Human Induced Pluripotent Stem Cell-Derived Cardiac Progenitor Cells in Phenotypic Screening: A Transforming Growth Factor-β Type 1 Receptor Kinase Inhibitor Induces Efficient Cardiac Differentiation

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Key Words. Stem cells • Phenotypic screening • Proliferation • Differentiation • Drug discovery • High throughput screening • Assay development

ABSTRACT

Several progenitor cell populations have been reported to exist in hearts that play a role in cardiac turnover and/or repair. Despite the presence of cardiac stem and progenitor cells within the myocardium, functional repair of the heart after injury is inadequate. Identification of the signaling pathways involved in the expansion and differentiation of cardiac progenitor cells (CPCs) will broaden insight into the fundamental mechanisms playing a role in cardiac homeostasis and disease and might provide strategies for in vivo regenerative therapies. To understand and exploit cardiac ontogeny for drug discovery efforts, we developed an in vitro human induced pluripotent stem cell-derived CPC model system using a highly enriched population of KDR pos/CKIT neg/NKX2.5 pos CPCs. Using this model system, these CPCs were capable of generating highly enriched cultures of cardiomyocytes under directed differentiation conditions. In order to facilitate the identification of pathways and targets involved in proliferation and differentiation of resident CPCs, we developed phenotypic screening assays. Screening paradigms for therapeutic applications require a robust, scalable, and consistent methodology. In the present study, we have demonstrated the suitability of these cells for medium to high-throughput screens to assess both proliferation and multilineage differentiation. Using this CPC model system and a small directed compound set, we identified activin-like kinase 5 (transforming growth factor-β type 1 receptor kinase) inhibitors as novel and potent inducers of human CPC differentiation to cardiomyocytes. Stem Cells Translational Medicine 2016;5:164–174

SIGNIFICANCE

Cardiac disease is a leading cause of morbidity and mortality, with no treatment available that can result in functional repair. This study demonstrates how differentiation of induced pluripotent stem cells can be used to identify and isolate cell populations of interest that can translate to the adult human heart. Two separate examples of phenotypic screens are discussed, demonstrating the value of this biologically relevant and reproducible technology. In addition, this assay system was able to identify novel and potent inducers of differentiation and proliferation of induced pluripotent stem cell-derived cardiac progenitor cells.

INTRODUCTION

Myocardial infarction often leads to cardiomyocyte death and subsequent pathological remodeling of the heart involving fibrotic repair, structural changes, and altered electrical activity. This process generates a robust scar that can withstand elevated filling pressures but does not electrically couple or actively contract, a condition that can lead to heart failure and death over time, if left untreated. The only currently available curative treatment of end-stage heart failure is heart transplantation, with other treatment options merely slowing the progression of the disease.

During most of the 20th century, the heart was predominantly viewed as a postmitotic organ that soon after birth lost its ability to generate new cardiomyocytes. This dogma was supported by the apparent lack of regeneration after acute myocardial infarction and the inability to observe dividing cardiomyocytes in histological sections in studies conducted in the beginning of the 20th century [1]. However, recent reports have revealed that the adult mammalian heart is not a terminally differentiated organ and that new cardiomyocytes are generated at measurable rates throughout life, providing support for endogenous regenerative potential.
therapies [2]. Regenerative strategies currently being investigated include cell therapy from exogenous sources, such as stem cell-derived cell types, proliferation of pre-existing cardiomyocytes, and de novo generation of all cardiac cell types from endogenous multipotent stem or progenitor cell populations. During the past 10 years, a number of putative progenitor populations have been identified in the adult heart by many independent groups, often following cells from embryonic development, which is a complex process preserved across species, allowing for later translation from animal models to human models and vice versa [3–9]. For the detection of early cardiomyogenic commitment to the cardiomyocyte lineage, expression of transcription factors such as T-box transcription factor 5 (TBX5), NKX2.5, GATA4, and myocyte-specific enhancer factor 2C (MEF2C) are often used [10]. Despite the debate about the precise identity of progenitor cells in the adult heart, it is clear that cells exist that can play a role in cardiac repair via de novo creation of myocardium [11].

The progenitor cells in the myocardium are capable of differentiating into all the cardiac cell types thought to be necessary for successful cardiac repair, including cardiomyocytes, endothelial cells, and smooth muscle cells, and are therefore a promising cell population for use in identifying molecules that can be used to regenerate heart tissue in situ. However, despite the presence of these cells in vivo, the adult mammalian heart has little regenerative capacity, possibly owing to their rarity in the adult mammalian organism. In addition, owing to their scarcity and the difficulty of isolation, little is known about the molecular mechanisms required for their proliferation and differentiation. CPCs can also be generated from human pluripotent stem cells, because they differentiate into cardiomyocytes, permitting interrogation of relevant human biology [12–15]. One of the methods to isolate these cells is the expression of the cell surface receptor KDR (vascular endothelial growth factor [VEGF] receptor 2) and the lack of CKIT, and these cells are similar to their adult CPC counterparts in that they can generate cardiomyocytes, endothelial cells, and smooth muscle cells [7, 12–16]. In addition, when looking across many of the various populations of CPCs that have been described, Nkx2.5 emerges as a common marker [7, 13, 17]. From these data, human pluripotent stem cell-derived CPCs could be used as a relevant and robust phenotypic screening platform to identify small molecules capable of enhancing CPC proliferation and differentiation to the cardiac lineages. Ultimately, the molecules identified in these screens could be used to enhance the proliferation and differentiation of adult CPCs necessary for in situ regeneration of heart tissue.

**MATERIALS AND METHODS**

**Human iPSC Generation**

Human iPSCs were generated from peripheral blood using nonintegrating episomal vectors, as described previously [25]. In brief, expanded peripheral blood mononuclear cells were transduced with OriP constructs containing Oct4, Sox2, Nanog, Klf4, L-myc, Lin28, and T-antigen, as described previously [25]. The resultant clones were confirmed by morphology (supplemental online Fig. 1A), alkaline phosphatase staining, and TRA-1-81 and SSEA-4 expression (supplemental online Fig. 1B, 1C). The expanded clones were characterized for normal karyotype using G-banded cytogenetic analysis, to be T-cell and B-cell gene rearrangement free, and to confirm loss of episomal vectors, also as previously described [25]. Human primary cells were obtained in vitro from tissue samples from human donors with appropriate written informed consent provided.

**Differentiation of Human iPSCs to CPCs and Cryopreservation**

Human CPCs were obtained from Cellular Dynamics International (iCell Cardiac Progenitor Cells; Madison, WI, http://www.cellulardyamics.com) and manufactured from iPSCs, similar to the protocol previously described by Ma et al. [26]. iPSC aggregates were formed in Essential 8 medium (Life Technologies, Madison, WI, http://www.lifetechnologies.com) containing basic fibroblast growth factor (bFGF; custom; 200 ng/ml), H1152 (1 μM; EMD Millipore, Billerica, MA, http://www.emdmillipore.com), and gentamicin (25 μg/ml; Invitrogen, Life Technologies). Aggregates were cultured in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com) containing 5% or 10% fetal bovine serum (FBS; Thermo Fisher Scientific), H1152 (1 μM), bFGF (200 ng/ml), and gentamicin (25 μg/ml) and were transitioned to medium supplemented with activin A (6 ng/ml) and bone morphogenetic protein 4 (BMP4; 10 ng/ml; Fisher Scientific). After 8 days of culture, dissociated aggregates were cryopreserved.

**Flow Cytometry**

At passage 27, human iPSCs were harvested (0.05% Trypsin/EDTA; Life Technologies) and washed with fluorescence-activated cell sorting (FACS) buffer containing Dulbecco’s phosphate-buffered saline.
saline (DPBS; Life Technologies) and 2% FBS (Thermo Fisher Scientific). The stains were performed in FACS buffer, using the antibodies Dylight 488 conjugated mouse anti-TRA-1-81 (Stemgent, Cambridge, MA, http://www.stemgent.com) and fluorescein isothiocyanate (FITC)-conjugated mouse SSEA-4 (BD Pharmingen, San Diego, CA, http://www.bdbiosciences.com), or the respective isotype controls, Dylight 488-conjugated mouse IgM (Stemgent) or FITC-conjugated mouse IgG3-FITC. For flow cytometry staining of CPCs, the CPCs were harvested at the indicated time points using TrypLE (Thermo Fisher Scientific). For cell surface stains, the cells were washed, and stains were performed in staining buffer containing DPBS and 2% FBS (Thermo Fisher). The cells were stained with the following antibodies: mouse anti-human VEGF R2/KDR phycoerythrin monoclonal antibody (MAb; R&D Systems, Minneapolis, MN, http://www.rndsystems.com), mouse anti-human CD117 allophycocyanin MAb (Life Technologies), rabbit anti-PDGF receptor-α Alexa Fluor 647 MAb (Cell Signaling Technology, Danvers, MA, http://www.cellsignal.com). For intracellular staining, after harvesting, the cells were stained with LIVE/DEAD Fixable Green Dead Cell Stain Kit (Life Technologies), if staining for cardiac troponin T (cTnT) only. The cells were then washed with DPBS and fixed with 4% formaldehyde solution (36.5% formaldehyde solution; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) diluted in DPBS. The cells were then washed in permeabilization buffer (0.1% saponin in staining buffer; Sigma-Aldrich) and stained with primary antibodies in permeabilization buffer containing 1% wt/vol dry milk. The cells were stained with the following antibodies: mouse anti-troponin T, cardiac isoform Ab-1 MAb (Thermo Scientific), rabbit anti-α-smooth muscle actin (Abcam, Cambridge, U.K., http://www.abcam.com). The cells were then washed and stained in permeabilization buffer with the following antibodies: Alexa Fluor 647 donkey anti-mouse and Alexa Fluor 488 donkey anti-rabbit (Life Technologies). The cells were analyzed using a flow cytometer (BD Accuri; BD Bioscience, Ann Arbor, MI, http://www.bdbiosciences.com).

Assay Development

CPC Proliferation

384-Well black imaging plates (Corning, Corning, NY, http://www.corning.com) were coated with human fibronectin (1 mg/ml; Roche Applied Science, Indianapolis, IN, https://www.roche-applied-science.com) for 1 hour at 37°C. The CPCs were thawed and immediately plated at 6,000 per well in basal medium (William’s E Medium; Gibco) with 5% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml gentamicin, 0.1% dimethyl sulfoxide (DMSO) and 0.1% nonessential amino acids (NEAA) as the positive control. For 96-well plates, the cells were plated at 10,000 per well in 25 μl of basal medium. For 96-well plates, the cells were plated at 50,000 cells per well in basal medium or basal medium with XAV939 (10 μM). The T-150 tissue culture flasks were seeded with 17.5 × 10^6 CPCs in basal medium with XAV939 (10 μM). The medium was changed to basal medium every 48 hours for the duration of the experiments. For flow cytometry, the cells were fixed and stained using the methodology described above (flow cytometry). For imaging, the cells were fixed for 20 minutes in formaldehyde on the indicated day, after plating, and stained with mouse anti-cardiac troponin T (Thermo Fisher), rabbit anti-NKX2.5 (Abcam), rabbit anti-α-smooth muscle actin (Abcam), or rabbit anti-CD31 (Abcam) overnight before being counterstained with the following antibodies: Alexa Fluor 488 donkey anti-rabbit and Cy5 donkey anti-mouse (Life Technologies) for 1 hour and Hoechst 33342 for 10 minutes. The plates were imaged on an ImageXpress Micro system (Molecular Devices) at ×4, and the images were quantified using MetaXpress software (Molecular Devices). Thirty-five sites were imaged for each of six wells of each condition or time point. For each site, the percentage of cells positive for Hoechst 33342 and cardiac troponin T or NKX2.5 was quantified using MetaXpress software (Molecular Devices). The averages for each well and condition were determined, followed by the averages and SDs across time points.

Real-Time Polymerase Chain Reaction Assays

Cell samples were collected using lysis solution (nucleic acid purification; catalog no. 4305895; Thermo Fisher Scientific). Total RNA was extracted using a semiautomated prepsystem (6100 Nucleic Acid PrepStation; Thermofisher Scientific). The samples were added to a RNA purification tray (catalog no. 4305673; Thermo Fisher Scientific) and processed using the RNA Cell Method with the RNase Wash protocol, as specified by the distributor. RNA was transcribed to cDNA using high-capacity cDNA reverse transcription kit (catalog no. 4368814; Thermo Fisher Scientific). Quantitative reverse transcription-polymerase chain reaction (PCR) were performed in technical triplicate using a 7900 real-time PCR detection system (Thermo Fisher Scientific) using the TaqMan Gene Expression System (catalog no. 4369016; Life Technologies), with glyceraldehyde-3-phosphate dehydrogenase as the reference gene. Primer sequences are described in supplemental online Table 1.

RESULTS

Isolating Human Cardiac Progenitor Cells

Experimentally accessing cardiac ontogeny and CPC biology requires a model system in which CPCs can be screened for compounds that promote proliferation and/or differentiation to the cardiac lineages (Fig. 1A). CPCs represent one of the earliest commitment steps to the cardiac lineages [27–29] and can be prospectively identified through
relative protein expression patterns, primarily KDR-positive, CKIT-negative, and NKX2.5-low (KDR<sup>pos</sup>CKIT<sup>neg</sup>NKX2.5<sup>low</sup>). Therefore, the time points during CPC differentiation were analyzed to identify an enriched population of CPCs that could be removed from the process, cryopreserved, reanimated, and, ultimately, placed back into the differentiation process within a screening environment. First, to identify mesodermal and CPC formation, differentiating iPSCs were analyzed by quantitative PCR for markers consistent with mesoderm (MESP1 and GATA4) [30, 31] and CPC generation (ISL1 and NKX2.5) [32] (Fig. 1B). MESP1 and GATA4 expression were first detected in the differentiating cultures at day 5 of differentiation and continued to increase to day 8. These were closely followed by increases in ISL1 and NKX2.5 expression. By day 8 of differentiation, we observed a strong expression of genes consistent with CPC emergence. Subsequent analysis of the differentiating cultures for cell surface markers consistent with CPCs [15] showed an enriched (>50%) KDR<sup>pos</sup>CKIT<sup>neg</sup> population by day 8 (Fig. 1C), consistent with the emergence of CPCs according to our quantitative PCR results.

To enable an efficient workflow for large-scale experiments, CPCs were generated at a scale of 9.5 × 10<sup>6</sup> ± 0.3 × 10<sup>6</sup> cells per liter from cells validated as iPSCs (supplemental online Fig. 1), cryopreserved at day 8 of differentiation, and their cardiac competency tested after reanimation. On thawing, these cells were viable (>90%; data not shown) and maintained their KDR<sup>pos</sup>CKIT<sup>neg</sup> profile (Fig. 1C). In addition, when plated into wells of a fibronectin-coated 96-well plate at 15,800 cells per cm<sup>2</sup>, these cells formed adherent monolayers within 24 hours (Fig. 1D). Two days after thawing and plating, markers consistent for CPCs had increased compared with day 8. At that point, the cultures were 85% KDR<sup>pos</sup>CKIT<sup>neg</sup> and 83% KDR<sup>pos</sup>PDGFR<sup>alpha</sup><sup>pos</sup> [14] (Fig. 1E), consistent with a highly enriched population of CPCs. In addition, at this time point, the cultures were enriched for expression of NKX2.5 (>75%) when analyzed using high content imaging (Fig. 1F). The expression of markers used to identify CPCs was consistent across manufacturing batches: 84.8% ± 3.4% KDR<sup>pos</sup>CKIT<sup>neg</sup>, 83.0% ± 2.7% KDR<sup>pos</sup>PDGFR<sup>alpha</sup><sup>pos</sup>, and 76.5% ± 6.0% NKX2.5<sup>pos</sup> cTnT<sup>neg</sup>. These results indicate that isolated CPCs can be enriched and cryopreserved while maintaining their phenotypic profile.

CPCs Differentiate Into Cardiac Lineages Under Defined Conditions

In order to adapt the system for higher throughput screening, protocols for CPC differentiation down the cardiomyocyte lineage were optimized. The CPCs were thawed and plated with XAV939, a small molecule inhibitor of tankyrase 1 and 2 and hence an inhibitor of Wnt signaling, known to promote the differentiation of CPCs to the cardiomyocyte lineage [33]. XAV939 was added to the cultures for 2 days at 0.1, 1, and 10 µM. After incubation for 2 days, the cultures were changed to basal medium alone. The cultures were harvested and analyzed for cTnT expression by flow cytometry 7 days after plating. As expected, XAV939 promoted differentiation of CPCs to the cardiomyocyte lineage in a dose-dependent manner (Fig. 2A).

The duration of XAV939 presence in the cultures was tested and found to be necessary for at least 2 days to optimize differentiation to the cardiomyocyte lineage (Fig. 2B). In addition, the optimal time point for XAV939 addition was determined to be at day 0 (after thawing), because the addition after day 0 showed a dramatic reduction in cardiomyocyte potential when analyzed at day 7 (Fig. 2C). Maintaining XAV939 for longer than 2 days (range, 4–6) might enhance cardiomyocyte differentiation to a greater extent, although this was not rigorously tested. From these data, we have determined that cardiomyocyte differentiation from cryopreserved CPCs can be controlled in a dose-dependent manner with XAV939, thus establishing a regulated screening substrate and phenotype.

The temporal expression patterns of NKX2.5 and cTnT with or without the treatment of XAV939 were then determined using high content imaging. Just as seen in Figure 2D and 2E, with the addition of XAV939, cTnT expression was first detected at day 4 after plating and the fraction of cTnT<sup>pos</sup> cells increases up to day 7 of differentiation. cTnT expression is not detected at high levels at any tested time point without the addition of XAV939 (basal conditions). In addition, conditions containing XAV939 resulted in only 20% more cells than conditions without (48,200 ± 1,100 and 40,000 ± 1,000 cells, respectively). This number was not sufficient to account for the dramatic difference in cTnT expression between the conditions (59.3% ± 1.9% and 0.09% ± 0.02%, respectively). The robust expression of cTnT in cultures treated with XAV939 compared with conditions without yielded Z<sup>‘</sup> values >0.5 when assayed at day 7 of differentiation, demonstrating that the assay is suitable for high throughput screening [34]. The expression of NKX2.5 was also analyzed to determine whether the fraction of cells of cardiac lineage changed with or without the addition of XAV939 (Fig. 2D, 2F, 2G). As seen in Figure 2D, conditions with XAV939 present appeared to have a higher fraction of NKX2.5<sup>pos</sup> cells at days 6 and 7 of differentiation. However, when quantified, this was an increase in the average NKX2.5 intensity per cell (Fig. 2F), consistent in timing with the increasing fraction of cTnT<sup>pos</sup> cells. In addition, the percentage of NKX2.5<sup>pos</sup> cells stayed consistent, at nearly 80%, at each time point assayed and in each condition (Fig. 2G). In total, these data show that the use of the described CPC differentiation assay is suitable for identifying molecules that promote the differentiation of CPCs to cardiomyocytes.

In addition to cardiomyocytes, CPCs are capable of generating other cells of the cardiac lineage, including endothelial and smooth muscle cells. Consistent with native biology, we detected that >10% of the cTnT<sup>pos</sup> population expressed smooth muscle actin (SMA), and 3% of the cTnT<sup>pos</sup> fraction also expressed SMA, when CPCs are cultured in conditions that support cardiomyocyte development (Fig. 2H). In addition, when cultured with bFGF, CD31 can be detected on a large proportion of the culture, indicating endothelial cell development (Fig. 2I). These data demonstrate that the CPCs retain their multipotency for cardiac differentiation.

iPSC-Derived CPCs Have Proliferative Capacity Amenable to Screening

In order to identify compounds that can enhance the proliferative ability of CPCs, we developed a proliferation assay suitable for screening in a 384-well format. As a positive control, we used bFGF [35]. After 3 days of incubation with bFGF, the nuclei number and NKX2.5 expression were measured (Fig. 3A). On stimulation with bFGF, an increase in nuclei number and NKX2.5 expression was observed (Fig. 3A, 3B) compared with the unstimulated basal control. Using 100 ng/ml bFGF, we could generate a reproducible signal-to-noise window (1.5-fold), with a Z<sup>‘</sup> of 0.4 (average across 100 plates; Fig. 3C).

CPCs Have Multilineage Differentiation Capacity

In order to determine multiple phenotypes of interest in a single assay, in this case, differentiation down different cardiac lineages, we developed a high content imaging approach. To optimize for screening, the assay was miniaturized to a 384-well format and semiautomated, with culture conditions set for 2 days of compound dosing as described and 4 additional days of culture (Fig. 4A). This allowed for...
the identification of compounds that induce cardiac differentiation as assessed by cTnT expression (Fig. 4B).

In the conditions developed for screening, we verified that the multilineage differentiation capacity of the cells was maintained. Coupled with the imaging, we examined gene expression at various time points throughout the differentiation process using quantitative PCR and were able to observe an increase in cardiac and smooth muscle genes within 2 days after XAV939 treatment at 10 μM (Fig. 4C). This corresponds to the imaging results, in which expression of both cTnT and α-SMA is seen after stimulation with XAV939 (Fig. 4D; supplemental online Fig. 2).

To demonstrate the capacity of the CPCs to differentiate into endothelial cells, we tested various small molecules and growth factors to enhance differentiation. Stimulation with VEGF alone was sufficient to induce small numbers of the CPCs to become endothelial cells in phenotype, as demonstrated by the expression of CD31 (Fig. 4B).

In the wells treated with a combination of XAV939 (10 μM) and VEGF (100 ng/ml), differentiation down both cardiomyocyte and endothelial lineages was observed (Fig. 4D; supplemental online Fig. 2). We saw an increase in the gene expression of endothelial markers CD31 and KDR after treatment with VEGF, with CD31 expression peaking at day 4 and KDR expression continuing to increase (Fig. 4E–4F). Some CD31 expression was seen early (day 2) with XAV939 treatment, which disappeared at later time points, although low KDR expression was maintained. However, if XAV939 was coupled with VEGF stimulation, CD31 was expressed at all time points (Fig. 4E, 4F).
Screening for Molecules Inducing Cardiomyocyte Differentiation

A small library of 24 compounds (supplemental online Table 2), reported to contribute to the differentiation, dedifferentiation, or proliferation of different cell types, including stem and progenitor cells, was used to establish optimal assay conditions for phenotypic screening. The assay endpoint was the percentage of cells expressing cTnT (Fig. 5A–5F). Total nuclear count was also measured to understand and differentiate compounds that had cytotoxic effects and, in total, four compounds, including XAV939, were identified as inducers of cardiac differentiation. The positive control used was XAV939, as described, which efficiently induced cardiac differentiation by day 6 (Fig. 5D, 5H) with an EC50 value of approximately 2 μM.
Differentiation increased over time, leading to more than 90% induction of cTnT at later time points (days 8–12; data not shown). Additional inhibitors of Wnt pathway signaling were also tested, including KY02111 and the porcupine inhibitor IWP-2 (Fig. 5B, 5C) [36, 37]. Both compounds induced cardiac differentiation, although to different extents compared with XAV939, with a maximum induction of 100% for IWP-2 (Fig. 5J) and 25% for KY02111 (data not shown), using XAV939 as the 100% control. We also observed high levels of cardiac differentiation on treatment of dorsomorphin, an inhibitor of AMP-activated protein kinase (AMPK) and BMP signaling (50% cardiac induction at approximately 1.5 μM; Fig. 5E), which has previously been shown to induce cardiomyogenic differentiation, although this compound resulted in a significant reduction in cell count under these conditions at higher doses (5 and 10 μM), as demonstrated by a lower nuclei count (Fig. 5I) [14]. The assay established is robust and reproducible, with a Z’ value >0.5, a large assay window, and a good signal-to-noise ratio, because the background level of differentiation under basal conditions is low (Fig. 5G).

Identification of Novel Compounds That Induce Cardiac Differentiation

Although the CPC assay was validated with known inducers of cardiac differentiation, the true test of assay utility lies in the identification of unknown compounds that can achieve the phenotype of interest. To that end, an additional small molecule compound library consisting of diverse molecules with annotated mechanisms of action was screened, and novel differentiation-inducing compounds were identified. One interesting finding was the identification of the potent inhibitor of the TGF-β type 1 receptor ALK5 known as RepSox (Fig. 6A) [38]. The efficacy and potency of RepSox was less than we observed for XAV939, reaching 50% efficacy at the top concentration of 10 μM compared with that achieved with XAV939 (Fig. 6D). To build additional support for the inhibition of TGF-β type 1 receptor signaling via the receptor kinase ALK5 in cardiomyocyte differentiation from CPCs, we investigated additional structurally diverse inhibitors of ALK5. If ALK5 is the functional target of RepSox, it was hypothesized that these inhibitors should also display these effects. The small molecule SB-525334 (Fig. 6B) has been reported to be a potent inhibitor of ALK5, and SB-431542 (Fig. 6C) has been documented as a potent inhibitor of ALK4/5/7. Both compounds have been shown to inhibit TGF-β1 signaling in different cellular systems [39, 40]. In addition, both compounds do not inhibit BMP signaling, the mechanism of action for dorsomorphin, and show good selectivity in kinase selectivity screening [40, 41]. The treatment of CPCs with either SB-525334 or SB-431542 induced cardiomyocyte differentiation. However, the extent of differentiation was different for both compounds, with SB-431542 giving 35% cardiac differentiation at a 10-μM compound concentration relative to XAV939. In contrast, SB-525334 was more potent and gave higher efficacy, with a maximal effect of 60% at approximately 5 μM (Fig. 6E, 6F). The different potency and efficacy achieved with the three ALK5 inhibitors could have resulted from a number of factors, including potency of the compound at the target in this cell type, differences in cellular permeability and efflux, and compound stability in the cellular environment or under the assay conditions.
addition, the contribution of modulating other targets cannot be ruled out. However, the finding that three structurally diverse and potent inhibitors of ALK5 are active suggests that this mode of action is playing a significant role in the observed cardiomyogenic activity. These results are consistent with the idea that inhibition of TGF-β signaling via the receptor kinase ALK5 plays a significant role in the cardiomyocyte differentiation mechanism of these compounds. This is the first report of an inhibitor of TGF-β type 1 receptor signaling showing differentiation of human CPCs to cardiomyocytes and highlights the utility of the described assay to identify novel compounds and targets for human cardiomyocyte differentiation.

**DISCUSSION**

The identification of novel compounds that can be used to induce cardiac regeneration is of significant interest; however, a limiting factor has been the identification and validation of a human relevant, robust, and reproducible cellular assay that is amenable for compound hit finding. Using existing knowledge of biological signals that can efficiently guide cardiac differentiation from iPSCs, a population of CPCs was generated and isolated in significant numbers and quality to be suitable for compound screening and drug discovery. We have shown that a population of CPCs can be isolated and maintained during the differentiation process of iPSCs to cardiomyocytes. These cells express established markers, including KDR, PDGFR-α, and NKX2.5 and have been shown to maintain these characteristics with time and freezing. In addition, these cells can be expanded under proliferative conditions and differentiated to cardiomyocytes, endothelial cells, and smooth muscle cells. Assays were developed to identify compounds that could preferentially direct differentiation of the progenitor cells toward cardiac and endothelial lineages, both of which play a critical role in regenerating myocardium after injury.

Using a set of small molecules, we were able to validate a cardiac differentiation screening assay and identify new compounds and targets that influence cardiac differentiation. The assay highlights and supports earlier reports that additional inhibitors of the Wnt signaling pathway, including IWP-2 and KY02111 and dorsomorphin, an inhibitor of AMPK and BMP signaling, can effectively cause differentiation to human cardiomyocytes. However, interestingly, the assay also identified that inhibition of the TGF-β pathway, specifically inhibition of ALK5 with the ALK5 inhibitor RepSox, can increase cardiac differentiation of human iPSC-derived CPCs. Although the importance of TGF-β signaling in cardiogenesis has been previously documented [42], this earlier research focused on cardiomyocyte differentiation from uncommitted mesodermal progenitors in mouse and human embryonic stem cells and the role of the TGF-β type 2 receptor in this process. Specifically, the identified compounds were shown to induce the proteasome-mediated degradation of the cell surface TGF-β type 2 receptor through a ubiquitin-independent mechanism. Although a role for TGF-β signaling in cardiac development has been established, the role of ALK5 is poorly understood. The additional findings that inhibition of ALK5 with the structurally diverse molecules SB-525334 and SB-431542 can also induce cardiomyocyte differentiation of human...
iPSC-derived CPCs provides further support for the importance of TGF-β signaling in this context. SB-431542, the weakest of the three ALK5 inhibitors to induce cardiomyocyte differentiation identified in the present study, has been shown to enhance cardiac differentiation of human iPSCs [14]. However, this was combined with the inhibitor of BMP signaling, dorsomorphin, showing that inhibition of both TGF-β and BMP signaling pathways was required. Hsueh et al. [9] have recently reported that RepSox increased the number of cardiomyocytes derived from endogenous CPCs in old mice, suggesting that high TGF-β1 activity might negatively regulate cardiomyocyte replenishment in vivo in aged hearts. However, whether the increase in CPC-derived cardiomyocytes was due to enhanced proliferation or differentiation of CPCs was not studied. In a more recent publication, Chen et al. [17] also showed that A83-01, an alternative inhibitor of ALK5, could induce both proliferation and differentiation of mouse Nkx2.5+ cardiomyoblasts in vitro. When this compound was administered to mice after myocardial infarction, it also led to an increase in the number of newly formed cardiomyocytes and an improvement in ventricular elasticity and stroke work, leading to improved contractility. These recently reported findings suggest that the compounds and biological targets discovered from the human iPSC-derived CPC assays we have described will also translate to in vivo rodent models of heart failure. This translation from in vitro to in vivo is an important step in understanding and developing novel compounds and mechanisms that will be suitable for the human situation and also demonstrates the power of using these cells and assay system to discover novel mechanisms for cardiac repair. The findings we have reported in the present study are the first to show that inhibition of the ALK5 kinase and inhibition of the TGF-β pathway alone, with the inhibitors RepSox, SB-525334, or SB-431542 causes differentiation of human CPCs to cardiomyocytes, which starts to shed light on potential effects of ALK5 inhibition within a human heart. RepSox and SB-525334 are novel reagents for additional investigations of cardiac differentiation of human relevant cells.

**CONCLUSION**

The use of the described human iPSC-derived CPCs and the disclosed robust phenotypic screening platform can provide opportunities to discover novel biology and molecular agents and build understanding of new molecular pathways and targets important to enhancing CPC proliferation and differentiation to the cardiac lineages. Ultimately, these findings could lead to the identification of regulators of cardiac development, discovery of novel
strategies for endogenous cardiac repair, and potentially yield new therapeutic agents to treat patients.

**AUTHOR CONTRIBUTIONS**

L.D. and Q.-D.W.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; C.K.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; S.P.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; A.J.: collection and/or assembly of data, data analysis and interpretation; A.T.P.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript; S.J.K.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; H.A.: collection and/or assembly of data, data analysis and interpretation; B.A.: conception and design, collection and/or assembly of data, data analysis and interpretation; B.J.S.: conception and design, collection and/or assembly of data, data analysis and interpretation; G.B.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

L.D., S.P., A.J., A.T.P., H.A., Q.-D.W., and G.B. are employees of AstraZeneca. C.K., S.J.K., B.A., and B.J.S. are employees of Cellular Dynamics International.
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