Small-Molecule Inhibitors of the Wnt Pathway Potently Promote Cardiomyocytes From Human Embryonic Stem Cell–Derived Mesoderm

Erik Willems, Sean Spiering, Herman Davidovics, Marion Lanier, Zebin Xia, Marcia Dawson, John Cashman, Mark Mercola

**Rationale:** Human embryonic stem cells can form cardiomyocytes when cultured under differentiation conditions. Although the initiating step of mesoderm formation is well characterized, the subsequent steps that promote for cardiac lineages are poorly understood and limit the yield of cardiomyocytes.

**Objective:** Our aim was to develop a human embryonic stem cell–based high-content screening assay to discover small molecules that drive cardiogenic differentiation after mesoderm is established to improve our understanding of the biology involved. Screening of libraries of small-molecule pathway modulators was predicted to provide insight into the cellular proteins and signaling pathways that control stem cell cardiogenesis.

**Methods and Results:** Approximately 550 known pathway modulators were screened in a high-content screening assay, with hits being called out by the appearance of a red fluorescent protein driven by the promoter of the cardiac-specific MYH6 gene. One potent small molecule was identified that inhibits transduction of the canonical Wnt response within the cell, which demonstrated that Wnt inhibition alone was sufficient to generate cardiomyocytes from human embryonic stem cell–derived mesoderm cells. Transcriptional profiling of inhibitor-treated compared with vehicle-treated samples further indicated that inhibition of Wnt does not induce other mesoderm lineages. Notably, several other Wnt inhibitors were very efficient in inducing cardiogenesis, including a molecule that prevents Wnts from being secreted by the cell, which confirmed that Wnt inhibition was the relevant biological activity.

**Conclusions:** Pharmacological inhibition of Wnt signaling is sufficient to drive human mesoderm cells to form cardiomyocytes; this could yield novel tools for the benefit of pharmaceutical and clinical applications. (Circ Res. 2011;109:360-364.)

**Key Words:** human embryonic stem cells ■ Wnt inhibitors ■ cardiogenesis ■ small molecules

Heart disease often leads to cardiomyocyte death and pathological remodeling, and the only replacement option is heart transplantation, but its clinical complexity and the limited number of donors have prompted research into stem cell–based alternatives. Stem cell–based approaches include both cell transplantation and mobilization of an endogenous stem and progenitor pool, which show promise for therapeutic regeneration. Development of stem cell–based replacement therapies, however, is limited by incomplete understanding of the factors that drive differentiation of cardiomyocytes from either human embryonic stem cells (hESCs) or endogenous cardiac stem and progenitor cells. Therefore, we sought to develop high-throughput screens for simultaneous testing of small molecules for cardiogenic potential in order to identify cellular signals that are required or need to be inhibited at different stages of cardiac development. Moreover, such a chemical biology approach would generate small-molecule tools to improve the yield in hESC differentiation protocols and could even result in drug leads for cardiac regeneration should they target hESC-derived progenitors that resemble adult cardiac progenitor cells. Here, we describe the development and implementation of an hESC-based assay that probes the signals that drive the conversion of platelet-derived growth factor receptor-α (PDGFR-α)−mesoderm posterior 1 homolog (MESP1)−cardiogenic cells to cardiomyo-
cytes, as well as one of the hits that arose from screening pathway modulator libraries.

**Methods**

MYH6-mCherry hESC were propagated as described previously and were differentiated with growth factors as outlined in Figure 1A. For high-content screening (HCS), day 4 embryoid bodies (EBs) were dissociated and plated in the presence of small molecules. For analysis, red fluorescence from the cardiac-specific MYH6 reporter was imaged with a high-throughput microscope and was quantified with an image analysis software package.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Results**

To develop an HCS assay for small molecules that drive mesoderm to differentiate into cardiomyocytes in hESCs, we first profiled cardiac marker expression during differentiation in EBs (Figure 1A, upper half), when cardiac-inducing signals are provided to mesodermal cells by closely juxtaposed endodermal and potentially other cell types present in early embryos (Online Figure I). Mesoderm was induced in EBs by addition of activin A and bone morphogenetic protein 4, which resulted in mesoderm induction between days 1 and 4 (Figure 1A, blue full line), and cardiomyocytes appeared spontaneously from day 6 onward (Figure 1A, red dashed line).

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>53AH</td>
<td>structural analog of IWR-1</td>
</tr>
<tr>
<td>EB</td>
<td>embryoid body</td>
</tr>
<tr>
<td>HCS</td>
<td>high-content screen</td>
</tr>
<tr>
<td>hESC</td>
<td>human embryonic stem cell</td>
</tr>
<tr>
<td>InhibitorSelect</td>
<td>small-molecule collection of 244 kinase inhibitors</td>
</tr>
<tr>
<td>IWR</td>
<td>inhibitor of Wnt response, IWR-1</td>
</tr>
<tr>
<td>IWP</td>
<td>inhibitor of Wnt production, IWP-3</td>
</tr>
<tr>
<td>StemSelect</td>
<td>small-molecule collection of 305 pathway modulators</td>
</tr>
<tr>
<td>XAV</td>
<td>inhibitor of tankyrase, XAV939</td>
</tr>
</tbody>
</table>

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**Figure 1. A high-content screen for cardiogenesis in hESCs identifies a Wnt inhibitor.**

A, In EBs, after treatment with the indicated growth factors, mesoderm was induced efficiently from days 1 to 4 (assessed by mRNA; upper half, blue full line), and cardiomyocytes occurred spontaneously from day 6 onward (assessed by mRNA; upper half, red dashed line). For the HCS assay, day 4 EBs were dissociated, plated, and treated with molecules or growth factors as depicted in the lower half of panel A. B, Day 4 cells reached maximum mesoderm levels and were further characterized for early cardiac markers by reverse transcription quantitative polymerase chain reaction for MESP1 and for PDGFRα by flow cytometry (average ±SD; n=3). C and D, HCS assay was screened against a collection of 244 kinase inhibitors (InhibitorSelect; C) and a collection of 305 pathway modulators (StemSelect; D) in 3 concentrations (0.3, 1, and 3 μmol/L). All compounds with a z score greater than 2 are shown for the StemSelect library (D). E, Fluorescence image of cells expressing the MYH6-mCherry reporter under vehicle- or IWR-treated cultures. Bmp4 indicates bone morphogenetic protein 4; bFGF, basic fibroblast growth factor; Vegf, vascular endothelial growth factor; SFM, serum free media; and T3, triiodothyronine.
Because mesoderm induction appeared to be maximal at day 4, we further characterized the day 4 population for the early cardiac markers MESP1 and PDGFR-α (Figure 1B). Because this population appeared to be highly enriched for both markers, we developed an HCS assay that would probe these cells and determine what signals would drive differentiation toward cardiomyocytes (Figure 1A, lower half). HCS assays are image-based and have better dynamic range and sensitivity than traditional plate-reader–based assays. Day 4 EBs were collected and dispersed into 384-well plates and subsequently exposed to 3 concentrations (0.3, 1, and 3 μmol/L) of 2 small collections of well-characterized pathway modulators, namely, approximately 244 protein kinase inhibitors (InhibitorSelect; EMD Millipore, Billerica, MA) and 305 pathway agonists and antagonists (StemSelect; EMD Chemicals). In the InhibitorSelect collection, no compound induced cardiogenesis (Figure 1C), whereas in the StemSelect collection, 1 extremely potent hit was identified (Figure 1D). The hit IWR-1, a recently published inhibitor of the canonical Wnt signaling response (IWR),6 induced beating foci from monolayer cultures, whereas none were observed in dimethyl sulfoxide conditions (Figure 1E; Online Movies I and II). Inhibitors of endothelial growth factor, vascular epidermal growth factor, transforming growth factor-β, insulin, and Sonic hedgehog signaling did not result in increased cardiogenesis, which suggests that Wnt inhibition alone is sufficient for cardiac induction (Online Table I). Moreover, agonists of the Wnt, vascular endothelial growth factor, and Sonic hedgehog pathways failed to promote cardiac fate (Online Table I).

Further characterization revealed that maximal cardiac induction by IWR occurs when IWR is added from day 4 to day 5, with cardiac induction levels decreasing as the treatment window is shifted up toward day 10 of differentiation (Figure 2A). The EC50 to induce cardiogenesis at the day 4 time window was 2241 nmol/L (Figure 2B). Flow cytometry analysis demonstrated that IWR yielded up to 30% cardiomyocytes, a 200-fold increase over vehicle-treated cultures (Figure 2C). Reverse transcription quantitative polymerase chain reaction analysis was performed to study the effect of
IWR on mesoderm, cardiac progenitors, and mesoderm derivatives such as cardiomyocytes, endothelial cells, and smooth muscle (Figure 2D). The mesoderm-specific gene T/BRACHYURY had a downward tendency as soon as 24 hours after IWR exposure, although this was not statistically significant on most days (Figure 2D). The earliest markers of cardiac fate, MESP1 and kinase insert domain receptor, were not affected in the first few days after IWR treatment, which suggests that IWR drives MESP1+/kinase insert domain receptor− cardiac mesoderm cells toward cardiomyocytes (Figure 2D). MEF2C and NKX2.5, 2 cardiac transcription factors, as well as the cardiomyocyte structural genes MYH6, ACTN2, and TNNT2, were increased dramatically by IWR (Figure 2D). Further characterization of other mesodermal lineages revealed smooth muscle lineages were not increased (ACTA2 and TGLN) and endothelial cell markers (CD31 and kinase insert domain receptor) even decreased. Flow cytometry confirmed a >50% reduction in CD31+ cells (Online Figure II).

We next questioned whether other inhibitors of the Wnt pathway were also able to induce cardiogenesis in this assay (Figure 3A). We tested 3 more small-molecule inhibitors of Wnt signaling: (1) the PORCN inhibitor IWP-3 (IWP)6; (2) the more potent IWR-1 analog 53AH; and (3) the tankyrase inhibitor XAV939 (XAV), which is cardiogenic in mouse embryonic stem cells.7 All 3 compounds promoted cardiogenesis, with 53AH having an EC50 below the micromolar range (Figure 3B). Because EC50 measurements are not indicative of the efficacy of each compound, we ran maximal effect doses of each compound, as well as the biological Wnt inhibitor DKK1 (Dickkopf-related protein 1), per comparison to evaluate their maximum efficacy in terms of cardiogenesis. IWR and its analog 53AH were the most efficacious, followed by IWP and XAV, and any of the small-molecule inhibitors were at least 40 times more effective in inducing cardiogenesis than DKK1, a purified recombinant Wnt inhibitor protein (Figure 3C).

Discussion

Definition of the pathways that control stem cell cardiogenesis is important to efficiently derive cardiomyocytes from human pluripotent stem cells and might be useful to mobilize endogenous cardiac stem cells. To discover new molecules and pathways that would direct cardiac differentiation and/or regeneration, we developed an hESC-based HCS assay that allows small-molecule screens in serum-free conditions, on a scale that has not been reported for any hESC-based assay.

We prepared cardiogenic mesoderm (platelet-derived growth factor receptorα−MESP1+ cells) in bulk EB culture (Figure 1B) and dispersed and plated the cells in a monolayer with the intent of identifying small molecules that are able to replace the natural signals provided in the 3-dimensional EB, thus yielding insight into signals that direct mesodermal progenitors to form cardiomyocytes.

By screening approximately 550 pathway modulators, we identified a small-molecule inhibitor of the β-catenin−dependent canonical Wnt pathway. Although it was not entirely unexpected that a Wnt inhibitor would be identified as a
cardiac inducer, given that the natural Wnt inhibitor DKK1 is capable of directing cardiogenesis in Xenopus and hESCs, the present study showed that Wnt inhibition was sufficient to drive the hESC-derived mesoderm to a cardiac fate in the absence of other signaling modulators, and no other inhibitors had comparable activity (Online Table I). Moreover, Wnt inhibition drove cardiomyocytes specifically and did not increase other mesoderm lineages, which suggests that Wnt inhibition specifically drives a mesoderm progenitor toward cardiomyocytes (Figures 2D and 2E).

To further explore inhibition throughout the Wnt pathway, we evaluated structurally diverse Wnt inhibitors that target different cellular components of the pathway (Figure 3D). Importantly, all of the small molecules were much more cardiogenic than DKK1 (Figure 3C). The most interesting finding, however, was that IWP, an inhibitor that prevents cells from producing Wnt, was also potent, which revealed that endogenous Wnt activity must be blocked. This is an important finding, because it suggests not only that exogenous Wnt signals need to be inhibited to direct the formation of cardiomyocytes from a mesoderm progenitor. In summary, HCS of an hESC-based assay identified Wnt inhibition as critical for cardiogenesis. Active compounds function by blocking Wnt secretion and stabilize Axin to destabilize β-catenin. The present data further suggest that endogenous Wnt signals need to be inhibited to direct the formation of cardiomyocytes.

**Acknowledgments**

The authors would like to thank Dennis Schade for functional confirmation of hits in a Wnt assay and critical reading of the manuscript.

**Novelty and Significance**

**What Is Known?**

- Human embryonic stem cells (hESCs) show great promise as a source for generating myocardial cells for use in cell transplantation therapies.
- hESCs form cardiomyocytes if they are treated appropriately; however, the yield is typically low, because the mechanisms that drive hESC toward a cardiac myocyte phenotype are poorly understood.

**What New Information Does This Article Contribute?**

- We developed an hESC-based high-throughput small-molecule screen assay using a cardiac myocyte-specific fluorescent reporter to identify molecules that drive hESCs to cardiac myocytes.
- Small-molecule inhibitors of the Wnt signaling pathways were identified as potent inducers of cardiac myocytes. They increased cardiac myocyte yield dramatically over recombinant protein inhibitors. Many other pathway modulators were inactive. Thus, Wnt inhibition is uniquely important for directed cardiogenesis.

The development of a high-throughput assay for hESC cardiogenesis was deemed essential because it allowed simultaneous probing of the many signals that may drive hESCs to cardiac myocytes. Screening of more than 500 pathway modulators demonstrated that inhibition of the Wnt pathway alone was sufficient to drive cardiac cell formation specifically and did not induce other mesodermal derivatives, such as endothelial or smooth muscle cells. Small-molecule inhibitors of the Wnt pathway are thus useful tools for increasing cardiac myocyte yield. These molecules have great potential benefits for clinical and pharmaceutical applications.
Small-Molecule Inhibitors of the Wnt Pathway Potently Promote Cardiomyocytes From Human Embryonic Stem Cell–Derived Mesoderm

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doi: 10.1161/CIRCRESAHA.111.249540

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Supplement Material

Extended materials and methods

*Human embryonic stem cell culture and embryoid body differentiation assay*

Human embryonic stem cell (hESC) H9 lines carrying MYH6-mCherry reporters were routinely maintained as described previously. In brief, cells were grown on a mouse embryonic fibroblast coated matrigel plate in Knock Out DMEM (Gibco) media supplemented with 20% Knock Out Serum Replacement (GIBCO) and 8ng/ml bFGF (Sigma). For embryoid body (EB) differentiation, hESC were mechanically passaged onto gelatin-coated dishes preseeded with mouse embryonic fibroblasts. After 4 days hESC colonies were lifted off with a 10 minute 1mg/ml collagenase IV treatment (GIBCO) and directly differentiated in StemPro 34 (GIBCO) with addition of growth factors as depicted in Figure 1A. hrActivin A, hrBMP4 and hrDKK1 were from R&D Systems, and VEGF from the Biological Research Bank NCI-Frederick. XAV939, a tankyrase inhibitor, was purchased from Selleck Chemicals. At day 4 of differentiation, EB were plated onto gelatin-coated dishes. Media changes were done at days 1, 5, 7, 10, 12 with StemPro 34 and the indicated growth factors unless indicated otherwise.

*High content screening assay*

For the screening assay, EB were grown as described above until day 4 and were dissociated gently to single cells with TrypLE (GIBCO). Single cells were then transferred in gelatin coated 384-well optical plates (Greiner Inc.) in StemPro 34 with 5ng/ml FGF at a seeding density of 250000 cells/cm². StemSelect Small Molecule Regulator and InhibitorSelect Protein Kinase Inhibitor libraries (EMD/Millipore) were transferred to a final concentration of 0.3, 1 and 3µM using an Echo 555 liquid dispenser (Labcyte Inc.). At day 10, media with compound was removed and exchanged for a serum free media (SFM) that is described elsewhere. T3, the thyroid hormone analog triiodothyronine (Sigma), was added at day 10 to increase the red signal driven by the MYH6 promoter for more reliable imaging. For RT-qPCR analysis T3 treatment was omitted to avoid interference with the biological mRNA expression of MYH6. Media was changed to PBS at day 14 and red fluorescence was imaged on an InCell 1000 (GE Healthcare) high throughput microscope. For the quantification of cardiac induction, image analysis was performed with the Cyteseer Image Analysis Software package (Vala Sciences Inc.) by measuring the total area and intensity of the MYH6-mCherry reporter in each well. The MYH6-expression levels indicated in the graphs represent the multiplication of the total area and intensity of red fluorescence.

For toxicity assessment we engineered the hESC cell line for screening with a H2B-GFP reporter, allowing quantification of the cell number. Using Cyteseer, we quantified total GFP signal in each well. Wells with a GFP reduction of 20% or more (relative to vehicle only controls) were flagged for toxicity and were manually confirmed under a bright field microscope for evidence of cell death. Toxicity was consistent across replicates, and doses that caused toxicity were removed from the dataset displayed in the figures.
Flow cytometry

Day 4, day 14 or day 16 cultures from the screening assay were harvested and dissociated with TrypLE or enzyme free cell dissociation buffer (GIBCO) for receptor analyses. mCherry protein was read directly and for receptor analysis, cells were stained live using a PDGFRα antibody (kind gift from W. Stallcup) coupled to a secondary APC labeled antibody (Invitrogen) and a directly labeled CD31 antibody (eBioscience). A PBS suspension of single cells was run on a LSRFortessa or FACSCanto Flow cytometer (BD Biosciences) and data was analyzed with FlowJo (Treestar Inc.).

Reverse transcription quantitative PCR (RT-qPCR)

Samples were collected in Trizol (Invitrogen) at the indicated time points. Total RNA was extracted according to the manufacturers instructions and 1µg was treated for DNA removal and converted into cDNA using the Quantitect Reverse Transcription kit (Qiagen). SYBR Green qPCR was run on a LightCycler 480 (Roche) using the LightCycler 480 SYBR Green Master I Kit (Roche). Data was analyzed using the ddCt method, using a stable reference gene as normalization control, which was determined as described previously 6. Primers were designed to have an efficiency of 95% or more and sequences are listed below:

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene bank accession</th>
<th>Marker for</th>
<th>Sequence</th>
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<tr>
<td>ACTA2</td>
<td>NM_001613</td>
<td>Smooth muscle</td>
<td>F: CAGGGCTGTTCATCCATGTCC&lt;br&gt; R: GCCATGTTCTATCGGTTACTTC</td>
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<td>NM_001102</td>
<td>Cardiomyocytes</td>
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<td>T</td>
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<td>Mesoderm</td>
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<td>CD31</td>
<td>NM_000442</td>
<td>Endothelial cells</td>
<td>F: AACAGTGGACGATAGGAGGCC&lt;br&gt; R: TGAAAACAGCAGTCATCCTT</td>
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<tr>
<td>KDR</td>
<td>NM_002253</td>
<td>Endothelial cells/cardiogenic mesoderm</td>
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<tr>
<td>MEF2C</td>
<td>NM_002397</td>
<td>Cardiac mesoderm</td>
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<td>MESP1</td>
<td>NM_018670</td>
<td>Cardiogenic mesoderm</td>
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<td>Cardiomyocytes</td>
<td>F: CAGAGCAGGAAATGGAGAG&lt;br&gt; R: TTCGATTCTCTTCCAGGAGA</td>
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**Statistical analysis**

For the screens, Z scores were calculated using the fluorescence readout for each concentration normalized to DMSO treated wells. Integrated Z scores over all concentrations were calculated by adding the Z scores of the 3 concentrations tested. Any compound with an integrated Z score higher than 6 was assumed a hit.

Data standardization and statistical analysis for RT-qPCR was performed as described\(^7\).

Dose curves and EC\(_{50}\) values were obtained with the Prism statistical analysis package (GraphPad Software Inc.) using the (log)agonist vs. normalized response (variable slope) equation. Data used for dose curves consisted of quadruplicate reads for three biological dose response (24 points, 1nM to 10000nM) experiments and were normalized to the compound concentration with the highest induction level (designated as 100%). Toxic doses were excluded from analysis.
References for materials and methods


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5. Kucharova K, Stallcup WB. The ng2 proteoglycan promotes oligodendrocyte progenitor proliferation and developmental myelination. *Neuroscience*. 2010;166:185-194


Supplementary Figures

Online Figure I: Time course gene expression analysis of EB differentiated in serum free conditions as outlined in Figure 1A. Markers included are for mesoderm (T, BRACHYURY), cardiogenic mesoderm (KDR and MESP1), cardiac mesoderm (NKX2.5) and cardiomyocytes (TNNT2 and MYH6).
Online Figure II: (A) Example of flow cytometric analysis of day 16 cells using CD31 as a marker for endothelial cells in DMSO and IWR treated samples. (B) Histogram plot reflecting average and standard deviation of six experiments. Asterisk indicates a p-value < 0.001.
Supplementary file legends

Supplementary Movie 1: Bright field recording of beating cardiomyocytes (day 14) that arise after treating with the Wnt inhibitor IWR-1.

Supplementary Movie 2: Recording of the MYH6-mCherry reporter in beating cardiomyocytes (day 14) that arise after treating with the Wnt inhibitor IWR-1.

Online Table I: Overview of all specific pathway modulators screened in the described assay. The table lists both inhibitors and agonists, with the compound name as found in the EMD/Calbiochem Catalog, the % induction (normalized to IWR), the primary target of the compound and the affected signaling pathway.