Endothelial Cells Regulate Cardiomyocyte Development From Embryonic Stem Cells

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ABSTRACT

The molecules and environment that direct pluripotent stem cell differentiation into cardiomyocytes are largely unknown. Here, we determined a critical role of receptor tyrosine kinase, EphB4, in regulating cardiomyocyte generation from embryonic stem (ES) cells through endothelial cells. The number of spontaneous contracting cardiomyocytes, and the expression of cardiac-specific genes, including α-MHC and MLC-2V, was significantly decreased in EphB4-null ES cells. EphB4 was expressed in endothelial cells underneath contracting cardiomyocytes, but not in cardiomyocytes. Angiogenic inhibitors, including endostatin and angiostatin, inhibited endothelial cell differentiation and diminished cardiomyogenesis in ES cells. Generation of functional cardiomyocytes and the expression of cardiac-specific genes were significantly enhanced by co-culture of ES cells with human endothelial cells. Furthermore, the defects of cardiomyocyte differentiation in EphB4-deficient ES cells were rescued by human endothelial cells. For the first time, our study demonstrated that endothelial cells play an essential role in facilitating cardiomyocyte differentiation from pluripotent stem cells. EphB4 signaling is a critical component of the endothelial niche to regulate regeneration of cardiomyocytes. J. Cell. Biochem. 111: 29–39, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: EMBRYONIC STEM (ES) CELLS; CARDIOMYOCYTES; ENDOTHELIAL CELLS; EPHB4; EPHRIN

Functional cardiomyocytes in the adult heart are terminally differentiated and unable to reenter the cell cycle for regeneration. The damage to cardiomyocytes resulting from ischemic injury is irreversible and leads to the development of progressive heart failure. Recently, therapies based on cardiac progenitor cells have emerged as potential cardiac therapeutics. Cardiac stem or progenitor cells can be isolated from early embryos or pluripotent stem cells, and cultured to differentiate into functional cardiomyocytes. A small amount of cardiac stem or progenitor cells are identified in the adult myocardium [Beltrami et al., 2003; Oh et al., 2003; Matsuura et al., 2004; Laugwitz et al., 2005; Pfister et al., 2005; Wang et al., 2006; Oyama et al., 2007]. However, the quantity of cardiac progenitor cells in the adult heart may not be sufficient for regeneration after myocardial injury. Therefore, different non-cardiac cells, including skeletal myoblasts [Menasche et al., 2003], bone marrow cells [Jackson et al., 2001], endothelial progenitor cells [Kocher et al., 2001], and mesenchymal stem cells [Makino et al., 1999], were examined as potential cell

sources to repair the injured heart after cardiac damage [Davani et al., 2005; Laflamme and Murry, 2005]. One of the major obstacles for using these cells in transplantation study is the low efficiency of myogenic differentiation in vivo and the modest effect on cardiac function.

Pluripotent embryonic stem (ES) cells are able to differentiate into cardiomyocytes [Laflamme and Murry, 2005]. Transplantation of cardiomyocytes derived from human or mouse ES cells improves cardiac function in animal models [Klug et al., 1996; Xue et al., 2005; Caspi et al., 2007]. Although ES cells have the potential to be renewable sources of cell-based therapy, efficiently generating cardiomyocytes from ES cells is a major challenge because the condition and factors to direct ES cells differentiation into cardiomyocytes are largely unknown. Several studies demonstrate that endothelial progenitor cells harvested from the bone marrow [Takahashi et al., 1999; Kocher et al., 2001; Schachinger et al., 2004], circulating peripheral blood [Asahara et al., 1999; Urbich and Dimmeler, 2004], and ES cells [Li et al., 2007] can contribute to angiogenesis and improve the function of ischemic myocardium. The developmental relationship of endothelial cells and cardiomyocytes, and mechanisms by which endothelial cells improve cardiac function after ischemic injury are not well understood.

During embryonic heart development, endothelial cells and cardiomyocytes arise from the same cardiac mesodermal precursors [Linask and Lash, 1993; Narumiya et al., 2007]. There are two types of cardiac endothelial cells in the heart: (i) endocardial endothelial cells and (ii) myocardial capillary endothelial cells (MyoCapE) [Brutsaert, 2003]. Cardiac endothelial cells release some factors, such as nitric oxide, endothelin-1, prostanoids, adenyl purines, and natriuretic peptides [Shah, 1996]. Interaction between endothelial cells and cardiomyocytes in the heart regulates normal heart functions, such as cardiac growth, contractile performance, and rhythmicity [Brutsaert, 2003; Kuruvilla and Kartha, 2003; Hsieh et al., 2006]. The receptor tyrosine kinase, EphB4, and its ligand, ephrinB2, are key regulators of vascular development [Gale and Yancopoulos, 1999; Conway et al., 2001]. Signaling between Eph receptors and their ephrin ligands is restricted to sites of direct cell–cell contact, and can induce reciprocal bidirectional events between interacting cells [Davis et al., 1994; Bruckner and Klein, 1998; Gale and Yancopoulos, 1999]. The bidirectional signaling of EphB4 and ephrinB2 regulates arteriovenous asymmetry in the developing vasculature [Gale and Yancopoulos, 1999; Conway et al., 2001]. During embryonic development, EphB4 is predominantly expressed in the venous endothelium, whereas ephrinB2 is primarily expressed in arterial endothelial cells [Gerety et al., 1999; Wang et al., 1998]. Null mutations in either EphB4 or ephrinB2 genes result in embryonic lethality, and display identical defects in forming capillary connections between arterial and venous networks of the head and yolk sac [Gerety et al., 1999; Wang et al., 1998]. Arrested heart development at E9.5 to E10, such as failure to increase in size and incomplete cardiac looping, occurs in EphB4-null and ephrinB2-null mice, which corresponds to early defects of vascular development [Gerety et al., 1999].

We previously demonstrated that EphB4 signaling regulates hematopoietic and endothelial development. The differentiation of ES cells into cardiomyocytes is also impaired in EphB4$^{-/-}$ ES cells [Wang et al., 2004]. In this study, we investigated the effect of EphB4 on ES cell differentiation into cardiomyocytes. Although differentiated cell masses from ES cells, called embryoid bodies (EBs), are far less organized than the actual embryo, they can partially mimic the spatial organization of the embryo. Our data demonstrate that generation of functional cardiomyocytes is dependent on endothelial cells, which may provide a "niche" in dishes to direct ES cell differentiation. EphB4, which is expressed in endothelial cells at regions containing functional cardiomyocytes, is a critical component for endothelial cells promoting ES cell differentiation into cardiomyocytes. The cardiomyocyte differentiation defect in EphB4$^{-/-}$ ES cells is rescued by human endothelial cells. Our studies, for the first time, demonstrate that interaction of endothelial cells and ES cells facilitates cardiomyocyte differentiation, in which EphB4 signaling is essential.

MATERIALS AND METHODS

CELL CULTURE

CGR8-GFP mouse ES cell line was generously provided by Dr. Richard T. Lee (Harvard Medical School, Boston, MA). EGFP expression in CGR8-GFP cells is under the control of cardiac muscle specific α-myosin heavy chain (MHC) promoter [Takahashi et al., 2003]. EphB4$^{+/-}$ and EphB4$^{-/-}$ ES cell lines were generously provided by Dr. David Anderson (California Institute of Technology, CA). Mouse ES cells were cultured as we described previously [Wang et al., 2004]. Briefly, mouse ES cells were maintained on a mouse feeder cell line (SNL) in Dulbecco's modified Eagle medium (DMEM) containing 15% defined-fetal bovine serum (FBS; HyClone, Logan, UT), 2 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acid, 150 μM monothioglycerol (MTG; Sigma–Aldrich, St. Louis, MO), 50 U/ml penicillin, 50 μg/ml streptomycin, and 10 ng/ml leukemia inhibitory factor (LIF; Chemicon International). The ES cells were cultured on gelatin-coated plates for two passages to remove feeder cells before EB differentiation.

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (Cambrex, Walkersville, MD). HUVEC and human bone marrow microvascular endothelial cells (BMEC) [Rafii et al., 1994; Candal et al., 1996] were cultured on gelatin-coated dishes in EGM-2 media (Cambrex). NIH3T3 cells and mouse embryonic fibroblast (MEF) cells were cultured in DMEM with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acid, 50 U/ml penicillin, and 50 μg/ml streptomycin. To generate conditioned medium (CM), BMEC were cultured in ES cell differentiation medium for 48 h, and then filtered through a 0.22 μm Steriflip filter (Millpore, Billerica, MA).

CARDIOMYOCYTE DIFFERENTIATION OF ES CELLS

ES cell differentiation into EBs was performed as described previously [Maltsev et al., 1994]. Briefly, 400 ES cells were suspended in hanging-drop cultures in 30 μl differentiation medium containing Iscove modified Dulbecco's medium (IMDM), 15% defined-FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. After 5 days of differentiation in the presence or the absence of 100 μM ascorbic acid (vitamin C), the individual EBs

were transferred to a gelatin-coated well in 48-well plates. Differentiation medium was changed every 3 days. After 24 h, the EBs were attached to the surface of the wells. After 7 to 8 days of differentiation, GFP+ cardiomyocytes from CGR8-GFP ES cells were observed under a fluorescent microscope. Spontaneously, contracting areas in beating EBs were observed after 8 to 9 days of differentiation. In general, the GFP+ cardiomyocytes formed cell clusters that generated spontaneously beating areas after 24 h.

ES cells were cocultured with endothelial cells, NIH3T3 cells, and MEFs. Undifferentiated ES (400 cells) and cocultured cells (100 to 400 of HUVEC, BMEC, NIH3T3, or MEF cells) were formed into EBs in 30 μl of ES cell differentiation medium using the hanging-drop method. ES cell differentiation was carried out with or without endostatin (2 μg/ml), angiostatin (1 μg/ml), or TNYL-RAW (5 μM). The TNYL-RAW peptide (TNYLFSPNGPIARAW) is an antagonist of ephrinB2 binding to EphB4 [Koolpe et al., 2005]. The control peptide (WYPSNTRPGILNAFA) contains a scramble sequence of TNYL-RAW. Both TNYL-RAW and control peptide are synthesized by EZBiolab (www.ezbiolab.com; Westfield, IN). Endostatin, angiostatin, TNYL-RAW, or control peptide was added into the differentiation medium at different times, depending on experiment design.
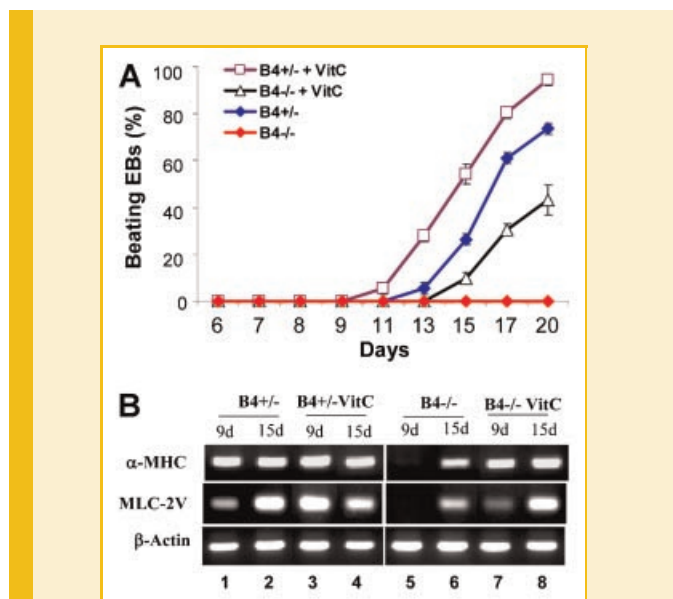
RT–PCR AND QUANTITATIVE PCR (Q-PCR) ANALYSES

Undifferentiated ES cells and differentiated EBs were harvested at different time points. Total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies). One microgram RNA was used to generate cDNA in 20 μl by reverse transcription (RT) using SuperScript III reverse transcriptase (Invitrogen). Specific primers used for PCR are listed in Supplemental Table 1.

For quantitative PCR (real-time PCR), 0.5 μl of cDNA was added to 19.5 μl of master mix containing 1× SYBR Green (Bio-Rad Laboratories, Hercules, CA) and 25 μM sense and antisense primers. Triplicates of the cDNA were amplified for 40 cycles with the iCycler iQ real-time thermal cycler (Bio-Rad Laboratories). The PCR product level was expressed as Ct, the amplification cycle at which the emission intensity of the product rises above an arbitrary threshold level. Specific primers used for Q-PCR are listed in Supplemental Table 2.
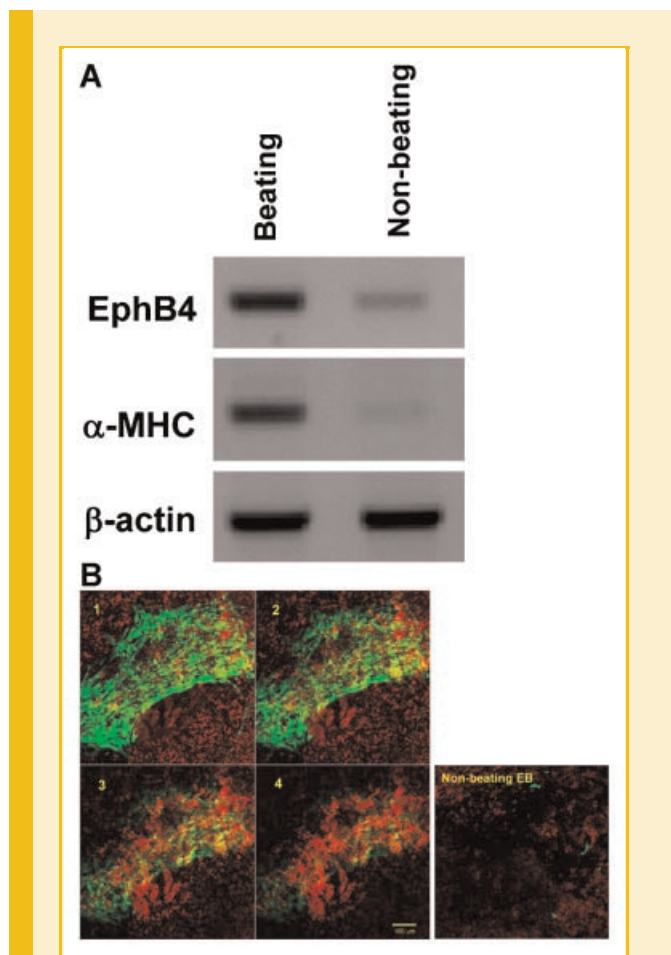

Fig. 1. Defect of cardiomyocyte differentiation in EphB4–null ES cells. Four hundred undifferentiated ES cells were differentiated in hanging-drops. Individual EBs were transferred to gelatin-coated wells at day 5. Spontaneous contracting EBs (beating EBs) were observed after EB attachment. A: Undifferentiated EphB4$^{+/-}$ ES cells (B4$^{+/-}$) or EphB4$^{-/-}$ ES cells (B4$^{-/-}$) formed EBs in hanging-drop with or without ascorbic acid (vitamin C). After 5 days, individual EBs were transferred to 48-well plates. Beating EBs were counted at different time points. For each example, percentile of beating EB was calculated from total of 24 wells. Data are representative from three independent experiments. B: RT-PCR analysis of cardiac genes, α–MHC and MLC-2V, in EphB4$^{+/-}$ or EphB4$^{-/-}$ ES cells. ES cells were differentiated in the presence or absence of ascorbic acid for 5 days. RNA samples were harvested at day 9 or 15. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 2. EphB4 expression in beating EBs. α–MHC–GFP ES cells were differentiated into EBs as in Figure 1. A: RNA samples were isolated from beating and non-beating EBs. RT-PCR was performed using primers listed in Supplemental Table 1. B: EphB4 localization was detected by immunohistochemistry with PE-conjugated EphB4 antibodies. A beating EB was analyzed by confocal microscopy at 10 μm intervals (from 1 to 4). GFP+ cells were abundant on the upper layer (1 and 2), and a high density of EphB4+ cells was observed underlying the GFP+ layer (3 and 4). A non–beating EB is showed for comparison in the right panel. The scale bar = 100 μm.

FLOW CYTOMETRIC ANALYSIS AND IMMUNOSTAINING FOR CONFOCAL MICROSCOPE ANALYSIS

Differentiated EBs at different time points were trypsinized for 2 min, and passed through a 30 μm pre-separation filter (Miltenyi Biotec Inc., Auburn, CA) to produce a single cell suspension. Cells (1×10^6) were then stained with primary antibodies as follows: VE-Cadherin (VE-Cad) at 1:100 dilution, Flk-1 (VEGFR-2) at 1:100 dilution, rat IgG2a (control antibodies) at 1:100 dilution. PE- or APC-conjugated secondary antibodies were used for flow cytometric analysis. Dead cells were gated out by a DNA dye (7AAD staining). All antibodies were purchased from BD Bioscience (Franklin Lakes, NJ).

Differentiated EBs cultured in chamber slides were fixed in 4% paraformaldehyde for confocal microscopy (Leica microscope LTCS-SP; Heidelberg, Germany). The following primary antibodies were used: CD31 (PE-CAM; Serotec, Oxon, United Kingdom) at 1:100 dilution, VE-Cad at 1:100 dilution (Bioscience), EphB4 at 1:100 dilution (R&D System, Minneapolis, MN), and ephrin B2 at 1:100 dilution (R&D System). Nuclei were labeled with TO-Pro-3 iodide (Invitrogen). Expression of human EphB4 and ephrinB2 was analyzed using a rabbit polyclonal IgG anti-human EphB4 at 1:100 and a rabbit polyclonal IgG anti-human ephrinB2 at 1:100 (both from Santa Cruz Biotechnology, CA). Nuclei were labeled with DAPI (Invitrogen).

STATISTICAL ANALYSIS

The results were subjected to statistical analysis by the Student's *t*-test. For all analyses, values of $P < 0.05$ were considered statistically significant.

RESULTS

DIFFERENTIATION INTO CARDIOMYOCYTES IS IMPAIRED BY INHIBITION OF EPHB4 SIGNALING

Our previous study indicated that beating cardiomyocytes were significantly decreased in EphB4-null (EphB4$^{-/-}$) ES cells [Wang et al., 2004]. To further characterize the role of EphB4 signaling in cardiomyocyte differentiation, we used the hanging-drop method to generate embryoid bodies (EBs) in a controlled manner during ES cell differentiation. After 5 days of initial aggregation of ES cells in hanging-drops, individual EBs were transferred into gelatin-coated wells for further cardiomyocyte differentiation. Ascorbic acid (vitamin C) has been shown to enhance cardiomyocyte differentiation, which is not enhanced by the other antioxidants [Takahashi et al., 2003]. To test whether ascorbic acid promotes cardiogenesis in EphB4$^{-/-}$ ES cells, we measured the kinetic of beating EB at different times with or without ascorbic acid. As shown in Figure 1A, no beating EBs were observed from EphB4$^{-/-}$ ES cells up to day 20 of differentiation without ascorbic acid, whereas addition of ascorbic acid increased the number of beating EBs in both EphB4$^{+/-}$ ES cells and EphB4$^{-/-}$ ES cells. By RT-PCR analysis of EBs at days 9 and 15, the expression of the muscle specific genes, α-MHC and MLC-2V, were negative at day 9, but detected at day 15 in EphB4$^{-/-}$ EBs (Fig. 1B, lanes 5 and 6). The addition of ascorbic acid increased cardiac gene expression (Fig. 1B, lanes 5 and 7) in EphB4$^{-/-}$ ES cells. These data demonstrated that EphB4 signaling can be compensated for by ascorbic acid. The mechanisms of ascorbic acid enhancing cardiogenesis in ES cells have not been defined. Whether EphB4 and ascorbic acid share downstream signaling pathway in cardiac differentiation is under investigation.
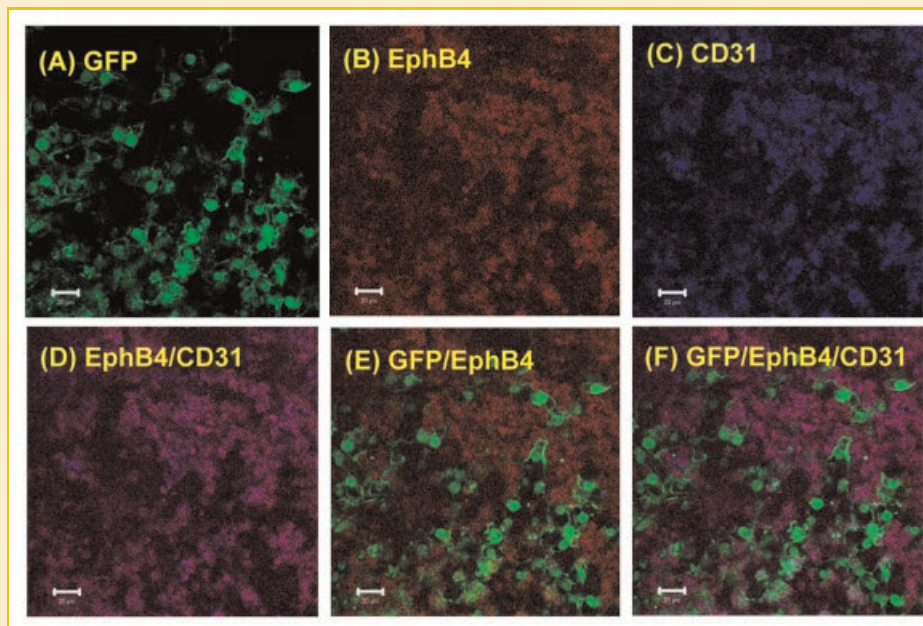

Fig. 3. EphB4 expression in endothelial cells. α-MHC-GFP ES cells was differentiated into EBs. At day 11 of differentiation, EBs were immunostained with PE-conjugated EphB4 (B) and APC-conjugated CD31 (C), and analyzed by confocal microscopy using different filters (wavelengths). GFP (A), EphB4 (B), and CD31 (C) were analyzed by single wavelength in the same beating EB. The expression of EphB4 and CD31 (D), GFP and EphB4 (E), and GFP, EphB4 and CD31 (F) in the same EB were analyzed by multi-wavelengths. The scale bar = 20 μm. The data represent one of five EBs analyzed.

EPHB4 AND EPHRINB2 ARE EXPRESSED IN BEATING EBS

To determine whether EphB4 signaling is directly involved in regulating cardiomyocyte beating function, we analyzed EphB4 expression in CGR8-GFP mouse ES cells, in which GFP expression is controlled by the α-cardiac MHC promoter [Takahashi et al., 2003]. During ES cell differentiation, GFP is exclusively expressed in cardiomyocytes in CGR8-GFP ES cells. High expression of EphB4 in the beating EBs was observed by RT-PCR analysis (Fig. 2A). To further determine whether EphB4 is expressed in functional cardiomyocytes, beating and non-beating EBs were analyzed by confocal microscopy. Within a beating EB, GFP+ cardiomyocytes were clustered together in sheets, and were located on the upper layer of beating EBs (Fig. 2B). EphB4 was expressed in cell aggregates underneath the cardiomyocytes (Fig. 2B-3,4), but not in GFP+ cardiomyocytes (Fig. 2B-1,2). EphB4 was rarely expressed in non-beating EBs (Fig. 2B, right panel). These results indicate that EphB4+ cells are colocalized in regions of beating cardiomyocytes within a beating EB.

EPHB4+ CELLS UNDERNEATH CARDIOMYOCYTES EXPRESSED THE ENDOTHELIAL MARKER, CD31

EphB4 plays an essential role in angiogenesis during embryonic development [Wang et al., 1998]. Our previous study demonstrated that endothelial development is impaired in EphB4$^{-/-}$ ES cells [Wang et al., 2004]. To determine whether EphB4-expressing cells underneath GFP+ clusters were endothelial cells, we analyzed individual beating EBs by a confocal microscopy with APC-conjugated CD31 antibodies and PE-conjugated EphB4 antibodies. As shown in Figure 3, GFP (A), EphB4 (B), and CD31 (C) were expressed in the beating EB under a single fluorescence wavelength. Analyses of the beating EB with multi-wave lengths indicated that EphB4+ cells also expressed the endothelial cells marker, CD31 (D). However, neither EphB4 (E) nor CD31 (F) was expressed in GFP+ cardiomyocytes.

ENDOTHELIAL CELLS ENHANCE CARDIOMYOCYTE DEVELOPMENT IN ES CELLS

Communication of endothelial cells and cardiomyocytes regulates cardiomyocyte function, including the contractile state of the adult heart [Brutsaert, 2003]. It is unclear whether endothelium provides signals to facilitate stem cells to differentiate into functional cardiomyocytes. Ascorbic acid promotes collagen synthesis during cardiomyocyte differentiation of ES cells [Sato et al., 2006]. In addition, ascorbic acid is essential in collagen synthesis by endothelial cells during angiogenesis [Telang et al., 2007]. To test the hypothesis that endothelial cells play an essential role in ES cell differentiation into cardiomyocytes, we cocultured endothelial cells with CGR8-GFP mouse ES cells in hanging-drop EB formation. Human endothelial cells (BMEC or HUVEC) or non-endothelial cells (NIH3T3 or MEF) were mixed and aggregated with 400 ES cells. By day 13 of ES cell differentiation, the EBs containing either BMEC or HUVEC endothelial cells dramatically increased the number of beating EBs (Fig. 4A), as compared to ES cells alone (control). In contrast, the addition of MEF cells moderately decreased the number of spontaneous beating EBs, whereas no spontaneous beating EB was observed with the addition of NIH3T3 cells (Fig. 4A). As shown
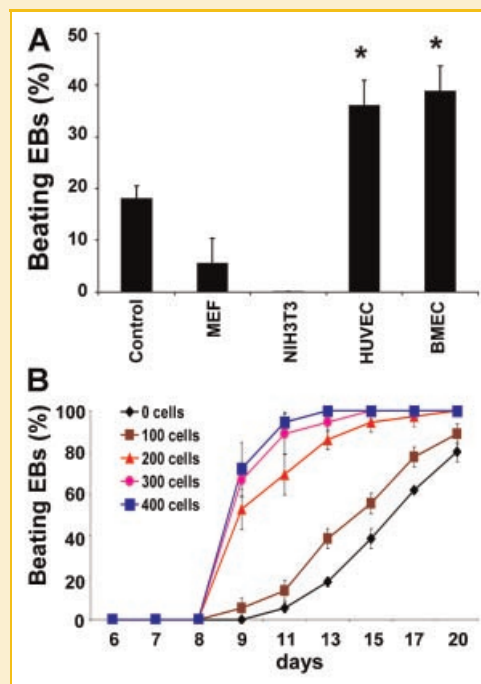

Fig. 4. Effects of endothelial cells on cardiomyocyte differentiation. A: Undifferentiated α-MHC-GFP ES cells formed EBs in hanging-drops (400 ES cells/drop) without 200 exogenous cells (control) or with MEF, NIH3T3, HUVEC, or BMEC cells. After 5 days, individual EBs were transferred to individual wells in 24-wells coated with gelatin. The wells containing beating EBs were counted at day 13 of differentiation. B: Undifferentiated ES cells formed EBs in hanging-drops (400 ES cells/drop) containing 0, 100, 200, 300, or 400 BMEC cells. The wells containing beating EBs were counted on different days. Data are representative from three independent experiments. Error bars represent standard deviation ($^*P < 0.05$ vs. control). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in Figure 4B, for each EB containing 400 of ES cells, the addition of 200 or more of BMEC cells dramatically accelerated beating EB formation, indicating that the capacity of endothelial cells to promote cardiomyocyte generation is dependent on cell number.

CARDIOMYOCYTE DIFFERENTIATION IS IMPAIRED BY ANGIOGENIC INHIBITORS

Our data indicated that the presence of exogenous endothelial cells promotes cardiomyogenesis during mouse ES cell differentiation. To investigate whether endogenous endothelial cells are essential for cardiomyogenesis during ES cell differentiation, we used angiogenic inhibitors to inhibit endothelial cells during ES cell differentiation. Endostatin and angiostatin predominantly interfere with the molecular pathways involved in endothelial cell migration, proliferation, and survival [O'Reilly et al., 1997; Tabruyn and Griffioen, 2007]. As showed in Figure 5A, the addition of either endostatin or angiostatin delayed formation of spontaneous beating EBs, and dramatically decreased the number of beating EBs. Furthermore, addition of endostatin partially abolished BMEC-promoted cardiomyocyte differentiation (Fig. 5B).
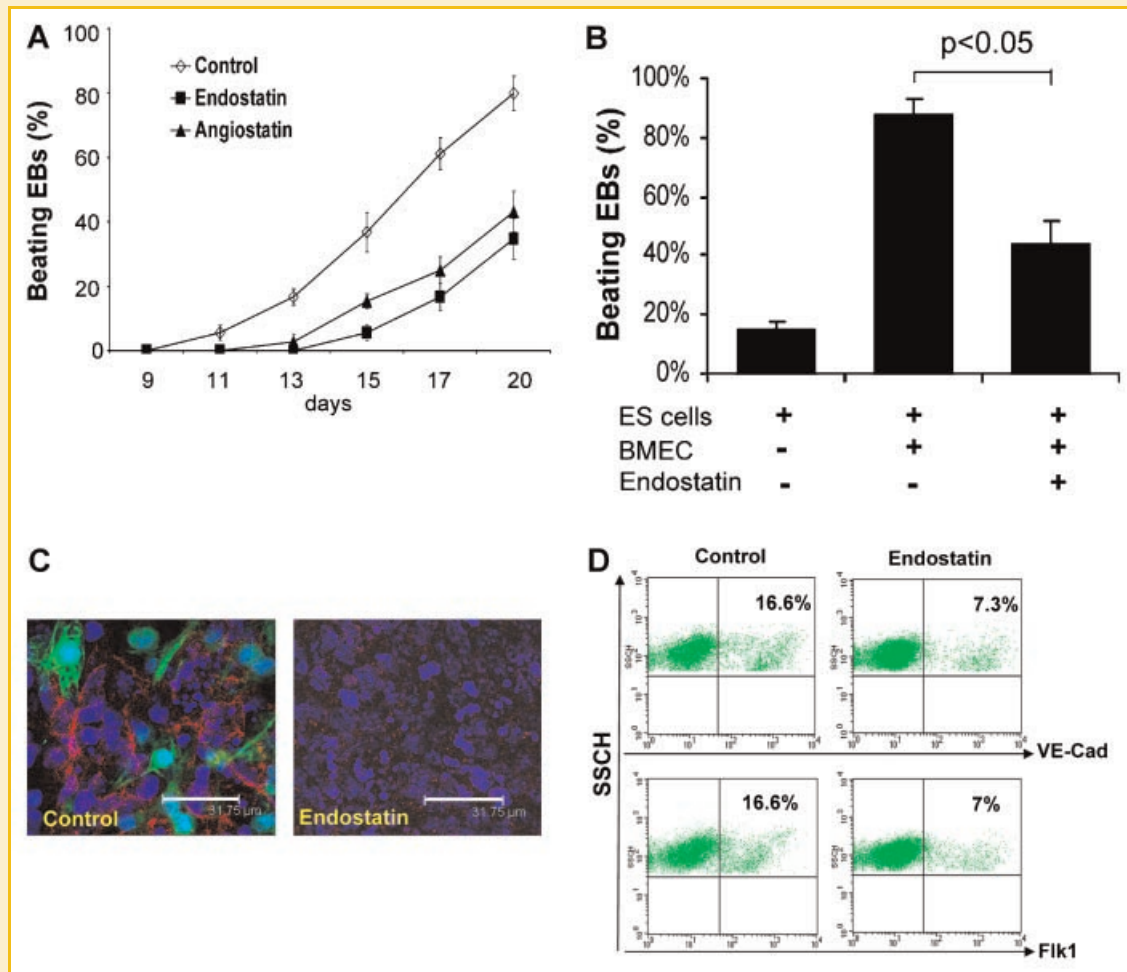
Fig. 5. Inhibition of cardiomyogenesis by angiogenic inhibitors. A: Differentiation of αMHC–GFP ES cells was induced without (control) or with the angiogenic inhibitors, endostatin (2 μg/ml), or angiostatin (1 μg/ml). Percentages of beating EBs were quantified over time as in Figure 1. Data are representative of three independent experiments. Error bars represent standard deviation. B: Cardiomyogenesis of αMHC–GFP ES cells was induced without (control) or with 200 of BMEC cells in the absence (+BMEC) or in the presence of endostatin (+BMEC/+Endostatin). Beating EBs were quantified at day 13 of differentiation. Data are representative of three independent experiments. Error bars represent standard deviation ($P < 0.05$). C: Cardiomyogenesis of αMHC–GFP ES cells was carried out in the absence (control) or presence of endostatin. At day 11 of differentiation, EBs were immunostained for VE–Cad expression with PE-conjugated antibodies (red). The VE–Cad+ endothelial cells and GFP+ cardiomyocytes were analyzed using confocal microscopy. Cell nuclei were stained with Topro–3 (blue). The scale bar = 31.75 μm. D: Cardiomyogenesis of αMHC–GFP ES cells was induced without (control) or with endostatin. At day 11 of differentiation, VE–Cad+ cells and Flk1+ cells were analyzed by flow cytometry with PE-conjugated antibodies recognizing each antigen.

To examine whether angiogenic inhibitors inhibit endothelial cell-mediated differentiation of ES cells, we analyzed cardiomyocytes and endothelial cells by confocal microscopy during differentiation. As showed in Figure 5C, GFP+ cardiomyocytes and VE-Cad+ endothelial cells were decreased in the presence of endostatin during ES cell differentiation. The decreased VE-Cad+ and Flk1+ endothelial cells were confirmed by flow cytometric analysis (Fig. 5D). RT-PCR analysis of differentiated ES cells indicated that expression of endothelial cell specific genes (CD31, Flk1, VE-Cad, EphB4, and ephrinB2) and cardiac specific genes (GATA4, Nkx2.5, α-MHC, and α-MHC) in ES cells were decreased by endostatin (Fig. 6A). The expression of an endodermal (FoxA2) and ectodermal genes (MAP-2, FGF-5, and Nestin) were not affected by endostatin treatment of ES cells (Fig. 6A). The inhibition of endothelial and cardiac gene expression by endostatin treatment was confirmed by real-time PCR at different time points of ES cell differentiation (Fig. 6B). Expression of Flk1, VE-Cad, EphB4, ephrinB2, Nkx2.5, and GATA4 was initiated as early as day 2, and gradually increased during ES cell differentiation in control ES cells, whereas endostatin treatment blocked the expression of these genes during ES cell differentiation. These data suggest that endostatin inhibits endothelial cell development in ES cells, resulting in restraining cardiomyocyte differentiation.

EPHB4–EXPRESSING ENDOTHELIAL CELLS RESCUES CARDIOMYOCYTE-DEFECT IN EPHB4$^{-/-}$ ES CELLS

To further evaluate whether endothelial cells compensate EphB4 signaling to rescue cardiomyocyte differentiation-defects in EphB4$^{-/-}$ ES cells, we cocultured BMEC cells with EphB4$^{-/-}$ ES cells, and induced cardiomyocyte differentiation in hanging-drop EB formation. Immunohistochemistry and RT-PCR analysis confirmed EphB4 and ephrinB2 expression in BMEC and HUVEC
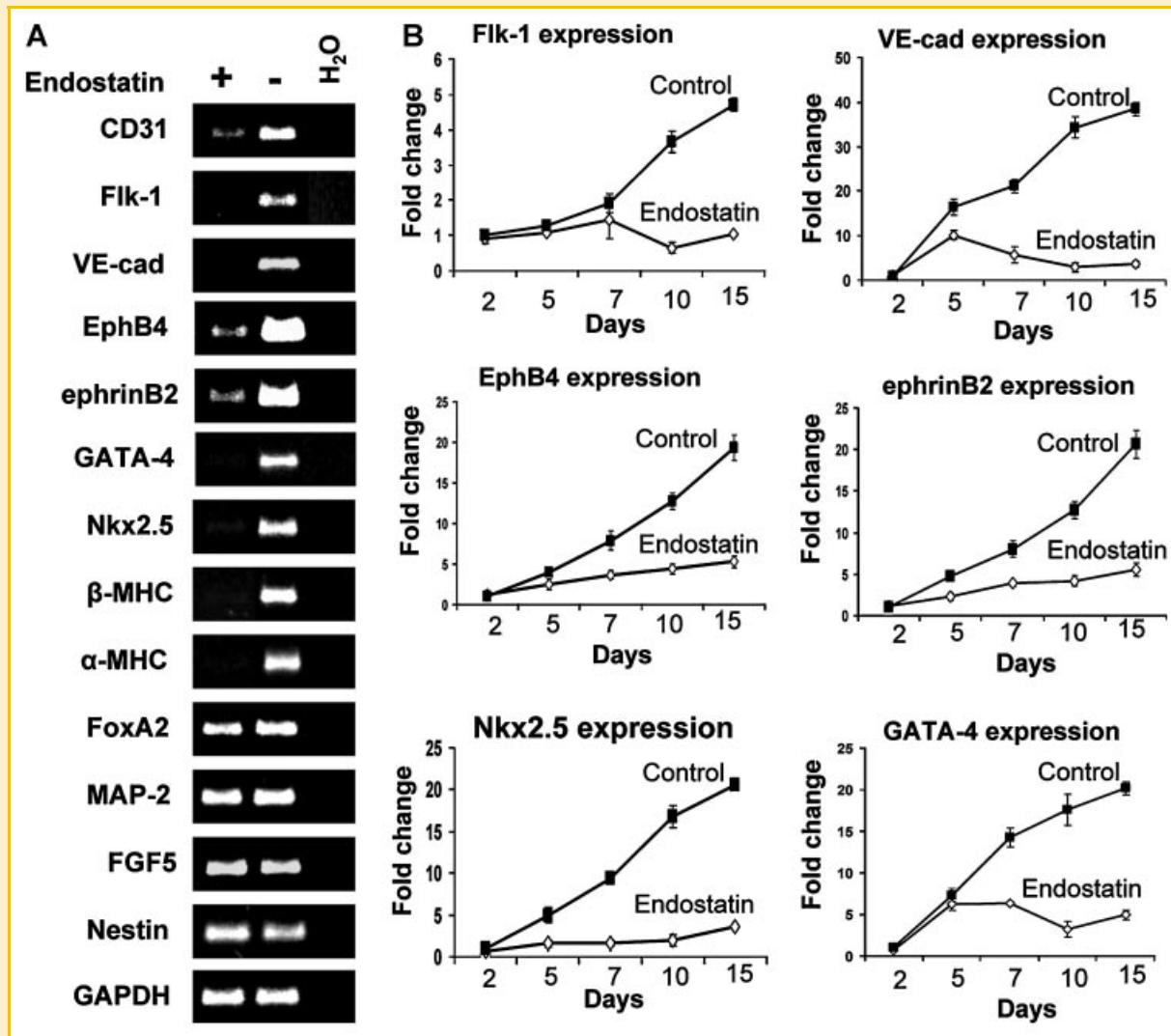
Fig. 6. Expression of endothelial and cardiomyocyte genes during ES cell differentiation. Differentiation of αMHC–GFP ES cells was carried out without (control) or with 2 μg/ml endostatin. A: At day 10 of ES cell differentiation, RNA samples were harvested from EBs in the presence (+) or absence (−) of endostatin. RT–PCR was used to analyze gene expression. B: RNA samples were harvested from EBs in the presence (Endostatin) or absence (Control) of endostatin at day 2, 5, 7, 10, and 15 of differentiation. Real–time PCR was performed with the primers listed in Supplemental Table 2. GAPDH was used as a standard.

(Fig. 7A,B). EphB4$^{-/-}$ ES cells lost beating activity without BMEC cells. The addition of BMEC fully restored beating activity (Fig. 7C), suggesting that EphB4-expressing endothelial cells provide a microenvironment that directs ES cell differentiation into cardiomyocytes in vitro.

DISCUSSION

Eph receptor tyrosine kinases and their ligands, ephrins, play important roles in a variety of processes during embryonic development, including the targeting behavior of migratory neurons, vascular cell assembly, and angiogenesis [Gale and Yancopoulos, 1999]. Fourteen different Eph receptors have been catalogued into EphA or EphB subclasses based on their affinity for ligands. Eight different ephrins have been identified to date. They

are membrane proteins of either glycerophosphatidylinsital (GPI)-linked (ephrinA) or transmembrane (ephrinB) [Committee, 1997]. Rather than long range communication, signaling from Eph receptors and their ligands is restricted to sites of direct cell–cell contact and is capable of inducing reciprocal bidirectional events between interacting cells [Bruckner and Klein, 1998]. Essential roles for Eph receptor tyrosine kinases and their ephrin ligands emerged from studies of embryonic development [Himanen et al., 2007], including cardiovascular development [Adams and Klein, 2000; Kuijper et al., 2007]. EphB4 is specifically expressed at the venous endothelium, while the cognate ligand of EphB4, ephrinB2, is specifically and reciprocally expressed on arterial endothelial cells at the earliest stages of vascular development [Wang et al., 1998]. Mice lacking either EphB4 or ephrinB2 display identical defects in angiogenesis by both arteries and veins in the capillary networks of the head and yolk sac [Wang et al., 1998; Gerety et al., 1999].
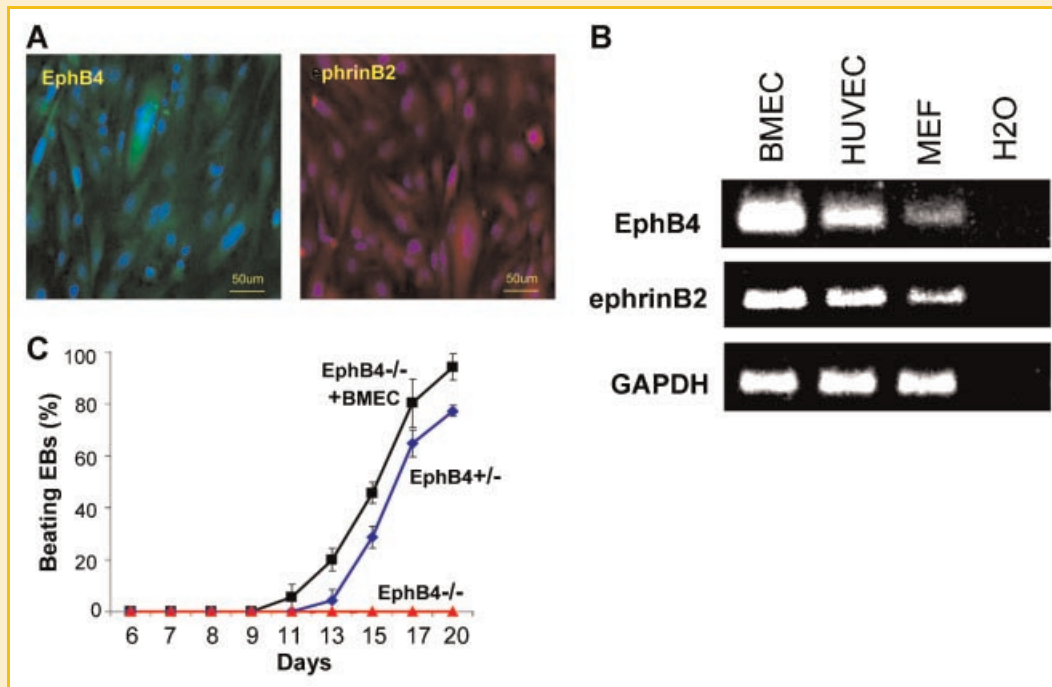
Fig. 7. Rescue of cardiomyocyte–defect in EphB4$^{-/-}$ ES cells by human endothelial cells. A: Immunostaining was performed on BMEC cells to localize human EphB4 and ephrinB2 (rabbit polyclonal anti–human antibodies), using FITC–conjugated and PE–conjugated secondary antibodies, respectively. B: RT–PCR analysis of EphB4 and ephrinB2 expression in endothelial cells compared to MEF. C: EphB4$^{-/-}$ cells (400 cells) were induced to differentiate with (EphB4$^{-/-}$ and BMEC) or without (EphB4$^{-/-}$) BMEC cells (200 cells) in hanging–drops. EphB4$^{+/-}$ ES cells were used as a control. The percentiles of beating EBs were quantified over time. Data are representative from three independent experiments. Error bars represent standard deviation.

EphB4-null or ephrinB2-null mice are also evident for heart defects [Gerety et al., 1999]. It is unclear whether the heart defects are due to direct impairment of cardiogenesis or from indirect impairment of angiogenesis. We previously demonstrated that differentiation of ES cells into endothelial cells and cardiomyocytes is impaired in EphB4$^{-/-}$ ES cells [Wang et al., 2004]. In this study, we provided evidences that EphB4 signaling on endothelial cells regulates ES cell differentiation into cardiomyocytes.

Ascorbic acid promotes collagen synthesis of endothelial cells, and enhances cardiomyocyte differentiation of ES cells [Takahashi et al., 2003; Sato et al., 2006; Telang et al., 2007]. We found that ascorbic acid partially restored cardiomyocyte differentiation in EphB4$^{-/-}$ ES cells, suggesting the overlapping function of ascorbic acid and EphB4 signaling pathways. Interestingly, EphB4 is expressed in endothelial cells at regions containing functional cardiomyocytes in beating EBs, but not in cardiomyocytes. Whether ephrinB2 ligand is involved in regulating cardiomyocyte differentiation in ES cells is under investigation.

Vascular endothelial niches play important roles in regulating self-renewal and differentiation of neural and hematopoietic stem cells in vivo [Shen et al., 2004, 2008; Li and Li, 2006]. Although, cardiac stem or progenitor cells are identified in vivo and in vitro [Beltrami et al., 2003; Oh et al., 2003; Matsuura et al., 2004; Laugwitz et al., 2005; Pfister et al., 2005; Wang et al., 2006; Oyama et al., 2007], it is unclear whether endothelial cells mediate cardiac regeneration from stem cells in the adult heart. In the adult heart, every cardiomyocyte in myocardium is surrounded by the capillary network of endocardial endothelial cells that provides not only oxygen and nutrients, but also local communication between endothelial cells and cardiomyocytes [Hsieh et al., 2006]. Interaction between endothelial cells and cardiomyocytes regulates normal function of the heart, such as cardiac growth, contractile performance, and rhythmicity [Brutsaert, 2003]. Recent studies demonstrate that implantation of endothelial cells from different sources to an infarcted heart improves cardiac function [Kocher et al., 2001; Badorff et al., 2003; Li et al., 2007]. Several mechanisms by which endothelial cells improve cardiac function have been proposed after transplantation: endothelial cells may transdifferentiate into functional cardiomyocytes in vivo [Badorff et al., 2003]; neovascularization by endothelial cells may promote cardiomyocyte survival and cardiac reorganization by direct interaction with endothelial cells and cardiomyocytes [Narmoneva et al., 2004]; and implanted cells may secrete angiogenic cytokines to activate endogenous cells that accelerate repair of infarcted myocardium [Yoshioka et al., 2005]. Our study explored the hypothesis that endothelial cells provide signals to direct cardiomyocyte differentiation of ES cells. Directing differentiation of adult cardiac stem cells in vivo may be particularly significant for the treatment of patients with various heart diseases including chronic heart failure. It will be interesting to further investigate whether co-transplantation of ES cell-derived endothelial cells and cardiomyocytes into infarcted hearts can enhance cardiac regeneration in vivo.

Endocardial endothelial cells and cardiomyocytes are both derived from lateral plate mesoderm at about the same time during

development, suggesting that myocardial and endocardial lineages have a common precursor [Linask and Lash, 1993]. Reciprocal signaling is evident between the endocardium and myocardium [Ramsdell and Markwald, 1997; Dor et al., 2001]. The interdependence of endothelial and myocardial cells during embryonic heart development is still largely unknown. ES cells not only have enormous potential as a source of therapeutic tissues, but also provide a unique system for studying embryonic development, including cardiovascular lineage commitment. Recent studies in ES cells demonstrate that common cardiovascular progenitors have the potential to differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells in the heart [Kattman et al., 2006; Moretti et al., 2006; Wu et al., 2006]. We found that exogenous human endothelial cells promoted the differentiation of spontaneously contracting cardiomyocytes from ES cells. Enhancement of cardiomyocyte differentiation by endothelial cells occurs in a dose-dependent manner, suggesting that signaling from endothelial cells specifically regulates the kinetics of cardiomyogenesis. Cardiac endothelial cells may regulate myocardial function through paracrine pathways, such as nitric oxide, endothelin-1, prostanoids, adenyl purines, and natriuretic peptides [Shah, 1996]. A recent study showed that paracrine factors of bovine aortic endothelial cells facilitate ES cell differentiation into Nkx2.5+ cells [Kado et al., 2008]. We will investigate whether conditioned medium from human bone marrow endothelial cells enhances generation of beating EBs, or a cell autonomous endothelial cell mechanism facilitates cardiomyocyte differentiation. Nevertheless, our study demonstrated that inhibition of endogenous endothelial cells with endostatin or angiostatin resulted in retardation of cardiogenesis, indicating that cardiogenesis does not occur to its full extent in the absence of endothelial cells.

To understand the mechanism of endothelial cell regulation of cardiomyocyte differentiation from pluripotent stem cells, we investigate the role of EphB4 in regulating cardiomyocyte differentiation. Our study demonstrated that EphB4 was highly expressed in endothelial cells that were underneath beating EBs. Human bone marrow endothelial cells rescued the defect of cardiomyocyte differentiation from pluripotent stem cells in EphB4-null ES cells. These data suggest that EphB4-expressing endothelial cells provide a niche for cardiomyogenesis from ES cells. It is unclear whether endothelial cells promote cardiac differentiation at the level of the pluripotent stem cells, at level of mesodermal development, or at the level of the cardiomyocyte progenitor cells. Further dissecting the role of endothelial cells in cardiomyocyte differentiation will be significant for cardiac regeneration after ischemic injury.

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