Stage-Specific Role of Endogenous Smad2 Activation in Cardiomyogenesis of Embryonic Stem Cells

Ryoji Kitamura, Tomosaburo Takahashi, Norio Nakajima, Koji Isodono, Satoshi Asada, Hikaru Ueno, Tomomi Ueyama, Toshikazu Yoshikawa, Hiroaki Matsubara, Hidemasa Oh

Abstract—The role of Smads and their specific ligands during cardiomyogenesis in ES cells was examined. Smad2 was activated bimodally in the early and late phases of cardiac differentiation, whereas Smad1 was activated after the middle phase. Nodal and Cripto were expressed in the early stage and then downregulated, whereas transforming growth factor-β and activin were expressed only in the late phase. Suppression of early Smad2 activation by SB-431542 produced complete inhibition of endodermal and mesodermal induction but augmented neuroectodermal differentiation, followed by poor cardiomyogenesis, whereas inhibition during the late phase alone promoted cardiomyogenesis. Inhibitory effect of Smad2 on cardiomyogenesis in the late phase was mainly mediated by transforming growth factor-β, and inhibition of transforming growth factor-β–mediated Smad2 activation resulted in a greater replicative potential in differentiated cardiac myocytes and enhanced differentiation of nonmyocytes into cardiac myocytes. Thus, endogenous Smad2 activation is indispensable for endodermal and mesodermal induction in the early phase. In the late phase, endogenous transforming growth factor-β negatively regulates cardiomyogenesis through Smad2 activation by modulating proliferation and differentiation of cardiac myocytes. (Circ Res. 2007;101:78-87.)

Key Words: embryonic stem cells | cardiomyogenesis | Smad2 | TGF-β | differentiation

Embryonic stem (ES) cells are well-established pluripotent stem cells and capable of self-renewal and differentiation into derivatives of all 3 primary germ layers. With appropriate culture conditions, ES cells can differentiate into specialized cells including cardiac myocytes in vitro. The in vitro differentiation of ES cells into cardiac myocytes not only provides unique opportunities to study development of cardiac myocytes but also proposes the potential use of ES cell–derived cardiac myocytes for many medical applications such as cell transplantation therapy against various heart conditions. With the use of different sets of type I receptors and receptor-regulated Smad (R-Smad), the superfamily members can be classified into 2 major branches: (1) the TGF-β/activin/Nodal branch and (2) the bone morphogenetic protein (BMP)/growth and differentiation factor (GDF) branch. The TGF-β/activin/Nodal branch activates activin receptor–like kinase (ALK)-4, -5, and -7, which phosphorylate Smad2 and -3, whereas Smad1, -5, and -8 are substrates for the BMP/GDF branch through ALK-1, -2, -3, and -6. The role of Smad1/5/8-activating BMP in cardiogenesis is relatively well documented. In chick embryo, BMP2 is able to induce expression of myocardial lineage markers in ectopic locations in vitro, and anterior lateral mesoderm explant cultures in vitro, and Noggin, one of soluble BMP antagonists, prevents myocardial differentiation of lateral mesendoderm culture in vitro. The dependence of myocardial specification on BMP signaling is evolutionally conserved, although several members of BMP have overlapping function in murine cardiac differentiation. In murine teratocarcinoma P19CL6 cells, myocardial differentiation is shown to depend on functional BMP signals, supporting the essential role for BMP signaling in cardiac specification. However, little is yet known about the function of Samd2/3-activating TGF-β, activin, and Nodal in cardiac differentiation.

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In the present study, we hypothesized that Smad2 activation is involved in cardiomyogenesis and analyzed the roles of Smad2 and its specific ligands during in vitro differentiation of ES cells into cardiac myocytes.

Materials and Methods
An expanded Materials and Methods section can be found in the online supplement at http://circres.ahajournals.org.

ES Cell Culture and Differentiation
CGR8 mouse ES cells and ES cells stably transfected with $\alpha$-myosin heavy chain (MHC) promoter-driven enhanced green fluorescent protein (EGFP) were used, and differentiation was induced by forming embryoid bodies (EBs) in the hanging drop suspension culture.7

Replication-Defective Recombinant Adenoviruses
Adenoviral vectors expressing a soluble type II TGF-$\beta$ receptor (sTGF-$\beta$ IIIR), dominant negative mutant of Smad2 (Smad2 DN), or $\beta$-galactosidase (LacZ) were prepared as described.8,9

Flow Cytometric Analysis and Cell Sorting
$\alpha$-MHC–EGFP ES cells were analyzed with a FACSCalibur Flow Cytometer or sorted with FACSaria cell sorter.

Immunostaining
Cells were stained with the primary antibody against sarcomeric $\alpha$-actinin or $\beta$III-tubulin (Sigma). Sorted cells were centrifuged onto polylysine slides and stained with anti–phospho-histone H3 antibody (Millipore).

5-Bromodeoxyuridine Incorporation Assay
Cells were labeled with 5-bromodeoxyuridine (BrdUrd) for 2 hours at day 8. Sorted cells were stained with anti-BrdUrd antibody (Roche).

Results
Cardiac Differentiation Programs in ES Cells
In vitro differentiation of ES cells was induced by forming cellular aggregates called EBs through the hanging drop method,7 and spontaneously contracting cell clusters developed within EB outgrowths at day 7 (attached culture for 2 days after 5 days of hanging drop suspension culture). Early mesodermal markers, Brachyury and Mesp1, were transiently expressed around day 5 (Figure 1A). Wnt-3 expression was upregulated transiently between day 3 and 5, and Wnt-11 expression was upregulated with the differentiation (Figure 1B). Among the transcription factors crucial for cardiac differentiation, GATA4 expression was first detected at day 5, followed by expression of Nkx2.5 and Tbx5 (Figure 1C). The genes for myocardial structural proteins $\alpha$-MHC and cardiac troponin (cTnI) and a cardiac-specific peptide, atrial natriuretic peptide (ANP), were expressed after day 8 (Figure 1D). These results were consistent with the previous reports1,10 and indicated that the in vitro differentiation of ES cells recapitulates the developmental program of cardiac myocytes and that during the formation of EB in suspension culture, mesodermal induction occurs, and, thereafter, the specification and maturation of cardiac myocytes are executed.

Smad2 Shows Bimodal Activation in the Early and Late Phases, Which Is Mediated by Nodal/Cripto and TGF-$\beta$/Activin, Respectively
To assess how Smads were regulated during ES cell differentiation, expression and phosphorylation of Smad proteins were examined by immunoblot analysis with total and phospho-specific antibodies against Smad2 (Smad2 DN), or $\beta$-galactosidase (LacZ) were prepared as described.8,9

Figure 1. Cardiac differentiation programs in ES cells. ES cells were cultured in hanging drop suspension cultures for 5 days and then on gelatin-coated dishes for 7 more days. Cells were harvested at the indicated time points. Gene expression was analyzed by real-time kinetic PCR (A through D). A, Brachury, Mesp1. B, Wnt-3, Wnt-11. C, GATA4, Nkx2.5, Tbx5. D, $\alpha$-MHC, cTnI, ANP. The results were expressed relative to the level of 18S ribosomal RNA and plotted as percentages of the maximum.

Smad2 activation is involved in cardiomyogenesis and analyzed the roles of Smad2 and its specific ligands during in vitro differentiation of ES cells into cardiac myocytes.
expression of TGF-β superfamily ligands, which can activate the Smad2 signaling pathway. Nodal and its coactivator Cripto were abundantly expressed in undifferentiated ES cells, whereas TGF-β and activin A were undetectable in these cells (Figure 2C). On induction of differentiation, the expression of Nodal and Cripto declined and was almost undetectable at day 5 (Figure 2C). In contrast, TGF-β and activin were markedly induced at days 5 and 8, respectively (Figure 2C). TGF-β isoforms showed different expression patterns during ES cell differentiation: the expression of TGF-β1 and TGF-β2 peaked at day 8 and then declined, whereas TGF-β3 continued to be upregulated until day 12 (Figure 2C). These results suggested that Smad2 was activated by Nodal/Cripto in the undifferentiated and early stages, whereas TGF-β and activin were responsible for the late activation of Smad2.

**Smad2 Activation Has Stage-Specific Opposing Effects on Cardiomyogenesis in ES Cells**

The role of Smad2 activation in cardiomyogenesis of ES cells was analyzed using SB-431542, a specific and potent inhibitor of ALK-4, -5, and -7.11 To date, no target of SB-431542 other than Smad2-activating ALK-4, -5, and -7 has been reported, and SB-431542 has no effect on other ALK family members, extracellular signal-regulated kinase, c-Jun N-terminal kinase, or p38 mitogen-activated protein kinase at the concentration used in this study.11 As Smad2 was activated biphasically in the early and late phases, cells were divided into 4 groups (Figure 3A): control; no treatment; SB-431542 treated throughout differentiation, SB-431542; treated for only the first 5 days, SB-431542; treated after day 5. Treatment with SB-431542 completely abolished the phosphorylation of Smad2 throughout the course of differentiation (Figure 3C). Using ES cell clones that express EGFP under the transcriptional control of a cardiac-specific α-MHC promoter,7 we examined the effects of phase-specific inhibition of Smad2 activation on cardiomyogenesis by analyzing the proportion of EGFP-positive cardiac myocytes (Figure 3A and 3B). When cells were treated in the early phase, cardiomyogenesis was markedly inhibited compared with the untreated control, although it was significantly augmented when cells were treated in only the late phase (Figure 3A). In untreated control cells, the proportion of cardiac myocytes was 4.1% (Figure 3B). On treatment with SB-431542 in the early phase, EGFP-positive cells decreased in a concentration-dependent manner, regardless of treatment in the late phase, and almost completely diminished at 5 μmol/L SB-431542 (Figure 3B). When treated in only the late phase,
cardiomyogenesis was markedly augmented in a dose-dependent manner. At 10 \( \mu \text{mol/L} \), SB-431542, the proportion of cardiac myocytes reached 13.2\%, a more than 3-fold increase over the control (Figure 3B). A vehicle control, DMSO, did not influence the proportion of cardiac myocytes (Figure 3B). Inhibition of the late activation of Smad2 did not alter general growth properties, as treatment of cells with SB-431542 in the late phase alone did not alter the total viable cell number of the cultures (Figure 3D). These results indicated that SB-431542 treatment in the late phase alone results in a 3-fold increase in the yield of cardiac myocytes from the same scale of culture. These EGFP-positive cells were cardiac myocytes, as stained positively with antibody against sarcomeric \( \alpha \)-actinin or cTnI and showing a myofibrillar structure (data not shown).

Analysis of cardiac gene expression in nontransfected naïve ES cells revealed that expression of cardiac-specific genes such as \( \alpha \)-MHC, cTnI, and ANP was almost completely inhibited by SB-431542 when used in the early phase, whereas the expression of these genes showed an \( \approx 3 \) -fold increase when treated only in the late phase (Figure 3E). Furthermore, protein expression of sarcomeric myosin and \( \alpha \)-actinin exhibited the results consistent with the gene expression analysis (Figure 3F). These results clearly demonstrated that Smad2 activation in the early phase is essential for cardiomyogenesis, whereas the late activation of Smad2 negatively regulates cardiomyogenesis of ES cells.

**Treatment With SB-431542 in the Early Phase Inhibits Mesodermal and Endodermal Induction but Augments Neuroectodermal Differentiation**

As treatment of cells with SB-431542 in the early phase inhibited cardiac differentiation, the effect of SB-431542 on expression of markers for 3 germ layers was analyzed (Figure 4A through 4D). Mesodermal markers such as Brachyury and Flk1 and endodermal markers such as Mixl1, Foxa2, and \( \alpha \)-fetoprotein were downregulated by SB-431542 (Figure 4A and 4B), whereas the expression of neuroectodermal markers such as Pax6, neurogenic differentiation 1 (Neurod1), and neurogenin 2 (Neurog2) was upregulated (Figure 4C and 4D). Immunostaining with sarcomeric \( \alpha \)-actinin and \( \beta \)III-tubulin (Figure 4E), and counting the percentages of EBs positively stained with \( \beta \)III-tubulin or containing the beating area (Figure 4F), also revealed that SB-431542 treatment in the
early phase resulted in impaired cardiac differentiation and enhanced neuronal differentiation. These results indicated that, in the early phase, activation of Smad2 is indispensable for mesodermal and endodermal differentiation, and once this pathway is inhibited, neuroectodermal differentiation is enhanced.

Tbx5 Is Markedly Upregulated by SB-431542 Treatment in the Late Phase

The effect of SB-431542 treatment in the late phase on the expression of cardiac transcription factors such as Nkx2.5, GATA4, Tbx5, Tbx20, Mef2C, myocardin, and isl1 was examined at day 8 (Figure 5A). Among these factors, GATA4, Tbx5, Tbx20, and Mef2C were significantly upregulated by treatment with SB-431542. The upregulation of Tbx5 was remarkable and reached a level ~6-fold that of the untreated control, implying a role of increased Tbx5 expression in augmented cardiomyogenesis by Smad2 inhibition in the late phase.

Endogenous TGF-β and Activin Negatively Regulate Cardiomyogenesis in the Late Phase

To determine the responsible endogenous ligands for the inhibition of cardiomyogenesis through Smad2 activation in the late phase, effects of neutralizing antibodies against TGF-β and activin on cardiomyogenesis were examined (Figure 5B). Although treatment with either anti-TGF-β or anti-activin antibody in the late phase resulted in a significant increase in cardiomyogenesis, anti–TGF-β neutralizing antibody was clearly more potent than anti-activin antibody, exhibiting a 2-fold increase in cardiomyogenesis (Figure 5B). To further reveal the role of endogenous TGF-β and Smad2, the effect of a soluble TGF-β type II receptor (sTGF-βIIR) and a dominant negative mutant of Smad2 (Smad2 DN) was analyzed (Figure 5C through 5F). Cells were infected with an adenovirus expressing sTGF-βIIR or β-galactosidase at day 0 or 5 and analyzed for cardiomyogenesis. Inhibition of TGF-β action by sTGF-βIIR indeed attenuated Smad2 phosphorylation in the late phase (Figure 5D) and augmented cardiomyogenesis, as assessed by the expression of cardiac genes (Figure 5D). Even when the cells were infected at day 0 with an adenovirus expressing sTGF-βIIR, cardiomyogenesis was increased to the similar extent to the cells infected at day 5 (Figure 5D). Furthermore, infection of cells with an adenovirus expressing Smad2 DN at day 5, which indeed inhibited Smad2 activation in the late phase (Figure 5E), enhanced cardiomyogenesis (Figure 5F). Thus, endogenous
TGF-β and activin act as negative regulators of cardiomyogenesis in the late phase, and TGF-β–mediated Smad2 activation plays a major role in the negative control of cardiomyogenesis.

TGF-β–Smad2 Signaling Negatively Modulates Both Proliferation and Differentiation of Cardiac Myocytes

As TGF-β–Smad2 signaling is known to be a multifunctional factor that regulates proliferation as well as differentiation in various cell types, we sought to determine whether the inhibition of TGF-β–Smad2 signaling enhanced cardiomyogenesis by increasing proliferation of cardiac myocytes or directing the cells to differentiate into cardiac myocytes. To analyze cardiac myocytes and nonmyocytes individually, we sorted α-MHC–EGFP ES cells as EGFP-positive cardiac myocytes and EGFP-negative nonmyocytes at day 8. The proliferative activities were assessed by BrdUrd labeling and phospho–histone H3 staining. EGFP-positive cardiac myocytes retained greater replicative potential in ES cells expressing sTGF-β IIR or treated with SB-431542 than control cells, as the percentages of BrdUrd-incorporated and phospho–histone H3–positive cells were significantly higher in the cells expressing sTGF-β IIR or treated with SB-431542 (Figure 6A). The proliferation of EGFP-negative nonmyocytes was not altered by these interventions (Figure 6A). These results indicated that endogenous TGF-β–Smad2 signaling negatively modulates the proliferation of differentiated cardiac myocytes. As diverse signaling mediators have been implicated in the antiproliferative effect of TGF-β–Smad2 signaling including N-myc, c-myc, cdc25A, and cyclin-dependent kinase inhibitors,2 expression of these genes in cardiac myocytes and nonmyocytes was examined. sTGF-β IIR expression or SB-431542 treatment in the late phase upregulated N-myc, c-myc, cdc25A, and cyclin-dependent kinase inhibitors in cardiac myocytes, but not in nonmyocytes (Figure 6B). Furthermore, downregulated expression of p57KIP2, but not other cyclin-dependent kinase inhibitors such as p15INK4B, p21WAF1/CIP1, and p27KIP1, was observed specifically in cardiac myocytes (Figure 6B). In nonmyocytes, expression of p21WAF1/CIP1 was suppressed by sTGF-β IIR expression or SB-431542 treatment (Figure 6B).

To examine the role of TGF-β–Smad2 signaling in the differentiation process, EGFP-negative nonmyocytes were sorted at day 8 from ES cell culture with or without sTGF-β IIR or SB-431542 treatment (Figure 7A). Expression of α-MHC–EGFP in cardiac myocytes and nonmyocytes was examined at day 12. Expression of α-MHC–EGFP was analyzed at day 8. *P<0.05 vs control. #P<0.05 vs anti-activin antibody. C and D, Naïve ES cells were infected with an adenovirus expressing sTGF-β IIR or LacZ at day 0 or day 5 and cultured until day 12. Expression of sTGF-β IIR and phosphorylation of Smad2 (C), and expression of α-MHC and cTnI (D) was analyzed. *P<0.05 vs control. E and F, Naïve ES cells were infected with an adenovirus expressing Smad2 DN or LacZ at day 5 and cultured for 7 more days. Phosphorylation of Smad2 (E) and expression of α-MHC and cTnI (F) were analyzed. *P<0.05 vs control.
7B through 7D) and cultured for 7 more days. In the case of SB-431542 treatment, cells were treated as indicated in Figure 7B: control; not treated, control to SB; treated only after sorting at day 8, SB; treated from day 5 to day 15, SB-431542 to (-); treated only from day 5 to day 8.

Cardiomyogenesis was monitored by the expression of cardiac genes (Figure 7A and 7C), and the proportion of EGFP-positive cardiac myocytes (Figure 7D). Although the differentiation of EGFP-negative nonmyocytes into EGFP-positive cardiac myocytes was observed in control cells, more EGFP-positive cells were apparent in the cells expressing sTGF-β IIR or treated with SB-431542. Expression of cardiac genes α-MHC and cTnI was significantly enhanced by sTGF-β IIR expression (Figure 7A). Treatment with SB-451542 enhanced not only the expression of cardiac genes (Figure 7C) but also the proportion of cardiac myocyte-
differentiated cells (Figure 7D), although treatment after cell sorting was critical for SB-431542 to augment cardiomyogenesis. These results suggested that inhibition of TGF-β–Smad2 signaling in the late phase resulted in not only augmented proliferation of cell that already differentiated into cardiac myocytes but also enhancement in cardiac myocyte differentiation.

**Discussion**

In this study, we found that Smad2 was activated bimodally in the early and late stages of ES cell differentiation, and gene expression of Smad2-activating ligands suggested that Smad2 activation in the undifferentiated and early phase was mediated by Nodal/Cripto. The roles of these components in early mammalian embryogenesis have been studied with genetic modifications in mice. Null mutants of Smad2 or activin receptor IIB result in a malformed primitive streak and failure of mesoderm to form.12,13 Mice deficient in both Smad2 and Smad3 display a similar but more severe developmental phenotype than Smad2-null mutants, with a complete failure of gastrulation.14 Our study showed that the early activation of Smad2 in ES cell differentiation was dispensable for mesodermal and endodermal induction, and its inhibition led to enhanced neuroectodermal induction. By using Cripto-deficient ES cells, Cripto is shown to be essential for cardiogenesis especially in the initial few days of ES cell differentiation.15 A blockade of Nodal signaling by antagonists such as Lefty and Cerberus-short leads to extensive neuroectodermal development,16 whereas Nodal gain-of-function experiments result in inhibition of neuroectodermal development.17 Thus, our results with others indicated that Nodal/Cripto-dependent Smad2 activation is required for endodermal and mesodermal induction, and once this pathway is inhibited, neuroectodermal induction is augmented, supporting the neuroectoderm default model.17

Once inactivated at day 5, Smad2 was activated again at day 8, which was evident until day 12. During this period, TGF-βs and activin were induced to be expressed, suggesting that Smad2 activation in the late phase was stimulated by TGF-βs and activin. Our results with the inhibition of Smad2 activation in this late phase indicated that the late-phase activation is inhibitory to cardiomyogenesis of ES cells. Furthermore, with the neutralizing antibodies against TGF-β and activin, it was shown that both ligands are inhibitory to cardiomyogenesis, although TGF-β clearly plays a major role in suppressing cardiomyogenesis in the late phase. This was also demonstrated by the experiments with an adenovirus expressing sTGF-β IIR, in which blockade of TGF-β action resulted in enhanced cardiomyogenesis to a similar extent as anti–TGF-β neutralizing antibody. Enhanced cardiomyogenesis by the expression of sTGF-β IIR was observed even when cells were infected at day 0, suggesting TGF-β plays a major role in cardiomyogenesis in the late phase. In heart development, TGF-β has been shown to be critically involved in the formation of atrioventricular valvuloseptal and endocardial cushion tissues through a mechanism of epithelial–mesenchymal transition,18 whereas less is known about the functions of TGF-β in cardiac myocyte differentiation. Furthermore, infection of cells with an adenovirus expressing Smad2 DN at day 5, which indeed inhibited Smad2 activation in the late phase, enhanced cardiomyogenesis. Our results demonstrated that Smad2 activation by endogenous TGF-β and activin in the late phase suppresses cardiac differentiation. This seems in conflict with previous reports indicating that the priming of undifferentiated ES cells with TGF-β enhances mesodermal and cardiac differentiation19 and treatment with TGF-β2 augments cardiac differentiation of ES cells.20 However, as the functional signaling pathways including the receptors for TGF-β exist in the initial phase of ES cell differentiation,19,21 and our results indicated that Smad2 activation in the initial phase was indispensable for mesodermal induction, the effect of exogenous TGF-β in these studies might be through enhancing the induction of mesodermal lineages. Furthermore, TGF-β is secreted by extraembryonic tissues and also is expressed in early embryos at the stage of blastocysts in vivo.22 As the interaction between the extraembryonic cell types and the primitive ectoderm, from which ES cells are derived, plays an integral role in mammalian embryonic development,23 TGF-β from the extraembryonic tissues might be involved in mesodermal induction in vivo. However, as mouse ES cells are shown to irreversibly commit to an epiblast lineage and rarely spontaneously differentiate into trophoectoderm and primitive endoderm derivatives,23,24 autocrine Nodal and Cripto play an essential role in mesodermal induction in vitro ES cell differentiation.

Tbx5 expression was markedly upregulated by SB-431542 treatment in the late phase. Tbx5 is a member of T-box gene family, whose mutations are responsible for human Holt–Oram syndrome, a disease involving a congenital heart malformation.25 Tbx5 is shown to be expressed in cardiac crescent of murine embryos and regulates several cardiac genes such as ANP and connexin 40.25 Although loss of Tbx5 alone does not prevent cardiac differentiation, overexpression of Tbx5 in P19Cl6 cells accelerates cardiac differentiation but is not sufficient to promote the commitment to cardiac lineage in the absence of the inducing agent DMSO.25 Furthermore, a recent study revealed that Tbx5 regulates the embryonic proliferation of cardiac myocytes,26 whereas the effect of Tbx5 on cardiac cell growth might be phase specific.25,27 Thus, augmented expression of Tbx5 could be involved in the enhanced cardiomyogenesis induced by Smad2 inhibition, although further studies are needed to elucidate the functional relationship between Tbx5 expression and enhanced cardiomyogenesis.

TGF-β superfamily is known to regulate a plethora of biological responses such as cell growth, differentiation, and matrix production, and the effects of TGF-β depend on the type and status of the cells, sometimes producing opposing effects such as enhancing or suppressing cellular growth.1 Several growth factors such as fibroblast growth factors, neuregulin, and insulin-like growth factor I have been shown to trigger the proliferation of myocardial cells.28,29 However, less is known about the endogenous negative regulators of cardiac cell proliferation. By analyzing cardiac myocytes and nonmyocytes separately, we showed that TGF-β–Smad2 signaling is an endogenous negative regulator of cardiac cell proliferation. The increase in proliferative capacity in cardiac myocytes was associated with an upregulation of the expres-
sion of several cell cycle regulators, which have been shown to be targets of TGF-β signaling, such as N- and c-myc, cyclin A2, cdc25A, and p57KIP2. Transgenic expression of c-myc or cyclin A2 in the heart leads to enhanced hyperplastic growth of cardiac myocytes.28 Mice deficient in N-myc show a hypocellular myocardium.30 p57KIP2 is first detectable in the developing heart at embryonic day (E)10.5 among multiple cell cycle regulators,31 and elevated expression of p57KIP2 is associated with a dramatic reduction in proliferative activity in cardiac myocytes in BMP-10–null mice.28 Transgenic expression of a constitutive active mutant of ALK5 in mice hearts causes hypoplastic hearts in addition to arresting cardiac looping morphogenesis.28 These results indicated that inhibition of TGF-β by aspartic acid and endodermal induction and the subsequent cardiac differentiation through the control of cell cycle regulators, although the effects might not be exclusive to cardiac myocytes, as the nonmyocyte population contained various types of cells. Furthermore, inhibition of TGF-β function led to enhanced cardiac differentiation of nonmyocytes, suggesting that inhibition of TGF-β-Smad2 signaling directed the cells to differentiate into cardiac myocytes. However, as spontaneous differentiation from nonmyocytes to cardiac myocytes was observed in control cells, it could not be excluded that TGF-β–Smad2 inhibition modulated the proliferation of spontaneously differentiated cardiac myocytes, leading to augmented cardiac differentiation.

Our study demonstrated that ES cell differentiation exhibited a unique bimodal activation of Smad2 pathway, in which Nodal/Cripto and TGF-β/activin were responsible for the early and late activation, respectively (Figure 8). Furthermore, the early activation was indispensable for the mesodermal and endodermal induction and the subsequent cardiac differentiation, and the late activation, especially mediated by TGF-β, was inhibitory to cardiomyogenesis through attenuating the proliferation and differentiation of cardiac myocytes. Thus, endogenous TGF-β/Nodal/activin–Smad2/3 signaling has temporally distinct, stage-specific roles in cardiomyogenesis in ES cells. These findings revealed the novel roles of Smad2 signaling in cardiac differentiation of ES cells and may contribute to the efficient production of cardiac myocytes from ES cells for various applications.

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Disclosures
None.

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Materials and methods

ES Cell Culture and Differentiation

CGR8 mouse ES cells and ES cells stably transfected with α-myosin heavy chain (MHC) promoter-driven EGFP were cultured as described previously, except substituting 15% Knockout Serum Replacement (Invitrogen) for fetal bovine serum. Differentiation was induced by forming embryoid bodies (EB) in the hanging drop suspension culture. SB-431542 was purchased from Tocris. Neutralizing monoclonal antibodies against Activin A and TGF-βs were obtained from R&D systems.

RT-PCR and Immunoblot Analysis

Gene expression was analyzed by semi-quantitative PCR or kinetic real time PCR. For sorted cells, total RNA was exacted using RNeasy Micro kit (Qiagen) and analyzed with SuperScript III Platinum SYBR Green One-Step qPCR kit (Invitrogen). The primer sequences and TaqMan Gene Expression Assays used in this study are available upon request. Immunoblot analysis was performed with a primary antibody against Smad1, Smad2/3, TGF-β receptor type II (Millipore), phospho-Smad1/5/8, phospho-Smad2 (Cell Signaling Technology), sarcomeric myosin (MF20;
Developmental Studies Hybridoma Bank), α-actinin (EA-53; Sigma) or GAPDH (Chemicon). For TGF-β receptor type II, the conditioned medium from the cells infected with an adenovirus was analyzed. Densitomeric analysis was performed using ImageJ software.

**Replication-defective Recombinant Adenoviruses**

Adenoviral vectors expressing a soluble type II TGF-β receptor (sTGF-βIIR), dominant negative mutant of Smad2 or β-galactosidase (LacZ) were prepared as described previously. Adenoviral titer was determined by scoring the cytopathic effect in 293T cells using the tissue culture infectious dose 50 method. Cells were infected with adenoviruses at an MOI of 100 in day 0 and at an MOI of 500 in day 5, which produced an almost 100% rate of infection.

**Flow Cytometric Analysis and Cell Sorting**

α-MHC-EGFP ES cells were dissociated with collagenase type 2 (Worthington), washed, and then immediately analyzed with a FACSCalibur Flow Cytometer using CellQuest acquisition and analysis software (BD Biosciences). Total events of 20,000
were analyzed in each sample. Cell sorting was performed with FACSaria cell sorter using FACSDiva software (BD Biosciences).

**Immunostaining**

Cells were stained with the primary antibody against sarcomeric α-actinin or βIII-tubulin (Sigma), followed by Alexa Fluor 555-conjugated rabbit anti-mouse IgG secondary antibody (Invitrogen). Nuclear staining was performed with DAPI. For phospho-histone H3 staining, sorted cells were centrifuged onto polylysine slides (Cytospin, Shandon), and stained with anti-phospho-histone H3 antibody (Millipore) and Alexa Fluor 568-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen).

**BrdU Incorporation Assay**

Cells were labeled with 10 μmol/L BrdU for 2 hours at day 8. After being washed, cells were dissociated with collagenase type 2, sorted, centrifuged onto polylysine slides, fixed in 100% ethanol, and stained with anti-BrdU antibody (Roche) and Alexa Fluor 555-conjugated rabbit anti-mouse IgG.
Statistical Analysis

All experiments were performed at least 3 times, and data were expressed as mean ± standard deviation, and analyzed by Student’s t test or One-way ANOVA with post hoc analysis. A value of $P<0.05$ was considered statistically significant.

References


