A Nodal-to-TGFβ Cascade Exerts Biphasic Control Over Cardiopoiesis

Wenqing Cai,* Rosa M. Guzzo,* Ke Wei, Erik Willems, Herman Davidovics, Mark Mercola

Rationale: The transforming growth factor-β (TGFβ) family member Nodal promotes cardiogenesis, but the mechanism is unclear despite the relevance of TGFβ family proteins for myocardial remodeling and regeneration.

Objective: To determine the function(s) of TGFβ family members during stem cell cardiogenesis.

Methods and Results: Murine embryonic stem cells were engineered with a constitutively active human type I Nodal receptor (caACVR1b) to mimic activation by Nodal and found to secrete a paracrine signal that promotes cardiogenesis. Transcriptome and gain- and loss-of-function studies identified the factor as TGFβ2. Both Nodal and TGFβ induced early cardiogenic progenitors in embryonic stem cell cultures at day 0 to 2 of differentiation. However, Nodal expression declines by day 4 due to feedback inhibition, whereas TGFβ persists. At later stages (days 4–6), TGFβ suppresses the formation of cardiomyocytes from multipotent Kdr<sup>+</sup> progenitors while promoting the differentiation of vascular smooth muscle and endothelial cells.

Conclusions: Nodal induces TGFβ, and both stimulate the formation of multipotent cardiovascular Kdr<sup>+</sup> progenitors. TGFβ, however, becomes uniquely responsible for controlling subsequent lineage segregation by stimulating vascular smooth muscle and endothelial lineages and simultaneously blocking cardiomyocyte differentiation. (Circ Res. 2012;111:876-881.)

Key Words: Nodal ■ Cripto ■ TGFβ2 ■ Kdr ■ cardiogenesis

Embryonic stem cells (ESCs) and induced pluripotent stem cells hold great potential as sources of cardiomyocytes and as models to understand how cardiomyocytes, vascular smooth muscle, and endothelial cells arise from common cardiopoietic progenitors. Defining the signals that control cardiopoietic differentiation will be important for numerous applications, including regenerative medicine.

The divergent transforming growth factor-β (TGFβ) family member Nodal is critical for the formation of the heart and other visceral organs. Nodal activates a heteromeric complex of type I [Acvr1b (Alk4) or Acvr1c (Alk7)] and type II (Acvr2a and b) serine/threonine kinase receptors, leading to phosphorylation of Smad2 and Smad3, which then activate target genes. Mouse embryos lacking Acvr1b, Smad2, or Nodal, and double knockout of the 2 type II receptors (Acvr2a and Acvr2b) fail to gastrulate or form mesendoderm. Genetic deletion of Cripto, an essential Nodal co-receptor in most contexts, is less severe, such that embryos form mesendoderm but are severely deficient in cardiogenic progenitor cells. The cardiogenesis deficit inherent in Cripto<sup>−/−</sup> ESCs can be rescued either by incorporation into chimeric (Cripto<sup>−/−</sup>:wild-type [WT]) embryos or by a constitutively active mutant human ACVR1b receptor, demonstrating the existence of yet unknown paracrine effectors that propagate the signal from cell to cell.

We used murine ESCs (mESCs) to model cardiogenesis and found that TGFβ2 is induced by Nodal and propagates the cardiogenic signal. The essential nature of TGFβ for...
cardiogenesis is based on resistance to the feedback inhibitors Lefty1, Lefty2, and Cerberus1 (Cer1) that block Nodal. Consequently, both Nodal and TGFβ induce early cardiogenic progenitors, but Nodal expression declines due to feedback inhibition whereas TGFβ expression persists in Kdr–/– cardiopoietic precursors. In this population, TGFβ suppresses cardiomyocyte differentiation while promoting vascular smooth muscle and endothelial cell formation. Thus, a Nodal-to-TGFβ cascade, including feedback inhibition, provides biphasic control over cardiopoietic cell fate.

Methods

Protocols and primer sequences are described in the Online Data Supplement.

Results

Cardiogenic Rescue Implicates a Diffusible Factor Downstream of Nodal/Avcr1b

Cripto–/– mESCs are deficient in production of cardiogenic progenitors, exhibiting low Kdr and Mesp1 expression,4,5 (Online Figure IA) and are thus ideal for a cell-mixing study to identify paracrine factors that initiate cardiogenesis downstream of Nodal/Avcr1b (Figure 1A). A constitutively active human ACVR1b receptor (caACVR1b) was stably introduced into Cripto–/– mESCs to activate downstream signaling (Cripto–/– caACVR1b, inducers) (Online Figure IA). Coculture (Figure 1A and 1B) of these cells dramatically restored Kdr and Mesp1 expression in eGFP-labeled Cripto–/– mESCs (responders) (Figure 1C). Coculture also increased the number of Kdr–/– cells among the responder (eGFP–/–) population, from 3.34 ± 0.06% to

Figure 1. Paracrine signaling downstream of Nodal. Schematic (A) and images (B) of the cell-mixing experiment. Kdr and Mesp1 expression (C) and proportion of Kdr–/– cells (D) in FACTS-isolated populations from cocultures. Mesp1 expression in FACTS-isolated Kdr–/–/GFP–/– cells (E). Note induction by coculture. F, Myh6, Mef2c, and Tbx5 in FACTS-isolated populations from cocultures; representative of >3 trials (Online Figure II). G and H, Cell-nonautonomous signaling induced cardiogenesis. Schematic of the experiment (G) and representative confocal image of Myh6-mCherry reporter (H). Online Movie II shows multiple optical planes. I through K, Coculture from day 0 to day 5 is sufficient for cardiogenesis in responder cells. L, Schematic of experiment. Quantitative real-time reverse transcription PCR analysis of Myh6 expression (J) and image of Myh6-mCherry reporter (K) after 10 days of iso-culture. *P < 0.05, unpaired Student t test. Error bars indicate SEM; n = 3.
21.28 ± 1.37% after 5 days (Figure 1D). FACS-isolated GFP+ Kdr+ cells (responders) coexpressed Mespl (Figure 1E). By day 9, the induced cells expressed cardiomyocyte markers (Figure 1F) and beat rhythmically (Online Movie I). Residual Cripto−/− caACVR1b cells contaminating the responder population after FACS (0.5%) were insufficient to account for this level of rescue (Online Figure II). Finally, the rescue occurred cell-nonautonomously, since mixtures of eGFP-labeled Cripto−/− caACVR1b inducers with Myh6-mCherry responders revealed clearly distinct patterns of eGFP and mCherry expression (Figure 1G and 1H and Online Movie II).

To test if the induced Kdr+ progenitors autonomously form cardiomyocytes, aggregated responder (Cripto−/−, Myh6-mCherry, eGFP+) and inducer (Cripto−/− caACVR1b) cells were separated by FACS at day 5, reaggregated separately, and cultured for an additional 15 days (Figure 1I). The responder cells expressed Myh6 (Figure 1J) and mCherry (Figure 1K), showing that the paracrine factor(s) initiate cardiogenesis before day 5. Since Cripto−/− cells negligibly respond to Nodal (Online Figure IB), the factor is neither Nodal nor a shed version of Cripto.

TGFβ2 Acts Downstream of Nodal to Induce Cardiogenic Mesoderm

Microarray analysis (not shown) showed that caACVR1b upregulated mRNAs encoding TGFβ1, TGFβ2, TGFβ3, and inhibins. Of these, Tgfb2, Tgfb3, and Inhba were greatly upregulated by caACVR1b transfection in Cripto−/− mESCs (Figure 2A). Since E5.5 to E7.5 mouse embryos express mRNAs encoding Tgfb2 but not Tgfb3 and Inhibins, TGFβ2 emerged as an attractive candidate for the paracrine factor. Indeed, TGFβ2 treatment from days 0 to 2 gave a dose-dependent induction of genetic markers of mesoderm (Mespl, Mix1, Aplnr, and Gsc) and mesoderm derivatives (Myh6, Pecam1, Tagln, Cdh5, and Acta2) and the Myh6-mCherry reporter in Cripto−/− ESCs (Figure 2B and 2C) and even enhanced Mespl, Kdr, and Myh6 in WT cells (Figure 2D), revealing a functional relationship.

To test if TGFβ is necessary downstream of Nodal/ Acvr1b, Cripto−/− responder ESCs were transfected with siRNA against Tgfr1 before coculture with Cripto−/− caACVR1b ESCs (Figure 2F). Tgfr1 siRNAs blocked induction of Kdr transcripts (to about 20% of negative control siRNA), establishing TGFβ2 as a paracrine mediator of Nodal signaling.

TGFβ2 Suppresses Cardiomyocyte Differentiation During a Late Stage of Differentiation

The preceding showed that TGFβ2 induces cardiogenic progenitors before day 5. Tgfb2 mRNA, however, continues to rise between days 4 to 8 (Figure 3A), whereas Nodal mRNA declines, suggesting that TGFβ but not Nodal plays a role as Kdr+ progenitors differentiate. To understand the basis for the shift from Nodal to Tgfb2, we examined expression of Lefty1, Lefty2, and Cer1, encoding Nodal inhibitors. All 3 became expressed concomitantly with the decline in Nodal levels (Figure 3A), and each was induced by Nodal/TGFβ signaling (Figure 3B and 3C). Moreover, Nodal and TGFβ both induced Nodal (Figure 2A and 3C). The fact that Cer1 and Lefty1 and Lefty2 do not
Figure 3. Biphasic role of TGFβ2 in cardiogenesis. A, Temporal expression profiles of Tgfb2, Nodal, Cer1, Kdr, and Myh6 during mESC differentiation. B, Lefty1 expression by quantitative real-time PCR (qRT-PCR) in WT and Cripto−/− mESCs and induction by caACVR1b. C, Induction profile of Nodal cascade genes in response to recombinant TGFβ2. Cripto−/− EBs were used to provide low basal levels of expression. Note induction of Nodal but not TGFβ2. D and E, siRNAs against Tgfbr1 and Tgfbr2 transfected at day 4 enhanced expression of Myh6 mRNA (D) as well as Myh6-GFP reporter (E) without effects on Pecam1 and Myh11 (day 16) (D). F, Kdr+ cells express Tgfb2, by qRT-PCR. G and H, Contrasting effects of SB-431542 treatment of WT CGR8 mESCs at early (days 0–2) (G) and of isolated Kdr+ progenitors at late (days 4–6) (H) stages of differentiation. I through L, TGFβ2 treatment between days 4 to 6 attenuated expression of cardiomyocyte markers [Myh6 mRNA (I), Tbx5 and Mef2c protein (L)] and Myh6 immunostaining (K) but increased markers of vascular endothelial and smooth muscle [Pecam1 and Myh11 mRNA (I) and immunostaining (J and K)]. *P<0.05, unpaired Student t test. Error bars indicate SEM; n=3.
block TGFβ1 probably accounts for the persistence of Tgfβ2 after the decline in Nodal. Interestingly, TGFβ2 does not induce Tgb1 or Tgfβ2 and only minimally induced Tgb3 (Figure 3C), making the cascade inherently self-limiting.

We next asked whether TGFβ influences cardiopoietic differentiation. siRNAs to either Tgfrb1 or Tgfrb2 transfected at day 4 unexpectedly increased expression of Myh6, as well as eGFP driven by the Myh6 promoter (Figure 3D and 3E). At this time, Tgb2 mRNA predominates in Kdr+ cells (Figure 3F), suggesting autocrine repression of cardiomyocyte differentiation.

To gain further insight into the bimodal function of TGFβ, we treated ESC cultures with SB-431542, a small-molecule inhibitor of Acvr1b/1c and Tgfbr1, at early and late time windows (Figure 3G and 3H). Treatment between 0 and 6 days abolished Mesp1 expression (Figure 3G). Treatment at 4 to 6 days, in contrast, markedly enhanced Myh6 levels in Kdr+ derivatives (Figure 3H). Conversely, recombinant TGFβ2 between days 4 to 6 suppressed Myh6 mRNA as well as Mef2c and Tbx5 protein but increased Pecam1 and Myh11 mRNAs and the level of Pecam1 and Myh11 immunostaining (Figure 3I through 3L). We conclude that a Nodal-to-TGFβ cascade enhances production of cardiogenic mesoderm before day 4 and that TGFβ persists to suppress cardiomyocyte differentiation of Kdr+ cells while biasing their differentiation toward endothelial and smooth muscle lineages.

Discussion

Genetic and stem cell experiments have shown that Nodal acts positively and negatively in cardiogenesis, depending on the developmental stage; however, the identity and function of downstream mediators were unknown.4,6,8,9 Our results define a Nodal-to-TGFβ signaling cascade that exerts positive and negative effects on progenitor induction and cardiomyocyte differentiation, respectively (Figure 4). The biphasic function resembles that of Wnts and BMPs, both of which promote formation of cardiogenic progenitors (eg, Mesp1+, Kdr+) during the period when mesoderm is induced but suppress the subsequent formation of cardiac progenitors (eg, Nkx2.5+), and at least BMP acts positively again once Nkx2.5+ progenitors arise.1

Mechanistically, the cascade incorporates auto-induction and inhibition properties that regulate Nodal and TGFβ expression within narrowly delimited developmental times. Nodal is well known for activating its own transcription as well that of its antagonists Lefty1, Lefty 2, and Cer1, yielding an auto-induction cascade that is feedback inhibited. However, TGFβ cannot auto-induce (Figure 2A and 3C) nor is inhibited by Cer1 and Lefty. Consequently, Tgb2 expression is induced by Nodal and persists after Nodal expression declines.

Considering the possible functions for a time-resolved Nodal-TGFβ cascade led to the finding that TGFβ suppresses cardiomyocyte differentiation while simultaneously enhancing formation of endothelial and smooth muscle lineages (Figure 3E through 3L). The only other factors known to impart cardiopoietic fate are Wnts, which also suppress cardiomyocyte differentiation at the same developmental stage.1

A specific requirement for TGFβ in cardiac differentiation has implications for understanding congenital heart defects. Genetic deletion of Tgfr1 in mice causes severe cardiovascular defects,10 and mutation of the latent TGFβ-binding protein 3, which regulates TGFβ bioavailability, impairs differentiation of second heart field cells in zebrafish.11 It will be important to determine if altered TGFβ signaling at the time of cardiac progenitor specification underlies human congenital heart disease, such as the cardiac defects that can present in Loey-Dietz syndrome caused by mutated TGFBR1 or TGFBR2.

Acknowledgments

We thank Youv Altman, Joseph Russo, and Dr Ed Monosov (SBMRI) for expert assistance.

Sources of Funding

This study was supported by the National Institutes of Health (P30AR061303) and California Institute for Regenerative Medicine (RC1-000132).

Disclosures

None.

References


Novelty and Significance

What Is Known?

- The divergent TGFβ protein Nodal is well known to play a role in specifying cardiac tissue during early development and is commonly used to generate cardiac cell types, including cardiomyocytes, from pluripotent stem cells.
- The cardiogenic activity of Nodal is propagated from cell to cell by unknown paracrine signals, although a shed version of the Nodal co-receptor Cripto has been suggested to be involved.

What New Information Does This Article Contribute?

- Nodal induces TGFβ2, and both induce the formation of cardiogenic progenitors in embryonic stem cell cultures.
- Nodal expression declines as cardiogenic progenitors form; TGFβ persists and suppresses cardiomyocyte differentiation while simultaneously promoting vascular smooth muscle and endothelial lineages.

TGFβ superfamily members are important for cardiogenesis as well as fibrosis and inflammation associated with myocardial injury. We describe a regulatory cascade that controls the production of TGFβ. TGFβ initially promotes the formation of multipotent cardiac progenitors but subsequently inhibits their differentiation to cardiomyocytes. TGFβ might play a similarly bimodal role in myocardial regeneration.
A Nodal-to-TGFβ Cascade Exerts Biphasic Control Over Cardiopoiesis
Wenqing Cai, Rosa M. Guzzo, Ke Wei, Erik Willems, Herman Davidovics and Mark Mercola

Circ Res. 2012;111:876-881; originally published online August 7, 2012;
doi: 10.1161/CIRCRESAHA.112.270272

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/111/7/876

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/08/07/CIRCRESAHA.112.270272.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Supplemental Material

Cai et al.,

A Nodal to TGFβ Cascade Exerts Biphasic Control of Cardiopoiesis

Contents:

1. Extended Materials and Methods
2. Online Table I
3. Online Figures
4. Movie Descriptions
Extended methods

mESCs differentiation
Mouse ESCs were differentiated either in 10% serum containing Iscove’s Modified Dulbecco Media (IMDM) or serum free IMDM as embryoid bodies (EBs), hanging drops or as monolayer. In serum conditions, IMDM was supplemented with 10% FBS, 2mmol/L glutamine, 4.5x10^{-4} mol/L monothioglycerol, 0.5 mmol/L ascorbic acid, 200 µg/mL transferrin (Roche), 5% protein-free hybridoma media (PFHM-II; Invitrogen) and Penicillin/Streptomycin; Serum free IMDM was supplemented with 25% DMEM/Ham’s F-12, 2 mmol/L glutamine, 4.5x10^{-4} mol/L monothioglycerol, 0.5 mmol/L ascorbic acid, B27 supplement without Vitamin A (Gibco), N2 supplement (Gibco) and Penicillin/Streptomycin.

Cell mixing assays
R1 Cripto⁻/⁻ ESCs engineered to express GFP were mixed with Cripto⁻/⁻ caACVR1b ESCs at a ratio of 5:1 in hanging drops. Hanging drops were also generated from pure populations of Cripto⁻/⁻ ESCs and Cripto⁻/⁻ caACVR1b ESCs as controls. Cells were maintained as hanging drops for two days, then as EBs in suspension for remaining days. EBs were then subjected to either FACS analysis at differentiation day 5 for Kdr expression or qRT-PCR analysis on day 9 for the expression of cardiac markers. In some cases, FACS isolated single cells were re-aggregated for subsequent culture. Dr. Malcolm Whitman (Harvard School of Dental Medicine) provided the caACVR1b-HA cDNA and Dr. Eileen Adamson (Sanford-Burnham Medical Research Institute) provided Cripto⁻/⁻ ESCs.

Flow cytometric analysis and cell sorting
Day 5 EBs were dissociated into single cells by 0.25% Trypsin (Gibco, Invitrogen), stained with phycoerythrin (PE)–conjugated anti-mouse Kdr (1:100, eBioscience), and then immediately analyzed with a FACSCanto (BD Biosciences). Total events of 100,000 were analyzed in each sample. Cell sorting was performed with FACS Vantage-Diva sorter (BD Bioscience) for GFP⁺ and GFP⁻ fractions or GFP⁺ /PE⁺ and GFP⁺ /PE⁻ fractions as indicated in figures and text. Dead cells were identified by staining of 0.1 µg/mL propidium iodide (PI) (Sigma-Aldrich) and excluded from analysis.

RNA extraction and qRT-PCR
Total RNA was extracted with TRIzol (Invitrogen) and reverse transcribed to cDNA with QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions.
cDNA samples synthesized from 500ng of total RNA by Quantitect RT kit (Qiagen) were subjected to qRT-PCR with LightCycler 480 SYBR Green I Master kit (Roche) performed with LightCycler 480 Real-Time PCR System (Roche). Primer sequences are listed in Supplementary Table 1.

**siRNAs transfection**
Pre-designed siRNAs against *Tgfbr1* and *Tgfbr2* (Ambion) and validated siRNA control (Ambion) were transfected at 100nmol/L final concentration into Cripto−/−, R1 or CGR8 cells by Lipofectamine RNAiMAX (Invitrogen), as per manufacturer’s instruction.

**Western blotting**
Cell pellets were lysed with RIPA buffer supplemented with protease and phosphatase inhibitors (Sigma) on ice and were mixed with 2X sample buffer (Invitrogen). Protein samples were then run on 10% SDS-tris glycine pre-cast gels (Invitrogen) and transferred onto a 45 µm PVDF membrane. Antibodies anti phospho-Smad2/3 (1:500, Cell Signaling), total Smad2/3 (1:500, Cell Signaling), Mef2c (1:1000, Aviva), Tbx5 (1:1000, Santa Cruz) and β-actin (1:5000, Sigma) were applied on blots and detected by ECL Plus detection kit (Abcam).

**Data analysis and statistics**
In qRT-PCR data analysis, values are expressed either as $2^{\Delta\Delta Ct}$, with $\Delta\Delta Ct$ defined as the difference in crossing threshold (Ct) values between experimental and control samples, using *Gapdh* as a control gene.

Each experiment was repeated at least two times using a minimum of three biological replicates per condition. Statistical analysis was performed with unpaired Student’s T-test, Asterisk in figures represents $P<0.05$. Error bars indicate the S.E.M.
### Online Table I. qRT-PCR Primer Sequences

<table>
<thead>
<tr>
<th>GENE (Accession No.)</th>
<th>FORWARD PRIMER</th>
<th>REVERSE PRIMER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oct3/4</strong> (NM_013633)</td>
<td>tcaacctggctagagaagg</td>
<td>tgacgggaacagagggagaag</td>
</tr>
<tr>
<td><strong>T/Brachyury</strong> (NM_009309)</td>
<td>acgtctggctagggctgcaaa</td>
<td>cgagtcgcttggtgatgtag</td>
</tr>
<tr>
<td><strong>Goosecoid</strong> (NM_010351)</td>
<td>accaatctcagatgacgc</td>
<td>ctggctcgggctgcttaaac</td>
</tr>
<tr>
<td><strong>Kdr</strong> (NM_010612)</td>
<td>tggctgaattctaatctgttg</td>
<td>aaatcaggactacattccttg</td>
</tr>
<tr>
<td><strong>Mesp1</strong> (BC012689)</td>
<td>aatgcaacgcagatgtg</td>
<td>agctgtactactattgg</td>
</tr>
<tr>
<td><strong>Gata4</strong> (NM_008092)</td>
<td>catcaatatcagagccct</td>
<td>aagcaagcaagactgtcct</td>
</tr>
<tr>
<td><strong>Mef2c</strong> (NM_025282)</td>
<td>agataacccaacaccaactgaggct</td>
<td>cattatcctcagaggtctgcct</td>
</tr>
<tr>
<td><strong>Myh6</strong> (M76601)</td>
<td>cactgcaggtgcagacct</td>
<td>ccatcactctgtacctgcc</td>
</tr>
<tr>
<td><strong>Pax6</strong> (NM_013627)</td>
<td>gcctcttcatactctgtgg</td>
<td>cgtctcttaacccgcca</td>
</tr>
<tr>
<td><strong>GFP</strong> (YP_002302326)</td>
<td>ctgcgtccgcacacaaaccatcgtactcttcttg</td>
<td>tggcatgcgctctggttgg</td>
</tr>
<tr>
<td><strong>Gapdh</strong> (K0_1458)</td>
<td>aatggtagacgctagccct</td>
<td>tggcagcgaactttlttag</td>
</tr>
<tr>
<td><strong>Nodal</strong> (NM_013611)</td>
<td>ccaagacacgcagacactgagggctg</td>
<td>aagcaagccgaggagtcct</td>
</tr>
<tr>
<td><strong>Lefty1</strong> (NM_010094)</td>
<td>ctcgatcaacgcctctt</td>
<td>cataaacacacactagctc</td>
</tr>
<tr>
<td><strong>Lefty2</strong> (NM_177099)</td>
<td>agttctgctagtggagct</td>
<td>cttctagggctactaggt</td>
</tr>
<tr>
<td><strong>Inhibina</strong> (NM_010564)</td>
<td>caggcttactctttccacacc</td>
<td>gggatggccggaactacatag</td>
</tr>
<tr>
<td><strong>Inhibinb</strong> (NM_008381)</td>
<td>atagggaggagcagactgcagggctgtaaa</td>
<td>cagctctactacgacaggg</td>
</tr>
<tr>
<td><strong>Cerberus1</strong> (NM_009887)</td>
<td>gcagacctatctgtgga</td>
<td>atgacacatgatgctt</td>
</tr>
<tr>
<td><strong>Tgfb1</strong> (NM_011577)</td>
<td>attcctggctcactcaatgctcttg</td>
<td>acctttttagctatgatgcttcgc</td>
</tr>
<tr>
<td><strong>Tgfb2</strong> (NM_009367)</td>
<td>tcggcatgtcgtctttggtggct</td>
<td>ccttgtagctatgtatagatgg</td>
</tr>
<tr>
<td><strong>Tgfb3</strong> (NM_009368)</td>
<td>cctggcgcgctggaactctg</td>
<td>gacgtggtgtcatcaccggtatc</td>
</tr>
<tr>
<td><strong>Pecam1/CD31</strong> (NM_008816)</td>
<td>gcaccacatcactacccacc</td>
<td>cttcatccacgccccgctc</td>
</tr>
<tr>
<td><strong>Myh11</strong> (NM_013607)</td>
<td>aagctgctgccgctagaggtta</td>
<td>agctcttttggaagttctcttc</td>
</tr>
<tr>
<td><strong>Aplnr</strong> (NM_011784.3)</td>
<td>ggttaaacactatggtgctgctgta</td>
<td>actggtagcctcttttg</td>
</tr>
<tr>
<td><strong>Cdh5</strong> (NM_009868)</td>
<td>tgccccttttgatgcaaa</td>
<td>tgtgcagctttggatgat</td>
</tr>
<tr>
<td><strong>Tagln</strong> (NM_013607)</td>
<td>agggatcagacagcagaga</td>
<td>aactgtgctcatcacttccttc</td>
</tr>
<tr>
<td><strong>Acta2</strong> (NM_007392)</td>
<td>gcaccaagcagaaaacactat</td>
<td>tccatactcttggaagaag</td>
</tr>
<tr>
<td><strong>Mixl1</strong> (NM_013729)</td>
<td>acgcagtgctttccaaaacc</td>
<td>cccgcaagttggagttgcttg</td>
</tr>
</tbody>
</table>
Supplementary Figures

Online Fig. I. Constitutively active, ligand independent Nodal signaling restores cardiogenesis in *Cripto*<sup>−/−</sup> ESC.

A) Expression of mesoderm and cardiac markers is restored in Cripto<sup>−/−</sup> ESCs stably transduced with caACVR1b. qRT-PCR analysis for each gene marker was performed at developmentally relevant time points during mESC differentiation for markers of stem state (*Oct3/4*); mesoderm (*T*/*Brachyury, *Kdr, Goosecoid*); cardiogenic mesoderm (*Gata4, Mef2c*); cardiomyocytes (*Myh6*) and neural tissue (*Pax6*).

B) Relative mRNA expression of *Lefty2* in R1 and *Cripto*<sup>−/−</sup> ESCs, which were transiently transfected with either control empty plasmid vector (pCS2+) or plasmids directing expression of Activin A, Nodal (NHN), or a Nodal fusion protein (BHN) that contains the BMP2/4 pro-region in place of the native pro-region to facilitate processing. Note that Nodal (BHN, NHN) is insufficient to induce *Lefty2* expression in *Cripto*<sup>−/−</sup> ESCs whereas Activin A, which signals independently of Cripto, restored downstream genes expression to levels.

*P* < 0.05, unpaired Student’s T-test. Error bars indicate the S.E.M.; n=3.
Online Fig. II. Residual inducer cells are insufficient to account for cardiac *Myh6* gene expression in FACS-isolated responder population.

GFP<sup>−</sup> inducer and GFP<sup>+</sup> responder cells were combined without co-culture and tested for gene expression by qRT-PCR, yielding a positive linear correlation for *Myh6* mRNA and negative for GFP mRNA (inset) relative to the GFP<sup>−</sup> inducer cells. The actual *Myh6* expression in the co-culture experiment (Fig. 1F) would require 73.9% percent contamination. This value differs from the 0.5% actual residual value derived from re-sorting and results in $P = 1.5 \times 10^{-17}$ for the experiment from $\chi^2$-squared distribution (Table inset).
Online Fig. III. Validation of Myh6-mCherry fluorescent reporter ESC line.

A) Schematic representation of the cardiac-specific Myh6 promoter driven mCherry reporter.

B) Coincident α-actinin immunostaining (green) with Myh6-mCherry expression, validating reporter fidelity.

C) Flow cytometry profiles of parental R1 and Myh6-mCherry reporter cells at day 9 in EB culture yielding 4.41% mCherry⁺ cells in the reporter line.

D) qRT-PCR analysis of mCherry and Myh6 in day 9 FACS sorted mCherry⁺ and mCherry⁻ cells (C) showed Myh6 mRNA expression localized primarily in the mCherry⁺ cells.

*P< 0.05, unpaired Student's T-test. Error bars indicate the S. E. M.; n=3.
Movie Descriptions

Online Movie I. Cell non-autonomous rescue of beating cardiomyocytes in Cripto⁺ ESCs.

Cripto⁺ ESCs with the cardiac specific \textit{Myh6}-mCherry reporter (responder) were mixed with GFP⁺ caACVR1b Cripto⁺ ESCs (inducer) and imaged at day 10 of differentiation.

Online Movie II. 3-dimensional reconstruction of confocal images shows cell non-autonomous rescue of cardiogenesis in Cripto⁺ ESCs.

Cripto⁺ ESCs with the cardiac specific \textit{Myh6}-mCherry reporter (responder) were mixed with GFP⁺ caACVR1b Cripto⁺ ESCs (inducer) and analyzed by confocal microscopy at day 9 of differentiation. Confocal imaging demonstrated that the mCherry⁺ cardiomyocytes induced in the Cripto⁺ population were distinct from GFP⁺ caACVR1b inducer cells.