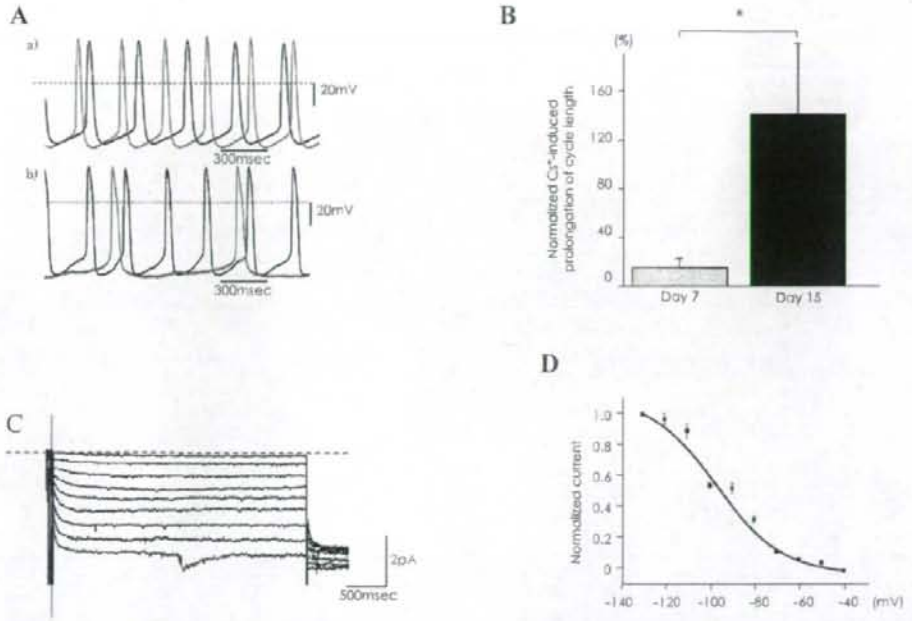

Fig. 4 Changes in both Cs^+-sensitive automaticity and I_f currents in Nkx2.5-positive cells on days 7 and 15 of their differentiation. A: Spontaneous action potentials recorded by perforated patch-clamp in the presence (red) and absence (black) of 2 mM Cs^+ in Nkx2.5/GFP(+) cells on days 7 (a) and 15 (b) of their differentiation. Dotted lines indicate the potential at 0 mV. B: Summary on the effect of 2 mM Cs^+ on the length of spontaneous action potential cycle in Nkx2.5/GFP(+) cells on days 7 and 15. Values (% increase) are the mean ± SE of 15 independent experiments. $^*P < 0.05$. C: Representative I_f currents in Nkx2.5/GFP(+) cells on day 15 after differentiation elicited every 2 s by 2000 ms step hyperpolarizing pulses from a holding potential of −40 mV to test potentials of −40 to −130 mV at 10 mV intervals. D: Voltage-dependent activation curve of I_f currents in Nkx2.5/GFP(+) cells on day 15 after differentiation. The curve was fitted using Boltzmann's equation (Eq. 1) given in the Materials and Methods section. Values are the mean ± SE of 6 independent experiments.

DISCUSSION

In the present study, we tracked changes in the sensitivity of automaticity to Cs^+, immunoreactivity of HCN proteins, and I_f channel activity in ES cell-derived Nkx2.5/GFP(+) progenitor cells during differentiation through EBs formation. Nkx2.5/GFP(+) cells showed an increment of HCN1 and 4 transcripts and proteins, Cs^+-sensitive automaticity, and I_f channel activity during their differentiation.

The present data indicated that although spontaneous beating of EBs was detected in the early stage of their differentiation, I_f currents were not detectable at that stage; this finding explained at least in part the low sensitivity of beating rate and action potential frequency to Cs^+. It has been reported that a slower rhythmic release of intracellular calcium through IP3-sensitive stores may contribute to beating in mouse ES cells that do not possess I_f channels (10). HCN channels confer faster rhythmicity to beating EBs, as reported by Qu *et al.* (19). The activity of I_f channels in mouse ES cell-derived cardiomyocytes isolated from EBs was found to be small during the early stage of differentiation, and increased in late stages (1). Another report of experiments that used human ES cells demonstrated the presence of I_f currents and HCN gene mRNAs in the undifferentiated state and their decrease in the late stages of cardiac differentiation (22). EBs contain cell types with different chamber specificities (5). Qu *et al.* (19) found that in mouse ES cells, the proportion of cells expressing I_f channels and the density of I_f channels in these cells increased during differentiation, which is consistent with our present data. This different chamber specification makes it difficult to study the changes in I_f channel activities and HCN gene transcription during differentiation. Tracking the markers of stem cells is an appropriate

technique to evaluate changes in cardiac progenitor cells during their differentiation. By tracking a marker of stem cells, it was found that mouse ES cell-derived Flk1(+) mesoderm cells cultured on OP9 stroma cells gave rise to spontaneously beating cardiomyocytes (25). Yanagi *et al.* (26) investigated developmental changes in automaticity, I_f channel activity and HCN gene transcription in ES cell-derived Flk1(+) progenitor cells cultured on OP9 stroma cells (on days 9.5 and 23.5). They found that their spontaneous beating rate, I_f channels and expression of HCN1 and 4 were significantly decreased on day 23.5. There is a discrepancy between the report by Yanagi *et al.* and our present results. In the present study, we found that most of the population of Nkx2.5(+) cells did not overlap with the population of Flk1(+) cells. These findings together with the fact that Flk1(+) cells did not express HCN genes even in the late stage of their differentiation, indicated that Flk1(+) cells were not involved in the changes in Cs^+-sensitive automaticity, HCN1 and 4 transcription and expression or I_f channel activity of Nkx2.5(+) cells under the present experimental conditions. Instead of differentiation through co-culture with OP9 stroma cells, we differentiated ES cells into cardiac cells through EBs formation, which is the reason most of the Nkx2.5(+) cell population did not overlap with the population of Flk1 (+) cells.

This study showed that Nkx2.5(+) cardiac progenitor cells expressed both HCN1 and 4 genes in the late stage of their differentiation. Since sino-atrial nodal cells express HCN1 and 4 mRNA (5, 6), the present result is consistent with that of a previous study in which a fraction of Nkx2.5(+) cells showed electrophysiological properties mimicking those of sino-atrial nodal cells as well as the atrial and ventricular cells after long-term culture (5). Mommersteeg *et al.* recently reported that Nkx2.5 suppressed HCN4 gene transcription in a genetic mice model (14), which differs from the present data. Although Nkx2.5 suppressed the HCN4 gene transcript after E10.5, Nkx2.5(+) cells overlapped with HCN4-positive cells in E9.5 (14), indicating the possibility that *in vivo* Nkx2.5 suppresses the transcription of HCN4 in Nkx2.5-positive cardiac precursor cells after E10.5. Further experiments are necessary to elucidate this possibility.

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Paracrine factors of vascular endothelial cells facilitate cardiomyocyte differentiation of mouse embryonic stem cells

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ABSTRACT

For myocardial regeneration therapy, the low differentiation capability of functional cardiomyocytes sufficient to replace the damaged myocardial tissue is one of the major difficulties. Using Nkx2.5-GFP knock-in ES cells, we show a new efficient method to obtain cardiomyocytes from embryonic stem (ES) cells. The proportion of GFP-positive cells was significantly increased when ES cells were cultured with a conditioned medium from aortic endothelial cells (ECs), accompanied by upregulation of cardiac-specific genes as well as other mesodermal genes. The promotion was more prominent when EC-conditioned medium was added at an early stage of ES cell differentiation culture (Day 0–3). Inhibitors of bone morphogenic protein (BMP), cyclooxygenase (COX), and nitric oxide synthetase (NO) prevented the promotion of cardiomyogenesis by EC-conditioned medium. These results suggest that supplementation of EC-conditioned medium enables cardiomyocytes to be obtained efficiently through promotion of mesoderm induction, which is regulated by BMP, COX, and NOS.

Regeneration therapy by cell transplantation is a new challenging option in the treatment of severe heart failure refractory to conventional therapies. Autologous transplantation of skeletal myoblasts, mesenchymal stem cells, or induced pluripotent stem (iPS) cells have an advantage over allograft transplantation in terms of ethical problems and allograft rejection. Differentiation of skeletal myoblasts or mesenchymal stem cells into cardiac myocytes is, however, minimal or negligible. It has been revealed recently that iPS cells have a considerable analogy with ES cells in their morphological, functional and growth properties [1–3]. The information available about the cardiomyogenesis of iPS cells is still limited, and the data obtained from ES cells are considered to be quite useful in the future progress of myocardial regeneration therapy using iPS cells [2].

In the ES cell-derived embryoid bodies (EBs) under normal culture condition, proportion of cardiomyocytes is only several percent, and the low efficiency of cardiomyogenesis limits the usage of ES cells in transplantation therapies. Recently, several reports have shown that various paracrine factors such as bone morphogenic proteins (BMP), transforming growth factor-beta2, nitric oxide, and wnt11 facilitate cardiomyogenesis of ES cells [4–6]. Noggin, a naturally occurring inhibitor of BMP, was shown to cause a prominent enhancement of cardiomyogenesis of ES cells when applied at the very early stage of their differentiation [7].

During the embryonic growth, differentiation and development of mesodermal organs including the heart is preceded by vasculogenesis and/or angiogenesis [8] prior to the initiation of effective circulation. It is, therefore, conceivable that certain paracrine factors released from vascular endothelial cells may affect the initiation and progression of cardiomyogenesis. The present study aims to test this hypothesis. We examined the effects of conditioned medium obtained from cultured bovine aortic endothelial cells (EC-conditioned medium) on the cardiomyocyte differentiation of murine ES cells. The results have revealed a potent facilitation of cardiomyogenesis of ES cells by the medium.

Material and methods

Cell culture and differentiation. The mouse hcgp7 cell line [a cell line of Nkx2.5-GFP knock-in ES (Nkx2.5-GFP ES cells)] established from a 129/Ola-derived ES cell line ht7 was cultured without

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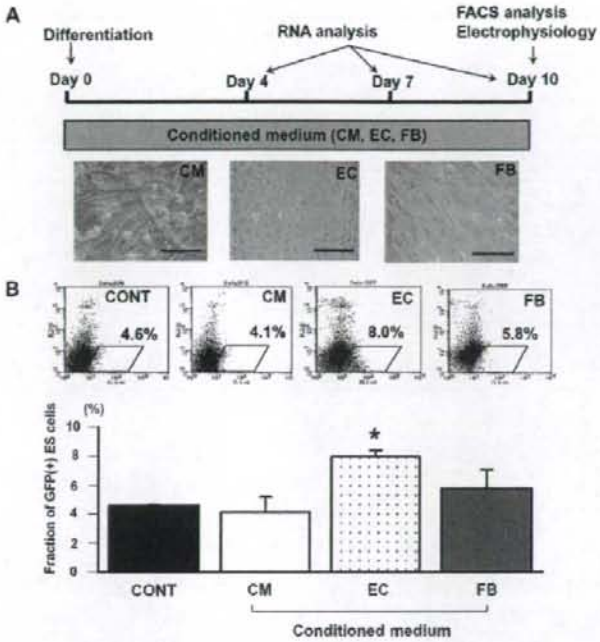

Fig. 1. Effects of conditioned medium on cardiac differentiation of ES cells. (A) Experimental protocols. Conditioned medium from neonatal cardiomyocytes (CM), bovine aortic endothelial cells (EC) or embryonic fibroblasts (FB) was added to the differentiation medium of Nkx2.5-GFP ES cells throughout the entire process of EBs (from Day 0 to Day 10). Lower panels demonstrate representative pictures of cultured CM, EC and FB. Bars indicate 10 μm. (B) Flow cytometric analysis of EBs at Day 10. *Upper panels*: representative experiments (scatter plots) of Nkx2.5-GFP ES cells cultivated without conditioned medium (CONT) and with a conditioned medium from CM, EC or FB. The numbers in each fraction indicate percentages of GFP(+) cells among entire FSC/SSC-gated cell population. In each panel, abscissa and ordinate indicate the fluorescent intensity of GFP and propidium iodide, respectively. *Lower panel*: pooled data obtained from each 4–7 experiments. Values (fraction of GFP(+) cells) are means ±SE (*$p < 0.05$ vs. Control).

feeder cells, and then differentiated as described previously [9]. Detailed methods are presented in Supplementary materials. The ES cells digested by trypsin were cultured for 3 days in small drops (each 20 μl containing 1600–2000 cells) suspended from the lid of the culture dish ("hanging-drop") to form spheroids (embryoid bodies: EBs). EBs were then transferred to tissue culture dishes (50 EBs per dish) and further cultivated for 7 days. Supernatants of culture media of (1) bovine aortic endothelial cells (EC) (2B2-C75: Cell systems, Kirkland, USA); (2) mouse embryonic fibroblasts (FB) (CRL-1503: ATCC, Manassas, USA); (3) ventricular cardiomyocytes of 1 day-old mouse neonates (CM) were applied to the ES-cell differentiation medium for 10 days throughout all the stages of differentiation unless otherwise specified. In experiments to quantify the expression of gene transcripts, GFP-free ES cells (hr7 cells) were used instead of Nkx2.5-GFP ES cells to avoid the influence of GFP knock-in [9].

In experiments to specify the substance involved in the paracrine effect of EC-conditioned medium, the following five compounds were applied to the conditioned medium: an angiotensin II type-1 receptor blocker (losartan: 1 μM, provided by Merck), an endothelin-1 receptor blocker (BQ123: 1 μM, Sigma–Aldrich, St. Louis, USA), a naturally occurring inhibitor of bone morphogenic proteins (Fc noggin: 1 μg/mL, R&D systems, Minneapolis, USA), an inhibitor of cyclooxygenase (prostaglandin-I: 2 μM, Sigma–Aldrich) and an inhibitor of NO synthetase (NG-nitro-L-arginine methyl ester [L-NAME]: 1 μM, Sigma–Aldrich).

Flow Cytometry. EBs at Day 10 were dispersed with trypsin–EDTA (0.25%) (GIBCO-BRL, Gainthersberg, USA), and their cardiac differentiation was estimated by flow cytometry using a dual laser FACSCalibur (BD Biosciences, San Jose, USA). The ES cells were excited with a 488 nm argon laser and a 530 nm band-pass filter was used to detect GFP positive cells. Data were analyzed using the CellQuest acquisition software (BD Biosciences). The extent of cardiac differentiation was expressed as a percentage of GFP-positive cells among all the ES cells.

Gene expression analysis by real-time reverse transcription-polymerase chain reaction. Gene transcripts for cardiac differentiation (*Nkx2.5, Gata4, Mef2c*), cardiac contractile proteins (myosin light chain 2a: *Myl7*, myosin heavy chain beta: *Myh7*), mesodermal/endothelial markers (brachyury: *Brachyury*, flk1: *Kdr*, VE-cadherin: *Cdh5*) and maintenance of embryonic stem cells in undifferentiated state (Oct3/4: *Pou5f1*) were detected by a reverse transcription-polymerase chain reaction (RT-PCR) method and quantified on a real-time thermal cycler (7700 Sequence Detector Systems, Applied Biosystems) with QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) for *Nkx2.5, Gata4, Mef2c, Myh7*,

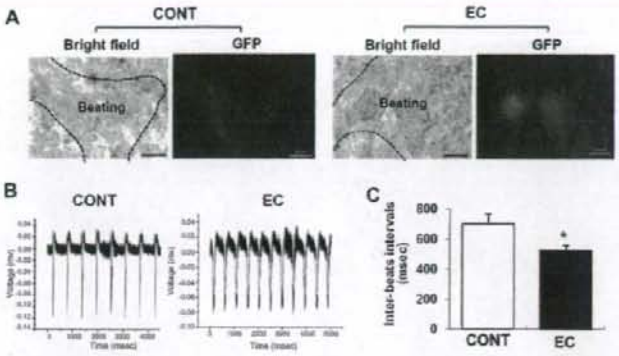

Fig. 2. Spontaneous beating of embryoid bodies differentiated from ES cells. (A) Spontaneously beating areas (inside of dotted line) of EBs at Day 10 in the absence (CONT) and in the presence of EC-conditioned medium (EC). Discernible fluorescence in the Nkx2.5-GFP EBs was detected in both groups. Solid bars indicate 50 μm. (B,C) Extracellular potentials of spontaneously beating EBs were recorded using multi-electrode culture dishes. Bar graphs show the summarized data of inter-beat intervals in the control and the EC-treated groups. (Values are means ± SE ($n = 4–6$, $^*p < 0.05$ vs. Control). Note the EC-treated group showed faster spontaneous beating.

Myl7,*Kdr and Cdh5*; and with TaqMan EZ-RT PCR kit (Perkin-Elmer, Waltham, MA, USA) for *Brachyury* and *Pou5f1*. GenBank accession nos of these genes are provided in Supplementary Table 1. Total RNA extracted from the EBs was treated with RNAse-free DNAse I, and reverse transcribed using Superscript II enzyme (GIBCO-BRL, Gainthersberg, MD, USA) with random hexamer. To ensure the fidelity of the mRNA extraction and reverse transcription, all samples were subjected to PCR amplification using hypoxantine-guanine phosphoribosyltransferae 1 (*Hprt1*) primers and the data were normalized with Hprt1 mRNA. The primer sequences or assay ID numbers are listed in Supplementary Table 1.

Extracellular potential recording. The effects of EC-conditioned medium on the automaticity of ES cell-derived EBs were examined using an extracellular potential recording system (MED64 system, Alpha MED Sciences, Kadoma, Japan). EBs of Nkx2.5-GFP ES cells were transferred to a culture dish with multielectrodes (MED-P515A, Alpha MED Sciences) at Day 3, and further cultivated for 7 days. The EC-conditioned medium was added to the ES-cell differentiation medium (EC group) for the entire culture period, compared to the control medium without EC-conditioned medium (CONT group). At Day 10, extracellular potentials of spontaneously beating EBs were recorded. Data were sampled at 20 kHz and inter-beat intervals were calculated using Conductor software (Conductor, Alpha MED Sciences).

Data presentation and statistics. Values are presented as means ± SE unless otherwise specified. The number of data presented in each figure indicates the number of dishes used for the culture of EBs. The statistical significance was determined by the one-way ANOVA or unpaired *t* test. Differences were considered statistically significant at $p < 0.05$.

Results

Supernatant from culture medium of endothelial cells promoted cardiac differentiation of ES cells

We examined the effects of the supernatants of culture media of ECs, FBs, and CMs added separately to the ES cell differentiation medium throughout the entire process (from Day 0 to Day 10) (Fig. 1A). In these experiments, EBs typically started spontaneous beating around Day 7. The proportion of GFP-positive cells significantly increased in the conditioned medium from EC culture (EC 8.0 ± 1.3% vs. Control 4.6 ± 1.0%, $n = 7$, $p < 0.05$) (Fig. 1B). In contrast, the conditioned media from CM or FB did not affect the proportion; the values for CM-medium (4.1±0.4%, $n = 7$) and for FB-medium (5.8 ± 1.0%, $n = 7$) were comparable to the control (NS).

Extracellular potentials of spontaneous beatings were recorded at Day 10 (Fig. 2) (Movies of spontaneously beating EBs are also available in Supplementary materials). As summarized in Fig. 2C, the EC-treated EBs showed significantly shorter inter-beat intervals compared to control EBs (control 702 ± 63 vs. EC 526 ± 31 ms, $p < 0.05$, $n = 4$).

We also checked the expression of transcripts related to cardiac differentiation (*Nkx2.5, Gata4 and Mef2c*), contraction (*Myh7, Myl7*), mesodermal/endothelial markers (*Brachyury, Kdr, Cdh5*) and maintenance of embryonic stem cells in undifferentiated state (*Pou5f1*) in EBs harvested at Day 4, Day 7 and Day 10 (Fig. 3). As for the genes related to cardiac differentiation, they increased with the progress of ES differentiation. At Day 7, transcript levels of *Nkx2.5* and *Gata4* in EBs cultivated with EC-conditioned medium were significantly larger than those of control (by 161% and 1450%, respectively, $n = 3–7$, $p < 0.05$); At Day 10, transcript levels of *Nkx2.5, Gata4* and *Mef2c* in the presence of EC-conditioned medium were significantly larger than controls (by 219%, 820%, and 131%, respectively, $n = 3–7$, $p < 0.05$). As for the genes related to cardiac contraction, at Day 10 the transcript level of *Myl7* in the presence of EC-conditioned medium was significantly larger than control (by 322%, $n = 7$, $p < 0.05$). Transcript level of *Myh7* at Day 10 in the presence of EC-conditioned medium tended to be larger than control, but the difference did not reach a statistical significance. As for mesodermal/endothelial markers, *Brachyury, Kdr*, and *Cdh5* were significantly increased by EC-conditioned medium at Day 7 (*Brachyury* by 6362%; *Kdr* by 21800%; *Cdh5* by 1160%, $p < 0.05$, $n = 3–5$). As for *Pou5f1*, there was no significant difference between the two groups in between the absence (control) and presence of EC-conditioned medium throughout the whole period of EB growth.

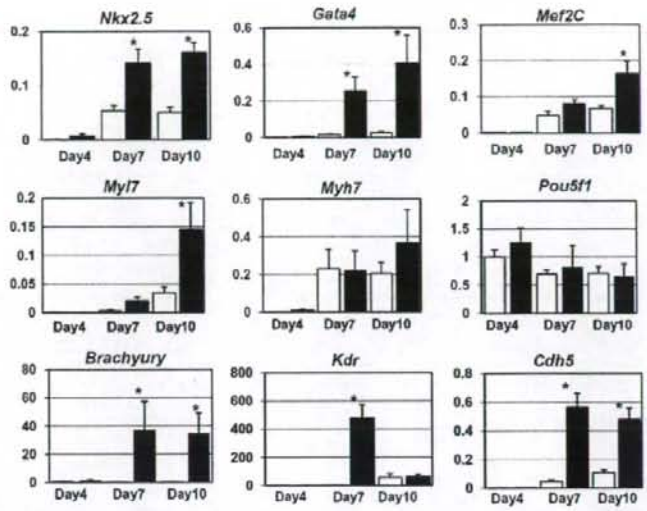

Fig. 3. Gene expression profiles of lineage markers during ES differentiation. RNA was harvested from cultured EBs at different stages of differentiation (Day 4, Day 7, and Day 10) in the absence (Control, open columns) and in the presence of EC-conditioned medium (solid columns). Real-time RT-PCR analysis was carried out for genes related to cardiac differentiation (*Nkx2.5, Gata4 and Mef2c*), cardiac contraction (*Myh7, Myl7*), maintenance of undifferentiated state (*Pou5f1*) and mesodermal/endothelial markers (*Brachyury, Kdr, Cdh5*). Total mRNA was normalized with *Hprt1* mRNA. In each panel, ordinate indicates arbitrary unit. Values are means ± SE ($n = 4$–7, $^*p < 0.05$ vs. Control).

Cardiac differentiation was not promoted when EC-conditioned medium was applied at later stage

In a series of experiments, we examined the cardiomyogenic effects of EC-conditioned medium applied at different stages from the initiation of ES cell differentiation. The whole differentiation period (10 days) was divided into four stages; Stage 1 for Day 0–3, Stage 2 for Day 3–5, Stage 3 for Day 5–7 and Stage 4 for Day 7–10 (Fig. 4A). When the EC-conditioned medium was applied during Stage 1, the proportion of GFP-positive ES cells in the flow cytometry increased significantly (by 65.5%, $n = 7$, $p < 0.05$ vs. Control): the promotion of cardiac differentiation was comparable to that obtained by application of the EC-conditioned medium during the whole process (by 88.2%) (Fig. 4A). Application of the EC-conditioned medium at later stages (Stage 2–4) had no significant effect on cardiac differentiation of ES cells.

EC-conditioned medium-induced facilitation of cardiac differentiation is attenuated by BMP inhibitor, COX inhibitor, and NOS inhibitor, but not by ATII antagonist or ET-1 antagonist

To specify the substance involved in the cardiomyogenic paracrine effects of the EC-conditioned medium, we examined the effects of application of the following compounds together with EC-conditioned medium throughout the entire stages of differentiation; angiotensin II type 1 receptor blocker (losartan), endothelin-1 receptor blocker (BQ123), a naturally occurring inhibitor of bone morphogenic proteins (noggin), an inhibitor of cyclooxygenase (prostaglandin I; PG-I) and an inhibitor of NO synthetase (L-NAME). Cardiomyogeneis in EBs at Day 10 was estimated by a proportion of GFP-positive cells in flow cytometry (Fig. 4B). Among these agents, noggin, PG-I and L-NAME significantly inhibited the cardiomyogenic effects of EC-conditioned medium by 26.9%, 26.8%, and 30.9%, respectively ($n = 4$–9, $p < 0.05$ vs. EC-conditioned medium alone) (Fig. 4B). In contrast, the addition of losartan or BQ123 to EC-conditioned medium did not show any significant changes compared to the use of EC-conditioned medium only (Fig. 4B).

Discussion

BMP2 has been shown to promote cardiac myogenesis through the activation of smad1/5/8 [10]. COX may be involved in the cardiomyogenesis through its effect in increasing BMP production [11]. Since both BMP and COX are known to be produced by vascular endothelial cells, it is reasonable to speculate that EC-conditioned medium may promote cardiomyogenesis through BMP action directly or indirectly. NOS, like BMP and COX, is produced abundantly in vascular endothelial cells. Previous studies reported that NOS promotes cardiomyocyte differentiation from ES cell [12], and that NOS induces apoptosis of ES cells which are not committed to cardiac differentiation, giving rise to an increase of a fraction of cardiomyocyte-committed cells [5]. In the present study, the fraction of cells undergoing apoptosis was found to be significantly increased with EC-conditioned medium (Supplementary Figure 1). It is suggested that reactive oxygen species or pro-oxidants are involved in the NOS-induced apoptosis, but the detailed mechanisms causing the different susceptibilities between the ES cells committed and those not committed to cardiac differentiation remains to be clarified [5].

Recently, Zhu et al. have reported that insulin-like growth-factor-binding protein (IGFBP4) plays an essential role in the cardiomyogenesis of ES cells [13]. Using signal sequence trap method, they first identified several candidate proteins secreted from OP9

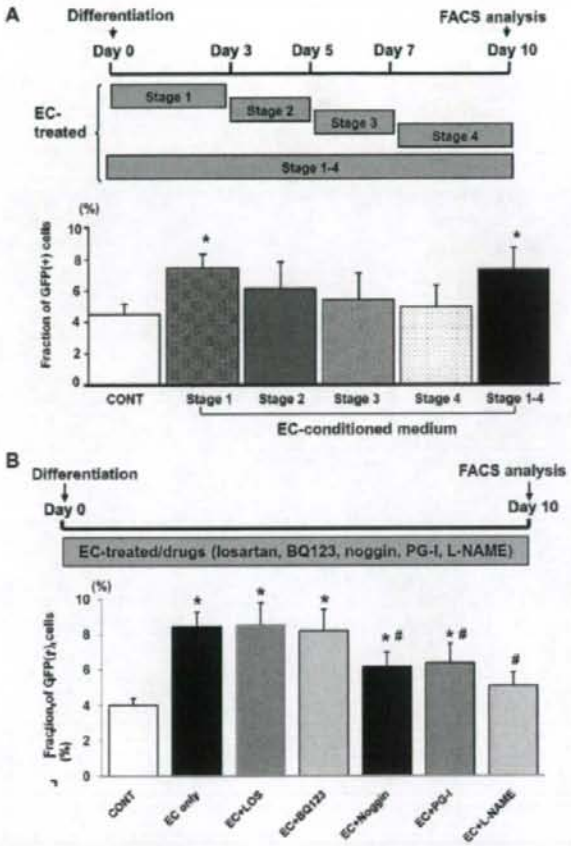

Fig. 4. Underlying mechanisms of EC-conditioned medium induced-promotion of cardiogenesis. (A) Stage-dependent effects of EC-conditioned medium on cardiac differentiation of ES cells. The whole EB-culture period from the initiation of differentiation were divided into four stages; Stage1 for Day 0–3, Stage 2 for Day 3–5, Stage 3 for Day 5–7 and Stage 4 for Day 7–10 (*upper panel*). Conditioned medium from aortic endothelial cells (EC) were applied at different stages of differentiation of ES cells. Bar graph shows the GFP-positive fraction in EBs from seven series of experiments. Values are means ± SE ($n = 7$. $^{*}p < 0.05$ vs. Control) (*lower panel*). (B) Effects of inhibitors of signal transduction on the facilitation of cardiac differentiation by EC-conditioned medium. EC-conditioned medium were added throughout all of the differentiation stages (1 through 4) in the absence (EC only) and the presence of five inhibitors of signal transduction. EBs cultured in the absence of EC-conditioned medium and any inhibitors were employed as Control (CONT). The proportion of GFP-positive cells among entire EBs was obtained in each group of 7 experiments, and the data were normalized to Control. Values are means ± SE. ($^{*}p < 0.05$ vs. Control, $^{\#}p < 0.05$ vs. EC only).

stromal cells, which facilitate cardiogenesis from ES cells. Among these candidate proteins, they confirmed that IGFBP4 plays a key role in promoting the cardiac differentiation through an inhibition of Wnt signals. A similar signal sequence trap approach would be useful in recognizing candidate proteins secreted from vascular endothelial cells. Encompassing investigation using high-throughput devices such as "cell chip" would also be useful [14] to screen off the candidate molecules involved in cardiomyogenesis.

The paracrine factors seemed to affect during the periods when the cell lineage is determined. It is still unclear if the EC-conditioned medium facilitated differentiation into entire mesoderm organs or specifically into the heart.

Since iPS cells were established from skin fibroblasts, a lot of studies are conducted for the development of autologous transplantation therapy of regenerative tissue including the heart. The results obtained in the present study could be applicable to efficiently differentiate iPS cells to cardiomyocytes, because there is a

considerable analogy between ES cells and iPS cells in their morphological, functional and growth properties [1–3].

In the near future, it is conceivable that there may be increasing demands for myocardial regeneration therapy to treat severe heart diseases. Thus, it is of great importance to clarify the mechanisms affecting the promotion of differentiation of ES cells to cardiomyocytes. Further experimental studies will be required to elucidate the point.

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.2008.09.160.

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Supplementary Table 1 Sequences of primers used for RT-PCR

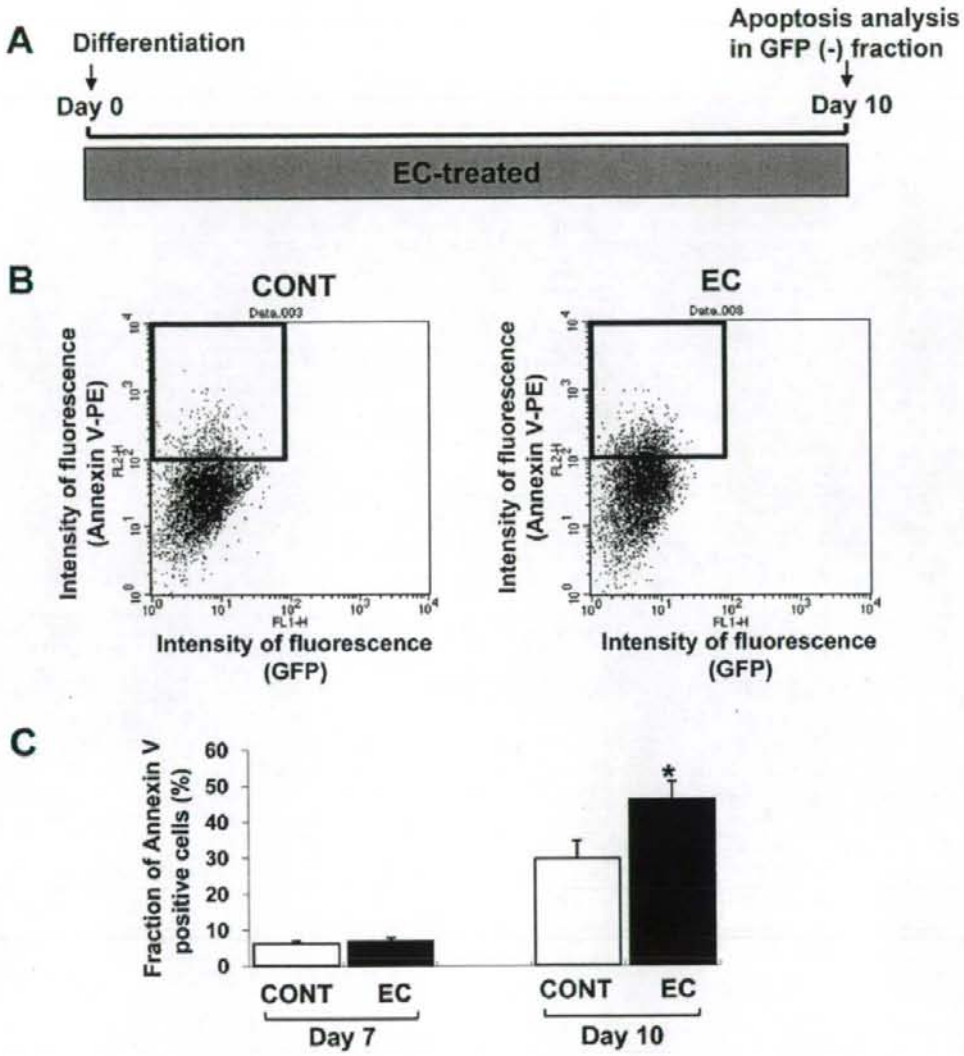
Gene	GenBank Accession nos	Primer sequence
Nkx2.5 (Nkx2.5)	NM_008700	Forward: ACCCAGCCAAAGACCCTC Reverse: GACAGGTACCGCTGTTGCTT
Gata4 (GATA binding protein 4)	NM_008092	Forward: TCTCACTATGGGCACAGCAG Reverse: GCGATGTCTGAGTGACAGGA
Mef2c (Myocyte enhancer factor 2c)	NC_000079	Forward: ACTGGGAAACCCCAATCTTC Reverse: ATCAGACCGCCTGTGTTACC
Myl7 (myosin light chain 2a)	NM_022879	Forward: TCAGCTGCATTGACCAGAAC Reverse: AAGACGGTGAAGTTGATGGG
Myh7 (myosin heavy chain beta)	NM_080728	Forward: CTACAGGCCTGGGCTTACCT Reverse: TCTCCTTCTCAGACTTCCGC
Kdr (flk1, VEGFR2)	NM_010612	Forward: GGCGGTGGTGACAGTATCTT Reverse: CTCGGTGATGTACACGATGC
Cdh5 (VE-cadherin)	NM_009868	Forward: AGACACCCCCAACATGCTAC Reverse: GCAAACTCTCCTTGGAGCAC
Hprt1 (HPRT)	NM_013556	Forward: ATCAGTCAACGGGGGACATA Reverse: AGAGGTCCTTTTCACCAGCA
Pou5f1 (Oct3/4)	NM_013633	Assay ID: Mm00658129_gH (the primer sequence: not disclosed)
Brachyury Brachyury	NM_009309	Assay ID: Mm00436877_m1 (the primer sequence: not disclosed)

Supplementary methods

A cell line of Nkx2.5-GFP knock-in ES cells (Nkx2.5-GFP ES cells) was used to investigate their cardiomyogenesis. The Nkx2.5-GFP ES cell lineage was established from a 129/Ola-derived ES cell line ht7 (GFP-free cell) (provided by Dr. Hitoshi Niwa, RIKEN Center for Developmental Biology, Kobe, Japan) as described previously (9). To maintain undifferentiated Nkx2.5-GFP ES cells, the cells were grown without feeder cells on gelatinized dishes by using "undifferentiation maintaining medium" [Glasgow-modified Eagle's medium (GMEM, Sigma-Aldrich, St Louis MO), FBS (v/v) (10% v/v, Gibco Invitrogen), L-glutamine (2 mM, GibcoBRL), penicillin (50 U/ml, Sigma-Aldrich), streptomycin (50 μg/ml, Sigma-Aldrich), sodium pyruvate (1mM, GibcoBRL), 2-mercapto ethanol (100 μM , GibcoBRL), and leukemia inhibitory factor (LIF) (10 mU/ml GibcoBRL)]. The undifferentiated Nkx2.5-GFP ES cells were then digested with trypsin-EDTA and suspended in the LIF-free differentiation medium (the same composition as described above except for the absence of LIF). The ES cells were cultured in small drops (each 20 μl containing 1,600-2000 cells) hanged from the lid of culture dish ("hanging-drop") to form spheroids (embryoid bodies: EBs) for 3 days. EBs were then transferred to tissue culture dishes (50 EBs per dish) and further

cultivated for 7 days. Supernatants of culture media of [1] bovine aortic endothelial cells (ECs), [2] mouse embryonic fibroblasts (STO cells) (FB), [3] ventricular cardiomyocytes of 1 day-old mouse neonates (CMs) were applied to the ES-cell differentiation medium for 10 days throughout the whole stages of differentiation unless otherwise specified. Nkx2.5-GFP ES cells cultured in the absence of these conditioned media were used as control.

Supplementary Figure 1. Effects of EC-conditioned medium on the induction of apoptosis in non-cardiac cells. (A) Protocol. Apoptosis was assessed by Annexin V-PE(phycoerythrin) apoptosis detection kit (BD Biosciences Pharmingen, San Diego, CA, USA). EBs of Nkx2.5-GFP ES cells at Day 10 were dispersed with trypsin-EDTA (0.25%), and GFP-positive and -negative cells were collected separately using a cell sorter (FACSVantage, BD Biosciences, San Jose, CA, USA). The collected cells were stained with annexin V-PE and subjected to flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA). Annexin V-positive living cells were considered undergoing apoptosis. (B) Representative scatter plots of annexin V-PE -positive cell population in the GFP-negative fraction (non-cardiac cells) from EBs on Day 10 cultivated in the absence (CONT) and in the presence of the EC-conditioned medium throughout differentiation (EC). Abscissa and ordinate indicates the intensity of GFP and PE fluorescence, respectively. Cells inside the thick square were considered annexin V-positive. (C) The proportion of the GFP positive cardiac cells with or without the EC-conditioned medium (Data are mean ± SE. $^{*}p < 0.05$ vs. CONT. $n = 7$.). Note that the EC-treatment increased the proportion of cells undergoing apoptosis in non-cardiac committed cells, compared to CONT at Day 10, whereas the fraction of annexin V-positive cells were low in both CONT and EC groups.


A
Differentiation
Day 0
Apoptosis analysis
in GFP (-) fraction
Day 10
EC-treated
B
CONT
EC
Intensity of fluorescence
(Annexin V-PE)
Intensity of fluorescence
(GFP)
C
Fraction of Annexin V
positive cells (%)
60
50
40
30
20
10
0
*
CONT
EC
Day 7
CONT
EC
Day 10

「ES 細胞の現状と問題点：基礎と応用」

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Summary
ES 細胞は自律拍動する心筋細胞へと分化し、障害を受けた心臓の機能回復にむけた応用が期待されるが、実現への道筋は平坦ではない。たとえ利用可能な細胞が得られたとしても、移植片化の工夫、良い足場、共存すべき細胞種などの知見は不十分であり、ES 細胞由来心筋細胞の効率的な大量調製法の確立も必要である。一方、iPS 細胞の樹立は、ヒト ES 細胞についての倫理的問題の多くが回避されるとして注目されるが、この細胞を用いることができるとしても、幹細胞からの心筋細胞の効率的な分化・純化・大量調製法にむけた研究の進展が必要である。従って、ES 細胞についての研究はまだまだ発展させる必要がある。

Point
・ES 細胞は自律拍動する心筋細胞へと分化するが分化制御法はまだ未確立である。
・iPS 細胞は注目されるが、研究利用においてもまだ ES 細胞の代替にはならない。
・細胞分化過程での、心筋・心筋前駆細胞のマーカー探索とその活用は大きな課題である。

Key Words：ES 細胞、心筋細胞、細胞分化、心筋マーカー

はじめに

ES 細胞は無限増殖能と様々な細胞への多分化能の両者を備えた細胞であり、動物では 1 個の ES 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、発生分化の細胞モデルとして、また、遺伝子操作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 年にはヒト ES 細胞が樹立[1]され、他の方法で治療が困難である組織の機能障害を補うための再生医療の細胞ソースとしての期待が高まっている。以来、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てきた。しかしながら、ES 細胞は受精卵からできあがる胚を壊して作製する必要があり、研究成果への期待とともに、胚を操作するという倫理的な問題や、そもそも他人の細胞であることによる免疫拒絶の問題など、ES 細胞の臨床応用や実用化には乗り越えなければならない課題がまだまだ多い。一方で、近年、体細胞に複数の遺伝子を発現させることにより、ES 細胞と同様な性質を有する iPS 細胞の樹立がマウス、ついでヒトでも可能となった[2,3]。iPS 細胞の詳細については別項に譲るが、胚を操作するという倫理的な問題を回避し、かつ、論理的には本人由来の幹細胞の調製が可能であり免疫拒絶の問題も回避できることから、臨床応用・実用化可能な幹細胞がついに現実的になった、と考えられるようになった。もっとも、iPS 細胞を作製する方法の改良、たとえば遺伝子改変なしの手法の開発、また、iPS 細胞から作製可能な生殖細胞の扱いの検討など、現時点では iPS 細胞にも未解決の課題は少なくない。

こうしたことから、現時点では、無限増殖能と様々な細胞への多分化能の両者を備えた細胞について基礎的な検討や応用に向けた検討を行うには ES 細胞を用いて行うことがまずは必要であり、ES 細胞研究の重要性は減っていない。著者らも、生後はほとんど細胞分裂せず一生涯機能する必要があるが、一旦傷害をうけると再生しない心筋につき、その再生を最終目標に、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[4]。その中、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上昇する遺伝子群を同定した[5]。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うる候補遺伝子も含まれており、現在、これらの因子・マーカーの利用により、効率的な分化誘導法、単離法の確立と大量調製に向け、研究を実施している。本稿では、これまでに ES 細胞の心筋分化に関して行った研究で明らかにした知見と問題点を他の ES 細胞研究の現状とともに論ずる。

I. ES 細胞の臨床応用は大きな期待があるが超えるべきハードルも高い

はじめに述べたように、1998 年にヒト ES 細胞が樹立され、ES 細胞の細胞移植ソースとしての期待が高まった。しかし、ES 細胞を用いた臨床応用については実現の道のりはまだ遠い。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をもつテーラーメイド細胞が利用できるということになり、まさに、移植ソースとしては免疫拒絶のない理想的なものとなりうる（図1）。現時点ではクローン胚よりヒトES細胞を作成することは成功していないものの、将来的には成功するだろうと考えられが、クローン胚樹立のために核移植を行う際には、必然的に卵子を破壊する必要があり、その樹立ごとに倫理的な問題を伴うことはクローン胚由来ヒトES細胞の樹立の大きな障害となる。さらに、樹立の際にコストと時間がかかりすぎることも、このアプローチは現実的ではないとして、ES細胞の臨床応用に対しての否定的な考え方につながる。一方、こうしたテーラーメイドのES細胞の樹立に対して、ES細胞のバンク化を行えばクローン胚樹立せずともES細胞の臨床応用は可能である、という考え方もある（図1）。その根拠として、200ものES細胞株を樹立すれば、組織適合性抗原のミスマッチは最小限に抑えることができ、ほとんどの患者に対応可能となり、臨床応用は可能になるだろうというものである[6]。むろん、これだけの数のES細胞株の樹立が現実的に可能なのか、余剰胚の提供が今後どのくらい進むのか、現時点では未知数の部分が多く、判断はまだ困難である。ただし、私たちは、研究者の立場として、基礎研究の成果を医療応用に向けて有意義なものとして示すことによって、一般的な理解とコンセンサスが一層高まり、理解が得られるよう努力していきたいと考えている。

II. レポーター導入によりES細胞から心筋細胞へと分化した細胞は純化できる

私たちは、これまで数年来にわたり、マウスES細胞を用いて、ES細胞から心筋分化を誘導し、それを単離するための系を構築してきた。ES細胞は細胞塊を作らせると胚様体と呼ばれる3次元構造を構築し、おそらくは細胞同士の間接あるいは直接的相互作用等によって、一部が自動拍動する心筋細胞を生ずる。私たちは心筋特異的ホメオボックス転写因子Nkx2-5遺伝子座に緑色蛍光タンパク質EGFPをノックインしたマウスES細胞株を樹立し、その細胞を元に、セルソーターを用いてES細胞由来心筋細胞を単離することに成功した[4]（図2）。その中で、単離心筋細胞を培養皿にて培養すると、最初はほとんどがペースメーカー型の活動電位を示すのに対し、培養を続けることによって、心室筋型、心房筋型のパターンを示すものも現れることを明らかにした。また、レチノイン酸刺激を加えることにより、心房筋型細胞の割合が増える、という変化が確認された。こうして、EGFPなどの「(標識)レポーター」を導入することにより、すなわち遺伝子改変を施すことにより、ES細胞から心筋細胞を純化することは可能であることを実証した。レポーターとしては蛍光タンパク質の他に、表面抗原として利用できるタンパク質（たとえばCD4など）も利用可能であるので、そうした方法で2次抗体の修飾を変えることによって、磁力による純化も可能であると考えられ、現在検討を行っている。また、レポーターを薬剤耐性遺伝子とした場合はさらなる大量調製が可能であると考えられるので、こうした手法を組み合わせることで、ES細胞からの心筋細胞の分化誘導を効率よく行って、目的とする心筋細胞を高純

度で大量に獲得することすらかなり現実に近いものになってきたといえる。

III. 網羅的発現遺伝子解析により心筋特異的遺伝子についての理解が進んだ

前項ではES細胞からの心筋細胞の分化研究の進捗と期待を述べたが、これまでの研究では、通常の血清添加培養条件下で 5-10%の細胞が心筋細胞となるのみであり、これだけでは心筋細胞を単離し利用するには不十分であって、高効率の細胞分化系の確立が必要と考えられてきた。そこで、Wnt11 などの既知の因子等の添加によって分化誘導の改善を試みるとともに種々の細胞培養条件を比較検討し[7]、その結果、一時的な血清の除去が心筋細胞への分化を 20-30%まで促進することを見出した[5]。この現象は最近ヒトES細胞でも報告されており、細胞株による効果の違いは若干あるものの、一般的な現象であると考えられた。そこで、私たちは、この心筋細胞を多く含む胚様体のマイクロアレイ解析により、どのような遺伝子が胚様体の心筋分化誘導に伴い発現しているかを網羅的に解析して、分化誘導促進作用のある遺伝子の探索を試みた。この解析では、既知の心筋で働く転写因子と心筋細胞特異的構造タンパク質遺伝子はほとんど全て Nkx2-5 遺伝子と同様な発現パターンを示すことが確認された。次いで、先の解析で候補として抽出された遺伝子のうち、胚発生過程で組織特異的発現について調べられていなかった遺伝子について、マウス Whole Mount In situ Hybridization を行い、その発現の組織特異性を確認したところ、Nkx2-5と同様なパターンを示す遺伝子はほとんどが胚発生において心臓特異的に発現していることが明らかとなった。このことは、私たちのマイクロアレイ解析データは発生過程での発現が心筋特異的である遺伝子を絞り込むのに有用であることを示唆している。また、こうした候補のうち、心筋特異的と考えられるパターンをとる転写因子遺伝子の中には原始心筒では必ずしも発現していないものも含まれていた。これらの中には第二の心臓系譜というべき Second Heart Field[8]で発現する Isl1, Tbx1 などが含まれていることが明らかになった。また、胚様体では心筋細胞だけでなく心内膜細胞に特徴的な遺伝子を発現する細胞の存在も明らかとなった[9]。この結果は、胚様体においても心筋分化に関わる複数の細胞系譜が存在している可能性を示唆するものであるとともに、これらの細胞系譜における共通した心筋分化誘導メカニズムの存在の可能性を示しているようであり、非常に興味深い。

IV. ES 細胞由来心筋細胞の大量調製・移植片化にむけて特異的マーカーの同定は重要である

前述したように、ES細胞から心筋細胞を大量に獲得するための方策には、薬剤耐性遺伝子を導入したり、表面マーカー遺伝子を外来より導入したりして、選択や純化に利用する方法が考えられる。しかしながら、将来、臨床応用の実用化に向けて、先に紹介したように仮に 200 種類のES細胞バンクを作ったとしよう。この場合、レポーターをそれぞれのES細胞株に安定に導入することは可能なのであろうか？また、遺伝子改変されたES細胞の安全性はどのようにすれば確保されるのであろうか？このようなことを考えると、やはり、

外来の選択マーカーの導入でなく、内在性の心筋細胞表面特異的なマーカーの同定とその利用が必須であると考えられる。しかしながら、現在のところ、心筋細胞には特異的な表面マーカーは知られていない。これまでには、心筋前駆細胞をFlk1などのマーカーを利用して心筋細胞を単離することの報告[10]はなされているが、Flk1陽性細胞は分化前の前駆細胞であり、心筋細胞のみならず血管内皮等の系譜にも分化するため、心筋細胞についての特異性を高く純化するにはそのままでは不十分であり工夫が必要と考えられる。そこで、私たちは、マイクロアレイ解析で得られた遺伝子から、細胞の表面で発現している可能性のある遺伝子の抽出を行った。結果はごくわずかな遺伝子のみが細胞表面で発現していた。血清除去という条件を用いたためか、これまでに他の細胞系譜の特異マーカーとして利用され、心筋についても期待された増殖因子受容体遺伝子の多くは、むしろ、その発現が減少する傾向にあった。現在、候補となった数個のマーカーについて、胚及び胚様体のFACS解析、in situ hybridizationなどで特異性を確認しているところであり、将来、その活用が応用につながる心筋細胞表面特異的なマーカーであることを期待して研究を進めている。

おわりに

Klugらが ES 細胞由来心筋細胞を mdx マウスに移植し、ES 細胞由来心筋細胞が宿主心臓に取り込まれたことを示して[11]、ES 細胞由来心筋細胞の臨床応用への期待が高まって以来、すでに10年の月日が経過した。しかしながら、これまでのところ、障害を受けた心臓の機能を移植した ES 細胞由来心筋細胞により回復させたという報告はごくわずかである。また、単離した心筋細胞をそのまま生体に移植しても多くが生着せず、細胞はほとんど死んでしまうという報告もある[12]。大量に純化した心筋細胞が得られてとしても、ただ注入するだけでは心臓の機能はあまり回復しないのではないだろうか？ではどのように移植片化することが必要なのであろうか？温度感受性シートやマトリゲルなどの足場が良いのだろうか？単に純化するだけでなく共存すべき細胞種があるのであろうか？こういった疑問を解決すべく、私たちは基礎実験を推進しているが、そのためにも、ES 細胞由来心筋細胞は、今こそ、しかも大量に、必要な状況である。ところで、最近、胚由来でない細胞に4つの遺伝子を導入することで、ES 細胞に類似した性質を有する多能性幹細胞(iPS 細胞)が樹立されうることが報告された[13]。この報告は、途中で述べたヒト ES 細胞についての倫理的問題、とくにクローン胚由来 ES 細胞をめぐる倫理的問題の多くが回避される可能性を示唆するものとしても注目される。しかし、この細胞を用いることができるとしても、心筋細胞を分化・純化して大量に使用するとすれば、ここで述べた研究の進展がやはり必要である。私たちも、今後とも分化誘導の効率化、調製の大量化に向けて取り組み、ES 細胞由来心筋細胞を材料としてより使いやすいものにし、さまざまな基礎実験に対応できるような系を構築して、臨床応用・再生医療実現に近づけるよう日夜努力しているところである。

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