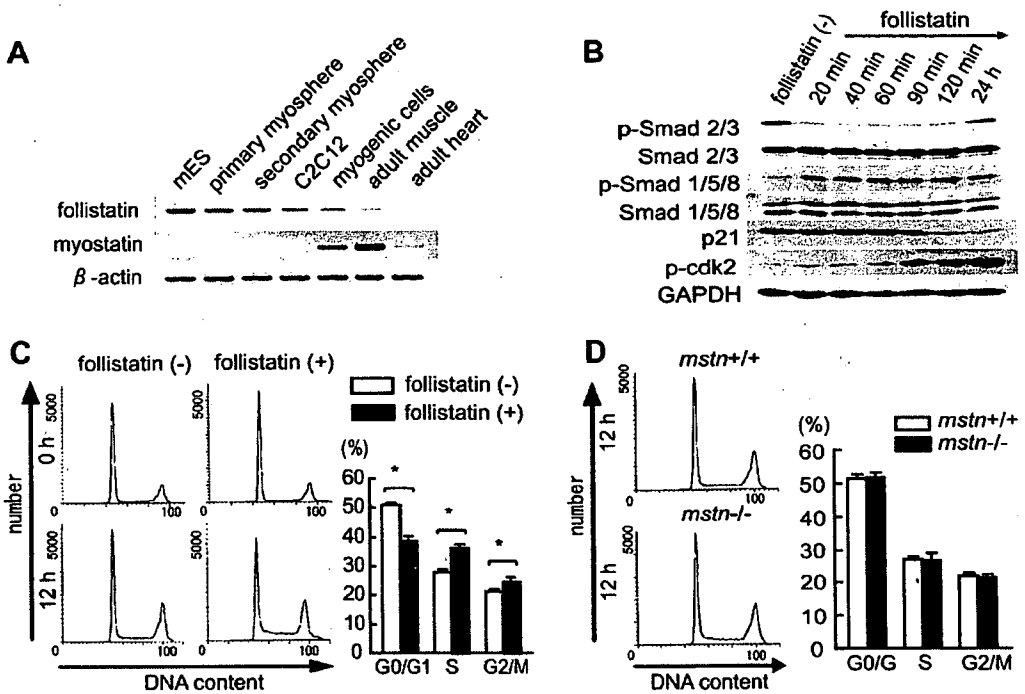

Fig. 2. Intrinsic myostatin is not the follistatin-sensitive ligand to regulate the growth of MDPCs through inhibition of Smad2/3 phosphorylation. (A) RT-PCR for myostatin and its antagonist, follistatin, in myospheres. (B) Actively proliferating myospheres treated with follistatin were characterized by the inhibition of Smad2/3 phosphorylation, down-regulation of p21, and activation of Smad1/5/8 and cdk2. (C) FACS analysis. MDPCs were cultured with or without follistatin (20 nM) for 7 days followed by 24 h serum-free starvation. The cells were then harvested at 0 and 12 h after cell culture in growth medium and subjected to flow cytometry analysis ($n = 5$). G0/G1, S, and G2/M phases of cell cycle for each treatment are summarized on the right. $^*p < 0.01$. (D) FACS analysis. MDPCs were isolated from *mstn*−/− or *mstn*+/+ mice and were grown in the growth medium for 12 h before harvesting. Cell cycle distribution is summarized on the right ($n = 5$).

cell cycle progression in MDPCs cultured in growth medium for 12 h (Fig. 2C).

Since it is known that follistatin inhibits the binding of myostatin to ActRII, we next focused on myostatin for further investigation because of its potential role in negative regulation of skeletal muscle growth [12,15]. For this purpose, myospheres were generated using the cells isolated from myostatin null (*mstn*−/−) and from age-matched littermate controls (*mstn*+/+) and examined the cell cycle distribution of the MDPCs. We subjected synchronous, actively proliferating MDPCs cultured in growth medium for 12 h to FACS analysis and found that MDPCs isolated from *mstn*−/− and littermate *mstn*+/+ mice had a similar cell cycle progression profile from mitosis through G1 to the next S phase (Fig. 2D).

ActA and GDF11 negatively regulate MDPC growth

Because Smad1/5/8 are thought to be specific for bone morphogenetic protein (BMP) 2/4/7 signaling [16], we next investigated whether endogenous activin and TGF-β family signaling that might be blocked by follistatin and might regulate myosphere proliferation via Smad2/3 phosphorylation [10,11,16]. RT-PCR analysis showed that myospheres were positive for activin A/B and GDF11 but not nodal expression (Fig. 3A), suggesting that the follistatin-induced inhibitory interaction of ActRII to activin and GDF11 binding might produce the rapid progression of myospheres.

To directly test whether the ActA and GDF11 signaling is required for the MDPC development, we targeted endogenous expression of ActA and GDF11 by using siRNA technology. We first assessed the efficacy of the siRNAs by testing their ability to inhibit endogenous protein levels of ActA and GDF11. The specificity of the siRNAs for ActA and GDF11 was verified by the use of uninfected MDPCs and Mock controls (Figs. 3B and C). The most effective siRNA for each was selected for use in the following experiments. MDPCs infected with siRNA of either ActA or GDF11 showed a significant increase in the replicative potential during 21 days in culture, in comparison to the Mock control (Fig. 3D).

Knockdown of ActA and GDF11 accelerates cell cycle progression in MDPCs

To quantitate the effects of ActA and GDF11 on the MDPC development, we next carried out FACS analysis. We synchronized the MDPCs in G0 phase by culturing the cells in serum-free conditions. When MDPCs were stimulated by serum treatment for 16 h, 46.3 ± 0.4% and 52.1 ± 0.8% of control and GDF11-silenced MDPCs,

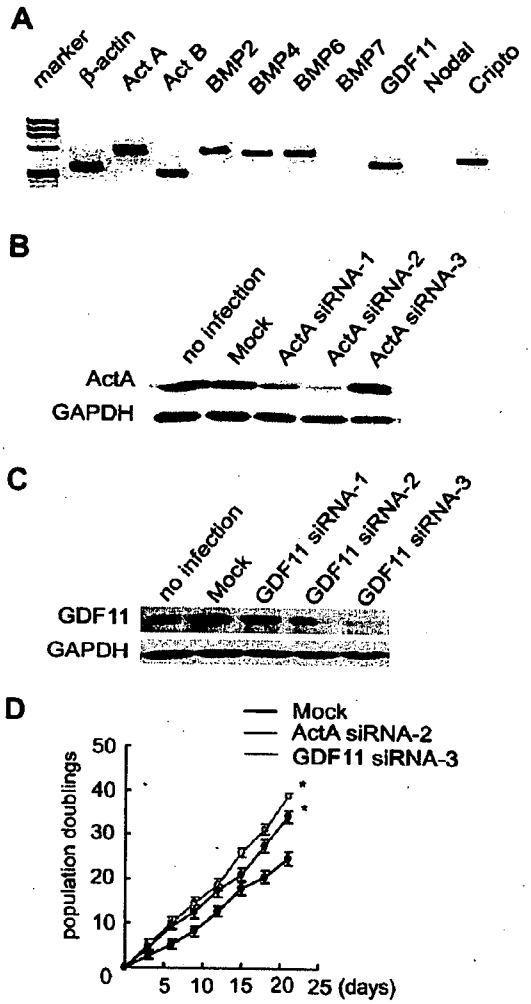

Fig. 3. ActA and GDF11 negatively regulate MDPC growth. (A) RT-PCR for the expression of ligands in activin and TGF-β family signaling. Protein levels of ActA (B) and GDF11 (C) following specific-siRNA infection. Three different siRNAs, as indicated, were tested for each. (D) Growth kinetics of MDPCs infected with the respective siRNAs as indicated. Data were obtained for 3 independent clones from MDPCs treated with each siRNA. $^{*}p < 0.01$.

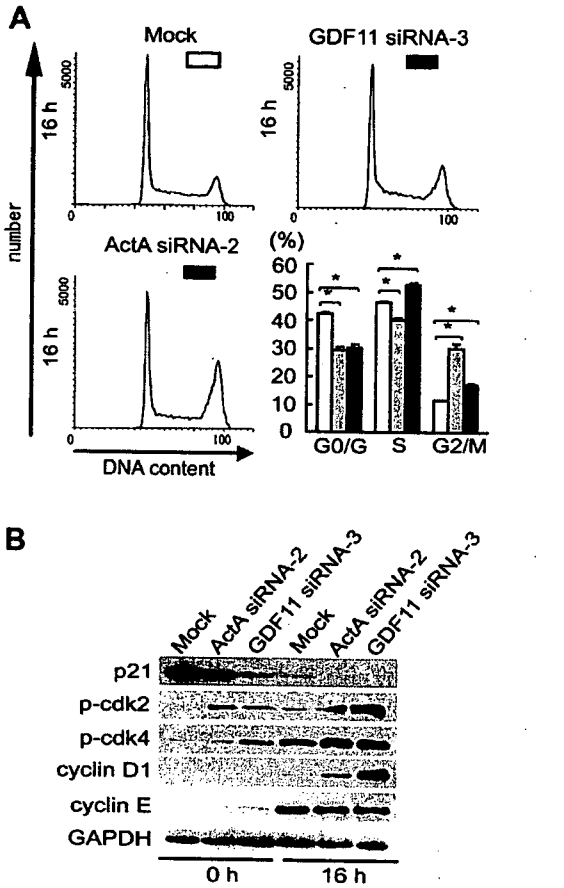

Fig. 4. Knockdown of ActA and GDF11 accelerates cell cycle progression through down-regulation of p21, activation of cdk2/4, and cyclin D1 induction in MDPCs. (A) At the start of the experiment, MDPCs were infected with Mock, ActA, or GDF11 siRNA. After 24 h in serum-free conditions, the medium was changed to growth medium and the cells were cultivated for 16 h before being subjected to FACS. Mean values ± SE of 5 independent experiments are shown. $^{*}p < 0.01$. (B) Equal amounts of protein from MDPCs obtained from the cells infected as indicated were used to evaluate the expression of cell cycle regulators by Western blotting. Cell cycle arrest was induced by serum-free culturing followed by growth medium stimulation for 16 h. The results shown are representative blots obtained from 5 independent experiments.

respectively, entered in S phase (Fig. 4A, $p < 0.01$). Analysis of the cell cycle distribution at 16 h in ActA knockdown MDPCs showed that the cells had been driven into G2/M phase (30.2 ± 1.6%), whereas only a small control-cell population was present in mitotic phase (Fig. 4A; 11.4 ± 0.3%, $p < 0.01$).

Given that endogenous ActA and GDF11 regulate the process of cell cycle withdrawal of MDPCs, we speculated that these ligands may affect the cell-cycle-regulatory proteins. To examine this possibility, actively proliferating MDPCs infected with Mock or siRNA of ActA or GDF11 were cultured in growth medium for 16 h. Before serum stimulation (0 h), ActA and GDF11 siRNA-infected MDPCs demonstrated down-regulation of the p21 levels coincident with increased activity of cdk2/4 (Fig. 4B). These changes were significantly enhanced when the MDPCs were treated with serum for 16 h. In addition, undetectable or low levels of cyclin D1/E were present in MDPCs prior to the serum stimulation, and following exposure to serum for 16 h, the protein level of cyclin D1 was significantly increased in MDPCs with ActA or GDF11 knockdown (Fig. 4B).

Discussion

The enhancement of muscle regeneration by specific growth factors has been reported [8,9]; however, factors that might activate muscle-derived cardiogenic progenitor cells remain unknown. Previous studies have clearly shown that myosin light chain promoter-driven transgenic mice of follistatin or a dominant-negative form of ActRII and *mstn*−/− mice exhibited large increases in muscle mass

through satellite cell proliferation [12,15]. However, the absence of myostatin expression in myospheres highlighted the distinctness of their phenotype from that of satellite cells and also suggested that endogenous ActA and GDF11 appeared to be the plausible candidates to negatively regulate MDPC self-replication through Smad2/3 phosphorylation, which has been observed in ES cells [17].

As previously shown in other cell types, follistatin has been reported to activate hepatic satellite cells and neural progenitors through inhibiting the action of ActA and GDF11 [11,18]. Endogenously expressed ActA and/or GDF11 in myospheres, both signaling can be inhibited by follistatin, may act as negative regulators of the cardiogenic progenitor cell number, whereas follistatin can also induce myoblast fusion through neutralizing myostatin [19].

Our data demonstrated for the first time that follistatin induced cell cycle progression of MDPCs through inhibition of ActA and GDF11 signaling. Inhibition of the endogenous expression of ActA and GDF11 in MDPCs may increase cyclin D1 levels through Smad2/3 inactivation, which is in contrast to the recent report showing that exogenous myostatin signaling promoted cyclin D1 degradation independently of the Smad3 pathway in C_2C_{12} cells [20]. Myostatin is known to repress myogenic cell proliferation through up-regulation of p21 expression and inhibition of cdk2 activity [21]. The lack of alteration of cdk4 and cyclin D1 protein levels by myostatin treatment in C_2C_{12} cells highlighted the existence of distinct signaling pathway mediated through activin and GDF11 in MDPCs [22]. Our results suggest that follistatin may be an effective reagent to promote muscle-derived cardiogenic progenitor cell growth ex vivo for application in regenerative medicine.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.11.087.

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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 diseases and pharmacological testing on cardiac myocytes.[1] However, the molecular mechanisms governing differentiation of ES cells into specific lineages are poorly understood, and their comprehension would improve the efficiency of differentiation into specific cell types.

Members of transforming growth factor (TGF)-β superfamily are pleiotropic cytokines involved in many biological processes and signaling via heteromeric complexes of type I and type II serine/threonine kinase receptors.[2] On ligand binding and heterodimerization, the constitutively active type II receptor kinase phosphorylates the type I receptor, which in turn activates downstream signal transduction cascades including Smad pathways. According to the usage 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.[2] 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.[2,3] 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 vivo, and anterior lateral mesoderm explant cultures in vitro,[4] and Noggin, one of soluble BMP antagonists, prevents myocardial differentiation of lateral mesendoderm culture in vitro.[4] The dependence of myocardial specification on BMP signaling is evolutionally conserved, although several members of BMP have overlapping function in murine cardiac differentiation.[5] In murine teratocarcinoma P19CL6 cells, myocardial differentiation is shown to depend on functional BMP signals,[6] 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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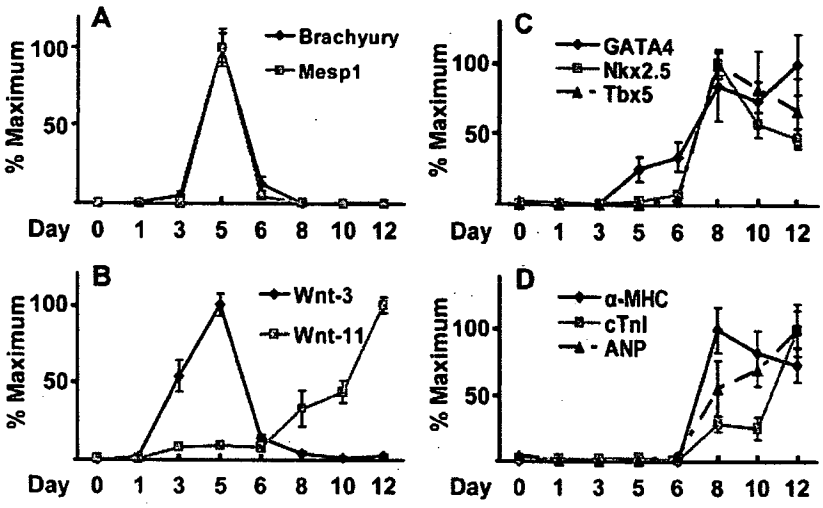

Figure 1. Cardiac differentiation programs in ES cells. ES cells were cultured in hanging drop suspension cultures for 5 days and then on gelatin-coated dishes for 7 more days. Cells were harvested at the indicated time points. Gene expression was analyzed by real-time kinetic PCR (A through D). A, Brachyury,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

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 α-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-β receptor (sTGF-β IIR), dominant negative mutant of Smad2 (Smad2 DN), or β-galactosidase (LacZ) were prepared as described.[8,9]

Flow Cytometric Analysis and Cell Sorting

α-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 α-actinin or βIII-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 α-MHC and cardiac troponin (cTn)I and a cardiac-specific peptide, atrial natriuretic peptide (ANP), were expressed after day 8 (Figure 1D). These results were consistent with the previous reports[1,10] 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-β/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 Smads (Figure 2A and 2B). In undifferentiated ES cells, although both Smad1 and Smad2 were expressed, only Smad2 was robustly phosphorylated. Phosphorylation of Smad1 was detected at day 5, which remained detectable thereafter. In marked contrast, Smad2 phosphorylation was decreased on induction of differentiation and disappeared at day 5. Phosphorylated Smad2 was detected again at day 8 and remained detectable until day 12. The expression of Smad2 was also regulated during ES cell differentiation. The expression of Smad2 was decreased during the initial phase and increased again in the late phase (Figure 2A and 2B), whereas the biphasic activation of Smad2 was still evident even after normalized with the expression of Smad2 (Figure 2B). These results indicated that Smad1 and Smad2 are differently regulated and that Smad2 shows unique bimodal activation in ES cell differentiation. As Smad1 activation has been shown to be involved in cardiomyogenesis, we focused on the role of Smad2 activation in cardiomyogenesis. We, therefore, analyzed the gene

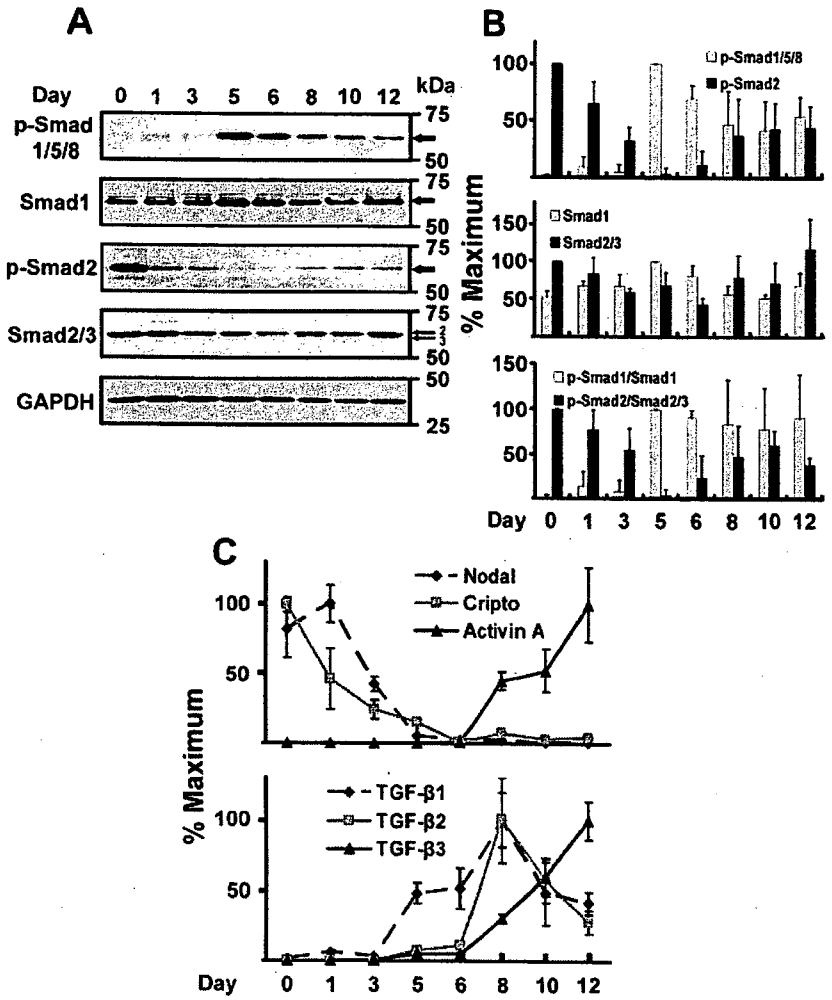

Figure 2. Activation of Smads and expression of TGF-βs, activin, Nodal, and Cripto during ES cell differentiation. A, Smads were analyzed by immunoblot analysis with the indicated antibodies. The corresponding bands are indicated with the arrows. B, Densitometric analysis was performed for Smads. The results are expressed as percentages of the maximum. C, Expression of Smad2-activating TGF-β family ligands Nodal, Cripto, activin, and TGF-βs was analyzed by real-time kinetic PCR. The results are expressed relative to the level of 18S ribosomal RNA and plotted as percentages of the maximum.

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-β_1 and TGF-β_2 peaked at day 8 and then declined, whereas TGF-β_3 continued to be upregulated until day12 (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 control, SB+/+; treated throughout differentiation, SB+/−; treated for only the first 5 days, SB−/+; 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,

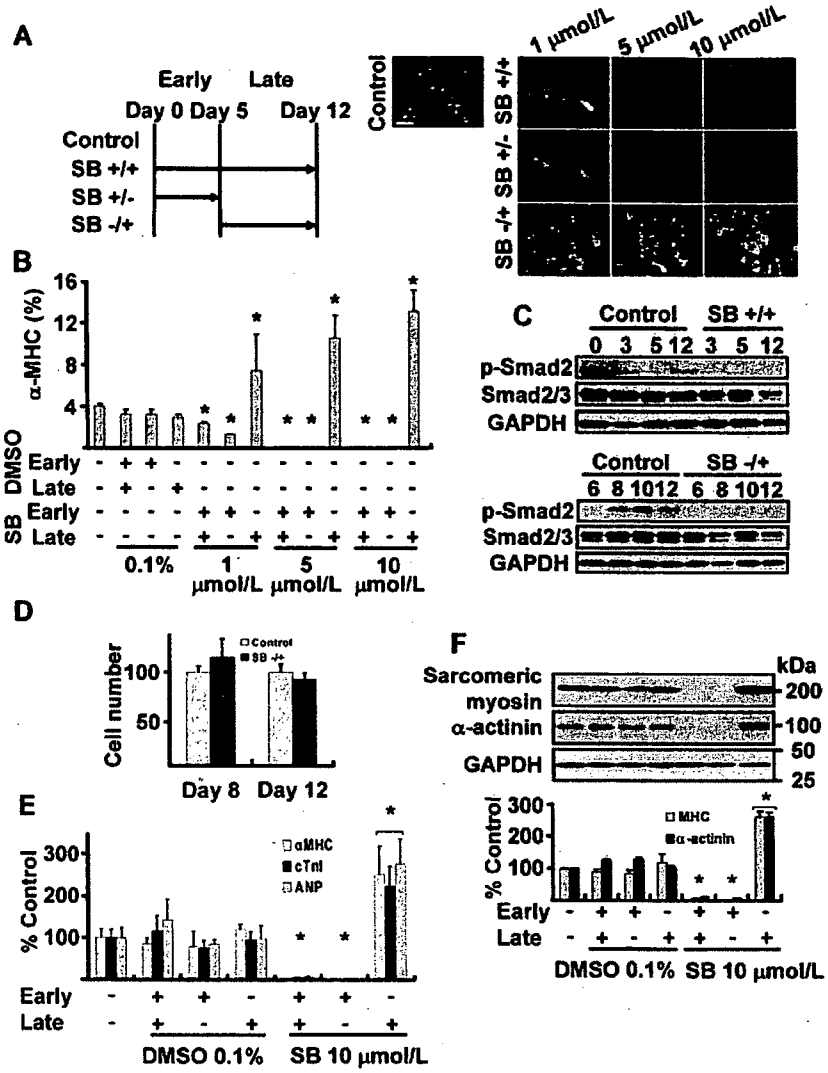

Figure 3. Effect of Smad2 inhibition on cardiac differentiation in ES cells. A, α-MHC–EGFP ES cells were treated with the various concentrations of SB-431542 (SB) for the indicated periods of time. Cardiac differentiation was monitored by the appearance of EGFP-expressing cells. B, The proportions of EGFP-positive cardiac myocytes were examined by flow cytometric analysis. C, The effect of SB-431542 on Smad2 phosphorylation was assessed by immunoblot analysis in naïve ES cells. D, Naïve ES cells were treated with or without SB-431542 as indicated, and the total number of viable cells was counted with trypan blue exclusion at days 8 and 12. E, Nontransfected naïve ES cells were treated with or without SB-431542 or DMSO as indicated, and the expression of α-MHC, cTnI, and ANP was analyzed. The results are plotted as percentages of the control value. F, Expression of sarcomeric myosin and α-actinin was examined by immunoblot analysis. *$P<0.05$ vs respective DMSO control.

cardiomyogenesis was markedly augmented in a dose-dependent manner. At 10 μmol/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 α-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 α-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 ≈3-fold increase when treated only in the late phase (Figure 3E). Furthermore, protein expression of sarcomeric myosin and α-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 α-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 α-actinin and βIII-tubulin (Figure 4E), and counting the percentages of EBs positively stained with βIII-tubulin or containing the beating area (Figure 4F), also revealed that SB-431542 treatment in the

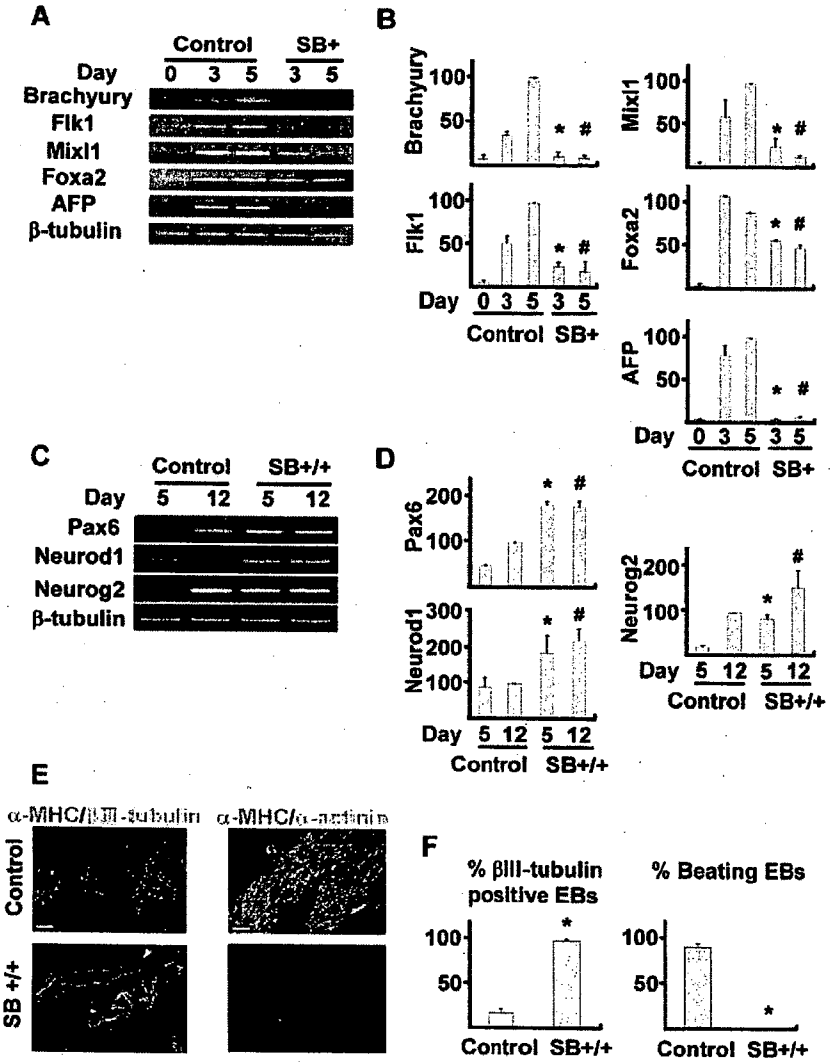

Figure 4. Inhibited mesodermal and endodermal formation and augmented neuroectodermal formation by treatment with SB-431542 in the early phase. Naïve ES cells were treated with or without SB-431542 and harvested at the indicated time points. A and B, The expression of mesodermal markers such as Brachyury and Flk1 was analyzed by RT-PCR. *$P<0.05$ vs control at day 3, #$P<0.05$ vs control at day 5. C and D, The expression of neuronal markers such as Pax6, Neurod1, and Neurog2 was analyzed. *$P<0.05$ vs control at day 5, #$P<0.05$ vs control at day 12. E, α-MHC–EGFP cells were treated with or without SB-431542 and immunostained with antibody against βIII-tubulin or sarcomeric α-actinin at day 12. F, The proportions of EBs positively stained with βIII-tubulin or containing the beating area were counted. *$P<0.05$ vs control.

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-β IIR)[9] and a dominant negative mutant of Smad2 (Smad2 DN)[8] was analyzed (Figure 5C through 5F). Cells were infected with an adenovirus expressing sTGF-β IIR or β-galactosidase at day 0 or 5 and analyzed for cardiomyogenesis. Inhibition of TGF-β action by sTGF-β IIR indeed attenuated Smad2 phosphorylation in the late phase (Figure 5C) 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-β IIR, 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

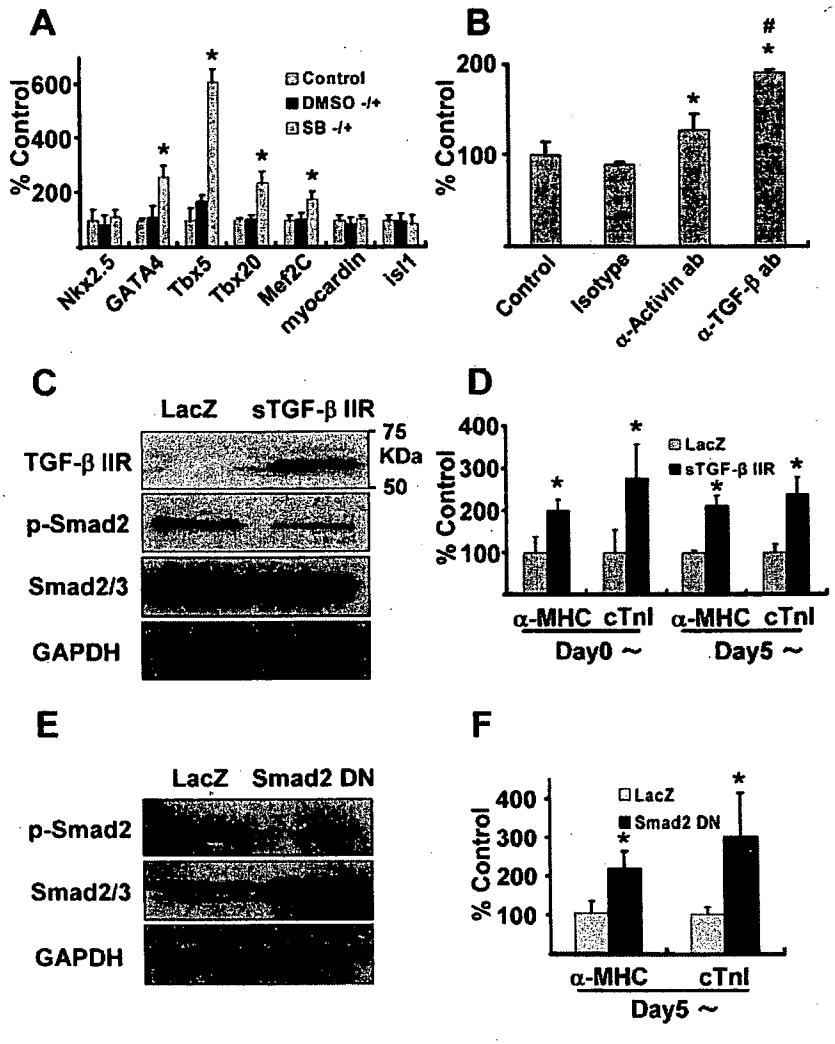

Figure 5. Effect of SB-431542 on cardiac transcription factor expression, effects of neutralizing antibodies against TGF-β and activin, and adenovirus-mediated expression of sTGF-β IIR and Smad2 DN. A, Naïve ES cells were treated with or without SB-435142 after day 5. The expression of Nkx2.5, GATA4, Tbx5, Tbx20, Mef2C, myocardin, and isl1 was analyzed at day 8. B, α-MHC–EGFP ES cells were treated with or without a neutralizing antibody against TGF-β or activin or isotype-matched control antibody after day 5, and the proportion of EGFP-expressing cardiac myocytes was analyzed at day 12. *$P<0.05$ 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.

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, cyclin A2, and cdc25A specifically in cardiac myocytes, but not in nonmyocytes (Figure 6B). Furthermore, downregulated expression of $p57^{KIP2}$, but not other cyclin-dependent kinase inhibitors such as $p15^{INK4B}$, $p21^{WAF1/CIP1}$, and $p27^{KIP1}$, was observed specifically in cardiac myocytes (Figure 6B). In nonmyocytes, expression of $p21^{WAF1/CIP1}$ 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 expression (Figure 7A) or SB-431542 treatment (Figure

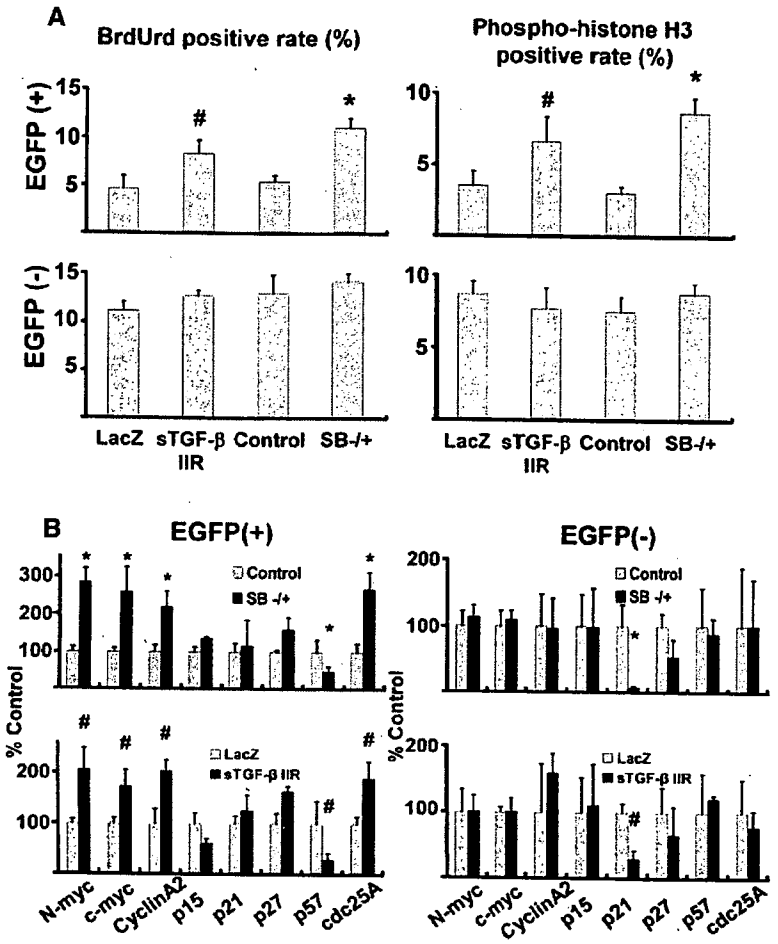

Figure 6. Inhibition of TGF-β–Smad2 signaling in the late phase enhances proliferation of cardiac myocytes. α-MHC–EGFP ES cells were infected with an adenovirus expressing sTGF-β IIR or LacZ at day 5, or treated with or without SB-431542 after day 5. Cells were labeled with BrdUrd at day 8 and then sorted as EGFP-positive cardiac myocytes and EGFP-negative nonmyocytes. A, Sorted cells were stained for BrdUrd and phospho–histone H3, and the proportion of BrdUrd-positive or phospho–histone H3–positive cells was calculated among EGFP-positive cardiac myocytes and EGFP-negative nonmyocytes. #$P<0.05$ vs LacZ, *$P<0.05$ vs control. B, Real-time kinetic PCR was performed with the indicated specific primers. The results are expressed relative to the level of β-tubulin and plotted as percentages of the control value. #$P<0.05$ vs LacZ, *$P<0.05$ 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-451542. 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–

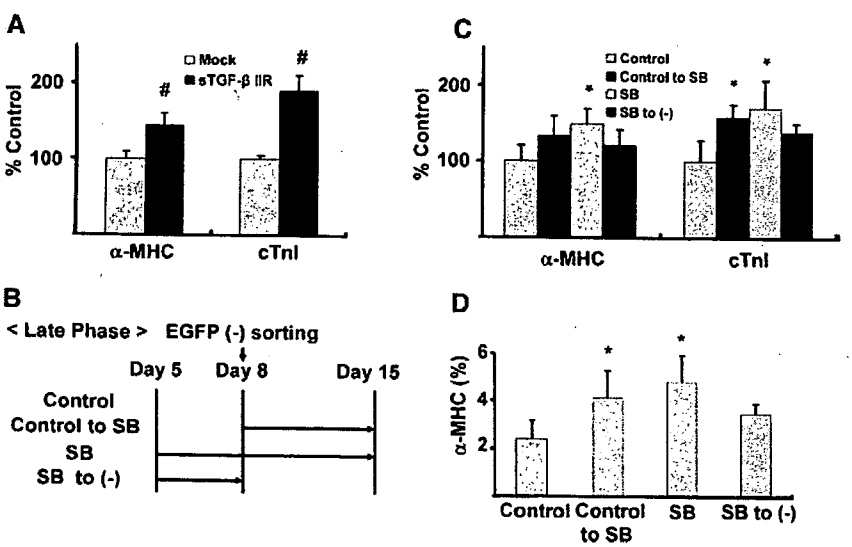

Figure 7. Inhibition of TGF-β–Smad2 signaling enhances the differentiation of EGFP-negative nonmyocytes into EGFP-positive cardiac myocytes. A, α-MHC–EGFP ES cells were infected with an adenovirus expressing sTGF-βIIR or LacZ at day 5. EGFP-negative nonmyocytes were sorted at day 8 and then cultured on gelatin-coated dishes for 7 more days. The expression of α-MHC and cTnI was examined. #$P<0.05$ vs LacZ. B, α-MHC–EGFP ES cells were treated with or without SB-431542. EGFP-negative nonmyocytes sorted at day 8 were cultured with or without SB-431542 for 7 more days as indicated. C, The expression of α-MHC and cTnI was examined. *$P<0.05$ vs control. D, The proportion of EGFP-positive cardiac myocytes was analyzed at day 15. *$P<0.05$ vs control.

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 indispensable 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 neuroectoderm development,[16] whereas Nodal gain-of-function experiments result in inhibition of neuroectoderm 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 differentiation[19] 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 trophectoderm and primitive endoderm derivatives,[23,24] autocrine Nodal and Cripto play an essential role in mesodermal induction in 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.[3] 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-

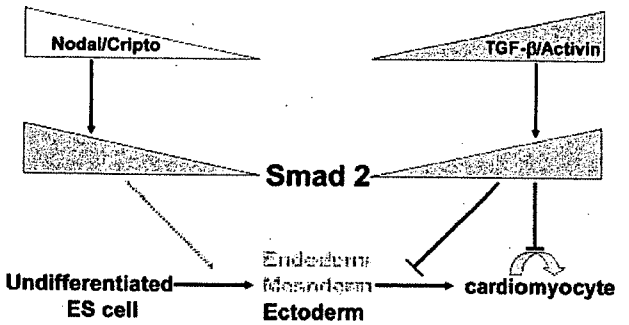

Figure 8. Schematic model of a stage-specific role of endogenous Smad2 activation in cardiomyogenesis of ES cells.

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 $p57^{KIP2}$. 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] $p57^{KIP2}$ is first detectable in the developing heart at embryonic day (E)10.5 among multiple cell cycle regulators,[31] and elevated expression of $p57^{KIP2}$ is associated with a dramatic reduction in proliferative activity in cardiac myocytes in BMP-10–null mice.[32] 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 endogenous TGF-β suppressed the proliferation of cardiac myocytes 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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Osteopontin is a myosphere-derived secretory molecule that promotes angiogenic progenitor cell proliferation through the phosphoinositide 3-kinase/Akt pathway

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Abstract

We have reported that skeletal myosphere-derived progenitor cells (MDPCs) can differentiate into vascular cells, and that MDPC transplantation into cardiomyopathic hearts improves cardiac function. However, the autocrine/paracrine molecules and underlying mechanisms responsible for MDPC growth have not yet been determined. To explore the molecules enhancing the proliferation of MDPCs, we performed serial analysis of gene expression and signal sequence trap methods using RNA isolated from MDPCs. We identified osteopontin (OPN), a secretory molecule, as one of most abundant molecules expressed in MDPCs. OPN provided a proliferative effect for MDPCs. MDPCs treated with OPN showed Akt activation, and inhibition of the phosphoinositide 3-kinase (PI3K)/Akt pathway repressed the proliferative effect of OPN. Furthermore, OPN-pretreated MDPCs maintained their differentiation potential into endothelial and vascular smooth muscle cells. These findings indicate an important role of OPN as an autocrine/paracrine molecule in regulating the proliferative growth of muscle-derived angiogenic progenitor cells via the PI3K/Akt pathway.

Keywords: Osteopontin; Skeletal muscle; Progenitor cells; Proliferation; Differentiation

Studies carried out in the past few years have shown adult skeletal muscle tissue contains stem cells able to differentiate into several lineages [1–5]. We have reported the isolation of multipotent progenitor cells from adult skeletal muscle tissue [6] on the basis of their proliferative potential to form floating-spheres (termed myospheres) [5]. Myosphere-derived progenitor cells (MDPCs) have phenotypic characteristics resembling mesenchymal stem cells and differentiate into endothelial and smooth muscle cells. When MDPCs were grafted into δ-sarcoglycan knockdown hearts that served as a model of cardiomyopathy, neoangiogenesis was enhanced and cardiac function was improved. Since skeletal muscle is an easily accessible tissue source, MDPCs have the potential for clinical application to treat patients with heart failure. For efficient autologous transplantation as a regenerative therapy, sufficient numbers of expanded MDPCs from small tissue samples will be required. However, little is known about the molecules and mechanisms controlling MDPC self-renewal.

In adult neural stem cells (NSCs), a glycosylated form of cystein C [7] and insulin-like growth factor-1 [8] are essential autocrine/paracrine molecules identified as cofactors of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), respectively. Stem cell-derived neural

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stem/progenitor cell supporting factor has been also identified as an autocrine/paracrine factor that facilitates the proliferative growth of adult NSCs [9]. Therefore, we anticipated that MDPCs could produce autocrine/paracrine factors that regulate their own proliferative growth. In the present study, we identified osteopontin (OPN, also referred to as secreted phosphoprotein 1), a secreted molecule that promotes the proliferative growth of MDPCs, and clarified the role of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway in MDPCs treated with OPN.

Materials and methods

MDPC isolation. MDPC isolation was performed as previously described [6]. Briefly, the primary hindlimb muscle cells were isolated from 8-week-old C57BL/6J mice (Shimizu Laboratories Supplies) using 470 U/ml collagenase type II (Worthington) for digestion. Cells were suspended in the isolation medium, which was DMEM/F12 (Invitrogen) supplemented with B27 (Invitrogen), 20 ng/ml EGF (SIGMA), and 40 ng/ml recombinant bFGF (Promega). Cell suspensions were then cultured onto a non-coated dish at 20 cells/μl density over 7 days to generate myospheres.

MDPC expansion and differentiation. Myospheres were picked and transferred into fibronectin-coated culture plates in an expansion medium composed of Advanced DMEM/F12 (Invitrogen), 200 μM L-glutamine (SIGMA), 2% fetal bovine serum (FBS), 20 ng/ml EGF, 10 ng/ml bFGF, and 10 ng/ml LIF (CHEMICON) to obtain MDPCs. Differentiation medium containing DMEM/F12 and 10% FBS supplemented with 10 ng/ml vascular endothelial growth factor (VEGF; R&D Systems) or 50 ng/ml platelet-derived growth factor-BB (PDGF; R&D Systems) was used to induce endothelial or smooth muscle cell differentiation, respectively.

Serial analysis of gene expression (SAGE). The SAGE libraries were constructed essentially following the I-SAGE long kit protocol (Invitrogen) using total RNA extracted from MDPCs. The double-stranded cDNA was digested with NlaIII and the restriction enzyme was replaced by MmeI after linker ligation. Ditags produced from 400 PCRs were isolated, cleaved with NlaIII, and cloned into pZErO. All sequence files were processed using the SAGE2000 ver. 4.5 software. The extracted tags were further processed to determine the identity of associated genes through several stringent filters using the CGAP website (http://cgap.nci.nih.gov/SAGE).

Signal sequence trap (SST). SST was performed as previously described [10,11]. Briefly, a library was constructed in the retrovirus vector pMX-SST employing cDNA derived from mRNA isolated from MDPCs. The interleukin-3 (IL-3)-dependent pro-B cell line Ba/F3 was infected with retrovirus, followed by seeding onto 96-multiwell plates in the absence of IL-3. Genomic DNAs extracted from IL-3-independent Ba/F3 clones were subjected to PCR to recover the integrated cDNAs using primers specific for the cloning vector. After electrophoresis of the PCR products, DNA was recovered and subjected to sequencing.

Immunofluorescent microscopy. Specimens were fixed in 4% paraformaldehyde and stained with rabbit monoclonal anti-OPN antibody (IBL, Fujioka, Japan), rat monoclonal anti-CD31 antibody (BD Biosciences), and mouse monoclonal anti-smMHC antibody (DAKO). Secondary antibodies were conjugated with Alexa Flour 555, and nuclei were visualized using 4′,6-diamino-2-phenylindole (DAPI; Invitrogen).

RNA extraction and quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR). Total RNA was extracted from MDPCs using the RNeasy mini kit (QIAGEN) and cDNA was synthesized by the SuperScript III kit (Invitrogen). Synthesized cDNA was analyzed by kinetic real-time PCR using the ABI Prism 7700 Sequence Detector system (Applied Biosystems) with SYBR Green Real-time PCR Master Mix (TOYOBO, Osaka). Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization, and the comparative threshold (C_T) method was used to assess the relative abundance of the targets. Primers used were as follows: OPN-f, GCAGACACTTTCACTCCAATCG; OPN-r, GCCCTTTCCGTTGTTGTCCTG; GAPDH-f, TTGTGATGGGTGTGAACCACGAGA; GAPDH-r, CATGAGCCCTTCCACAATGCCAAA.

MDPC proliferation assay. To test the proliferative effect of OPN or growth factor on MDPCs, recombinant OPN (R&D Systems), LIF, EGF or bFGF was added to the culture medium. Seventy-two hours after treatment, cell proliferation and/or viability effects were estimated using Cell Proliferation Reagent WST-1 (Roche Applied Science), which was added to the culture medium and measured the number of viable cells. Twenty-four hours after treatment, cell proliferation was also estimated by the measurement of BrdU incorporation into newly synthesized cellular DNA using Cell Proliferation ELISA, BrdU (colorimetric) (Roche Applied Science). To estimate the involvement of the PI3K/Akt pathway, MDPCs were pretreated with 1.0 μM LY294002 (CALBIOCHEM) or 5.0 μM Akt inhibitor (CALBIOCHEM) for 30 min.

Western blotting. Cell lysates were extracted with a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 1× protease inhibitor cocktail (Pierce), 1 mM Na_3VO_4, and 1 mM NaF. Cell lysates were electrophoresed on 10% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). Transferred membranes were incubated with primary antibodies against phospho-Akt (S473) and Akt (Cell Signaling). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (GE Healthcare) was used as a secondary antibody.

Statistical analysis. All experiments were performed at least three times. Data were expressed as means ± standard error and analyzed by one-way ANOVA with post hoc analysis. A value of $P < 0.05$ was considered significant.

Results

Identification of osteopontin

To explore the potential molecules regulating MDPC proliferation, we performed SAGE and SST using RNA isolated from MDPCs. SAGE provides a comprehensive approach for elucidation of quantitative gene expression patterns that does not depend on prior availability of transcript information [12], and SST is an efficient strategy to identify secreted and cell-surface molecules [11]. By using the two distinct methods, we identified OPN, a secretory molecule, as one of the most abundant molecules expressed in MDPCs. To confirm OPN expression in MDPCs, we performed immunostaining using an anti-OPN antibody. As shown in Fig. 1A, OPN protein was readily detected in myospheres and individual MDPCs.

When MDPCs were isolated and expanded, we used an isolation medium and an expansion medium, respectively. Both media contain bFGF and EGF, while LIF was also used in the expansion medium. Since bFGF has been reported to increase OPN mRNA expression in rat osteosarcoma cells [13], we speculated that the growth factors used might affect OPN expression during the isolation and expansion of MDPCs. To examine this possibility, quantitative RT-PCR for OPN mRNA was performed using total RNA isolated from MDPCs treated with or without the growth factors. Among the growth factors used in the present study, bFGF enhanced OPN mRNA expression in MDPCs (Fig. 1B), whereas EGF and LIF did not. The addition of exogenous OPN did not alter

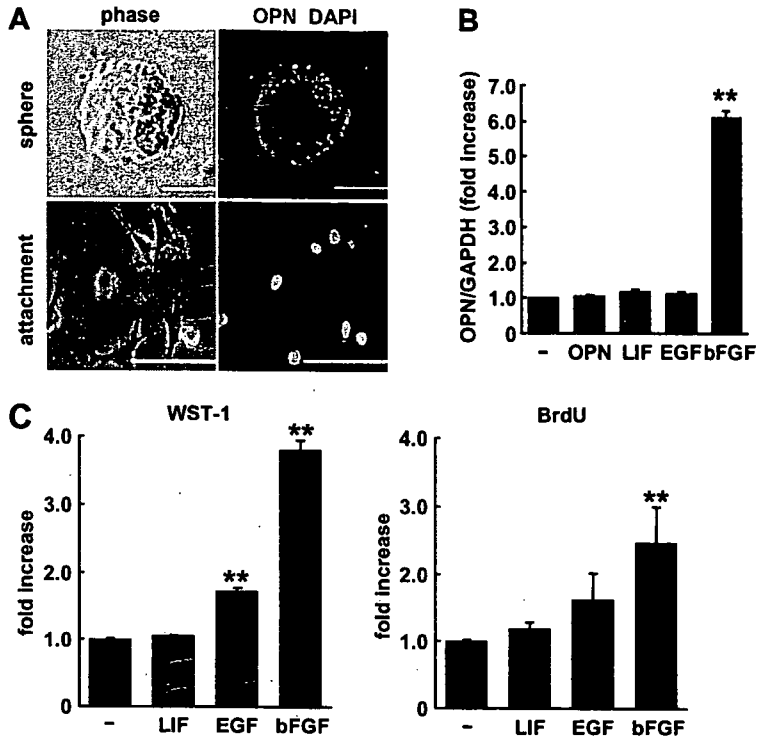

Fig. 1. OPN expression in MDPCs. (A) OPN expression in spheroid or attached MDPCs was examined by immunostaining using an anti-OPN antibody. Bar indicates 200 μm. (B) MDPCs were treated with or without the growth factors for 12 h. OPN mRNA expression in MDPCs was assessed by real-time RT-PCR. Concentrations of LIF, EGF, and bFGF were 10, 20, and 10 ng/ml, respectively. (C) Cell proliferation activity was assessed using the WST-1 and BrdU ELISA assay systems. MDPCs were treated with or without the growth factors. Values are means ± SEM. $^{**}P < 0.01$ vs control.

endogenous OPN mRNA expression in MDPCs, indicating that OPN has no feedback regulation on its own expression. Furthermore, to investigate which of these growth factors contributes to MDPC proliferation, we measured cell proliferation activity by using WST-1 and BrdU ELISA assays (Fig. 1C). Significant increases in the number of viable MDPCs estimated by the WST-1 assay were seen in EGF- and bFGF-treated MDPCs, and increased DNA synthesis measured by the BrdU ELISA assay was detected in bFGF-treated MDPCs, suggesting that both EGF and bFGF have proliferative effects for MDPCs. In contrast, LIF had no obvious proliferative effect on MDPCs.

OPN has a proliferative effect for MDPCs

We then examined the role of OPN on MDPC proliferation. When the recombinant OPN protein was added to the culture medium containing 2% FBS and growth factors (bFGF, EGF, and LIF) to mimic the culture condition for MDPCs expansion, slight but significant increases in the numbers of viable MDPCs and DNA synthesis were observed in 5.0 μg/ml OPN-treated MDPCs as estimated by WST-1 assay and BrdU ELISA assay, respectively (Fig. 2A). Even if FBS was removed from the above medium mimicking the culture condition for MDPC isolation, the addition of OPN at a concentration of 5.0 μg/ml also induced a significant increase in MDPC proliferation (Fig. 2B). These findings suggest that OPN cooperates with bFGF, EGF, and LIF to induce MDPC proliferation during isolation and expansion of MDPCs.

The findings showed in Fig. 1B suggest that OPN is secreted into the culture medium from MDPCs treated with the growth factors. This may have caused additional OPN treatment to show only a slight effect on growth factors-treated MDPC proliferation as shown is Fig. 2A and B. Furthermore, the results obtained above did not reveal whether OPN alone in the absence of the growth factors could have an effect on MDPC proliferation. Therefore, to clarify the proliferative effect of OPN alone, the recombinant OPN protein was added to the culture medium without FBS and the growth factors, and then cell proliferation activity was assessed. As shown in Fig. 2C, OPN significantly increased MDPC proliferation as assessed by the cell number (right panel) and DNA synthesis (left panel) in a dose dependent manner.

The proliferation of MDPCs induced by OPN depends on the PI3K/Akt pathway

OPN has been reported to activate various kinases such as PI3K, nuclear factor inducing kinase, protein kinase C,

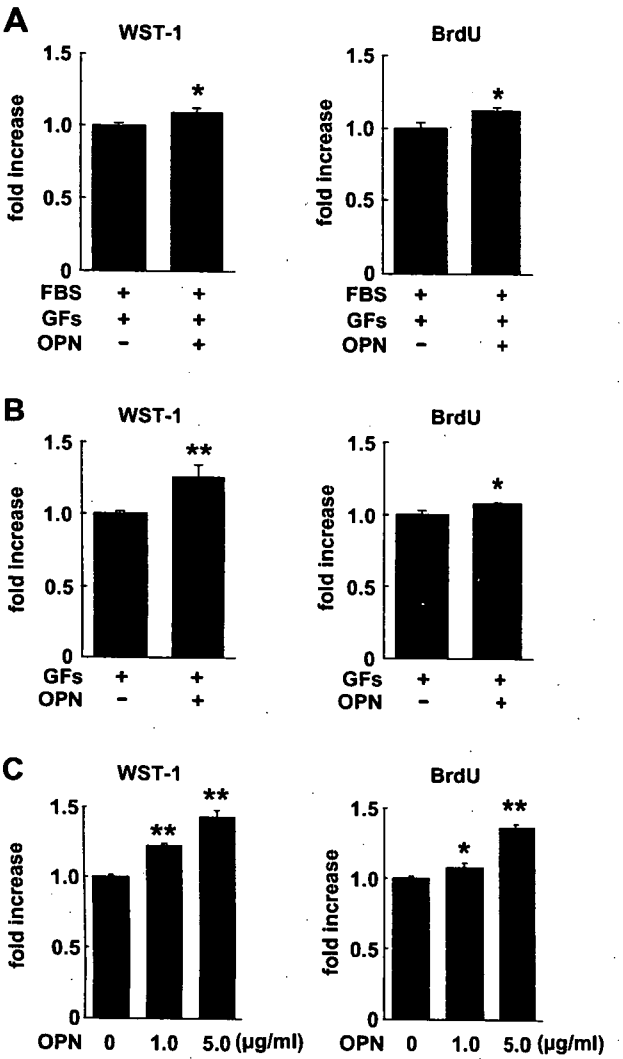

Fig. 2. Enhancement of proliferation effect on MDPCs by OPN. Cell proliferation activity was assessed by use of the WST-1 and BrdU ELISA assay systems. (A) MDPCs were treated with or without 5.0 μg/ml OPN in the culture medium containing 2% FBS and GFs. (B) MDPCs were treated with or without 5.0 μg/ml OPN in the culture medium containing GFs. (C) MDPCs were treated with indicated concentrations of OPN in the culture medium without FBS and GFs. Values are means ± SEM. GFs, growth factor mix (10 ng/ml LIF, 20 ng/ml EGF, and 10 ng/ml bFGF). $^{*}P<0.05$, $^{**}P<0.01$ vs control.

and MAP kinases [14]. Since we have shown that the proliferation of cardiac stem cells (CSCs) is dependent on the Akt pathway [15], we investigated whether OPN induces Akt activation in MDPCs. As shown in Fig. 3A, OPN-induced Akt phosphorylation was detected within 15 min after the addition of OPN, reached a maximum at around 30 min, and then declined. Since Akt is one of downstream target molecules of PI3K, we then examined whether OPN-induced activation of the PI3K/Akt pathway regulates MDPC proliferation. To define the role of the PI3K/Akt pathway, we used inhibitors for PI3K and Akt. OPN-induced Akt phosphorylation was inhibited by 1.0 μM of LY294002, a PI3K inhibitor, and 5.0 μM of Akt inhibitor in MDPCs (data not shown). Therefore, we treated MDPCs with 1.0 μM LY294002 or 5.0 μM Akt inhibitor, followed by the addition of OPN, and then measured cell proliferation activity. As shown in Fig. 3B, pretreatment with LY294002 inhibited OPN-induced MDPC proliferation as assessed by WST-1 and BrdU ELISA assays. Akt inhibition also reduced OPN-induced MDPC proliferation as assessed by WST-1 and BrdU ELISA assays (Fig. 3C). These results indicate that OPN-induced MDPC proliferation depends on the PI3K/Akt pathway.

OPN-treated MDPCs can differentiate into endothelial and smooth muscle cells

Finally, we examined the potential of MDPCs treated with OPN to differentiate into vascular cells. MDPCs that had been treated with 1.0 μg/ml of OPN for 4 days were induced into endothelial and smooth muscle cell differentiation using VEGF and PDGF, respectively. Immunostaining using an anti-CD31 antibody showed that CD31 positive cells appeared by 14 days after treatment with VEGF (Fig. 4, upper panel). When MDPCs treated by OPN were cultured in the differentiation medium containing PDGF for 14 days, smMHC positive cells were observed (Fig. 4, lower panel). These results indicate that OPN-treated MDPCs retained their replicative growth capacity and could maintain the commitment to differentiate into at least two different vascular lineages: endothelial and smooth muscle cells.

Discussion

OPN has been known to be involved in many physiological and pathological processes including cell adhesion, angiogenesis, apoptosis, inflammatory responses and tumor metastasis [16]. Here, we identify OPN as a myosphere-derived secretory molecule, and provide a novel role of OPN in regulating the proliferative growth of MDPCs through the PI3K/Akt signaling pathway.

bFGF and EGF have been revealed to affect the rates of proliferation for neural stem and progenitor cells [17]. Studies from single cell cultures demonstrated that EGF and bFGF are mitogens for neurospheres [18,19]. Both bFGF and EGF were also used for the isolation of CSCs as a cardiosphere from the heart [20]. Muscle-derived stem cells were isolated as a myosphere from skeletal muscle by Sarig et al. using bFGF and LIF [5]. Similarly, we have shown that MDPCs as well as CSCs can be isolated as a sphere and expanded using bFGF, EGF, and LIF [6,15]. Regarding the proliferation of MDPCs, we demonstrated here that proliferative effects were dependent on bFGF and EGF but not LIF, and that the effect of bFGF was greater than that of EGF. The previous report by Sarig et al. showed that LIF increased the proportion of Sca-1 expressing muscle-derived stem cells from 15% to 80% [5]. Since we have used MDPCs that highly express Sca-1

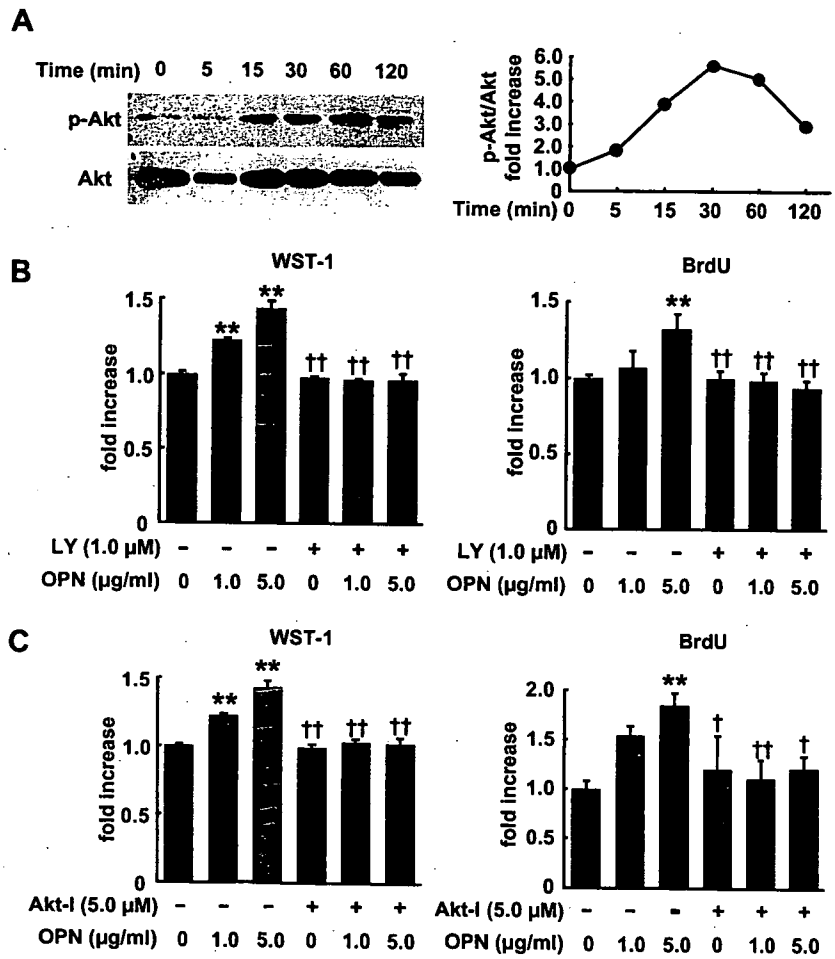

Fig. 3. OPN stimulates proliferation of MDPCs through the PI3K/Akt signaling pathway. (A) MDPCs were treated with 5.0 μg/ml OPN for various time periods. Cell extracts were subjected to Western blotting (left panel) using an anti-phospho-specific Akt antibody (p-Akt, upper panel) or an anti-Akt antibody (Akt, lower panel). Right graph shows the time course of relative phosphorylation of Akt during OPN treatment. The experiment shown represents one of three independent trials that gave nearly identical results. (B,C) Cell proliferation activity was assessed by use of the WST-1 and BrdU ELISA assay systems. Cell proliferation and DNA synthesis of MDPCs induced by OPN were inhibited by pretreatment with LY294004, a PI3K inhibitor (B), or Akt inhibitor (C). Values are means ± SEM. LY, LY294002; Akt-I, Akt inhibitor. $^{**}P < 0.01$ vs control; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs 5.0 μg/ml OPN.

(FACS analysis, 84.0 ± 2.6%) [6], the discrepant result between our study and Sarig et al. [5] regarding the effect of LIF might be due to the population of Sca-1 expressing cells. We also found that OPN expression was enhanced by bFGF but not EGF and LIF in MDPCs, and that OPN promotes MDPC proliferation. These findings suggest that OPN contributes to bFGF-induced MDPC proliferation.

OPN has a protease-hypersensitive site that separates the integrin- and CD44-binding domains and exerts its effects by interacting with various integrins and CD44 receptors [14]. We have shown that CD29 (also known as β1 integrin) and CD44 are expressed on MDPCs [6]. OPN has been shown to activate the PI3K/Akt signaling pathway through αvβ3 integrin- and CD44-mediated pathways [21,22]. Furthermore, CD44 has been demonstrated to cooperate with β1 integrin to bind OPN [23]. Therefore, our findings suggest that CD44 and β1 integrin may mediate OPN-induced activation of the PI3K/Akt signaling pathway and allow the proliferative growth of MDPCs.

Extensive research using OPN-knockdown by siRNA and OPN-null mutant mice has demonstrated that OPN promotes the growth or survival of tumor cells [14,24–27]. Similar to the effect of OPN on tumor cells, we showed a proliferative effect of OPN on MDPCs in the present study. We also demonstrated that OPN-treated MDPCs retained the ability to differentiate into vascular cells. OPN expression has been revealed to be up-regulated in regenerating skeletal muscle after injury [28]. A study using OPN-null mutant mice also demonstrated that OPN was required for efficient angiogenesis in ectopically implanted bone discs in skeletal muscle [29]. Taken together with our findings, these data suggest that OPN is involved in the proliferation of MDPCs and angiogenesis by MDPCs in skeletal muscle. However, in primitive hematopoietic stem cells (HSCs) OPN has been reported to be a critical component of the HSC niche within the bone marrow microenvironment, and to work as a negative regulator of HSC proliferation [30,31]. The reason why OPN has opposite