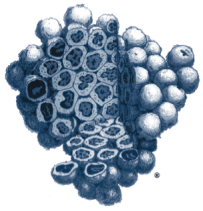

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Bone Morphogenetic Protein-4 Enhances Cardiomyocyte Differentiation of Cynomolgus Monkey ESCs in Knockout Serum Replacement Medium

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Key Words. Embryonic stem cells • Differentiation • Bone morphogenic protein • Primate

ABSTRACT

Despite extensive research in the differentiation of rodent ESCs into cardiomyocytes, there have been few studies of this process in primates. In this study, we examined the role of bone morphogenic protein-4 (BMP-4) to induce cardiomyocyte differentiation of cynomolgus monkey ESCs. To study the role of BMP-4, EBs were formed and cultured in Knockout Serum Replacement (KSR) medium containing BMP-4 for 8 days and subsequently seeded in gelatin-coated dishes for 20 days. It was found that ESCs differentiated into cardiomyocytes upon stimulation with BMP-4 in KSR medium, which resulted in a large fraction of beating EBs (~16%) and the upregulation of cardiac-specific proteins in a dose and time-dependent manner. In contrast, the addition of BMP-4 in FBS-containing medium resulted in a lower fraction of beating EBs (~6%). BMP-4 acted principally between mesendodermal and mesoderm progenitors and subsequently enhanced their expression. Ultrastructural observation revealed that beating EBs contained mature cardiomyocytes with sarcomeric structures. In addition, immunostaining, reverse transcription-polymerase chain reaction, and Western blotting for cardiac markers confirmed the increased differentiation of cardiomyocytes in these cultures. Moreover, electrophysiological studies demonstrated that the differentiated cardiomyocytes were electrically activated. These findings may be useful in developing effective culture conditions to differentiate cynomolgus monkey ESCs into cardiomyocytes for studying developmental biology and for regenerative medicine. STEM CELLS *2007;25:571–580*

INTRODUCTION

The loss of cardiomyocytes resulting from ischemic is irreversible and leads to the development of progressive heart failure. The heart under stress primarily responds with cell hypertrophy rather than proliferation because of a limited mitotic capacity of differentiated cardiomyocytes [1]. In this regard, cell transplantation is a potentially useful therapeutic strategy to replace damaged or lost myocardial tissue to restore cardiac function. ESCs are a promising source of cells for cardiac regeneration. However, despite much research, the clinical application of ESCs has been hindered by an inability to direct the differentiation of these cells in a uniform manner; by the risks of differentiated cells, such as their arrhythmogenic properties [2]; by potential formation of teratomas [3]; and by possible transmission of pathogens from mouse feeder cells [4]. So far, many studies have examined the differentiation of ESCs to cardiomyocytes. However, most of these studies have been limited to mouse models [5, 6]. Despite their merit, which includes the wide availability of knockout and knockin systems, mouse models cannot be used to address preclinical concerns such as postimplantation functionality, immune rejection, or the formation of teratomas. In addition, because of differences in rodent and primate embryonic development, as well as their underlying molecular regulation, primate-based models will be of great potential significance [7]. Therefore, the development of nonhuman primate ESC lines can be used to study early cardiogenesis in species that are more closely related to humans. A suitable primate model is cynomolgus monkeys (*Macaca fascicularis*), which are widely used for medical research [8] and have ESCs that are remarkably similar to human ESCs in many aspects, including morphology, cell surface marker expression, growth velocity, and dependence on feeder cells for self-renewal. A major challenge in ESC-based cardiac therapies, particularly for primate models, is that the differentiation efficiency of ESCs into myocardial cells is low. Previously, a number of growth factors and chemical compounds have been shown to induce cardiomyocyte differentiation of mouse ESCs, including transforming growth factor β (TGF-β) in combination with

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bone morphogenetic protein 2 (BMP-2) [9]. BMPs are members of the TGF-β growth factor superfamily that have been shown to play a pivotal role in most morphogenetic processes during development [10]. Emerging evidence suggests that BMP signaling required in mesoderm induction and cardiac differentiation [11, 12]. Moreover, embryos homozygous for the *BMP-4 null* mutation do not proceed beyond the egg cylinder stage and show little or no mesodermal differentiation [13]. The activity of BMP signaling on cardiac differentiation has been further confirmed by using inhibitors of BMP signaling, including the BMP inhibitor noggin, truncated versions of *type I* (tALK3) or *type II* (tBMPRII) BMP receptors, and inhibitory SMAD6 [14–16].

In this study, we developed an effective protocol for obtaining cardiomyocytes from cynomolgus monkey ESCs by modifying culture medium contents and stimulating ESCs with BMP-4. Using this culture system, we found that BMP-4 is an important factor in the enhancement of the cardiomyocyte differentiation of cynomolgus monkey ESCs in vitro.

Materials and Methods

Cell Line and Cell Culture of Cynomolgus ESCs

The cynomolgus monkey ESC line CMK6 [17] was cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) on mouse embryonic fibroblast (MEF) feeder layers derived from explanted day 11.5 fetuses (where day of vaginal plug was counted as the first day) of the MTL-Neo^r strain of mice that was mitotically inactivated with 10 μg/ml mitomycin C (Kyowa, Tokyo, Japan). The medium was supplemented with 0.1 mM 2-mercaptoethanol (Sigma-Aldrich; M7522), 2 mM L-glutamine (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 1% nonessential amino acids (Gibco, Grand Island, NY, http://www.invitrogen.com), 1 mM sodium pyruvate (Invitrogen), and 20% Knockout Serum Replacement (KSR) (Gibco). ESCs were passaged every 3 days to maintain their undifferentiated state. To induce differentiation, ESCs were dispersed into small clumps by using 1 mg/ml collagenase IV (Wako Chemical, Osaka, Japan, http://www.wako-chem.co.jp/english) and subsequently transferred to 100-mm plastic Petri dishes (Greiner Bio-One, Frickenhausen, Germany, http://www.gbo.com/en) in medium containing fetal bovine serum (FBS) (embryonic-stem-qualified; Gibco) and cultured for 2 days. During this period, the cells aggregated to form EBs, and the medium was changed, based on experimental models with KSR or FBS, for an additional 8 days. To determine the dose-dependent effect of BMP-4 (Sigma-Aldrich; B-2680) on cardiomyogenesis, BMP-4 was added to the culture medium at three final concentrations of 10, 50, and 100 ng/ml; subsequently, adherent cultures of differentiating ESCs were initiated by plating the EBs onto 0.1% gelatin-coated 10-mm culture plates at a density of 50 EBs/100-mm plate. These cultures were maintained for 20 days, and media in the cultures were changed every 3 days. Three experimental models were examined in this study: the FBS$^{-/-/-}$ model, containing KSR medium throughout culture; the FBS$^{+/+/+}$ model, containing FBS medium throughout culture; and the FBS$^{+/-/-}$ model, containing FBS medium for 2 days followed by culture with KSR medium. The appearance of spontaneous contractions and dipocytes containing lipid droplets was observed under the light microscope.

Immunohistological Analysis

Contracting regions within EBs (day 18) were mechanically dissected using a sterile micropipette and incubated with trypsin-EDTA. To dissociate the aggregates, cells were subsequently resuspended in medium and seeded onto surfaces that were coated with human fibronectin (BD Biosciences, San Diego, http://www.bdbiosciences.com). After 24 hours, cells were analyzed using immunocytochemistry as previously described [18]. Briefly, after fixation with 4% paraformaldehyde for 20 minutes, cells were incubated with one of the following primary antibodies: anti-mouse cardiac β-myosin heavy chain monoclonal antibody (Abcam, Cambridge, U.K., http://www.abcam.com; 1:200), mouse IgG1 monoclonal anti-sarcomeric α-actinin antibody (clone EA-53; Sigma-Aldrich; 1:200), mouse IgG2a monoclonal anti-troponin T antibody (clone CT3; Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/~dshbwww; 1:500), polyclonal rabbit IgG anti-atrial natriuretic factor (anti-ANF) antibody (clone T-4011; Bachem, Weil am Rhein, Germany, http://www.bachem.com; 1:1,000), and a mouse IgG1 monoclonal anti-myosin light chain 2 ventricular (anti-MLC2v) (clone F109.3E1; Biocytex, Marseille, France, http://www.biocytex.fr; 1:500). To visualize the primary antibodies, cells were incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com; 1:2,000). To stain EBs, they were first fixed in 4% paraformaldehyde for 45 minutes and then stained with anti-mouse cardiac β-myosin heavy chain monoclonal antibody.

Oil Red O Staining

The EBs were washed in 1× phosphate-buffered saline (PBS) and fixed with 10% formalin for 40 minutes. After being washed with PBS, the EBs were stained with oil red O (ORO) (Sigma-Aldrich) solution (ORO-saturated solution in isopropanol:water, 3:2) for 15 minutes. The EBs were then washed with 70% alcohol for 5 seconds to remove background staining and finally were rinsed in tap water, counterstained with Harris hematoxylin (10 seconds), and mounted in glycerol-PBS (9:1) for observation.

Transmission Electron Microscopy

To prepare the samples for transmission electron microscopy (TEM) analysis, day 18 contracting EBs were retrieved and washed with PBS and fixed using 2% glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for 2 hours. The samples were then washed and exposed to a secondary fixation process in 1% osmium tetroxide in the same buffer for 1.5 hours. The samples were washed and then dehydrated in increasing concentrations of ethanol (from 30% to ~100%) for 15 minutes each. Samples were then put into molds containing 100% Spurr's resin and polymerized at 70°C overnight. After polymerization, 80-nm sections were cut and stained with lead citrate for 8 minutes. Micrographs of transverse sections of ES and MEF cells were taken on plate films on a Zeiss EM900 (Oberkochen, Germany, http://www.zeiss.com) transmission electron microscope.

Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNA was prepared from EBs using TriZol (Sigma-Aldrich), according to the manufacturer's instructions. Polymerase chain reaction (PCR) conditions included denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and polymerization at 72°C for 1 minutes; after 35 cycles, an extension step of 10 minutes at 72°C was added. To exclude false-positive results based on contaminating MEFs, all primer pairs were designed to mismatch the corresponding murine sequences. Primers were also designed based on available sequences of *Homo sapiens*, *Macaca fascicularis*, *Pan troglodytes*, *Sus scrofa*, *Canis familiaris*, and *Bos taurus*. Primer sequences used in these studies are summarized in Table 1. Species specificity was validated by using RNA of adult mouse and cynomolgus monkey heart tissue.

Western Blotting

Whole cell lysates extracted from EBs were subjected to Western blotting for cardiac β-myosin heavy chain (MHC) (Abcam; 1:200) and cardiac α-actinin (Sigma-Aldrich; 1:500) and α-actin as loading controls, as described previously [18]. Samples were subjected to 10% SDS-polyacrylamide gel electrophoresis, and the separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes (PerkinElmer Life and Analytical Sciences, Boston, http://www.perkinelmer.com). Blots were incubated with primary antibodies against sarcomeric cardiac β-MHC and cardiac α-actinin, and the primary antibodies were detected using horserad-

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Table 1. Primer sequences

Target gene	Sequence
Brachyury T	Forward, 5′-CGGAACAATTCTCCAACCTATT-3′ Reverse, 5′-GTACTGGCTGTCCACGATGTCT-3′
Medoderm posterior factor 1	Forward, 5′-CTCGTCTCGTCCCCAGACT-3′ Reverse, 5′-AGCGTGCGCATGCGCCAGTT-3′
Nkx2.5	Forward, 5′-TGCCGCCGGAGAGCTCTCTG-3′ Reverse, 5′-ACCGCGATCCTGCGGGCAG-3′
GATA-4	Forward, 5′-GGAGGAAGGCTCTCACTGCC-3′ Reverse, 5′-GAGTGGGCCTCTCCTGTG-3′
Cardiac β-myosin heavy chain	Forward, 5′-TGCAAAGGCTCCAGGTCTGAGGGC-3′ Reverse, 5′-GCCAACACCAACCTGTCCAAGTTC-3′
Atrial natriuretic factor	Forward, 5′-GAACCAGAGGGGAGAGACAGA-3′ Reverse, 5′-CCCTCAGCTTGCTTTTTAGGAG-3′
Cardiac α-myosin heavy chain	Forward, 5′-AGGATCCTCTCAACGAGACT-3′ Reverse, 5′-GTGATCAATGTCCAGAGAGC-3′
Myosin light chain 2 ventricle	Forward, 5′-AGGAGGCCTTCACTATCATGG-3′ Reverse, 5′-GTGATGATGTGCACCAGGTTC-3′
Oct-4	Forward, 5′-GGCGTTCTCTTTGGAAAGGTGTTC-3′ Reverse, 5′-CTCGAACCACATCCTTCTCT-3′
β-Tubulin	Forward, 5′-CCGGACAGTGTGGCAACCAGATCGG-3′ Reverse, 5′-TCACTGTGCCTGAACTTACC-3′

ish peroxidase-labeled donkey anti-mouse immunoglobulin G followed by enhanced chemiluminescence (PerkinElmer).

Microelectrode Recording

To detect field potentials (FPs), we used substrate-integrated, planar multielectrode arrays (MEAs) (Multi Channel Systems, Reutlingen, Germany, http://www.multichannelsystems.com) for long-term recordings of the spontaneous electrical activity from cultures of cardiac myocytes and EBs [19]. Beating EBs were positioned in the middle of a sterilized MEA consisting of 60 titanium nitride-coated gold electrodes (diameter, 30 μm; interelectrode distance, 200 μm in a square grid). For recordings, a separate sterile Ag/AgCl electrode was temporarily inserted into the dish as the ground electrode. The MEA was connected to the amplifier and data-acquisition system (Multi Channel Systems), which included a heating device to maintain a constant temperature of 37°C. Data were recorded simultaneously from up to 60 channels (sampling frequency up to 40 kHz). The data were analyzed off-line with a customized toolbox programmed for MATLAB (Mathworks, Natick, MA, http://www.mathworks.com) to detect FPs. The frequency of contractions was calculated by measuring the distance between the respective FP minima (FP_{MIN}) and denoted interspike interval (ISI) [7]. FP_{MIN} represents the activity of Na^+ channels. A second, slowly occurring negative FP deflection (FP_{SLOW}), representing the activity of voltage-dependent Ca^+ channels, was analyzed to characterize the pharmacological modulation of cardiomyocytes cultures derived from ESCs.

Statistical Analysis

All experiments were performed at least three times, and data are expressed as mean ± SD and analyzed by Student's t test or one-way analysis of variance with post hoc analysis. A value of $p < .05$ was considered statistically significant.

RESULTS

Characterization and Differentiation of ESCs into Cardiomyocytes

To induce optimal differentiation of monkey ESCs into cardiomyocytes, we used a three-step process (Fig. 1A). In the first step, monkey ESCs (Fig. 1B) were induced to form aggregates by culturing ESCs in FBS medium on nonadhesive dishes for 2 days [17]. In the second step, ESC aggregates were induced to differentiate in KSR-containing medium for 8 days (Fig. 1C), and in the third step, the EBs were plated on gelatin-coated dishes for 20 days. A small fraction of EBs showed necrotic regions after long-term suspension cultures (10 days). The third step was used to minimize necrosis in EBs in suspension and to reduce complications regarding analysis and detection of beating cardiomyocytes. In each step, we used either FBS-containing medium or KSR medium, based on experimental models. KSR medium is a defined formulation that provides consistent growth conditions and has been shown to result in less differentiation compared with FBS-supplemented medium.

Formation of the first contracting regions was usually observed at day 8 of differentiation (floating EBs), and the proportion of EBs with contracting areas increased from day 8, reaching a maximal percentage of contractile EBs on day 18 of differentiation (10 days in suspension, followed by 8 days outgrowth in gelatin-coated dishes). Although the initial beating was irregular, the beating rhythm became consistent, to an average frequency of 53 ± 12 (Fig. 1D). Interestingly, contractile activity of cynomolgus monkey ESC-derived cardiomyocytes was sensitive to temperature compared with mouse ESCs and decreased during analysis under a microscope.

To enhance cardiomyocyte differentiation, we used BMP-4 for 8 days, while EBs were in suspension. In addition, we used FBS- or KSR-supplemented medium at various stages of the protocol. We observed that only a few EBs, which had been maintained in KSR-supplemented medium ($FBS^{-/-/-}$), were beating even after 30 days in culture (despite the presence of BMP). We also found that in cultures that were maintained in FBS-supplemented medium throughout the study ($FBS^{+/+/+}$), the addition of BMP-4 decreased the number of EBs containing beating regions in a dose-dependent manner. In our studies, cultures that were initially supplemented with FBS and then cultured in KSR medium (i.e., $FBS^{+/-/-}$) were sensitive to BMP-4 in a dose-dependent manner. We observed that low concentrations of BMP-4 (10 ng/ml) induced cardiomyocyte differentiation. To determine whether longer stimulation by BMP-4 enhance ESC-derived cardiomyocytes, EBs at day 2 of differentiation were stimulated for 10 and 20 days by BMP-4. The results indicated that induction of cardiomyocyte gene expression was not enhanced (data not shown). To confirm whether beating EBs were cardiomyocytes, beating EBs at day 18 were analyzed using TEM. TEM images showed the pres-

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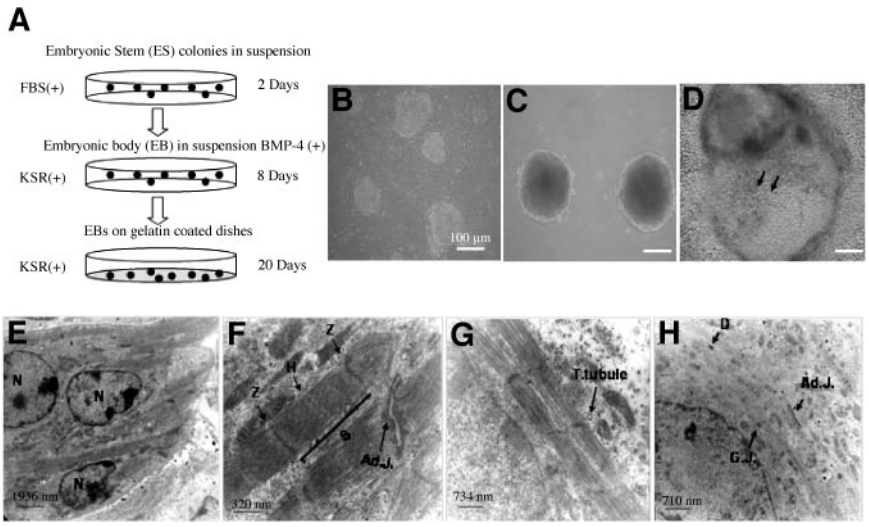

Figure 1. Schematic of experimental procedures and light micrographs of cynomolgus monkey ESCs. **(A):** Schematic of the protocol used to induce cardiomyocyte differentiation of ESCs. **(B):** Morphology of undifferentiated cynomolgus monkey ESC colonies on feeder cells. **(C):** EBs generated by culturing ESC aggregates in suspension. **(D):** Beating EB on gelatin dish (arrowheads). Ultrastructural analysis of ES-derived cardiomyocytes. **(E):** Mononucleated cells. **(F):** Higher magnification of an organized sarcomere. **(G):** High-power electron micrograph showing a T-tubule. **(H):** G.J., D, and Ad.J. Abbreviations: Ad.J., adjacent junctions; BMP, bone morphogenic protein; D, desmosome; FBS, fetal bovine serum; G.J., gap junctions; H, H band; KSR, Knockout Serum Replacement; N, nucleus; S, sarcomere; Z, Z disc.

ence of the nucleus cells (Fig. 1E, indicated by N) and mature sarcomeric organization, such as distinct Z bands and H bands (Fig. 1F). The diameters of the Z line corresponded to the diameter of the myofibrillar arrangements and were spaced approximately 2 μm apart. In addition, T-tubules (Fig. 1G), spot desmosomes, and adjacent junctions (Fig. 1H) could be visualized.

BMP-4 Acted upon Early Mesoderm and Enhanced the Expression of Cardiac Mesoderm and Cardiomyocyte Genes in KSR-Supplemented Culture Medium

To determine the stage of cardiomyocyte development that was influenced by exogenous BMP-4, we performed reverse transcription (RT)-PCR analysis for expression of various mesoderm markers. BMP-4-treated ESCs (undifferentiated) did not express brachyury T (Fig. 2A). On the other hand, the $FBS^{+/-/-}$ model treated with BMP-4 expressed high levels of brachyury T on day 4 of EB formation, which increased by day 6 of differentiation and was subsequently maintained (Fig. 2A, black columns). In contrast, in the $FBS^{-/-/-}$ model, there was a weak expression of brachyury T on day 2 of differentiation, which decreased at subsequent time points (Fig. 2A, hatched columns). Brachyury T in the $FBS^{+/+/+}$ model was expressed at day 2 of differentiation but did not significantly change with time (Fig. 2A, white columns). These results suggest that FBS induces the expression of the early mesendodermal marker brachyury T, and the subsequent addition of BMP-4 enhances this pattern. In addition to an increase in brachyury T levels in ESCs treated with BMP-4 in the $FBS^{+/-/-}$ model, the expression of the mesoderm marker (Mesp1) and cardiac mesoderm markers (NKx2.5 and GATA-4) was also markedly increased by day 4 and was maximal by day 8 of differentiation (Fig. 2B, 2C, and 2D, respectively, black columns). In contrast, the exogenous addition of BMP-4 in the $FBS^{+/+/+}$ model significantly decreased the expression of mesoderm and cardiac markers (Fig. 2B, 2C, and 2D, respectively, white columns). In addition, BMP-4-treated ESCs in the $FBS^{+/-/-}$ model had increased level of expression of the cardiomyocyte markers (β-MHC and ANF) from day 4 of differentiation (Fig. 2E, 2F, black columns) in comparison with BMP-4-treated ESCs in the $FBS^{+/+/+}$ model. Moreover, ESCs in the $FBS^{-/-/-}$ model weakly expressed cardiac mesoderm and cardiomyocyte markers (Fig. 2C, 2D, 2E, and 2F, respectively, hatched columns). Taken together, these data suggest that BMP-4 acts principally between the early mesendodermal progenitors (brachyury T-positive state) and mesodermal state (Mesp1).

BMP-4 Enhanced Differentiation of ESCs into Cardiomyocytes Based on Doses Applied and Culture Conditions

To compare the effect of BMP-4 on cardiac differentiation in KSR- or FBS-supplemented culture media, various BMP-4 concentrations (10, 50, and 100 ng/ml) were used during the EB step (8 days stimulation). Figure 3A represents the effects of BMP-4 stimulation in cultures containing FBS throughout the culture (i.e., $FBS^{+/+/+}$) As illustrated, the addition of 10 ng/ml BMP-4 decreased the frequency of beating EBs after 16 days in culture compared with control samples (3.1% and 5.7%, respectively). Furthermore, 50 and 100 ng/ml BMP-4 strongly inhibited the number of beating EBs. This finding suggests that exogenous addition of BMP-4 in medium supplemented with FBS inhibit cardiomyocyte differentiation of ESCs. Figure 3B shows the effect of BMP-4 in the $FBS^{+/-/-}$ model. In these cultures, a significant increase in the frequency of beating EBs was observed in cultures containing 10 ng/ml BMP-4 ($\sim$12% on day 14 and 16% on day 18 of differentiation compared with control, 6.4%). Interestingly, higher concentrations of BMP-4, such as 50 and 100 ng/ml, did not increase the frequency of beating EBs.

To further analyze the effect of BMP-4 for differentiation of ESCs into cardiomyocytes, we tested ESCs in the $FBS^{+/-/-}$ model after 18 days using different doses of BMP-4 (10, 50, and 100 ng/ml). As can be seen in Figure 3C, OCT-4 was strongly expressed in undifferentiated cells, and its expression was lost as ESCs differentiated. Cardiac-specific proteins (including ANF, β- and α-MHC, and MLC-2v) were strongly expressed with 10 ng/ml of BMP-4, and expressions of these proteins decreased at high BMP-4 concentrations (Fig. 3C). Specifically, at 100 ng/ml BMP-4, the expression of α-MHC, MLC2v, and ANF was inhibited, and expression of β-MHC had decreased. To validate RT-PCR results, Western blot analysis was performed. These results confirmed that EBs treated with BMP-4 (10 ng/ml) expressed cardiac β-MHC and cardiac α-actinin at levels that were 2–4-fold higher (Fig. 3D, lane 3) than those seen in the other lanes (Fig. 3D, lanes 4 and 5). In contrast, the cardiomyocyte content was markedly lower in $FBS^{+/+/+}$ and $FBS^{-/-/-}$ models with stimulation of BMP-4 at different applied doses (data not shown).

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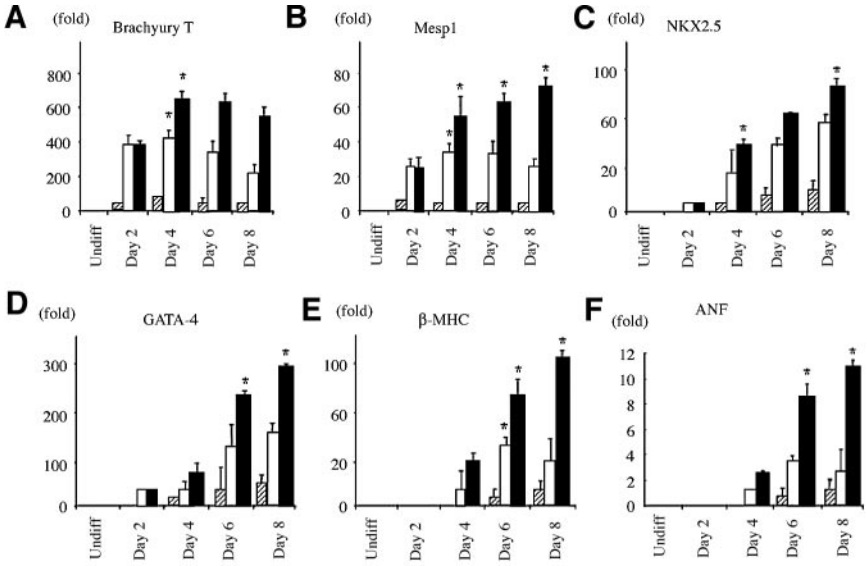

Figure 2. mRNA expression of early mesoderm, cardiac mesoderm, and cardiomyocyte from differentiated ESCs. Shown are quantitative reverse transcription-polymerase chain reaction of early mesendoderm marker **(A)**, mesoderm marker **(B)**, cardiac mesodermal markers **(C, D)**, and cardiac-specific proteins **(E, F)**. Hatched columns represent the fetal bovine serum$^{-/-/-}$ ($FBS^{-/-/-}$) model, black columns represent the $FBS^{+/-/-}$ model, and white columns represent the $FBS^{+/+/+}$ model. Each column was normalized by β-tubulin. *, $p < .05$/control. Abbreviations: ANF, atrial natriuretic factor; MHC, myosin heavy chain; Undiff, undifferentiated.

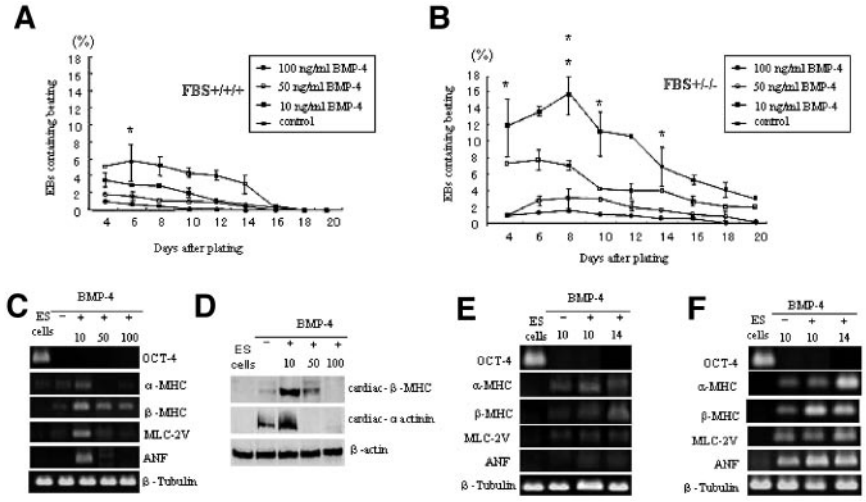

Figure 3. Effect of BMP-4 on cardiogenesis in a dose-, time-, and culture conditions-dependent manner. **(A, B):** Effect of BMP-4 concentration on the frequency of spontaneously beating EBs in the $FBS^{+/+/+}$ model **(A)** and in the $FBS^{+/-/-}$ model **(B)**. **(C):** Reverse transcription-polymerase chain reaction (RT-PCR) of cardiac-specific proteins (α-MHC, β-MHC, MLC-2v, and ANF) and OCT-4 were performed in the $FBS^{+/-/-}$ model. **(D):** Western blotting analysis for cardiac β-MHC and cardiac α-actinin of $FBS^{+/-/-}$ model. **(E, F):** RT-PCR analysis of cardiac-specific proteins of the $FBS^{+/+/+}$ and $FBS^{+/-/-}$ models of EBs treated with BMP-4 on days 10 and 14 of differentiation, respectively. *, $p < .05$; **, $p < .001$/$FBS^{-/-/-}$ model. Abbreviations: ANF, atrial natriuretic factor; BMP, bone morphogenic protein; ES, embryonic stem; FBS, fetal bovine serum; MHC, myosin heavy chain; MLC-2V, myosin light chain 2 ventricular.

To gain further understanding about the role of BMP-4 in directing the differentiation of ESCs to cardiomyocytes, we analyzed the expression of a panel of genes associated with ESCs (OCT-4), as well as early and late cardiomyogenesis (α-MHC, β-MHC, MLC-2v, and ANF) by RT-PCR. As shown in Figure 3E, after 10 and 14 days, EBs that were cultured in FBS culture medium (with or without exogenous BMP-4) weakly expressed cardiac-specific proteins, whereas ESCs cultured in BMP-4-supplemented KSR medium expressed both early and late cardiac genes from day 10 to day 14 (Fig. 3F).

Effect of BMP-4 on Different Lineages Based on Doses Applied and Culture Conditions

To further validate the incidence of cardiomyocyte induction in the three experimental models, ESCs treated with BMP-4 (10 ng/ml) were immunostained for cardiac-specific proteins and analyzed. Most ESCs treated with BMP-4 (10 ng/ml) stained for cardiac α-actinin, MLC2v, ANF, and cardiac troponin T in the $FBS^{+/-/-}$ model (Fig. 4A, bottom panels). In contrast, fewer ESCs stained for these markers in the $FBS^{+/+/+}$ and $FBS^{-/-/-}$ models (Fig. 4A, middle and top panels, respectively). Also, EBs stained with cardiac β-MHC showed results similar to those obtained for individual cells (Fig. 4A, right column). We also performed immunostaining for cardiac β-MHC (Fig. 4B, brown positive cells) to count the number of cardiomyocytes in each EB. Figure 4C summarizes the results of the three experimental models. For all three conditions, we administrated BMP-4 (10 ng/ml) from day 3 to day 10 (8 days). We found that in the $FBS^{+/-/-}$ model, BMP-4 significantly increased the percentage of cardiomyocytes per EB (21.07 ± 2.01) and inhibited cardiac induction in the $FBS^{+/+/+}$ model (9.33 ± 1.40). In contrast, BMP-4 did not augment cardiac induction significantly in the $FBS^{-/-/-}$ model (1.19 ± 1.1). Cell number/EB induction in the $FBS^{+/+/+}$ model was higher compared with the $FBS^{+/-/-}$ and $FBS^{-/-/-}$ models ($p < .001$), and the addition of BMP-4 did not influence these numbers. These findings emphasize that (under different culture conditions) an appropriate concentration of BMP-4 was required to promote cardiac-specific protein expression during ESC differentiation into cardiac myocytes.

We also studied the effect of BMP-4 on adipogenesis. Figure 4D shows an EB outgrowth with some small droplets and some large droplets of fat. Figure 4E shows an EB outgrowth stained with ORO, a specific lipid-soluble stain. We found that in the $FBS^{-/-/-}$ model, untreated EBs did not show adipogenic staining. Instead, differentiated cells in the untreated ESCs were identified as neural-like cells with the appearance of axons and

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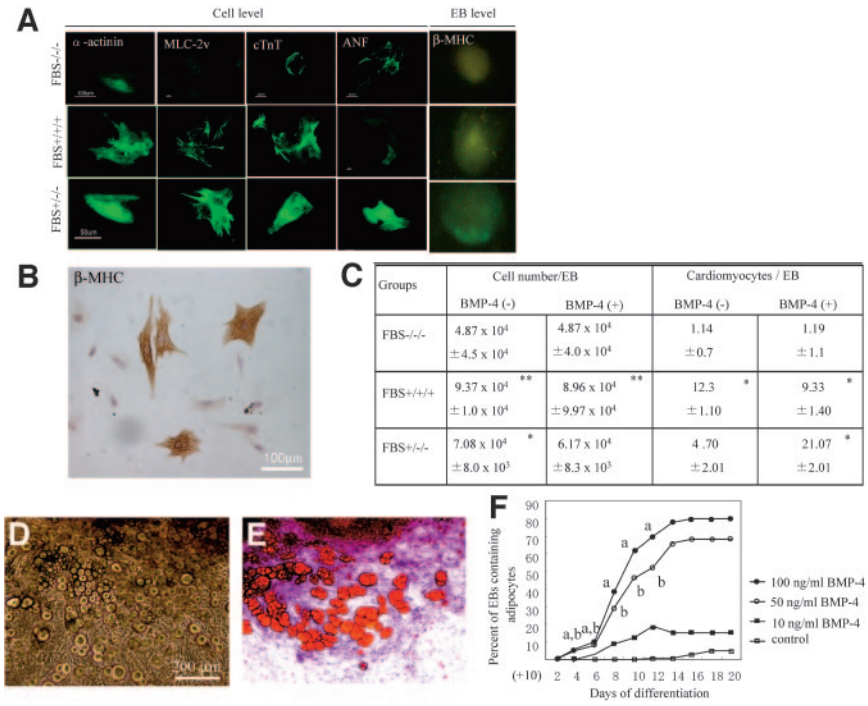

Figure 4. Stimulation of EBs with BMP-4 induced different lineages based on dose and culture condition. **(A):** EBs in three experimental models at day 18 were dissociated into single cells followed stained for cardiac-specific proteins. **(B):** ESCs also were stained for cardiac β-MHC. Brown, positive cells represent cardiac-MHC. **(C):** Number of cells/EB and number of positive cardiomyocytes (β-MHC cells)/EB. **(D):** An EB containing fat droplets under the light microscope. **(E):** Oil red O staining of an EB containing fat droplets (red stain). **(F):** Percentages of EBs containing adipocytes at different day of differentiation. *, $p < .05$/control; **, $p < .001$/control for **(A)** and **(B)**. **(A, B):** 100 ng/ml BMP-4 **(A)** and 50 ng/ml BMP-4 **(B)** versus 10 mg/ml BMP-4 group are significant ($p < .05$). Abbreviations: ANF, atrial natriuretic factor; BMP, bone morphogenic protein; cTnT, cardiac troponin T; FBS, fetal bovine serum; MHC, myosin heavy chain; MLC-2v, myosin light chain 2 ventricular.

dendrites, which were observed throughout edges of EBs on day 10 and increased until day 30 (data not shown). The percentage of EBs that stained with ORO after treatment with BMP-4 at a low concentration (10 ng/ml) was 6%; at a high concentration (50 ng/ml), it was 20%. In the $FBS^{+/-/-}$ model, the untreated ESCs were similar to the $FBS^{-/-/-}$ model except that the appearance of fine fat droplets in the cells were observed from day 24 of differentiation (Fig. 4F). In this model, stimulation of EBs with BMP-4 (10 ng/ml) resulted in low-level adipogenesis (13% at day 30 of differentiation), whereas stimulation with 50 and 100 ng/ml increased the number of EBs containing adipocytes (76% and 79% at day 30 of differentiation, respectively) (Fig. 4F). In the $FBS^{+/+/+}$ model, there was no detectable adipogenesis until 30 days after plating either in untreated ESCs or in cells that had been treated with BMP-4 at any of the three concentrations. Indeed, stimulation of ESCs by BMP-4 shifted the differentiation lineage into hematopoietic-like clusters at day 17 after plating (data not shown).

Effect of BMP Antagonists and Growth Factors on Differentiation of ESCs into Cardiomyocytes

To determine whether BMP antagonists such as noggin and chordin could be used to inhibit the differentiation of ESCs into cardiomyocyte, we stimulated EBs that were treated with BMP-4 in KSR medium with noggin (150 ng/ml) and chordin (150 ng/ml) throughout the 8 days of suspension culturing followed by outgrowth in gelatin-coated dishes and assessed the EB beating frequency of the control, BMP-4-treated (10 ng/ml), noggin-treated, and chordin-treated groups at day 18 of differentiation. Chordin and noggin significantly decreased the number of beating EBs (noggin, 2.2%; chordin, 1.4%) compared with the BMP-4 group (16%) and control group (6%) (Fig. 5A). The samples were also analyzed for protein expression using Western blotting analysis. As shown in (Fig. 5B), the expression of cardiac-MHC was increased by BMP-4 but was decreased dramatically by the addition of noggin and chordin.

The exogenous stimulation of ESCs with different growth factors was also analyzed. Figure 5C represents the stimulation of ESCs in the $FBS^{+/+/+}$ model (white columns) and $FBS^{+/-/-}$ model (black columns) with various growth factors. It was found that the exogenous addition of either FGF or insulin-like growth factor-1 (IGF-1) in the $FBS^{+/+/+}$ model inhibited the differentiation of ESCs into cardiomyocytes, whereas in the $FBS^{+/-/-}$ model, cardiomyocyte differentiation was slightly increased.

A number of growth factors and chemical compounds induce cardiomyocyte differentiation of mouse ESCs, including ascorbic acid (fivefold increase) [20], reactive oxygen species (2.7-fold increase) [21], and TGF-β plus BMP-2 (threefold increase) [9], as well as oxytocin, retinoic acid, and dimethyl sulfoxide [22–24]. To our knowledge, however, no previous protocol for increasing cardiomyocyte differentiation is as efficient as our protocol (approximately 80-fold increased in the number of cardiomyocytes compared with control).

Cynomolgus Monkey ESC-Derived Cardiomyocytes Were Electrically Active Based on Electrophysiological Studies by MEAs

To determine whether the electrophysiological properties of the multicellular cardiac structure follow cardiac maturation of ESC-derived cardiomyocytes, an MEA system was used to measure the extracellular electrical activity. By plating EBs on the MEA, we examined electrical activity from spontaneously beating cardiac myocytes. The development of visible beating coincided with the occurrence of negative deflection in the FP recorded with the MEA. Figure 6A illustrates an example of FP recorded from EB at day 18 of differentiation. In the magnification of the FP recorded from electrode 56, the FP parameters analyzed are depicted (Fig. 6A, left panel): the ISIs, the size of largest negative peak (FP_{MIN}), the last positive peak in a cycle (FP_{MAX}), the duration of FP (FP_{dur}, defined as the interval between FP_{MIN} and FP_{MAX}), and the FP descent phase (FP_{rise}, defined as the time from the onset of the FP [baseline] to FP_{MIN}). These parameters were chosen according to previous analysis and interpretation of FP, which directly correlated FP parameters to simultaneously recorded action potentials [25–27]. MEA records revealed FP morphological properties that are comparable to the P wave and the QRS complex of electrocar-

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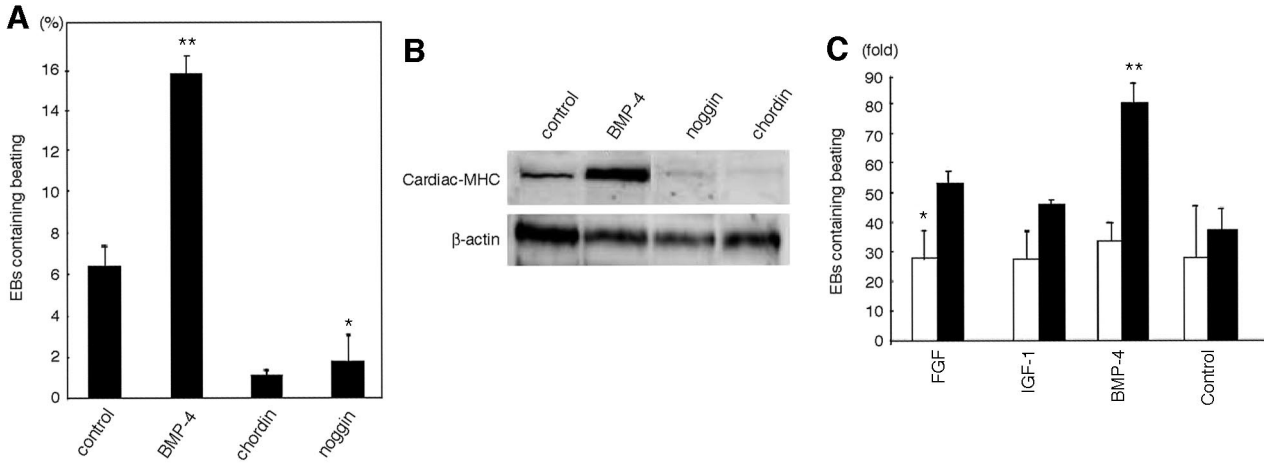

Figure 5. BMP antagonists decreased cardiomyocyte induction from ESCs. EBs were stimulated with BMP-4 (10 ng/ml), noggin (150 ng/ml), and chordin (150 ng/ml) for 8 days. **(A):** Percentages of EBs containing beating cardiomyocytes were counted at day 14. **(B):** Western blotting analysis was performed for EBs by using whole cell extract for cardiac-MHC protein. Lane 2 indicates 10 ng/ml BMP-4-treated EBs. Lanes 3 and 4 indicate Noggin-treated (150 ng/ml) and chordin-treated (150 ng/ml) EBs, respectively. **(C):** Other factors, including IGF-1 and FGF, were tested. Black columns are $FBS^{+/-/-}$-treated ESCs, and white columns are $FBS^{+/-/-}$-treated ESCs. *, $p < .05$; **, $p < .001$/control. Abbreviations: BMP, bone morphogenic protein; FGF, fibroblast growth factor; IGF, insulin-like growth factor; MHC, myosin heavy chain.

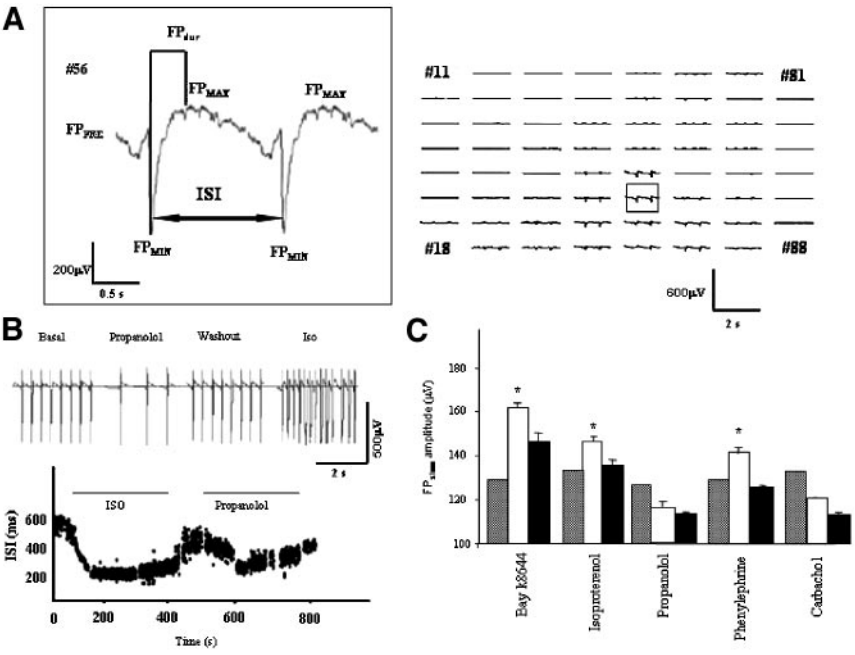

Figure 6. Pharmacological analysis of cardioactive agents on rhythmical and electrical activity of ESC-derived cardiac clusters. **(A):** Right panel, FP recorded from EB (day 18) with multielectrode arrays. The 8 × 8 arrangement of the traces reflects the arrangement of electrodes. The parameters analyzed are indicated for the magnified FP (left panel). **(B):** FP recording derived from a representative cynomolgus monkey ESC cluster under treatment of 10^{-5} M propranolol and 10^{-5} M Iso. **(B):** Lower part indicates ISI plotted against the time of recording. Propranolol antagonized reversibly the positive chronotropic effect induced by Iso. **(C):** Statistical analysis of the FP_{SLOW} amplitude. White columns, EBs treated with bone morphogenic protein 4 (BMP-4) in the $FBS^{+/-/-}$ group; black columns, EBs treated with BMP-4 in the $FBS^{+/+/+}$ group; gray columns, baseline level. For statistical evaluation, analysis of variance was performed. *, $p < .05$/control. Abbreviations: FP, field potential; FP_{dur}, duration of field potential; FP_{MAX}, last positive peak in a cycle; FP_{MIN}, size of largest negative peak; FP_{SLOW}, slowly occurring negative FP deflection; ISI, interspike interval; Iso, Isoproterenol; s, seconds.

diograms by the plating of two beating EBs on the MEA (data not shown). These two beating EBs were connected by a conductive tissue bridge, and it is thought that the first EB may serve as a primary pacemaker, continuously driving the activity of the second beating EB. Another source of data, typical for cardiac cells, would be to consider the chronotropic response to cardioactive agents, such as Bay k8644 (Ca^{2+} ionic channel activator) [28], isoprenaline (agonist of β1-adrenoceptors) [29], phenylephrine (agonist of α1-adrenoceptors) [30], propranolol (antagonist of β-adrenoceptors) [30], and carbachol (agonist of muscarinic cholinoceptor) [31], on differentiated ESCs in culture. A negative chronotropic effect in the heart is mediated by propranolol via the inhibition of β-adrenoceptors. To test whether the susceptibility of the beating frequency to agonist and antagonist adrenergic stimulation in ESCs modeled this change during the differentiation process in culture, we perfused the ESC-derived cardiomyocytes with the β-adrenergic agonist isoprenaline or the β-adrenergic antagonist propranolol at 10^{-5} M alone or in the presence of BMP-4. In these studies, the application of propranolol decreased the beating frequency, whereas administration of Isoproterenol (ISO) (10^{-5} M) increased it (Fig. 6B, upper panel). On the other hand, the negative chronotropic effect of ISO is reflected in a significant decrease of ISI, whereas propranolol is reflected in a small increase of the ISI (Fig. 6B, lower panel), indicating a stable increase of beating frequency until washout of propranolol was performed, similar to the effect of washout of ISO. The beating frequency in EBs treated with BMP-4 in the $FBS^{+/+/+}$ model was lower than that in nontreated EBs in response to agonist and antagonist adrenergic stimulation, whereas the beating frequency in EBs treated with BMP-4 in the $FBS^{+/-/-}$ model was higher than that in the $FBS^{+/+/+}$ model (data not shown). These results suggest that the development of ion channels, adrenoreceptors α and β, and functional cholinoreceptors in EBs treated with BMP-4 in the

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$FBS^{+/-/-}$ model is more complete than in the $FBS^{+/+/+}$ model. As previously reported [32], FP_{SLOW} can be taken as a direct readout for L-type Ca^{+} channel activity. We examined effect of Bay k8644 on FP_{LOW} amplitude as well as ISO and propranolol since we expected that it would enhance L-type Ca^{+} channel activity and increase conduction velocities. We found that FP_{SLOW} amplitude was significantly increased after application of Bay k8644 (Fig. 6C). ISO also increased the FP_{SLOW}, whereas propranolol decreased FP_{SLOW}. As shown in Figure 6C, the FP_{SLOW} amplitude in EBs treated with BMP-4 in the $FBS^{+/-/-}$ model in response to the Ca^{+} channel activator, as well as to the (β-adrenergic) agonist, and antagonist was better than EBs treated with BMP-4 in the $FBS^{+/+/+}$ model (Fig. 6C). These results correspond to those shown in Figure 6B.

DISCUSSION

We demonstrate for the first time the potential of nonhuman primate (cynomolgus monkey) ESCs to differentiate into cardiomyocytes in vitro. Using our new protocol, we were able not only to successfully generate functional cardiomyocytes from cynomolgus monkey ESCs with efficiencies comparable to those of human ESCs but also to enhance their differentiation rates. This technique may be useful since, based on their evolutionary origin, cynomolgus monkey ESCs may be similar to human ESCs [33]. Thus, the cardiac differentiation of these ESCs provides an excellent model to study the early steps in human cardiogenesis in vitro or as an in vivo model for ESC-based cardiac repair.

The developmental fate of ESCs is determined by a complex array of growth factors, signaling molecules, and extracellular matrix proteins [34]. Therefore, determining the key molecular pathways and signaling cues that determine ESC differentiation is a critical to developing ESC-based therapies. So far, no culture conditions exist to produce a pure population of cardiomyocytes from ESCs. To direct the differentiation of ESCs into cardiomyocytes, it is critical to identify the inductive or inhibitory effect of various growth factors. A number of growth factors and chemical compounds have been shown to induce cardiomyocyte differentiation of mouse ESCs, including TGF-β with BMP-2 [9], IGF [35], FGF [36], oxytocin [37], dimethyl sulfoxide [38], and ascorbic acid [20]. Here, we found that the exogenous cardiac inducer BMP-4 in KSR medium could be used to induce cardiac differentiation of monkey ESCs. The exact role of BMPs in cardiac development is unclear. Recently, Yuasa et al. [40] indicated that the BMP antagonist noggin is transiently expressed in the heart-forming area and induces cardiomyocyte differentiation of mouse ESCs. Moreover, they reported that inhibition of BMP signaling by noggin in ESCs enhances cardiomyocyte differentiation. However, there is no information about the involvement of BMP signaling in the cardiac differentiation of nonhuman primate ESCs. Although BMPs were originally identified as peptides that induce bone and cartilage formation in ectopic extraskeletal sites in vivo, they are involved in most morphogenetic processes during development. Depending on culture conditions, BMP signaling is involved in mesodermal induction and cardiac formation in mice [11, 12]. Also, efficient hematopoietic development and maintenance of hematopoietic precursors from rhesus monkey ESCs required the presence of exogenous BMP-4 when the culture medium environment was supplemented with animal serum [39], thereby suggesting that serum supplements in combination with BMP-4 would be sufficient to support hematopoietic differentiation. Furthermore, the role of BMP in adipocyte differentiation was previously suggested using an in vitro system in which cultures were maintained under differentiating conditions [41]. In another study on mouse ESCs, BMP-4 inhibited neurogenesis in serum-free medium [42]. In the present study, we stimulated cynomolgus monkey ESCs in suspension cultures with BMP-4 in a modified manner. We administrated serum (FBS) during EB formation for 2 days to mimic transient and strong expression of mesoderm at the early stage. Further outgrowth of EBs by stimulation with BMP-4 in KSR medium directed them to complete mesoderm formation and cardiomyocyte induction.

In our model, we used KSR medium as a serum-free replacement. The use of serum-free media is desired since they eliminate the batch-to-batch variability associated with normal serum and minimize the risk of pathogens and foreign molecules. The KSR serum was originally developed for the maintenance of ESCs in an undifferentiated state within in vitro culture. Although the exact chemical composition of KSR is not available because it is a protected trade secret, it has been reported to be completely devoid of undefined growth factors or differentiation-promoting factors. The results of the present study indicated that in the $FBS^{-/-/-}$ model (i.e., KSR medium throughout the 30-day experiment), a small fraction of beating EBs was observed in both control EBs and EBs treated with BMP-4. Therefore, BMP-4 alone is not sufficient to induce cardiomyocyte differentiation. Stimulation of EBs in the $FBS^{+/+/+}$ model with BMP-4 inhibits the spontaneous beating of EBs. Therefore, we suggest that the concentration of BMP-4 presented in serum is at an ideal concentration to promote cardiogenesis and that the addition of exogenous BMP-4 resulted in a negative feedback that inhibited cardiogenesis. In the $FBS^{+/-/-}$ model, in which EBs were formed in FBS-containing medium followed by following suspension and plating with KSR medium, stimulating EBs with a low-dose of BMP-4 induced maximum cardiomyocyte differentiation. These results suggest that BMP-4, as a cardiac inducing factor, act in synergy with other factors. It appears that in early states of differentiation (EB formation for 2 days), additional growth and differentiation factors in FBS are required to promote the differentiation of ESCs into a primed state from which BMP-4 can induce further differentiation into cardiac lineages. Since BMP-4 is present in FBS, perhaps the lack of FBS in early stages of differentiation prevents mesoderm induction in comparison with ectoderm and endoderm. The appearance of abundant neural cells in the $FBS^{-/-/-}$ model confirmed the prevalence of cellular differentiation into ectodermic cells. Thus, the starting point of BMP-4 for cardiac differentiation may be downstream of mesoderm induction, which correlates with a previous report that proposed BMP signaling is essential for at least two steps in the cardiomyocyte induction process: mesoderm induction and cardiomyocyte differentiation. However, between these steps, a transient block of intrinsic BMP signaling may be the most important step for cardiomyogenic differentiation [40]. Another recent report indicated that BMPs are not required for expression of early transcriptional regulators of cardiac fate but are essential for migration and/or fusion of the heart primordia and cardiomyocyte differentiation [43]. In this report, the embryos in which BMP signaling was selectively blocked expressed early transcription cardiac markers, such as Nkx2.5 and GATA-4. They showed that BMP signaling was required for maintenance of Nkx2.5 expression and heart formation, but it did not inhibit the early expression of Nkx2.5. Taken together, these results demonstrate that BMPs do not contribute to the early expression of Nkx2.5 but rather its subsequent maintenance. Thus BMP might act downstream of Nkx2.5 and GATA-4. Our results indicate that BMP-4 acted upon mesendodermal precursors and promoted them to give rise to mesodermal progenitors. In medium supplemented with FBS, addition of

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BMP-4 inhibited cardiomyocyte differentiation in a dose-dependent manner.

Ultrastructure analysis revealed that in the $FBS^{+/+/+}$ model, EBs treated with BMP-4 had irregular myofibril arrays and unaligned densely packed sarcomeres and lacked transfer tubules, desmosomes, gap junctions, and adjacent junctions. This may be due to a direct effect of BMP-4 on cardiomyocyte differentiation that delays or inhibits the formation of substructurally matured cardiomyocytes, but in the $FBS^{+/-/-}$ model, BMP-4 not only induced mature myofibrillar bundle organization but also induced abundant intercalated discs, including adherence junctions, gap junctions, and desmosomes. These characteristics indicate that the development of ion channels, functional receptors, and intercalated discs was induced by BMP-4. These findings suggest that ESC-derived cardiomyocytes cultured in KSR medium supplemented with BMP-4 matured more rapidly both in terms of chronotropic characteristics and subcellular organelle development.

Electrophysiological studies were also performed to obtain further proof of the functionality of the generated cardiomyocytes. With the differentiating EBs plated on a MEA, we were able to continuously monitor the spatial and temporal structure and the dynamics of electrical activity of a developing multicellular structure of ESC-derived cardiac myocytes. In differentiating EBs, cells of all three germ layers were detected, although the distribution of different cell types inside an EBs needs to be further analyzed. Oyamada et al. [44] reported that no dye coupling takes place between cardiac and noncardiac cells in the EBs, indicating that cardiac cells are electrically isolated from the surrounding tissue. In addition to the analysis of the beating frequency and the velocity of excitation spread by means of the FP, β-adrenergic signaling as a specific feature of cardiomyocytes versus other muscle cell types, including skeletal muscle and smooth muscle cells, was investigated by stimulation with the β-adrenergic agonist and antagonist (ISO and propranolol, respectively). The electrophysiological basis of the increased beating frequency could depend on multiple factors. In isolated ESCs, intracellular Ca^+ oscillation, ATP-dependent K^+ channels (I_{to}), and the pacemaker current (*If*) have been described to play an important role in the development of pacemaker activity. Therefore, we performed an analysis of a positive and negative chronotropic effect of ISO and propranolol on beating frequency of EBs. As expected, increased beating and FP frequency was recorded with ISO and decreased with propranolol. Comparing the beating frequency following administration of a β-adrenergic agonist and antagonist revealed that the beating frequency in EBs treated with BMP-4 was higher in the $FBS^{+/-/-}$ model and lower in the $FBS^{+/+/+}$ model (data not shown). This result indicated that the development of ion channels, α-β adrenoreceptors, and functional cholinoreceptors in ESCs treated with BMP-4 in the $FBS^{+/-/-}$ model was more complete than in EBs treated with BMP-4 in the $FBS^{+/+/+}$ model. Our TEM results corresponded to the response of ESC-derived cardiomyocytes to β-adrenergic stimuli. Moreover, the FP_{SLOW} amplitude as an indirect readout for voltage-dependent Ca^+ channel activity was significantly increased after administration of ISO, Bay k8644, and phenylephrine, especially in EBs treated with BMP-4 in the $FBS^{+/-/-}$ model. Therefore, due to the development of spontaneous electrical activity and beating, as well as positive chronotropic effects and increased FP_{SLOW} in response to L-type Ca^+ channel activity, we suggest that cynomolgus monkey ESC-derived cardiomyocytes obtained a functional characteristic similar to that of embryonic cardiomyocytes.

In conclusion, our results indicate that BMP-4 had a suppressive effect on ESC differentiation into cardiomyocytes in FBS culture medium, as it decreased the incidence of EBs containing beating and the cardiomyocyte responsiveness to chronotropic drugs, especially in early and intermediate stages, as well as ultrastructural differentiation of cardiomyocytes and downregulated cardiac-specific gene expression. Thus, we suggest that to induce mesodermal layer, FBS culture medium is required at the early stages of EB formation and that BMP-4 enhanced differentiation of cardiomyocytes from ESCs only when the culture medium was replaced with KSR. This induction system may provide new approaches for elucidating the mechanisms regulating primate cardiac development and differentiation during embryogenesis.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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Bone Morphogenetic Protein-4 Enhances Cardiomyocyte Differentiation of Cynomolgus Monkey ESCs in Knockout Serum Replacement Medium

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