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A Nodal to TGFβ Cascade Exerts Biphasic Control Over Cardiopoiesis

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Abstract

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—Determine the function(s) of TGFβ family members during stem cell cardiogenesis.

Methods and Results—Murine embryonic stem cells (mESCs) 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 ESC cultures at day 0–2 of differentiation. However, Nodal expression declines by day 4 due to feedback inhibition whereas TGFβ persists. At later stages (day 4–6), TGFβ suppresses the formation of cardiomyocytes from multipotent Kdr^+ 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^+ progenitors. TGFβ, however, becomes uniquely responsible for controlling subsequent lineage segregation by stimulating vascular smooth muscle and endothelial lineages and simultaneously blocking cardiomyocyte differentiation.

Keywords

Nodal; Cripto; TGFβ2; Kdr; cardiogenesis

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DISCLOSURES
None.

INTRODUCTION

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) 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[1]. 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 -3 that then activate target genes[2]. Mouse embryos lacking *Acvr1b, Smad2*, or *Nodal*, and double knockout of the two type II receptors (*Acvr2a* and *Acvr2b*) fail to gastrulate or form mesendoderm[3]. 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[4, 5]. The cardiogenesis deficit inherent in *Cripto*$^{-/-}$ ESCs can be rescued either by incorporation into chimeric (Cripto$^{-/-}$:wildtype (WT)) embryos[4], or by a constitutively active mutant human ACVR1b receptor[6], demonstrating the existence of yet unknown paracrine effectors that propagate the signal from cell to cell.

We used 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 while 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 in online supplemental materials.

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 (Online Fig. IA and[4, 5]), and are thus ideal for a cell-mixing study to identify paracrine factors that initiate cardiogenesis downstream of Nodal/Avcr1b (Fig. 1A). A constitutively active human ACVR1b receptor (*caACVR1b*) was stably introduced into *Cripto*$^{-/-}$ mESCs to activate downstream signaling (*Cripto*$^{-/-}$*caACVR1b*, inducers) (Online Fig. IA). Co-culture (Fig. 1A,B) of these cells dramatically restored *Kdr* and *Mesp1* expression in eGFP-labeled *Cripto*$^{-/-}$ mESCs (responders) (Fig. 1C). Co-culture also increased the number of Kdr^{+} progenitors among the responder (eGFP^{+}) population, from 3.34% ± 0.06% to 21.28% ± 1.37% after 5 days (Fig. 1D). FACS-isolated GFP^{+}, Kdr^{+} cells (responders) co-expressed *Mesp1* (Fig. 1E). By day 9, the induced cells expressed cardiomyocyte markers (Fig. 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 Fig. II). Finally, the rescue occurred cell non-autonomously, since mixtures of eGFP-labeled *Cripto*$^{-/-}$*caACVR1b* inducers with

Myh6-mCherry responders revealed clearly distinct patterns of eGFP and mCherry expression (Fig. 1G,H 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, re-aggregated separately, and cultured for an additional 15 days (Fig. 1I). The responder cells expressed *Myh6* (Fig. 1J) and mCherry (Fig. 1K), showing that the paracrine factor(s) initiate cardiogenesis prior to day 5. Since *Cripto*$^{-/-}$ cells negligibly respond to Nodal (Online Fig. 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*, *Tγϕβ3* and *Inhba* were greatly upregulated by *caACVR1b* transfection in *Cripto*$^{-/-}$ mESCs (Fig. 2A). Since E5.5 to E7.5 mouse embryos express mRNAs encoding *Tgfb2*, but not *Tgfb3* and *Inhibins*[7], TGFβ2 emerged as an attractive candidate for the paracrine factor. Indeed, TGFβ2 treatment from days 0–2 gave a dose-dependent induction of genetic markers of mesoderm (*Mesp1, Mixl1* and *Gsc*) and mesoderm derivatives (*Myh6, Pecam1, Aplnr,Tagln, Cdh5 and Acta2*), and the *Myh6*-mCherry reporter in *Cripto*$^{-/-}$ ESCs (Fig. 2B,C) and even enhanced *Mesp1, Kdr* and *Myh6* in WT cells (Fig. 2D), revealing a functional relationship.

To test if TGFβis necessary downstream of Nodal/Acvr1b, *Cripto*$^{-/-}$ responder ESCs were transfected with siRNA against *Tgfbr1* prior to co-culture with *Cripto*$^{-/-}$ *caACVR1b* ESCs (Fig. 2F). *Tgfbr1* 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 prior to day 5. *Tgfb2* mRNA, however, continues to rise between days 4–8 (Fig. 3A) while *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[3]. All three became expressed concomitantly with the decline in *Nodal* levels (Fig. 3A) and each was induced by Nodal/TGFβsignaling (Fig. 3B, C). Moreover, Nodal and TGFβboth induced *Nodal* (Figs. 2A and 3C). The fact that Cer1 and Lefty1,2 do not block TGFβ[3] likely accounts for the persistence of *Tgfb2* after the decline in *Nodal*. Interestingly, TGFβ2 does not induce *Tgfb1* or *Tgfb2*, and only minimally induced *Tgfb3* (Fig. 3C), making the cascade inherently self-limiting.

We next asked whether TGFβ influences cardiopoietic differentiation. siRNAs to either *Tgfbr1* or *Tgfbr2* transfected at day 4 unexpectedly increased expression of *Myh6*, as well as eGFP driven by the *Myh6* promoter (Fig. 3D,E). At this time, *Tgfb2* mRNA predominates in Kdr$^+$ cells (Fig. 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 (Figs 3G and H). Treatment between 0–2 days of culture abolished *Mesp1* expression (Fig. 3G). Treatment at 4–6 days, in contrast, markedly enhanced *Myh6* levels in Kdr$^+$ derivatives (Fig. 3H). Conversely, recombinant TGFβ2 between days 4–6 suppressed *Myh6* mRNA as well as Mef2c and Tbx5 protein, but increased *Pecam1* and *Myh11* mRNAs and the level of Pecam1 and Myh11 immunostaining (Fig. 3I–L). We conclude that a Nodal to TGFβ2 cascade enhances production of cardiogenic mesoderm prior to day 4,

and that TGFβ persists to suppress cardiomyocyte differentiation of Kdr^+ cells while biasing their differentiation towards 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 (Fig. 4). The biphasic function resembles that of Wnts and BMPs, both of which promote formation of cardiogenic progenitors (*e.g.* $Mesp1^+$, Kdr^+) during the period when mesoderm is induced, but suppress the subsequent formation of cardiac precursors (*e.g.* $Nkx2.5^+$), 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, 2 and Cer1, yielding an auto-induction cascade that is feedback inhibited. However, TGFβ cannot auto-induce (Figs. 2A and 3C) nor is inhibited by Cer1 and Lefty. Consequently, *Tgfb2* 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 (Fig. 3E–L). The only other factors known to apportion 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 *Tgfbr1* 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 (SHF) 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 Loeys-Dietz syndrome caused by mutated *TGFBR1* or *TGFBR2*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations

Acvr1b	Alk4, activin A receptor, type IB
Tgfbr1	Alk5, transforming growth factor, beta receptor I
Acvr1c	Alk7, activin A receptor, type IC

Acvr2a	activin receptor IIA
Acvr2b	type 2 Activin receptorb
Cer1	cerberus 1 homolog (Xenopus laevis)
Inhba	inhibin beta-A
Inha	Inhibin alpha
Kdr	Flk1, kinase insert domain protein receptor
Lefty1	left right determination factor 1
Lefty2	left right determination factor 2
Mesp1	mesoderm posterior 1
Myh6	αMHC, myosin, heavy polypeptide 6, cardiac muscle, alpha
Myh11	sm-MHC, myosin, heavy polypeptide 11, smooth muscle
Pecam1	CD31, platelet/endothelial cell adhesion molecule 1
qRT-PCR	quantitative real-time reverse transcription PCR
T	Bra/T, brachyury
Tgfbr2	transforming growth factor, beta receptor IIb

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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 (ESC) cultures.
- Nodal expression declines as cardiogenic progenitors form; TGFβ persists and suppresses cardiomyocyte differentiation while simultaneously promoting vascular smooth muscle and endothelial lineages.

TGFβ uperfamily members are important for cardiogenesis, as well as fibrosis and inflammation associated with myocardial injury. Here we describe a regulatory cascade that controls the production of TGFβ. TGFβinitially promotes the formation of multi-potent cardiac progenitors, but subsequently inhibits their differentiation to cardiomyocytes. TGFβ might play a similarly bimodal role in myocardial regeneration.

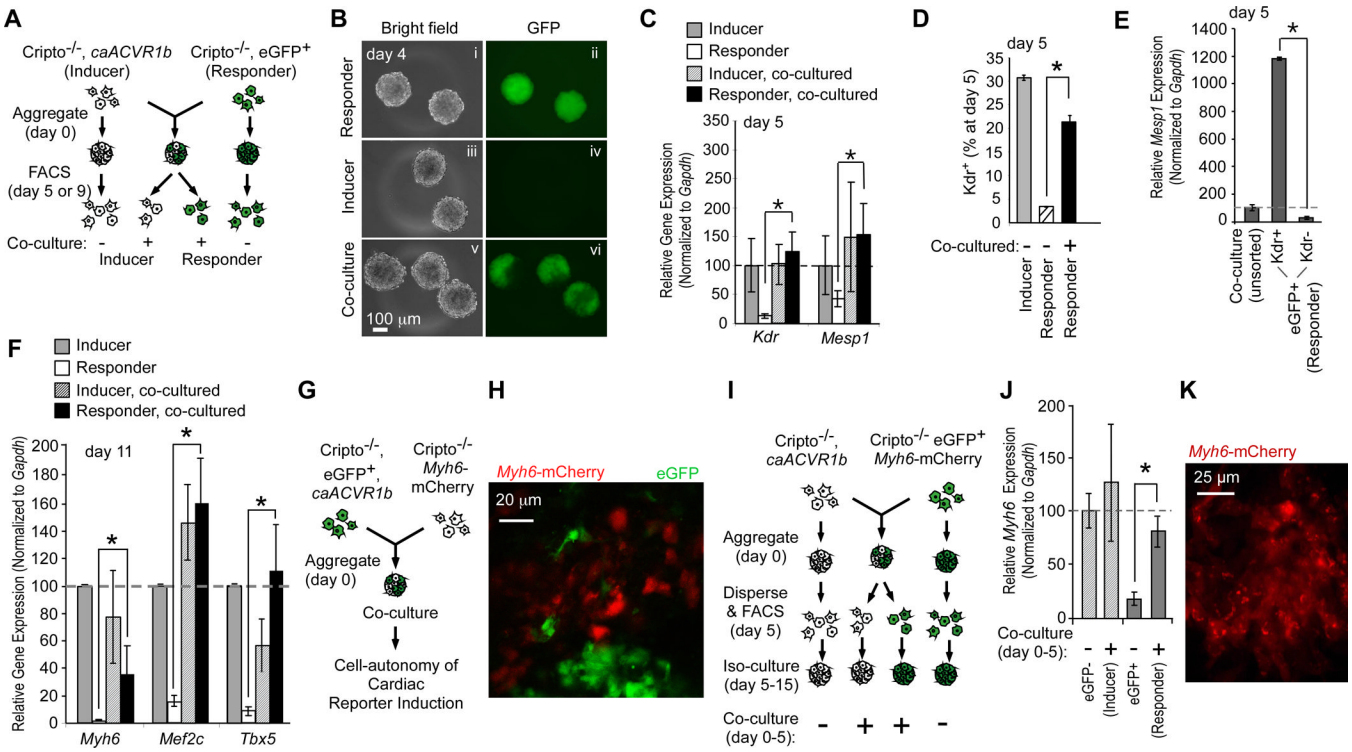

Fig. 1. Paracrine signaling downstream of Nodal

A, B, Schematic (**A**) and images (**B**) of the cell mixing experiment. **C–E**, *Kdr* and *Mesp1* expression (**C**) and proportion of Kdr$^+$ cells (**D**) in FACS-isolated populations from co-cultures. *Mesp1* expression in FACS-isolated Kdr$^+$/GFP$^+$ cells (**E**). Note induction by co-culture. **F**, *Myh6, Mef2c, and Tbx5* in FACS-isolated populations from co-cultures; representative of >3 trials (see Online Fig. II). **G, H**, Cell non-autonomous signaling induced cardiogenesis. Schematic of the experiment (**G**) and representative confocal image of *Myh6*-mCherry reporter (**H**). Movie II shows multiple optical planes. **I–K**, Co-culture from day 0–5 is sufficient for cardiogenesis in responder cells. Schematic of experiment (**I**). Quantitative real-time reverse transcription PCR (qRT-PCR) analysis of *Myh6* expression (**J**) and image of *Myh6*-mCherry reporter (**K**) after 10 days iso-culture. **P< 0.05*, unpaired Student's T-test. Error bars indicate the S. E. M.; n=3.

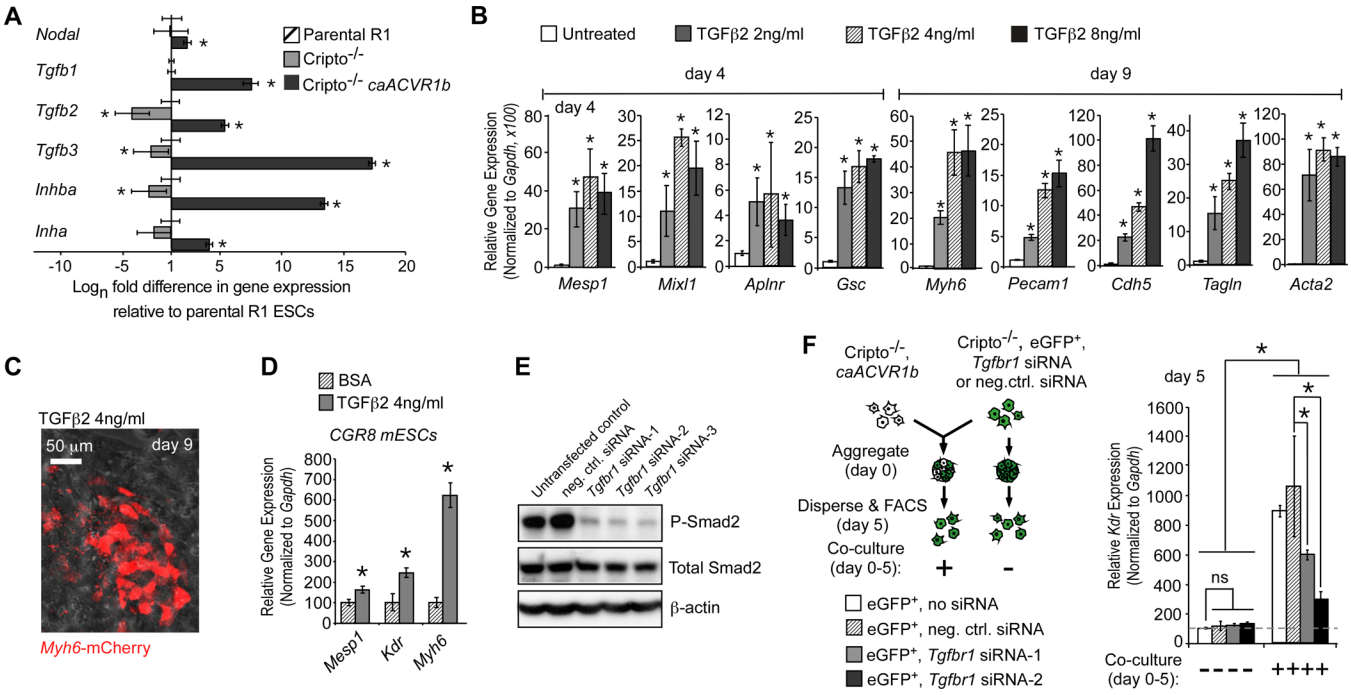

Fig. 2. TGFβ2 acts downstream of Nodal/Acvr1b to induce Kdr$^+$ progenitors

A, qRT-PCR analysis of TGFβsuperfamily members in R1, Cripto$^{-/-}$ and Cripto$^{-/-}$ caACVR1b ESCs. **B–D**, Treatment of *Cripto*$^{-/-}$ (**B,C**) and WT (**D**) ESCs treated with TGFβ2 between days 0–2 of differentiation under defined conditions analyzed for gene (**B,D**) and *Myh6*-mCherry expression (**C**). **E–F**, Effect of siRNA knockdown of *Tgfbr1* on *Kdr*. Western blot of R1 ESCs showing efficacy of Tgfbr1 siRNAs (**E**). Schematic protocol and qRT-PCR analysis of *Kdr* in responder cells (**F**). **P< 0.05*, unpaired Student's T-test. Error bars indicate the S. E. M.; n=3.

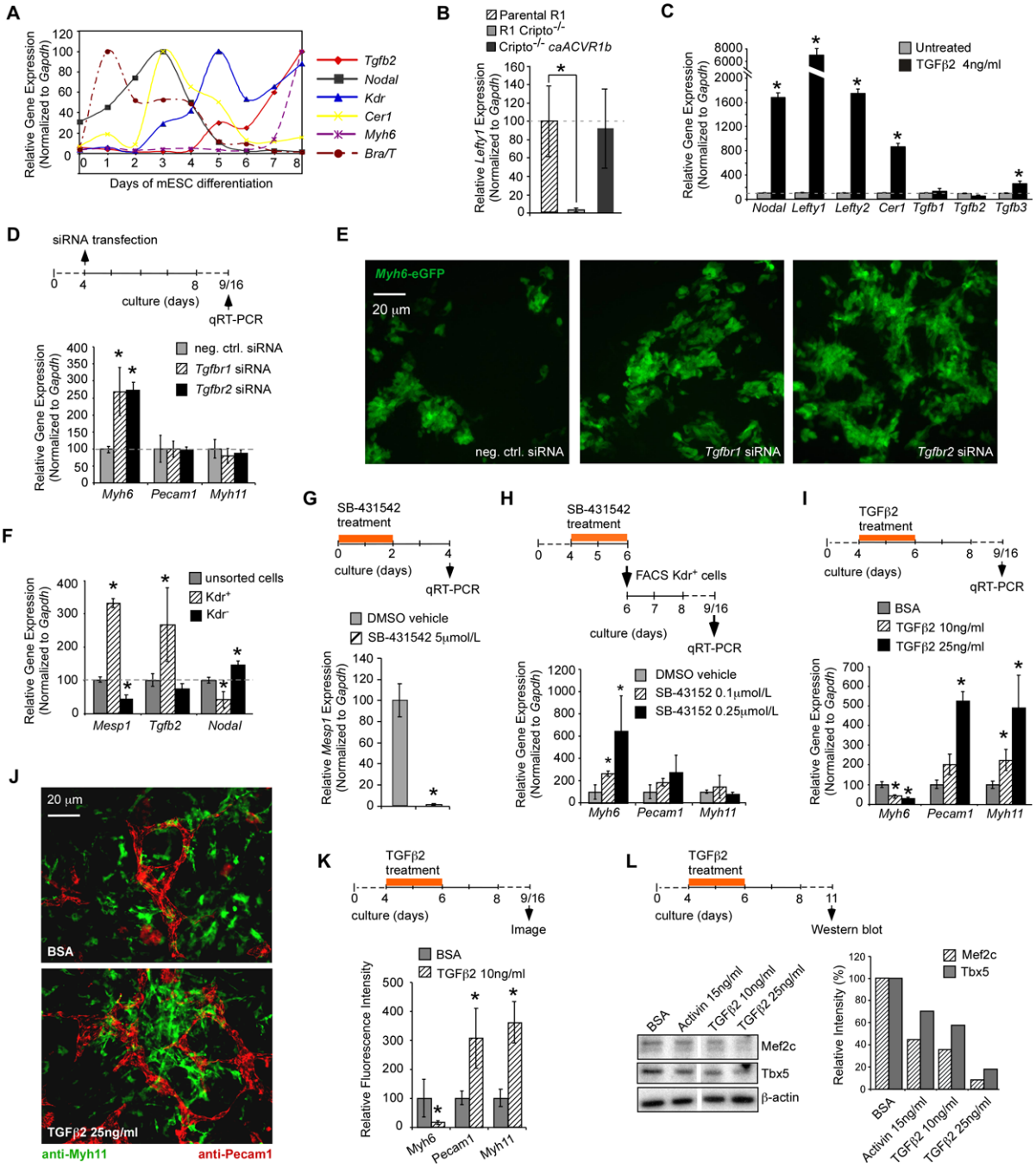

Fig. 3. Biphasic role of TGFβ2 in cardiogenesis

A, Temporal expression profiles of *Tgfb2, Nodal, Cer1, Kdr* and *Myh6* during mESC differentiation. **B**, *Lefty1* expression by 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 TGFβ. **D,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–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–L**, TGFβ2 treatment between days 4–6 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,K**)]. **P< 0.05*, unpaired Student's T-test. Error bars indicate the S. E. M.; n=3.

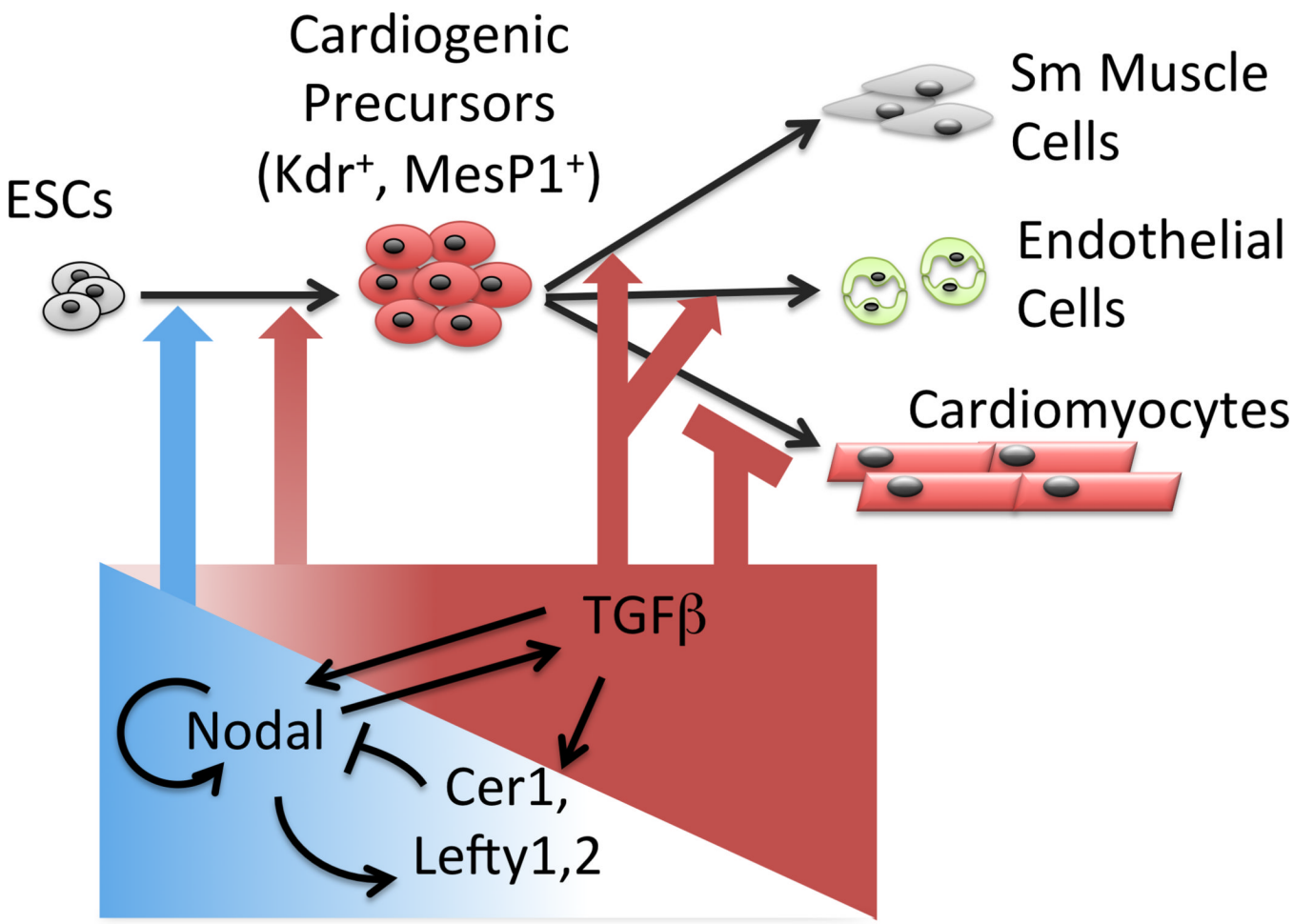

Fig. 4. Summary

Nodal and TGFβ induce early cardiogenic progenitors. Subsequently, feedback inhibition blocks Nodal, allowing persistence of TGFβ which inhibits cardiomyocyte differentiation and promotes formation of vascular smooth muscle and endothelial cells.