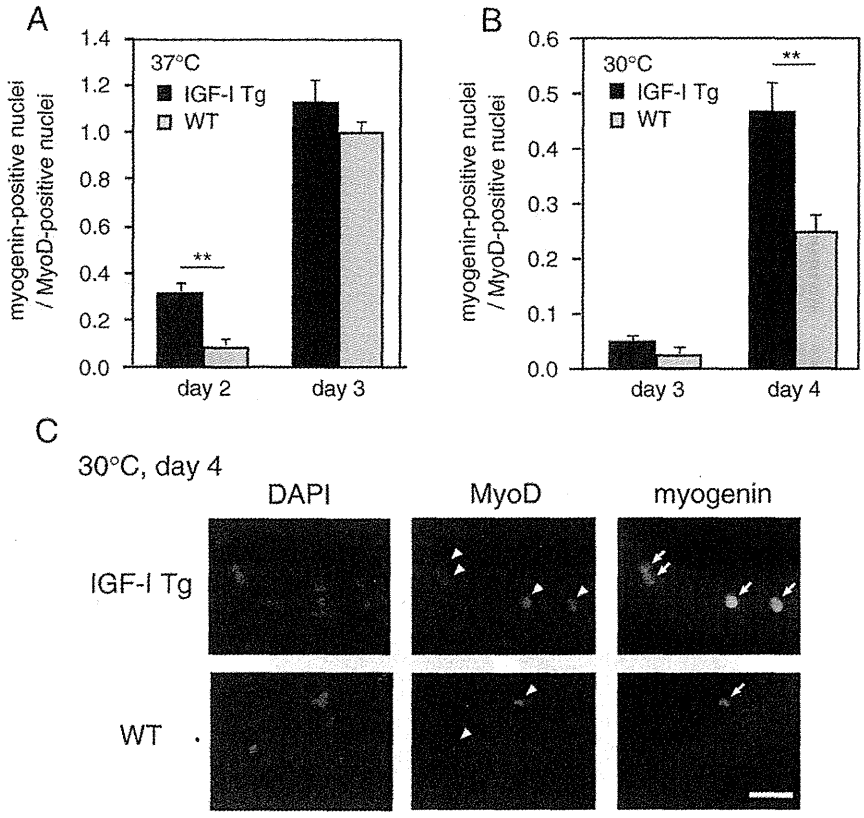


**Fig. 6 – MyoD and myogenin expression in single-fiber culture of IGF-I Tg mice and WT mice. A and B. Myofibers were isolated from EDL muscles of adult mice and cultured in 20% FBS/DMEM without VC at 37 °C (37 °C culture) or 37 °C for the first 24 h to activate the satellite cells and subsequently at 30 °C (30 °C culture). The ratios of myogenin-positive nuclei to MyoD-positive nuclei per myofiber in the 37 °C culture (A) or in the 30 °C culture (B) were calculated and are presented as the mean ± SEM. The satellite cells of IGF-I Tg mice expressed myogenin at a higher level than those of WT mice on day 2 in the 37 °C culture and on day 4 in the 30 °C culture. $^{**}p<0.01$. C. The satellite cells on the myofibers from IGF-I Tg and WT mice in the 30 °C culture were immunostained for MyoD (arrowhead) and myogenin (arrow) 4 days after the activation. Cell nuclei were stained with DAPI. Scale bar: 50 μm.**

temperature, IGF-I stimulates myogenin expression through the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway [29,30]. Although it has not been clear yet if IGF-I promotes myogenin expression via the identical signaling pathway at 30 °C, it is likely that the same mechanism is responsible for both temperatures. On the other hand, acting as an antioxidant, VC also plays multiple important roles *in vivo*, which includes promoting the formation of collagen matrix and proteoglycan [31]. These extracellular matrices (ECM) act as reservoirs of various cytokines and growth factors and produce microenvironments for cells *in vivo*, which commit cells to a specific differentiation fate. Our study showed that VC enhanced the effect of IGFs for myogenin expression and myotube formation at 30 °C. A conceivable mechanism of the observed synergistic effects of IGFs and VC involves the stimulation of ECM production by VC, which then serves to efficiently concentrate IGFs around cells thereby enhancing its effect. It is also possible that VC acts as a reducing agent on a signaling pathway acting downstream of IGFs and enhances myogenin expression.

We anticipate that the rescue experiments of myogenic differentiation with IGFs and VC at low temperatures will be a useful tool for the investigation of the molecular events during myogenic differentiation, which normally progresses so rapidly at normal culture temperature that it is difficult to study the minute details of the signaling pathway. In the low-temperature cell culture system described here, the differentiation speed can be controlled by varying the culture temperature and concentrations of IGFs and VC in the culture medium and therefore each of the cell differentiation steps can be studied separately.

MicroRNAs examined in this study were reported to be involved in the regulation of myogenic differentiation or muscle regeneration [25]. It was shown that MyoD and myogenin bind the regulatory regions of miR-1, -133 and -206 and are likely to control their expressions [32]. Therefore, it is probable that IGF-I and VC promoted the expressions of these miRNAs at 30 °C as a result of rescuing myogenin expression. On the other hand, miR-181 was reported to express upstream of MyoD and to induce MyoD expression through downregulation of Hox-A11 [33]. In our study, IGF-I and VC also rescued miR-181 expression at 30 °C, suggesting that these factors might promote myogenic differentiation such as expression of miR-181 through the other signaling pathway, which was not directly activated by MyoD or myogenin.

In the single-fiber culture, satellite cells of both IGF-I Tg and WT mice expressed myogenin by day 4 at 30 °C. This trend was entirely different from C2C12 cells, which hardly expressed myogenin at 30 °C even after 10 days of culture in DM (data not

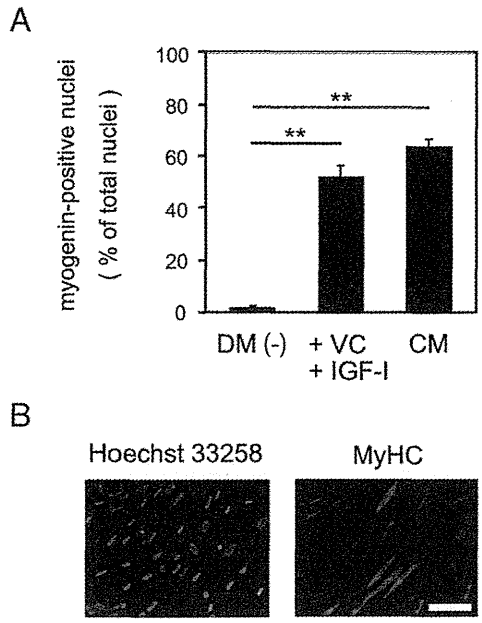


**Fig. 7 – Effects of conditioned medium of differentiated C2C12 cells at 30 °C. A. C2C12 cells were cultured for 6 days at 30 °C in DM with or without 100 ng/ml IGF-I and 200 μM VC or conditioned medium (CM) of C2C12 cells differentiated at 38 °C and immunostained for myogenin. Myogenin expression was rescued with CM at the same level as that with both IGF-I and VC. The percentages of myogenin-positive nuclei to the total nuclei are presented as the mean ± SEM. $^{**}p<0.01$. B. C2C12 cells were cultured in CM for 10 days at 30 °C and immunostained for MyHC. The cells expressed MyHC but did not form multinucleated myotubes. Bar: 100 μm.**

shown). This discrepancy may have been due to the secretion of certain biological factors into the culture medium by cells associated with the isolated WT mouse myofibers other than satellite cells, such as myofibroblasts, or myofibers themselves which could rescue myogenin expression.

To confirm this hypothesis, we cultured C2C12 cells at 30 °C in conditioned medium (CM) of C2C12 cells differentiated at 38 °C and found that CM promoted myogenin expression at 30 °C at the almost same level as that with both IGF-I and VC. On the other hand, myotubes were not formed though the cells were cultured in CM for 10 days. The conceivable reason of this observation is that CM did not have sufficient nutritional quality for myotube formation or that CM contained some factors preventing myoblast fusion such as transforming growth factor-β (TGF-β) [34].

Our result demonstrates that differentiated myogenic cells secrete a factor (or factors) which can restore myogenin expression at 30 °C. Although we have yet to identify the specific factor(s), it is probable that one of the factors contained in CM is IGF-II, because skeletal muscle cells secrete IGF-II endogenously during differentiation [35,36]. It is likely that innate factors exist *in vivo* which serve as buffers to low temperature and help normal muscle cell differentiation even in parts of the body with temperatures lower than 30 °C [3].

We examined whether or not human skeletal muscle cells were also inhibited to differentiate at 30 °C and further IGF-I and VC have a rescuing effect. Human cells showed the same tendency as C2C12 mouse myoblast cells, but did not form myotubes under the culture condition described in Materials and methods. Therefore, we just evaluate their myogenin expression, which is a marker of myogenic differentiation in the early stage.

Another instance in which the body experiences low temperatures is during hypothermia treatment, which involves maintaining the body at 32–34 °C for several days and is known to be effective for improving survival and neurological outcome after brain injury, such as cerebral ischemia and hemorrhage [37], and sudden cardiac arrest [38]. The protective mechanism of hypothermia includes reduction of brain metabolism by depressing its electrical activity, inhibition of programmed cell death by reducing excitatory amino acid release and attenuating oxidative stress. However, this treatment strategy can also have negative effects for patients as the suppression of the immune system occasionally results in serious infections and death. Therefore, the administration of drugs which enhance local biological activities in patients whose body temperature are lower than 35 °C to levels observed at normal body temperature during hypothermia treatment could help to improve survival rates. As IGF-I and VC promoted differentiation of human skeletal muscle cells at 30 °C, we speculate that they may function as *in vivo* buffers to low temperatures and are good drug candidates for compensating against the defects of hypothermia treatment in not only skeletal muscles, but also in other organs.

## Conclusions

At 30 °C, mouse skeletal muscle cells neither express myogenin nor fuse into multinucleated myotubes, while the myoblasts continuously expressed Id3 and do not upregulate muscle-specific miRNAs. Human skeletal muscle cells also did not express myogenin at 30 °C. However, the low temperature-induced inhibition of myogenic differentiation is effectively alleviated by the addition of either IGFs (IGF-I and IGF-II) or VC into culture medium. Satellite cells from IGF-I overexpressing Tg mice swiftly

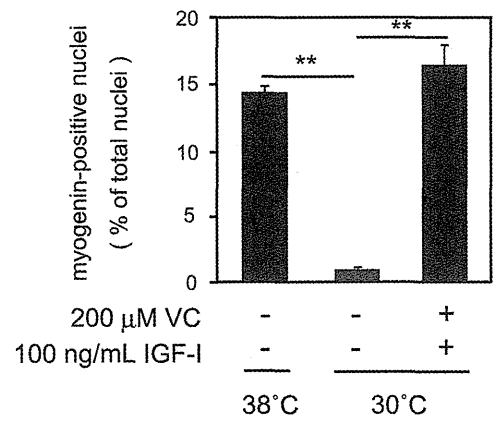


**Fig. 8 – Myogenin expression in human skeletal muscle cells cultured with IGF-I and VC at 30 °C. Human skeletal muscle cells were cultured in DM at 38 or 30 °C and in DM containing 100 ng/ml IGF-I and 200 μM VC at 30 °C for 6 days and then immunostained for myogenin. Myogenin expression in the human muscle cells was inhibited at 30 °C, but was rescued by treatment with IGF-I and VC. The percentage (%) of myogenin-positive nuclei to the total nuclei is presented as the means ± SEM. $^{**}p<0.01$.**

differentiated at both 37 and 30 °C in single-fiber culture. Our findings that IGFs and VC promote myogenic differentiation at lower temperatures than body temperature suggest that the sensitivity of myogenic cells to low temperature could be buffered by certain physiological factors *in vivo*.

## Acknowledgments

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# Muscle injury-induced thymosin β4 acts as a chemoattractant for myoblasts

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**Yuka Tokura[1,2,*], Yuki Nakayama[1,3,*], So-ichiro Fukada[4], Noriko Nara[1], Hiroshi Yamamoto[4], Ryoichi Matsuda[2] and Takahiko Hara[1,†]**

[1]Stem Cell Project Group, The Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506; [2]Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902; [3]Priority Organization for Innovation and Excellence, Kumamoto University, 2-39-1 Kurokami, Kumamoto-shi, Kumamoto 860-8555; and [4]Department of Immunology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita-shi, Osaka 565-0871, Japan

*These authors contributed equally to this work.

†Takahiko Hara, Stem Cell Project Group, The Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan. Tel: +81 3 5316 3310, Fax: +81 3 5316 3226, email: hara-tk@igakuken.or.jp

**Thymosin β4 (Tβ4) is a major intracellular G-actin-sequestering peptide. There is increasing evidence to support important extracellular functions of Tβ4 related to angiogenesis, wound healing and cardiovascular regeneration. We investigated the expression of 'Tβ4' and 'thymosin β10', a closely related peptide, during skeletal muscle regeneration in mice and chemotactic responses of myoblasts to these peptides. The mRNA levels of 'Tβ4' and 'thymosin β10' were up-regulated in the early stage of regenerating muscle fibres and inflammatory haematopoietic cells in the injured skeletal muscles of mice. We found that both Tβ4 and its sulphoxized form significantly accelerated wound closure and increased the chemotaxis of C2C12 myoblastic cells. Furthermore, we showed that primary myoblasts and myocytes derived from muscle satellite cells of adult mice were chemoattracted to sulphoxized form of Tβ4. These data indicate that muscle injury enhances the local production of Tβ4, thereby promoting the migration of myoblasts to facilitate skeletal muscle regeneration.**

*Keywords*: chemotaxis/myoblast/regeneration/skeletal muscle/thymosin beta4.

*Abbreviations*: bFGF, basic fibroblast growth factor; CT, cardiotoxin; DIG, Digoxigenin; DMEM, Dulbecco's modified Eagle's medium; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; ILK, integrin-linked kinase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; TA, tibialis anterior; Tβ4, thymosin β4; Tβ4so, thymosin β4 sulphoxized; Tβ10, thymosin β10.

Skeletal muscles are damaged and repaired daily, and support the locomotor functions of life. During muscle regeneration, various cytokines and secretary peptides are transiently produced by inflammatory immune cells and regenerating muscle fibres. These factors play an important role in the cell cycle entry of satellite cells (muscle stem cells), the differentiation of myoblasts and in myotube formation (*1*). Since some factors, such as insulin-like growth factor-1 (*2*) and vascular endothelial growth factor (*3*), have been reported to ameliorate the damaged skeletal muscles in mouse models of muscular dystrophy, it is important to extend our knowledge of these muscle injury-induced secretory molecules.

We and others reported previously that the expression of 'thymosin β4 (Tβ4)' mRNA is up-regulated in the skeletal muscles of dystrophin-deficient *mdx* mice (*4*, *5*) and in a *mdx*-derived myoblastic cell line (*6*) compared with their respective wild-type controls. Tβ4 is an N-terminally acetylated polypeptide of 4.9 kDa and contains 43 amino acid residues (*7*). Tβ4 sequesters intracellular monomeric G-actin and inhibits the assembly of actin fibres within cells (*8*). However, a substantial amount of Tβ4 is released from cells, where it serves as a paracrine factor to support angiogenesis, wound healing, hair growth and cardiac regeneration (*9–14*). It has been reported that Tβ4 is chemotactic for endothelial cells (*9*), keratinocytes (*12*) and cardiomyocytes (*14*). In contrast, Tβ4 is inhibitory for the chemotaxis of neutrophils (*15*). Notably, Tβ4 sulphoxide (Tβ4so), in which the sixth methionine residue is oxidized, has an even greater ability to inhibit the migration of neutrophils (*15*). Moreover, a closely related peptide, thymosin β10 (Tβ10) (*16*, *17*) may have similar functions to Tβ4.

Recently, two groups reported a critical role for Tβ4 and its cleavage product in cardiac regeneration (*13*, *14*). Tβ4 was shown to be physically associated with the LIM (Lin-1, Isl-1, and Mec-3) domain protein, PINCH (particularly interesting new Cys-His protein) and with integrin-linked kinase (ILK) to activate Akt kinase in cells (*13*). Tβ4 promoted the survival and repair of cardiomyocytes after cardiac injury through this signalling pathway, in addition to stimulating the migration of cardiomyocytes and endothelial cells.

The expression level of Tβ4 is reported to be high in wound fluid and in regenerating tissues (*18–20*). Based on the fact that 'Tβ4' expression is up-regulated in the skeletal muscles of *mdx* mice (*4*, *5*) and injured porcine muscles (*20*), we hypothesized that it may play an important role in skeletal muscle regeneration. In this study, we show that 'Tβ4' and 'Tβ10' mRNAs are induced in regenerating muscles and inflammatory haematopoietic cells. More importantly, we

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demonstrate that Tβ4 serves as a chemoattractant for myoblasts.

## Materials and Methods

### *Muscle injury models*

First experimental model was set up as described earlier (*21*). Briefly, 100 μl of cardiotoxin (CT, 10 μM in 0.9% NaCl; Wako, Osaka, Japan) was injected into the tibialis anterior (TA) muscles of 6-week-old male C57BL/6 mice (Nihon SLC, Hamamatsu, Japan) using 27G needles. The CT-treated TA muscles were then harvested at various time points (from 6 h to 14 days), fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4, frozen, sectioned and stained with haematoxylin and eosin.

Alternatively, TA muscles were quickly frozen in liquid nitrogen for RNA extraction. A second muscle crush-injury model was established by puncturing the gastrocnemius muscles of 8-week-old male C57BL/10 mice (Nihon SLC) with 23G needles. At different time points (from 5 h to 14 days) after injury, the gastrocnemius muscles were isolated, frozen in liquid nitrogen and subjected to RNA extraction.

Mice were maintained under a 12-h light/dark cycle in a pathogen-free animal facility. All experimental procedures involving the mice were pre-approved by the ethical committee of the institute.

### *Reverse transcription–polymerase chain reaction (PCR)*

Total RNAs were prepared from skeletal muscle tissues using Trizol (Invitrogen, Carlsbad, CA, USA). Five micrograms of RNA from each sample was reverse-transcribed using the SuperScript II pre-amplification system for first strand cDNA synthesis with oligo(dT) primers (Invitrogen). Part of the cDNA mixture (1/125) was used in a PCR reaction with an annealing temperature of 56°C, ExTaq DNA polymerase (Takara, Otsu, Japan), and the following primer sets: 5′-TCTGACAAACCCGATATGGCT-3′ and 5′-CGAT TCGCCAGCTTGCTTCTCT-3′ for detection of 'Tβ4' (PCR product: 129 bp), 5′-GCAGACAAGCCGGACATGGGG-3′ and 5′-GG AGATTTCACTCCTCTTTTCC-3′ for 'Tβ10' (PCR product: 129 bp) and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCA CCACCCTGTTGCTGTA-3′ for 'glyceraldehyde 3-phosphate dehydrogenase' ('Gapdh'; PCR product: 452 bp), respectively. 'Gapdh' was used as a template control. Real-time PCR was performed with SYBR® premix ExTaq II (Takara). The $\Delta\Delta C_t$ method was used to determine relative mRNA expression levels.

### In situ *hybridization*

The PCR-amplified 3′-untranslated regions of the 'Tβ4' and 'Tβ10' cDNAs were cloned into the PCR-Script Amp SK(+) vector (Stratagene, La Jolla, CA, USA). Digoxigenin (DIG)-labelled RNA probes were prepared by using the DIG RNA labelling kit (Roche Diagnostics, Indianapolis, IN, USA). The CT-treated TA muscles from C57BL/6 mice were dissected on the fifth day after injection and frozen in isopentane pre-cooled in liquid nitrogen. Cryostat cross sections (10 μm) were prepared, fixed in 4% paraformaldehyde in PBS, and treated with 1 μg/ml proteinase K (Wako) in PBS at room temperature for 7 min. After acetylation with acetic anhydride in triethanolamine (Wako), the sections were hybridized with a DIG-labelled anti-sense or sense RNA probe at 65°C for 18 h and the signals were detected colorimetrically (*22*).

### *Cells and reagents*

Mouse myoblast-derived C2C12 cells (American Type Culture Collection, CRL-1772) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO, USA) containing 10% fetal calf serum (Invitrogen) and 0.5% penicillin-streptomycin (Sigma). Myotube differentiation of C2C12 cells was carried out as previously described (*23*). Muscle satellite cells were separated from 8-week-old female C57BL/6 mice by using SM/C-2.6 antibody as described earlier (*24*) and sub-cultured for 7 days in 20% fetal calf serum, human basic fibroblast growth factor (bFGF, 5 ng/ml; PeproTech, Rocky Hill, NJ, USA) and 0.5% penicillin-streptomycin on matrigel (BD Biosciences, San Jose, CA, USA)-coated plates. SM/C-2.6$^+$ cells were sorted on a FACSAria (BD Biosciences). Approximately $4 \times 10^5$ cells of SM/C-2.6$^+$ fraction were obtained from skeletal muscle of two adult mice.

Tβ4 was chemically synthesized by the Peptide Institute Inc. (Osaka, Japan) and oxidized in the presence of 30% $H_2O_2$. Unoxidized Tβ4 was separated from Tβ4so by high-performance liquid chromatography.

### *Scratch wound closure assay*

Confluent monolayers of C2C12 cells in 6-well plates were scratched with a blue tip to generate a gap (~5 × 1 mm). Cells were incubated with DMEM containing 10 mM thymidine (Sigma) at 37°C for 8 h in the presence or absence of various concentrations of Tβ4 or Tβ4so. The medium was replaced after 4 h of incubation. The scratched areas were photographed under a microscope both before and after culture, and the width of each gap measured.

### *Chemotaxis assay*

C2C12 cells or muscle satellite cells-derived myoblasts/myocytes were washed and resuspended at a concentration of $10^6$ cells/ml (C2C12) or $3.6 \times 10^5$ cells/ml (primary myoblasts/myocytes) in DMEM containing 0.1% fatty acid-free bovine serum albumin (Sigma) and 20 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid pH 7.3 (Invitrogen). The wells of a 24-well culture plate were filled with 550 μl of Tβ4, Tβ4so or bFGF at various concentrations. Chemotaxicell chambers (8 μm pore size; Kurabo, Osaka, Japan) were pre-coated with bovine fibronectin (100 μg/ml; Wako) for 2 h at room temperature and placed in each well. Cells (200 μl) were then added to the upper chamber. The plate was then incubated for 6 h at 37°C, after which the cells remaining in the upper chamber were scraped off. The cells that had migrated to the bottom surface of the membrane were stained with Diff-Quik (Kokusai Shiyaku, Kobe, Japan) and counted using a microscope.

### *Statistical analyses*

All statistical analyses were performed using ANOVA (analysis of variance) repeated measures analysis (Statview J5.0, Abacus Concepts, Berkeley, CA, USA). $P<0.05$ was considered significant for the unpaired Student's *t*-test.

## Results

### *Expression of Tβ4 and Tβ10 mRNAs is enhanced in skeletal muscles after injury*

First, we investigated the expression patterns of 'Tβ4' and 'Tβ10' mRNAs during the recovery of injured skeletal muscles. As previously demonstrated (*21*), injection of CT into TA muscles caused the extensive degeneration of muscle fibres within 6 h (Fig. 1A). On Days 3–5, many inflammatory haematopoietic cells were recruited into the interstitial spaces. From Day 7 after CT injection, regenerating muscle fibers with central nuclei were gradually formed and the entire TA muscles were eventually repaired by Day 14 (Fig. 1A). Expression of 'Tβ4' mRNA was increased within 1 day, reaching its highest level on Day 3, and decreasing to base line levels by Day 14 after CT injection (Fig. 1B). Although the relative amount of 'Tβ10' mRNA was smaller than that of 'Tβ4', its expression was also maximal around Day 3 (Fig. 1B). In the crush injury model, 'Tβ4' mRNA was similarly up-regulated until Day 4 (when inflammatory reactions are initiated) and then gradually decreased by Day 14 (Fig. 1C).

### *Localization of Tβ4 and Tβ10 mRNAs in the regenerating skeletal muscles*

We next determined the type of cells that produce 'Tβ4' and 'Tβ10' during the regeneration of TA muscles by *in situ* hybridization. 'Myosin light chain' was utilized as a marker for the regenerating muscle fibres. As shown in Fig. 2, 'Tβ4' and 'Tβ10' mRNAs were

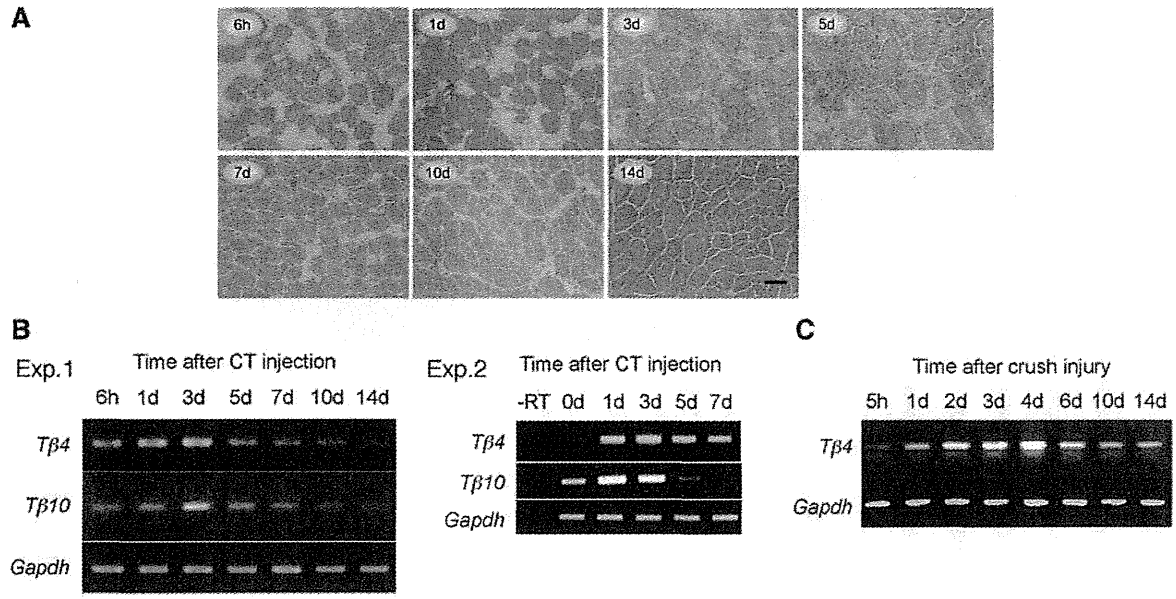


**Fig. 1 Up-regulation of 'Tβ4' and 'Tβ10' mRNAs during skeletal muscle regeneration.** (A) Histological appearance of skeletal muscle during the course of regeneration after CT injection. TA muscles were recovered from CT-injected mice at indicated time points (6 h to 14 days). Their frozen sections were stained with haematoxylin and eosin. Scale bar, 50 μm. (B and C) The mRNA levels of the indicated genes were analysed by reverse transcription–PCR at various time points (5 h to 14 days) after CT injection (B) or crush injury (C). Base line expression levels (Day 0) of 'Tβ4' and 'Tβ10' mRNAs were shown in the second CT injection experiment. DNA was visualized by ethidium bromide staining. 'Gapdh' was used as a template control.

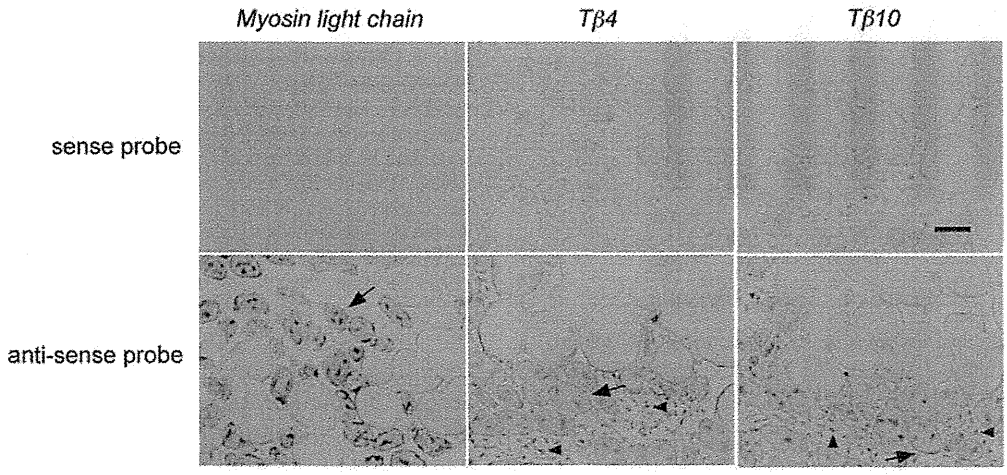


**Fig. 2 Location of 'Tβ4' and 'Tβ10' mRNAs in the regenerating skeletal muscle.** Transverse cryosections of TA muscles were harvested 5 days after CT injection and were hybridized with DIG-labelled sense (upper panels) or anti-sense (lower panels) cRNA probes for 'Myosin light chain', 'Tβ4' and 'Tβ10', respectively. Gene-specific signals in cytoplasm were visualized as light brown paints in the centrally nucleated muscle fibres (arrows) and interstitial haematopoietic cells (arrowheads). Scale bar, 50 μm.

detectable in centrally nucleated regenerating muscle fibres, as well as in the haematopoietic cells present in the interstitial spaces (Fig. 2). These staining patterns were not observed with the sense probes, confirming the specificity of signals.

### *Migratory responses of C2C12 cells to Tβ4 and Tβ4so*

As Tβ4 is known to promote the migration of endothelial cells and cardiac myocytes (*9*, *14*), we examined the migratory responses of myoblasts to both Tβ4 and its oxidized form. As shown in Fig. 3A, both forms significantly enhanced the wound closing capacity of C2C12 cells in a dose-dependent manner. Maximum activity was observed at 100 pg/ml for Tβ4so, whereas Tβ4 exhibited a constantly high activity at concentrations >100 pg/ml. In contrast, the chemotactic responses of C2C12 cells to both Tβ4 and Tβ4so were very similar in terms of dose dependency (Fig. 3B). The total number of cells migrated in response to Tβ4 and Tβ4so was comparable to that seen with bFGF, a known chemotactic factor for C2C12 cells (*25*). When Tβ4 or Tβ4so was included in the upper chamber, or both the upper and lower chambers, of Chemotaxicell, enhancement of cell migration was cancelled (Fig. 3C). Therefore, Tβ4 and Tβ4so induce chemotaxis, but not chemokinesis to C2C12 cells.

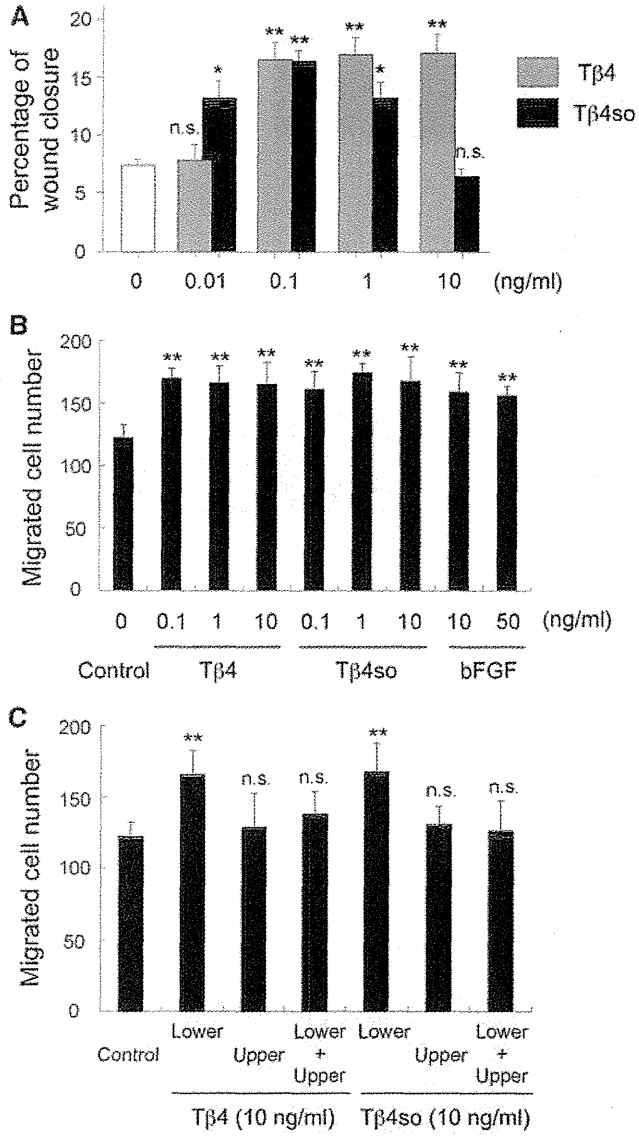


**Fig. 3 Wound closure and chemotactic responses of C2C12 cells by exogenous Tβ4 and Tβ4so.** (A) Recovery ratio of scratched wounded areas of C2C12 monolayer was measured in the presence or absence of indicated concentrations of Tβ4 or Tβ4so. (B) Chemotactic responses of C2C12 cells to various concentrations of Tβ4 and Tβ4so. bFGF was used as a positive control. (C) Evaluation of chemokinetic responses of C2C12 cells to Tβ4 and Tβ4so. In (B) and (C), assays were performed using Chemotaxicell (8 μm pore size) with indicated reagents added in the upper and/or lower chambers. After 6 h of incubation, cells that had migrated to the bottom surface of the membrane were stained and subjected to microscopic observation and cell counting. Each value represents the mean ± S.D. ($n = 8$–9). $^{*}P < 0.05$; $^{**}P < 0.01$. n.s., not significant compared to the value of medium control.

### *Chemotactic responses of satellite cells-derived primary myoblasts/myocytes*

To confirm that Tβ4-responsiveness is a general property of myoblasts and not a C2C12-specific phenomenon, we prepared primary myoblasts and used them for the chemotaxis assay. For this purpose, we isolated SM/C2.6$^{+}$ muscle satellite cells from adult mice and cultured them for *in vitro* differentiation. As previously demonstrated (*24*), they vigorously proliferated and gave rise to myoblasts, myocytes and myotube-like structures (Fig. 4A). When we applied these cells to

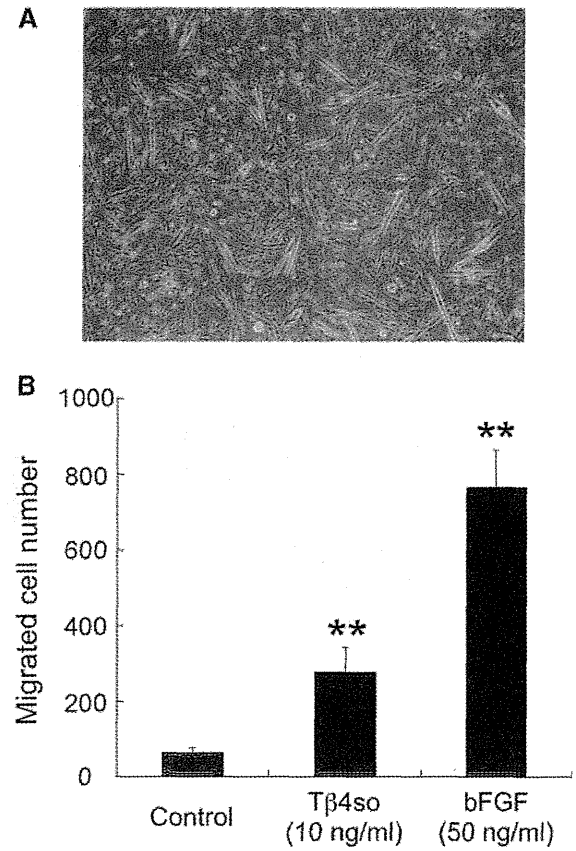


**Fig. 4 Chemotaxis of satellite cells-derived myoblasts/myocytes to exogenous Tβ4so.** (A) Morphological appearance of primary myoblasts and myocytes derived from muscle satellite cells of adult mice. (B) Chemotactic responses of primary myoblasts/myocytes to Tβ4so and bFGF. Cells and indicated reagents were added in upper and lower chambers of Chemotaxicell (8 μm pore size), respectively. After 6 h of incubation, cells that had migrated to the bottom surface of the membrane were stained and counted. Each value represents the mean ± S.D. ($n = 3$). $^{**}P < 0.01$ compared to the value of medium control.

Chemotaxicell, significant number of cells migrated across the membrane in response to Tβ4so (Fig. 4B). These data demonstrated the responsiveness of primary myoblasts/myocytes to Tβ4so. It is noteworthy that satellite cells-derived primary cells were more strongly chemoattracted to bFGF than to Tβ4so (Fig. 4B) when compared to C2C12 cells (Fig. 3B). This is probably related to a higher frequency of myocytes in the primary cell population.

### *Up-regulation of Tβ4 and Tβ10 mRNAs during myotube differentiation*

To understand whether expression of 'Tβ4' and 'Tβ10' mRNAs is changed between myoblasts and myofibers, we compared their expressions in C2C12 cells before and after myotube differentiation *in vitro*. As shown in Fig. 5, both 'Tβ4' and 'Tβ10' mRNAs were significantly increased by the myotube differentiation.

## Discussion

Previous reports from several laboratories noted that the expression levels of 'Tβ4' in the skeletal muscle of *mdx* mice are significantly higher than

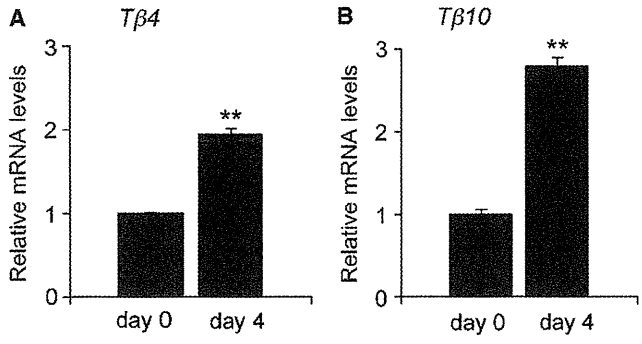


**Fig. 5 Increase of 'Tβ4' and 'Tβ10' mRNAs during myotube differentiation of C2C12 cells.** (A) C2C12 cells were cultured in the differentiation media to induce myotube-like structures. Expression levels of 'Tβ4' (A) and 'Tβ10' (B) mRNAs before (Day 0) and after (Day 4) the myotube differentiation were determined by real-time RT–PCR. 'Gapdh' was used to normalize the relative abundance of 'Tβ4' or 'Tβ10' mRNA. In each panel, value of Day 4 was expressed as a relative amount to that of Day 0. Each value represents the mean ± S.E. ($n = 3$). **, $P < 0.01$ compared to the value of Day 0.

those of wild-type mice (*4*, *5*). We also found that larger amounts of 'Tβ4' mRNA are produced by the *mdx*-derived myoblastic cell line (*6*) and the skeletal muscles of *mdx* mice (Nakayama, Y. and Hara, T., unpublished data). *In situ* hybridization data show that both 'Tβ4' and 'Tβ10' mRNAs are present in regenerating muscle fibres, but are absent from mature skeletal muscles. The specific localization of Tβ4 protein in regenerating muscles was shown in a very recent report using *mdx* mice (*26*). In addition to immature muscles, we found that haematopoietic cells in the interstitial spaces also produced 'Tβ4' and 'Tβ10' mRNAs. In the early stages (3–5 days) of muscle regeneration after CT injection, large amounts of inflammatory blood cells, including macrophages and T cells, infiltrate into the degenerated areas of skeletal muscle. These cells play an important role in tissue repair by secreting a number of cytokines. Accordingly, the levels of 'Tβ4' and 'Tβ10' mRNAs in the TA muscles were at their highest on Day 3 after CT injection.

Tβ4 is known to promote wound healing in skin (*11*), hair growth (*12*) and cardiac repair (*13*, *14*). However, the physiological relevance of this up-regulation of 'Tβ4' and 'Tβ10' in *mdx* muscles and injured skeletal muscles remains to be clarified. In this study, we demonstrated that Tβ4 and its oxidized form, Tβ4so, are capable of stimulating the migration of both C2C12 cells and skeletal muscle-derived primary myoblasts/myocytes. Tβ4so was more potent in promoting the migration of C2C12 cells in the scratch wound assay than Tβ4, as was shown for human umbilical vein endothelial cells (*15*). As the chemotaxis-inducing activity of Tβ4 and Tβ4so was very similar, these two assays might detect a distinct biological activity of Tβ4so. In this regard, it is interesting that Tβ4so, but not Tβ4, is a potent inducer of neutrophil locomotion, and suppresses footpad swelling (*15*). However, we cannot rule out the possibility that an action range of the chemotaxis assay is broader than that of wound closure assay.

As mentioned above, 'Tβ4' mRNA is expressed in myoblastic cell lines and its expression level is higher in *mdx*-derived myoblastic cells than that in C57BL/10 mice-derived myoblasts (*6*). If Tβ4 acts as a chemoattractant for myoblasts, how Tβ4 gradient is generated in the regenerating skeletal muscles? We demonstrated that both Tβ4 and 'Tβ10' mRNAs were markedly up-regulated during myotube differentiation of C2C12 cells. Therefore, newly formed myofibers as well as inflammatory haematopoietic cells would produce larger amounts of Tβ4 and Tβ10 locally, thereby facilitating mobilization of myoblasts into the regenerating skeletal muscle regions.

Given the fact that Tβ4 and Tβ4so mobilize myoblasts to injured muscles, it may also facilitate the regeneration of injured skeletal muscles. In fact, a very recent report by Spurney *et al.* (*26*) shows that Tβ4-treated *mdx* mice have significantly increased numbers of regenerating muscle fibres compared with control *mdx* mice. Considering the role of Tβ4 in cardiac regeneration, it is likely that Tβ4 mediates the recruitment and survival of myoblasts via the PINCH-ILK-Akt pathway. This is supported by the fact that ILK and Akt are activated in C2C12 cells in response to Tβ4 (*14*). Alternatively, a reduction of NF-κB activity induced by Tβ4 might play a role in the anti-apoptotic effects seen in skeletal muscles (*27*).

Unfortunately, the impaired skeletal muscle strength and fibrosis seen in *mdx* mice were not ameliorated after chronic administration of Tβ4 in the study of Spurney *et al.* (*26*). In transgenic mouse lines overexpressing Tβ4 in their skeletal muscles, we failed to observe significant differences in the kinetics of muscle regeneration after CT injection (Nakayama, Y. and Hara, T., unpublished data). In this case, it is possible that the concentrations of endogenous Tβ4 and Tβ10 were sufficiently high to mobilize myoblasts in the injured muscles of these transgenic mice. Regarding the clinical application of Tβ4 for the treatment of muscular dystrophies, the combination of Tβ4 with other regeneration-promoting cytokines would be beneficial.

Only endothelial cells (*9*), keratinocytes (*12*), cardiomyocytes (*14*) and skeletal myoblasts (this study) are chemoattracted to Tβ4. Fibroblasts, smooth muscle cells, monocytes and neutrophils do not respond to Tβ4 (*9*, *15*). Interestingly, both Tβ4 and Tβ4so are rather inhibitory for neutrophil chemotaxis elicited by N-formyl-methion-leucyl-phenylalanine (*15*). Therefore, Tβ4 modulates cell migration in a cell-type specific fashion to either promote tissue regeneration, or attenuate inflammatory responses. Future investigation of putative Tβ4 receptors would provide critical information for understanding how extracellular Tβ4 exerts its biological activities in cells.

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**Conflict of interest**
None declared.

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# FGF2 Induces ERK Phosphorylation Through Grb2 and PKC during Quiescent Myogenic Cell Activation

**Yosuke Nagata[1,2*], Yusuke Honda[2], and Ryoichi Matsuda[2]**

*[1]Komaba Organization for Educational Development, College of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan, [2]Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan*

*ABSTRACT.* **Satellite cells are muscle-resident stem cells, which are located beneath the basement membrane of myofibers. Because the number of satellite cells is normally constant, there must be a tight regulation of satellite cell activation and self-renewal. However, the molecular mechanisms involved in satellite cell maintenance are largely unknown, and thus have become the subject of extensive study these days. Although RNA interference with a small interfering RNA has been widely used to investigate the role of specific gene products, inefficient knockdown of Grb2 expression occurred in quiescent reserve cells, a model for quiescent satellite cells, by ordinary transfection protocol. In this study we report that pretreatment with trypsin greatly enhanced siRNA delivery into quiescent reserve cells, resulting in efficient silencing of Grb2 expression. By applying a combination of Grb2-silencing and protein kinase C inhibitors, we demonstrated that extracellular signal-regulated kinase (ERK) phosphorylation induced with fibroblast growth factor 2 (FGF2) was dependent on both Grb2 and protein kinase C (PKC) with different kinetics. We concluded that the PKC-mediated pathway contributes to rapid initiation and termination of ERK phosphorylation, while the Grb2-mediated pathway contributes to delayed and sustained ERK phosphorylation.**

**Key words:** C2C12/satellite cell/Grb2/PKC/siRNA

## *Introduction*

Satellite cells are muscle-resident stem cells, which are located beneath the basement membrane of myofibers (Mauro, 1961). Adult myofibers are formed by fusion of hundreds of mononucleated myoblasts produced by proliferation of satellite cells. Satellite cells are normally quiescent, but become active in response to certain stimuli, such as injury, overload, and exercise. Activated satellite cells then enter the cell cycle to produce large numbers of myogenic precursor cells, which fuse with existing myofibers or each other to form new myofibers (reviewed in Zammit *et al.*, 2006). Because the number of satellite cells is normally constant, there must be tight regulation of satellite cell activation and self-renewal (reviewed in Charge and Rudnicki, 2004). Certain growth factors, such as fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF) are reported to participate in satellite cell activation (Johnson and Allen, 1995; Tatsumi *et al.*, 1998; Sheehan and Allen, 1999; Yablonka-Reuveni *et al.*, 1999; Rochat *et al.*, 2004). However, the molecular mechanisms involved in satellite cell activation are largely unknown.

Extracellular signal-regulated kinase (ERK) plays an important role in the control of various cellular responses, including cell proliferation, differentiation, and survival. Ligand binding to growth factor receptors stimulates the receptor tyrosine kinase activity. An adaptor protein Grb2 interacts with activated receptor tyrosine kinases either directly or indirectly via other proteins such as Shc (Margolis, 1999), insulin receptor substrate (Sun *et al.*, 1993) and fibroblast growth factor receptor substrate (Kouhara *et al.*, 1997), and recruits the guanine nucleotide releasing factor Sos. The Grb2/Sos complex facilitates the

*To whom correspondence should be addressed: Yosuke Nagata, Room 25B, Building 101, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan.
Tel: +81–3–5465–8820, Fax: +81–3–5465–8821
E-mail: cynagata@mail.ecc.u-tokyo.ac.jp

Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle Medium; dsRNA, double strand RNA; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FGF2, fibroblast growth factor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; PBS, phosphate buffered saline; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; siRNA, small interfering RNA; sMyHC, sarcomeric myosin heavy chain.

exchange of GDP for GTP on small G-protein Ras at the plasma membrane, resulting in the activation of Raf/ MEK/ ERK pathway (reviewed in Schlessinger, 2000). Although the Grb2/Sos complex plays a central role in ERK activation, other factors, including phosphoinositide 3-kinase (PI3K) /Akt and phosphorlipase C (PLC)/protein kinase C (PKC), could also contribute to the phosphorylation of ERK1/2 via distinct pathways (Grammer and Blenis, 1997; Shu *et al.*, 2002). Because the precise mechanisms of reserve cell activation are largely unknown, understanding what signaling pathways are responsible for the activation of ERK1/2 is of great importance.

These days, RNA interference has been used widely to investigate the function of genes of interest. Successful transfection of cells with a small interfering RNA (siRNA) causes suppression of specific gene expression, thus enabling gene function analysis. siRNA-mediated RNA interference in quiescent myogenic cells would make a significant contribution to the understanding of the precise mechanisms in satellite cell activation. Most studies, however, have attempted siRNA transfection using proliferating cells (Elbashir *et al.*, 2002). In other words, there are few studies using siRNA transfection of quiescent cells. There are several works achieving gene silencing in quiescent cells by transfection of proliferating cells with siRNAs followed by making cells quiescent by serum withdrawal (Asano *et al.*, 2005; Tullai *et al.*, 2007); however, this procedure is not suitable for silencing the genes which are involved in cell proliferation or survival because it will cause significant reduction in the number of quiescent cells. Therefore, an efficient means of siRNA delivery in quiescent cells was required for the purpose. In fact, it was shown that gene silencing did work well in quiescent human bladder carcinoma cells (Nabatiyan and Krude, 2004). However, they also reported that the higher concentrations of siRNA required caused cell death probably by off-target effect. Therefore, we sought a condition for efficient transfection for quiescent myogenic cells with lower concentrations of siRNA. In this study, we demonstrated that pre-treatment with trypsin dramatically improved the efficiency of siRNA delivery and eventually made it possible to silence gene expression in quiescent reserve cells. The efficiency of siRNA delivery was monitored with fluorescent-labeled siRNA, and gene silencing was carried out with siRNAs at 5 nM against Grb2.

In this study, we showed that FGF2 induced DNA synthesis in quiescent reserve cells, a model of satellite cells, in a manner dependent on ERK1/2. Furthermore, we demonstrated that ERK phosphorylation induced with FGF2 was dependent on both the Grb2- and the PKC-mediated pathway with different kinetics by efficient gene silencing of Grb2 in quiescent reserve cells.

## *Materials and Methods*

### *Cell culture and drug treatment*

C2C12 myogenic cells (Yaffe and Saxel, 1977; Blau *et al.*, 1983) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) containing 20% fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX), 25 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37°C in 5% $CO_2$. To induce differentiation and produce reserve cells, $5\times10^4$ cells were seeded on a 35-mm tissue culture dish in growth medium, and 24 h later, the medium was replaced with serum-free differentiation medium (DMEM supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 1 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO), and 25 mM HEPES). To isolate reserve cells from myotubes, C2C12 cells were cultured in differentiation medium for 4–5 days and then only the myotubes were detached with 0.05% trypsin (Gibco) in phosphate buffered saline (PBS) containing $CaCl_2$ and $MgCl_2$ (PBS(+)), for 5 min at 37°C.

### *siRNA transfection*

Two sets of pre-designed siRNA duplexes (Stealth siRNA) as well as a negative control duplex were obtained from Invitrogen (Carlsbad, CA). Transfections were performed with Lipofectamine RNAiMAX (Invitrogen) on 35-mm-dishes according to the manufacturer's instructions. siRNA diluted in Opti-MEM and Lipofectamine RNAiMAX diluted in Opti-MEM were mixed and added to cells. The final concentrations of siRNA in the culture media were 5 nM. The sequences for Grb2 siRNAs were as follows: 5'-CCGTGTCCAGGAACCAGCAGATATT-3' (#1) and 5'-CCTGTGGGTGGTGAAGTTTAATTCT-3' (#2). BLOCK-iT red fluorescent oligo was used instead of these siRNAs to monitor the delivery of siRNA into cell nuclei.

### *Western blotting*

Cells were fixed with 10% trichloroacetic acid, and then lysed in SDS-sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.0025% bromophenol blue), followed by boiling for 3 min. Ten micrograms of protein were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Inc., Lincoln, NE) and incubated overnight with primary antibodies. Membranes were washed three times and incubated with Alexa Fluor 680 or IRDye-conjugated secondary antibodies and analyzed with an Odyssey Infrared Imaging System (LI-COR). Primary antibodies used were monoclonal mouse anti-Grb2 (clone 81/GRB2; BD Transduction Laboratories, San Jose, CA), monoclonal mouse anti-α-tubulin (clone DM1A, Sigma-Aldrich), polyclonal rabbit anti-ERK1/2 (Promega, Madison, WI), monoclonal mouse anti-phospho-ERK1/2 (clone E10, Cell Signaling Technology, Danvers, MA), and polyclonal rabbit anti-phospho-PKC (pan) (Cell Signaling Technology).

### *RT-PCR*

Total RNA was isolated with RNAisoPlus reagent (Takara Bio Inc., Shiga, Japan) as specified by manufacturer. One microgram of RNA was used for the reverse transcription reactions using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). Aliquots of cDNA were placed in a total volume of 10 μl in Platinum Blue PCR SuperMix (Invitrogen). The PCR parameters were 94°C for 15 s, 55°C for 15 s, and 72°C for 15 s, for 25 cycles (Grb2) or 30 cycles (Grb2). Five microliter of the PCR products was electrophoresed on 2% agarose gel containing SYTO60 dye (Invitrogen). The gel was scanned and analyzed with an Odyssey Infrared Imaging System (LI-COR). Primers used in this paper were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-AACTTTGGCATTGTGGAGG-3' and 5'-ACACATTGGGGGTAGGAACA-3'; Grb2, 5'-GAGCCAAGGCAGAAGAAATG-3' and 5'-CTTCACCACCCACAGGAAAT-3'.

### *Immunofluorescent staining*

Cells were fixed with 10% formalin in PBS for 15 min, and then permeabilized with 0.5% Triton X-100. Cells were then incubated with mouse anti-sarcomeric myosin heavy chain (sMyHC) antibody (clone MF20). After washes in PBS, primary antibody binding was visualized with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Eugene, OR) for 30 min before washing and mounting in Mowiol mounting medium containing 100 ng/ml Hoechst 33258.

### *Bromodeoxyuridine incorporation*

C2C12 cells were fed with fresh DMEM containing 10 μM 5-bromo-2'-deoxyuridine (BrdU). After incubation for 24 h, cells were fixed with 4% paraformaldehyde in PBS, followed by permeabilization with 0.5% Triton X-100. After 2 N hydrochloric acid treatment for 10 min at room temperature, cells were immunostained for BrdU with rat anti-BrdU antibody (clone BU1/75, Abcam, Cambridge, MA) as described above. In each experiment, 5 randomly selected fields were photographed and the numbers of total and BrdU-positive nuclei were counted. Each field contained approximately 200 nuclei on the average.

## *Results*

Quiescent satellite cells play central roles in skeletal muscle regeneration and repair. C2C12 cells, the most commonly used skeletal muscle cell line, generate quiescent satellite cell-like cells as well as differentiated myotubes (Lindon *et al.*, 1998; Yoshida *et al.*, 1998). Reserve cells are mitotically quiescent but can be activated to enter the cell cycle with FBS, judging from BrdU incorporation (Fig. 1A). Reserve cells would presumably be activated with certain growth factors, such as FGF, HGF, and IGFs, which was reported to participate in satellite cell activation (Johnson and Allen, 1995; Tatsumi *et al.*, 1998; Sheehan and Allen, 1999; Yablonka-Reuveni *et al.*, 1999; Rochat *et al.*, 2004). As shown in Fig. 1A, reserve cells were also activated with FGF2, a well-known regulator of myogenic cell proliferation (Olwin *et al.*, 1994; Sheehan and Allen, 1999; Yablonka-Reuveni *et al.*, 1999), in the presence of insulin (Fig. 1A). FGF2-induced BrdU incorporation in reserve cells was significantly suppressed with U0126, an inhibitor for MEK, suggesting the involvement of ERK1/2 in reserve cell activation induced with FGF2 (Fig. 1B).

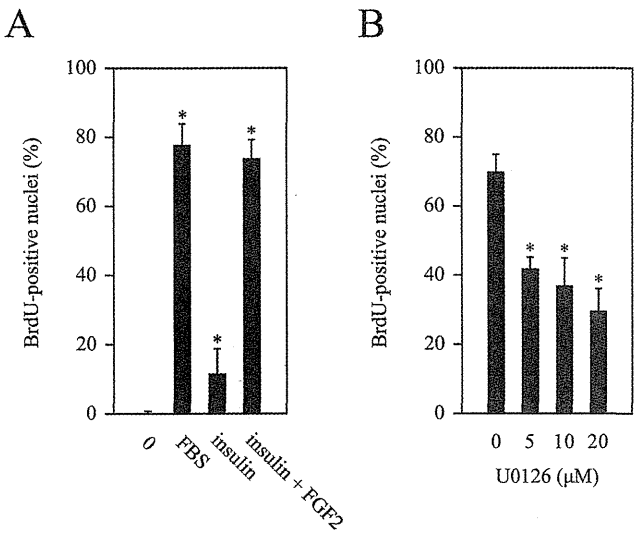


**Fig. 1.** FGF2-induced cell cycle entry of C2C12 reserve cells. Reserve cells were prepared by culturing C2C12 cells in serum-free differentiation medium for 5 days, and fed with BSA/DMEM containing 10 μM BrdU in the absence or presence of 10% FBS, 10 μg/ml insulin or 25 ng/ml FGF2. Cells were fixed with 10% formaldehyde after 24 h in culture, and then subjected to immunodetection of BrdU (A). After reserve cells were treated with MEK inhibitor U0126 at indicated concentrations, they were stimulated with a combination of 10 μg/ml insulin and 25 ng/ml FGF2. BrdU incorporation was examined after 24 h (B). Data presented are the mean percentage of BrdU positive nuclei±SD from three independent experiments. Asterisks indicate that data are statistically significant using a *t* test ($P<0.01$) compared to unstimulated cells (A) or U0126-untreated cells (B).

The aim of this study was to define the signaling systems responsible for the initiation and maintenance of ERK1/2 activation induced with FGF2 in reserve cells. Here, we especially focused on the PKC- and Grb2-dependent pathways by using inhibitor and/or siRNA. siRNA-mediated gene silencing was easily and reproducibly carried out in proliferating/differentiation C2C12 cells. We first tried to transfect proliferating C2C12 cells with siRNAs and confirmed efficient silencing of the target. Two independent sequences of siRNA duplexes against Grb2 (#1 and #2) were tested in this study. After cultured in serum-rich growth medium for 24 h, C2C12 cells were transfected with siRNAs in serum-free differentiation medium. At 48 h after transfection, C2C12 cells were harvested for detection of

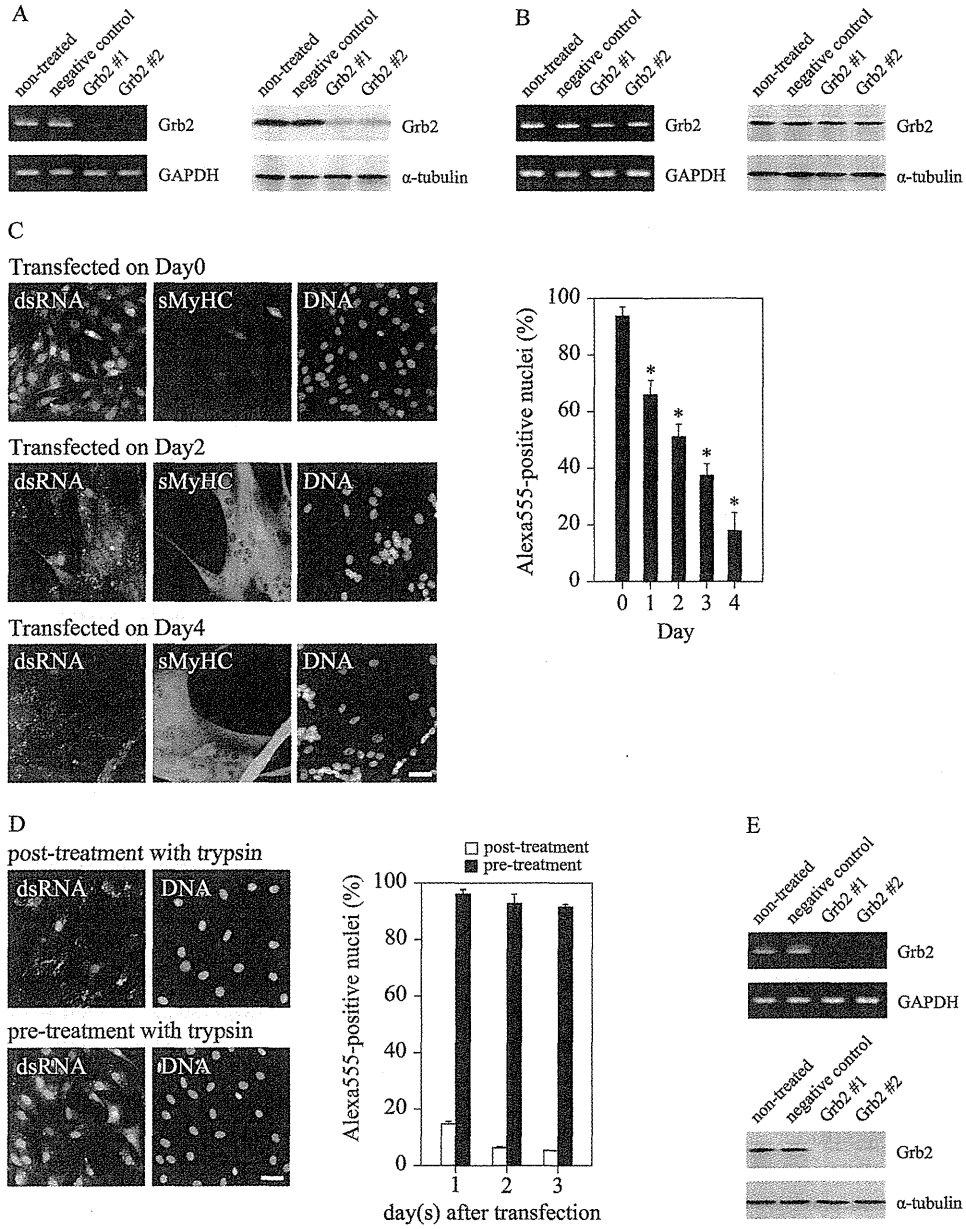


**Fig. 2.** Transfection of Grb2 siRNA into proliferating or quiescent C2C12 cells. (A and B) Proliferating C2C12 cells (A) or quiescent C2C12 cells cultured for 4 days in differentiation medium were transfected with siRNAs, and harvested at 2 days after the transfection for RT-PCR or Western blotting analysis. In contrast to clear decrease in Grb2 expression in proliferating cells, the effect of gene silencing was negligible. siRNAs used in experiment were negative control and 2 independent sequences for Grb2 (#1 and #2). GAPDH and α-tubulin were used as internal control for RT-PCR and Western blotting, respectively. (C) To monitor the efficiency of siRNA delivery, C2C12 cells were transfected with fluorescent labeled dsRNA at the same time or 1–4 day(s) after serum withdrawal. Cells were fixed at 24 h after transfection and immunostained for sarcomeric myosin heavy chain (sMyHC) as well as counterstained for DNA. Although transfection on Day0 resulted in efficient dsRNA delivery, the efficiency dropped significantly on Day2 and became much less on Day4. Bar: 50 μm. The percentage of red fluorescent oligo positive nuclei after the transfection was determined and represented as a graph. Bars indicate SD from three independent experiments. Asterisks indicate that data are statistically significant using a *t* test ($P<0.01$) compared to the transfection on Day0. (D) C2C12 cells cultured in differentiation medium for 4 days were transfected with fluorescent labeled dsRNA. At that time, C2C12 cells formed multinucleated myotubes and mononucleated reserve cells. Mild trypsinization detaches myotubes only, thus leaving reserve cells on culture plate. When trypsin treatment was carried out after the transfection, just before the fixation, only a few cells incorporated siRNA into their nuclei. In contrast, trypsin treatment before transfection greatly enhanced the efficiency of siRNA delivery. Bar: 50 μm. The percentage of fluorescent dsRNA oligonucleotide-positive cells treated with trypsin after transfection (blank column) and cells treated with trypsin before transfection (filled column) were represented as a graph. Bars indicate SD from three independent experiments. (E) Reserve cells were transfected with siRNAs as described above, harvested 3 days thereafter, and analyzed for Grb2 expression by RT-PCR and Western blotting. As expected, gene silencing of Grb2 was clearly observed at both mRNA- and protein-level.

Grb2 at mRNA level by RT-PCR as well as at protein level by Western blotting analysis. As shown in Fig. 2A, mRNA expression was markedly reduced with Grb2-specific siRNA. Negative control siRNA, which was designed to minimize sequence homology to any known vertebrate transcript, showed virtually no effect. Furthermore, Western blotting analysis showed Grb2 protein was effectively reduced in Grb2-specific siRNA transfected cells (Fig. 2A).

In order to define the signaling systems responsible for the initiation and maintenance of ERK1/2 activation induced with FGF2 in reserve cells, we needed to achieve efficient gene silencing in quiescent reserve cells. In contrast to successful siRNA transfection into proliferating cells (Fig. 2A), the transfection of quiescent reserve cells, which were cultured in differentiated serum-free medium for 4 days, resulted in poor silencing of Grb2 expression at both the mRNA- and protein-level (Fig. 2B).

To evaluate the efficiency of siRNA delivery into C2C12 cells, we used fluorescent-lableled double strand RNA (dsRNA) oligomer. The fluorescent dsRNA oligomer would be incorporated into cell nuclei when the transfection was achieved successfully. We examined on which day C2C12 cells can be transfected with siRNA after the induction of differentiation. C2C12 cells were induced to differentiate, and then siRNA transfections were carried out at the same time on 1–4 days thereafter with fluorescent dsRNA oligomer. Cells were fixed at 24 h after the transfection, and the uptake of fluorescent dsRNA oligomer was assessed. As shown in Fig. 2C, fluorescent signals were readily observed in nuclei when transfection was carried out at the same time of the induction of differentiation. The presence of differentiated myotubes, which expressed sMyHC, became apparent on Day2 or later (Fig. 2C). The efficiency of transfection, as well as fluorescence intensity, was severely reduced in cells cultured in differentiation medium (Fig. 2C). Only 18.1% of cells incorporated fluorescent dsRNA oligomer when transfection was carried out on Day4 (Fig. 2C). It is worth noting that aggregated fluorescence signals were frequently observed outside cells when siRNA transfection resulted in poor efficiency (Fig. 2C). We also examined how long siRNA remained after C2C12 cells were transfected with fluorescent dsRNA oligomer on Day0, and found that only 31.4% of cells possessed fluorescent signal after 5 days (data not shown). Taken together, these results suggest that the ordinary procedure does not work for transfecting quiescent reserve cells with siRNA because of severe reduction in siRNA delivery in these cells.

Mild trypsinization was reported to be useful as to obtain pure reserve cell cultures (Kitzmann *et al.*, 1998). In fact, we have also utilized the technique with some modification and established reproducible isolation (Nagata *et al.*, 2006a). Because conventional transfection did not work for siRNA transfection of reserve cells, we next tried transfection soon after reserve cell isolation. C2C12 cells were allowed to differentiate for 4 days in differentiation medium. At this time point, approximately 70% of the cells form multinucleate myotubes, while the rest of the cells remain in an undifferentiated quiescent state (Kitzmann *et al.*, 1998; Yoshida *et al.*, 1998). Cells were treated with 0.05% trypsin in PBS (+) for 5 min at 37°C. Only myotubes were detached from the cell culture plate by this treatment, while reserve cells remain attached to cell dishes. Cells were rinsed with PBS (+) to wash away detached myotubes and remaining trypsin, then fed with DMEM containing siRNA-liposome complexes. Although trypsin-treatment after the transfection did not make any improvement, nuclear-localized fluorescent signals were clearly observed in cells pre-treated with trypsin, and as a consequence, the percentage of fluorescent siRNA-positive cells was maintained at more than 90% on Day3 after transfection. (Fig. 2D)

Because fluorescent-labeled siRNA were successfully delivered into quiescent reserve cells by trypsin pretreatment, we then tried transfection with siRNAs against Grb2. Reserve cells were harvested for the analysis of the expression of Grb2 at 3 days after transfection. The decrease in mRNA expression was observed at maximum on Day3 (Fig. 2E), and concomitantly, the silencing of gene expression was also observed at protein level (Fig. 2E). Importantly, the efficient silencing of Grb2 expression in quiescent reserve cells was achieved with siRNAs at a concentration of 5 nM. There was no obvious cytotoxicity in that condition.

Since we established an efficient siRNA transfection into quiescent reserve cells, we next examined the signaling pathway of reserve cell activation. To examine the involvement of Grb2 and PKC in FGF2-induced reserve cell activation, serum-starved reserve cells were transfected with siRNA against Grb2 as described above, and then after 3 days, cells were stimulated with either FGF2 or phorbol 12-myristate 13-acetate (PMA) in the presence or absence of PKC inhibitors. Initially, reserve cells were stimulated with PMA. PMA is a well-known activator of PKC, and thus used to examine the potential role of PKC on ERK1/2 phosphorylation in reserve cells. PMA caused rapid and sustained phosphorylation of ERK1/2 (Fig. 3A), suggesting the presence of a PKC-mediated pathway leading to ERK phosphorylation in reserve cells.

We used a combination of two PKC inhibitors, namely, Gö6976 (Martiny-Baron *et al.*, 1993) and Gö6983 (Gschwendt *et al.*, 1996), to inhibit almost all isoforms of PKC as reported (Shu *et al.*, 2002). After 30 min pretreatment with PKC inhibitors, reserve cells were stimulated with PMA for 15 min and then harvested for Western blotting analysis. In reserve cells, the combination of Gö6976 and Gö6983 almost completely suppressed PMA-induced phosphorylation of ERK1/2 (Fig. 3B). Although expression levels of Grb2 were reproducibly decreased to less than 10% of normal level in Grb2-siRNA treated cells, the silencing of Grb2 did not have any effect on ERK phosphorylation in PMA-stimulated reserve cells (Fig. 3B),

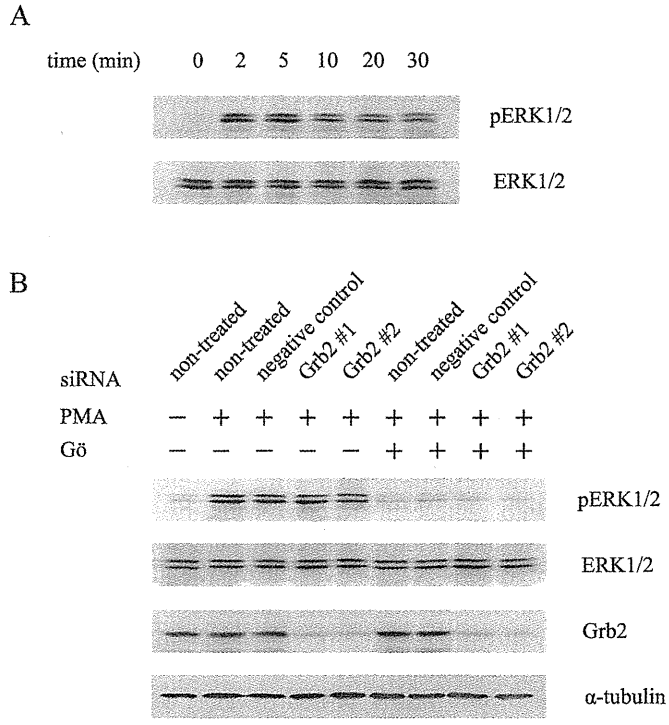


**Fig. 3.** Analysis of PKC- and Grb2-dependent ERK phosphorylation in PMA-stimulated reserve cells. (A) Serum-starved reserve cells were transfected with siRNA against Grb2 after pretreatment with trypsin, and then 3 days later, cells were fed with 100 nM PMA. After incubation for 2–30 min, cells were harvested for the analysis of ERK phosphorylation. (B) Before PMA was added, reserve cells transfected with siRNAs were treated with a combination of two PKC inhibitors, 1 μM Gö6976 and 1 μM Gö6983. Fifteen minutes after PMA stimulation, cells were harvested for Western blotting analysis. Expression levels of phospho-ERK1/2, ERK1/2, Grb2, α-tubulin were detected with their specific antibodies.

demonstrating that PKC phosphorylated ERK independent of Grb2.

PKC has been reported to mediate peptide growth factor induced signaling pathways (Presta *et al.*, 1989; Nishizuka, 1992; Kim *et al.*, 2003; Clerk *et al.*, 2006).

Indeed, FGF2, as well as PMA, caused phosphorylation of PKC especially with higher molecular weight (Fig. 4A). The PKC antibody used in this study recognizes α, βI, βII, δ, ε, η, and θ isoforms only when phosphorylated at an autophosphorylation site (Keranen *et al.*, 1995). The similar band patterns were reported previously (Sweeney *et al.*, 2001).

FGF2 caused robust and transient phosphorylation of ERK1/2 in reserve cells, detectable within 2 min after the stimulation, peaked around 5 min (Fig. 4B). The suppression of ERK phosphorylation by PKC inhibition was prominent at 2 min. Interestingly, higher levels of phosphorylated ERK1/2 were detected at later time points in Gö-treated cells compared to untreated cells (Fig. 4B). Gene-silencing of Grb2 resulted in decreased levels of ERK phosphorylation at any time point, but certain levels of phosphorylated ERK1/2 were detectable (Fig. 4B).

For close examination of FGF2-induced ERK phosphorylation, cells were treated with the combination of siRNA and PKC inhibitors, and then sampled at 2, 15, and 30 min after FGF2 stimulation. At 2 min after FGF2 stimulation, phosphorylation of ERK1/2 was significantly suppressed with either siRNA against Grb2 or PKC inhibitors. Almost complete suppression of ERK phosphorylation was observed when cells were transfected with Grb2-specific siRNA followed by treatment with PKC inhibitors (Fig. 4C). Although ERK phosphorylation which was dependent on Grb2 still existed at high levels, sensitivity of PKC inhibition was not observed at 15 min (Fig. 4C). Only weak signals of phosphorylated ERK1/2 were detectable at 30 min in normal conditions, but PKC-inhibition resulted in higher levels of ERK phosphorylation (Fig. 4C), suggesting the presence of PKC-mediated suppression of receptor-coupled tyrosine kinase activity (Cochet *et al.*, 1984) at later time points. Notably, as shown in Fig. 4C, phosphorylation of ERK1/2 at 30 min was dependent on Grb2, in contrast to PMA-induced ERK phosphorylation (see Fig. 3B).

Collectively, these results demonstrated that FGF2 induced phosphorylation of ERK1/2 in a manner dependent on both Grb2 adapter protein and PKC with distinct kinetics.

## *Discussion*

Quiescent satellite cells must be initially activated before participating in skeletal muscle regeneration and repair. Although it is widely known that certain stimuli, such as injury, overload, and exercise, cause activation of satellite cells (reviewed in Charge and Rudnicki, 2004), the molecular mechanisms in the control of satellite cell activation is largely unknown. FGF2 is an established mitogen for myogenic cells and implicated in satellite cell activation (Olwin *et al.*, 1994; Sheehan and Allen, 1999; Yablonka-Reuveni *et al.*, 1999). We showed FGF2 made reserve cells enter the cell cycle partially through the ERK pathway. ERK plays an important role in the control of various cellular responses, including cell proliferation, differentiation, and survival. A variety of growth factors and cytokines are known to activate ERK1/2 via a complicated signaling network. In addition to the Grb2/Sos-mediated pathway, other distinct signaling cascades such as a PLC/PKC- or a PI3K/Akt-mediated pathway could also contribute to ERK activation (reviewed in Schlessinger, 2000).

We showed FGF2 induced phosphorylation of ERK1/2 was mostly dependent on both Grb2 and PKC because ERK phosphorylation was almost, if not completely, suppressed by a combined treatment with Grb2-specific siRNA and PKC inhibitors. Therefore, delayed and sustained phosphorylation of ERK in the presence of PKC inhibitors would reflect mostly the Grb2-mediated pathway. Higher levels of

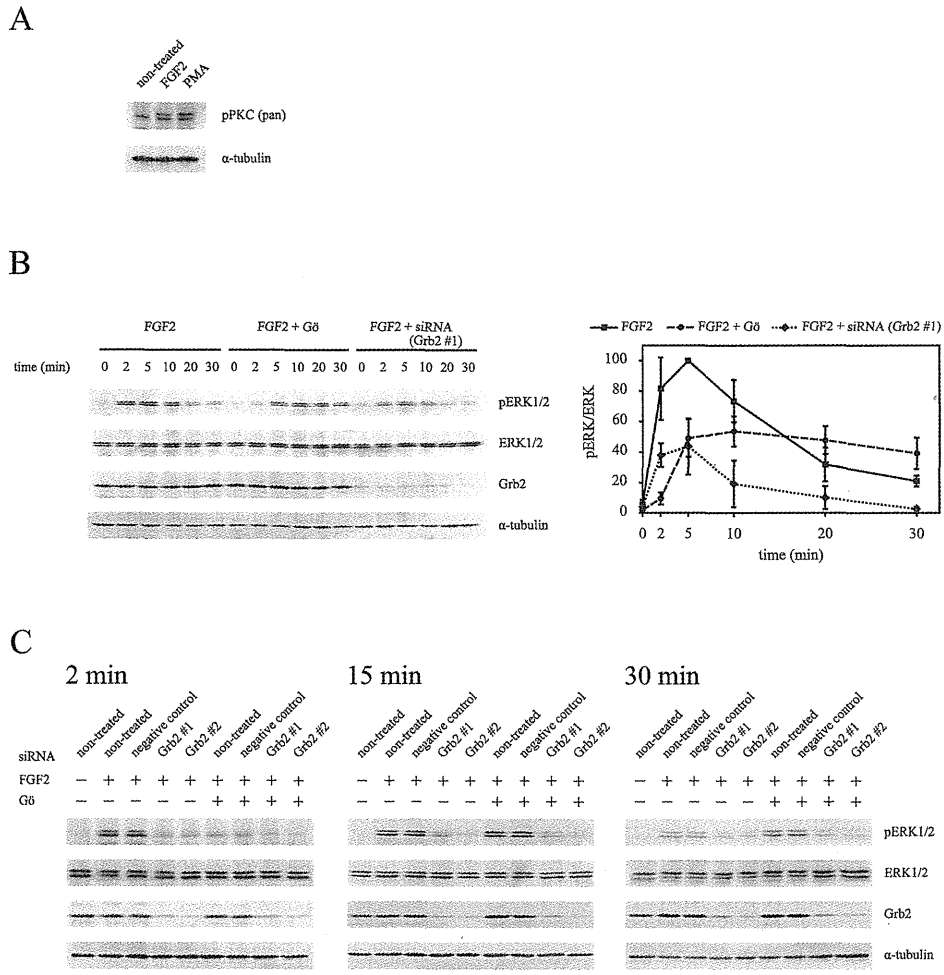


**Fig. 4.** Analysis of PKC- and Grb2-dependent ERK phosphorylation in FGF2-stimulated reserve cells. (A) Reserve cells were incubated with either FGF2 for 5 min or PMA for 15 min, and then harvested for the analysis of PKC phosphorylation by Western blotting. The PKC antibody used in this study recognizes α, βI, βII, δ, ε, η, and θ isoforms only when phosphorylated at an autophosphorylation site. (B) FGF2-induced ERK phosphorylation was examined in reserve cells which were in normal condition, treated with PKC inhibitors, or transfected with siRNA against Grb2 (#1). At 2–30 min after stimulation, cells were harvested for the analysis of ERK phosphorylation. The intensities of phosphor-ERK/ERK were measured and normalized to the value of phosphor-ERK/ERK at 5 min in FGF2-stimulated reserve cells in normal conditions. Bars indicate SD from three independent experiments. (C) Reserve cells transfected with siRNAs were treated with a combination of PKC inhibitors, and then fed with FGF2. Cells were harvested for Western blotting analysis at 2, 15 or 30 min after the stimulation. Expression levels of phospho-ERK1/2, ERK1/2, Grb2, and α-tubulin were detected with their specific antibodies.

ERK phosphorylation at later time points would demonstrate PKC-mediated suppression of receptor-coupled tyrosine kinase activity (Cochet *et al.*, 1984) in normal conditions. On the other hand, rapid phosphorylation of ERK observed in cells transfected with Grb2 specific siRNAs would mostly demonstrate the PKC-mediated pathway. Taken together, we would conclude that FGF2-caused ERK phosphorylation is dependent on both the Grb2- and the PKC-mediated pathway, and that the PKC-mediated pathway contributes to rapid initiation and termination of ERK phosphorylation, while the Grb2-mediated pathway contributes to delayed and sustained ERK phosphorylation.

In this study, we achieved efficient siRNA transfection of quiescent reserve cells. For siRNA transfection, exponentially growing cells are preferentially used (Elbashir *et al.*, 2002). In fact, we showed that inefficient knockdown of Grb2 expression occurred in quiescent reserve cells by common transfection protocol (Fig. 2B). That is because of poor delivery of siRNA into nuclei as the delivery of fluorescent labeled siRNA was significantly reduced after cells were induced to differentiation by serum withdrawal. Surprisingly though, remarkable improvement of siRNA delivery was observed when transfection was carried out after mild trypsinization. Furthermore, knockdown of Grb2 expression has also occurred efficiently in the trypsin-treated cells before transfection. Several studies have reported siRNA-

mediated gene silencing in quiescent cells of other cell types by transfection of proliferating cells with siRNAs followed by making cells quiescent by serum withdrawal (Asano *et al.*, 2005; Tullai *et al.*, 2007). However, this protocol was unsuitable for a loss-of-function analysis of Grb2 in reserve cells since the absence of Grb2 in proliferating C2C12 cells resulted in significant loss of reserve cells (data not shown). Because reserve cells are prepared by serum withdrawal, which causes apoptosis as well as differentiation, it is predicted that the suppression of gene products which control cell proliferation, differentiation, or apoptosis would cause abnormalities in the formation of reserve cells. In contrast, our technique had much less effect on reserve cell formation, thus enabling reliable loss-of-function analyses in reserve cells. It was shown that efficient knockdown was carried out in quiescent human bladder carcinoma cells without extra manipulation except that they used 50 nM of siRNA, five times higher concentrations than normally used, which caused significantly more cell death possibly because of off-target effect (Nabatiyan and Krude, 2004). On the other hand, our method does not require high concentration of siRNA and apparent cytotoxicity was not observed. Because aggregated fluorescent signals were observed only when siRNA transfection resulted in poor efficiency, we conjectured that siRNA/liposome complexes are trapped extracellularly in such conditions. We also speculated trypsin-treatment removed sticky molecules existing in the differentiated cell culture, which thus made siRNA/liposome complexes accessible to reserve cells. We have previously shown that the expression level of sphingomyelin was decreased as satellite cells were activated (Nagata *et al.*, 2006a), and that sphingosine-1-phosphate, one of sphingomyelin metabolites, contributed to the transition of satellite cells from quiescent to proliferative (Nagata *et al.*, 2006b). Detailed analysis of signaling pathways in the activation of reserve cells, as a model of satellite cells, would become possible by applying siRNA-mediated gene silencing in quiescent reserve cells.

In this study, we revealed that FGF2 caused ERK phosphorylation in a manner dependent on both Grb2 and PKC with different kinetics by efficient gene silencing of Grb2 in quiescent reserve cells. Successful gene silencing in reserve cells would be applicable to other gene products, thus would be useful to examine the molecular mechanisms in the activation of quiescent satellite cells as well as in the maintenance of quiescent state.

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# Relationship of negative mood with prefrontal cortex activity during working memory tasks: An optical topography study

Ryuta Aoki[a,b,*], Hiroki Sato[c,**], Takusige Katura[c], Kei Utsugi[d], Hideaki Koizumi[c], Ryoichi Matsuda[a], Atsushi Maki[c]

[a] Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan
[b] Japan Society for the Promotion of Science, 8 Ichibancho, Chiyoda-ku, Tokyo 102-8472, Japan
[c] Advanced Research Laboratory, Hitachi, Ltd., 2520 Akanuma, Hatoyama, Saitama, 350-0395, Japan
[d] Systems Development Laboratory, Hitachi, Ltd., 292 Yoshida-cho, Totsuka-ku, Yokohama, Kanagawa 244-0817, Japan

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ABSTRACT

Mood has a substantial impact on cognitive functions. Although studies have shown that the interaction between mood and cognition is mediated by the prefrontal cortex (PFC), little is known about how naturalistic mood in everyday life is associated with PFC activity during cognitive tasks. We investigated whether inter-individual variation in perceived mood under current life situations (recent week) is related to PFC activity during working memory (WM) tasks in healthy adults. Levels of positive and negative moods were quantified with the Profile of Mood States (POMS) questionnaire. PFC activities during verbal and spatial WM tasks were measured by optical topography (OT), a non-invasive low-constraint neuroimaging tool, to minimize experimental intervention in participants' moods. Group-average analysis showed significant activations in the bilateral dorsolateral PFC in both WM tasks. Correlation analysis revealed that the participants reporting higher levels of negative moods showed lower levels of PFC activity during the verbal WM task but not during the spatial WM task. This relationship was significant even after controlling for possible confounding factors such as age, gender, and task performance. Our results suggest that verbal WM is linked with naturalistic negative mood and that the PFC is involved in the mood–cognition interaction in daily circumstances.

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## 1. Introduction

In everyday life, the way we think and act is substantially affected by our mood, regardless of whether we recognize it or not. Psychologists have long investigated the relationship between mood and cognition, revealing that many cognitive functions are actually modulated by mood (Mitchell and Phillips, 2007; Robinson and Sahakian, 2009). An important cognitive function in the mood-cognition interaction is working memory (WM), a mental process well documented in both psychology and neuroscience (Baddeley, 2003). Behavioral studies have shown that performance on various cognitive tasks requiring WM (e.g., word span task, Tower of London planning task) is affected by the participant's mood Mitchell and Phillips (2007). Psychopharmacological studies and theoretical models have also suggested that mood and relevant neurotransmitters (e.g., dopamine and serotonin) are linked with WM performance (Ashby et al., 1999; Luciana et al., 1998).

Neuroimaging research has begun to elucidate the underlying neural mechanisms of the mood-cognition interaction in WM. As the prefrontal cortex (PFC) has been demonstrated to play a crucial role in WM (Smith and Jonides, 1999; Smith et al., 1996), the effect of mood on WM is likely mediated by the PFC function. Indeed, a functional magnetic resonance imaging (fMRI) study showed that activity in the dorsolateral PFC (DLPFC) during a WM task (numerical N-back task) was reduced when the participants were exposed to acute psychological stress (induced by viewing aversive movie clips), which led to increased negative mood (Qin et al., 2009). This finding is consistent with the results of other fMRI studies showing that WM-related activity in the DLPFC is attenuated by affective modulation using negative emotional stimuli (Anticevic et al., 2010; Perlstein et al., 2002). These results provide valuable insight into the role of the PFC in the interaction between mood and WM.

However, experimentally induced mood may be different from naturalistic mood in its relationship with cognition. Characteristics of induced moods (e.g., intensity, duration, and whether

* Corresponding author. Tel.: +81 3 5454 6637; fax: +81 3 5454 4306.
** Co-corresponding author. Tel.: +81 49 296 6111; fax: +81 49 296 5999.
*E-mail addresses:* cc097702@mail.ecc.u-tokyo.ac.jp, ryuta.aoki.mu@hitachi.com (R. Aoki), hiroki.sato.ry@hitachi.com (H. Sato).

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