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Biochemical and Biophysical Research Communications 355 (2007) 47–53
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Efficient capture of cardiogenesis-associated genes expressed in ES cells

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Received 27 December 2006
Available online 30 January 2007

Abstract

Cardiogenesis can be induced *in vitro* in ES cells, though it is difficult to distinguish cardiac-specific genes, since embryoid bodies simultaneously differentiate into multiple lineages. In the present study, transient serum removal during culture greatly enhanced cardiogenesis, and reduced generation of endothelial and hematopoietic cells. Using DNA microarray analysis of 24 differentiated sample cultures including cardiogenesis-enhanced cells, we successfully selected genes up-regulated in embryoid bodies that had undergone cardiogenic differentiation. Besides contractile protein genes, cardiac transcriptional regulatory genes, such as Nkx2-5, Gata4/5, Mef2c, and Myocd, were primary constituents of the first 100 genes chosen as cardiogenesis-associated genes. Further, whole mount *in situ* hybridization analysis of 13 genes containing non-characterized ones confirmed that most of them were specifically expressed in the heart region of mouse embryos from E9.5–10.5. Based on our results, we consider that the present profiling method may be useful to identify novel genes important for cardiac development.

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

ES cells are differentiated into the three embryonic germ layers through the formation of three-dimensional structures called embryoid bodies (EBs) [1]. Cardiogenesis is easily detected by spontaneous contraction of EBs and has been suggested to mimic that which occurs in embryos. Among the different types of cardiac lineage cells, atrial, ventricular, and conduction system cell types differentiate as EBs, which has been demonstrated by gene expression pattern, immunological, electrophysiological, and pharmacological analyses [2]. Although ES cells are considered as a promising source of seed cells for tissue engineering, and many factors and molecules have been reported to enhance cardiogenesis *in vitro*, it is still difficult to completely control the direction of differentiation, partially because of the clonal differences in differentiation potential of different ES cell lines. Thus, it is important to elucidate the mechanism by which cardiomyocytes are generated during embryogenesis.

Cardiac development is controlled by an evolutionarily conserved network of transcription factors, including *Nkx2-5*, *Gata4/5/6*, *Mef2c*, and *Hand2*, which connects signaling pathways with genes for muscle growth, patterning, and contractility [3,4]. Mutations in components of the cardiac gene network cause congenital heart disease, the most common human birth defect. Among them, the homeodomain-containing transcription factor *Nkx2-5*, a vertebrate homolog of the Drosophila homeobox gene *tinman*, is one of the earliest markers of the mesoderm. Expression of *Nkx2-5* is first detected in cardiogenic mesoderm tissue termed the cardiac crescent and then maintained in cardiomyocytes throughout heart development [5,6]. In a

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doi:10.1016/j.bbrc.2007.01.109

previous study, knock-in of EGFP in one of the *Nkx2-5* loci successfully identified and isolated cardiomyocytes derived from ES cells [7].

Recent high-throughput analyses using DNA microarray methods have uncovered the profiles of genes expressed during EB differentiation, demonstrating that mesoderm induction precedes cardiogenesis. However, it has been difficult to distinguish cardiomyocyte-specific genes from others in these kinds of studies [8], since multiple cell lineages differentiate simultaneously following mesoderm induction. These types of cells include other mesoderm derivatives, such as hematopoietic and endothelial cells, as well as endoderm derivatives. Notably, these cells are generated in a spatially related manner in embryos [9]. On the other hand, use of purified cells that are committed to cardiomyocytes for gene expression profiling may make it difficult to identify genes transiently expressed during the process of differentiation.

In the present study, we found that transient serum removal greatly enhanced cardiogenesis in EBs, which resulted in EB populations composed of up to 30% cardiomyocytes, instead of decreased populations of hematopoietic and endothelial cells. Those findings prompted us to also analyze cardiomyocyte-rich EBs using microarray analysis. In addition to use of the cardiomyocyte-rich EBs, the multiple culture conditions utilized enabled us to efficiently select genes expressed in the early stages of cardiogenesis, including most of the well-characterized cardiac-specific transcription factor genes that are relevant for heart development. We also identified novel genes that are specifically expressed in the embryonic heart. The resultant microarray database should enable identification of genes potentially important for cardiomyocytes as well as cardiac function.

Materials and methods

ES cell culture and differentiation. The mouse ES cell line ht7 and its derivative hcgp7 (Nkx2-5 EGFP knock-in ES cells) were cultured without feeder cells, and then differentiated as described previously [7,10]. For serum removal, floating EBs were collected once into a 50-ml test tube and transferred to a bacterial Petri dish filled with serum-free medium on day 4 (d4). To determine the time window of effect of serum removal, serum was removed on d3. To determine beating EBs, floating EBs were transferred onto gelatin-coated dishes on d6 and cultured further for 4 days to observe spontaneous contraction.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from ES cell-derived cells using Trizol reagent (Invitrogen, Carlsbad, CA) and then reverse-transcribed to cDNA with SuperScript III (Invitrogen). PCR was performed with a QuantiTect SYBR Green PCR kit (Qiagen, Chatsworth, CA) using a real-time thermal cycler (7700 Sequence Detector Systems, Applied Biosystems, Foster City, CA). To ensure the fidelity of the mRNA extraction and reverse transcription processes, all samples were subjected to PCR amplification using Rodent GAPDH Control Reagent (Applied Biosystems), with the data normalized with respect to those values. The primers used, described in detail previously [7].

DNA microarray analysis. To obtain samples for DNA microarray analyses, 3 independent differentiation experiments were performed under 3 different differentiation conditions. (1) Normal condition: floating EBs were cultured in the presence of 10% fetal calf serum. (2) Cardiogenesis-enhanced condition: serum was removed on d4 and total RNA extracted on d7. (3) Cardiogenesis-inhibited condition: floating EBs were treated with 10^{-7} M retinoic acid from d3 to d7. More than 5 μg of total RNA was isolated from each EB sample using an RNeasy kit (QIAGEN). As a negative control, a fibroblast cell line established from an embryonic heart was also included. Each fluorescent labeled cDNA pool was hybridized on an Affymetrix Mouse Expression Chip (Mouse Expression Set 430) and acquired data regarding gene expression were visualized using eXintegrator software (http://www.cdb.riken.go.jp/scb/documentation/). Similarities to the specified profiles were calculated as the mean of the Euclidean distance to the set of probe pair profiles for each probe set.

Whole-mount in situ hybridization. Complementary DNA fragments of cardiogenesis-associated genes (see Supplemental Table 1) were generated by PCR amplification using total cDNA from d8 EBs as the template, with specific primers (primer sequences are available upon request). Hybridization was performed using mouse embryos under standard conditions with an automated *in situ* hybridization system (Genemaster ISH-W, Aloka Co., Ltd., Tokyo) [11]. No signals were observed with the sense probes (data not shown).

Immunohistochemistry and determination of apoptotic cells. The EBs were cryo-sectioned at 8 μm, then cardiomyocytes and apoptotic cells were detected using fluorescence microscopy. The sections were fixed in 1% paraformaldehyde, followed by cell permeabilization with 0.1% Triton X-100 and 0.1% sodium citrate (pH 7.4). After washing in PBS, the sections were incubated with a TUNEL reaction mixture (In Situ Cell Death Detection Kit TMR red, Roche Diagnostics, Basel, Switzerland) and the anti-sarcomeric myosin antibody MF20 (Developmental Studies Hybridoma Bank). MF20 was visualized with anti-mouse IgG-Alexa488 (Invitrogen). Thereafter, the cells were observed under a BX51 microscope (Olympus Corporation, Tokyo) and photographed using a DP70 digital camera (Olympus Corporation).

Flow cytometry and cell sorting. For determination of endothelial/hematopoietic/myocardial cells in the EBs, Nkx2-5/EGFP ES cell-derived EBs were dissociated into single cells using trypsin/EDTA. The cells were incubated with the antibody against phycoerythrin (PE)-conjugated CD31 or CD45 (eBioscience, San Diego, CA). Stained cells were analyzed using a FACS Calibur (BD Biosciences, San Jose, CA) with CellQuest software (BD Biosciences). For determination of myosin heavy chain-expressing cells by flow cytometry, dissociated cells were fixed and permeabilized using FIX & PERM Cell Permeabilization Reagent (Caltag Laboratories, Burlingame, CA). The cells were then washed and incubated in staining buffer containing MF20, following incubation with anti-mouse IgG-Alexa488.

Results

Effects of serum removal on differentiation of embryoid bodies

Using an Nkx2-5/EGFP ES cell line, we recently reported that Wnt11 can facilitate cardiac differentiation of ES cells [10]. In the course of our studies, we discovered that transient serum removal increased the population of Nkx2-5/EGFP cells in EBs (Fig. 1A and B). To confirm that finding, we monitored cardiac differentiation by counting the number of spontaneously beating EBs. When serum was removed from d4, most of the EBs started to beat by d7, earlier than control EBs (Fig. 1C). Consistent with those results, transcripts of the cardiac transcription factor genes *Gata4*, *Mef2C*, *Nkx2-5*, as well as the structural protein genes, *Myl2* and *Myl7*, were enhanced after serum-free treatment (Fig. 1D). That enhancement is likely to be stage-specific, as no beating EBs were observed and the levels of cardiac marker genes were decreased when serum was

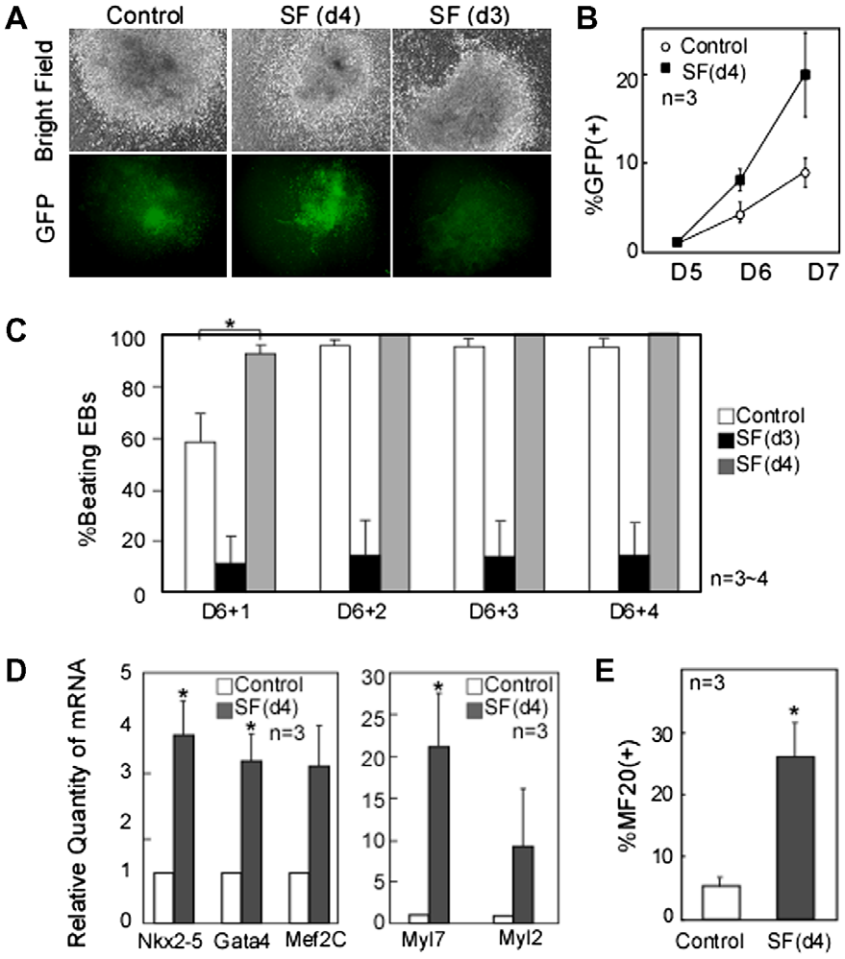

Fig. 1. Transient serum removal enhances cardiac differentiation of ES cells. (A) GFP fluorescence of Nkx2-5/EGFP ES cell-derived EBs differentiated after transient serum removal. Serum was removed at d4 or at d3. EBs were attached on gelatin-coated plates at d6 and further cultured for 2 days. (B) Quantification of Nkx2-5/EGFP-positive cell in EBs. Trypsin-dissociated cells were analyzed by flow cytometry. (C) The number of spontaneously beating EBs after transient serum removal. EBs were transferred to a 24-well gelatin-coated dish on d6 and the numbers of beating EBs were counted daily (mean ± SE, $*P < 0.05$). (D) Quantification of mRNA level of cardiac transcription factor genes total RNA was extracted from EBs and real time RT-PCR analysis was performed (mean ± SE, $*P < 0.05$). (E) Quantification of MyHC-positive cells in EBs. Trypsin-dissociated cells were fixed, permeabilized, and stained with MF20 and anti-mouse IgG conjugated with Alexa488. MF20-positive cells were analyzed by flow cytometry (mean ± SE, $*P < 0.05$).

removed from d3. On the other hand, *Nes*, a marker of neural differentiation, was up-regulated in that condition (data not shown), consistent with the observation that ES cells differentiated into neuroectoderm tissue in the absence of serum [12]. To further quantify the number of cardiomyocytes that had differentiated from EBs, we analyzed EBs by flow cytometry. The population of sarcomeric myosin heavy chain-positive ($MF20^+$)cells in serum-free treated EBs was considerably higher than that in control EBs (Fig. 1E). Immunohistochemical analyses of frozen sections revealed that the $MF20^+$ area was much larger in serum-free treated EBs as compared to control EBs (Fig. 2A).

EBs usually develop apoptotic cells under a normal serum-containing culture condition, which may contribute to cavity formation [13]. On the other hand, serum removal is generally believed to cause cell death in cultured cells. In the present experiments, serum removal did not increase the number of apoptotic cells, shown as TUNEL positive cells (Fig. 2A). Overall, the number of cells incubated without serum was about half that in the control EB cultures (Fig. 2B), while the number of cells with hematopoietic ($CD45^+$) or endothelial cell ($CD31^+$) lineage was much lower with the serum-free condition (Fig. 2C), and we could not detect dying cells or apoptotic cells of $CD45^+$ or $CD31^+$ lineage (data not shown). Thus, the fate of EBs appeared to change from a hematopoietic/endothelial lineage to a cardiac lineage, perhaps through selective differentiation of the cells.

Microarray analysis of genes expressed in embryoid bodies

Using serum-free treated cardiomyocyte-rich EBs, we profiled the genes expressed during cardiac differentiation of EBs with DNA microarray analysis. EBs became differentiated using a standard protocol with 10% serum, a cardio-

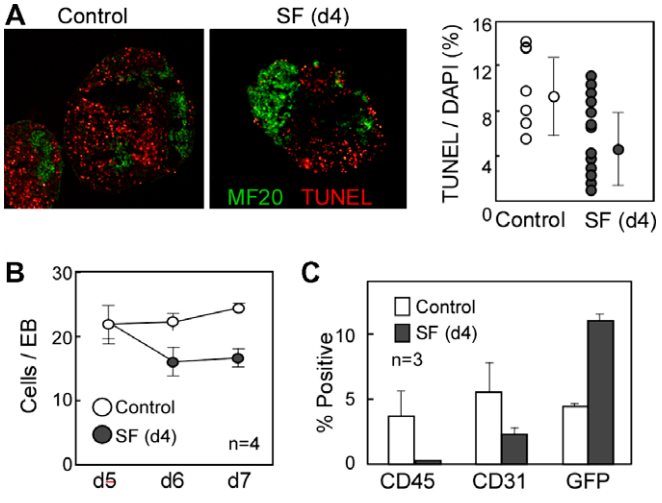

Fig. 2. Transient serum removal changes the fate of EBs by reducing generation of endothelial/hematopoietic cells. (A) Apoptosis of EBs after culturing in serum-free medium. Frozen sections of EBs were analyzed for TUNEL-positive cells, and the frequency of TUNEL-positive cells was calculated. (B) Number of cells in EBs after culturing in serum-free medium. (C) Endothelial/hematopoietic cell lineage analysis of EBs. Trypsin-dissociated Nkx2-5/EGFP ES cell-derived EBs on d7 were stained with PE-conjugated CD45 or CD31. Quantification of CD45, CD31, and GFP positive cells was performed by flow cytometry.

genesis-enhancing protocol with transient serum removal, and a cardiogenesis-inhibiting protocol with retinoic acid [7,14]. Using 24 of the EB samples (18 time course samples from d3 to d8, and 3 each of cardiogenesis enhanced and repressed samples from d7), along with a fibroblast sample as a negative control, total RNA was extracted, cDNA was labeled and hybridized on a DNA chip, and eXintegrator software was utilized to visualize the expression patterns (Fig. 3A) [15]. Cardiac-specific genes exhibited a similar pattern, as shown in Fig. 3, including the transcription factors *Nkx2-5*, *Myocd*, *Smyd1*, *Gata4*, *Gata5*, *Hand2*, and *Smarcd3*, as well as contractile protein genes such as *Myl4*, *Myl7*, *Tnnc1*, *Tnni1*, and *Tpm1*. In contrast, endothelial/hematopoietic genes (*Tal1*, *Kdr*, and *Hbb-bh1*) and a smooth muscle gene (*Tagln*) exhibited distinct patterns, with a decreased expression in the serum-free condition. Endoderm-related genes (*Sox17*, *Hhex*, *Krt1-19*, and *Afp*) were also expressed, but not in the serum-free condition. Nascent mesoderm and mesoendoderm-related genes (*Mixl1*, *T*) were induced transiently on d3 and d4, then declined before cardiac differentiation began. The derivatives of neuroectoderm-related genes (*NeuroD3*, *Pax6*) were not differentiated efficiently in our default condition with serum, unless retinoic acid was added to the EBs. Thus, our profiling data were demonstrated to distinguish cardiac cell lineages from others in differentiating EBs.

Expression of cardiogenesis-associated genes in mouse embryos

The eXintegrator software package allows the user to compare different samples in the database on the basis of the given probe sets. Using that software, we chose 130 probe sets with the greatest similarity to *Actc1*. After eliminating probe sets without a single Gene ID and those with the suffix "s_at" or "x_at", 100 genes were remaining (Supplemental Table 1). This extraction method may not cover all of the cardiogenesis-associated genes, since we were unable to set a threshold. However, the selected genes included many transcriptional regulatory factors that were related to cardiac morphogenesis and differentiation (*Nkx2-5*, *Mef2c*, *Myocd*, *Gata4*, *Gata5*, *Hand2*, *Smyd1*, *Foxc1*, *Lbh*, *Smarcd*, *Csrp2*, *Csrp3*, and *Hdac5*), suggesting that we were able to successfully select genes expressed in the early stages of cardiogenesis with this method. Next, we selected 13 genes containing non-characterized ones and analyzed them for their expression in embryos using whole-mount *in situ* hybridization. Remarkably, nearly all of those genes including *Rcsd1* were found to be preferentially expressed in the heart region of E9.5–E10.5 mouse embryos (Fig. 4 and Supplemental Fig. 1). Further, *Ppp1r14c*, *Fbxo32*, *and Klhdc8b* were expressed in the heart in a regional manner, suggesting their region-specific functions. Cardiac-specific expression of these genes was also confirmed with quantitative RT-PCR analysis of the heart and other regions dissected from E8.5 and E9.5 embryos (data not shown).

Discussion

An EB system is useful to study cardiac differentiation during the early stages of heart development, which is considered to be a difficult period for defining progenitor cells in embryos. However, EBs simultaneously generate multiple cell lineages in addition to cardiomyocytes. Under the differentiation conditions used in the present study, mesodermal cells (cardiomyocytes, endothelial cells, and hematopoietic cells) and endodermal cells were preferentially differentiated as compared to neural cells. On the other hand, neuroectodermal cells were preferentially induced by retinoic acid treatment. While our default condition caused nearly 100% of the EBs to start beating by d8 or d9, we found that transient serum removal further enhanced cardiogenesis, partially by reducing the populations of other cell lineages such as endothelial/hematopoietic cells. Similar phenomena have been observed in other mouse ES cell lines as well as in human ES cells [16,17]. Thus, transient serum removal is thought to trigger a conserved pathway to induce cardiogenesis in EBs. Since it has been reported that ES cells can be committed toward a cardiac lineage by TGFβ superfamily [18–20], it is important to determine how much level the serum contains TGFβ, its related factors, and inhibitory factors.

We used cardiomyocyte-rich EBs to identify cardiogenesis-associated genes, though the mechanism by which serum removal treatment enhanced cardiogenesis remains to be elucidated. By combining different culture conditions and time courses, we classified the genes expressed

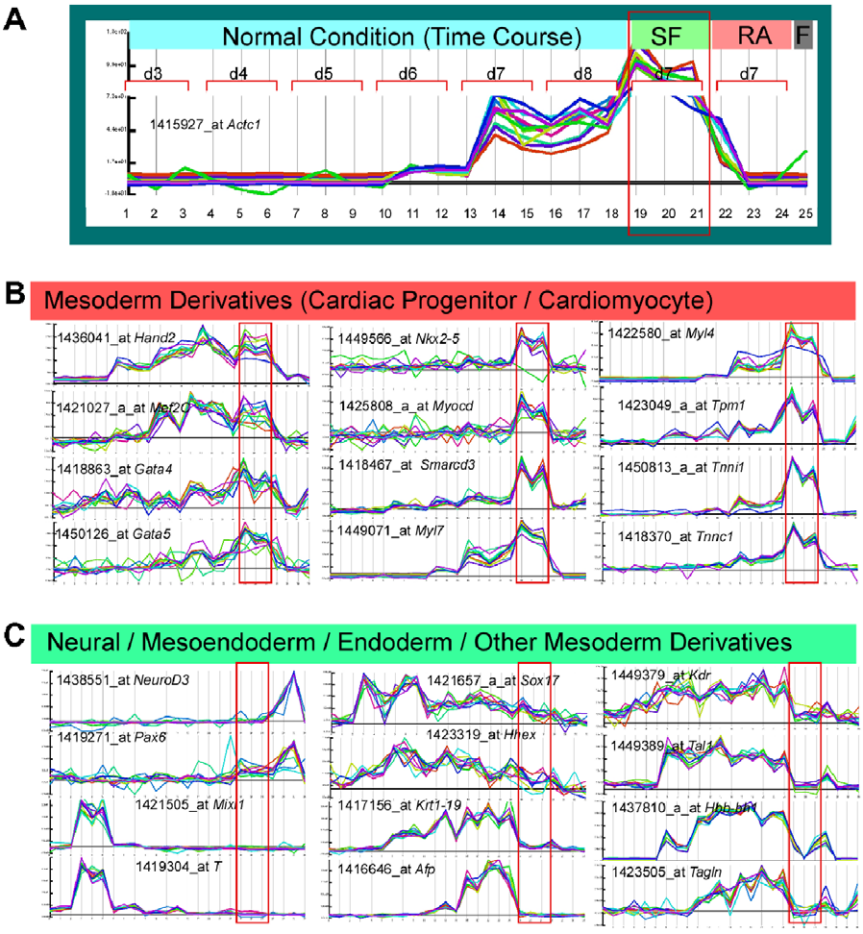

Fig. 3. Microarray analysis distinguishes cardiogenesis-associated genes from others in EBs. eXintegrator analysis of microarray data. (A) Representative eXtingrator analysis data of *Nkx2-5* are shown. Embryoid bodies were cultured in a normal condition with 10% fetal calf serum, a cardiogenesis-enhanced condition treated transiently with serum-free (SF) medium, or a cardiogenesis-inhibited condition treated with retinoic acid (RA). In the 3 independent differentiation experiments, EBs cultured in the normal condition were sampled on d3 (lanes 1–3), d4 (lanes 4–6), d5 (lanes 7–9), d6 (lanes 10–12), d7 (lanes 13–15), and d8 (lanes 16–18), while those cultured in the cardiogenesis enhanced condition (lanes 19–21) and in the cardiogenesis-inhibited condition (lanes 22–24) were sampled on d7. As a negative control, a fibroblast cell line (F) derived from an embryonic heart was also included (lane 25). (B) Cardiogenesis-associated genes. (C) Neuroectoderm, mesoendoderm, endoderm, and other mesoderm derivatives.

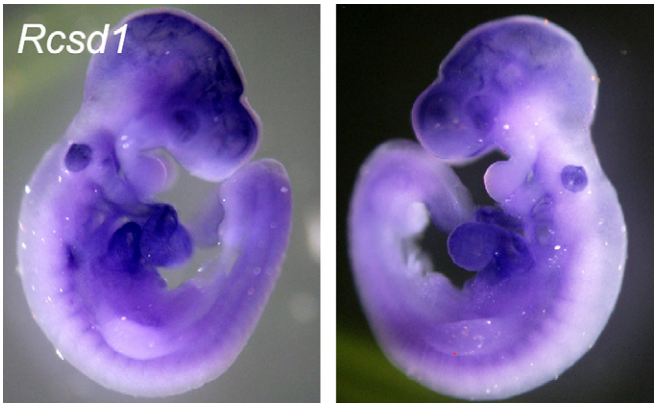

Fig. 4. Expression patterns of cardiogenesis-associated genes in mouse embryos. Whole-mount *in situ* hybridization revealed that the Rcsd1, one of the genes that exhibited an expression pattern similar to *Actc1*, was preferentially expressed in the mouse embryonic (E9.5) heart region. Expression in the heart region (heart and others) was also confirmed by real time RT-PCR of dissected embryos (data not shown).

in EBs. As reported previously, genes expressed in the primitive streak, such as *T* and *Mixl1*, were transiently expressed in EBs at 3 or 4 days after the induction of differentiation, followed by the expression of definitive endoderm and mesoderm derivatives [21]. While those genes were simultaneously induced, we found that only cardiomyogenesis-related genes were enhanced by transient serum removal. From the top 100 genes that showed a similar expression pattern to *Actc1*, 13 uncharacterized genes were found to be expressed specifically in the heart region. Thus, the present profiling method is considered useful to preferentially identify novel genes expressed in cardiomyocytes.

Among the top 100 cardiogenesis-associated genes, many known cardiac transcriptional regulators have been found, including *Nkx2-5*, *Mef2c*, *Myocd*, *Gata4*, *Gata5*, *Hand2*, *Smyd1*, *Foxc1*, *Lbh*, *Smarcd*, *Csrp2*, *Csrp3*, and *Hdac5* [3,22–26]. Although similar results have been

reported, the methods used may not have recaptured genes expressed at the early stages of cardiac differentiation, since only a few cardiac transcription factor genes were extracted [27], which may have been due to the usage of different differentiation time points. Those 100 genes also include the signaling molecule genes *Wnt2, Mdk*, and *Pgf*. Interestingly, *Mdk* and *Pgf* may have a protective role against cardiac ischemia [28], and might be potentially important for cardiac development by functioning in an autocrine or a paracrine manner, though additional studies will be required. In addition, the 13 non-characterized genes found to be expressed in the embryonic heart in the present study include potential signal transduction factors, such as *Asb2*, *Diras2*, *Ppp1r14c*, *Sorbs2*, and *Fbxo32*. Since signaling pathways involved in cardiogenesis have not been well elucidated, it is important to analyze these genes *in vivo* as well as *in vitro* in future studies.

Acknowledgments

We express our thanks to Dr. Hitoshi Niwa for providing the ht7 ES cells, and Ms. Sachiko Suzuki and Ms. Shoko Miyamoto for assistance with the ES cell cultures. This work was supported in part by a grant from Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR) of Japan, by Research Grants for Cardiovascular Diseases from the Ministry of Health, Labour and Welfare, Japan, and by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.01.109.

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