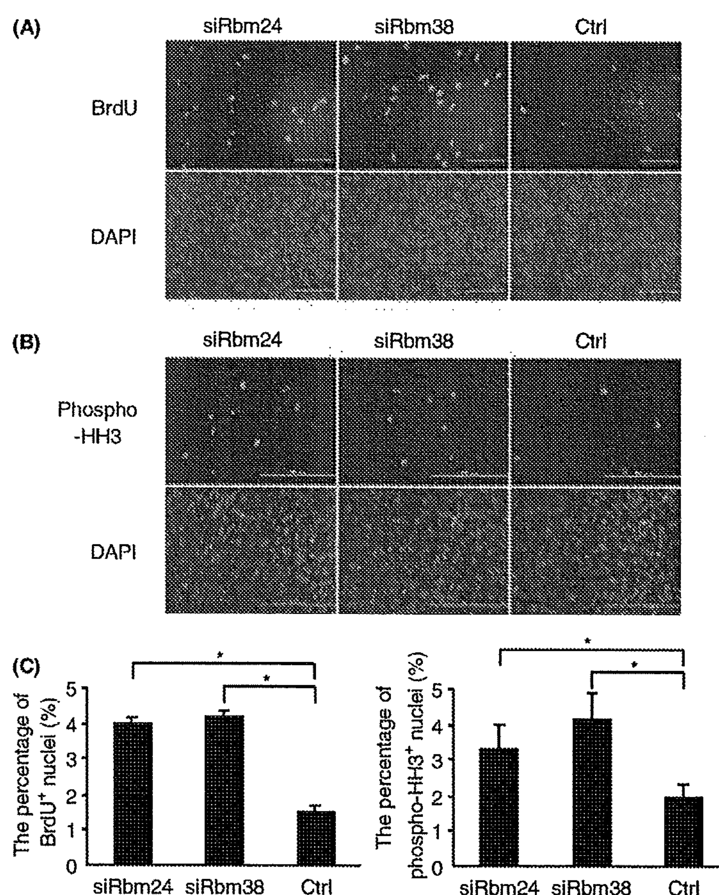

Figure 3 Knockdown of Rbm24 and Rbm38 activates DNA synthesis or mitosis, and inhibits cell cycle arrest. Immunofluorescence staining was carried out to determine the proliferative state of cells transfected with Rbm24 or Rbm38 siRNA. (A) C2C12 cells were treated with siRNA on the day of differentiation 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

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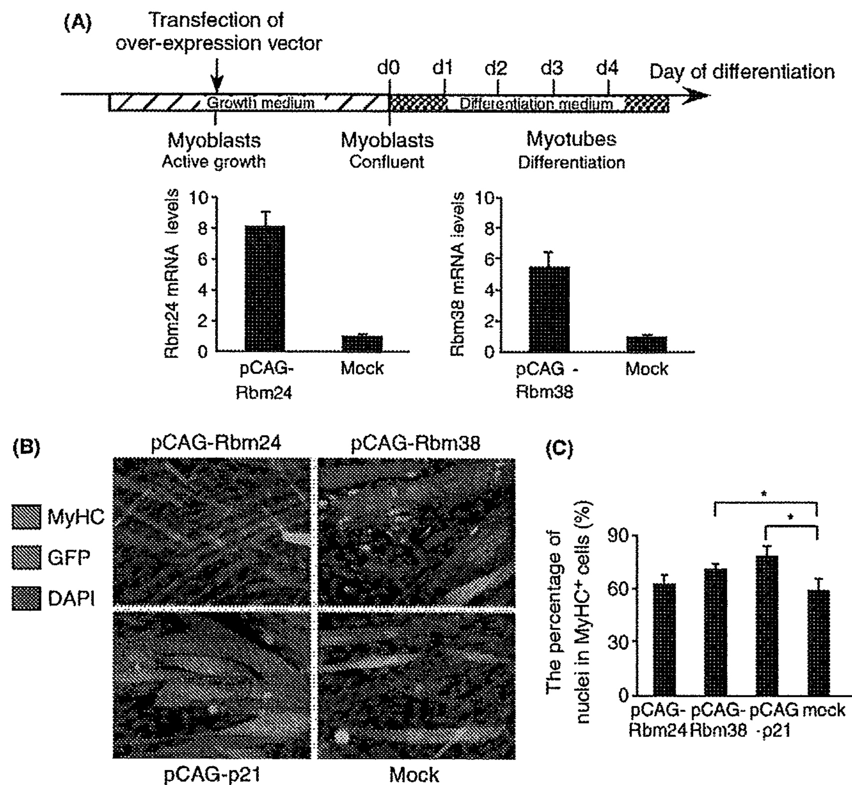

Figure 4 Over-expression of Rbm24 and Rbm38 promote myotube formation during C2C12 differentiation. (A) Schematic diagram of plasmid transfection protocol during C2C12 cell differentiation. Growing C2C12 myoblast cells (70%–80% confluent, 1 day before differentiation) were transfected with an Rbm24- 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

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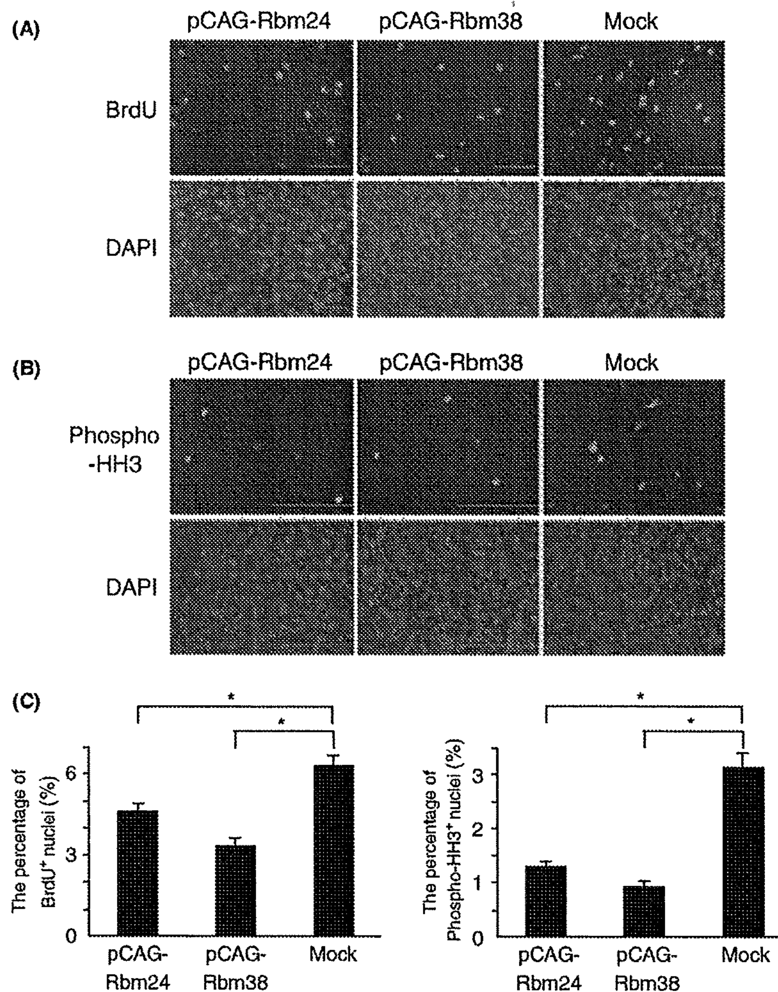

Figure 5 Over-expressed Rbm24 and Rbm38 inhibit DNA synthesis, and promote cell cycle arrest. Immunofluorescence staining was carried out to determine the proliferative state of cells transfected with Rbm24- and Rbm38-expressing plasmids. (A) C2C12 cells were transfected with an Rbm24- or Rbm38-expressing 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

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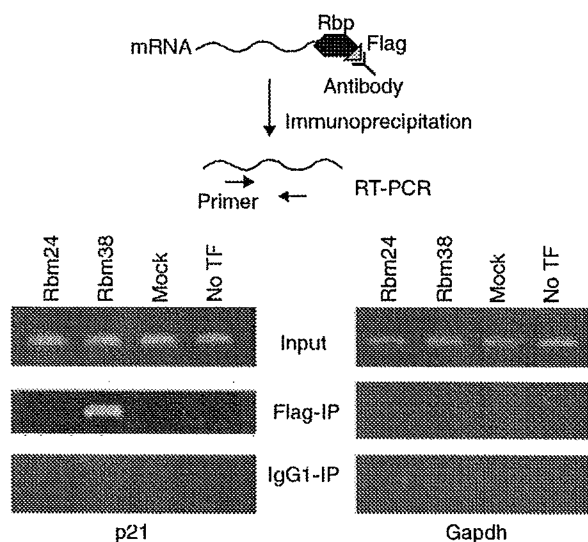

Figure 6 Rbm38, but not Rbm24, binds to p21 transcripts *in vivo*. An RNA immunoprecipitation assay was carried out using extracts from C2C12 cells transiently expressing Flag-tagged Rbm24, Rbm38 or mock. The anti-Flag antibody was used to immunoprecipitate potential Rbm24- and Rbm38-RNA complexes, whereas 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

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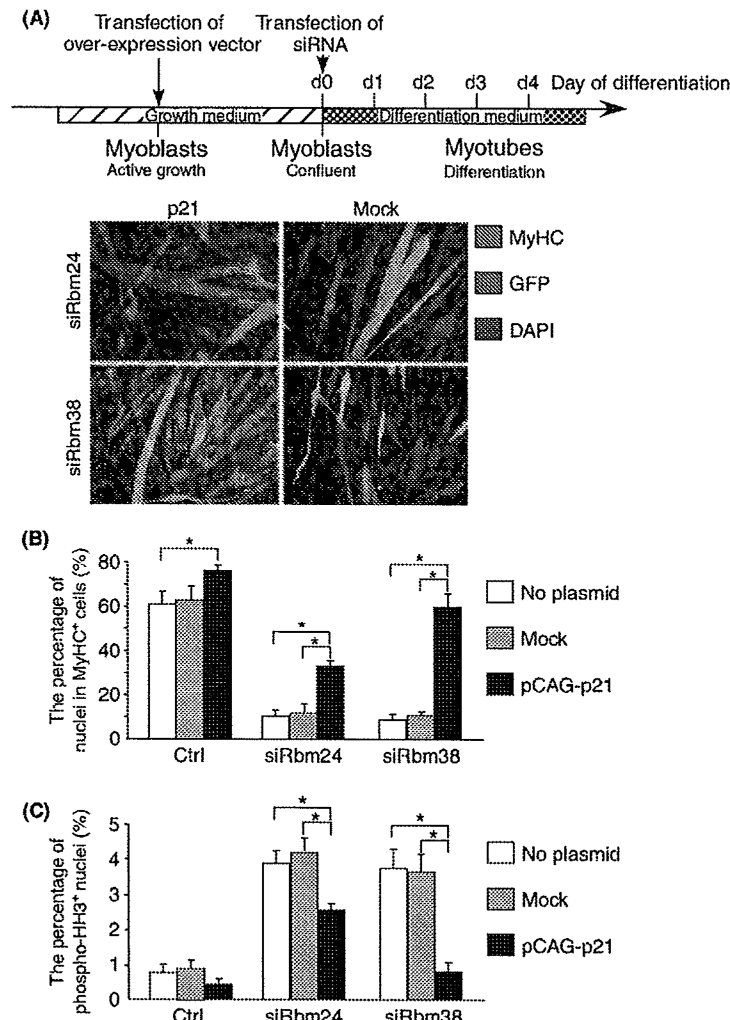

Figure 7 Differentiation of C2C12 with silenced expression of Rbm38 is rescued by p21 expression. (A) Schematic diagram of rescue experiments conducted during the course of myogenic differentiation of C2C12 cells. Growing C2C12 myoblast cells were transfected with a p21-expressing plasmid or control 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

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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

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by incubation in PBS-MT (PBS, 2% skim milk, 0.1% Triton X-100) for 30 min at room temperature, the cells were incubated with primary antibodies in PBS-MT for 1 h at room temperature, followed by incubation with secondary antibodies. The primary antibodies used were as follows: MyHC mouse monoclonal antibody (clone MF20, Hybridoma Bank) to detect differentiated cells, anti-GFP mouse monoclonal antibody (Invitrogen) to detect transfected cells, anti-phosphorylated histone H3 (phospho-HH3) rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA) to detect mitotic nuclei, and anti-BrdU mouse monoclonal antibody (Zymed, South San Francisco, CA, USA) to detect DNA synthesis. The secondary antibodies were as follows: AlexaFluor488-conjugated anti-mouse IgG and AlexaFluor546-conjugated anti-rabbit IgG antibodies (Molecular Probes, Eugene, OR, USA). For a BrdU assay, cell cultures were maintained for 2.5 h with 1 μM of BrdU. 4′,6-Diamidino-2-phenylindole (DAPI) was used to see the nuclei (0.5 μg/mL).

Analysis of interactions between proteins and mRNAs

C2C12 cells were transfected with over-expression vectors expressing FLAG-tagged Rbm24, Rbm38 or mock for 5 h, then immunoprecipitation was carried out as previously described (Peritz *et al.* 2006). The antibody-antigen-RNA binding protein complexes were subjected to immunoprecipitation with protein G agarose beads, then DNase (Invitrogen) treatment was carried out completely before RNA extraction. Five percent of the cell extract was used directly for total RNA isolation, with the remaining portion incubated with Protein G agarose beads (Invitrogen) conjugated with monoclonal anti-Flag M2 (Sigma) or anti-IgG1 control antibody (BD Pharmingen, San Diego, CA, USA) for 4 h. Reverse transcription was carried out using Super Script III (Invitrogen). The sense primer, 5′-CCCTCTCCCAGTCTCCAA-AC-3′ and antisense primer, 5′-TAAGGGCCCTACCGT-CCTAC-3′, were designed to amplify the entire p21 ORF, which was carried out for 1 min at 94 °C, 30 s at 55 °C and 1 min at 72 °C for 35 cycles. The primers used to amplify the GAPDH transcripts were as follows: forward primer, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and reverse primer, 5′-CATGTGGGCCATGAGGTCCACCAC-3′.

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Lecture

解　説

ES細胞*

森﨑隆幸**　日高京子**

Key Words: ES cells, cardiogenesis, differentiation, cardiac marker, progenitor

はじめに

ES細胞は無限増殖能とさまざまな細胞への多分化能の2つの特徴を備えた細胞で，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可能である．1980年にマウスES細胞が樹立され，発生分化の細胞モデルとしての活用や，遺伝子操作を施しての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．1998年にはヒトES細胞が樹立[1]され，ほかに治療方法のない組織の機能障害に対して，再生医療の細胞ソースとして利用できるのではないかとの期待が高まっている．それ以来，研究の推進により，ヒトES細胞を用いた研究成果の蓄積は著しい．しかし，ES細胞の樹立には受精卵からできあがる胚を壊して作製する必要があるため，胚を操作する過程が必須であり，倫理的な問題がある．また，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，研究成果への期待の高まりと同時に，ES細胞の臨床応用や実用化に向けては乗り越えなければならない課題がまだまだ多い．

最近，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，次いでヒトでも可能となった[2)3)]．iPS細胞の詳細は他稿に譲るが，iPS細胞の樹立により胚操作といった倫理的な問題を回避し，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しうることから，臨床応用・実用化可能な幹細胞の利用がついに現実的になった，と考えられるようにもなった．しかし現時点では，iPS細胞にも作製手法をはじめとして未解決の課題は少なくない．したがって現時点では，無限増殖能とさまざまな細胞への多分化能の両者を備えた細胞(幹細胞)について，基礎的な検討や臨床応用に向けての検討には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依然として高い．以上をふまえ本稿では，循環器疾患の再生医療との関連の深いES細胞について，その性質や基礎研究，応用との関係について概説する．

再生医療とES細胞

循環器疾患に対する再生医療は血管新生と心筋再生に大別される．心筋は，生後ほとんど細胞分裂せず一生涯機能する必要があり，いったん傷害を受けると再生はほぼしない．また，心筋細胞分化は発生の最も早期に開始するため，心筋へと分化が可能な細胞はES細胞，胚性腫瘍細胞など限定された細胞のみであり，成体の組織にも心筋細胞へと分化可能な幹細胞の存在は報告されているが，ただちに再生医療への応用が想定されるような分化能の高い細胞はいまだ知られていない．ES細胞はその多くの細胞への分化能の一つとして比較的容易に心筋細胞へと分化することから，循環器疾患との関係では，心血管系への*in vitro*分化系として利用されてき

* ES cells.

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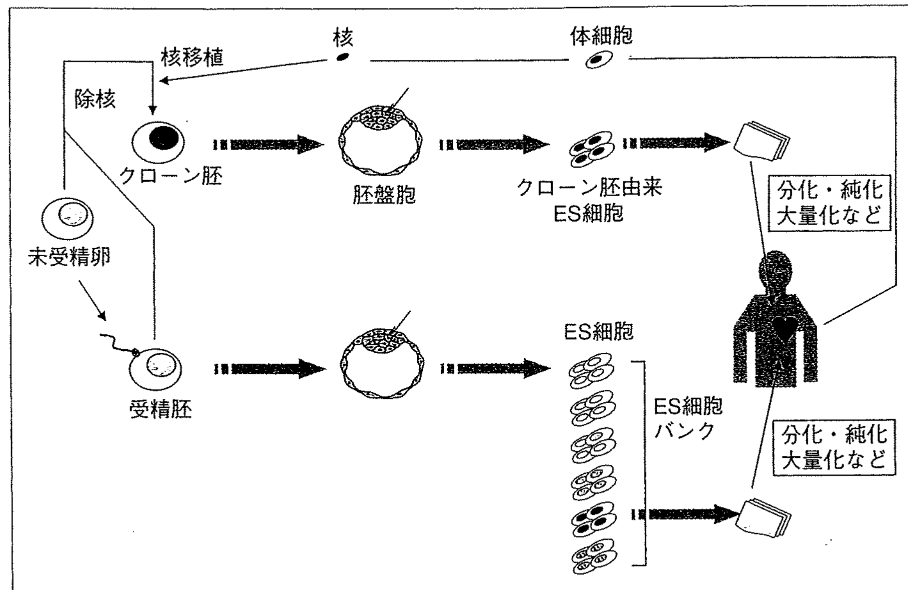

図1　臨床応用にむけたES細胞樹立とその利用
免疫拒絶を克服するには患者本人の遺伝情報を持つクローン胚作製を通してES細胞を作る(クローン胚由来ES細胞)ことが考えられるが，費用や時間などの問題は多い．一方，さまざまなHLAタイプを持ち安全性の担保されたES細胞のバンクがあれば，問題を乗り越えることが可能との考えがある．いずれにせよ，臨床応用に向けて，多数のES細胞株の樹立と，樹立されたES細胞の性質の確認や目的に応じた再現性の高い分化誘導法の確立が必要である．

た．一方，ES細胞は心筋細胞へと分化するとはいえ，ほかの細胞への分化も同時に生じ，心筋細胞のみへ分化を誘導することはまだ可能ではなく，分化した心筋細胞の機能的特徴(心室筋，心房筋，洞房筋)を制御することも困難である．さらに，再生医療へ応用するためには必要な細胞を必要な数だけ準備することも重要な事項となってくる．こうした事項への取り組みについても後述する．

ES細胞の臨床応用への期待と課題

はじめに述べたが，1998年にヒトES細胞が樹立されたのを契機に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実現へのハードルはまだ高い．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」に由来する細胞であり，生体から取り出され，試験管内で樹立された培養細胞であり，無限の増殖能を有する．また原理的には，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可能である．したがって，クローン胚由来ES細胞の樹立が実用化されれば，患者本人と同一の遺伝情報をもつテーラーメイド細胞の利用が可能となり，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，ヒトではクローン胚からES細胞を作製することは成功していないが，将来的には成功するであろうと考えられている．しかし，クローン胚樹立のために核移植を行うために，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．従来，クローン胚樹立には未受精卵子を用いる必要があるとされてきたが，最近，受精胚由来除核卵を用いてもクローン胚樹立が可能との研究成果がマウスで報告されて，3前核胚由来の除核卵を用いてクローン胚樹立を行うことが検討されるなど，倫理的な問題は一部軽減されてきている．しかし，クローン胚樹立にはコストと時間がかかりすぎることは依然として問題であり，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，ES細胞のバンク化を行えばクロー

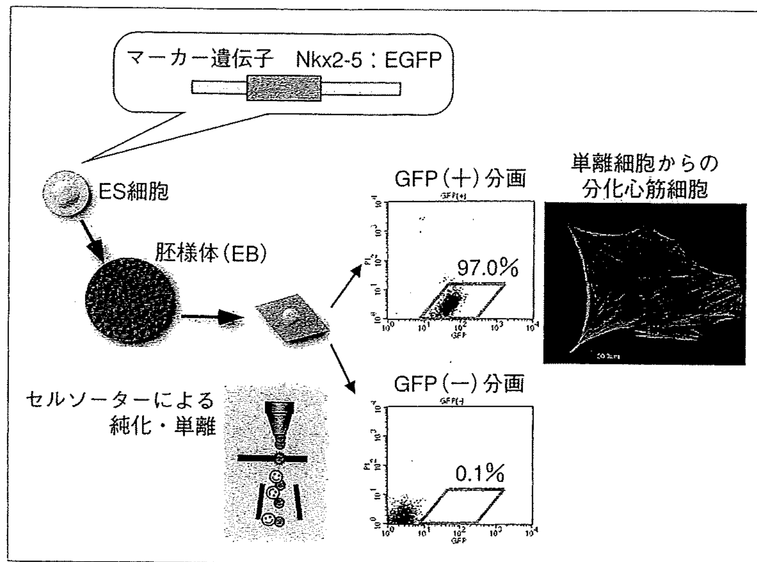

図2 マーカー遺伝子導入とセルソーターによるES細胞由来心筋細胞の単離
心筋ホメオボックス遺伝子Nkx2-5の遺伝子座に蛍光蛋白質遺伝子をノックインしたES細胞を用いると，分化誘導後にセルソーターを利用して心筋細胞を単離することができる．単離した細胞は培養を継続すると心筋収縮蛋白質を発現する．

ン胚を樹立しなくても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)．すなわち，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であって，臨床応用は可能になるであろうというものである[4]．実際には，これだけの数のES細胞株の樹立が本当に可能なのか，余剰胚の提供が今後どのくらい進むのかについては未知の部分も多く，判断はまだ困難である．研究者の立場としては，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し，一般の理解とコンセンサスが高まり，研究の方向性が理解されるよう努力しなければならないと考えている．

ES細胞由来心筋細胞の純化選別：レポーターの利用

先に述べたように，ES細胞は心筋細胞へと分化するが，心筋細胞のみへ分化を誘導することはまだ可能ではない．したがって，ES細胞から心筋分化を誘導し，心筋細胞を単離する系の確立が望まれる．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などによって一部が自動拍動する心筋細胞を生ずる．血球細胞などは細胞特異的な膜蛋白質(膜抗原)が知られるので，目的とする細胞はそれらのマーカーにより分離分別することが可能であるが，心筋細胞にはそのような特異マーカーは知られていない．そこで，心筋特異的に発現する蛍光蛋白質などのマーカー遺伝子を，ES細胞を導入し，マーカーを発現する細胞を認識して分化細胞を分離分別する手法が検討された．筆者の研究グループは，ホメオボックス転写因子Nkx2-5遺伝子座に緑色蛍光蛋白質EGFPをノックインしたマウスES細胞株を樹立し，その細胞をもとに，セルソーターを用いてES細胞由来心筋細胞の単離を検討した[5](図2)．その結果，単離した心筋細胞の培養を続けていくと，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細胞が出現することが明らかとなった．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，心筋細胞の性質の変化が確認された．こうして，EGFPなど「(標識)レポーター」の導入により，

ES細胞から心筋細胞を純化することは可能であり，さらにこうして得られた心筋細胞の性質の変化を追跡しうることが明らかとなった．レポーターとしては蛍光蛋白質のほかに種々の工夫も可能で，磁力による純化も可能であると考えられ，また，磁気を利用するなどして大量調製が可能となるシステムも考えられることから，心筋細胞を高純度で大量に獲得することは，今やかなり現実に近いものになってきたといえる．

網羅的発現遺伝子解析とES細胞の心筋細胞への分化誘導

ES細胞由来の心筋細胞の分化研究は進捗し期待されてはいるが，そのままでは10％内外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り，高効率の細胞分化系の確立が必要である．こうした中，われわれは，Wnt11などの既知の因子などの添加によって分化誘導の改善を試み種々の細胞培養条件を比較検討し[6]，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[7]．この現象はほかの研究者によりヒト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，この方法を活用してES細胞由来の心筋細胞の濃縮が行われる．しかしながら，まだ十分な数のES細胞由来の心筋細胞だけを獲得することは可能でなく，心筋細胞へと分化途上にある細胞の性質はまだブラックボックスの中にあり，血液細胞で分化途上の細胞を見分けるような細胞表面のマーカーの報告はまだない．私たちは，前述の手法で心筋細胞を多く含む胚様体を得て，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行い，発生過程で心筋特異的に発現する遺伝子の絞り込みを行っている．こうした手法を活用することで，ES細胞をモデルに初期の心筋分化過程で重要な働きをする遺伝子，マーカーとして活用が可能な遺伝子の探索が行えており，今後，ES細胞や胚様体における心筋分化にかかわる細胞系譜の情報が明らかになるものと期待している．

ES細胞由来心筋細胞の大量調製・移植片化の現状と課題

前述したが，ES細胞から心筋細胞を大量に得るためには，薬剤耐性遺伝子や表面マーカー遺伝子の導入を行う方法が考えられる．しかし，将来の臨床応用の実用化を考えると，多数のES細胞バンクについて，遺伝子をそれぞれのES細胞株に安定に導入することができるのか，ES細胞の安全性はどう担保すべきか，などが問題となる．やはり，内在性の心筋細胞表面特異的なマーカー同定と利用が必要であろう．これまでに，心筋前駆細胞をFlk1などのマーカーを利用した報告[8]はあるが，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る．われわれは前項で述べたように，マーカー候補遺伝子の絞り込みを進めており，胚および胚様体のFACS解析，*in situ* hybridizationなどで特異性を確認しているが，今後，候補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

ES細胞由来の心筋細胞がmdxマウスに移植され，ES細胞由来の心筋細胞が宿主心臓に取り込まれることが示され[9]，ES細胞由来の心筋細胞の臨床応用へ期待が高まって以来，10年の月日が過ぎた．しかし，障害を受けた心臓の機能が移植したES細胞由来心筋細胞により回復したという報告はこれまでほとんどない．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すらある[10]．大量に純化した心筋細胞が得られても，注入だけでは心臓の機能はあまり回復しないとも考えられる．したがって，どのように移植片化すべきなのか，温度感受性シートやマトリゲルなどの足場の必要性はどうか，単に純化するだけでなく共存すべき細胞種が必要か，など，多くの疑問があるものの，一部は解決されつつあるようにみえる．しかし，その確認はなお必要であり，そのためにも，ES細胞由来心筋細胞は，今こそ，しかも大量に必要であると考えられる．さらに，ES細胞由来心筋細胞についての

研究は，ES細胞をiPS細胞に置き換えた研究，さらにその応用につながりうるので，iPS細胞が話題となっていればなおさら，ES細胞からの心筋前駆細胞・心筋細胞の分離・純化に向けた研究を推進する必要があると考える．

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