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High-content phenotypic assay for proliferation of human iPSC-derived cardiomyocytes identifies L-type calcium channels as targets

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Abstract

Over 5 million people in the United States suffer from heart failure, due to the limited ability to regenerate functional cardiac tissue. One potential therapeutic strategy is to enhance proliferation of resident cardiomyocytes. However, phenotypic screening for therapeutic agents is challenged by the limited ability of conventional markers to discriminate between cardiomyocyte proliferation and endoreplication (e.g. polyploidy and multinucleation). Here, we developed a novel assay that combines automated live-cell microscopy and image processing algorithms to discriminate between proliferation and endoreplication by quantifying changes in number of nuclei, changes in

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AUTHOR CONTRIBUTIONS

LAW, ATP, QDW, and JJS conceived the research. LAW, MD, HA, and LD performed the experiments. LAW, ST, MD, ATP, OE, IB, and MF performed data analysis. ST, PA, MJW, SB, DLB, QDW and JJS contributed to experimental design, data interpretation, and manuscript revision. LAW and JJS wrote the manuscript.

The remaining authors declare no competing financial interests.

Data availability

The data supporting the findings of the study are available in this article, its Supplementary Information files, or from the corresponding author upon request. Data corresponding to treatments named in the manuscript (control, FGF2, BIO, CHIR99021, AR-A014418, verapamil, nitrendipine, felodipine, nimodipine, diltiazem, and cinnarazine) are fully available. Data corresponding to proprietary small molecules unnamed in the manuscript are available with coded compound IDs.

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number of cells, binucleation, and DNA content. We applied this assay to further prioritize hits from a primary screen for DNA synthesis, identifying 30 compounds that enhance proliferation of human induced pluripotent stem cell-derived cardiomyocytes. Among the most active compounds from the phenotypic screen are clinically approved L-type calcium channel blockers from multiple chemical classes whose activities were confirmed across different sources of human induced pluripotent stem cell-derived cardiomyocytes. Identification of compounds that stimulate human cardiomyocyte proliferation may provide new therapeutic strategies for heart failure.

Keywords

Cardiomyocyte proliferation; Phenotypic screen; Human iPSC-derived cardiomyocytes; High-content imaging; L-Type calcium channel blockers

1. INTRODUCTION

Heart failure affects 5.7 million Americans, with a projected 46% increase in prevalence by 2030 [1]. Most pathologies that lead to heart failure, including myocardial infarction (MI) and several cardiomyopathies, cause irreversible loss of cardiac muscle. As a result, current therapies can only slow or reverse limited aspects of cardiac dysfunction [2]. Adult mice and humans exhibit a low ~1% cardiomyocyte (CM) renewal rate [3, 4] with a small amount of increased CM proliferation after myocardial infarction [4] or mechanical unloading [5]. In contrast, newts [6], zebrafish [7], and neonatal mice [8] demonstrate remarkable cardiac regeneration due to dedifferentiation and proliferation of pre-existing CMs [9].

Recent studies highlight the potential for enhancing CM proliferation as a therapeutic strategy. For example, neuregulin 1, FGF1, and p38 MAPK inhibitors enhance proliferation in neonatal and adult rodent CMs, enhance cell cycle entry in mice, and improve cardiac function post-MI [10, 11]. Transient overexpression of the neuregulin 1 co-receptor ErbB2 following MI induces CM cell cycle activity, cytokinesis and reverses cardiac dysfunction in mice [12]. Several other perturbations have also demonstrated increased CM proliferation and cardiac function post-MI, including gradual hypoxia [13] and upregulation of members of the Hippo-YAP pathway [14, 15]. Indeed, there is emerging consensus that CM proliferation underlies endogenous CM renewal and may be stimulated therapeutically for cardiac regeneration [16–18].

These individual examples motivate screens to identify perturbations that may therapeutically enhance CM proliferation. A high-content screen of microRNAs (miRNAs) in neonatal rat CMs identified ~50 miRNAs that increased DNA synthesis, with two miRNAs improving function post-MI [19]. A high-content screen of kinase inhibitors in mouse embryonic stem cell-derived CMs identified four compound clusters that enhanced proliferation, including those that inhibit glycogen synthase kinase 3 (GSK3), p38 MAPK, CaM kinase II, and ERK signaling [20]. However, more comprehensive screens in human induced pluripotent stem cell-derived CMs (hiPSC-CMs) are needed to identify targets and compounds most likely to be effective in clinical trials [21]. Indeed, hiPSC-CMs have been used effectively to develop human disease models and to predict clinical drug responses [22]. While hiPSC-CMs have been used primarily to study cardiotoxicity, electrophysiology

or contractility, they may also provide a powerful platform with which to identify pathways and compounds that drive human CM proliferation [23, 24].

Previous screens for CM proliferation have assayed for DNA synthesis and endpoint counting of the number of nuclei [19, 20, 24, 25]. However, such measurements also reflect other forms of cell cycle activity including multi-nucleation and polyploidy [18, 26]. To address these limitations, we designed a new hybrid live/fixed CM proliferation assay that discriminates between proliferation and endoreplication. We applied this assay to perform secondary screening of top hits from a primary screen for compounds that induce DNA synthesis. Many of these top compounds increase hiPSC-CM proliferation, with limited binucleation or nuclear polyploidy. Among the top performing compounds are clinically-approved and structurally distinct L-type calcium channel (LTCC) blockers.

2. RESULTS

2.1 Discovery of novel compounds that enhance DNA synthesis in hiPSC-CMs

Measures of DNA synthesis are highly sensitive and robust, making them well suited for high-throughput screening to discover potential regulators of proliferation. To identify an initial list of candidates that initiate cell cycle activity in hiPSC-CMs, we performed a high-content screen of 5,094 chemical compounds selected from the AstraZeneca compound collection. This collection includes biologically annotated clinical, pre-clinical, and experimental tool compounds. Compounds were selected from a larger collection based on a combination of criteria including balancing the number of external/internal (known and propriety) compounds, diversity of annotated targets to cover greater than 1,500 biological targets, and known targets associated with cell proliferation. The primary screen employed a liquid handler to plate and culture hiPSC-CMs in 384-well plates before the cells were treated with the library of compounds at three concentrations (0.1, 1.0, and 10.0 μ M) for 48 hours. As shown in Fig 1a, this primary screening assay quantified DNA synthesis via 5-ethynyl-2-deoxyuridine (EdU) incorporation over a 48 hour treatment period. The cells were then fixed, stained, and microscopically scanned using a high-content imaging system. Automated image analysis software was used to segment the nuclei based on the DAPI channel, extract nuclear counts/morphology, threshold the integrated EdU intensity within each nuclei, and determine the fraction of EdU-positive nuclei within each well. The assay used DMSO and FGF2, as our negative and positive controls, respectively (Fig 1b and 1c). To reduce inter-plate variation, the fraction of EdU⁺ nuclei was normalized relative to the on-plate negative (DMSO as 0% of control) and positive (25 ng/ml FGF2 as 100% of control) controls.

The primary screen identified 68 compounds with an average DNA synthesis response greater than that of FGF2 (Fig 1d). On the other hand, 82 of the compounds tested exhibited signs of potential hiPSC-CM toxicity evidenced by reducing the number of nuclei by > 50%. To identify compounds that preferentially induced proliferation in hiPSC-CMs, we used human cardiac fibroblasts to assess 804 of the 5,094 compounds that were included in the initial primary screen (including the 68 compounds with a response greater than FGF2). Cardiac fibroblast proliferation was measured using EdU incorporation and endpoint cell

numbers as in our previous study [27]. 145 of the 804 compounds increased EdU incorporation of human cardiac fibroblast proliferation with EC₅₀ = 14.5 μ M (Fig 1e).

Among the most active compounds (top 1%) that increased hiPSC-CM DNA synthesis in the primary screen were well characterized and clinically used blockers of LTCCs. Cinnarazine, diltiazem, felodipine, nimodipine, nitrendipine, and verapamil are annotated in Fig 1d. These compounds span the dihydropyridine (felodipine, nimodipine, nitrendipine), phenylalkylamine (verapamil), benzothiazepine (diltiazem), and other (cinnarazine) chemical classes [28].

2.2 Microscopic image-based hiPSC-CM proliferation assay

While measures of DNA synthesis are highly sensitive, they are well recognized to have limited specificity for CM proliferation because they encompass other non-mitotic responses, including bi-nucleation and polyploidy [26, 29]. Furthermore, CM proliferation rates are typically low (<10%) which makes counting of cell numbers insufficiently sensitive for screening. Even modest variation in initial cell numbers further obscures changes in cell number in response to treatment. To address these challenges of sensitivity and specificity for hiPSC-CM proliferation, we developed a novel assay that combines automated live-cell microscopy and image analysis to accurately identify changes in the number of hiPSC-CMs. The method simultaneously extracts multiple features related to proliferation including changes in the number of cells, binucleation, and nuclear ploidy events (Fig 2). HiPSC-CMs were treated with fresh compound solutions every 48 hours over 6 days. To track changes in the population, the cells were stained with a low non-toxic concentration of Hoechst 33342 for 1 hour [30]. Using this as a live nuclear stain, the same microscopic field of view was imaged pre- and post treatment. At the end of the assay, the cells were fixed and stained for DAPI, cardiac troponin T, and Ki67, and imaged again. We developed image analysis scripts in MATLAB (see Methods) to extract information relevant to cell proliferation - DNA content, binucleation events, and changes in the numbers of nuclei and cells over 6 days. Description of assay optimization including positive controls and time points is included in the Supplementary Data (Supplementary Fig 1).

This assay was validated using a growth factor and small molecule inhibitors known to induce proliferation in CMs - namely, FGF2 and three GSK3 inhibitors [20, 31, 32]. These treatments have been shown previously to reproducibly induce DNA synthesis and enhance cell cycle progression. Each elicited a proliferation response in our primary screen (Supplementary Fig 1a & 1b). However, our secondary assay was able to further discriminate which treatments increased the number of nuclei and actual number of hiPSC-CMs, not just increased DNA synthesis. While GSK3 inhibitor BIO caused the greatest increases in fraction of EdU⁺ and Ki67⁺ cells, treatment over extended periods caused loss of CMs (Supplementary Fig 1c). The small molecule GSK3 inhibitor CHIR99021 was chosen as the positive control for secondary screening as it induced the greatest increase in number of nuclei and number of CMs over 6 days. CHIR99021 induced comparable levels of proliferation in unlabeled cells (without Hoechst incubation on day 0) counted manually from digital phase contrast and brightfield images (Supplementary Fig 1d & 1e). Manual

counts were strongly correlated with automated counts of the same cells stained with Hoechst at the end of the experiment (Supplementary Fig 1f).

2.3 Performance of top candidate compounds in the cell proliferation assay.

The compounds in the primary screen were rank-ordered based on the hiPSC-CM DNA synthesis response and the top 48 candidate compounds were selected for secondary screening based on: 1) hiPSC-CM DNA synthesis response in the primary screen, 2) lack of proliferation in the cardiac fibroblast counter-screen, 3) diversity of putative compound targets and 4) known effects on signaling pathways and different primary and stem cell populations. Our hiPSC-CM live/fixed assay was used to measure 5-point concentration responses (0.1, 0.3, 1.0, 3.2, and 10.0 μ M) in the change in number of hiPSC-CM nuclei from day 0 to day 6 for these 48 compounds (Fig 3a). We observed reproducible concentration-dependent effects across replicate wells (Supplementary Fig 2). Similar to the primary screen, compounds were scored by normalizing the average change in number of nuclei to the negative (0.16% DMSO) and positive (1 μ M CHIR99021) controls across the five compound concentrations. This secondary screen identified 28 of the 48 compounds that significantly increased the average number of hiPSC-CM nuclei, while four compounds exhibited decreased number of hiPSC-CM nuclei at the highest concentration, presumably due to toxicity (Fig 3b). Calcium channel blockers verapamil and nitrendipine increased the number of hiPSC-CM nuclei (Fig 3c & 3d) and were among the top performers in this secondary screen. To test the robustness of these findings, we tested three chemically distinct classes of calcium channel blockers (diltiazem, nitrendipine, and verapamil) as well as our negative (DMSO) and positive controls (CHIR99021) in hiPSC-CMs from a different commercial source (Ncardia Cor.4U CMs). Similar to the response we observed in the iCell CMs, the calcium channel blockers and CHIR99021 induced DNA synthesis and a concentration-dependent proliferative response in the Cor.4U CMs. (Supplementary Fig 3).

To further assess the importance of the live assay in studying proliferation in CMs, we compared the secondary assay to common end-point proliferation metrics - nuclear counts and EdU incorporation. For the 48 compounds tested in both primary and secondary screens, we compared DNA synthesis at 48 hours assessed by EdU incorporation to our live tracking method that measured changes in nuclear counts over 6 days. While there was moderate positive correlation between these proliferation metrics in the primary and secondary screens ($r = 0.34$), there were multiple more discordant responses that support the complementary nature of these two assays (Supplementary Fig 4a). Tracking the change in numbers produces an output measurement that normalizes endpoint counts to initial counts. This is particularly important since endpoint nuclear counts exhibited a stronger positive correlation with initial plating density (Supplementary Fig 4b). Furthermore, the live tracking assay exhibited decreased coefficient of variation compared to measuring endpoint nuclear counts (Supplementary Fig 4c). Thus, live image tracking increases the sensitivity of proliferation measurements and accounts for typical levels of variability in initial cell plating densities.

2.4 Extracting additional endpoints from the proliferation tracking assay.

Previous studies have indicated that proliferative CMs maintain a mononucleated diploid phenotype [10, 33–35]. Although measuring changes in nuclear counts indicates the cells are

completing karyokinesis, increased nuclear counts could result from either completed mitosis or multi-nucleation without cytokinesis. To address these complicating factors, we developed image analyses to measure nucleation and nuclear ploidy (Fig 4a).

To identify binucleated CMs, cells were stained with DAPI and α -Actinin to distinguish individual cells and neighbors and thereby correctly identify binucleated cells (Fig 4b). In addition, we used a combination of nuclear morphology and inter-nuclear distance measurements to reproducibly and robustly identify binucleated cells (see Methods). This set of parameters was validated by comparison against a set of images in which cells were manually classified. Applying this analysis to our controls showed that there was a significant increase in binucleation rates after treatment with CHIR99021 (Fig 4c).

Nuclear ploidy analysis was performed using a k-means clustering algorithm on the integrated intensities of the DAPI signal to automatically classify nuclei into three ploidy states - 2N, 4N, >4N as shown in Fig 4e (see Methods). While treatment with CHIR99021 increased the number of CMs (Fig 4d), it did not increase the proportion of CMs that were classified as 4N or greater than 4N. As Ki67 positivity had largely returned to baseline by day 6 for all but one compound (Supplementary Fig 5), 4N nuclei were classified as polyploid rather than as cells doubling their DNA content by completing S phase.

The addition of the binucleation and nuclear polyploidy analyses to our assay provides a more comprehensive evaluation of the extent to which compounds induce CM proliferation vs. endoreplication. Of the 48 compounds assessed in the proliferation tracking assay, only 12 compounds significantly increased binucleation compared to the DMSO control condition (Fig 5a). The remaining compounds either did not significantly change or decreased the fraction of binucleated CMs at day 6. Nitrendipine and verapamil were among the 36 compounds that did not increase binucleation. Similar to binucleation, 47 of the 48 compounds in the secondary screen maintained or even increased their fraction of diploid nuclei (Fig 5b). Thus, most compounds that increased the number of nuclei did not significantly increase binucleation or nuclear polyploidy. Nitrendipine significantly increased the fraction of diploid hiPSC-CMs over 6 days, while verapamil did not significantly change the fraction of diploid nuclei compared to baseline conditions.

Applying binucleation analysis to the nuclear count data further enabled assessment of the change in the number of CMs during the proliferation tracking assay. Indeed, many of the compounds that induced increases in the number of nuclei also increased cell proliferation (Fig 6a). Changes in cell number were strongly correlated with changes in number of nuclei in that same condition ($r = 0.98$), with small deviations for those conditions that enhanced binucleation (Fig 6b). Additionally, both calcium channel blockers nitrendipine (rank 3) and verapamil (rank 4) moved up in rank in terms of increase in cell number compared to increase in number of nuclei. Nitrendipine and verapamil both increased the number of CMs while increasing or maintaining the proportion of diploid nuclei and mononucleated CMs. Taken together, these data indicate a novel role of clinically utilized compounds and LTCCs in promoting CM proliferation.

3. DISCUSSION

The ability to enhance proliferation of CMs for therapeutic benefit remains limited, particularly in human cells [23, 36]. In this study, we used high-content screens to identify calcium channel blockers as potential agents to enhance proliferation of human iPSC-derived CMs. We developed a novel high-content proliferation tracking assay that provides automated measurement of changes in number of nuclei, changes in number of CMs, binucleation, and nuclear polyploidy. Automated imaging of living cells provides increased sensitivity and reproducibility for measurement of CM proliferation. Most compounds that strongly induced DNA synthesis increase the number of nuclei and number of CMs. However, several growth factors and small molecules that enhance DNA synthesis did not induce hiPSC-CM proliferation. Multiple clinically used compounds that target LTCCs stimulated CM proliferation. For example, nitrendipine and verapamil induced strong increases in DNA synthesis, number of nuclei and cell proliferation without increasing binucleation or polyploidization.

Previous phenotypic screens for CM proliferation imaged cells fixed at a single endpoint, which is convenient for evaluating a large number of agents. In screens for miRNAs that enhance CM proliferation, Eulalio et al. and Diez-Cunado et al. performed high-content imaging of rat or human CMs immunolabeled for EdU or Ki67 at 48 hr following treatment [19, 37]. Titmarsh et al. performed high content imaging of hiPSC-CMs using Ki67 at 24 h to assess responses to combinations of four agonists implicated in CM proliferation [25]. An alternative approach was taken by Uosaki et al., who measured the number of mouse embryonic stem cell- derived CM nuclei 5 days after treatment with a panel of kinase inhibitors [20]. They confirmed proliferation in a secondary screen of seven inhibitors using flow cytometry. A limitation of such endpoint assays is that the number of cells or nuclei is sensitive to variability in cell seeding density (Supplementary Fig 4). Diez-Cunado et al. addressed this issue by developing a secondary screening assay based on dilution of carboxyfluorescein succinimidyl ester (CFSE), which occurs during cell division [37]. Although CFSE labeling can quantify cell division events, it does not provide additional proliferation information such as binucleation or ploidy events.

Our new proliferation tracking assay followed cells over 6 days using low non-toxic concentrations of Hoechst that allow for time-lapse imaging. This permits delineating a product-precursor relationship for cells undergoing mitosis. Fixing and performing immunofluorescence imaging of the same fields of view from multiple days further enables additional automated analyses of compound-induced CM endoreplication-binucleation and polyploidization. We previously used inter-nuclear distances to detect binucleated CMs as a pre-processing step for automated measurement of cell area [38]. Here we further increased the robustness of this approach by applying morphological operations iteratively, incorporating analyses of nuclear shape, and showing that increases in binucleation of cells could be measured in response to stimuli. In contrast to CMs from adult rodents that primarily are binucleated with diploid nuclei, most adult human CM nuclei are polyploid [3]. Previous studies assessed polyploidy by using flow cytometry based on DNA content of CMs [39, 40], or in some cases of isolated CM nuclei [3]. Here we found that as shown for other cell types [41, 42], wide-field microscopy can be used to quantify CM nuclear DNA

content with distinct modal distributions. Furthermore, automated clustering of DNA content profiles allowed objective assessment of nuclear polyploidy. One advantage of these image analyses is that they can distinguish binucleation from nuclear polyploidy, which is not possible with traditional flow cytometry. To our knowledge the live-cell tracking approach developed here is a novel automated high-content assay to measure change in the number of CM nuclei and change in the number of CMs.

In primary screening we found that six clinically used calcium channel blockers (verapamil, nitrendipine, diltiazem, felodipine, nimodipine and cinnarazine) induced DNA synthesis of hiPSC-CM. Both calcium channel blockers tested in secondary screening (verapamil and nitrendipine) increased hiPSC-CM numbers, nuclei, and did not induce multinucleation or polyploidization. To address variability due to batch or cell source reported in many studies using hiPSC-CMs [43, 44] we confirmed the proliferative responses from three structurally distinct classes of calcium channel blockers (verapamil: phenylalkylamine, diltiazem: benzothiazepine, nitrendipine: dihydropyridine) in a different commercially sourced line of hiPSC-CMs. Despite reported differences in electrophysiology and ion channel gene expression [43, 44], both iCell and Cor.4U hiPSC-CMs responded in a concentration-dependent manner to our calcium channel blockers. The structural diversity of these very well characterized calcium channel blockers across phenylalkylamine, benzothiazepine, and dihydropyridine groups strongly implicates LTCCs as negative regulators of hiPSC-CM proliferation [28, 45].

Given the immature state of hiPSC-CMs, further studies are needed to test whether LTCC blockers enhance adult CM proliferation in vitro and in vivo, which would be aided by the favorable pharmacokinetic properties of these drugs. While LTCCs have not previously been described to regulate CM proliferation, LTCC blockade has been shown to suppress CM differentiation and maturation [46, 47]. Several miRNAs have been reported to regulate CM proliferation [19, 37]. Profiling of miRNA-mRNA interactions in human cardiac tissue identified three miRNA families that target CACN1AC (mRNA for alpha 1C subunit of LTCC), including the mir-30 family [48]. A separate phenotypic screen recently found multiple mir-30 family members that induce hiPSC-CM DNA synthesis and cell division [37].

A variety of related calcium pathways have been linked to aspects of maturation that are relevant to proliferation. Knockout of the alpha1G subunit of T-type calcium channels increases the fraction of mononucleated, diploid CMs [39]. In contrast, intercellular calcium propagation promoted redifferentiation of previously dedifferentiated adult CMs via calcineurin and NFAT [49]. Indeed, CM dedifferentiation precedes proliferation in multiple studies of cultured adult CMs [49, 50], zebrafish [9], and mice [8, 12]. Thus LTCC blockers may enhance hiPSC-CM proliferation in part by promoting dedifferentiation. Further studies are needed to determine whether LTCC blockers enhance hiPSC-CM proliferation by inhibiting downstream calcineurin or CaM kinase II pathways [20, 49].

Verapamil, nitrendipine, diltiazem, felodipine, nimodipine and cinnarazine have all been shown previously to be cardioprotective in acute myocardial infarction or heart failure animal models [51–54]. While several established mechanisms contribute to beneficial

effects of calcium channel blockers post-MI (e.g. reduced mitochondrial calcium overload, hypertrophy and arrhythmia) [55], examination of proliferation markers may be warranted. Clinical trials following acute MI largely found that calcium channel blockers did not reduce mortality or major cardiovascular events, in part due to pharmacokinetics and hemodynamic effects of early generation drugs [56, 57]. The American College of Cardiology and American Heart Association guidelines for the treatment of heart failure classifies calcium channel blockers as Class III: No Benefit (based on data from multiple older randomized clinical trials) for the treatment of heart failure with reduced ejection fraction and Class III: Harm for the treatment of stage B heart failure [58]. Adverse effects observed with first and second generation calcium channel blockers have been primarily attributed to their negative inotropic effect and hypotension. However, meta-analyses including larger and more recent clinical trials with calcium channel blockers have indicated reduction in all-cause mortality and prevention of heart failure compared with placebo [59]. Overall, the complex pleiotropic effects of calcium channel blockers have limited their use to post-MI or nonischemic heart failure patients unable to take front-line therapies such as angiotensin converting enzyme inhibitors or beta blockers.

In summary, this study provides a new high-content assay for tracking proliferation of CMs that will enable future studies to identify mechanisms underlying human CM proliferation and new therapeutic agents for cardiac regeneration. The study also identifies multiple drugs targeting LTCCs that enhance hiPSC-CM proliferation, which suggests that a newer generation of calcium channel blockers that induce proliferation without negative inotropic effects may be useful in the treatment of MI and heart failure.

4. MATERIALS AND METHODS

4.1 Reagents

All compounds used in the primary and secondary screen were supplied by AstraZeneca. The human iCell CMs (Lot# 1290129 and 1294270), iCell plating medium, and iCell maintenance medium were purchased from Cellular Dynamics International (CDI). The human Cor.4U CMs (Lot# CB891CL), Cor.4U complete culture medium, and BMCC serum-free medium were purchased from Ncardia. Williams E medium (A1217601), cocktail B supplement (CM4000), Penicillin-Streptomycin (P/S), 5-ethynyl-2'-deoxyuridine (EdU; A10044), DAPI, Hoechst 33342, AlexaFluor 488 antibody, AlexaFluor 568 antibody, and AlexaFluor 680 antibody, 488 Click-It EdU Imaging Kit were purchased from Life Technologies. α -Actinin antibody (A7811), GSK3 inhibitors BIO (B1686), AR-A014418 (A3230), and CHIR99021 (SML1046) were purchased from Sigma. Human FGF2 (100–18B) was purchased from Peprotech. Human neuregulin 1 (5218SC), p38 MAPK inhibitor SB203580 (5633), and Ki67 antibody (9129) were purchased from Cell Signaling.

4.2 Cell culture

Human iCell CMs were thawed and plated in 384-well plates (Corning #3683) at either 3500 (primary screen) or 2000 (secondary screen) cells/well in iCell plating medium supplemented with 100 units/mL of P/S. After 2 days of culture, the medium was replaced with iCell maintenance medium, and the cells were cultured for an additional 2 days before

beginning treatment. Cells were serum starved for 4 hours and treated in serum-free media composed of William's E medium supplemented with cocktail B.

Human Cor.4U CMs were thawed and plated in 96-well plates coated with 50 µg/mL Fibronectin at 7500 cells/well in Cor.4U complete culture medium supplemented with 100 units/mL of P/S. After 24 hours, the cells were treated with 2 µg/mL Puromycin in Cor.4U complete culture medium for 16 hours. Cells were cultured for an additional 2 days with daily media exchanges before beginning treatment. Cor.4U CMs were serum starved for 4 hours and treated in BMCC serum-free medium supplemented with 100 units/mL of P/S.

4.3 Primary Screen

4.3.1 DNA Synthesis Assay—Cells were treated with compound and 2 µM EdU in serum-free media for 2 days. The compounds were added to the treatment medium in 80 nL using the Echo 550 liquid handler (Labcyte). DMSO (0.16%) and FGF2 (25 ng/mL) were used as the negative and positive controls. After 2 days of treatment, the cells were fixed in 4% paraformaldehyde (PFA) and fluorescently labeled with the Click-It EdU AlexaFluor 488 Imaging Kit and DAPI. Images were acquired using an Arrayscan HCS imaging platform (Cellomics) and a 5× objective. DAPI and EdU-positive cells were counted using Thermo Scientific HCS Studio 2.0 Cell Analysis software.

4.3.2 Cardiac fibroblast counter-screen—Human cardiac fibroblasts were treated with compounds 24 hours after plating at 10 concentrations spanning 0.7 nM to 14.5 µM and with 2 µM EdU 3 days after treatment. On day 9 the cells were fixed in 4% PFA and stained for DAPI to measure total cell counts and EdU using the Click-It EdU AlexaFluor 488 Imaging Kit to measure DNA synthesis. Compounds with an EdU response EC50 less than or equal to the highest concentration (14.5 µM) were considered active in cardiac fibroblasts.

4.4 Secondary Screen

4.4.1 Live Tracking Proliferation Assay—Cells were treated with compounds diluted in serum-free media every 48 hours over the course of 6 days. DMSO (0.16%) and CHIR99021 (1 µM) were used as the negative and positive controls. Prior to treating the cells with compounds on Day 0, the cells were stained with 0.02 µg/mL Hoechst 33342 and imaged on either an inverted microscope (Olympus IX81) with a 10X UPlanSApo 0.4 NA objective or the Operetta CLS high-content imaging system (Perkin-Elmer) using a 10× 0.3NA objective. For images acquired on the Olympus microscope, additional journal scripts were developed in MetaMorph to automatically acquire 2×2 mosaics per well for the entire plate. After 6 days of treatment on Day 6, the cells were stained again with 0.02 µg/mL Hoechst 33342 and the same field of view was imaged as on Day 0.

4.4.2 Immunofluorescence and Imaging—At the end of the experiment, the cells were fixed with 4% PFA and stained with DAPI, monoclonal anti-α-Actinin, and anti-Ki67. The same field of view acquired on Days 0 and 6 were again imaged using the methods described in the previous section. The 2×2 mosaic captured on average 422 ± 78 cells per well.

4.4.3 Imaging Processing—Image analysis scripts were developed in MATLAB to automatically segment and classify the cells. Nuclear segmentation methods adapted from Bass et al were applied to images of nuclei stained with Hoechst 33342 or DAPI [38]. Briefly, a median filter with a window size of three pixels was applied to smooth the images, and the blurred nuclei were segmented using an Otsu threshold. Next, we identified clumps of nuclei by measuring the circularity of the segmented nuclei (<0.75). Objects with a low circularity factor were further separated by first applying an erosion operation followed by a watershed transform. Finally, objects that were outside the range of hiPSC-CM nuclei and those touching the image borders were removed. A mask of these objects was used to measure the integrated intensities of DAPI, Hoechst 33342, α -Actinin, and Ki67. Thresholds for determining whether a nuclei was positive for a label were calculated as a fraction of the standard deviation from the mode of the integrated intensities.

Binucleated cells were identified by dilating and then eroding the segmented nuclei using the same structural element. This sequence of morphological operations merged neighboring segmented nuclei separated by fewer than three pixels. Merged nuclei were classified as binucleated cells.

We used the integrated intensities of the DAPI signal to measure DNA content in the nuclei. Nuclei that had an area greater than three times the interquartile range of the data were filtered out as outliers. To automatically identify 2N, 4N, and greater than 4N nuclei in each condition, k-means clustering with 3 clusters was applied to the integrated intensity measurements. To enhance the robustness of the method to variability in the $> 4N$ population, we then set thresholds based on the centroids of the clusters. The threshold separating the 2N and 4N nuclei was set as the midpoint between the centroids of the 2N and 4N populations; the $> 4N$ population threshold was set by adding half the distance between the 2N and 4N population to the centroid of the 4N population.

4.5 Statistics

All experiments except for the primary screen had at least 3 replicates. The controls for the secondary screen had 36 replicate wells. 200–800 nuclei were counted per well in the secondary screen experiments. Error bars represent standard error of the mean. Statistical significance was determined using 2-sample unpaired Student's t-test, 2-sample Welch's t-test, 1-way ANOVA followed by Dunnett's post-hoc test, or multiple hypothesis testing correction using Benjamini-Hochberg procedure as noted in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Primary screen of hiPSC-CM DNA synthesis

(a) Schematic of primary screening strategy measuring DNA synthesis in hiPSC-CMs. (b) Representative images of hiPSC-CMs treated with 0.16% DMSO (neg. control) or 25 ng/mL FGF2 (pos. control) in the presence of 2 μ M EdU for 48 hours; stained for DNA (DAPI, blue) and EdU (magenta). Scale bar is 100 μ m. (c) Quantitative analysis of (b) ($n = S$, two-tailed Student's t -test). (d) Primary screen of 5,094 compounds measuring DNA synthesis of hiPSC-CMs at three concentrations (0.1, 1, and 10 μ M). The normalized EdU responses were averaged across the three concentrations for each compound. Calcium channel blockers

are highlighted in red: cinnarazine (Cin), diltiazem (Dil), felodipine (Fel), nimodipine (Nim), nitrendipine (Nit), and verapamil (Ver). **(e)** Rank ordering data in (d) of 804 compounds included in a counter-screen for proliferation of human cardiac fibroblasts. Compounds having no change (inactive) or inducing proliferation (active) in fibroblasts are shown in blue and red, respectively. Error bars represent mean $\pm$ s.e.m. * $P < 0.05$.

Figure 2. Hybrid live/fixed assay for hiPSC-CM proliferation
(a) Schematic of secondary screening strategy using live imaging and measuring multiple proliferation-related metrics in hiPSC-CMs. (b) Representative images of hiPSC-CMs treated with 0.16% DMSO (neg. control) or 1 μ M CHIR99021 (positive control) over 6 days. Live imaging tracked changes in nuclei stained with 0.02 μ g/mL Hoechst over six days (columns 1 and 2). On day 6 (column 3), cells were fixed and stained for DNA (DAPI, blue) and α -actinin (green). Scale bar is 100 μ m. (c) Quantitative analysis of (b) (n = 36, Wilcoxon rank-sum test). Error bars represent mean $\pm$ s.e.m. *** P <0.001.

Figure 3. Validation of the top 48 candidate compounds using the hiPSC-CM proliferation assay. (a) 5-point concentration response of hiPSC-CMs treated with each of the top 48 candidate compounds over 6 days. The relative change in the number of nuclei (day 6 / day 0) was normalized to the mean difference between the 1 μM CHIR99021 and 0.16% DMSO control- treated conditions (n = 3). (b) Compounds were ranked by the normalized change in number of hiPSC-CM nuclei, averaged over the 5 concentrations (0.1, 0.32, 1, 3.2, and 10 μM). 28 compounds significantly increased the mean number of hiPSC-CM nuclei compared to the untreated DMSO control (n = 36 for control and n = 3 for compounds; one-sided

Welch's t-test followed by Benjamini-Hochberg multiple testing correction; filled data points indicate false discovery rate (FDR) < 0.05). **(c)** hiPSC-CMs stimulated with 1 μ M CHIR99021, 1 μ M Ver, and 1 μ M Nit significantly increased the number of nuclei over 6 days compared to the untreated DMSO control (n = 36 for control and CHIR99021; n = 3 for Ver and Nit; 1-way ANOVA with Dunnett post-hoc test). **(d)** Representative images of hiPSC-CMs treated with 0.16% DMSO (neg. control), 1 μ M Ver, or 1 μ M Nit over 6 days. Live imaging tracked changes in nuclei stained with 0.02 μ g/mL Hoechst over six days (columns 1 and 2). On day 6 (column 3), cells were fixed and stained for DNA (DAPI, blue) and α -actinin (green). Scale bar is 100 μ m. Data from hiPSC-CMs treated with Nit and Ver are highlighted with red arrows. Error bars represent mean $\pm$ s.e.m. *** P <0.001.

Figure 4. Automated image analysis of CM binucleation, nuclear ploidy, and proliferation
(a) Schematic of extended image analysis pipeline for secondary screen including binucleate and nuclear ploidy analysis. **(b)** Example images of binucleate analysis. Composite images were used to identify binucleated hiPSC-CMs (white arrows) for parameter validation. White outlines represent automated segmentation results of nuclei and red outlines represent segmentation after binucleate analysis. Binucleated hiPSC-CMs are identified by two nuclei that have been merged into a single nuclei. Scale bar is 50 μ m. **(c)** 6-day treatment with 1 μ M CHIR99021 increases binucleation rate compared to 0.16% DMSO control condition (n

= 36; Wilcoxon rank-sum test). **(d)** 1 μ M CHIR99021 treatment induces cell division (n = 36; Wilcoxon rank-sum test). **(e)** Thresholds for nuclear ploidy analysis overlaid on top of the histogram of the integrated intensities of DAPI. Note that the median values for each category are approximately 2 $\times$ the previous category. **(f)** CHIR99021 treatment induces proliferation without increasing nuclear ploidy compared to DMSO control. Error bars represent mean $\pm$ s.e.m. *** P <0.001.

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Figure 5. Binucleate and nuclear ploidy analysis applied to the secondary screen.

(a) Proportion of binucleated cells across top 48 compounds ($n = 3$; Benjamini-Hochberg procedure used on p-values calculated from Welch's t-test; FDR < 0.05). (b) Nuclear ploidy analysis for top 48 compounds. FDR-adjusted p-values were calculated for 2N distribution across 48 conditions compared to the 0.16% DMSO control ($n = 3$; Benjamini-Hochberg procedure used on p-values calculated from Welch's t-test; *FDR < 0.05). Compounds were ordered as in Figure 3b, based on their average change in # of nuclei. Nitrendipine (Nit) and verapamil (Ver) are indicated by red arrows. Error bars represent mean $\pm$ s.e.m.

Figure 6. Compounds in the secondary screen increase the number of cardiomyocytes
(a) Quantifying cell proliferation in the secondary screen using additional binucleate analysis. 30 compounds significantly increased the number of hiPSC-CMs compared to the untreated control ($n = 3$; Benjamini-Hochberg procedure used on p-values calculated from one-sided Welch's t-test; $FDR < 0.05$). **(b)** Live tracking method measuring changes in nuclei counts is strongly correlated ($p = 0.98$) with changes in cell counts. Error bars represent mean $\pm$ s.e.m.