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Endocardiogenesis in embryoid bodies: Novel markers identified by gene expression profiling

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Abstract

Endocardial cells and cardiomyocytes differentiate from the cardiogenic mesoderm at about the same time during development. Although *in vitro* embryonic stem (ES) cell systems have been used to study the differentiation of various types of cell lineages, including cardiomyocytes, smooth muscle cells, and vascular endothelial cells, differentiation of endocardial cells, or endocardiogenesis, has not been well reported, because of a lack of specific molecular markers. In our search for cardiogenesis-associated genes expressed in embryoid bodies, we found several genes expressed in the heart region of mouse embryos, but not in cardiomyocytes. To identify the cell types expressing these genes, $CD31^+$ cells were taken from mouse embryos on embryonic day (E)8.5 and E9.5 and sorted, then their transcripts were analyzed using quantitative RT-PCR analyses. In those embryos, Gata4 and Nfatc1, as well as newly identified Cgnl1 and Dok4 were found to be preferentially expressed in endocardial cells, but not in yolk sac endothelial cells, while Cdh5 and Kdr were expressed in both cardiac and yolk sac endothelial cells. Immunohistochemical analyses of embryoid bodies revealed that some $CD31^+$ cells co-expressing Gata4 and Nfatc1 were located in close proximity to cardiomyocytes. These results suggest that embryoid bodies express endocardial specific genes and likely generate endocardial cells along with cardiomyocytes. Further, they indicate that these new marker genes are useful to study the origin and induction of endocardial cells, and identify other endocardial markers.

Keywords: Cardiogenesis; Embryonic stem cells; Embryoid body; Endocardium; Differentiation; Development; Gene expression; Microarray

Cardiac endothelial cells, i.e., endocardial cells and myocardial capillary endothelial cells, play essential roles in cardiac growth, contractile performance, and rhythmicity [1]. During the process of heart development, the primitive heart tube, which consists of endocardial cells of the inner layer and cardiomyocytes of the outer layer, is initially formed from the cardiogenic mesoderm, and further develops into a looped and then chambered heart by formation of a trabecular myocardial layer, and an endocardial cushion with valve and septum, along with growth and thickening of a compact ventricular chamber wall [2]. During each of these stages, reciprocal signaling pathways between the cardiac endothelial cells and cardiomyocytes play an obligatory role. For example, myocardial-derived VEGF and angiopoietin, as well as endocardial derived neuregulin are important for myocardial trabeculation. Although both cardiomyogenic and endocardial progenitors are derived from lateral plate mesoderm, the relationships among these lineages in the embryo are not well understood, though retroviral cell tracking analysis has demonstrated that they are separated before the primary heart tube is formed [3].

Embryonic stem (ES) cells have a remarkable capacity to generate a broad spectrum of cell lineages, thus providing

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new opportunities to study the earliest stages of lineage induction and specification [4]. For example, hemangioblasts, common progenitor cells of hematopoietic and vascular endothelial lineages, have been identified in the earliest stages of hematopoietic commitment using an ES cell differentiation model [5]. Likewise, a common progenitor of vascular smooth muscle and endothelial cells has been identified by an ES cell system [6]. ES cells also differentiate into cardiac lineages, which can be easily detected by the appearance of contracting cells that display characteristics of cardiomyocytes [7]. However, despite intensive studies of cardiomyogenesis with ES cell systems, the development of endocardial cells has been poorly studied, in contrast to hematopoietic and vascular systems, probably because of a lack of molecular markers specifically expressed in endocardial cells. Thus, the developmental relationships between cardiomyocytes and endocardial lineages have not been well reported, though the requirements of growth factors such as neuregulin and VEGF have been demonstrated in studies of ES cell cardiogenesis [8,9].

In a previous search for cardiogenesis-associated genes expressed in embryoid bodies (EBs), we found that several genes whose expression rose up during the development of EBs were also expressed in embryonic hearts [10]. These included genes expressed in endocardial cells, but not in yolk sac endothelial cells, suggesting their utility as novel markers specific to endocardial cells. In EBs cultured as floating cells, GATA4- and Nfatc1-expressing endothelial cells exist in close proximity with cardiomyocytes, suggesting that EBs also generate endocardial cells. Thus, we considered that our ES cell system would be able to identify genes expressed in endocardial cells, which are potentially important for heart development and morphogenesis.

Materials and methods

Whole mount in situ hybridization. cDNA fragments were generated by PCR amplification using total cDNA from EBs and cloned into pBlue-Script SKII+ (Stratagene) or pCR4-TOPO (Invitrogen). The cloned cDNA fragments were Cgnl1 (NM_026599, 3659...4003) and Dok4 (NM_053246, 749...1323). Hybridization was performed using mouse embryos under standard conditions.

Real time RT-PCR. Total RNA was extracted from mouse embryos and EBs using Trizol reagent (Invitrogen, Carlsbad, CA), then reverse-transcribed to cDNA with SuperScript III (Invitrogen). PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and a real-time thermal cycler (7700 Sequence Detector Systems, Applied Biosystems). All samples were subjected to PCR amplification using Rodent GAPDH Control Reagent (Applied) and data were normalized with respect to that value. The primers used are listed in the Supplemental Table.

Cell sorting. For isolation of embryonic heart and yolk sac derived cells, mouse embryos were dissected on embryonic day (E)8.5 and E9.5, then dissociated with Trypsin-EDTA and incubated with phycoerythrin (PE)-conjugated CD31 (Pharmingen). Stained cells were sorted with a FACS Aria (Becton Dickinson), then 5000–20,000 $CD31^+$ and $CD31^-$ cells were collected separately, and used for preparation of total RNA. For isolation of EB-derived cells, Nkx2-5 EGFP ES cells were differentiated in hanging drops (2500 drops) and cultured for 8 days. EBs were dissociated with Trypsin–EDTA and incubated with PE-CD31, after which 20,000–50,000 GFP+$CD31^-$, GFP–$CD31^+$, and GFP–$CD31^-$ cells were collected.

Immunocytochemistry. For frozen sections, cytospin preparations, and cultured cells, samples were fixed in 1% paraformaldehyde, followed by cell permeabilization with 0.1% Triton X-100. After washing in PBS, the samples were incubated with mouse monoclonal anti-sarcomeric myosin heavy chain (MyHC) (MF20, Hybridoma Bank), rat monoclonal anti-CD31 (Pharmingen), rabbit polyclonal anti-GATA4 (Santacruz), and mouse monoclonal anti-NFATc1 (Santacruz) antibodies, followed by incubation with secondary antibodies conjugated with Alexa-350, 488, and 546 (Molecular Probes). The anti-smooth muscle actin (SMA) antibody conjugated with Cy3 (1A4, Sigma) was also used. Cells were observed using an Olympus IX70 or BX51 microscope, and images were captured with an Olympus DP70 digital camera.

Results

Profiling of cardiogenesis-associated genes in EBs for identification of cardiac endothelial genes

We recently reported that transient serum removal generated highly cardiomyogenic EBs [10]. Prior to that discovery, we screened for cardiogenesis-related genes whose expression profiles were closely related to those of BMP2 or BMP4 during time course differentiation of EBs, since Nkx2-5 is known to be regulated by BMP signaling [11]. Whole mount *in situ* hybridization data indicated that the initially selected 10 genes (2010011I20Rik, D130058I21Rik, Car4, Fgd5, Asb4, Pcgf5, Mum1l1, Cgnl1, Frmd6, and Dok4) were preferentially expressed in the heart region of mouse E9.5 embryos (see Supplemental Fig. 1). However, 8 of those genes (2010011I20Rik, Car4, Fgd5, Asb4, Pcgf5, Mum1l1, Cgnl1, and Frmd6) were not up-regulated in cardiomyogenic EBs, as their expression was not increased by transient serum removal (data not shown). These results suggest that those genes are expressed in the heart region, but not preferentially in cardiomyocytes.

To determine more precisely which cell types express these genes, we sorted cells from E9.5 mouse embryos based on their expression of the endothelial-specific CD31 marker and examined mRNA levels in the sorted cells. As shown in Fig. 1A, $CD31^+$ cells derived from the heart could be considered as endocardial cells and $CD31^-$ cells as myocardial cells, based on their specific expression of Cdh5 and Myl2, respectively. While Nkx2-5 was specifically expressed in $CD31^-$ myocardial cells, Gata4 was preferentially expressed in $CD31^+$ endocardial cells. This was somewhat surprising, because Gata4 has been frequently used as a marker for cardiomyocytes. However, consistent with previous reports, our findings suggest that Gata4 is prominently expressed in endocardial cells at this stage of embryonic heart development [12,13]. In addition, Nfatc1 was expressed only in cardiac $CD31^+$ cells. Among the cardiogenesis-associated genes described above, cingulin-like 1 (Cgnl1) and docking protein 4 (Dok4) were expressed preferentially in cardiac $CD31^+$ cells, but not in yolk sac $CD31^+$ cells, while FYVE, RhoGEF, and PH domain containing 5 (Fgd5) was expressed in

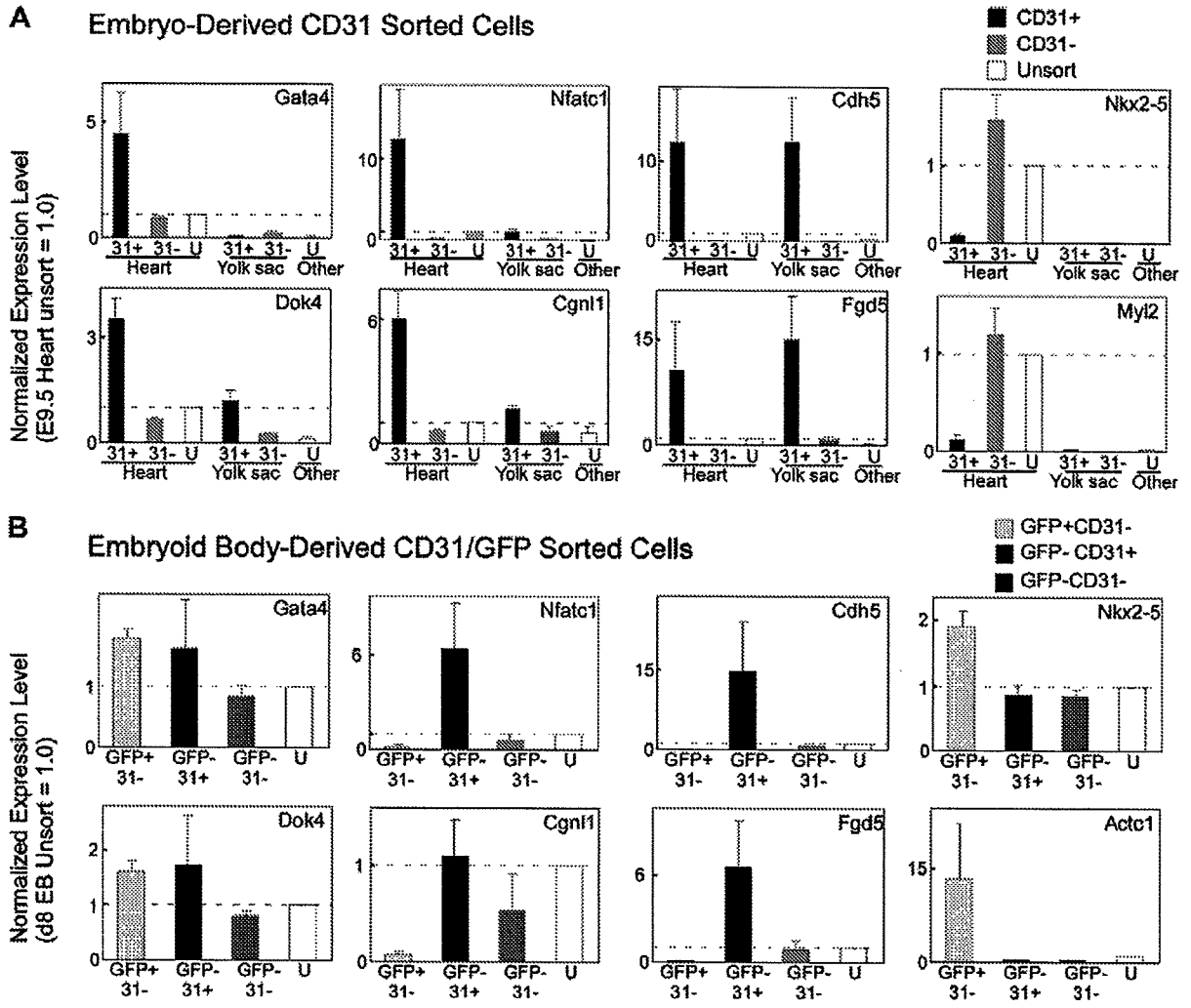

Fig. 1. Expression of cardiogenesis-associated genes in $CD31^+$ endothelial cells from embryos and embryoid bodies. (A) Real time RT-PCR analysis of cells sorted from mouse embryos. $CD31^+$ (+), $CD31^-$ (−), and unsorted (U) cells were obtained from the heart, yolk sac, and other parts of E9.5 mouse embryos. The mean ± SE of 3 separate experiments is shown. (B) Real time RT-PCR analysis of cells sorted from Nkx2-5-EGFP ES cell-derived embryoid bodies. GFP+$CD31^-$, GFP−$CD31^+$, GFP−$CD31^-$, and unsorted cells were obtained from Nkx2-5-EGFP embryoid bodies on day 8. The mean ± SE of 3 separate experiments is shown.

both cardiac and yolk sac $CD31^+$ cells. Although Nrg1 has been shown to be expressed in endocardium tissues, we detected it in both heart and yolk sac-derived $CD31^+$ cells (data not shown), indicating that embryo bodies are able to capture the earlier stages of cardiogenesis. To determine which cell types express these markers, we sorted EBs derived from Nkx2-5 EGFP ES cells, with GFP+ cells used to represent cardiomyocytes (Fig. 1B). While Cgnl1 and Nfat1 were expressed solely in $CD31^+$ cells, Dok4 and Gata4 were expressed in both $CD31^+$ cells and GFP^+ cardiomyocytes.

EBs generate endothelial cells near clusters of cardiomyocytes

Many studies have reported that EBs produce a vessel-like structure consisting of vascular endothelial cells and mural cells, which have been identified as alpha smooth muscle actin (SMA)-expressing cells [14]. However, SMA is also known to be expressed in developing cardiomyocytes [15]. To test our hypothesis that EBs can generate endocardial cells, we first examined the expression of SMA in floating EBs (Fig. 2A and B). Surprisingly, SMA and sarcomeric MyHC were co-expressed in populations of beating EBs, indicating that these cells were developing cardiomyocytes and not smooth muscle cells. Thus, at least in the early stage and under our experimental conditions, EBs can be considered to be avascular, though they generated SMA+$MyHC^-$ cells during later stages or after attachment to the culture dish (Fig. 2C). Next, we examined the expression of CD31, GATA4, and NFATc1 in beating EBs. The Nfatc1+ area was mostly included in the $CD31^+$ area, as shown in Fig. 3A, and was often found near the MyHC+ area, where cardiomyocytes were

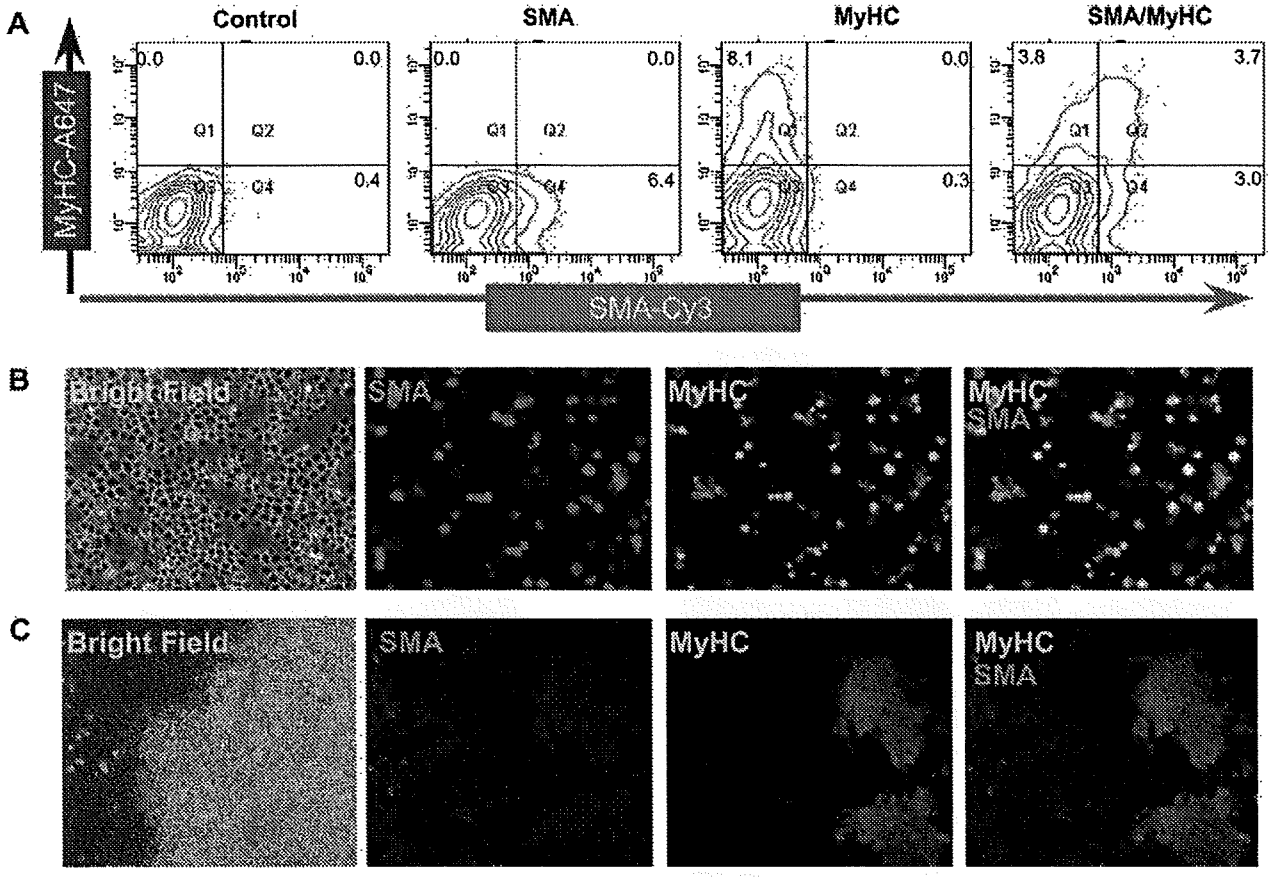

Fig. 2. Smooth muscle actin expression in cardiomyocytes in EBs. Co-expression of sarcomeric myosin heavy chain and smooth muscle alpha actin in EBs. (A) Floating EBs (d9) were dissociated, then fixed and stained with anti-SMA-Cy3, anti-MyHC, and anti-mouse IgG2b-Alexa647. (B) Cytospin preparations of dissociated cells (d8) were analyzed by immune fluorescence using anti-SMA-Cy3, anti-MyHC, and anti-mouse IgG2b-Alexa488. (C) EBs were attached to gelatinized plates on day 5 and cultured for an additional 3 days (d5+3), then stained with anti-SMA-Cy3, anti-MyHC, and anti-mouse IgG2b-Alexa488.

differentiated. The expression of GATA4 was mainly found in the nuclei of MyHC+ cardiomyocytes in EBs. In addition to cardiomyocytes, GATA4 was also detected in some Nfatc1+ cells located near cardiomyocytes (Fig. 3B). Further, co-expression of GATA4 and CD31 was confirmed by staining single $CD31^+$ cells sorted from EBs with GATA4 (Fig. 4C).

These results indicated that the EBs cultured in a floating condition were avascular, though they expressed endothelial and cardiomyocyte markers in the earliest stages. Further, some endothelial cells were found in close proximity to cardiomyocytes in the EBs and a novel endocardial cell specific marker, Cgnl1, was identified in EBs, supporting the hypothesis that EBs can differentiate into endocardial cells.

Developmental expression of Cgnl1 and Dok4 in mouse embryos

To elucidate the expression patterns of Cgnl1 and Dok4 in the early developmental stages of mouse embryos, we performed whole-mount *in situ* hybridization analysis (Fig. 4). Although it was previously reported that Cgnl1 and Dok4 are expressed in multiple tissues in adult mice [16,17], we observed that their transcripts were concentrated in the heart region of early embryos. On E7.5, Cgnl1 expression was observed throughout the neural ectoderm of the head fold and the cardiac mesoderm in the cardiac crescent (Fig. 4A and D). In addition, a strong expression of Cgnl1 was maintained in the heart at all stages examined (up to E10.5, data not shown). Consistent with the RT-PCR data, Cgnl1 expression was observed in the endocardium of the developing hearts and not in the myocardium (Fig. 4B, C, and E), and became more ubiquitous after E9.5.

Strong Dok4 expression was also observed in the developing endocardium from E8.25 (Fig. 4F–H) up to E10.5 (data not shown). RT-PCR examinations detected weak Dok4 expression in the myocardium (Fig. 1), while whole-mount *in situ* hybridization analysis demonstrated signals in the developing myocardium (Fig. 4H).

It has been reported that some endothelial cells differentiate into the developing vasculature on approximately E8.0 [18]. To determine whether the expression of Cgnl1

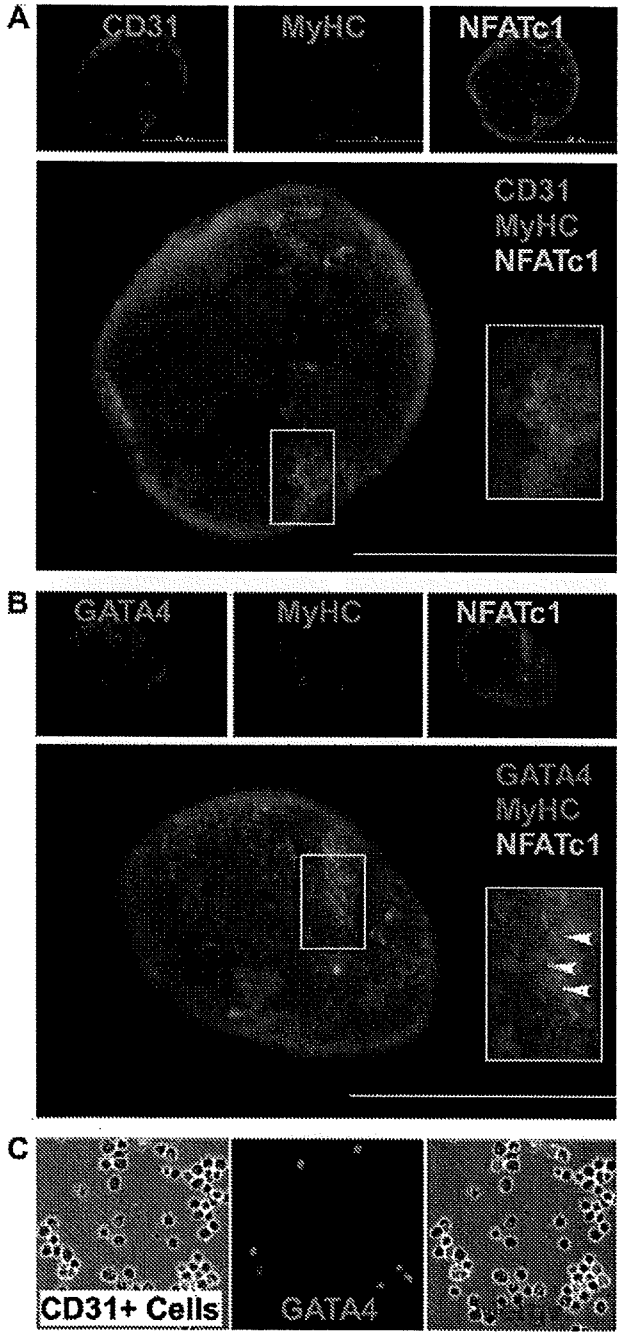

Fig. 3. Endocardiogenesis in EBs. Frozen sections of floating EBs (d8) were incubated with (A) anti-CD31, anti-MyHC, and anti-NFATc1 antibodies, or (B) anti-GATA4, anti-MyHC, and anti-NFATc1 antibodies. Signals were detected by anti-mouse IgG2b-Alexa350 (blue), anti-rabbit-Alexa546 or anti-rat-Alexa546 (red), and anti-mouse IgG1-Alexa488 (green). Gata4+ nuclei were found in NTATc1+ cells (arrows). (C) $CD31^{+}$ cells were sorted from day 8 EBs, then stained with anti-GATA4 and anti-rabbit IgG-Alexa546.

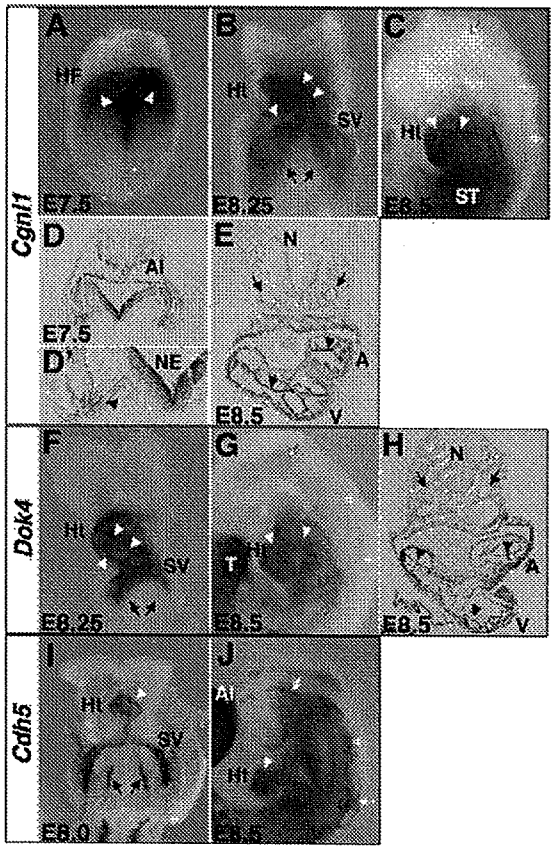

Fig. 4. Cgnl1 and Dok4 expression in developing endocardium. Whole-mount *in situ* hybridization and section analyses of Cgnl1, Dok4, and Cdh5 expression in E7.5–8.5 mouse embryos. Ventral views of embryos on (A) E7.5, (I) E8.0, and (B,F) E8.25. (C,G,J) Left-side views of embryos on E8.5. Arrows indicate the corresponding regions in mouse embryos: black, dorsal aorta; white, vascular networks in the head; and yellow, vascular networks in the trunk. (A–E) Cgnl1 Expression. Cgnl1 was expressed in the neural ectoderm of the head fold (arrowheads in A) and cardiac mesoderm of the cardiac crescent (arrowheads in D) on E7.5. (B,C,E; arrowheads) From E8.25, a strong expression of Cgnl1 was observed in the endocardium, however, not in the endothelium of the dorsal aorta (arrows in E), or vascular networks (arrows in C). Cgnl1 transcripts were also detected in the (C) allantois and (D) septum transversum. (F–H) Dok4 Expression. Dok4 was strongly expressed in the endocardium (arrowheads), however, not in the endothelium of other vascular networks in the head and trunk (arrows). Dok4 expression was also observed in some parts of the (G) myocardium and (H) tail. (I,J) Cdh5 Expression. Cdh5 was expressed in most of the vascular endothelium, including the endocardium (arrowheads), dorsal aorta (black arrows in I), and other vascular networks in the head (white arrows in J) and trunk (yellow arrows in J). A, atrium; Al, allantois; HF, head fold; Ht, heart; N, neural tube; NE, neural ectoderm; ST, septum transversum; SV, sinus venous; T, tail; V, ventricle; YS, yolk sac.

and Dok4 is restricted to cardiac endothelium, we compared the expression patterns of Cgnl1, Dok4, and Cdh5. Strong Cdh5 signals were detected in both the endocardium and developing vascular endothelium at all stages examined (Fig. 4I and J), however, no evidence of Cgnl1 or Dok4 expression was observed in the dorsal aorta or vascular networks in the head, trunk, and yolk sac from early embryos (Fig. 4C, E–H), while weak expression was seen in the umbilical veins and vitelline artery (data not shown). These results confirmed that Cgnl1 and Dok4

are concentrated in the endocardium during the early stages of mouse embryo development.

Discussion

Endocardial cells play an obligatory role in regulating and maintaining cardiac function. Although both endocardial cells and cardiomyocytes are derived from the lateral plate mesoderm, their relationship has not been well elucidated. Further, the molecular mechanism by which endocardial cells are differentiated remains to be explained. Although *in vitro* ES cell culture systems have identified a common progenitor of hematopoietic and endothelial lineages, as well as vascular smooth muscle and endothelial lineages, endocardial cell differentiation has not been thoroughly studied, partially because of a lack of molecular markers to distinguish endocardial cells from vascular endothelial cells.

With the present experimental system utilizing floating EB cells, we found that cardiogenesis predominantly occurred in the early stages of embryonic development, and that EBs generated GATA4+ and Nfatc1+ endothelial cells adjacent to clusters of cardiomyocytes. Further, Cgnl1 was expressed in endocardial cells and embryoid body-derived $CD31^+$ cells, but not in yolk sac endothelial cells. Together, these results suggest that EBs generate endocardial cells along with cardiomyocytes. It is intriguing that our *in vitro* ES cell differentiation system was seemingly able to recapture development of an organ that contains more than 2 cell lineages. As for intra-organ lineage relationships, recent studies have demonstrated that hepatocytes differentiate in close proximity to cardiomyocytes in EBs, indicating recapture of the inter-organ relationships during liver and heart development in embryos [19]. Although EBs do not possess defined structures like an embryo, microenvironments essential for the induction of differentiation are apparently conserved. Thus, the present ES cell system is able to provide important clues to identify the crucial factors involved with differentiation.

Recent studies have demonstrated that a single Flk1+ cell can give rise to both endothelial and myocardial cells [20,21], supporting the notion that EBs generate both endocardial cells and myocardial cells, and that they originate from a common cardiovascular progenitor. In the present experiments, we found that Gata4 and Dok4 were initially expressed in both myocardial cells and endocardial cells, and the molecular signatures suggested that both lineages were from a common progenitor. However, we cannot rule out the possibility that they were expressed in both lineages independently. Additional studies using a single cell tracing technique should be able to clarify the relationship between endocardial and myocardial lineages.

There is no previous report of a good marker for endocardial cells. The endocardial-specific markers newly identified in this study, such as Cgnl1, are considered to be useful and unique tools to study the relationship between endocardial and myocardial lineages, since vascular endothelial cells and endocardial cells are known to share common markers. In addition, identification of endocardial-specific genes will provide a novel opportunity to study the specific functions of genes and signaling pathways that are shared between endocardial and other endothelial cells, as well as those not shared. For example, recent studies have demonstrated that endothelial-specific knockout of Bmpr1-mediated signaling using cre-lox technology abolished the proper patterning of AV cushion development [22,23]. In those experiments, Kdr and Tie1 were used to drive the endothelial expression of Cre. Since the causes of death of mutant embryos include vascular defects, it will be intriguing to learn if endocardial-specific knockout specimens can be examined.

Cgnl1 encodes a plaque protein localized in the apical junctional complex and has been shown to be expressed rather ubiquitously. However, this is the first report of its specific expression in embryonic stages. Cell adhesion molecules play an important role in patterning embryonic hearts. Recent studies have demonstrated that overexpression of the tight junction protein Claudin-1 causes randomization of the direction of heart looping [24]. Although we could not detect asymmetrical expression of Cgnl1 in the present embryos, it is possible that it also has an effect on heart looping or other morphological events by regulating growth factor signaling.

Dok4, a signal adaptor protein, was previously reported to be expressed in endothelial cells [17], and unexpectedly exhibited an expression pattern similar to that of the cardiac-specific genes in the present EBs. Our *in situ* hybridization results indicate for the first time that Dok4 is expressed in the cardiac crescent of mouse embryos (data not shown). Although this protein has been reported to react with Tie2 in endothelial cells, it remains to be clarified which signaling pathway is mediated by Dok4 in cardiogenic progenitor cells. Additional studies including *in vivo* experiments are currently underway to explore the role of *Dok4* in heart development and differentiation.

In conclusion, the present newly reported marker genes will be useful to study the origin and induction of endocardial cells, as well as identify other specific gene functions in endocardial cells.

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.2007.04.030.

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Activation of MEK–ERK by heregulin-β1 promotes the development of cardiomyocytes derived from ES cells

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Abstract

We have previously shown that heregulin-beta1 (HRG-β1) was involved in the development and survival of cardiomyocytes derived from embryonic stem (ES) cells. This study was conducted to investigate the intracellular signal mechanisms by which HRG-β1 stimulates cardiogenesis in ES cells. The treatment with ErbB receptor inhibitor decreased the population of cardiomyocytes and transcripts levels of cardiac genes (Nkx2.5, β-MHC, cTnI, and MLC2a). The phosphorylation of ERK and development of cardiomyocytes by treatment with HRG-β1 was suppressed upon treatment with MEK1 inhibitor. Furthermore, cardiomyocytes and level of MHC protein were significantly increased by overexpression of wild type MEK1 or constitutive active MEK1, but not dominant negative MEK1. These results suggest that HRG-β1 promotes the development of cardiomyocytes predominantly by activation of MEK–ERK.

Keywords: Embryonic stem cell (ES) cell; Nkx2.5 transcription factor; Heregulin-beta1 (HRG-β1); Cardiomyocyte; Extracellular signal-regulated protein kinase (ERK); Mitogen-activated protein (MAP) kinase kinase (MEK)

Embryonic stem (ES) cells retain the potential for unlimited proliferation, which is essential for large-scale production and thus are considered one of the most promising sources of cells for transplantation therapy as well as a model system for elucidating mechanisms of cell differentiation in vitro [1–4].

Neuregulins/heregulin (NRG/HRG) are a family of growth factors that have been shown to binds to ErbB receptor and NRG-ErbB signaling axis has been shown to be a critical mediator cardiac development, and growing evidence supports a role for this system in developing heart [5,6]. HRG-β1 is known to be a ligand of ErbB2 and ErbB4, but not ErbB3 found on cardiomyocytes, and promotes the growth and survival of cardiomyocytes in culture through the activation of ErbB2 and ErbB4 receptors [7,8]. Specially, the rapid activation of mitogen-activated protein kinase (MAPK) and AKT kinase pathways via soluble HRG-β1 isoform has been implied in control development and repair of cardiomyocytes [9].

Nkx2.5 is an essential element in the early cardiac transcriptional regulatory machinery and is a central component of the transcription factor network that guides cardiac development [10,11]. We previously established the Nkx2.5GFP ES cell line, in which the GFP gene is knocked-in to the Nkx2.5 locus [12], a homeobox-containing cardiac transcription factor expressed in cardiomyocytes throughout the course of heart development [13]. Using this ES cell line, our previous study demonstrated ErbB4 and ErbB2, but not ErbB1 was predominantly expressed in ES-derived cardiomyocytes and HRG-β1 promoted development of cardiomyocytes from ES cells [3]. These reports indicated that HRG-β1-ErbB is essential

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for the growth of subjacent cardiomyocytes during development. It is, however, still unclear intracellular signaling pathway by HRG-β1 in regulating development of cardiomyocytes derived from ES cells.

In this study, we investigated the possible intracellular signaling pathway by which HRG-β1 promotes the development of cardiomyocytes derived from ES cells using the well-characterized Nkx2.5GFP ES cells. HRG-β1 increased the population of cardiomyocytes (Nkx2.5GFP cells) and induced extracellular signal-regulated kinase (ERK) phosphorylation. ErbB receptor inhibitor (AG1478) or MAP kinase kinase (MEK) 1 inhibitor (PD98059) suppressed the growth of cardiomyocytes induced by treatment with HRG-β1. Furthermore, MEK1 overexpression promoted the development of cardiomyocytes. These data suggest that MEK/ERK signaling pathway mediated by HRG-β1 is important in the development of cardiomyocytes from ES cells.

Materials and methods

Culture and differentiation of ES cells. Nkx2.5GFP knock-in ES cells (Nkx2.5GFP ES cells) were maintained and differentiated as previously described [12]. Briefly ES cells were grown routinely on gelatinized dishes without feeder cells by using culture medium containing Glasgow-modified Eagle's medium (GMEM, Sigma–Aldrich, St. Louis, MO) with 1000 U/ml leukemia inhibitory factor (ESGRO, Chemicon International, Inc., Temecula, CA), 100 μg/ml hygromycin (Invitrogen Corp., Carlsbad, CA). Differentiation of ES cells was induced through formation of embryoid body (EB).

Flow cytometry to quantify cardiomyocyte differentiation. Cardiomyocytes were observed by analysis of GFP and MHC using flow cytometry. For detection of Nkx2.5GFP cells, EBs were dissociated into single cells using 0.25% trypsin–EDTA and the single cells were resuspended in Hank's balanced salt solution containing 1% bovine serum albumin, and 2.5 μg/ml propidium iodide (PI) and analyzed GFP with flow cytometry (Calibur, BD Biosciences, San Jose, CA). For detection of MHC positive cell, cells were permeabilized with 70% ethanol for 30 min, incubated with mouse anti-myosin heavy chain (MHC) monoclonal antibody (F109.3E1, Alexis, San Diego, CA) at 4 °C for 1 h and were analyzed using flow cytometry after incubation with fluorescence-conjugated secondary antibody (anti-mouse IgG-Alexa488) for 1 h.

Effect of HRG-β1 on cardiac differentiation of embryoid bodies. EBs were cultured with the growth factor, HRG-β1 (Research & Diagnostic Systems, Minneapolis, MN), from day 5 to day 10 on gelatin-coated dishes. The influence of ErbB receptor inhibitor, AG1478 (Calbiochem–Novabiochem, San Diego, CA) and MEK1 inhibitor, PD98059 (Calbiochem–Novabiochem, San Diego, CA) on cardiomyocytes growth was determined by administration of reagents to 5-day-old EBs. Generation of cardiomyocytes in EBs was detected by flow cytometry.

Overexpression of MAP kinase kinase in embryoid body. The wild type (MEK1-WT), dominant negative (MEK-2A) rat MEK-1 vectors driven by cytomegalovirus promoters were used [14] and constitutively active rat MEK-1 (S218E, S222E; HA-MEK-CA) cloned into the pcDNA3.1-HA mammalian expression vector (Invitrogen) was used. For transient expression in EBs adhesion, recombinant MEK1 vectors were prepared in serum-free medium containing polyethyleneimine (PEI) (Sigma–Adlrich, St. Louis, MO) and then added to EBs attached in gelatin-coated plate. The overexpression of recombinant MEK protein was confirmed by Western blot using anti-MEK antibody, anti-phospho-MEK antibody (Cell Signaling Technology, Inc., Danvers, MA) and anti-HA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from EBs with TRIzol® Reagent (Invitrogen) and cDNA was synthesized by using SuperScript II reverse transcriptase (Invitrogen). Cardiac specific genes were compared in EBs at day 9 in the presence and absence of AG1478. Sequences of PCR primers designed previously were used [12]. GAPDH was used as internal standard.

Immunocytochemistry analysis. Cells were fixed in 4% paraformaldehyde. After treatment with a blocking solution containing 0.1% Triton X-100 and 2% skimmed milk, cells were incubated with mouse anti-myosin heavy chain (MHC) monoclonal antibody at room temperature for 2 h. Primary antibodies were visualized with anti-mouse IgG-Alexa546. Fluorescence signals were observed under a fluorescence microscope (IX70, Olympus Optical, Shinjuku-ku, Tokyo, Japan).

Western blot analysis. Cells were harvested and resuspended in 20 mM Tris–HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 1 μg/ml chymostatin). Proteins were separated on SDS–PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline and then incubated with primary antibodies. Blots were developed using peroxidase-conjugated secondary antibodies and visualized with enhanced chemiluminescence reagent (Amersham Biosciences, UK) according to the manufacturer's recommendations.

Statistical analysis. Data are presented as means ± SE and represent at least three independent experiments. Comparisons of means were performed using Student's t test. Values of $p \leqslant 0.05$ were accepted as significant.

Results

Spontaneous cardiac differentiation derived from ES cells

Spontaneous cardiac differentiation in vitro was assessed as the presence of rhythmically beating EBs under microscope, Nkx2.5GFP cells using flow cytometry and expression of heart-related protein, MHC using Western blot. We employed two kinds of culture system, EBs suspension and EBs adhesion. In EBs suspension system, spontaneously beating EBs first appeared at day 7, at which time approximately 46.7% of the EBs had contracting foci and the proportion of beating EBs gradually increased and reached over 90% at day 10 (Fig. 1A). MHC protein appeared around day 7 (Fig. 1B). EBs were adhered on gelatin at various time points (3, 4, 5, 6, and 7 days) after hanging drop EBs formation and then Nkx2.5GFP cells and MHC protein were observed at day 10. Previous our study reported cardiac transcription factor, Nkx2.5 was expressed in differentiating EBs at day 5, indicating initiation of cardiogenesis at day 5 [3]. In this study, the number of Nkx2.5GFP cells and the level of MHC protein were significantly decreased when EBs were adhered at day 4 or earlier (Fig. 1C and D). We checked other types of cell markers (transthyretin and GATA1—marker of endoderm derived cell, cdh5—marker of endothelial cell) using real time RT-PCR. Interestingly, our result showed early attachment of EBs delays of suppressed the differentiation of mesoderm derived cells (data were not shown). Collectively, these data indicate that cardiac differentiation programs in EBs start at day 4, before the typical EBs adhesion step and that signals of EBs adhesion inhibit spontaneous cardiogenesis. We here used EBs adhesion system to obtain more effective action of HRG-β1 on spontaneous cardiogenesis of ES cells.

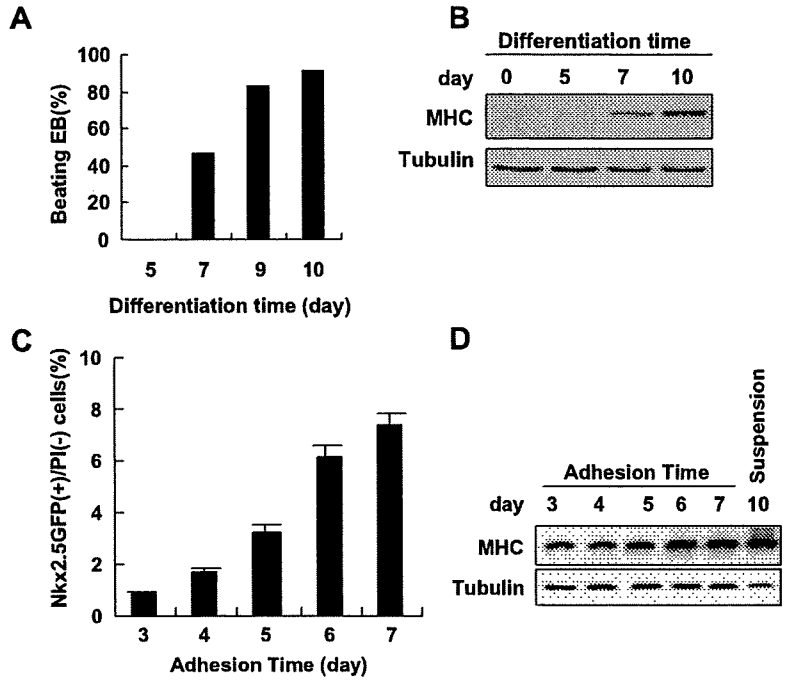

Fig. 1. Spontaneous development of cardiomyocytes in embryoid body (EB) adhesion was inhibited. (A) Rhythmical beating EB in suspension system under microscope was observed at day 7 ($n = 3$). (B) Western blot showed that MHC protein in EB suspension system appeared around day 7. After EB was adhered on gelatin at indicated time (3, 4, 5, 6, and 7 days), Nkx2.5GFP positive/propidium iodide (PI) negative cells at day 10 were analyzed by flow cytometry ($n = 5$) (C) and Western blot (D). All values are means ± SE of five independent experiments.

Effect of ErbB receptor inhibitor, AG1478 on cardiogenesis

In the adult heart, NRG/HRG receptors ErbB2 and ErbB4, but not ErbB3, are found on cardiomyocytes, whereas NRG has been detected in the endothelium [7]. Binding of NRG-1 to its receptor induces the formation of homo- and heterodimers, which is crucial for signaling [15]. Our previous report showed mRNA transcripts of ErbB1, ErbB2 and ErbB4 were increased in differentiating EBs at day 7–8 [3]. We first investigated the effect of ErbB receptor inhibitor on spontaneous cardiogenesis of EBs adhesion in the presence of 10% FBS. The number of Nkx2.5GFP cells or MHC-positive cells was analyzed by using flow cytometry after treatment with ErbB receptor inhibitors, AG1478. Low concentration of AG1478 ($IC_{50} = 3$ nM) is known to be a selective inhibitor of ErbB1, but high concentration of AG1478 inhibits the others. After treatment with AG1478 (1–10 μM) in EBs adhesion at day 4, EBs were further cultured for 4–6 days. Nkx2.5GFP cells were 1.03 ± 0.17, 0.82 ± 0.12, and 0.46 ± 0.04, respectively, in 9–10-day-old EBs treated with 1, 5, and 10 μM when compared with control (Fig. 2A). In addition, MHC-positive cells from EBs at day 9 were significantly decreased by treatment with 10 μM AG1478 (Fig. 2B). The level of cardiac genes (Nkx2.5, β-MHC, MLC2a, cTnI) in EBs treated with or without 10 μM AG1478 from day 4 to day 10 was analyzed by RT-PCR. EBs treated with AG1478 decreased the expression level of cardiac specific genes when compared with control (Fig. 2C). These results imply ErbB receptor-mediated signaling is important to promote spontaneous cardiogenesis of EBs.

MEK/ERK activation by HRG-β1 promotes cardiogenesis

Previously we reported HRG-β1 promoted the growth of cardiomyocytes and suppressed the death of cardiomyocytes [3]. This study was examined the intracellular signal mechanisms by which HRG-β1 stimulates cardiogenesis during EBs adhesion. 4-day-old EBs were attached to gelatin-coated plated in the presence of 2% FBS, treated with HRG-β1 (1, 10, and 100 ng/ml) and further cultured for 4–5 days. MEK inhibitor (PD98059) or ErbB receptor inhibitor (AG1478) was preincubated for 1 h before treatment with HRG-β1. Nkx2.5GFP cells from EBs treated with HRG-β1 were analyzed by flow. Consistent with our previous findings, HRG-β1 increased Nkx2.5GFP cells in a dose-dependent manner and Nkx2.5GFP cells increased about 2-fold in response to 50 ng/ml of HRG-β1 (Fig. 3A). We further examined HRG-β1-mediated intracellular signaling on EBs adhesion. HRG-β1 induced ERK phosphorylation dose-dependently, which is inhibited upon treatment with PD98059 (Fig. 3B). The phosphorylation of MEK in HRG-β1 (100 ng/ml)-treated EBs was decreased by treatment with AG1478 (10 μM) (Fig. 3B). Moreover, the pretreatment of PD98059 or AG1478 suppressed the increase of Nkx2.5GFP cells by HRG-β1 (Fig. 3C). These results suggested MEK/ERK

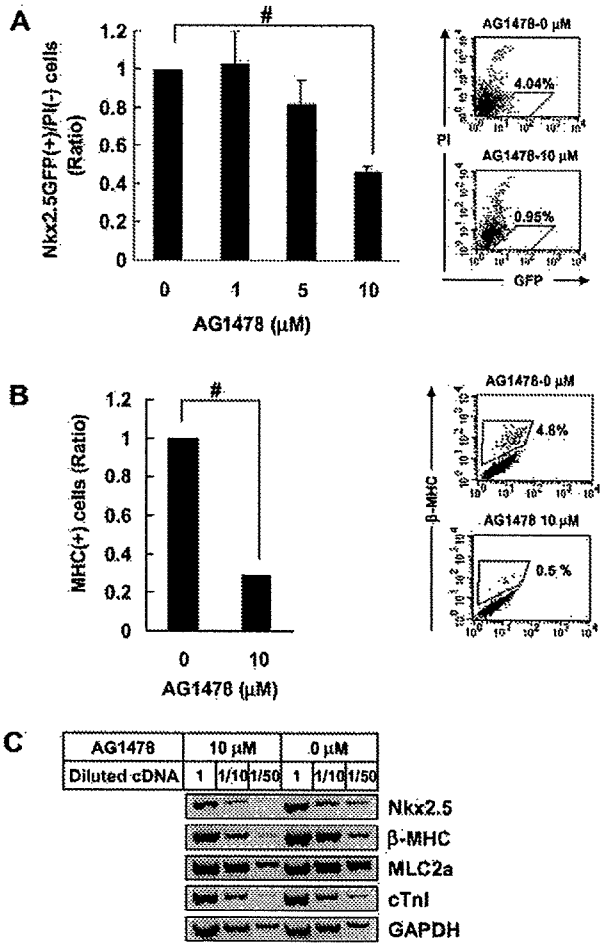

Fig. 2. Inhibition of ErbB receptor-mediated signaling pathway suppressed spontaneous development of cardiomyocytes in embryoid body (EB) adhesion. EB adhesion was differentiated for and additional 4–5 days in medium with 10% FBS after treatment of EB adhesion with ErbB receptor inhibitor (AG1478-1, 5, 10 μM) at day 4. (A) Nkx2.5GFP positive/propidium iodide (PI) negative cells were investigated by flow cytometry. (B) The cells stained with mouse anti-myosin heavy chain (MHC) monoclonal antibody were analyzed using flow cytometry. Values are means ± SE of three independent experiments ($^{\#}p < 0.05$). (C) The expression level of cardiac genes (Nkx2.5, β-MHC, MLC2a, and cTnI) was compared in EB treated with or without 10 μM AG1478 using by RT-PCR. cDNA concentration was diluted and GAPDH was used as internal controls in relative RT-PCR.

signal pathway might be involved in cardiomyocyte growth promoted by HRG-β1 via ErbB receptor.

MEK1 overexpression enhances spontaneous cardiogenesis

The treatment of ES cells with MAPK inhibitor decreased total ERK activity and expression of the mesodermal marker *brachyury*, suggesting that ERK plays an essential role in mesoderm differentiation *in vitro* [16]. We assessed the effect of MEK/ERK signaling activated by HRG-β1 on cardiogenesis of EBs. After transient transfection of recombinant MEK1 in EBs adhesion at day 5, we determined cardiomyocytes differentiation from EBs at day 9 or 10 by analysis of Nkx2.5GFP cells using flow cytometry and by analysis of MHC protein using Western blot. In EBs transfected with each recombinant MEK1, the level of cardiac specific protein, MHC protein was compared using Western blot. MEK1-WT and HA-MEK-CA increased the level of MHC protein when compared with vehicle vector (Fig. 4A). Next we investigated ERK that is downstream effector molecule of MEK1 after transfection with each plasmid. The phosphorylation of ERK was augmented by MEK1-WT and HA-MEK-CA but was rather suppressed by MEK-2A than vehicle vector (Fig. 4A). To confirm that overexpression of MEK1 enhances cardiomyocytes differentiation from EBs, we analyzed Nkx2.5GFP cell, and MHC positive cells. As shown in Fig. 4B and C, Nkx2.5GFP cells from EBs transiently overexpressing MEK1-WT (2.2 ± 0.34), but not MEK-2A (1.30 ± 0.17), were significantly increased when compared with vehicle vector. We employed immunostaining for MHC-positive cell dissociated from EBs at day 10 after transfection with MEK1-WT in EBs adhesion at day 5. Consistent with flow cytometry analysis, about 2-fold increase in population MHC-positive cells was shown in EBs transfected with MEK-WT (Fig. 4D). These results strongly suggest that MEK1 mediates activation of the ERK signaling pathway, leading to enhance cardiogenesis in ES cells.

Discussion

NRG/HRG and its receptors play an essential role during the development and growth of the heart as well as the maintenance of functional integrity of adult heart [7,8,17–19]. The importance of identifying the complexity of NRG's regulatory function in the heart is obvious considering the growing interest of new therapeutic opportunities targeting ErbB receptor-mediated signaling, and the fundamental role of this pathway in development of cardiomyocytes [20,21].

Recent investigations in the role of NRG-β1 in adult heart have focused on the identification of activated signaling pathways [8,9,22]. Our previous study demonstrated all ErbB receptor family expressed in developing EBs, specially, ErbB2 and ErbB4 expressed in cardiomyocytes isolated from EBs at day 8, whereas ErbB1 did not expressed in cardiomyocytes [3]. Moreover, HRG-β1 improved proliferation of cardiomyocytes and suppressed death of cardiomyocytes derived from ES cell [3]. Our present findings suggest some pathways by which HRG-β1 promotes stem cell efficacy for differentiation of cardiomyocytes derived from EBs adhesion. The main findings of the present study are the following: (1) Adhesion of EBs at day 3–4 inhibits spontaneous cardiogenesis of ES cells. (2) ErbB receptor inhibitor (AG1478) decreased phosphorylation of MEK and development of cardiomyocytes from ES cells. (3) HRG-β1 induced ERK phosphorylation, which is inhibited by MEK inhibitor (PD09859). (4) HRG-β1 significantly promoted the development of cardiomyocytes,

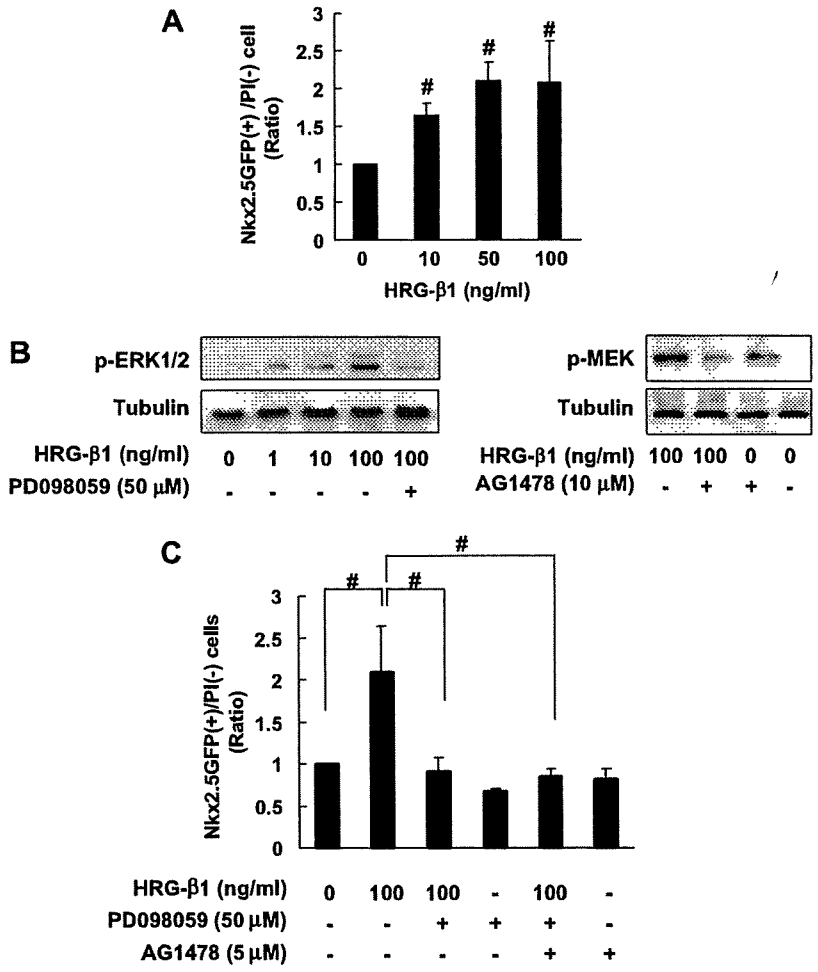

Fig. 3. Treatment with HRG-β1 activated MEK/ERK and promoted development of cardiomyocytes. (A) After treatment of 4-day-old embryoid body (EB) adhesion with HRG-β1 at indicated concentration (10, 50, and 100 ng/ml), EB adhesion was cultured in medium with 2% FBS for additional 5 days and then Nkx2.5GFP positive/propidium iodide (PI) negative cells were analyzed by flow cytometry. All values are mean ± SE of five independent experiments. (B) ERK and MEK phosphorylation by HRG-β1 was examined by Western blot. 4-day-old EB adhesion was cultured in serum-free medium for 2 days and was treated with HRG-β1 as indicated concentration (1, 10, and 100 ng/ml) for 2 h. MEK inhibitor (PD 50 μM) or ErbB receptor inhibitor (10 μM AG1478) was added for the 1 h of culture prior to treatment of 4-day-old EB adhesion with 100 ng/ml HRG-β1. (C) After treatment of 4-day-old embryoid body (EB) adhesion with HRG-β1 (100 μg/ml) in the presence of inhibitors (50 μM PD98059 or 5 μM AG1478) for 4–5 days, Nkx2.5GFP positive/propidium iodide (PI) negative cells were analyzed by flow cytometry. All values are means ± SE of five independent experiments ($^{\#}p < 0.05, n = 5$).

which were decreased by ErbB receptor inhibitor or MEK inhibitor. (5) MEK1 overexpression promoted the development of cardiomyocytes. These results indicate that HRG-β1 promotes cardiomyocytes development primarily by MEK-mediated ERK activation.

Other has reported the capability of NRG/HRG and ErbB receptor to promote muscle development [7,23]. Blocking ErbB-mediated signal by treatment with AG1478 in developing EBs significantly suppressed transcripts level of Nkx2.5, cTnI, β-MHC, and MLC2a and decreased the population of cardiomyocytes (Fig. 2). These results suggest ErbB mediated signal is important to regulate cardiogenesis in ES cells.

We further confirmed HRG-β1 signal promoted growth of cardiomyocytes derived from EBs adhesion. Increment of Nkx2.5GFP cells in EBs adhesion by treatment with HRG-β1 was shown (Fig. 3A). These findings are consistent with our previous findings as well as the other reports [7,17,24]. Specifically, the rapid activation of MAPK and AKT kinase pathways via soluble NRG-β1 isoform has been implied in control of development, and repair of cardiomyocytes [9,22,25]. In our study, HRG-β1 caused an activation of ERK in differentiating EBs, which was suppressed by the specific MEK inhibitor, PD98059 (Fig. 3B). HRG-induced phosphorylation of MEK was largely suppressed by treatment with AG1478 (Fig. 3B). Decrease in Nkx2.5GFP cells by treatment with PD98059 or AG1478 was detected (Fig. 3C). These are in line with the requirement of MAPK signaling for the enhanced development of cardiomyocytes by HRG-β1 in EBs. Thus, increase in cardiomyocytes from EBs treated with HRG-β1 appears to be mediated primarily via the MEK/ERK sig-

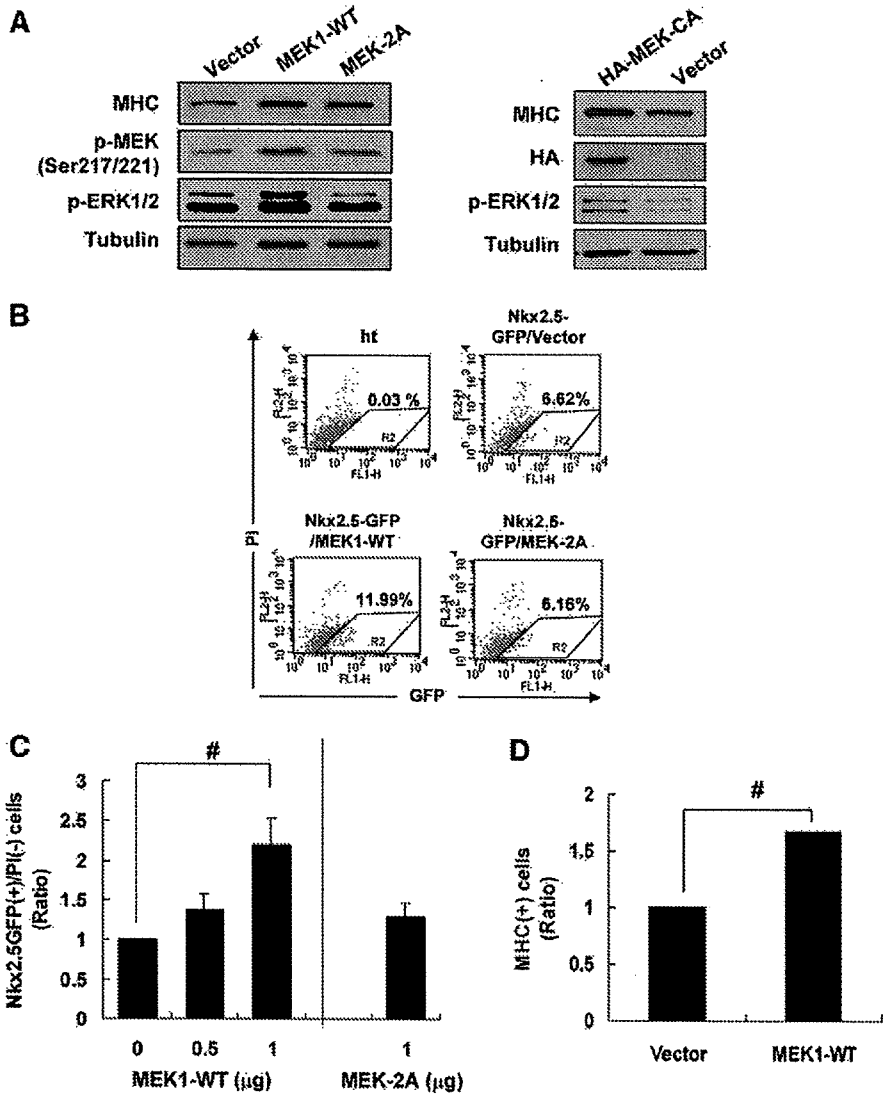

Fig. 4. MEK1 overexpression had effect on augmentation of ERK phosphorylation and promoted development of cardiomyocyte. Plasmid vectors to carry wild type MEK (MEK1-WT), dominant negative MEK (MEK2A), constitutive active MEK (HA-MEK-CA) were used. After transient transfection in embryoid body (EB) adhesion at day 4–5, EB adhesion was cultured for additional 4 days in medium with 10% FBS. (A) Development of cardiomyocytes was investigated by analysis of MHC protein using Western blot. (B) Representative flow cytometry of Nkx2.5GFP cells was shown. (C) Nkx2.5GFP cells in embryoid body (EB) adhesion transfected with MEK1-WT or MEK-2A were analyzed by flow cytometry. (D) For analysis of MHC protein using immunostaining, MHC-positive cells were counted from three areas under microscope. All values are means ± SE of three independent experiments ($^{\#}p < 0.05$, $n = 5$).

naling pathway. MAPK signaling pathway may play an important role in mesoderm induction at the time of gastrulation and is implicated as a modulator of cardiac function [26]. ERK1/2 activated by MEK was shown to be an essential signal molecule in mesoderm differentiation during embryonic development [16]. Intriguingly, overexpression with MEK1, upstream signal molecule of ERK induced an increase in cardiomyocytes as well as MHC protein in differentiating EBs (Fig. 4). This finding implies MEK/ERK is a central effector of HRG-β1 signaling to promote cardiogenesis in ES cells.

In conclusion, our results provide valuable insight on the mode of action of HRG-β1 in ES cell-derived cardiomyocytes. The present exploration is particularly helpful in unraveling the regulation of molecular pathways that are engaged in HRG-β1-promoted cardiogenesis of ES cells.

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Changes of HCN gene expression and I_f currents in Nkx2.5-positive cardiomyocytes derived from murine embryonic stem cells during differentiation

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ABSTRACT

Changes in the expression of hyperpolarization-activated cyclic nucleotide (HCN)-gated channels and I_f currents during the differentiation of embryonic stem cells into cardiac cells remain unknown. We examined changes of HCN genes in expression and function during the differentiation of Nkx2.5-positive cardiac precursor cells derived from mouse ES cells using cell sorting, RT-PCR, immunofluorescence and whole cell patch-clamp techniques. Cs^+-induced inhibition of automaticity and transcription of HCN genes increased during differentiation. Expressions of Nkx2.5, a marker of cardiac progenitor cell, and Flk1, a marker of hemangioblast, were mutually exclusive. Messenger RNA and proteins encoded by HCN1 and 4 genes were predominantly observed in Nkx2.5-positive cells on day 15, although Flk1-positive cells did not express genes of the HCN family on that day. Cs^+-induced prolongation of the cycle of spontaneous action potentials and I_f currents were predominantly observed on day 15. These results suggested that a fraction of Nkx2.5-positive cardiac precursor cells was committed to pacemaking cells expressing I_f channels predominantly encoded by HCN 1 and 4 genes.

Hyperpolarization-activated cyclic nucleotide-gated channels (I_f channels) encoded by a family of four HCN genes (HCN1-4) are widely expressed in heart cells (3) and neurons (21). I_f channels are best known for their prime role in the generation of automaticity in cardiac pacemaker cells in the adult sinus node and Purkinje system (3, 6, 17). Rabbit cardiac sinoatrial nodal cells express HCN1 mRNA together with strongly expressed HCN4 mRNA (7, 16) and rabbit Purkinje fibers contain almost equal amounts of HCN1 and HCN4 transcripts together with a small amount of HCN2 mRNA (23).

The activity of I_f channels is almost negligible in the adult ventricle, while the transcripts of HCN2

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and 4 are detected in the whole heart (8, 9, 24) together with traces of HCN3 mRNA (11). In contrast, I_f channels are present in the ventricle during the embryonic and perinatal stages: these currents have been observed in chick embryonic ventricle and cultured ventricular cells isolated from newborn rats as well as in differentiated adult rat ventricular cells (2, 9, 15, 16, 18, 20, 23). However, there are controversial reports regarding the developmental changes of I_f and HCN gene transcription. Yasui *et al.* reported that in mouse embryonic ventricles, HCN1 and 4 were predominantly expressed on day 9.5, while HCN2 was predominantly expressed in the neonatal period (27). This subtype switching appeared to underlie the attenuation of I_f activity and automaticity that takes place during embryogenesis. A recent large-scale analysis of ion channel expression in the mouse heart demonstrated that HCN1, 2 and 4 increased during development (4). However, the changes of I_f and HCN gene transcription during cardiac differentiation remain to be elucidated.

Automaticity is the most prominent characteristic of cardiomyocytes derived from ES cells, which can easily generate beating cardiac muscle by forming embryo-like cell aggregates called embryoid bodies (EBs). Using an ES cell differentiation system, it is possible to trace the cardiovascular differentiation process at the cellular level. Tracking the markers of stem cells is an appropriate technique to evaluate developmental changes of cardiac progenitor cells. Flk1, for instance, has been reported to be predominantly expressed in the lateral mesoderm and is a marker of hemangioblasts (25). Nkx2.5 (5) is expressed in the cardiac primordium, beginning in the cardiogenic mesoderm before alpha-actin and alpha-myosin heavy chain genes develop; thus, it is a marker of cardiac progenitor cells. Nkx2.5-positive cells (Nkx2.5/GFP(+) cells) are cardiac precursor cells derived from ES cells whose expression of GFP is driven by an Nkx2.5 promoter; these cells differentiate into sinus node- or conducting system-type cells as well as into myocytes (5). Since Nkx2.5-positive cells may differentiate into cells of any cardiac precursor lineage during heart development, Nkx2.5 expression can be used to identify various types of cardiomyocyte precursors in a mixture of ES cells. In the present study, we investigated changes in HCN genes and protein expression, Cs^+-sensitive automaticity and I_f channel currents during differentiation of Nkx2.5-positive cardiac precursor cells derived from murine ES cells through EBs formation *in vitro*.

MATERIALS AND METHODS

Culture of Nkx2.5/GFP(+) ES cells. Ht7 is an ES cell line derived from 129/Ola cells. Ht7 cells carry a hygromycin resistance gene in one of the Oct-3/4 loci, which allows selection of Oct-3/4-positive undifferentiated stem cells (5). Hcgp7 cells are Nkx2.5/GFP knock-in ES cells derived from Ht7 cells and carry the GFP reporter gene in one of the Nkx2.5 loci (14). Cells were grown and maintained on gelatinized dishes without feeder cells in Glasgow-modified Eagle's medium (GMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS, #6K0357; Equitech-Bio Inc., Kerrville, TX), 1 × non-essential amino acids (Invitrogen, Carlsbud, CA), 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1 g/mL penicillin, 0.1 mg/mL streptomycin, 100 μg/mL hygromycin (Gibco-BRB, Carlsbud, CA), and 1000 units/mL leukemia inhibitory factor (LIF) (ESGRO™; Chemicon International Inc., Temecula, CA). Differentiation of both Ht7 and Hcgp7 cells was induced through formation of EBs. Briefly, EBs were generated by plating 20 μL of cell suspension (final concentration of 2.5×10^4 cells/mL of growth medium: GMEM containing 10% FCS, 0.1 mg/mL penicillin, 0.1 mg/mL streptomycin, and 0.1 mM 2-mercaptoethanol) on Petri dishes, followed by incubation in hanging drops for 2 days. Sixty EBs were transferred to Petri dishes together with 10 mL of the growth medium and cultured as floating EBs until dissociation for FACS analysis. We used mainly floating EBs or cells sorted from floating EBs for most of the analyses. For experiments shown in Fig. 1A, embryoid bodies were transferred onto gelatin-coated dishes on day 5 and cultured to observe spontaneous beating.

Fluorescence-activated cell sorting. EB cells were dissociated by trypsinization and gentle pipetting, then resuspended in Hank's Balanced Salt Solution containing 1% bovine serum albumin and subjected to flow cytometry (EPICS XL; Beckman Coulter, Fullerton, CA) using WinMDI software. The sort gate for GFP(+) cells was established on the basis of the forward-scattered light, side-scattered light, and GFP fluorescence intensity of control ES cells (Ht7 cells). Hcgp7 cells were sorted into culture medium and reanalyzed to estimate purity. Typically, about 5×10^5 GFP(+) cells (Nkx2.5-positive cells) were obtained from 1×10^7 cells from EBs harvested on day 8. Flk1(+) cells (Flk1-positive cells) were also identified using anti-Flk1 antibody and PE-con-

jugated antibody and were subsequently sorted into culture medium and reanalyzed to estimate purity. Typically, about $3–4 \times 10^5$ Flk1(+) cells were obtained from 1×10^7 cells from EBs harvested on day 8.

Real-time reverse transcriptase—polymerase chain reaction (RT-PCR). Messenger RNA was collected from EBs on days 5, 7, 10 and 15 of their differentiation. For the analysis of mRNA in GFP(+) cells, the cells were obtained from EBs harvested on day 7 and sorted; then, they were further cultured on gelatin-coated dishes and analyzed on days 7 and 15 of their differentiation. Total RNA was isolated from EBs or sorted cells using an RNeasy Mini Kit (Quiagen, Valencia, CA) according to the manufacturer's instructions. RNA samples were treated with DNaseI (Promega, Madison, WC) to eliminate genomic DNA contamination, and cDNA was synthesized using SuperScript™ II reverse transcriptase (Gibco-BRL). Real-time PCR was performed using a Light Cycler SYBR Green I kit according to the manufacturer's instructions (Roche Diagnostics, Tokyo, Japan) and products were analyzed employing an ABI PRISM R 7700 Sequence Detection System (Applied Biosystems, Foster, CA). The gene-specific primers used in the experiments are shown in Table 1. PCR products were also separated by gel electrophoresis, stained with ethidium bromide and visualized in the UV transilluminator.

Electrophysiological recordings. Action potentials (APs) and I_f currents in GFP(+) cells were measured on days 7 and 15 of their differentiation using the patch-clamp technique (5, 12, 13). APs and I_f currents were recorded employing an Axopatch-200B amplifier (Axon Instruments). Command voltage pulse generation, data acquisition, and data analysis were performed using pCLAMP9 software (Axon Instruments). All experiments were carried out at 37°C.

APs were recorded according to the perforated-patch technique under current clamp mode. Cells were perfused with normal Tyrode's solution. The pipette solution contained (in mM) 140 K aspartate, 5 $MgCl_2$, 5 K_2ATP, 5 EDTA, 5 HEPES, and 250 μg/mL amphotericin B; pH was adjusted to 7.2 with KOH. Membrane perforation was monitored by changes in capacitance transients (*i.e.* changes in series resistance) in the voltage clamp mode. APs were triggered by application of 3 ms current pulses of 1 nA.

Whole cell I_f currents were recorded using the ruptured-patch technique under the voltage clamp mode. Cells were perfused with an external solution containing (in mM) 135 NaCl, 1 $MgCl_2$, 10 HEPES, 10 D-glucose, 2 $CaCl_2$, 5.4 KCl, 2 $BaCl_2$, and 0.2 $CdCl_2$ (pH 7.4 with NaOH). The internal pipette solution contained (in mM) 130 K-glutamate, 1 $MgCl_2$, 15 KCl, 5 NaOH, 5 HEPES, and 5 Mg-ATP (pH 7.3 with KOH). The recording pipettes had a tip resistance of < 5 MΩ when filled with the internal solution. To minimize voltage errors, recordings were not made until the series resistance became < 15 MΩ. The average cell membrane capacitance was 34.5 ± 3.5 pF (mean ± S.E.) in cells from EBs harvested on day 7, and 41.0 ± 4.8 pF (mean ± S.E.) in those from EBs harvested on day 15. I_f currents were elicited by 3 s hyperpolarizing pulses (from −130 to −40 mV) from a holding potential of −50 mV. An I_f activation curve was constructed from the amplitude of time-dependent inward currents measured at the end of the test pulses. I_f values were normalized to the current ampli-

Table 1 *Sequences of oligonucleotides used as real time RT-PCR primers*

Target	Accession number		Sequence (5'→3')	Size of PCR product (bp)
β-actin	X03672	Left Primer	CAACCGTGAAAAGATGAC	238
		Right Primer	CAGGATCTTCATGAGGTAGT	
HCN1	NM_010408	Left Primer	CTCCACTTTGATCTCCAGAC	253
		Right Primer	TTCTGCATCTGGGTCTGTAT	
HCN2	HM_008226	Left Primer	GACAATTTCAACGAGGTGCT	199
		Right Primer	CCATCTCACGGTCATATTTG	
HCN3	HM_008227	Left Primer	AACCCTCCATGCCAGCCTAT	177
		Right Rrimer	TTCCAGAGCCTTTACGCCT	
HCN4	AF_064874	Left Primer	CGACAGCGCATCCATGACTA	211
		Right Primer	GCTGGAAGACCTCGAAACGC	

RT-PCR: reverse transcriptase-polymerase chain reaction

tude at −130 mV and fitted by Boltzmann's equation

$$I_f = 1 / \{1 + \exp[(V_m - V_{1/2}) / k]\}, \quad (1)$$

where V_m is the membrane voltage, $V_{1/2}$ is the voltage at the half-maximal activation, and k is the slope factor.

Immunofluorescence. Cells were fixed in 4% paraformaldehyde for 10 min at 4°C and made permeable with 0.1% TritonX-100 for 10 min at 4°C. They were washed three times with PBS and treated with a blocking buffer (0.1% Triton X-100, 2% skim milk in PBS) at room temperature for 1 h. Anti-HCN1 and 4 (Alamone Lab, Jerusalem, Israel) staining of fixed cells was performed as described previously (13) using Alexa546-conjugated anti-mouse or anti-goat IgG (Alexa) as a secondary antibody, respectively. The nuclei were stained with DAPI. Images were collected employing a Bio-Rad MRC1024 confocal microscope.

Statistical analysis. For statistical analysis, one-way analysis of variance (ANOVA) and repeated measures analysis of variance (two-way ANOVA) were carried out. All data are expressed as the mean ± SE, with $P < 0.05$ being considered statistically significant.

RESULTS

Cs^+-sensitivity of automaticity and transcription of HCN genes during EBs differentiation

Figure 1A shows the effect of the authentic I_f inhibitor Cs^+ on spontaneous beating of EBs on days 7 and 15 of their differentiation. In the presence of 2 mM Cs^+, the frequency of spontaneous beating decreased by 40 ± 10% (mean value: 98 beats/min to 58 beats/min) in EBs on day 15, whereas at the same concentration Cs^+ barely affected their beating on day 7 (mean value: 64 beats/min to 57 beats/min). Since I_f currents encoded by HCN gene play a pivotal role in Cs^+-sensitive automaticity (3), we examined the transcription of HCN genes in EBs during their differentiation. Figure 1B shows the transcription of the HCN gene family (HCN1 to 4) in EBs on days 5, 7, 10 and 15 of their differentiation. The transcription of all HCN genes significantly increased, while beta-actin expression did not change over the entire phase of differentiation (data

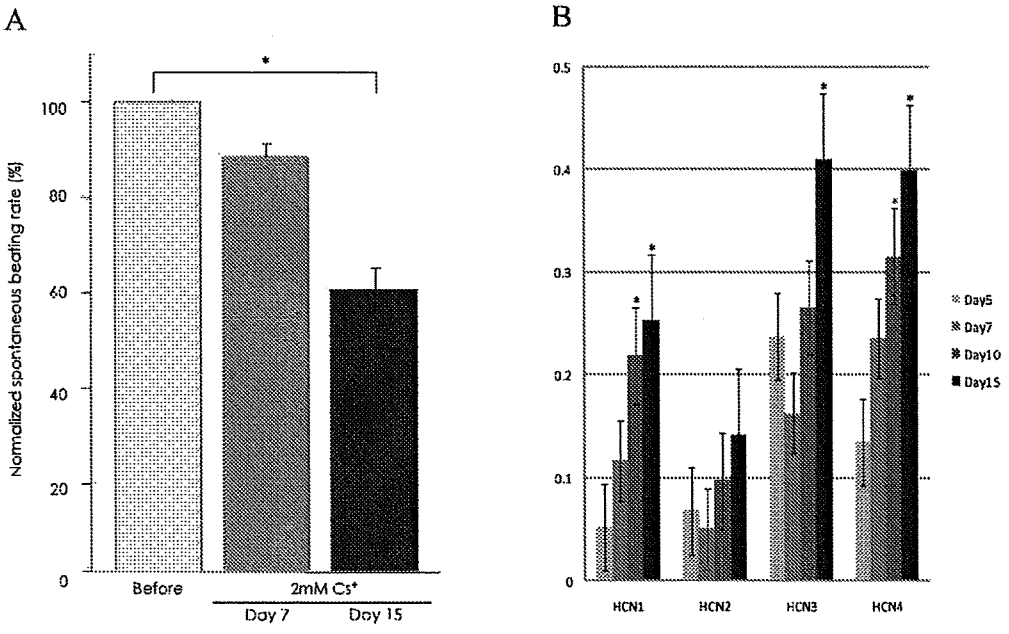

Fig. 1 Changes in Cs^+-sensitive automaticity and level of HCN gene transcription in EBs during their differentiation. A: Beating rates of EBs formed by Hcgp7 cells on days 7 and 15 of their differentiation in the presence and absence of 2 mM Cs^+. Before: before the addition of CsCl (open column). 2 mM Cs^+: in the presence of 2 mM CsCl. Values are the mean ± SE of 15 independent experiments. $^*P < 0.05$. B: Level of HCN gene transcription in EBs formed by Hcgp7 cells at the indicated time points during their differentiation. Y-axis indicates the normalized mRNA. Values are expressed as the mean ± SE of 15 independent experiments. $^*P < 0.05$.

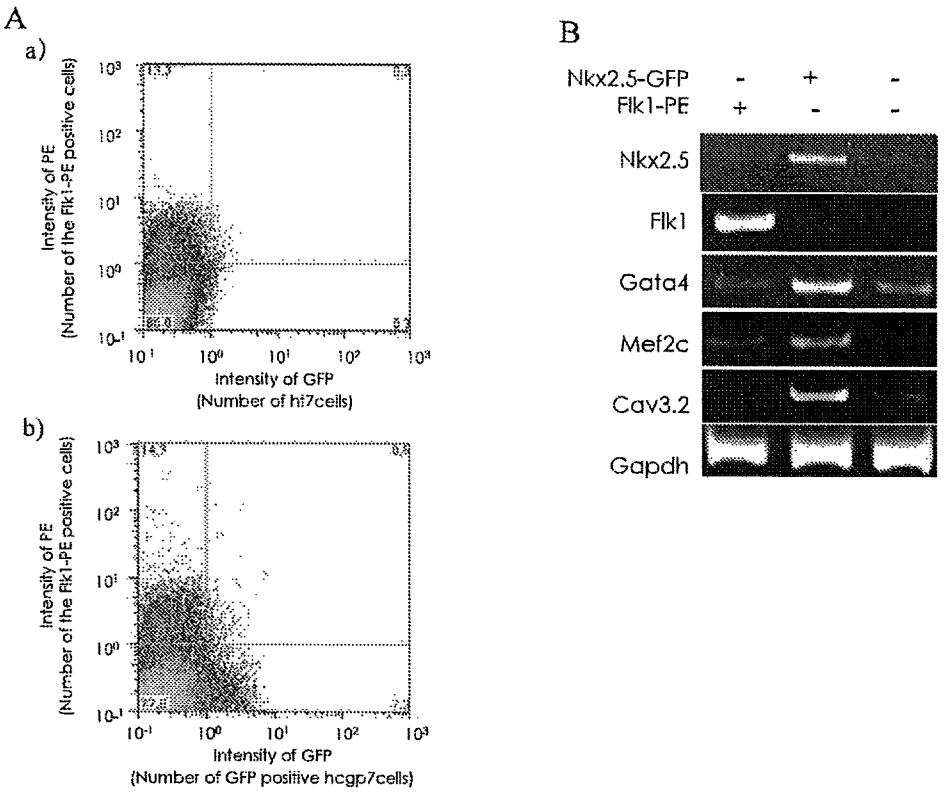

Fig. 2 Distribution of Nkx2.5-positive cells and Flk1-positive cells and gene expression. A: FACS Analysis of Flk1-positive and Nkx2.5/GFP(+) cells derived from EBs harvested on day 7 of their differentiation. Panel a), control Ht7 ES cells. Panel b), Flk1(+) cells (14.3% of all cells) and Nkx2.5/GFP(+) cells (7.3% of all cells) were identified by FACS analysis. B: Transcripts of cardiac specific genes in purified Nkx2.5-positive cells, Flk1-positive cells and double negative cells. Cells were sorted by FACS, and then mRNA was extracted from them. Each band corresponds to the transcript amplified using the indicated primers. (+): positive cell fraction, (−): negative cell fraction.

not shown).

Distribution of Nkx2.5/GFP(+) cells and Flk1(+) cells in EBs

In EBs, spontaneous beating cells responsive to Cs^{2+} include cardiomyocytes as well as other types of differentiated cells. Therefore, we examined the distribution of Nkx2.5/GFP(+) cardiac precursor cells in EBs based on the GFP fluorescence intensity of control Ht7 ES cells (Fig. 2Aa). It has recently been reported that Flk1-positive mesoderm cells give rise to spontaneous beating cardiomyocytes when cultured on OP9 stroma cells (25), suggesting the possibility that Flk1-positive cells (Flk1(+) cells) might be present in the population of Nkx2.5/GFP(+) cells. Our preliminary data indicated that the transcription of Flk1 started on day 4.5 to reach a steady level on day 7 and that of Nkx2.5 started on day 5.5 to reach a steady level on day 8.5 of the EBs differentiation (data not shown). As shown in Fig. 2Ab, on day 7 most Nkx2.5/GFP(+) cells did not overlap with the population of Flk1(+) cells. Fig. 2B shows the fluorescence intensity of the transcripts of cardiac specific genes in various fractions of sorted cells. Nkx2.5/GFP(+)-Flk1(−) cells expressed Nkx2.5, Gata4, Mef2c, and the T-type Ca^{2+} channel Cav3.2, which is consistent with our previous reports (12, 13). Nkx2.5/GFP(−)-Flk1(+) cells expressed mainly Flk1 and Gata4, and to a less extent Nkx2.5 and Mef2c, but they did not express Cav3.2; whereas, Nkx2.5/GFP(−)-Flk1(−) cells expressed Gata4 alone. These results suggested that the populations of Nkx2.5/GFP(+) and Flk1(+) cells were for the most part mutually exclusive.

Transcriptional level and immunoreactivity of HCN genes in Nkx2.5-positive cells

We purified Nkx2.5/GFP(+) cells from EBs harvested on days 7 and 15, and analyzed the level of HCN gene transcription by RT-PCR. Figure 3A