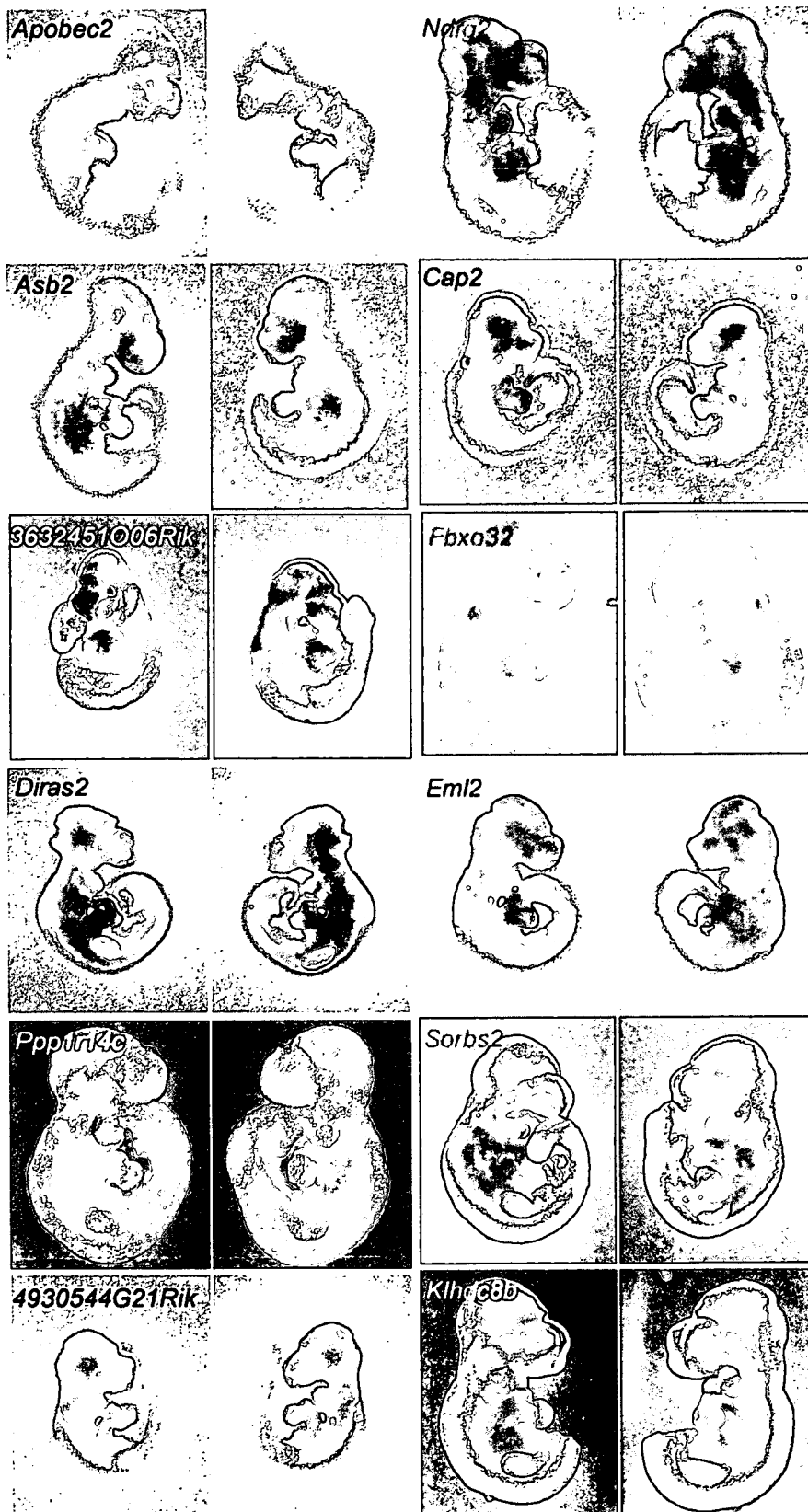


61	aldo-keto reductase family 1, member A4 (aldehyde reductase)	Akr1a4	1451035_a_at	
62	dapper homolog 1, antagonist of beta-catenin (xenopus)	Dact1	1417937_at	
63	junctophilin 2	Jph2	1455404_at	
64	popeye domain containing 2	Popdc2	1417806_at	
65	ankyrin repeat and SOCS box-containing protein 2	Asb2	1428444_at	(3)
66	small muscle protein, X-linked	Smpx	1418095_at	
67	single-stranded DNA binding protein 2	Ssbp2	1449815_a_at	
68	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3	Smarcd3	1418467_at	(1)
69	CAP, adenylate cyclase-associated protein, 2 (yeast)	Cap2	1423222_at, 1450910_at	(3)
70	RIKEN cDNA 3632451O06 gene	3632451 O06Rik	1450770_at	(3)
71	monocyte to macrophage differentiation-associated	Mmd	1423488_at, 1423489_at	
72	F-box only protein 32	Fbxo32	1448747_at	(3)
73	DIRAS family, GTP-binding RAS-like 2	Diras2	1455436_at	(3)
74	nexilin	Nexn	1435649_at	
75	heat shock protein 2	Hspb2	1429888_a_at	
76	ras homolog gene family, member U	Rhou	1449027_at	
77	adaptor-related protein complex 2, beta 1 subunit	Ap2b1	1452292_at	
78	phosphoglucomutase 2	Pgm2	1451149_at	
79	echinoderm microtubule associated protein like 2	Eml2	1428173_at	(3)
80	RIKEN cDNA 1700113I22 gene	1700113I 22Rik	1432073_at	
81	nebullette	Neb1	1438452_at	
82	protein phosphatase 1, regulatory (inhibitor) subunit 14c	Ppp1r14c	1417701_at	(3)
83	microfibrillar-associated protein 4	Mfap4	1424010_at	
84	RIKEN cDNA 4930544G21 gene	4930544 G21Rik	1454926_at	(3)
85	limb-bud and heart	Lbh	1429088_at	
86	kelch domain containing 8B	Klhdc8b	1428221_at	(3)
87	melanoma antigen, family D, 2	Maged2	1426306_a_at	
88	leucine rich repeat containing 39	Lrrc39	1453592_at	
89	brain glycogen phosphorylase	Pygb	1433504_at	

90	electron transferring flavoprotein, beta polypeptide	Etfb	1428181_at	
91	myocardin	Myocd	1425808_a_at, 1425978_at	(1)
92	unc-45 homolog B (C. elegans)	Unc45b	1436939_at	
93	peptide YY	Pyy	1424865_at	
94	alpha-kinase 2	Alpk2	1452478_at	
95	RCS domain containing 1	Rcsd1	1424194_at	(2)
96	spondin 1, (f-spondin) extracellular matrix protein	Spon1	1451342_at	
97	fusion, derived from t(12;16) malignant liposarcoma (human)	Fus	1451285_at	
98	sorbin and SH3 domain containing 2	Sorbs2	1437197_at	(3)
99	isocitrate dehydrogenase 2 (NADP+), mitochondrial	Idh2	1450048_a_at	
100	RIKEN cDNA 2610301F02 gene	2610301 F02Rik	1460125_at	

(1) Fig.3, (2) Fig.4, (3) Supplemental Fig.1



Supplemental Fig. 1



## 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<sup>+</sup> 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 Cgn1 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<sup>+</sup> 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.

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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 cush-

ion 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 Cgln1 (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<sup>+</sup> and CD31<sup>-</sup> 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<sup>-</sup>, GFP-CD31<sup>+</sup>, and GFP-CD31<sup>-</sup> 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, Mum11, Cgln1, 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, Mum11, Cgln1, 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<sup>+</sup> cells derived from the heart could be considered as endocardial cells and CD31<sup>-</sup> cells as myocardial cells, based on their specific expression of Cdh5 and Myl2, respectively. While Nkx2-5 was specifically expressed in CD31<sup>-</sup> myocardial cells, Gata4 was preferentially expressed in CD31<sup>+</sup> 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<sup>+</sup> cells. Among the cardiogenesis-associated genes described above, cingulin-like 1 (Cgln1) and docking protein 4 (Dok4) were expressed preferentially in cardiac CD31<sup>+</sup> cells, but not in yolk sac CD31<sup>+</sup> cells, while FYVE, RhoGEF, and PH domain containing 5 (Fgd5) was expressed in

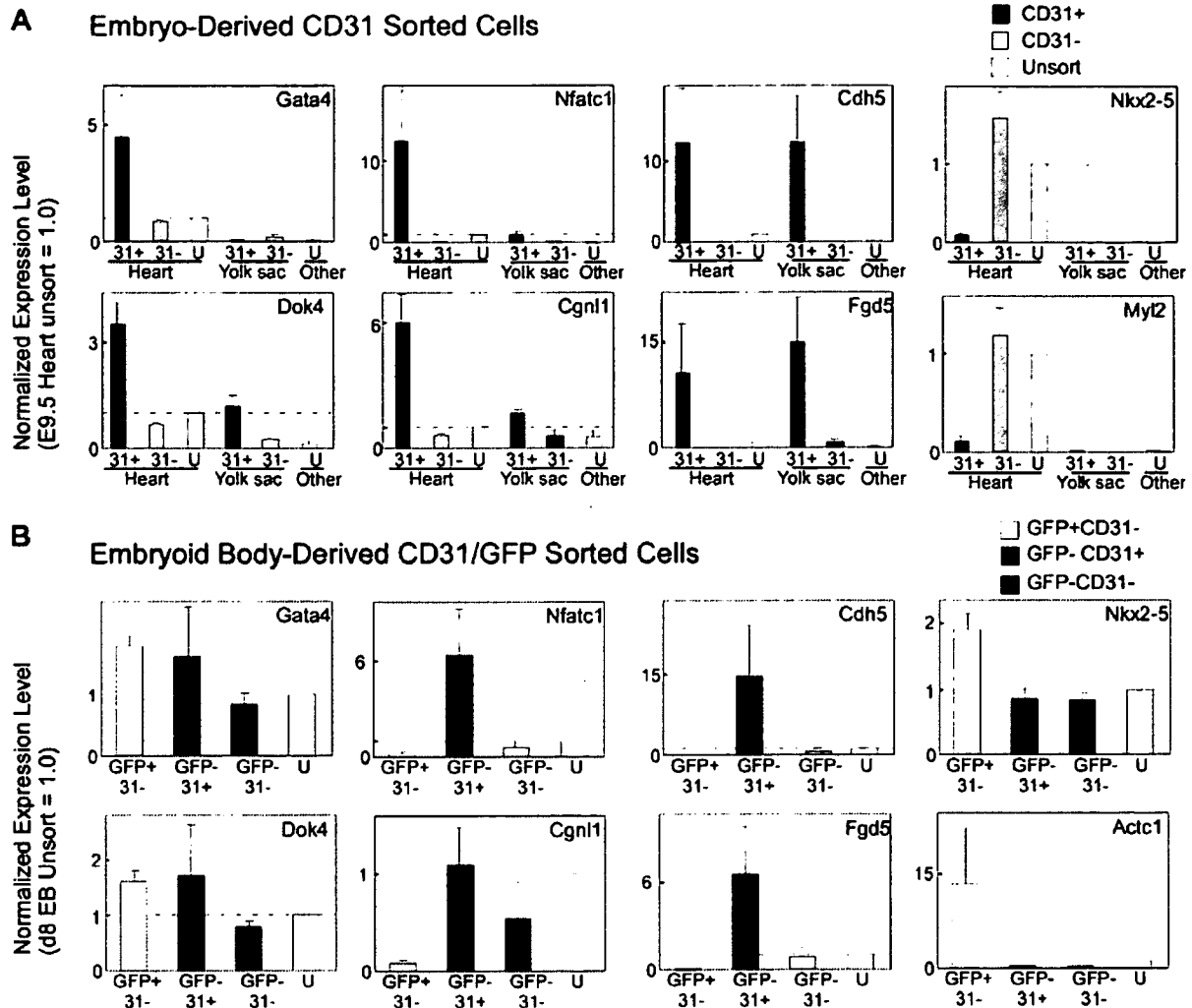


Fig. 1. Expression of cardiogenesis-associated genes in CD31<sup>+</sup> endothelial cells from embryos and embryoid bodies. (A) Real time RT-PCR analysis of cells sorted from mouse embryos. CD31<sup>+</sup> (+), CD31<sup>-</sup> (-), and unsorted (U) cells were obtained from the heart, yolk sac, and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

both cardiac and yolk sac CD31<sup>+</sup> cells. Although Nrg1 has been shown to be expressed in endocardium tissues, we detected it in both heart and yolk sac-derived CD31<sup>+</sup> cells (data not shown), indicating that embryoid 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<sup>+</sup> cells used to represent cardiomyocytes (Fig. 1B). While Cgml1 and Nfatc1 were expressed solely in CD31<sup>+</sup> cells, Dok4 and Gata4 were expressed in both CD31<sup>+</sup> cells and GFP<sup>+</sup> 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<sup>-</sup> 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<sup>+</sup> area was mostly included in the CD31<sup>+</sup> area, as shown in Fig. 3A, and was often found near the MyHC<sup>+</sup> area, where cardiomyocytes were

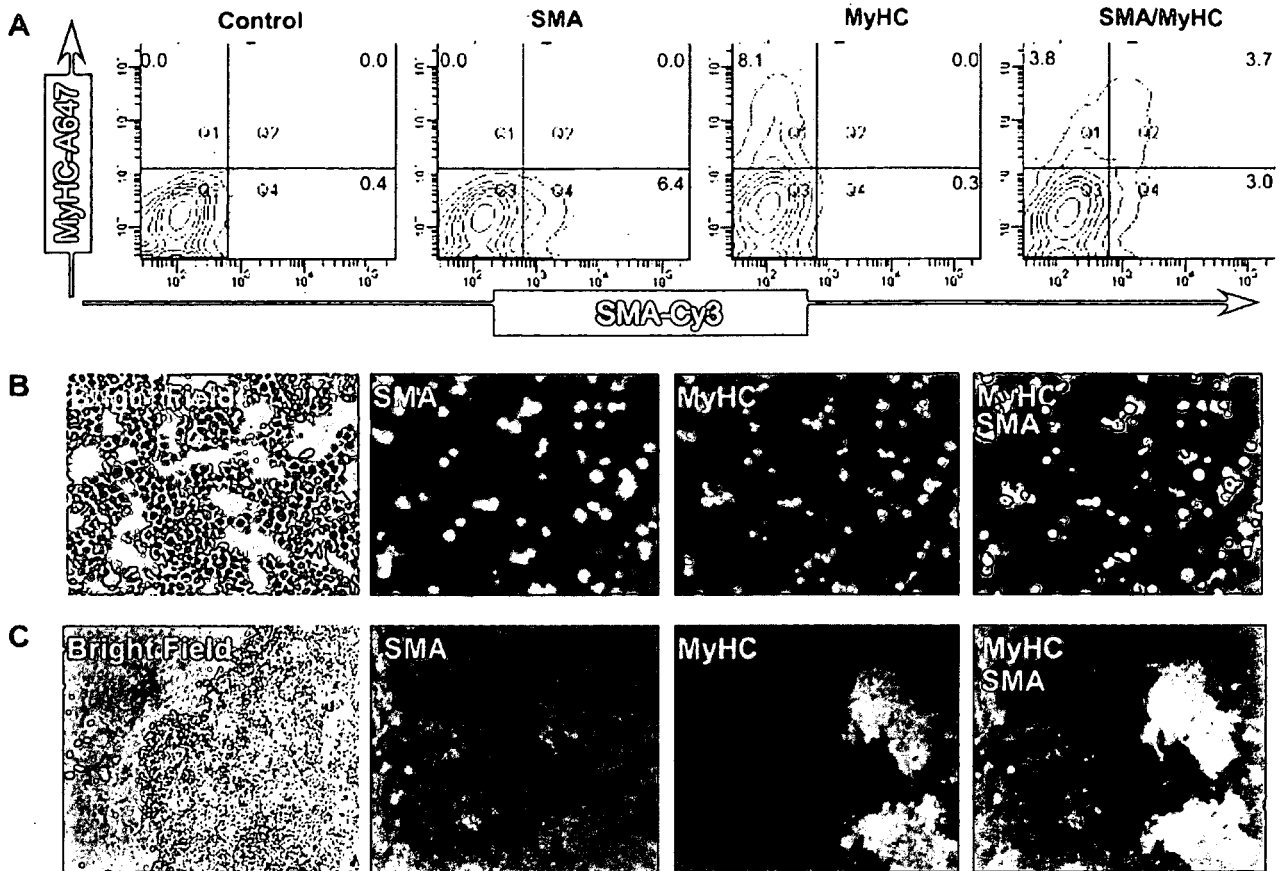


Fig. 2. Smooth muscle actin expression in cardiomyocytes in EBs. Co-expression of sarcomeric myosin heavy chain and smooth muscle alpha actin in EBs. (A) Floating EBs (d9) were dissociated, then fixed and stained with anti-SMA-Cy3, anti-MyHC, and anti-mouse IgG2b-Alexa647. (B) Cytopsin preparations of 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<sup>+</sup> cardiomyocytes in EBs. In addition to cardiomyocytes, GATA4 was also detected in some Nfatc1<sup>+</sup> cells located near cardiomyocytes (Fig. 3B). Further, co-expression of GATA4 and CD31 was confirmed by staining single CD31<sup>+</sup> 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, Cgln1, was identified in EBs, supporting the hypothesis that EBs can differentiate into endocardial cells.

#### Developmental expression of Cgln1 and Dok4 in mouse embryos

To elucidate the expression patterns of Cgln1 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 Cgln1 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, Cgln1 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 Cgln1 was maintained in the heart at all stages examined (up to E10.5, data not shown). Consistent with the RT-PCR data, Cgln1 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 Cgln1

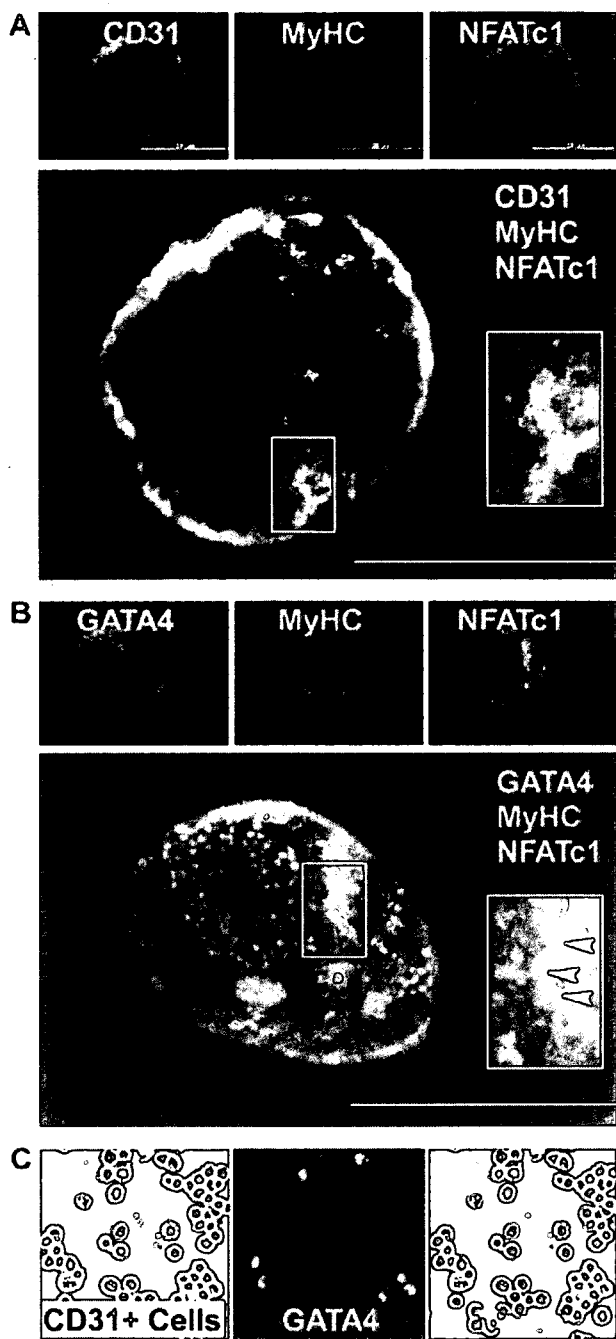


Fig. 3. Endocardiogenesis in EBs. Frozen sections of floating EBs (d8) were incubated with (A) anti-CD31, anti-MyHC, and anti-NFATc1 antibodies, or (B) anti-GATA4, anti-MyHC, and anti-NFATc1 antibodies. Signals were detected by anti-mouse IgG2b-Alexa350 (blue), anti-rabbit-Alexa546 or anti-rat-Alexa546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

and Dok4 is restricted to cardiac endothelium, we compared the expression patterns of Cgln1, Dok4, and Cdh5. Strong Cdh5 signals were detected in both the endocardium and developing vascular endothelium at all stages

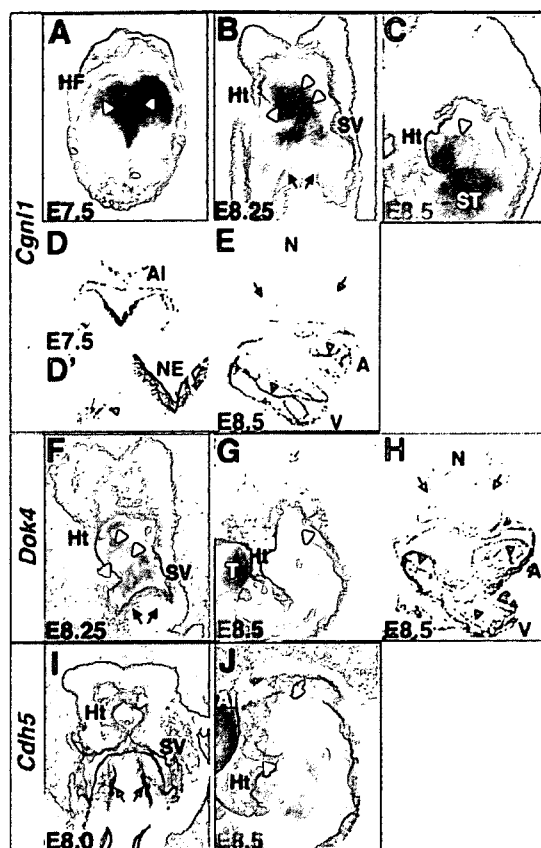


Fig. 4. Cgln1 and Dok4 expression in developing endocardium. Whole-mount *in situ* hybridization and section analyses of Cgln1, Dok4, and Cdh5 expression in E7.5–8.5 mouse embryos. Ventral views of embryos on (A) E7.5, (I) E8.0, and (B,F) E8.25. (C,G,J) Left-side views of embryos on E8.5. Arrows indicate the corresponding regions in mouse embryos: black, dorsal aorta; white, vascular networks in the head; and yellow, vascular networks in the trunk. (A–E) Cgln1 Expression. Cgln1 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 Cgln1 was observed in the endocardium, however, not in the endothelium of the dorsal aorta (arrows in E), or vascular networks (arrows in C). Cgln1 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 venosus; T, tail; V, ventricle; YS, yolk sac.

examined (Fig. 4I and J), however, no evidence of Cgln1 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 Cgln1 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, Cg11 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 Cg11, 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.

Cg11 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 Cg11 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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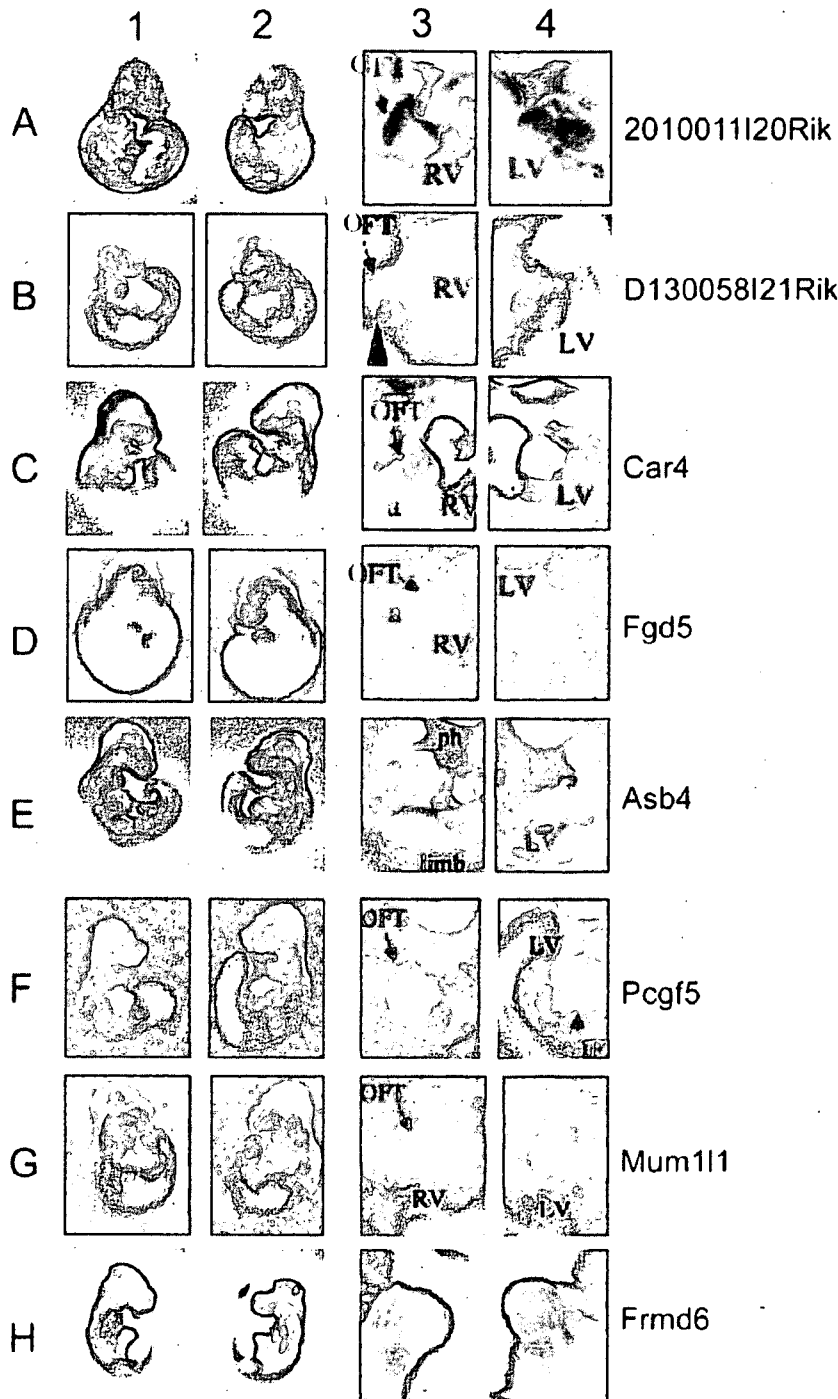
**Supplemental Table. Primers for RT-PCR**

Gene Symbol		Sequence
2010011I20Rik	fwd	5'-GTT CGG ATC AGC ATC ACC TT-3'
	rev	5'-TCA GAA GAG ACT GGC GTC AA -3'
D130058I21Rik	fwd	5'-CTT CAG CTC TGT CAC CCA CA-3'
	rev	5'-TTC TCA GTG GGA CTG GGA GT-3'
Car4	fwd	5'-AGA ATC AGC AGT CCC CCA TC-3'
	rev	5'-TTT TGG TCA TAG CCG ACG AG-3'
Fgd5	fwd	5'- AAG GTC TGT GAT GGC TGC TT-3'
	rev	5'-ATG CTG ACT GGA CGC TCT CT-3'
Asb4	fwd	5'- GGC TAT TGG TTG CCC AGT TA-3'
	rev	5'-TGG GAC GAC AGT TGA TGG TA-3'
Pcgf5	fwd	5'- AAG GCT GGA TAA CAC GTT GG-3'
	rev	5'-TCT TGC CCA TTT TCT TGA GG-3'
Mum111	fwd	5'- TTG GTT GAG GCA GAC ATG AG-3'
	rev	5'-GGT ACT CCT CCC TGG CTT TC-3'
Cgn1	fwd	5'- TCG ACC GAC TGG AAA GTT CT-3'
	rev	5'-CTC AGT GGG GCT TCG TAG AG-3'
Frmd6	fwd	5'-ACA GAG GTT GGT CCG AAA TG-3'
	rev	5'-ATT TGT GAA GGA GCC CAG TG-3'
Dok4	fwd	5'- CGG CTG GAG AAG TAT CCT GA-3'
	rev	5'- TTC TGC CTC CAG CTC TGA GT-3'

Legends for Supplemental Figure

Suppl Fig 1. Expression of cardiogenesis-associated genes in mouse embryos.

(A) Whole mount in situ hybridization of E9.5 mouse embryos. From left to right, left and right view of whole embryo, left and right view of heart region. A, 2010011120Rik, B, D130058I21Rik, C, Car4, D, Fgd5, E, Asb4, F, Pcgf5, G, Mum111, H, Cgnl1, I, Frmd6. OFT, out flow tract, a atrium, LV, left ventricle, RV, right ventricle, ph, pharyngeal arch



## Activation of MEK–ERK by heregulin- $\beta$ 1 promotes the development of cardiomyocytes derived from ES cells

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### Abstract

We have previously shown that heregulin-beta1 (HRG- $\beta$ 1) 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- $\beta$ 1 stimulates cardiogenesis in ES cells. The treatment with ErbB receptor inhibitor decreased the population of cardiomyocytes and transcripts levels of cardiac genes (Nkx2.5,  $\beta$ -MHC, cTnI, and MLC2a). The phosphorylation of ERK and development of cardiomyocytes by treatment with HRG- $\beta$ 1 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- $\beta$ 1 promotes the development of cardiomyocytes predominantly by activation of MEK–ERK.

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**Keywords:** Embryonic stem cell (ES) cell; Nkx2.5 transcription factor; Heregulin-beta1 (HRG- $\beta$ 1); 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- $\beta$ 1 is known to be a ligand of ErbB2 and ErbB4, but not ErbB3 found on cardiomyocytes, and pro-

motes 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- $\beta$ 1 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- $\beta$ 1 promoted development of cardiomyocytes from ES cells [3]. These reports indicated that HRG- $\beta$ 1-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- $\beta$ 1 in regulating development of cardiomyocytes derived from ES cells.

In this study, we investigated the possible intracellular signaling pathway by which HRG- $\beta$ 1 promotes the development of cardiomyocytes derived from ES cells using the well-characterized Nkx2.5GFP ES cells. HRG- $\beta$ 1 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- $\beta$ 1. Furthermore, MEK1 overexpression promoted the development of cardiomyocytes. These data suggest that MEK/ERK signaling pathway mediated by HRG- $\beta$ 1 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  $\mu$ g/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  $\mu$ g/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- $\beta$ 1 on cardiac differentiation of embryoid bodies.** EBs were cultured with the growth factor, HRG- $\beta$ 1 (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 polyethylenimine (PEI) (Sigma–Aldrich, 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<sup>®</sup> 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  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml pepstatin A, and 1  $\mu$ g/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  $\pm$  SE and represent at least three independent experiments. Comparisons of means were performed using Student's *t* test. Values of  $p \leq 0.05$  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 (transferrin 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- $\beta$ 1 on spontaneous cardiogenesis of ES cells.

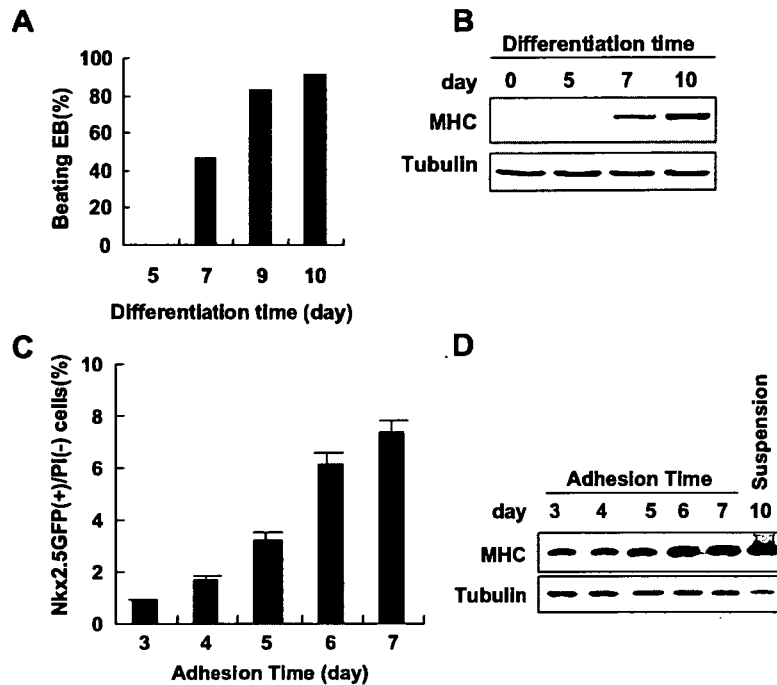


Fig. 1. Spontaneous development of cardiomyocytes in embryoid body (EB) adhesion was inhibited. (A) Rhythmical beating EB in suspension system under microscope was observed at day 7 ( $n = 3$ ). (B) Western blot showed that MHC protein in EB suspension system appeared around day 7. After EB was adhered 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  $\pm$  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  $\mu$ M) in EBs adhesion at day 4, EBs were further cultured for 4–6 days. Nkx2.5GFP cells were  $1.03 \pm 0.17$ ,  $0.82 \pm 0.12$ , and  $0.46 \pm 0.04$ , respectively, in 9–10-day-old EBs treated with 1, 5, and 10  $\mu$ M when compared with control (Fig. 2A). In addition, MHC-positive cells from EBs at day 9 were significantly decreased by treatment with 10  $\mu$ M AG1478 (Fig. 2B). The level of cardiac genes (Nkx2.5,  $\beta$ -MHC, MLC2a, cTnI) in EBs treated with or without 10  $\mu$ M 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- $\beta$ 1 promotes cardiogenesis

Previously we reported HRG- $\beta$ 1 promoted the growth of cardiomyocytes and suppressed the death of cardiomyocytes [3]. This study was examined the intracellular signal mechanisms by which HRG- $\beta$ 1 stimulates cardiogenesis during EBs adhesion. 4-day-old EBs were attached to gelatin-coated plated in the presence of 2% FBS, treated with HRG- $\beta$ 1 (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- $\beta$ 1. Nkx2.5GFP cells from EBs treated with HRG- $\beta$ 1 were analyzed by flow. Consistent with our previous findings, HRG- $\beta$ 1 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- $\beta$ 1 (Fig. 3A). We further examined HRG- $\beta$ 1-mediated intracellular signaling on EBs adhesion. HRG- $\beta$ 1 induced ERK phosphorylation dose-dependently, which is inhibited upon treatment with PD98059 (Fig. 3B). The phosphorylation of MEK in HRG- $\beta$ 1 (100 ng/ml)-treated EBs was decreased by treatment with AG1478 (10  $\mu$ M) (Fig. 3B). Moreover, the pretreatment of PD98059 or AG1478 suppressed the increase of Nkx2.5GFP cells by HRG- $\beta$ 1 (Fig. 3C). These results suggested MEK/ERK

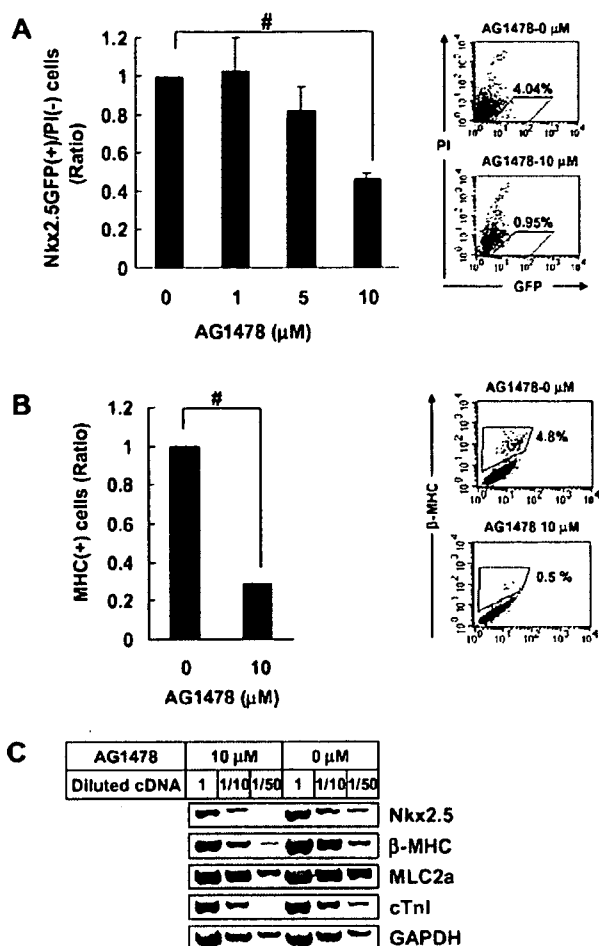


Fig. 2. Inhibition of ErbB receptor-mediated signaling pathway suppressed spontaneous development of cardiomyocytes in embryoid body (EB) adhesion. EB adhesion was differentiated for and additional 4–5 days in medium with 10% FBS after treatment of EB adhesion with ErbB receptor inhibitor (AG1478-1, 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 ( $2.2 \pm 0.34$ ), but not MEK-2A ( $1.30 \pm 0.17$ ), 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,



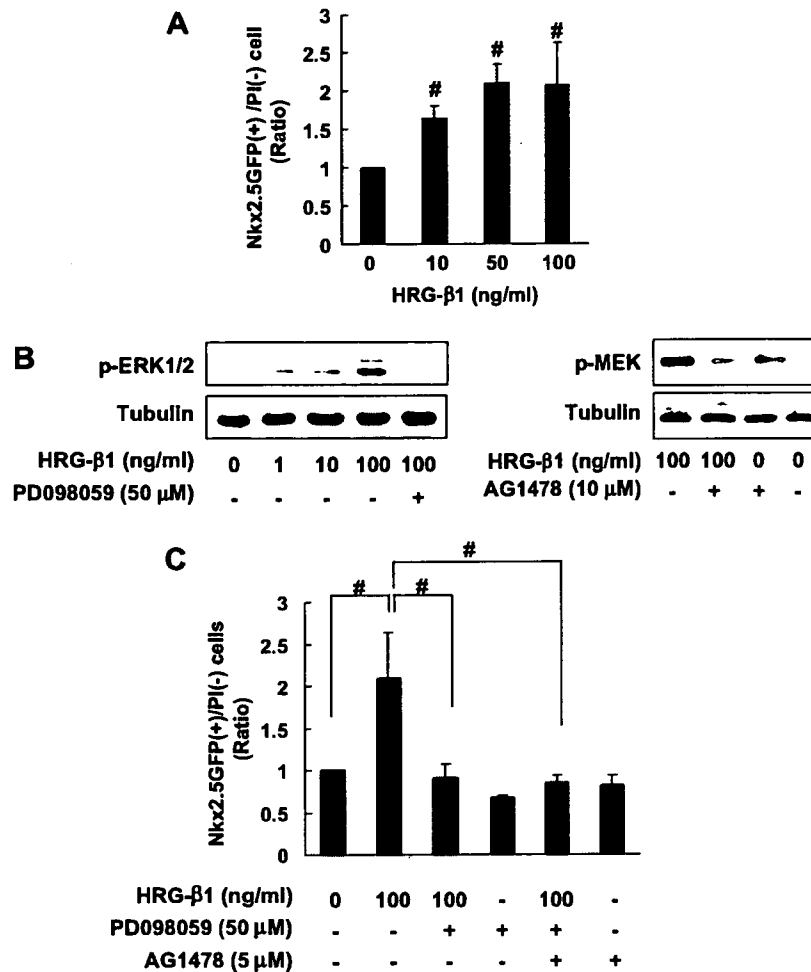


Fig. 3. Treatment with HRG-β1 activated MEK/ERK and promoted development of cardiomyocytes. (A) After treatment of 4-day-old embryoid body (EB) adhesion with HRG-β1 at indicated concentration (10, 50, and 100 ng/ml), EB adhesion was cultured in medium with 2% FBS for additional 5 days and then Nkx2.5GFP positive/propidium iodide (PI) negative cells were analyzed by flow cytometry. All values are mean  $\pm$  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  $\pm$  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-

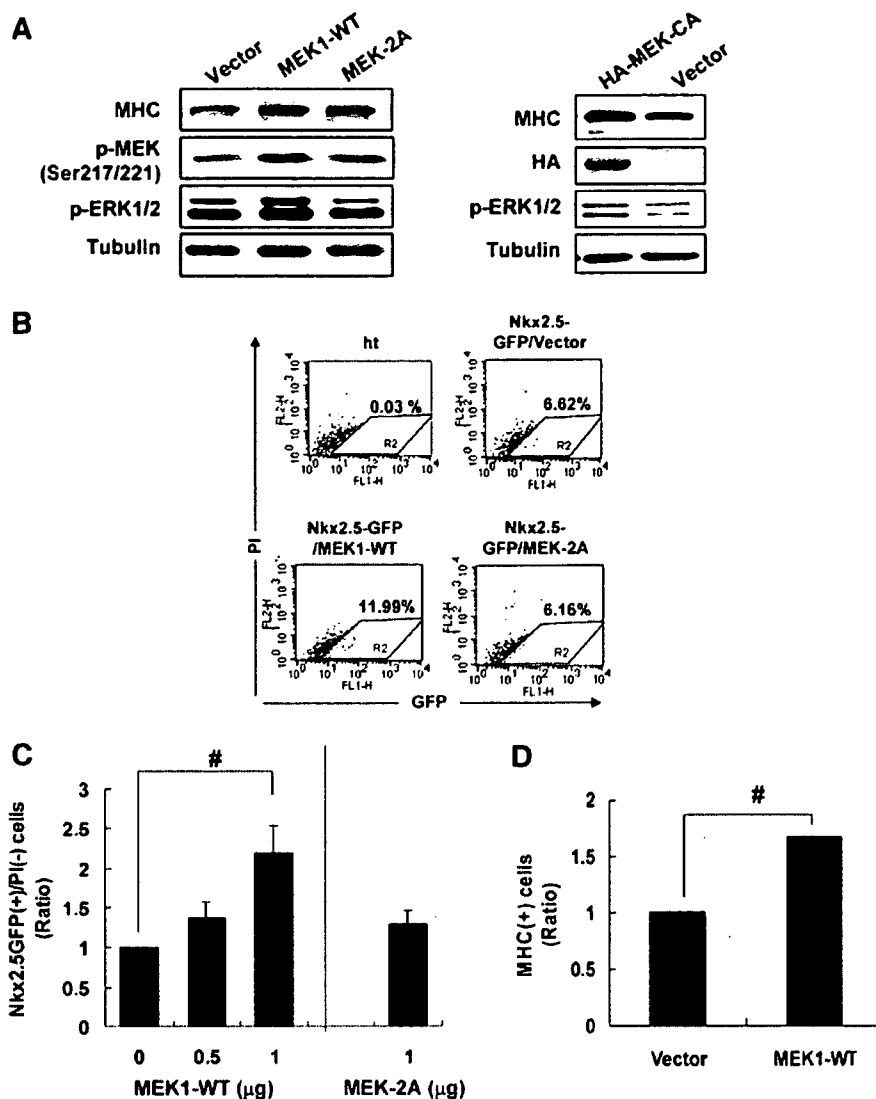


Fig. 4. MEK1 overexpression had effect on augmentation of ERK phosphorylation and promoted development of cardiomyocyte. Plasmid vectors to carry wild type MEK (MEK1-WT), dominant negative MEK (MEK2A), constitutive active MEK (HA-MEK-CA) were used. After transient transfection in embryoid body (EB) adhesion 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  $\pm$  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- $\beta$ 1 signaling to promote cardiogenesis in ES cells.

In conclusion, our results provide valuable insight on the mode of action of HRG- $\beta$ 1 in ES cell-derived cardiomyocytes. The present exploration is particularly helpful in unraveling the regulation of molecular pathways that are engaged in HRG- $\beta$ 1-promoted cardiogenesis of ES cells.

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## ES細胞の臨床応用：心血管系の再生医療実現に向けて

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### はじめに

ES細胞は、無限増殖能と様々な細胞に分化し得る多分化能を備えている細胞であり、動物では1個の細胞が個体の全構成細胞を構築することができる。1980年にマウスでES細胞が樹立され、その細胞特徴から、発生分化のモデルとして、また、遺伝子操作を施すことにより遺伝子改変動物作製の材料として研究に活用されてきた。さらに、1998年にヒトES細胞が樹立されて以来、傷害を受けた組織の機能を補うための再生医療の細胞ソースとしての期待がES細胞へ高まって研究が推進され、ヒトES細胞を用いた研究成果も次第に蓄積しつつある。しかしながら、他方、倫理的な問題や免疫拒絶の問題など、ES細胞の臨床応用に向けて乗り越えなければならぬ問題はまだまだ多い。

私たちは、ES細胞の多分化能に着目し、生後はほとんど細胞分裂せず、いったん傷害を受けると再生しない心筋について、その再

生に向けた方策を獲得することを最終目的に、これまで、ES細胞から心筋細胞を効率的に分化誘導し、それを単離するシステムの開発を行ってきた<sup>1)</sup>。その過程において、遺伝子の網羅的発現解析により、心筋細胞の分化とともに発現の増加する遺伝子群を同定することに成功した<sup>2)</sup>。これらの遺伝子群には、心筋細胞の分化形質を保つ上で必須な転写因子遺伝子のほか、細胞表面マーカーとなり得る候補遺伝子も含まれていた。現在、これらの因子・マーカーの利用により、さらに効率的な分化誘導法、単離法の確立が可能となることを期待して、大量調製に向けての準備を進めている。

本稿では、これまでに私たちがES細胞の心筋分化に関する研究の過程で明らかになった知見と問題点を近年の研究の進展とともに論ずる。

### I. ES細胞の臨床応用に向けての問題点

はじめに述べたように、1998年にヒトES細胞が樹立されて以来、ES細胞の細胞移植ソースとしての期待が高まっている。しかし、臨床応用実現に向けての道りはまだ遠いと言わざるを得ない。ES細胞は、受精卵が細胞分裂を経て生ずる「胚盤胞」の「内部細胞塊」に由来する細胞で、生体内から外に取り出され試験管内で樹立された培養細胞である。ES細胞は、核移植によって得られた「クローン胚」からも樹立することが原理的に可能であり、クローン胚由来ES細胞の樹立が実用化されれば、患者本人のDNAを持

#### Key word

ES cells  
Cardiomyocyte  
cell differentiation  
development

Application of ES cell-derived cardiomyocytes for the future cardiovascular regeneration therapy  
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