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研究代表者　日高　京子

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ヒトES細胞由来心筋細胞の表面マーカー探索および大量培養・純化システムの構築

研究代表者　日高京子　国立循環器病センター研究所　室長

研究要旨

高い増殖性と多能性を有する胚性幹細胞（ES 細胞）および人工多能性幹細胞（iPS 細胞）は、分化メカニズムの解明といった基礎研究の材料としてのみならず、薬効・毒性試験や細胞移植のソースとしての役割が期待されている。心臓の主たる構成細胞である心筋細胞は増殖能力の極めて低い細胞であり、ES 細胞や iPS 細胞から分化誘導させた心筋細胞の利用が大いに期待されている。心筋細胞にはこれまで特異的な細胞表面マーカーがほとんど知られておらず、単離を可能にするには遺伝子操作によるマーカーの導入が必要であった。そこでわれわれは、マウス ES 細胞の心筋分化誘導系の網羅的発現遺伝子解析より表面マーカーの候補としてプリオンタンパク質（PrP）を見出した。PrP は胚様体由来の心筋細胞およびマウス胚発生過程での心筋細胞に特異的に発現しているのみならず、心筋に分化する心筋前駆細胞にも発現していた。一方で、胚様体の大量作成法の開発にも取り組み、自動拍動する胚様体より磁気ビーズを用いて短時間かつ大量に PrP 陽性細胞を単離するシステムを構築した。単離後の PrP 陽性細胞は培養によって心筋トロポニンＩを発現する成熟した心筋細胞へと効率良く（＞９０％）分化し、心室筋あるいは心房筋に特徴的な電気生理学的性質を示した。これらの細胞は生体内への移植が可能で、移植後一ヶ月にわたって生存し腫瘍形成は観察されなかった。ヒト由来心筋細胞については自動拍動する iPS 細胞由来胚様体を用いてその発現確認を行った。以上のことから、PrP が特異的細胞表面マーカーとして多能性幹細胞由来心筋細胞の大量調製に有用であることがわかった。

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Ａ．研究目的

心臓は再生能力の低い臓器であり、不全に陥った心臓を救うことは容易ではない。心臓移植にはドナー不足による限界があり、新しい治療法の開発が待たれている一方、有効な薬剤の開発には薬効・毒性試験が必要であるが、用いることのできる細胞には限りがある。このような現状

において、多能性幹細胞（ES 細胞・iPS 細胞）をその細胞ソースとして利用することは大いに期待されていることであるが、心筋細胞には特異的な表面マーカーが知られておらず、効率的かつ大量に単離することが難しいため、実用化への足かせとなっている。ES 細胞・iPS 細胞の利用に向けては（１）目的細胞への選択的分化誘導法、（２）目的細胞の選別・純化法、（３）大量化へ向けたシステムの開発が基本となる。本研究においては胚様体網羅的発現遺伝子解析を出発点とし、（１）効率的分化誘導を行うための心筋特異的遺伝子の探索と分化メカニズムの理解、（２）心筋細胞特異的細胞表面マーカーの探索、（３）胚様体作成・培養・分離法の開発と改良を行った。

Ｂ．研究方法

Ｉ．ES 細胞の培養と分化誘導。マウス ES 細胞は胚様体形成を経て分化誘導させた。胚様体形成後は大量の培地（10%血清）に胚様体を浮遊させ、さらに４－５日培養させることにより自動拍動する胚様体を得た。心筋分化を促進する条件では４日目より血清を除去した培地に交換し培養を行った。胚様体を大量に作成するには、従来のハンギングドロップ法に加えて、懸濁培養法、自動分注機を用いた 384 ウェル法や専用デバイスを用いた EB チップ法（STEM バイオメソッド社製）などを検討した。

Ⅱ．フローサイトメトリー解析およびセルソーティング。PrP 抗原はプロテアーゼ感受性であるため、Collagenase Type II とそれに続く Dissociation Buffer (Invitrogen 社) 処理によって胚様体を解離させた。APC（蛍光色素の一つ）で標識した PrP 抗体と混合し、フローサイトメトリーにより陽性細胞を測定した。細胞内も同時に解析する場合は、PrP 抗体で染色した後、細胞を固定し細胞膜に穴を空け、細胞内抗原を認識する抗体を反応させた。セルソーティングは蛍光による分離（FACSAria, BD 社製）のほか、磁気ビーズを利用した分離 (AutoMACS、ミルテニー社製）を行った。

Ⅲ．RNA 発現解析。RT-PCR は細胞より抽出した RNA より cDNA を合成し、リアルタイム PCR によって発現量を測定した。マイクロアレイ解析の場合、マウス Expression Array 430 (Affymetrix 社)を用いた解析を行った。マウス胚においては whole mount in situ hyridization による解析を行った。

Ⅳ．胚様体由来心筋細胞の解析。PrP 陽性細胞は in vitro で培養後、培養後の細胞をフローサイトメトリーにより解析した。また、パッチクランプ法および多電極培養皿を用いた**電気生理学的解析を行った。PrP 陽性細胞を生体内にて解析する場合は細胞塊を形成させて腎被膜下への移植を行った。**

Ⅴ．胚様体由来心筋前駆細胞の解析。PrP および血小板由来成長因子受容体 (PDGDRa) でソートした細胞について、細胞塊の形成による分化能の解析のほか、メチルセルロース中での培養によるコロ

ニー形成を行った。

Ⅵ. ヒト iPS 由来の胚様体。ヒト iPS 由来の胚様体（自動拍動を確認したもの）はニプロより購入したものを使用した。

C. 研究成果

Ⅰ. 胚様体の網羅的発現遺伝子の解析。我々は以前一時的な血清除去が心筋への分化を促進し、高濃度のレチノイン酸処理により心筋分化が抑制されることを見出していた。そこで、(A) 通常の条件、(B) 心筋分化促進条件、(C) 心筋分化抑制条件で胚様体を培養し、どのような遺伝子が発現しているかマイクロアレイを用いて網羅的発現解析を行った。その結果、候補マーカー遺伝子としてプリオンタンパク質（PrP）が心筋分化促進条件の胚様体に発現していることがわかった。

Ⅱ. PrP の胚様体における発現特異性。胚様体を分化誘導させ、PrP の細胞表面における発現をフローサイトメトリーにより調べたところ、分化誘導後 5 日目（d5）の胚様体に PrP 陽性細胞が出現し、自動拍動開始（d7）やミオシン重鎖（MyHC）陽性細胞出現（d6）に先んじて発現することがあきらかとなった。PrP と MyHC をフローサイトメトリーにて解析したところ、生じたばかりの MyHC 陽性細胞は PrP 陽性であることが明らかとなった。

Ⅲ. PrP のマウス胚における発現特異性。マウス胚における発現を Whole Mount 免疫染色および in situ hybridizaitio n にて調べたところ、PrP は原始心筒およびそれ以前の心臓原基（Cardiac Crescent）で発現していることが分かった。PrP でソートした細胞は心筋マーカーを発現しており、発現特異性を支持する結果となった。

Ⅳ. 大量分化誘導系・大量分離系の開発。胚様体を効率よく作成するために、従来のハンギングドロップ法に加えて、懸濁培養法、384 ウェル法、EB チップ法などを検討したところ、どれも同等の効率で心筋細胞を分化誘導できることがわかった。懸濁培養法には培地の種類により効率に違いが見られ、最適な培地を選択する必要があった。384 ウェル法は自動分注機を用いるものであるが、ウェルから胚様体を取り出す際の効率に問題が残った。EB チップは胚様体形成に特化して開発されたもので、簡便に心筋細胞が誘導できることがわかった。

Ⅴ. PrP で分離した心筋細胞の性状解析。PrP 陽性細胞を in vitro、in vivo 両面において検討した。自動拍動開始直後（d7）の胚様体より分離した PrP 陽性細胞はほとんどが MyHC 陽性であるが、in vitro の培養によってさらに分化したマーカーである心筋トロポニン I（cTnI）を 90%以上の細胞において発現するようになった。一方、PrP 陽性細胞を再凝集させ、ヌードマウスの腎被膜下に移植したところ、一ヶ月以上にわたって生存し、心筋マーカーの発現も確認することができた。

Ⅵ. PrP で分離した心筋前駆細胞の解析。自動拍動開始２日前（d5）においては PrP

マーカーで将来心筋になる分画とそうでない分画を分離することは困難であった。そこで第二のマーカーとして中胚葉系で広く発現する PDGFRa を導入した。D5 で PrP/PDGFRa 二重陽性となった細胞を再凝集させると効率よく自動拍動する細胞塊を生じ、またこれらの細胞をメチルセルロース中で培養し、単一細胞由来のコロニーを作成したところ、一つのコロニー中に心筋および平滑筋細胞が混在し、PrP/PDGFRa 二重陽性細胞が両能性を持った細胞であることが明らかとなった。このことは心筋分化メカニズムを解明する上で大きな足がかりとなった。

Ⅶ. ヒト由来細胞における発現。

ヒト iPS 細胞由来の自動拍動する胚様体（少なくとも分化後 3 週間経過）をニプロ社より購入し、RT-PCR による発現の確認を行ったところ、PrP mRNA のヒト由来細胞における発現が確認できた。ヒト ES 細胞については心筋への分化誘導条件を検討中である。

(倫理面への配慮)

ヒト ES 細胞使用については研究機関内の倫理委員会の承認が得られ、文部科学省での確認の後、使用を開始した。遺伝子組換え実験は研究機関の委員会の承認を得て行われた。動物の取扱いについても研究機関の委員会の承認を得て行われ、また、動物愛護に配慮して実験を行った。

D. 考察

Ⅰ. 網羅的発現遺伝子探索。胚様体の培養条件を少しずつ変化させることによって、心筋で特異的に発現する遺伝子を効率よく見出すことができた。心筋細胞には特異的な表面マーカーがあまり知られていない。遺伝子の名前だけで表面に発現しているかどうかを見極めるのは容易ではないが、ウシ海綿状脳症 (BSE)との関連が報告されているプリオンタンパク質 (PrP) に CD230 の名前が付けられていることに着目した。CD (Cluster of differentiation)は白血球を主とする血液系細胞の表面マーカーである。PrP の他にも候補の探索を進め、これらを組み合わせることによってより確実な分離マーカーとすることを今後の検討課題としたい。

Ⅱ. PrP の心筋細胞における発現特異性。

胚様体は種々の細胞の集合体であるため、RT-PCR やウエスタンブロットといったバルクでの解析では発現の細胞特異性について正確な情報が得られない。一方、免疫染色法では分化途上のシグナルの弱い細胞を判断することが難しい。今回われわれは、フローサイトメトリーにより細胞表面の PrP と細胞内の構造タンパク質 (MyHC など)を同時に検出した。これにより PrP が生じたての心筋細胞に発現しているという結果を得ることができた。これと並行して PrP でのソーティングにより自動拍動する心筋分画とそうでない非心筋分画を確実に分離できるということを示したが、これは PrP の心筋特異的発現をさらに支持するものである。マウス初期胚での PrP の発現はこれまで詳細には解析されてこなかった。今回の解析で PrP が心臓原基で発現していることが明らかとなり、胚様体での発現特異性を裏付ける結果となった。以上のことは PrP を心筋マーカーとして用いるた

めの、重要な知見となると考えられた。

Ⅲ. PrP を指標とした心筋細胞分離法の開発。 PrP は細胞表面にあるので、磁気ビーズを用いた細胞の単離が可能である。AutoMACS による分離は FACS に比べると得られる純度の上で劣るが、カラムにかける回数を増やすことによって純度を改善することができた。自動拍動開始直後の胚様体が解離のしやすさ、シグナルの強さといった点で最も AutoMACS での単離に適しており、カラムを選べば 10^9 もの細胞の処理が可能となる。これは大量調製にむけての確かな足がかりとなった。MACS で得た PrP 陽性細胞は最初 cTnI を発現しない未熟な心筋細胞であるが、その後の場合によって 90%以上の細胞が cTnI を発現した。さらにこれらの細胞は電気生理学的にも心筋としての性質を示し、in vivo においても生存可能であった。これらのことは大量調製可能な PrP 陽性細胞が心筋細胞ソースとして有力なものとなることを示している。今後は心不全モデルへの PrP 陽性細胞の移植等により、応用へ向けた基礎的検討を行いたい。

E．結論

胚様体の発現遺伝子群の網羅的解析により、PrP が心筋細胞とその前駆細胞に特異的に発現していること、PrP を用いて心筋細胞を胚様体より効率よく分離できること、得られた PrP 陽性細胞は発現タンパク質の上で、あるいは電気生理学的にも心筋の性質を示し、生体内で生存可能であることなどが明らかとなった。一方、胚様体調製は専用デバイスを用いた大量化が可能であり、PrP は表面マーカーであるので磁気ビーズを用いた大量処理が可能であった。以上のことから PrP は多能性細胞由来の心筋細胞を大量に調製する上で強力な武器となりうることがわかった。

F．健康危険情報

なし。

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H．知的財産権の出願・登録状況（予定を含む）

なし。

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別添４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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雑誌

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Efficient capture of cardiogenesis-associated genes expressed in ES cells

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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.
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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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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

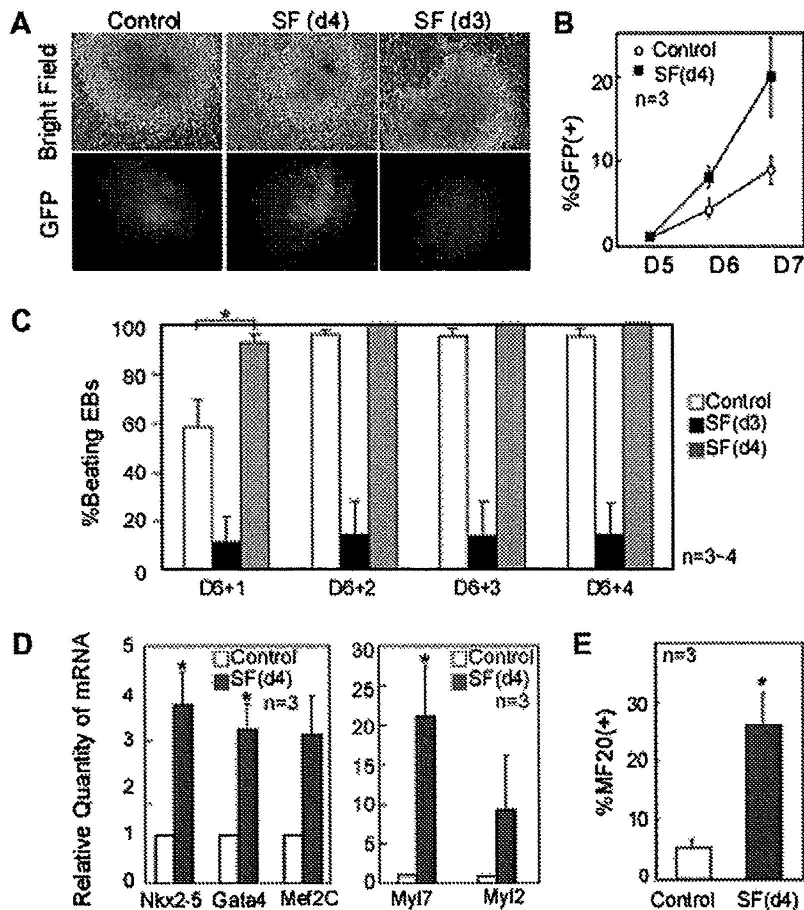

Fig. 1. Transient serum removal enhances cardiac differentiation of ES cells. (A) GFP fluorescence of Nkx2-5/EGFP ES cell-derived EBs differentiated after transient serum removal. Serum was removed at d4 or at d3. EBs were attached on gelatin-coated plates at d6 and further cultured for 2 days. (B) Quantification 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-

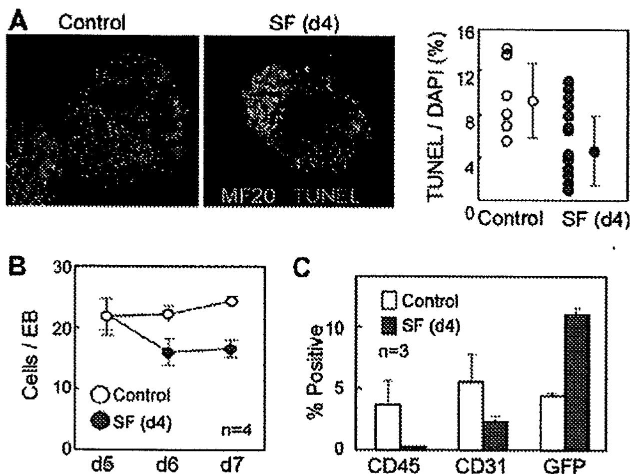

Fig. 2. Transient serum removal changes the fate of EBs by reducing generation of endothelial/hematopoietic cells. (A) Apoptosis of EBs after culturing in serum-free medium. Frozen sections of EBs were analyzed for TUNEL-positive cells, and the frequency of TUNEL-positive cells was calculated. (B) Number 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*, *Smydl*, *Gata4*, *Gata5*, *Hand2*, and *Smarcd3*, as well as contractile protein genes such as *Myl4*, *Myl7*, *Tnncl*, *Tnnil*, and *Tpml*. In contrast, endothelial/hematopoietic genes (*Tall*, *Kdr*, and *Hbb-bhl*) and a smooth muscle gene (*Tagln*) exhibited distinct patterns, with a decreased expression in the serum-free condition. Endoderm-related genes (*Sox17*, *Hhex*, *Krtl-19*, and *Afp*) were also expressed, but not in the serum-free condition. Nascent mesoderm and mesoendoderm-related genes (*Mixll*, *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 *Actcl*. 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*, *Smydl*, *Foxcl*, *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 *Rcsdl* were found to be preferentially expressed in the heart region of E9.5–E10.5 mouse embryos (Fig. 4 and Supplemental Fig. 1). Further, *Ppplrl4c*, *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

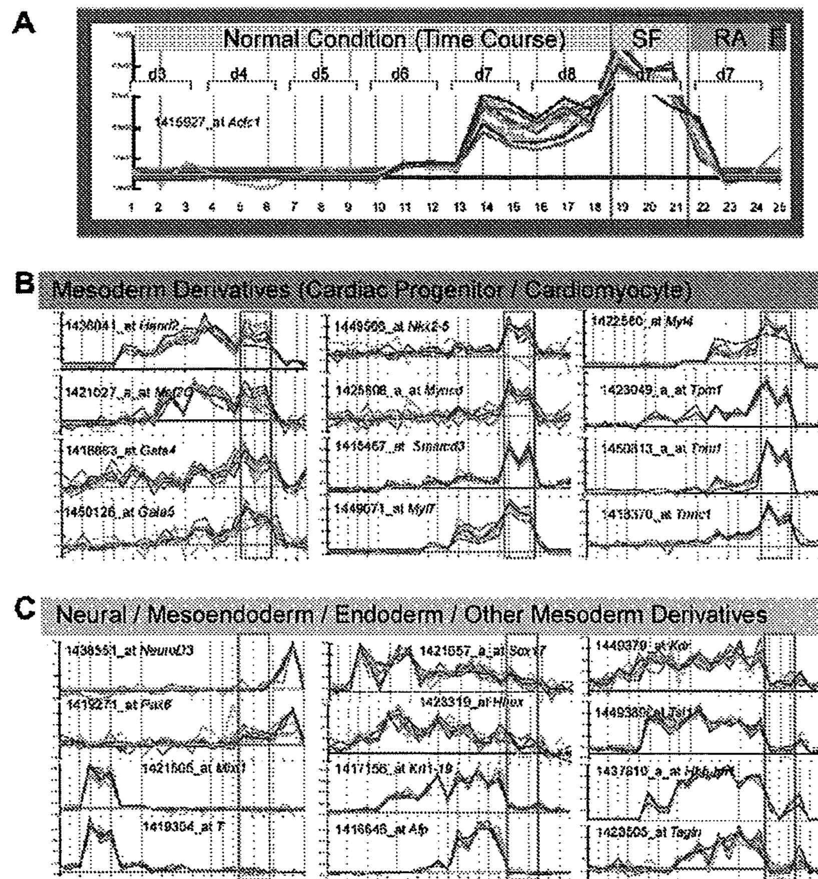

Fig. 3. Microarray analysis distinguishes cardiogenesis-associated genes from others in EBs. eXintegrator analysis of microarray data. (A) Representative eXtingrator analysis data of *Nkx2-5* are shown. Embryoid bodies were cultured in a normal condition with 10% fetal calf serum, a cardiogenesis-enhanced 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.

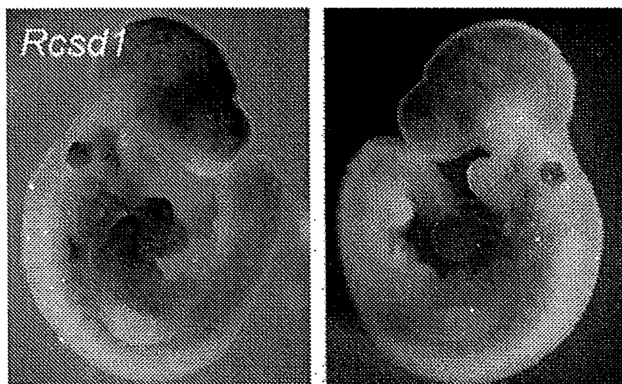

Fig. 4. Expression patterns of cardiogenesis-associated genes in mouse embryos. Whole-mount *in situ* hybridization revealed that the Rcsd1, one of the genes that exhibited an expression pattern similar to *Actcl*, was preferentially expressed in the mouse embryonic (E9.5) heart region. Expression in 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 *Actcl*, 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.

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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.2007.01.109.

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