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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.

Key words: embryonic stem cell; cardiac cell; vascular endothelial cell; paracrine factor; cardiomyogenesis; Nkx2.5; bone morphogenic protein; wnt; cyclooxygenase; nitric oxide synthase

Introduction

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 [4,5,6]. 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 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 1,600-2000 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 (ht7 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: 1μM, Sigma-Aldrich, St. Louis, USA), a naturally occurring inhibitor of bone morphogenic proteins (Fc noggin: 1μg/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, 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 Table1. 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 Table1.

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) (Figure 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$\pm$1.3% vs. Control 4.6$\pm$1.0 %, n=7, $p<0.05$) (Figure 1B). In contrast, the conditioned media from CM or FB did not affect the proportion; the values for CM-medium (4.1$\pm$0.4 %, n= 7) and for FB-medium (5.8$\pm$1.0 %, n=7) were comparable to the control (NS).

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

cantly 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.

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; Stage1 for Day 0-3, Stage2 for Day 3-5, Stage3 for Day 5-7 and Stage4 for Day 7-10 (Figure 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%) (Figure 4A). Application of the EC-conditioned medium at later stages (Stage2-4) 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 (Figure 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) (Figure 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 (Figure 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 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 recog-

nizing 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

Acknowledgment

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Figure Legends

Figure 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).

Figure 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.

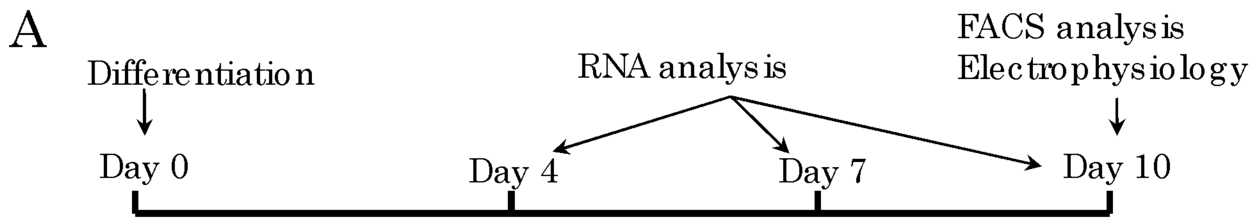
Figure 3. Gene expression profiles of lineage markers during ES differentiation. RNA was harvested from cultured EBs at different stages of differentiation (Day 4, Day 7 and Day 10) in the absence (Control, open columns) and in the presence of EC-conditioned medium (solid columns). Real-time RT-PCR analysis 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).

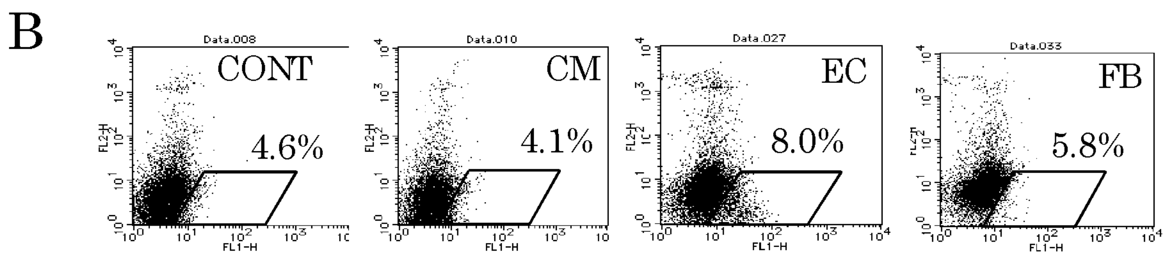
Figure 4. Underlying mechanisms of EC-conditioned medium induced-promotion of cardiogenesis. (A) Stage-dependent effects of EC-conditioned medium on cardiac differentiation of ES cells. The whole EB-culture period from the initiation of differentiation were divided into 4 stages; Stage1 for Day0-3, Stage 2 for Day3-5, Stage3 for Day5-7 and Stage4 for Day7-10 (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 7 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 5 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).

Figure 1

Figure 1





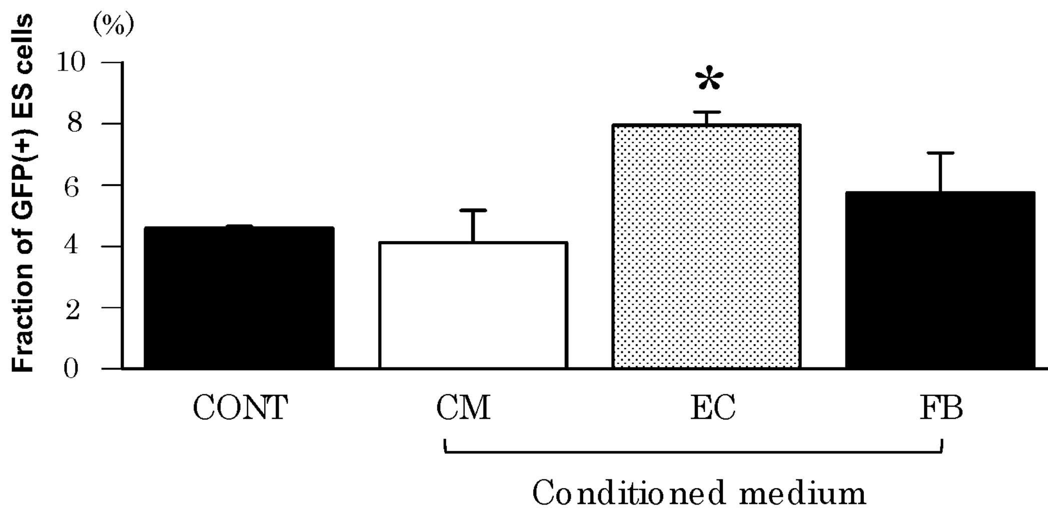


Figure 2

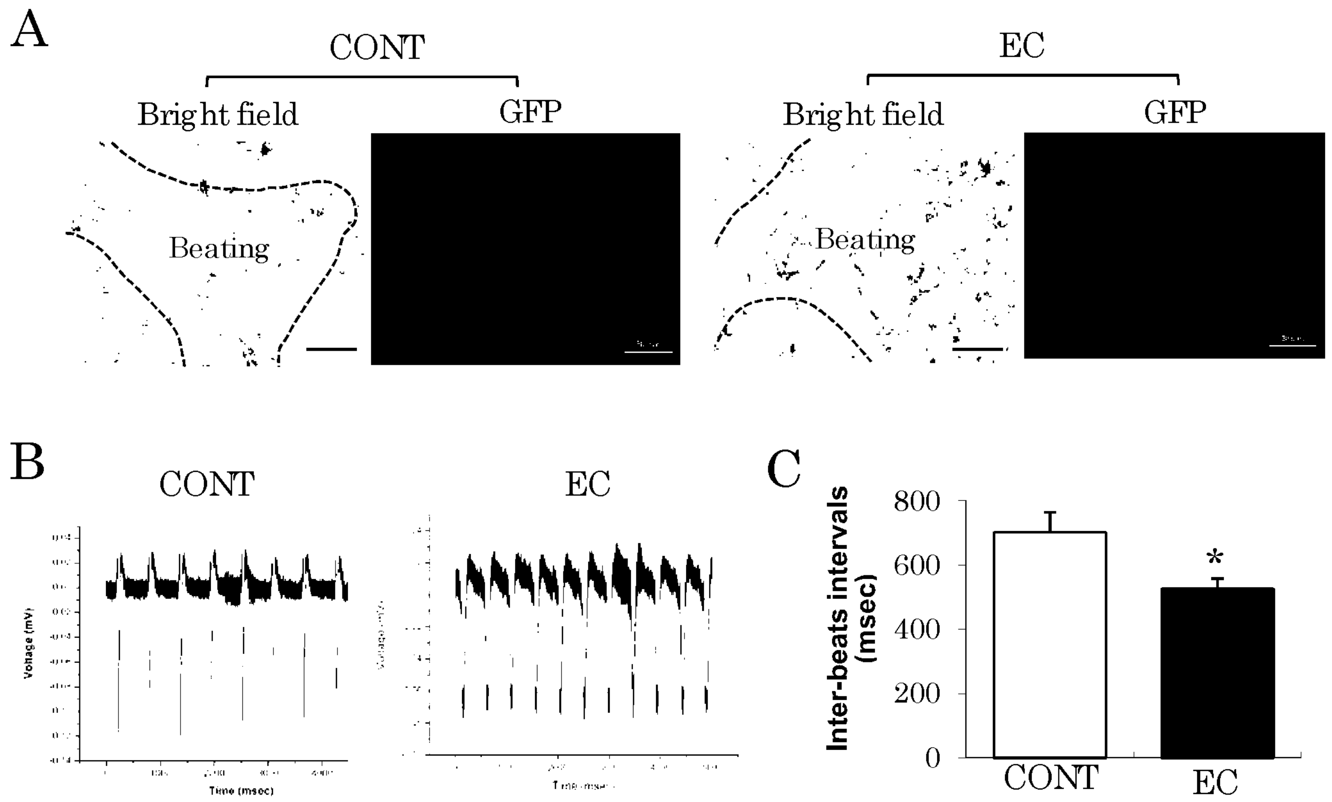
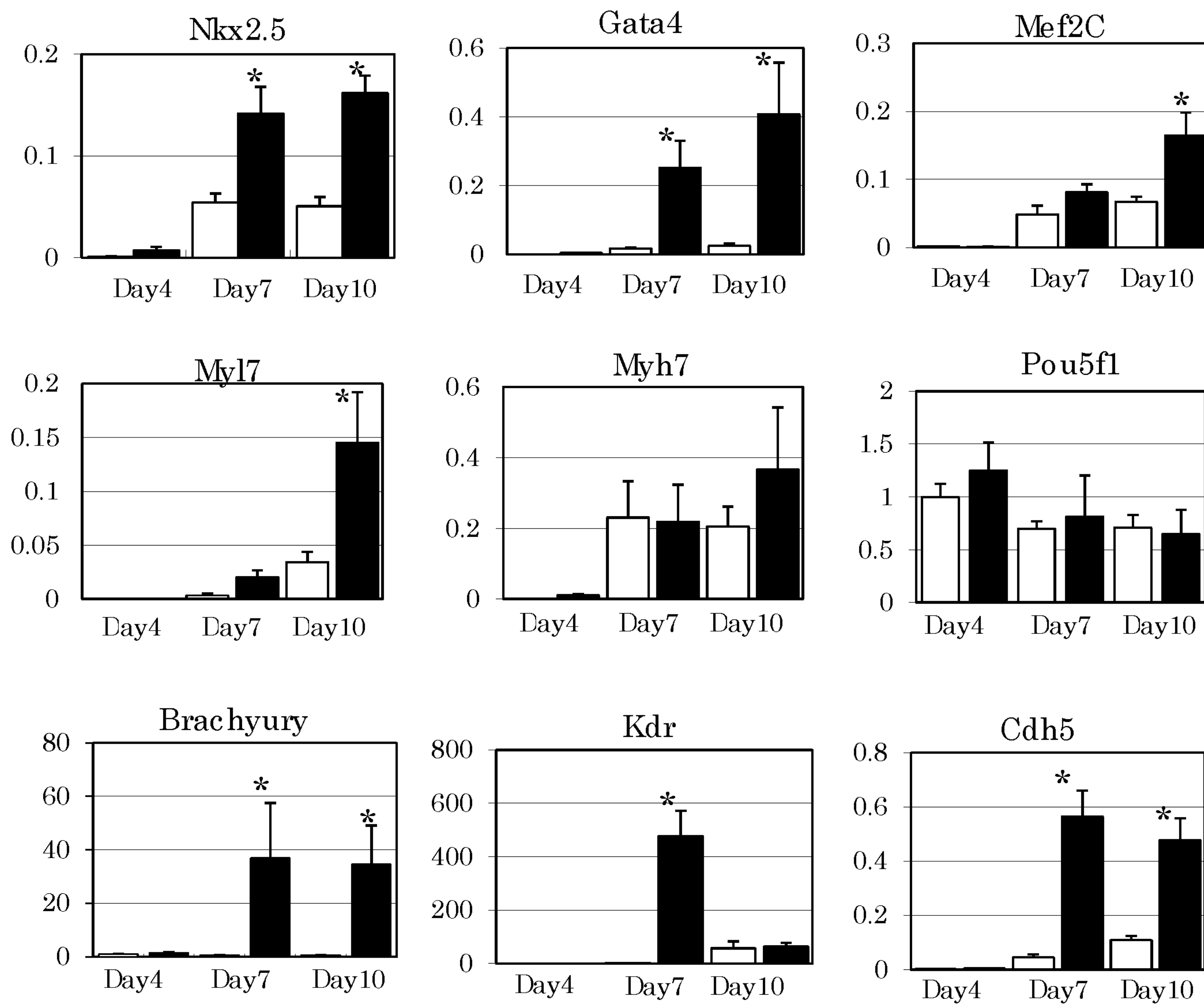


Figure 3

Figure 3



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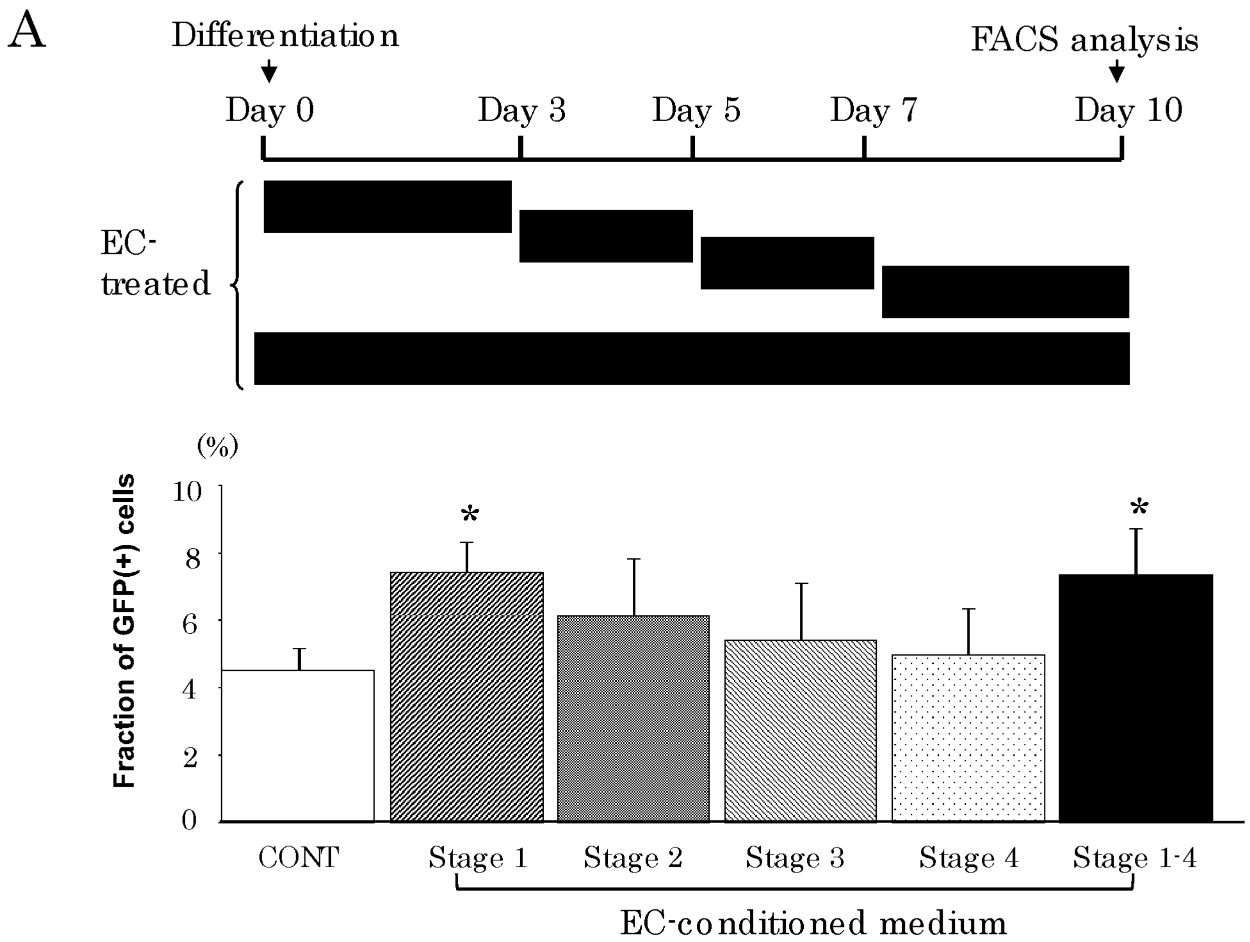

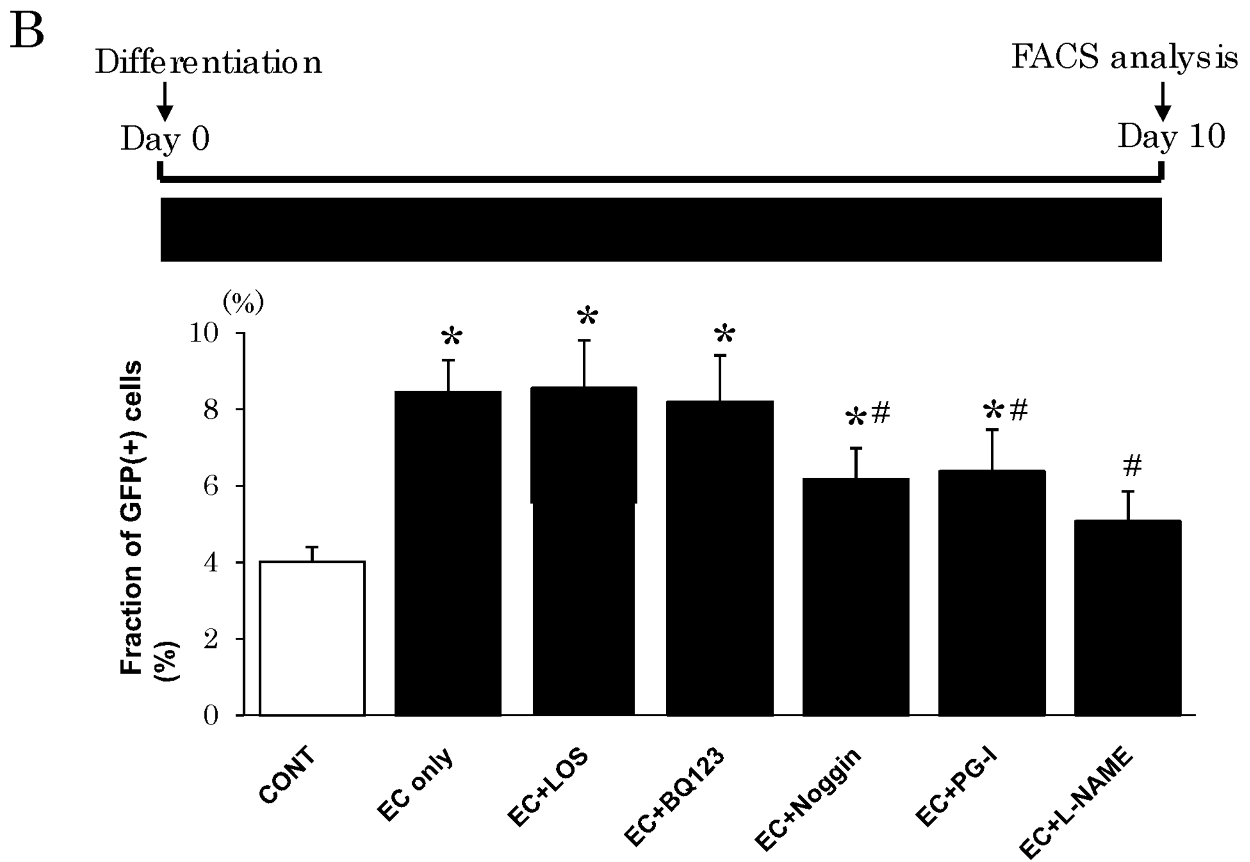

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