes (Table 1) [39], but in this study, we demonstrate that early intervention limited to the CPC stage accelerates cardiac gene expression in CPCs and the subsequent time course of hPSC-CM maturation. These results suggest that pIC treatment of CPCs resets a developmental clock controlling the kinetics of formation of hPSC-CMs and subsequent maturation. Developmental clocks have been described in other mesodermal-derived progenitors to regulate the precisely timed formation of somites and segmentation of the vertebrate body. This segmentation clock results from cyclic activation of Notch, Wnt/ β -catenin, and FGF signaling leading to phasic expression of downstream genes [66]. Species-specific differences in developmental kinetics also suggest developmental clock function in various progenitor populations [24]. In cardiac development, a developmental clock regulating differentiation and maturation of cardiomyocytes has not been well defined, but our results suggest that subsets of early cardiomyogenic and Notch gene expression programs are involved in this function. This is consistent with a recent study demonstrating that loss of *Jag1* disrupts ventricular cardiomyocyte development in the early embryonic heart leading to immature myocardium [60]. Further studies will be needed to define upstream clock mechanisms in cardiac development and how pIC treatment impacts these mechanisms. Whether pIC has similar effects on other tissue-specific progenitors is also an intriguing question for future studies.

Because CPC priming affects cardiac lineage maturation by accelerating the early developmental clock, it is fundamentally different than other described methods for advancing maturation by manipulating differentiated hPSC-CMs (Table 1). Manipulations to hPSC-CMs that have been shown to advance maturation include applying exogenous microRNA or chemical signals to trigger developmental pathways [6–8]; changing media to favor oxidative metabolism [1]; electrical interventions either by pacing or Kir2.1 ion channel overexpression to hyperpolarize the cells [2, 3, 9–13]; changing cardiomyocyte structural environment by mechanical loading, three-dimensional tissue construction, or constraining cell morphology into rod-like shapes [4, 13–20]; and extending cell culture over months [5, 21–23]. As these methods begin only after differentiation of hPSC-CMs, they entail costs of prolonged cell culture relative to CPC priming. As a simple single time-point additive, pIC is less laborious than other manipulations of hPSC-CMs. Early epigenetic CPC priming also provides the opportunity for synergy with later methods for further acceleration of maturation. Finally, certain of these methods, notably creation of structural or electrical microconstructs for cells, may have significant limitations in scalability, which can constrain drug testing and represent a major barrier to clinical applications requiring large numbers of cells.

The most ambitious goal of exogenous cell therapy for the infarcted or failing heart is remuscularization of areas of scar and diseased myocardium with adult-like cardiac muscle, but the best cell type or preparation for this purpose remains unknown. Comparison of cardiomyocyte grafts from neonatal and fetal versus adult rat hearts found superiority of the less mature populations in repairing a rat infarct model [67]. One explanation for such results is that immature cells have greater developmental or epigenetic plasticity. Furthermore, immature cardiomyocytes and CPCs have greater proliferation than mature cardiomyocytes

[68, 69], potentially leading to more successful grafts. These considerations suggest that cells that are developmentally early (such as CPCs) yet primed for mature tissue formation (such as that shown in our heterotopic transplants) may represent a bridge between optimal final graft composition and optimal starting cell sources for exogenous cell therapy of the heart. Priming CPCs with pIC also has potential clinical relevance given that CPCs are the first hPSCs derivative to be tested in a cardiovascular clinical trial [70]. In this regard, pIC is a synthetic, chemically defined polymer that has undergone safety evaluation as an FDA-approved agent for direct use in humans as an adjuvant in chemotherapy or vaccines. This makes priming CPCs with pIC practical to implement for clinical applications relative to other approaches to engineer cells such as genetic modification. Evaluation of the potency of primed CPCs to repair the injured heart is therefore an important future direction not addressed in the present study.

CONCLUSION

Epigenetic priming of hPSC-CPCs with pIC accelerates differentiation and cardiomyocyte maturation in vitro measured by structural, electrical, and metabolic assays. Heterotopic transplantation of pIC-primed hPSC-CPCs results in larger and more mature hPSC-CM grafts. Epigenetic and transcriptional profiling of primed CPCs reveals increased histone acetylation and earlier transcription of Notch and cardiomyogenic genes. This work reveals pathways regulating the time course of cardiac progenitor cell differentiation and cardiomyocyte maturation, providing a novel strategy to rapidly induce cardiomyocyte maturation for research and clinical applications.

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AUTHOR CONTRIBUTIONS

M.B.: conception/design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; W.C., D.L., J.H., L.P., Y.Z., B.N.N., A.M.R., M.E.B., M.T.W., A.S., T.H.: collection and/or assembly of data, data analysis and interpretation; A.C., D.G.I., D.P., T.A.H., W.C.C., W.J.B., A.V.G., Y.G.: data analysis and interpretation; T.J.K.: conception/design, financial

support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

T.J.K. is a consultant for Cellular Dynamics International, a stem cell biotechnology company. The other authors indicated no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Gene Expression Omnibus database [GEO:GSE98941] at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=avylwwgwwtmrlmz&acc=GSE98941>. Accession Numbers: Day 5 CPC RNAseq. Long time course GiWi protocol RNA from days 0–5, 8, 15, 30, and 60 PCBC synapseID: syn2582579 (laboratory of Bruce Conklin, UCSF).

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