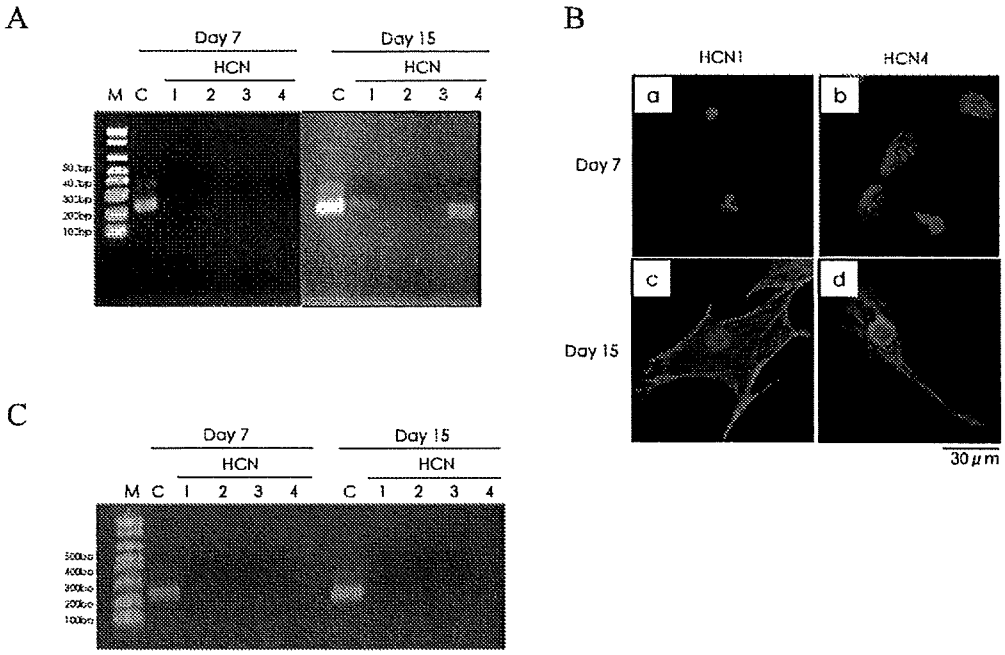

Fig. 3 Transcription and expression of HCN genes in Nkx2.5-positive cells and Flk1-positive cells on days 7 and 15 of their differentiation. A: Transcription of HCN genes in Nkx2.5/GFP(+) cells on days 7 and 15 of their differentiation. Cells were sorted by FACS, and then mRNA was extracted from them. M: Molecular marker, C: β-actin, 1: HCN1, 2: HCN2, 3: HCN3, 4: HCN4. B: Protein expression of HCN1 and 4 in Nkx2.5/GFP(+) cells on days 7 and 15 of their differentiation. a) and b): Nkx2.5/GFP(+) cells on day 7. c) and d): Nkx2.5/GFP(+) cells on day 15. Shown are representative images obtained employing a confocal microscope. Cells were sorted by FACS, then fixed and stained with the indicated antibody (red signal). Nuclei were stained using DAPI (blue signal). C: Transcripts of HCN genes in purified Flk1(+) cells on days 7 and 15 of their differentiation. Cells were sorted by FACS, and then mRNA was extracted from them. M: Molecular marker, C: β-actin, 1: HCN1, 2: HCN2, 3: HCN3, 4: HCN4.

shows the transcripts of HCN genes in Nkx2.5/GFP(+) cells. The bands at the predicted size of HCN1 and HCN4 genes were detected in Nkx2.5/GFP(+) cells from EBs harvested on day 15, although these transcripts were not detected in these cells on day 7. The immunoreactivity of HCN1 and 4 proteins was also detected in Nkx2.5/GFP(+) cells on day 15 of their differentiation (Fig. 3Bc, d), but not on day 7 (Fig. 3Ba, b). On the other hand, as shown in Fig. 3C, Flk1(+) cells did not express HCN gene transcripts on day 7 nor on day 15 under the differentiation conditions of these experiments, indicating that Nkx2.5/GFP(+) cells but not Flk1(+) cells experienced changes in the expression of HCN genes during their differentiation under the present experimental conditions.

Cs^+-sensitive automaticity and I_f currents in Nkx2.5-positive cells during differentiation

Figure 4A shows the effects of extracellular Cs^+ (2 mM) on action potentials (APs) in Nkx2.5/GFP(+) cells on days 7 and 15 of their differentiation. Cs^+ at 2 mM prolonged the cycle of spontaneous APs in the cells from EBs harvested on day 15 (panel b), but it had little effect on automaticity in the cells from EBs harvested on day 7 (panel a). The data obtained from multiple experiments (Fig. 4B) indicated that Cs^+ at 2 mM prolonged the cycle of spontaneous APs by 126.2 ± 50.3% (from 406.6 ± 77.2 ms to 851.1 ± 163.5 ms, $P < 0.05$) in cells from day 15, but only by 15.3 ± 6.7% (from 874.6 ± 176.5 ms to 1022 ± 221.2 ms, $P < 0.05$) in cells from day 7. We then compared the density and kinetics of I_f currents in Nkx2.5/GFP(+) cells on days 7 and 15 of their differentiation. I_f currents were not detected on day 7 of cell differentiation (data not shown), but they were significantly activated on day 15 (Fig. 4C) and comparable with the data of Cs^+-induced changes in APs in Nkx2.5/GFP(+) cells from day 15. The activation curve was determined by measuring tail currents at +15 mV upon fully activating I_f currents by applying 3 s hyperpolarizing voltage steps ranging from −40 to −130 mV, yielding a $V_{1/2}$ of −96.2 ± 2.2 mV and slope factor of 9.1 ± 0.7 (Fig. 4D).

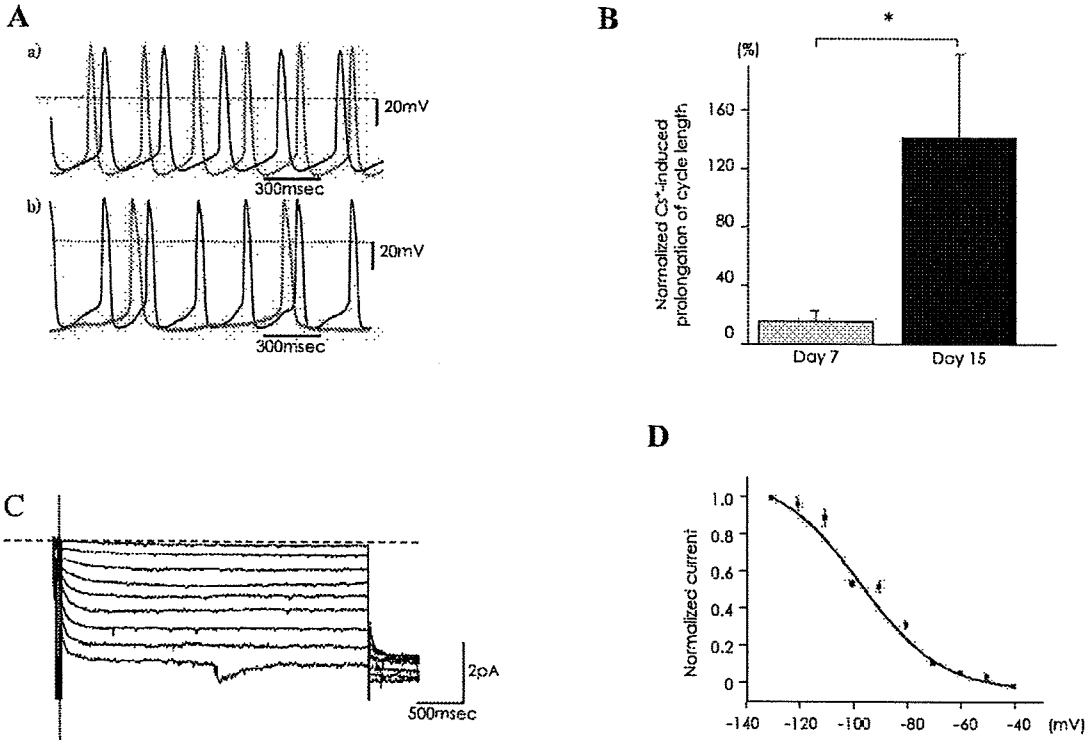

Fig. 4 Changes in both Cs^+-sensitive automaticity and I_f currents in Nkx2.5-positive cells on days 7 and 15 of their differentiation. A: Spontaneous action potentials recorded by perforated patch-clamp in the presence (red) and absence (black) of 2 mM Cs^+ in Nkx2.5/GFP(+) cells on days 7 (a) and 15 (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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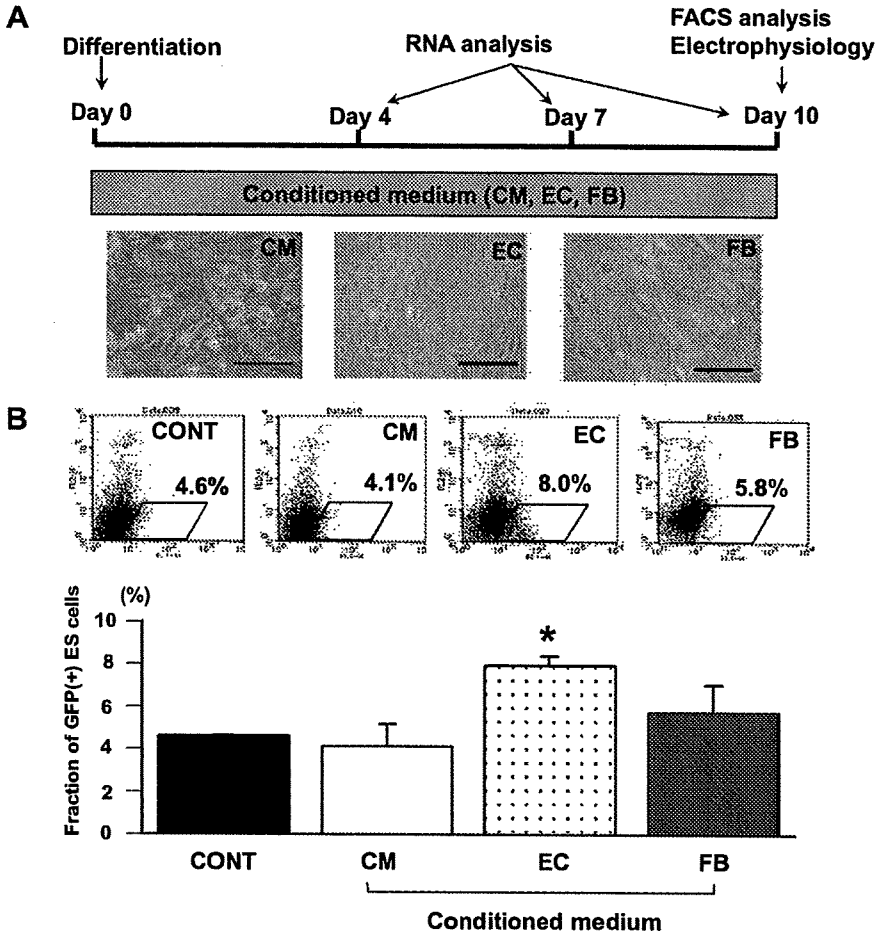

Fig. 1. Effects of conditioned medium on cardiac differentiation of ES cells. (A) Experimental protocols. Conditioned medium from neonatal cardiomyocytes (CM), bovine aortic endothelial cells (EC) or embryonic fibroblasts (FB) was added to the differentiation medium of Nkx2.5-GFP ES cells throughout 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 (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: 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*,

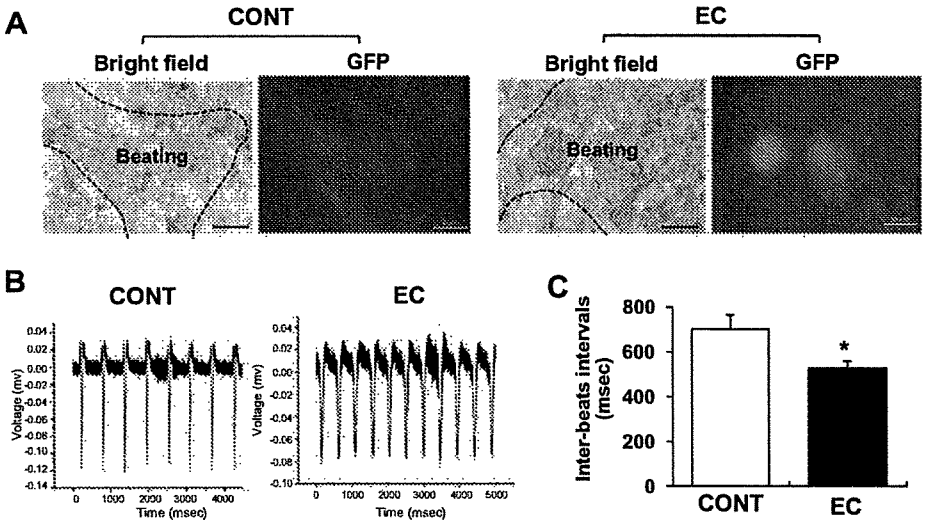

Fig. 2. Spontaneous beating of embryoid bodies differentiated from ES cells. (A) Spontaneously beating areas (inside of dotted line) of EBs at Day 10 in the absence (CONT) and in the presence of EC-conditioned medium (EC). Discernible fluorescence in the Nkx2.5-GFP EBs was detected in both groups. Solid 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.

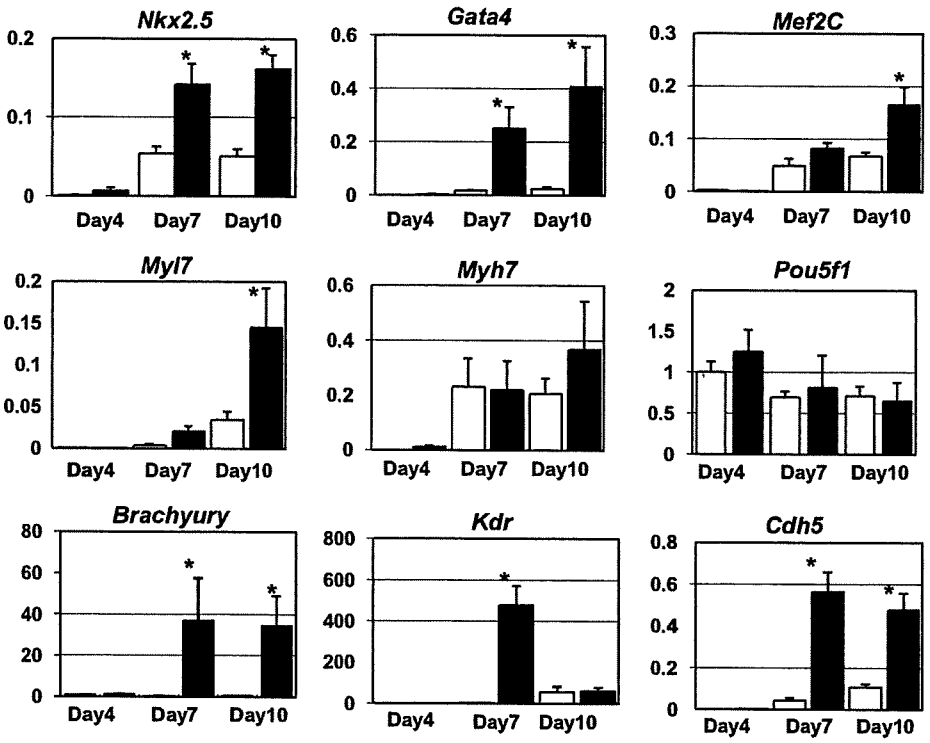

Fig. 3. Gene expression profiles of lineage markers during ES differentiation. RNA was harvested from cultured EBs at different stages of differentiation (Day 4, Day 7, and Day 10) in the absence (Control, open columns) and in the presence of EC-conditioned medium (solid columns). Real-time RT-PCR analysis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

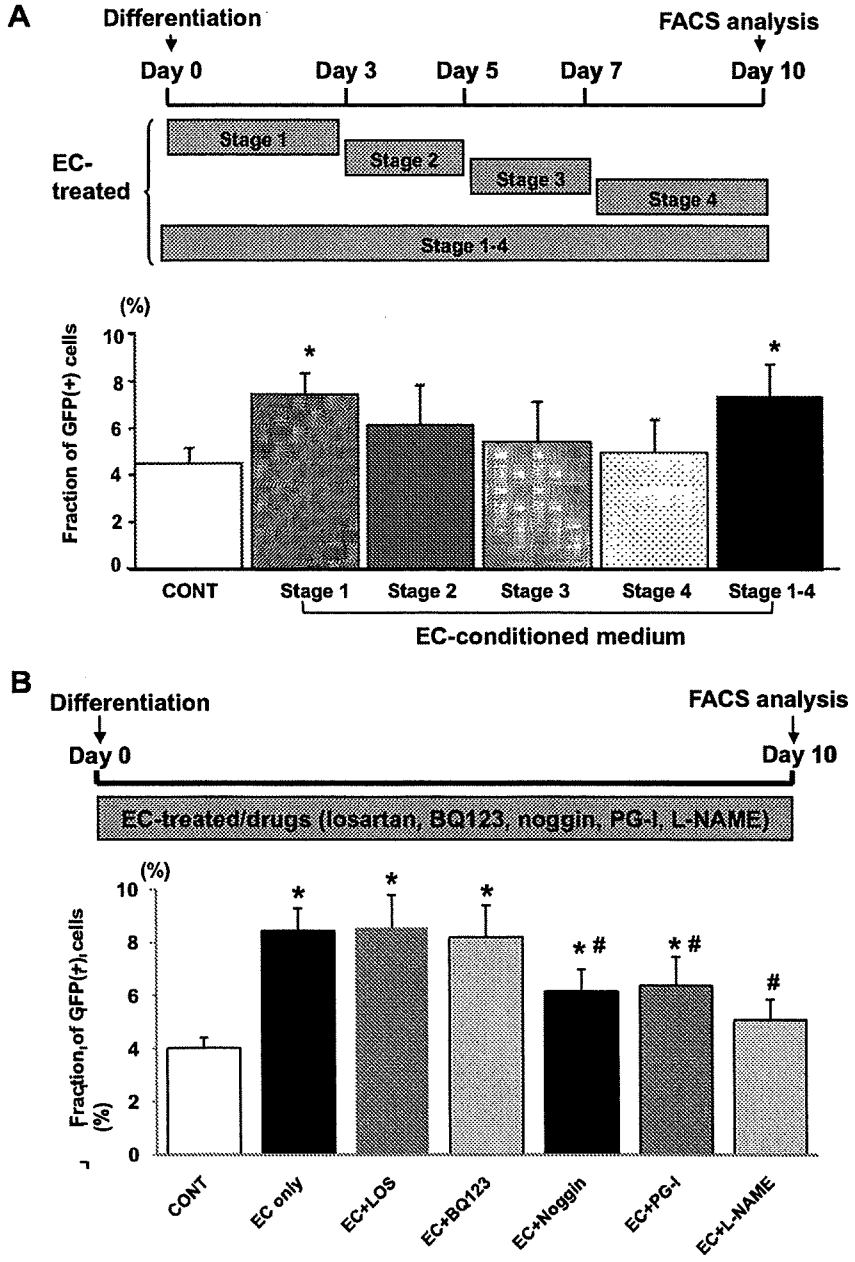

Fig. 4. Underlying mechanisms of EC-conditioned medium induced-promotion of cardiogenesis. (A) Stage-dependent effects of EC-conditioned medium on cardiac differentiation of ES cells. The whole EB-culture period from the initiation of differentiation were divided into four stages; Stage1 for Day 0–3, 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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RNA-binding proteins Rbm38 and Rbm24 regulate myogenic differentiation via p21-dependent and -independent regulatory pathways

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Skeletal muscle differentiation entails organized sequential events, including cell cycle arrest of proliferating myoblast cells and cell fusion, which lead to the formation of multinucleated myotubes. This process involves both transcriptional and post-transcriptional regulation of the gene expression of myogenic proteins, as well as cell-cycle related proteins. RNA-binding proteins bind to specific sequences of target RNA and regulate gene expression in a post-transcriptional manner. However, few tissue-specific RNA binding proteins have been identified. Herein, we report that the RNA binding proteins Rbm24 and Rbm38 were found to be preferentially expressed in muscle during differentiation *in vitro*. Further, knockdown of either by RNA interference suppressed cell-cycle arrest and delayed myogenic differentiation in C2C12 cells. In contrast, over-expression of Rbm24 or Rbm38 induced cell cycle arrest, and then had a positive effect on myogenic differentiation. Immunoprecipitation-RT-PCR analysis using tagged Rbm proteins indicated that Rbm38 binds to the p21 transcript *in vivo*. Consistent with this, differentiation of Rbm38 knockdown cells was rescued by over-expression of p21. Together, our results suggest that Rbm38 plays a crucial role in cell cycle arrest and myogenic differentiation via its binding to p21.

Introduction

In myogenic differentiation, proliferating myoblasts first exit from the cell cycle and are fused to form multinucleated myotubes with a contractile phenotype and then myofibers. These transition steps are known to be controlled by myogenic regulatory factors (MRFs), such as MyoD, Myf5, myogenin and MRF4. Indeed, muscle progenitor cells remain undifferentiated in independent myogenic compartments during embryonic development when these MRFs are missing (Kablar *et al.* 2003). It is known that MRFs and p21cip1 (p21), a cyclin-dependent kinase inhibitor, coregulate each other, and that p21 inhibits the activity of cyclin/cdk2 complexes and regulates mammalian cell cycle arrest (el-Deiry *et al.* 1993, 1994; Brugarolas *et al.* 1999), which is essential for myogenic differentiation. MyoD also activates p21 and induces the withdrawal of myoblasts from the cell cycle, an integral part of myogenic differentiation (Sorrentino *et al.* 1990; Guo *et al.* 1995). In myogenic cells, p21 is increasingly expressed during the G1 phase of the cell cycle, although a high level is required for myotube maintenance (Odelberg *et al.* 2000), while p21 induces myogenin expression during the myoblast-to-myotube transition (Halevy *et al.* 1995). Therefore, skeletal muscle differentiation entails the coordination of MRFs and terminal withdrawal from the cell cycle. Early studies using cultured myoblasts showed that cell-cycle exit and differentiation are coupled (Bischoff & Holtzer 1969; Nadal-Ginard 1978; Clegg *et al.* 1987). Mice lacking p21 undergo normal development, but are defective in G1 checkpoint control (Deng *et al.* 1995). Mice lacking both Cdk inhibitors, p21 and p57, display

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severe skeletal muscle defects, manifested as a failure to form myotubes, with increased proliferation of myoblasts (Zhang *et al.* 1999). However, the molecules controlling cell-cycle exit and the differentiation steps dependent on cell-cycle arrest are poorly understood.

In addition to transcriptional regulation, post-transcriptional regulation of muscle-specific genes has important roles in myogenesis. RNA-binding proteins are known to regulate gene expression in a post-transcriptional manner, such as for RNA splicing, transport, stability, polyadenylation and translation (Krecic & Swanson 1999), which have recently been noted to be critical mechanisms for gene regulation in mammalian cells. For example, HuR, which contains the RNA recognition motif (RRM), is known to be associated with the AU-rich element (ARE) in the 3′-UTR of MyoD, myogenin and p21 mRNA, and also contributes to the progression of myogenesis by stabilizing mRNA (Wang *et al.* 2000; Figueroa *et al.* 2003). Another RNA binding protein, NF90, containing two double-stranded RNA-binding domains, is also associated with and stabilizes the mRNA of its targets, MyoD and p21, by binding to the ARE in the 3′-UTR in developing muscle (Shim *et al.* 2002). However, expression of these RNA binding proteins is not muscle-specific, as HuR is also expressed in intestinal epithelial cells, where it modulates the stability of its target, activating transcription factor-2 (ATF-2) mRNA(Xiao *et al.* 2007). NF90 is also strongly expressed in testis and brain tissues, although it has moderate expression in the heart, spleen, lungs, liver and kidneys (Shi *et al.* 2005). Therefore, muscle-specific RNA binding proteins have never been reported in mammals and their mechanisms of post-transcriptional regulation during myogenesis remain unclear.

Previously, we identified several genes that are specifically expressed during the course of cell differentiation of ES cells using DNA microarray analysis (Terami *et al.* 2007), with Rbm24 shown to be one of these genes. A homology search with the deduced amino acid sequences showed that the Rbm24 gene product shares a significant similarity with that of Rbm38, suggesting that these genes are paralogues. A previous study showed that the *Caenorhabditis elegans* homologue of these genes, sup12, specifically regulated expression of a muscle-specific gene during myogenic development (Anyanful *et al.* 2004). Furthermore, human Rbm38 (also known as RNPC1) was previously investigated using a human colorectal cancer cell line and shown to induce cell cycle arrest in the G1 phase by regulating the stability of p21 mRNA. In the present study, we showed that Rbm24 and Rbm38 are RNA-binding proteins preferentially expressed in cardiac and skeletal muscle tissues, and then investigated the functions of these proteins for myogenesis, and found that both regulate myogenic differentiation by controlling the cell cycle in a p21-dependent or -independent manner.

Results

Expressions of Rbm24 and Rbm38 in cardiac and skeletal muscle tissues

We selected several genes specifically expressed during cell differentiation of multipotential ES cells using DNA microarray analysis (Terami *et al.* 2007) and considered them as candidate genes that function in cell differentiation processes for the present experiments. Rbm24, found to be increasingly expressed during cardiomyocyte differentiation, was selected as one of these candidate genes. This gene is an RNA binding protein that contains an RRM, which is the most prevalent type of eukaryotic RNA-binding motif (Dreyfuss *et al.* 1993). In addition, Rbm38 was also selected, because it is the paralogue of Rbm24. To investigate the expression profiles of Rbm24 and Rbm38, QRT-PCR analysis was carried out using various organs and tissues from adult mice (Fig. 1A,B). Consistent with the muscle-tissue specific expression of sup12 in *C. elegans*, both Rbm24 and Rbm38 were found to be preferentially expressed in cardiac and skeletal muscle tissues. Furthermore, we monitored the expression profiles of Rbm24 and Rbm38 during the course of myogenic differentiation of C2C12 myoblast cells (Yamaguchi 1995) using QRT-PCR analysis (Fig. 1C–F), which showed that their mRNA expression increased when the myoblast-to-myotube transition occurred in those cells.

Knockdown of Rbm24 and Rbm38 inhibits myogenic differentiation

To investigate whether Rbm24 and Rbm38 play roles in myogenic differentiation, a gene knockdown experiment was conducted using an RNAi method. C2C12 myoblast cells at 100% confluence were transfected with an siRNA duplex for Rbm24 or Rbm38, then differentiation was immediately induced by changing to differentiation medium (Fig. 2A). A non-specific siRNA duplex was also used as the control

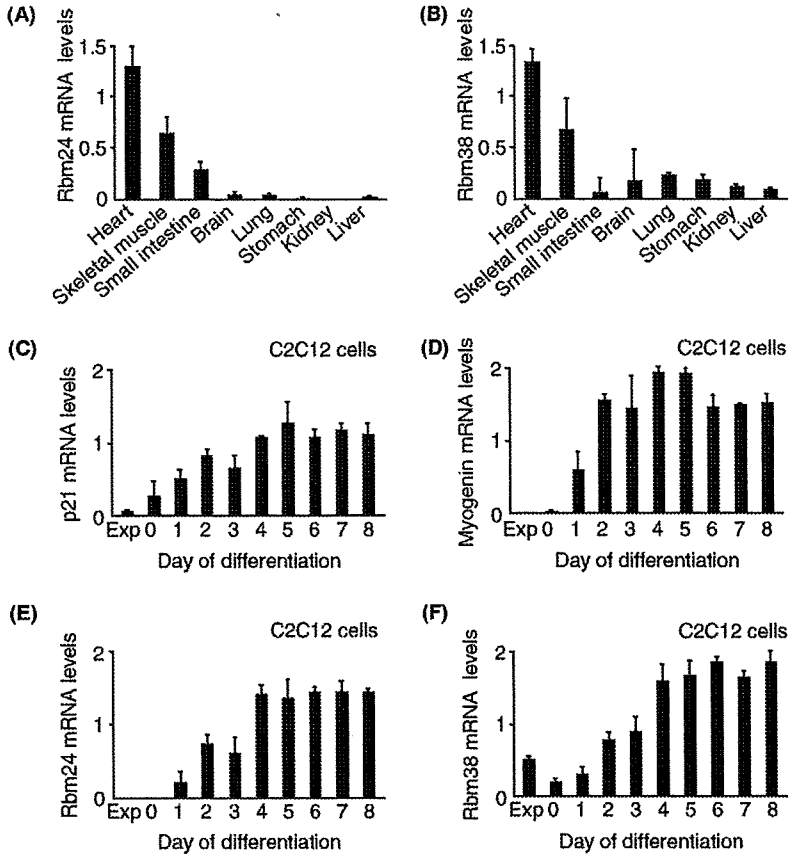

Figure 1 Preferential expressions of Rbm24 and Rbm38, RNA binding proteins, during myogenesis. (A, B) QRT-PCR analyses were carried out using various organs and tissues from adult mice. RNA was reverse-transcribed and then PCR-amplified using Rbm24- or Rbm38-specific primers. Both Rbm24 and Rbm38 were preferentially expressed in muscle tissues. (C, D) QRT-PCR was carried out during the course of myogenic differentiation of C2C12 myoblast cells to detect p21 and myogenin. Expressions of p21 and myogenin were highly maintained in differentiating C2C12 cells. (E, F) QRT-PCR was carried out to detect Rbm24 and Rbm38. The expressions of Rbm24 and Rbm38 were increased in differentiating C2C12 cells. Error bars indicate the standard error. Values shown are the average of three experiments.

siRNA to verify the specificity of the experiments. The inhibitory effects of the siRNA duplexes were examined by QRT-PCR. On day 2 of differentiation, the mRNA levels of Rbm24 and Rbm38 were decreased by more than 80% by transfection with Rbm24 siRNA and Rbm38 siRNA, respectively, as compared with the control siRNA. Next, to assess the effects of their decreased expression on myogenic differentiation, immunofluorescence staining for myosin heavy chain (MyHC) was carried out to check myotube formation on day 4 of differentiation (Fig. 2B,C). We found that MyHC-positive myotubes were significantly decreased in cells transfected with Rbm24 and Rbm38 siRNA as compared with those transfected with the control siRNA. These results suggest that Rbm24 and Rbm38 play important roles in myotube formation during myogenic differentiation.

The first step of myogenic differentiation in a model such as C2C12 cells is the cell cycle arrest of myoblasts, followed by cell fusion and multinucleated myotube formation. As human Rbm38 (RNPC1) is known to induce cell cycle arrest in the G1 phase of RKO cells, a human colorectal cancer cell line (Shu *et al.* 2006), we next investigated whether inhibition of Rbm24 and Rbm38 would affect DNA synthesis or mitosis by performing immunofluorescence staining with the anti-5-bromodeoxyuridine (BrdU) antibody, a marker of DNA synthesis, and the anti-phosphorylated histone H3 (phospho-HH3) antibody,

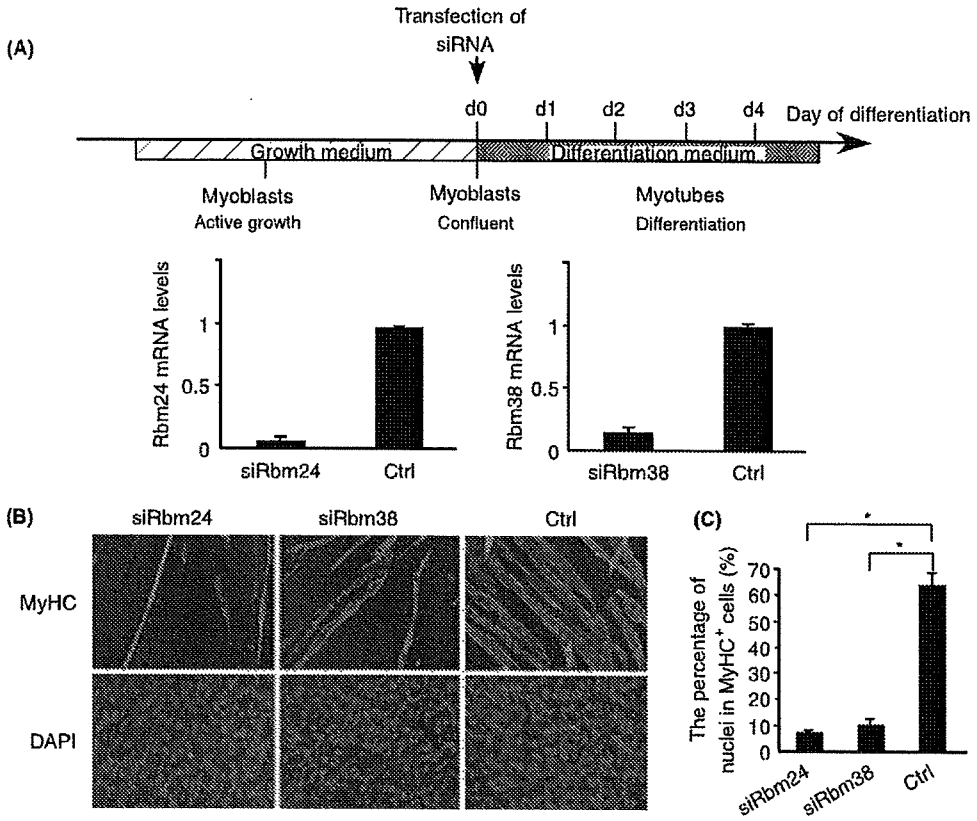

Figure 2 Knockdown of Rbm24 and Rbm38 inhibits myotube formation during C2C12 differentiation. (A) Schematic diagram of siRNA transfection protocol during C2C12 cell differentiation. C2C12 cells at 100% confluence were transfected with Rbm24 or Rbm38 siRNA on the day of differentiation induction. The inhibitory effects of siRNA duplexes were examined on day 2 by QRT-PCR. Error bars indicate the standard error. Values shown are the average of three experiments. (B) C2C12 cells were treated with siRNA on the day of differentiation and fixed on day 4. Cells were stained with anti-MyHC antibody and DAPI to determine their differentiation status. When cells were transfected with the Rbm24 or Rbm38 siRNA duplex, the number of MyHC-positive myotubes was significantly decreased as compared with the control siRNA. (C) The percentage of nuclei in MyHC-positive cells was calculated to assess differentiation efficiency of C2C12 cells treated with siRbm24 or siRbm38. Error bars indicate the standard error. Values shown are the average of three experiments ($*P < 0.005$). siRbm24, Rbm24 siRNA duplex; siRbm38, Rbm38 siRNA duplex; Ctrl, control siRNA duplex.

a marker of mitosis, on day 4 of differentiation (Fig. 3A,B). When the cells were transfected with Rbm38 siRNA, BrdU-positive and phospho-HH3-positive cells were increased by 180% and 110%, respectively, as compared with the control siRNA (Fig. 3C). When transfected with Rbm24 siRNA, BrdU-positive and phospho-HH3-positive cells were increased by 167% and 70%, respectively (Fig. 3C). By prolonging the BrdU incorporation time (24 hours), the percentage of BrdU-positive nuclei increased (17% of total nuclei in Rbm24-knockdown and 16% in Rbm38-knockdown cultures). However, we did not observe a robust increase in numbers of proliferating cells (data not shown). These results suggest that knockdown of Rbm24 and Rbm38 suppresses cell cycle arrest and delays myogenic differentiation, although their effects on cell cycles remain to be determined.

Over-expression of Rbm24 and Rbm38 promotes myogenic differentiation

To further investigate whether Rbm24 and Rbm38 have effects on myogenic differentiation, an experiment was employed utilizing their over-expression. To increase transfection efficiency, plasmids were introduced 1 day before differentiation induction, when the cells had reached 70%–80% confluence.

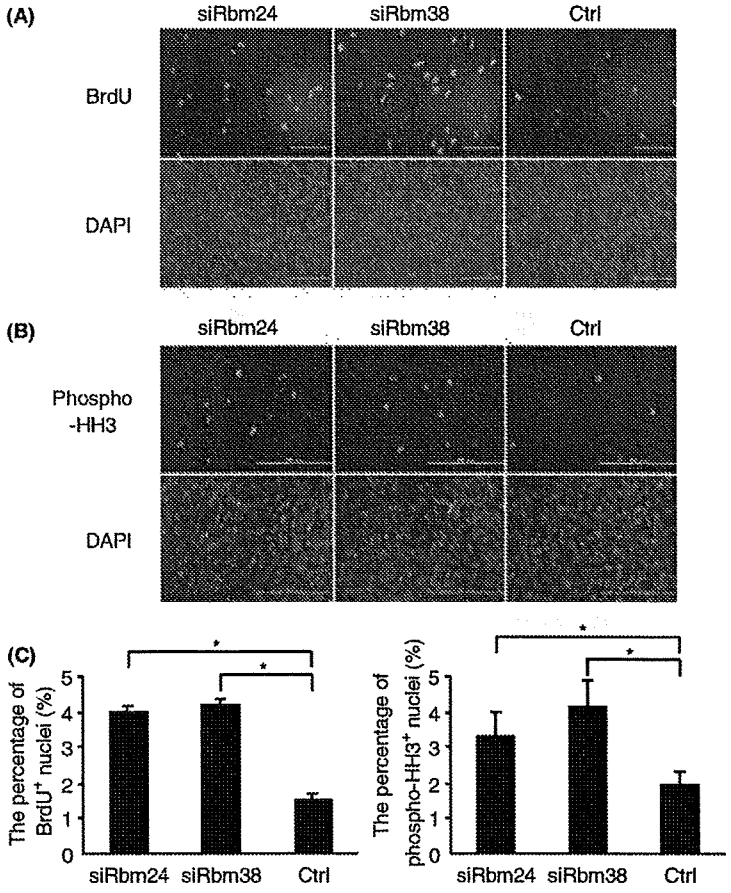

Figure 3 Knockdown of Rbm24 and Rbm38 activates DNA synthesis or mitosis, and inhibits cell cycle arrest. Immunofluorescence staining was carried out to determine the proliferative state of cells transfected with Rbm24 or Rbm38 siRNA. (A) C2C12 cells were treated with siRNA on the day of differentiation induction. On day 4 of differentiation, cells were exposed to BrdU for 2.5 h prior to fixation. Shown are anti-BrdU antibody and DAPI-stained images. (B) Shown are anti-phospho HH3 antibody and DAPI-stained images. (A, B) When cells were transfected with Rbm24 or Rbm38 siRNA, the proportions of BrdU- and phospho-HH3-positive cells were significantly increased as compared with the control. (C) The percentage of BrdU- and phospho-HH3-positive nuclei was calculated to assess the cell cycle status of C2C12 cells treated with siRbm24 or siRbm38. Error bars indicate the standard error. Values shown are the average of three experiments. siRbm24, Rbm24 siRNA duplex; siRbm38, Rbm38 siRNA duplex; Ctrl, control siRNA duplex.

mRNAs for Rbm24 and Rbm38 were each increased to greater than fivefold as compared to that following mock transfection with the control plasmid on day 4 of differentiation (Fig. 4A), after which the effects of over-expressed Rbm24 and Rbm38 on myogenic differentiation were evaluated by immunofluorescence staining to check myotube formation (Fig. 4B,C). In addition, over-expressed p21 in C2C12 cells caused a significant increase in myotube formation, as previously reported (Halevy *et al.* 1995). MyHC-positive myotubes were also significantly increased when the cells were transfected with pCAG-Rbm24 or pCAG-Rbm38, although myotubes with Rbm38 over-expression were longer and larger than those with Rbm24 over-expression, similar to those with p21 over-expression. These results suggest that over-expression of Rbm24 or Rbm38 promotes myogenic differentiation, although the latter has a stronger effect. Considering that 100% of our C2C12 cells did not differentiate into myotubes, these results indicate promotion of myogenic differentiation in nondifferentiating cells that lack differentiation potential.

Next, to investigate whether over-expressed Rbm24 and Rbm38 have effects on DNA synthesis or mitosis, immunofluorescence staining was carried

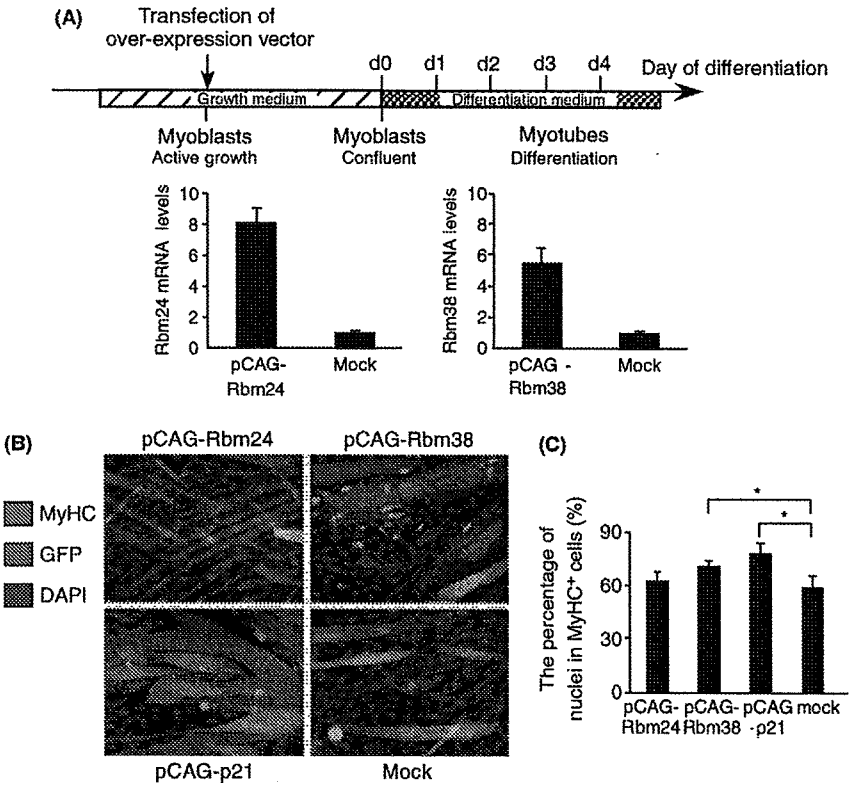

Figure 4 Over-expression of Rbm24 and Rbm38 promote myotube formation during C2C12 differentiation. (A) Schematic diagram of plasmid transfection protocol during C2C12 cell differentiation. Growing C2C12 myoblast cells (70%–80% confluent, 1 day before differentiation) were transfected with an Rbm24- or Rbm38-expressing plasmid, and the effects were examined by QRT-PCR on day 4 of differentiation. The expressions of both Rbm24 and Rbm38 were significantly increased as compared with the control. (B) C2C12 cells were transfected with an Rbm24-, Rbm38- or p21-expressing plasmid. Cells were stained with anti-MyHC and anti-GFP antibodies, and DAPI on day 4 of differentiation to determine their differentiation status. Shown are anti-GFP (green), anti-MyHC (red) and DAPI (blue) stained merged images. When cells were transfected with an Rbm24-, Rbm38- or p21-expressing plasmid, the number of MyHC-positive myotubes was significantly increased as compared with the control. (C) The percentage of nuclei in MyHC-positive cells was calculated to assess the differentiation efficiency of C2C12 cells treated with pCAG-Rbm24, pCAG-Rbm38 or pCAG-p21. Error bars indicate the standard error. Values shown are the average of three experiments ($^*P < 0.005$). pCAG-Rbm24, Rbm24-expressing plasmid; pCAG-Rbm38, Rbm38-expressing plasmid; pCAG-p21, p21-expressing plasmid; mock, control plasmid.

out using anti-BrdU and anti-phospho-HH3 antibodies on day 4 of differentiation (Fig. 5A,B). The number of BrdU-positive cells was decreased when the cells were transfected with the over-expression vector of Rbm24 or Rbm38, with similar results obtained following immunofluorescence staining for phospho-HH3. When the cells were transfected to over-express Rbm38, BrdU-positive cells were decreased by 48% and phospho-HH3-positive cells by 71% as compared with the control, and when the cells were transfected to over-express Rbm24, BrdU-positive cells were decreased by 27% and phospho-HH3-positive cells by 58% as compared with the control (Fig. 5C). These results suggest that over-expressions of Rbm24 and Rbm38 induce cell cycle arrest, which has a positive effect on myogenic differentiation.

Rbm38, but not Rbm24, binds directly to p21 transcripts

Human Rbm38 (RNPC1) is known to regulate the stability of p21 transcripts by binding to their 3′ UTRs (Shu *et al.* 2006). Therefore, mouse Rbm38 was also expected to bind directly to the p21 transcripts, resulting in induction of cell cycle arrest and regulation of myogenic differentiation. To confirm

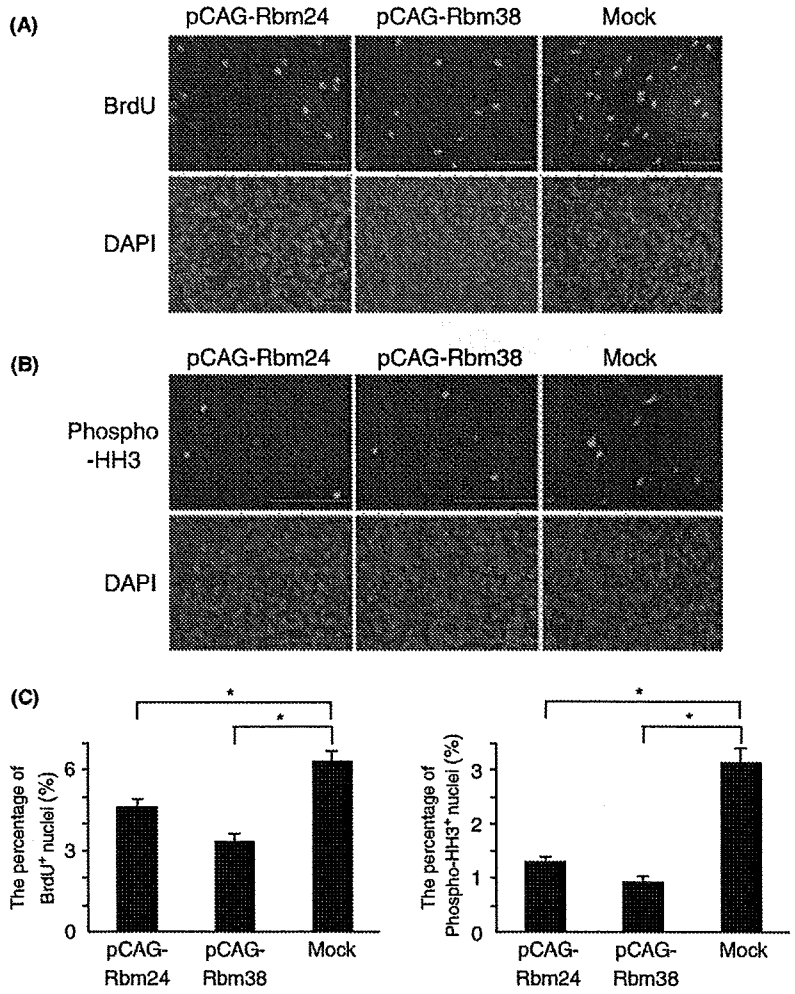

Figure 5 Over-expressed Rbm24 and Rbm38 inhibit DNA synthesis, and promote cell cycle arrest. Immunofluorescence staining was carried out to determine the proliferative state of cells transfected with Rbm24- and Rbm38-expressing plasmids. (A) C2C12 cells were transfected with an Rbm24- or Rbm38-expressing plasmid 1 day before differentiation induction. On day 4 of differentiation, cells were exposed to BrdU for 2.5 h prior to fixation. Shown are anti-BrdU antibody and DAPI-stained images. (B) Shown are anti-phospho HH3 antibody and DAPI-stained images. (A, B) When cells were transfected with Rbm24- and Rbm38-expressing plasmids, the proportions of both BrdU- and phospho-HH3-positive cells were significantly decreased as compared with the control. (C) The percentage of BrdU- and phospho-HH3-positive nuclei was calculated to assess the cell cycle status of C2C12 cells treated with pCAG-Rbm24 or pCAG-Rbm38. Error bars indicate standard error. Values shown are the average of three experiments. pCAG-Rbm24, Rbm24-expressing plasmid; pCAG-Rbm38, Rbm38-expressing plasmid, mock, control plasmid.

this, an RNA immunoprecipitation assay was carried out using cell extracts from C2C12 cells transiently expressing Flag-tagged Rbm24 or Rbm38. An anti-Flag antibody was used to identify Rbm24 and Rbm38 RNA complexes that underwent immunoprecipitation, while the anti-IgG1 antibody was used as a control (Fig. 6, upper). Following RT-PCR amplification, p21 transcripts were detected in association with Rbm38, but not Rbm24 (Fig. 6). These results indicate that Rbm38, but not Rbm24, binds to the p21 transcript and induces cell cycle arrest, resulting in a positive effect on myogenic differentiation.

Next, we carried out a p21 over-expression experiment with Rbm24 and Rbm38 siRNA-treated C2C12 cells to investigate whether their effects on

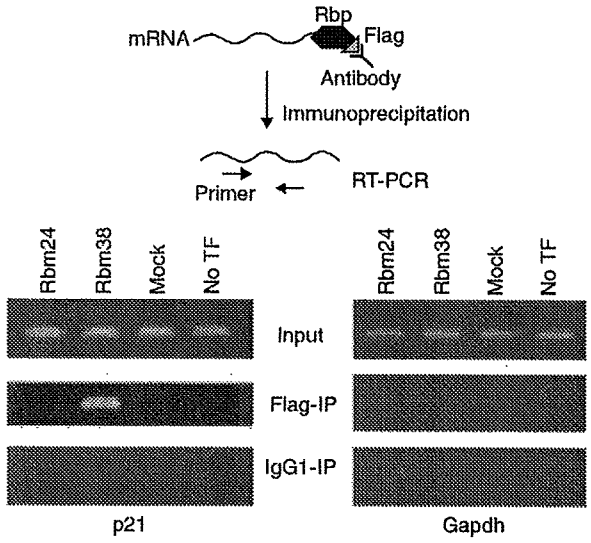

Figure 6 Rbm38, but not Rbm24, binds to p21 transcripts *in vivo*. An RNA immunoprecipitation assay was carried out using extracts from C2C12 cells transiently expressing Flag-tagged Rbm24, Rbm38 or mock. The anti-Flag antibody was used to immunoprecipitate potential Rbm24- and Rbm38-RNA complexes, whereas the anti-IgG1 antibody was used as the control. Upper: Precipitated Rbm24- and Rbm38-RNA complexes were treated with RNase-free DNase to remove trapped genomic DNA, then pull-down RNA was used for cDNA synthesis. Curved lines, target mRNA; black hexagon, RNA binding protein; gray triangle, Flag-tag; Y-shape, antibodies. Lower: p21 and Gapdh transcripts in potential Rbm38-RNA complexes were detected by RT-PCR. Five percent of the cell extract was used as an input control.

myogenic differentiation are mediated by p21. pCAG-p21 or a mock expression vector was transfected into exponentially growing C2C12 cells for 5 hours prior to transfection with Rbm24, Rbm38, or control siRNA (Fig. 7A, upper). Cells without plasmid transfection were used as a control for those with expression vectors. Immunofluorescence staining with anti-MyHC was then carried out to determine the differentiation status of the cells on day 4. Myotube formation was restored in Rbm38 siRNA-treated cells by transfection with a pCAG-p21 expression vector, whereas it was not restored in those cells transfected with the control expression vector or untransfected cells [Fig. 7A (lower), B]. In addition, phospho-HH3-positive nuclei in the cells were determined in the same field (Fig. 7C) to evaluate whether mitotic ability was changed in this rescue experiment. The percentage of phospho-HH3-positive nuclei was decreased by over 78% in Rbm38 siRNA-treated p21 over-expressed cells as compared with the Rbm38 siRNA-treated control cells. In contrast, myotube formation was not sufficiently restored in Rbm24 siRNA-treated cells and phospho-HH3-positive nuclei were not significantly changed by p21 over-expression. These results suggest that the effects of Rbm38 on cell cycle arrest and promotion of myogenic differentiation are correlated with p21 binding, and that Rbm24 regulates myogenic differentiation in a p21-independent manner.

Discussion

In a previous study, human Rbm38 (RNPC1) was identified as a factor that is induced by the p53 family and by DNA damage in a p53-dependent manner (Shu *et al.* 2006). Also, RNPC1 was shown to bind to the 3′ UTR of the p21 transcript and regulate its stability (Shu *et al.* 2006). However, Rbm38 has only been investigated in human cancer cell lines such as RKO cells, whereas its function with myogenesis has not been reported. It is generally known that p21 is transcriptionally regulated during the cell cycle transition from G1 to S by members of the p53 family, such as p53, p63 and p73 (el-Deiry *et al.* 1993; Zhu *et al.* 1998). In addition, post-transcriptional modulation of p21 is important for maintaining p21 function. For example, HuR, which is translocated from the nucleus to cytosol, is known to bind to the ARE in the 3′ UTR of the p21 transcript and enhance its stability (Wang *et al.* 2000; Giles *et al.* 2003; Yang *et al.* 2004). However, the post-transcriptional modulatory function of HuR is not tissue-specific, because it is also expressed and functions in intestine, thymus, spleen and testis tissues, in addition to skeletal muscles (Lu & Schneider 2004). Thus, we are the first to show that Rbm38 is an RNA binding protein that is preferentially expressed in muscle, and regulates the cell cycle and myogenesis through interaction with p21 transcripts.

A previously reported analysis suggested that RNPC1 is responsive outside of the conserved ARE in the 3′-UTR of p21 (Shu *et al.* 2006); therefore, it is possible that mouse Rbm38 may not share the binding region with HuR and NF90. Identifying the binding region of mouse Rbm38 for p21 is needed to further investigate its functions as an RNA binding protein.

In our previous report, we identified Rbm24 as a gene that is particularly expressed during the course of ES cell differentiation using DNA microarray analysis (Terami *et al.* 2007). Its expression during embryogenesis was investigated using whole mount *in situ* hybridization analysis, which showed that

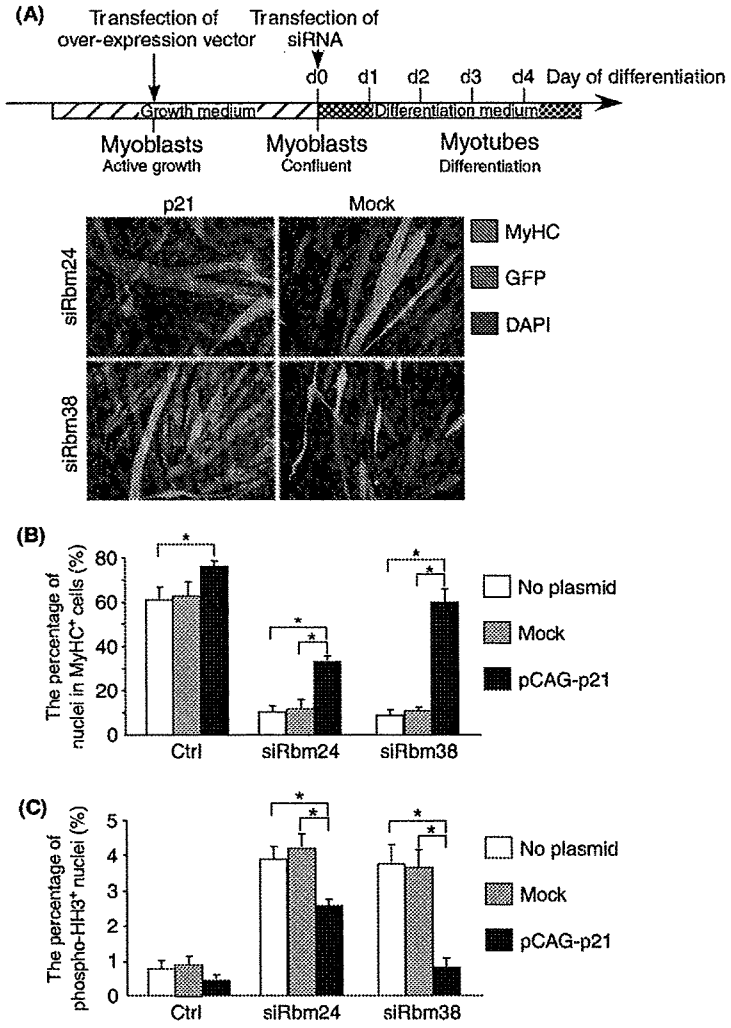

Figure 7 Differentiation of C2C12 with silenced expression of Rbm38 is rescued by p21 expression. (A) Schematic diagram of rescue experiments conducted during the course of myogenic differentiation of C2C12 cells. Growing C2C12 myoblast cells were transfected with a p21-expressing plasmid or control plasmid, and grown for 1 additional day. After the cells had reached 100% confluence, they were transfected with a Rbm24 or Rbm38 siRNA duplex, or control siRNA duplex and differentiation was induced. Immunofluorescence staining with anti-MyHC and anti-GFP antibodies was carried out to determine the differentiation status of the C2C12 cells on day 4 of differentiation. Shown are anti-MyHC (red), anti-GFP (green) and DAPI (blue) stained merged images. When cells were transfected with Rbm38 siRNA, myotube formation was restored by addition of a p21-expressing plasmid as compared with mock. In contrast, myotube formation was not restored in Rbm24 siRNA-treated cells by the addition of a p21-expressing plasmid. (B) The percentage of nuclei in MyHC-positive cells was calculated to assess the differentiation efficiency of C2C12 cells. Error bars indicate the standard error. Values shown are the average of three experiments ($*P < 0.005$). (C) The percentage of phospho-HH3-positive nuclei was calculated to assess the cell cycle status of C2C12 cells. Error bars indicate the standard error. Values shown are the average of three experiments. pCAG-Rbm24, Rbm24-expressing plasmid; pCAG-Rbm38, Rbm38-expressing plasmid; mock, control plasmid; siRbm24, Rbm24 siRNA duplex; siRbm38, Rbm38 siRNA duplex; Ctrl, control siRNA duplex; pCAG-p21, p21-expressing plasmid; mock, control plasmid; No plasmid, no plasmid.

Rbm24 is specifically expressed in the heart region on E8.25, and in the heart and somite regions on E 9.25 (data not shown), which were consistent with the findings of another recent report (Miller *et al.* 2008). In the present study, we found that the expression of Rbm24 is indeed muscle-specific and

that it regulates myogenesis by modulating the cell cycle. However, in contrast to Rbm38, Rbm24 did not bind to the p21 transcripts and Rbm24-knockdown myogenesis-suppressed cells were not sufficiently rescued by p21 over-expression, although Rbm24-knockdown cells inhibited cell cycle arrest and then prevented myotube formation. These findings suggest that Rbm24 is involved in control of the cell cycle and then regulation of myogenesis in a manner different from that of Rbm38. Although evidence is lacking, cyclin-dependent kinase inhibitors including p27 and p57 may be candidate targets controlled by Rbm24.

Although the effects of over-expressed Rbm24 and Rbm38 have not been verified *in vivo*, we are in the process of generating transgenic mice models and our preliminary results indicate that over-expression of Rbm24 causes embryonic lethality (data not shown). Such lethality might be triggered by an ectopic increase in Rbm24, resulting in acceleration of cell cycle arrest and interruption of muscle development, as shown *in vitro*. These preliminary results also support the notion that Rbm24, and probably Rbm38 as well, plays a critical role in myogenic development, although conditional expression model mice using Rbm24 will be required to further investigate its functions. We also intend to clarify the roles of Rbm24 and Rbm38 in skeletal muscle regeneration. According to results of DNA microarray analysis of quiescent and activated satellite cells, Rbm24 and Rbm38 are not expressed in those cells (Fukada *et al.* 2007). It would be interesting to examine whether Rbm24 and Rbm38 are up-regulated during the course of differentiation of satellite cells.

In conclusion, our findings are the first to show that the RNA-binding proteins Rbm24 and Rbm38 play critical roles in myogenic differentiation by regulating the cell cycle in mammals. Moreover, we found that Rbm38 induces cell cycle arrest by binding to p21 transcripts and regulating them. These results provide important information to understand myogenic mechanisms, myogenic diseases and tissue-specific RNA processing.

Experimental procedures

Plasmids

Expression vectors pCAG-Rbm24, pCAG-Rbm38 and pCAG-p21 were constructed as follows. cDNAs encoding Rbm24, Rbm38 and p21 were amplified separately by RT-PCR using total RNA from C2C12, with the following primers: sense primer for Rbm24, 5′-GGTATGCTCGAGATGCACACCACCCAGAAG-3′; antisense primer for Rbm24, 5′-GTGAGATATCGGGCCCTTACTACTGCATTC-3′; sense primer for Rbm38, 5′-GGTATGCTCGAGATGCTGCTGCAGCCCGCGT-3′; antisense primer for Rbm38, 5′-TATCGCGGCCGCGCATCACTGCATCCTGTCAGG-3′; sense primer for p21, 5′-GGTATGCTCGAGATGTCCAATCCTGGTGATG-3′; antisense primer for p21, 5′-TATCGCGGCCGCACTTCAGGGTTTTCTCTTGCA-3′.

These cDNAs were cloned into a pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA) and confirmed by sequencing. The entire coding region of each was subcloned into pCAG-FLAG-Hras-IRES-EGFP (Mochizuki *et al.* 2001) by replacing with an Hras sequence. The control plasmid was generated by removing the Rbm38 sequence from pCAG-Rbm38.

Cell culture

C2C12 cells (ATCC) were grown and maintained in Dulbecco's modified eagle medium (DMEM; Sigma, St Louis, MO, USA) containing 10% fetal bovine serum (MBL) and penicillin/streptomycin (Invitrogen), following the manufacturer's instructions (Invitrogen). First, 5×10^4 cells were seeded in 1.88-cm^2 wells, then differentiation was induced after 2 days, immediately after the cells had reached 100% confluence. To induce differentiation, growth medium was replaced with differentiation medium containing DMEM and 2% horse serum (Invitrogen). Although C2C12 cells do not differentiate as efficiently as satellite cells do, they are regarded as effective to investigate molecular functions in myogenesis.

Gene knockdown and over-expression

For the gene knockdown experiments with siRNA transfections, Lipofectamine RNAiMAX (Invitrogen) was used according to the manufacturer's protocol. Transfection with Rbm24 or Rbm38 siRNA, or nonspecific siRNA (Stealth RNAi; Invitrogen) was carried out after C2C12 cells had reached 100% confluence, then differentiation was induced by changing the medium to differentiation medium. For the over-expression experiments, transfection was carried out 1 day before the C2C12 cells had reached 100% confluence, in order to increase transfection efficiency. Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's instructions. Growing C2C12 cells (70%–80% confluent) were transfected with pCAG-Rbm24, -38, -p21 or the control plasmid, the cultured for one additional day until the cells became 100% confluent. Confluent cells were then differentiated by changing the medium to differentiation medium. For the rescue experiments, plasmid transfection was carried out 1 day before siRNA transfection and differentiation induction.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 minutes at 4 °C and washed with PBS three times for 5 min. After background binding was blocked