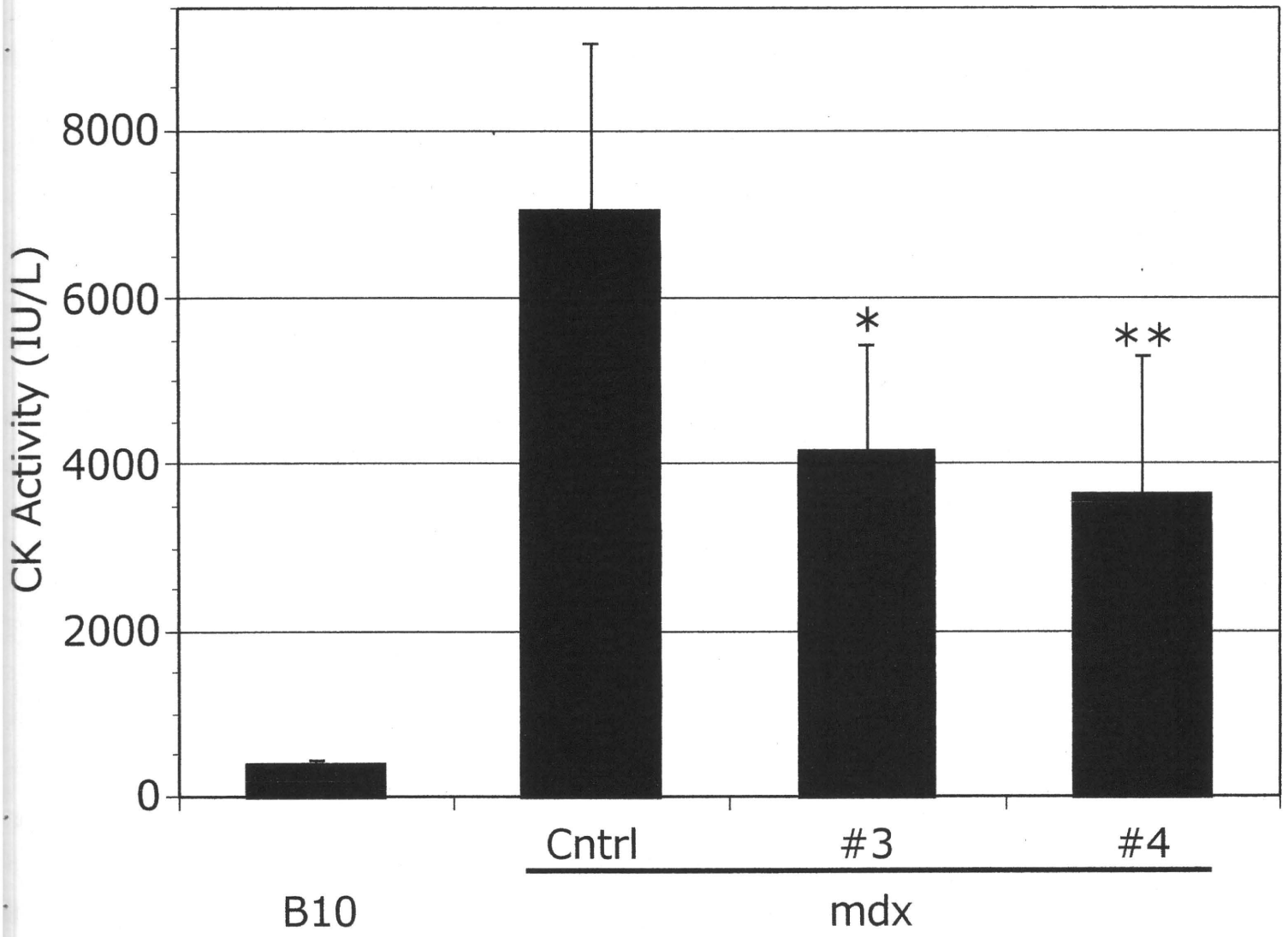
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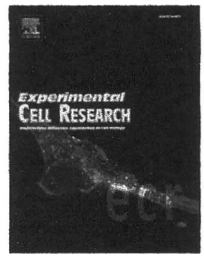

Research Article

IGF-I and vitamin C promote myogenic differentiation of mouse and human skeletal muscle cells at low temperatures

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ABSTRACT

In a previous study investigating the effects of low temperature on skeletal muscle differentiation, we demonstrated that C2C12 mouse myoblasts cultured at 30 °C do not express myogenin, a myogenic regulatory factor (MRF), or fuse into multinucleated myotubes. At this low temperature, the myoblasts continuously express Id3, a negative regulator of MRFs, and do not upregulate muscle-specific microRNAs. In this study, we examined if insulin-like growth factor-I (IGF-I) and a stable form of vitamin C (L-ascorbic acid phosphate) could alleviate the low temperature-induced inhibition of myogenic differentiation in C2C12 cells. Although the addition of either IGF-I or vitamin C alone could promote myogenin expression in C2C12 cells at 30 °C, elongated multinucleated myotubes were not formed unless both IGF-I and vitamin C were continuously administered. In human skeletal muscle cells, low temperature-induced blockage of myogenic differentiation was also ameliorated by exogenous IGF-I and vitamin C. In addition, we demonstrated that satellite cells of IGF-I overexpressing transgenic mice in single-fiber culture expressed myogenin at a higher level than those of wild-type mice at 30 °C. This study suggests that body temperature plays an important role in myogenic differentiation of endotherms, but the sensitivity to low temperature could be buffered by certain factors *in vivo*, such as IGF-I and vitamin C.

Introduction

Endotherms are able to maintain constant core body temperatures (36–39 °C, [1]) regardless of the surrounding environmental temperature. The high and constant body temperature has resulted in a finely tuned metabolism and high muscular power output in comparison with ectotherms [2]. However, human body temperature actually varies considerably from part to part; for

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Abbreviations: DM, differentiation medium; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EDL, extensor digitorum longus; FBS, fetal bovine serum; IGF, insulin-like growth factor; MEM, minimal essential medium; miRNA, microRNA; MRF, myogenic regulatory factor; MyHC, myosin heavy chain; PBS, phosphate-buffered saline; SEM, standard error of the mean; VC, vitamin C; WT, wild-type

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example, it was reported that the temperature of fingertips is 9 °C lower than the core body temperature (37 °C) in human at room temperature (20 °C) [3].

We have previously examined the effects of low temperature on cell differentiation using skeletal muscle cells as they are distributed throughout the entire body and are expected to be more influenced by surrounding temperature than cells of other organs located deeper inside the body. At a temperature of 30 °C, mouse myoblasts could neither express myogenin, a myogenic regulatory factor (MRF), nor fuse into multinucleated myotubes, and were observed to continuously express Id3, an inhibitory transcription factor for MRFs [4]. Although these results indicate that temperature plays an important role in myogenic differentiation, but there is still a question whether the myocytes completely lose the capacity to differentiate at 30 °C or whether they can differentiate even at low temperature if appropriate rescuers are added to the cell culture.

It is well known that insulin-like growth factor-I (IGF-I) plays multiple important roles during myogenesis by stimulating both growth and differentiation [5]. Transgenic mice which overexpress IGF-I in skeletal muscles display promoted adult muscle regeneration and hypertrophy via activation of muscle satellite cells [6–8]. Although there are no reports indicating that IGF-I is capable of inducing myogenin expression at low temperature, we prospected that IGF-I was a good candidate for promoting myogenin expression at 30 °C because it accelerates terminal myogenic differentiation by inducing myogenin expression at normal temperature [9,10]. We were additionally interested in examining IGF-II, as it also binds to IGF-I receptors [11] and induces accelerated myogenic differentiation when overexpressed [12].

Another molecule which affects myogenic differentiation is L-ascorbic acid (vitamin C (VC)). VC plays various essential roles *in vivo*, which includes serving as a cofactor for the hydroxylation of proline and lysine during collagen synthesis. Although L-ascorbic acid is unstable and rapidly degraded under the normal culture conditions [13], a phosphate derivative of L-ascorbic acid is considerably stable, with 85% still remaining after a 1-week incubation at 37 °C [14]. L-ascorbic acid phosphate was reported to increase myogenin expression in muscle cells and promote their myogenic differentiation by accelerating collagen synthesis at 37 °C [15,16]. In this study, we therefore used L-ascorbic acid phosphate instead of L-ascorbic acid as a VC source.

Here, we examined if IGFs (IGF-I and IGF-II) and VC could promote myogenic differentiation at lower temperatures than the normal body temperature of endotherms in C2C12 mouse myoblast cell line, satellite cells on mouse myofibers, and human skeletal muscle cells by immunostaining for myogenin and sarcomeric myosin heavy chain (MyHC) and RT-PCR for myogenin, Id3, and muscle-specific microRNAs (miRNAs). All the results suggested that IGF and VC could promote myogenic differentiation at low temperatures.

Materials and methods

Cell culture

The C2C12 cell line (ATCC, Manassas, VA) [17], which is a subclone of the C2 cell line isolated from the thigh muscle of an adult C3H mouse [18], was cultured in DMEM (high-glucose) (Nacalai Tesque, Kyoto, Japan) containing 20% FBS (SAFC Biosciences, Lenexa, KS), 50 U/ml penicillin and 50 μg/ml streptomycin on plastic dishes (AGC Techno Glass, Chiba, Japan) coated with 1% bovine skin gelatin (Sigma-Aldrich, St. Louis, MO) at 38 °C in 5% CO_2. At a point of near confluence, the medium was replaced with differentiation medium (DM) which consisted of MEM (Invitrogen, Carlsbad, CA) containing 10% horse serum (Invitrogen) and appropriate antibiotics and the cells were then cultured at either 25, 28, 30 or 38 °C. For rescue experiments, 10, 50 or 100 ng/ml IGF-I (R&D Systems, Minneapolis, MN), 100 ng/ml IGF-II (R&D Systems), or 200 μM L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako Pure Chemical Industries, Osaka, Japan) was added to DM. The medium was changed every other day.

Conditioned medium (CM) was collected every other day from well differentiated C2C12 cells cultured in DM at 38 °C and passed through 0.22 μm filter to exclude living cells. C2C12 cells were cultured in CM at 30 °C and the medium was changed with fresh CM every other day.

A human skeletal muscle biopsy was obtained under an IRB approved protocol at Children's Hospital of Philadelphia. Explant culture of the biopsy was then performed following the protocol of Decary et al. [19]. Myoblasts obtained from the explant cultures were used to produce immortalized clonal lines, following the procedures of Zhu et al. [20]. Human skeletal muscle cells were cultured in F-10 (Invitrogen) containing 20% FBS and appropriate antibiotics on plastic dishes coated with collagen I (AGC Techno Glass) at 38 °C. At a point of near confluence, the medium was replaced with the identical DM used in the C2C12 culture and the cells were then cultured at either 30 or 38 °C. Rescue experiments using IGF-I and VC were performed as described above for the C2C12 cells.

Single-fiber culture

Single muscle fibers of MLC/mIgf-1 transgenic (IGF-I Tg) mice (see [8], however, donors were not FBV but C57BL/6 mice) and C57BL/6 wild-type (WT) mice were prepared as described previously [21]. Briefly, the extensor digitorum longus (EDL) muscles were removed from euthanized adult male mice by excising the tendons, and were then treated with 0.1% collagenase type I (Sigma-Aldrich) in DMEM at 37 °C for 2 h. The fibers were cultured in DMEM (high-glucose, GlutaMAX-I+) (Invitrogen) containing 20% FBS (Thermo Fisher Scientific, Waltham, MA) and appropriate antibiotics at 37 °C for 3 days (37 °C culture) or at 37 °C for 1 day and then 30 °C for 3 more days (30 °C culture). Animal experimentation was carried out according to the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Immunofluorescence

Immunofluorescence was performed as described previously [4]. Briefly, cells or mouse muscle fibers were fixed with 10% formalin in PBS and treated with 100% methanol. The samples were then incubated with primary antibodies (mouse anti-myogenin, F5D (1:1, Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti-sarcomeric myosin heavy chain, MF20 (1:2, Developmental Studies Hybridoma Bank) and rabbit anti-MyoD (1:50, Santa Cruz Biotechnology, Santa Cruz, CA)) for 1 h, followed by Alexa Fluor dye-conjugated secondary antibodies (1:400,

Invitrogen) for 1 h. Cell nuclei were stained with either Hoechst 33258 (Sigma-Aldrich) or DAPI (Vector Laboratories, Burlingame, CA).

mRNA isolation and RT-PCR

Total RNA was isolated from C2C12 cells 4 days after the induction of differentiation using RNAZol B reagent (Tel-Test, Friendswood, TX) in accordance with the manufacturer's protocol. Reverse transcription of the isolated mRNA and amplification of cDNA were performed with 100 ng RNA using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen). The PCR conditions were 94 °C for 15 s, 60 °C for 30 s, and 55 °C for 45 s for 23 cycles. The sequences of the primers for myogenin, Id3 and β-actin were as follows: myogenin 5′-GAG CTG TAT GAG ACA TCC CC-3′ and 5′-GTA AGG GAG TGC AGA TTG TG-3′ [22]; Id3 5′-ACT CAG CTT AGC CAG GTG GA-3′ and 5′-CAT TCT CGG AAA AGC CAG TC-3′; β-actin 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ and 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′ [23]. The PCR products were resolved by electrophoresis on 2% agarose gels and stained with 1 μM SYTO60 Red Fluorescent Nucleic Acid Stain (Invitrogen) for 20 min. Band intensity was measured using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). The individual band intensity of myogenin and Id3 was standardized to that of β-actin in the same culture condition.

MiRNA isolation and RT-PCR

Small RNAs were isolated from C2C12 cells 4 days after the induction of differentiation using a *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX, USA) in accordance with the manufacturer's protocol. Reverse transcription of the isolated small RNAs and amplification of cDNA were performed with 10 ng RNA using a *mir*Vana qRT-PCR miRNA Detection Kit (Ambion) and *mir*Vana qRT-PCR Primer Sets (Ambion). The primers for microRNA (miR)-1, miR-133a, miR-181a, miR-206 and 5S rRNA were hsa-miR-1, hsa-miR-133a, hsa-miR-181a, hsa-miR-206 and 5S, respectively. The PCR conditions were 95 °C for 15 s and 60 °C for 30 s for 20 cycles. The PCR products were resolved by electrophoresis on 3.5% agarose gels and stained with 1 μM SYTO60 dye for 20 min. Band intensity was measured using the Odyssey Infrared Imaging System. The individual band intensity of each miRNAs was standardized to that of 5S rRNA in the same culture condition.

Statistical analysis

The cell nuclei expressing myogenin in the C2C12 and human skeletal muscle cells were counted in 3 or 4 different fields for each sample (totaling over 600 nuclei) and the percentages of myogenin-positive nuclei out of the total nuclei were calculated. The cell nuclei in MyHC-positive C2C12 cells were counted in more than 3 different fields for each sample (totaling over 400 nuclei) and the percentages to the total nuclei were calculated. For the single-fiber culture analysis, the ratios of myogenin-positive nuclei to MyoD-positive nuclei per myofiber were calculated. The number of analyzed myofibers was as follows: in the 37 °C culture, IGF-I Tg: $n=52$, WT: $n=34$ (day 2), IGF-I Tg: $n=46$, WT: $n=37$ (day 3); in the 30 °C culture, IGF-I Tg: $n=61$, WT: $n=37$ (day 3), IGF-I Tg: $n=45$, WT: $n=29$ (day 4). The percentages or ratios are presented as the mean ± SEM. The Student's *t*-test was used to analyze statistical significance.

Results

IGF-I and VC promote myogenin expression and myotube formation of C2C12 cells at 30 °C

At 38 °C, which is close to the normal body temperature of a mouse, C2C12 mouse myoblast cells undergo terminal differentiation immediately after being switched to DM and fuse into multinucleated myotubes. At 30 °C, however, myoblast cells express MyoD, but not myogenin, and do not form myotubes [4]. To elucidate that the lack of differentiation at low temperature was only due to temporary blocking and not a complete loss of the capacity to differentiate, we searched for factors that could promote myotube formation at 30 °C.

C2C12 cells in DM containing either IGF-I or L-ascorbic acid phosphate, a stable form of VC, expressed myogenin 4 days after the induction of differentiation (day 4) (Fig. 1A) and fused into myotubes by day 6 at 30 °C (Fig. 1B). Although the treatment with 100 ng/ml IGF-I or 200 μM VC promoted myogenin expression independently, the effect was synergetically enhanced when they were added in combination. Cells cultured in DM containing both IGF-I and VC formed multinucleated myotubes expressing MyHC, while only mononucleated myocytes or thinner myotubes were observed in DM containing IGF-I or VC alone (Fig. 1B). The percentage of nuclei in the MyHC-positive cells to the total nuclei was also significantly higher in the culture containing both IGF-I and VC than that in the culture containing either IGF-I or VC alone (Fig. 1C). IGF-I promoted myogenin expression in a dose-dependent manner, when the VC concentration was fixed at 200 μM (Fig. 1D). To examine whether the continuous addition of IGF-I was required to promote myogenic differentiation at 30 °C, the period of IGF-I treatment was changed while 200 μM VC was maintained in the medium throughout the culture. The treatment of 100 ng/ml IGF-I for only 1 or 2 days after the induction of differentiation could not promote the expression of myogenin at 30 °C (Fig. 1E), suggesting that the continuous presence of IGF-I for several days was required.

IGF-II also promotes myogenic differentiation at 30 °C

We also examined whether the addition of IGF-II, a homologue of IGF-I, could promote myogenic differentiation at 30 °C with or without the presence of VC. The percentage of myogenin-expressing nuclei to the total nuclei was higher when both IGF-II and VC were used to treat myoblasts than IGF-II alone (Fig. 2A). However, the percentage of myogenin-positive nuclei (11% ± 1.2 (−VC), 19% ± 3.2 (+VC)) was generally lower than that observed in IGF-I-treated cells (16% ± 0.8 (−VC), 33% ± 1.1 (+VC)) when the IGFs were added at identical concentrations (100 ng/ml). Multinucleated myotubes were formed in DM containing both IGF-II and VC, while thinner myotubes were formed when only IGF-II was added to DM (Fig. 2B and C).

Id3 expression is downregulated by IGF-I and VC

In a previous study [4], we reported that Id3, which acts as a negative regulator of MRFs and is downregulated upon the initiation of differentiation at 38 °C [24], was continuously expressed in myoblasts at 30 °C. Here, we examined whether the

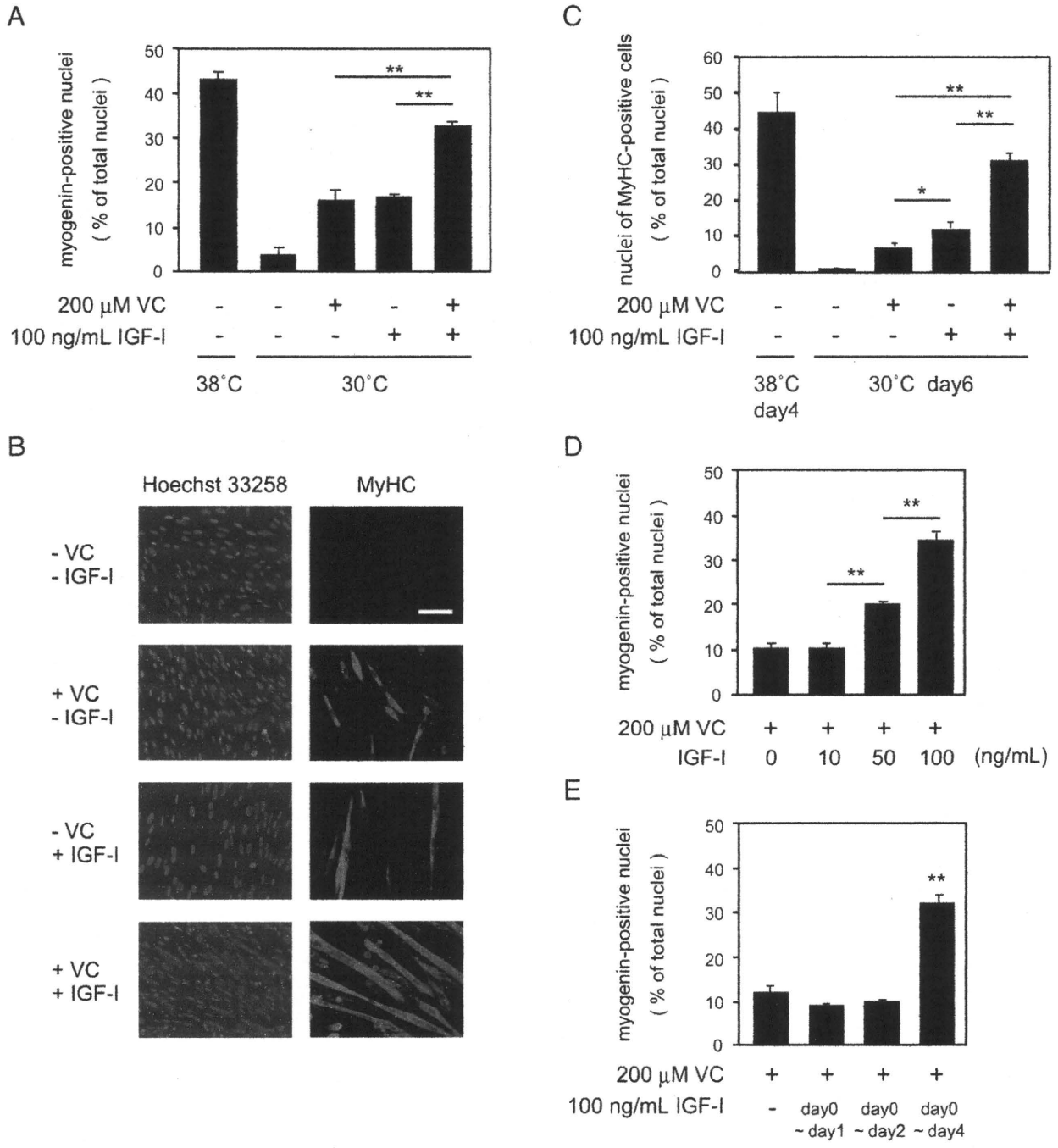

Fig. 1 – Effects of IGF-I and VC on myogenic differentiation of C2C12 cells at 30 °C. A. C2C12 cells were cultured in DM at either 38 or 30 °C and in DM containing 100 ng/ml IGF-I or 200 μM VC at 30 °C for 4 days prior to immunostaining for myogenin. Myogenin expression of the cells cultured in DM with both IGF-I and VC was significantly higher than that of the cells in DM with either IGF-I or VC alone. 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 DM with or without 100 ng/ml IGF-I and 200 μM VC at 30 °C for 6 days prior to immunostaining for MyHC. Cell nuclei were stained with Hoechst 33258. Thick multinucleated myotubes were formed in DM containing both IGF-I and VC, but only mononucleated myocytes or thinner myotubes expressed MyHC in DM with either IGF-I or VC alone. Scale bar: 100 μm. C. The percentages of nuclei in MyHC-positive cells to the total nuclei were determined by the immunostaining of C2C12 cells cultured at 38 °C for 4 days or at 30 °C for 6 days. MyHC expression of the cells cultured in DM with both IGF-I and VC at 30 °C was significantly higher than that of the cells in DM with either IGF-I or VC alone at 30 °C. $^{**}p<0.01$. $^{*}p<0.05$. D. C2C12 cells were cultured in DM with 0, 10, 50, 100 ng/ml IGF-I and 200 μM VC at 30 °C for 4 days. IGF-I rescued myogenin expression in a dose-dependent manner. $^{**}p<0.01$. E. The IGF-I treatment period was changed during the C2C12 culture with 200 μM VC at 30 °C for 4 days; no treatment (-), treatment from day 0 to day 1 (1 day), from day 0 to day 2 (2 days) or from day 0 to day 4 (4 days). The exposure to IGF-I for only 1 or 2 days could not promote myogenin expression. $^{**}p<0.01$ vs. 3 other conditions.

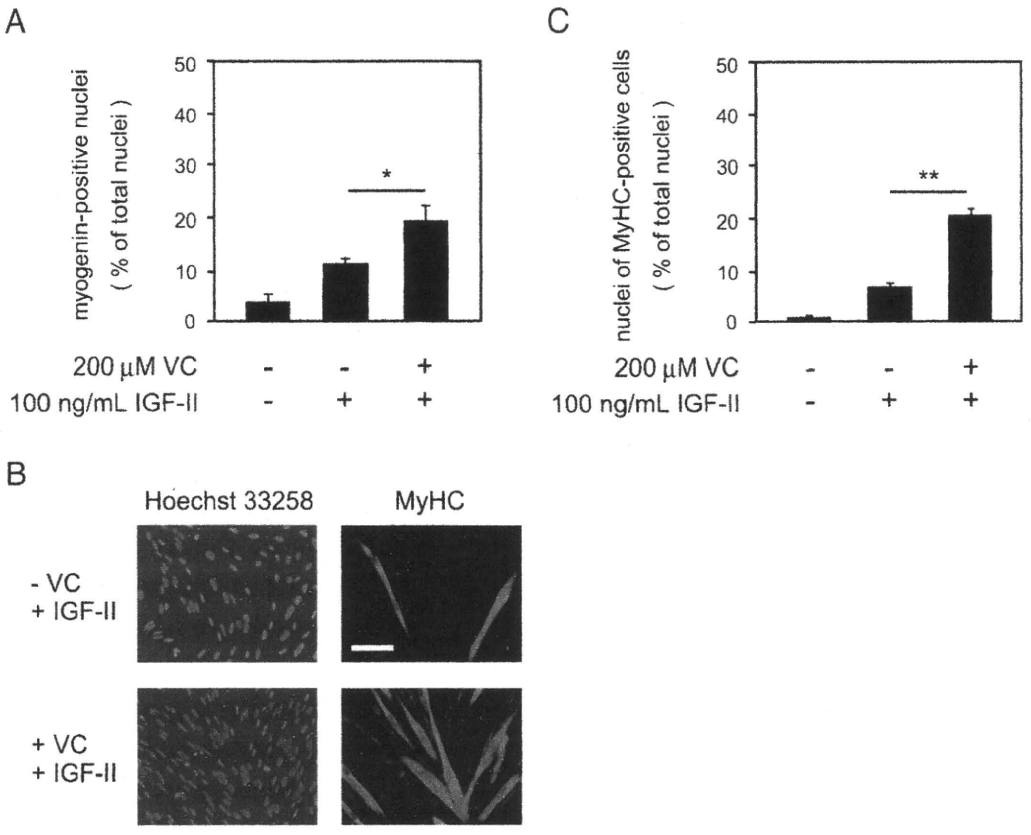

Fig. 2 – Effects of IGF-II and VC on myogenic differentiation of C2C12 cells at 30 °C. A. C2C12 cells were cultured in DM with or without 100 ng/ml IGF-II and 200 μM VC at 30 °C for 4 days and then immunostained for myogenin. Myogenin expression of the cells cultured in DM with both IGF-II and VC was significantly higher than that of the cells cultured in DM with IGF-II alone. The percentages of myogenin-positive nuclei to the total nuclei are presented as the mean ± SEM. $^{*}p<0.05$. B. C2C12 cells were cultured in DM with or without 100 ng/ml IGF-II and 200 μM VC at 30 °C for 6 days and then immunostained for MyHC. Cell nuclei were stained with Hoechst 33258. The cells in DM containing both IGF-II and VC formed numerous multinucleated myotubes, but only thin myotubes were observed in DM containing IGF-II alone. Scale bar: 100 μm. C. The percentages of nuclei in MyHC-positive cells to the total nuclei were determined by the immunostaining of C2C12 cells cultured at 30 °C for 6 days. MyHC expression of the cells cultured in DM with both IGF-II and VC was significantly higher than that of the cells in DM with IGF-II alone. $^{**}p<0.01$.

addition of IGF-I and VC would result in the decreased expression of Id3 at 30 °C. RT-PCR was used to examine the expression profiles of C2C12 cells cultured in either DM without adding IGF-I nor VC (DM(−)) at 38 and 30 °C or DM containing both 100 ng/ml IGF-I and 200 μM VC at 30 °C (Fig. 3).The individual band intensity of myogenin and Id3 was measured and standardized to that of β-actin in the same condition (Table 1). The presence of IGF-I and VC increased myogenin expression and decreased Id3 expression at 30 °C compared with those of the cells cultured in DM(−) (Fig. 3).

Expressions of muscle-specific miRNAs are upregulated by IGF-I and VC

Our previous study examining the temperature-dependent expression of the muscle-specific miRNAs miR-1, -133a, -181a, and -206 revealed that they were upregulated at 38 °C but not at 30 °C [4]. These miRNAs are specifically expressed or highly enriched in skeletal muscles and regulate myogenic differentiation [25]. RT-PCR revealed that the C2C12 cells cultured in DM containing both 100 ng/ml IGF-I and 200 μM VC at 30 °C expressed all of these miRNAs at nearly identical levels as the cells cultured in DM(−) at

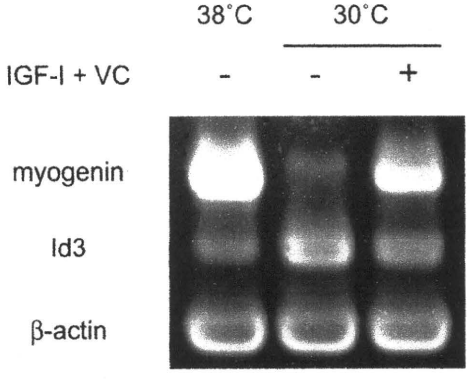

Fig. 3 – RT-PCR of myogenin and Id3 in C2C12 cells. Total RNA was isolated from C2C12 cells cultured in DM at 38 or 30 °C and in DM containing 100 ng/ml IGF-I and 200 μM VC at 30 °C for 4 days. IGF-I and VC increased myogenin expression and decreased Id3 expression at 30 °C compared with cells cultured without IGF-I or VC.

Table 1 – Band intensity of myogenin and Id3.

IGF-I + VC	38 °C	30 °C	
	−	−	+
Myogenin	3.13	0.21	1.43
Id3	0.35	1.00	0.55

Table 2 – Band intensity of microRNAs.

IGF-I + VC	38 °C	30 °C	
	−	−	+
miR-1	0.87	0.17	0.83
miR-133a	1.11	0.21	0.80
miR-181a	0.36	0.16	0.36
miR-206	1.04	0.22	0.92

38 °C (Fig. 4). The muscle-specific miRNAs were barely detectable in the cells cultured in DM(−) at 30 °C, though they expressed the 5S rRNA internal standard at levels similar to the cells cultured at 38 °C (Fig. 4). The individual band intensity of each miRNAs was measured and standardized to that of 5S rRNA in the same condition (Table 2).

IGF-I and VC can promote myogenic differentiation at temperatures lower than 30 °C

As IGF-I and VC were both shown to be able to prevent the low temperature-induced inhibition of myogenic differentiation, the ability of these factors to promote muscle cell differentiation at temperatures lower than 30 °C was examined. C2C12 cells were cultured in DM containing both 100 ng/ml IGF-I and 200 μM VC at either 25 or 28 °C and then immunostained for myogenin and MyHC. At both temperature, IGF-I and VC promoted expression of myogenin and MyHC (Fig. 5A and B), while no myoblasts expressed them when cultured in DM(−). Multinucleated myotubes were formed by day 10 at 28 °C, while only mono- or bi-nucleated myocytes were observed on day 11 at 25 °C (Fig. 5C).

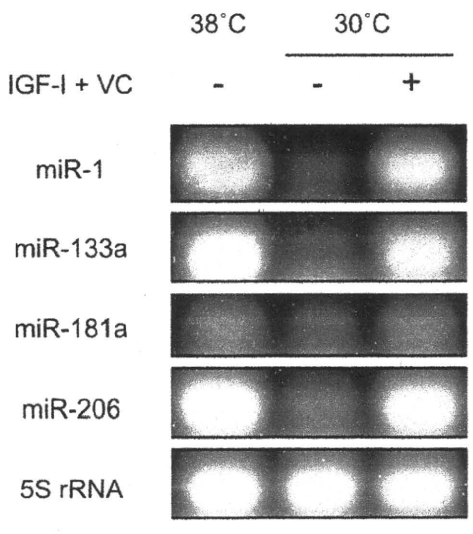

Fig. 4 – RT-PCR of muscle-specific miRNAs in C2C12 cells. Small RNAs were isolated from C2C12 cells cultured in DM at 38 or 30 °C and in DM with 100 ng/ml IGF-I and 200 μM VC at 30 °C for 4 days. RT-PCR for the muscle-specific miRNAs miR-1, -133a, -181a and -206 revealed that the cells cultured in DM with IGF-I and VC at 30 °C expressed all of these miRNAs at nearly identical levels as the cells cultured at 38 °C. The control cells cultured in DM(−) at 30 °C expressed 5S rRNA, the internal standard, but hardly expressed the muscle-specific miRNAs.

Satellite cells of IGF-I overexpressing Tg mice swiftly differentiate at both 37 °C and 30 °C

Satellite cells are myogenic stem cells [26] that are activated by signals released from crushed myofibers [27] to differentiate into myotubes which contribute to muscle regeneration [28]. In a previous study, we demonstrated that activated satellite cells expressed MyoD but not myogenin at 30 °C by day 3 [4]. Here, after the isolation of myofibers from IGF-I Tg mice, which display postnatal increases in muscle mass and strength [8], myogenin expression in the satellite cells was compared with the levels in WT mice without VC in 37 °C and 30 °C cultures. For both experimental conditions, myofibers were cultured at 37 °C for the initial 24 h in order to activate the satellite cells. To evaluate myogenin expression in satellite cells, we calculated the ratio of myogenin-positive nuclei to MyoD-positive nuclei per myofiber. A ratio greater than 1.0 indicated that a majority of satellite cells expressed myogenin, but had already downregulated MyoD expression. In the 37 °C culture, the ratio of myogenin-positive cells in IGF-I Tg mice was higher than that of WT mice on day 2, which suggests that the myogenic differentiation of satellite cells of IGF-I Tg mice was accelerated in comparison with WT mice. However by day 3, the difference in the ratios of myogenin-positive cells between IGF-I Tg mice and WT mice had disappeared (Fig. 6A). In the 30 °C culture, very few satellite cells of both IGF-I Tg and WT mice expressed myogenin on day 3, but on day 4, the ratio of myogenin-positive satellite cells of IGF-I Tg mice was significantly higher level than that of WT mice (Fig. 6B). A number of satellite cells of WT mice, however, expressed myogenin at 30 °C on day 4 (Fig. 6B and C), which was different from the observed expression pattern in C2C12 cells.

Conditioned medium from differentiated C2C12 cells can promote myogenin expression at 30 °C

We hypothesized that the reason why satellite cells of WT mice expressed myogenin at 30 °C without exogenous IGF-I and VC in the single-fiber culture was that some physiological factors which could rescue myogenin expression at 30 °C were brought or secreted into the medium from the myofibers. Therefore, we examined whether or not conditioned medium (CM) from C2C12 cells differentiated at 38 °C could rescue myogenin expression at 30 °C. C2C12 cells cultured in CM for 6 days expressed myogenin at almost the same level of the culture with 100 ng/ml IGF-I and 200 μM VC at 30 °C (Fig. 7A). The myoblasts cultured in CM at 30 °C expressed MyHC, but did not fuse into multinucleated myotubes even when cultured for 10 days, while the cell density gradually decreased (Fig. 7B).

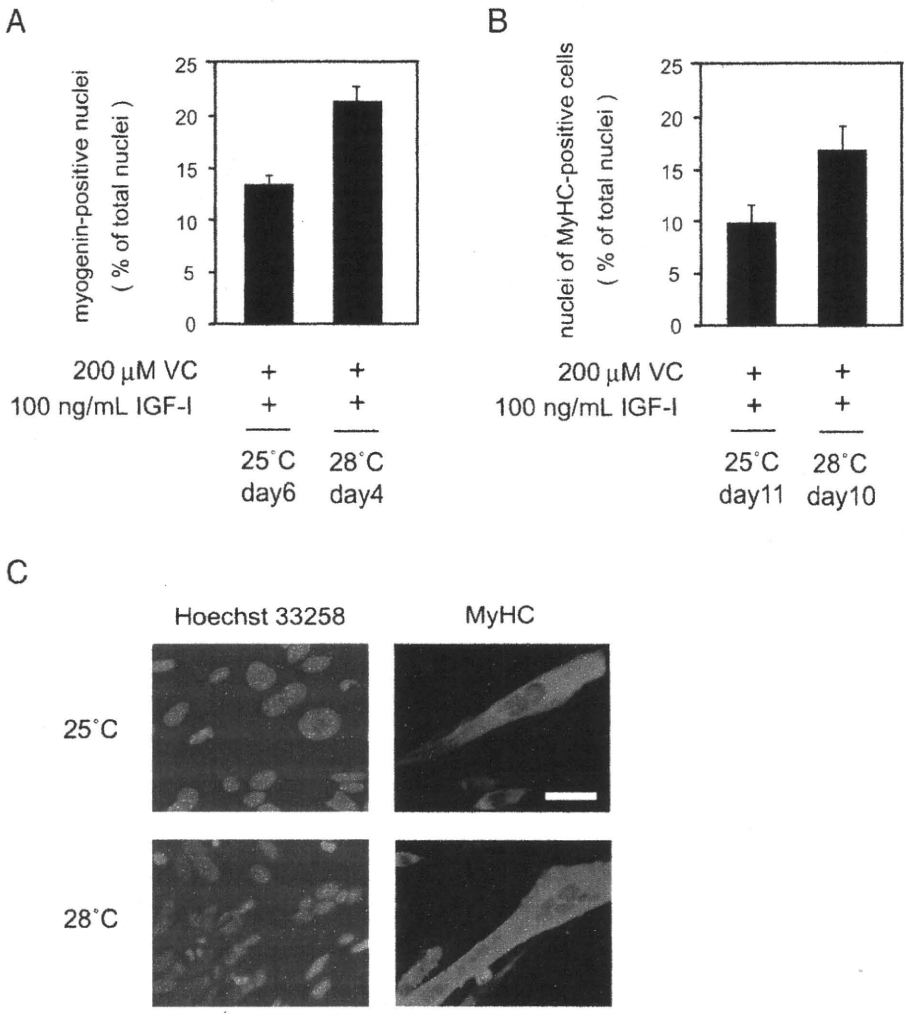

Fig. 5 – Effects of IGF-I and VC on myogenic differentiation of C2C12 cells at 25 and 28 °C. A. C2C12 cells were cultured in DM containing both 100 ng/ml IGF-I and 200 μM VC at 25 °C for 6 days or at 28 °C for 4 days and immunostained for myogenin. The percentages of myogenin-positive nuclei to the total nuclei are presented as the mean ± SEM. B. C2C12 cells were cultured in DM containing both 100 ng/ml IGF-I and 200 μM VC at 25 °C for 11 days or at 28 °C for 10 days and then immunostained for MyHC. The percentages of nuclei in MyHC-positive cells to the total nuclei are presented as the mean ± SEM. C. C2C12 cells were cultured in DM containing both 100 ng/ml IGF-I and 200 μM VC at 25 °C for 11 days or at 28 °C for 10 days and then immunostained for MyHC. Cell nuclei were stained with Hoechst 33258. Although the cells expressed MyHC at both temperatures, multinucleated myotubes were formed only at 28 °C, while mononucleated myocytes or binucleated myotubes were observed at 25 °C. Scale bar: 50 μm.

IGF-I and VC promote myogenic differentiation of human muscle cells at 30 °C

Finally, we examined whether human skeletal muscle cells could also be promoted to differentiate by treatment with IGF-I and VC at 30 °C. Although the percentage of myogenin-positive nuclei of human skeletal muscle cells was generally low (14.1 ± 0.8%) in comparison with C2C12 cells (42.9 ± 1.9%) even at 38 °C, the inhibition of myogenin expression at 30 °C (1.1 ± 0.3%) and the rescue effect with IGF-I and VC (16.2 ± 1.8%) was clearly demonstrated (Fig. 8).

Discussion

Mouse myoblasts do not differentiate into multinucleated myotubes at 30 °C, which is much lower than the core body temperature of most endotherms [4]. In this study, we demonstrated that at this temperature myoblasts do not completely lose the capacity to differentiate, as the treatment with exogenous IGFs (IGF-I and IGF-II) and VC stimulates myoblasts to proceed toward terminal differentiation. Although the addition of either IGF-I alone or VC alone could promote myogenin expression in C2C12 cells at 30 °C, elongated multinucleated myotubes which had almost the same appearance as the myotubes cultured at 38 °C were not formed unless both 100 ng/ml IGF-I and 200 μM VC were continuously administered over a period of several days. IGF-II, which also binds to IGF-I receptors, showed the same effects on myogenic differentiation at low temperature in the combination with vitamin C. These results show that appropriate chemical factors can overcome the inadequacy of temperature for myogenic differentiation, suggesting that the physical information such as external temperature shares the same downstream signaling pathways with the chemical information such as growth factors.

It is well established that IGF-I promotes both skeletal muscle growth and differentiation [5]. At normal physiological

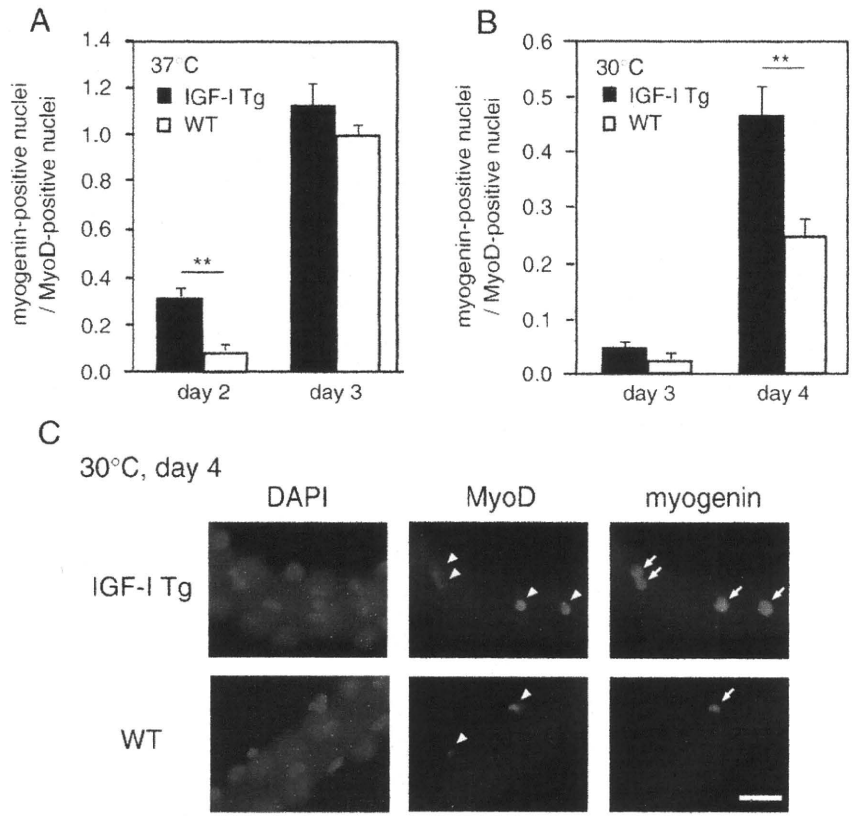

Fig. 6 – MyoD and myogenin expression in single-fiber culture of IGF-I Tg mice and WT mice. A and B. Myofibers were isolated from EDL muscles of adult mice and cultured in 20% FBS/DMEM without VC at 37 °C (37 °C culture) or 37 °C for the first 24 h to activate the satellite cells and subsequently at 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

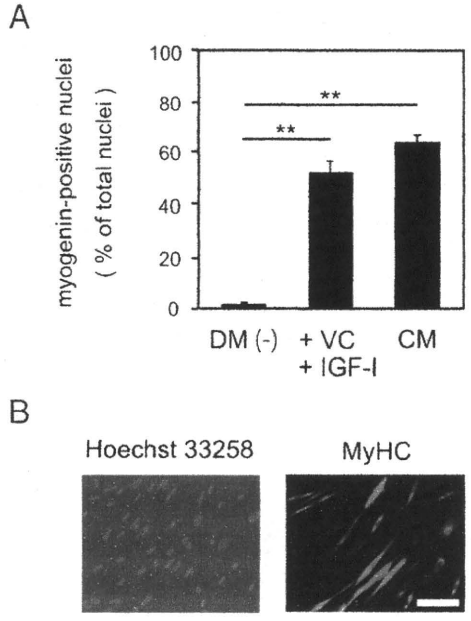

Fig. 7 – Effects of conditioned medium of differentiated C2C12 cells at 30 °C. A. C2C12 cells were cultured for 6 days at 30 °C in DM with or without 100 ng/ml IGF-I and 200 μM VC or conditioned medium (CM) of C2C12 cells differentiated at 38 °C and immunostained for myogenin. Myogenin expression was 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

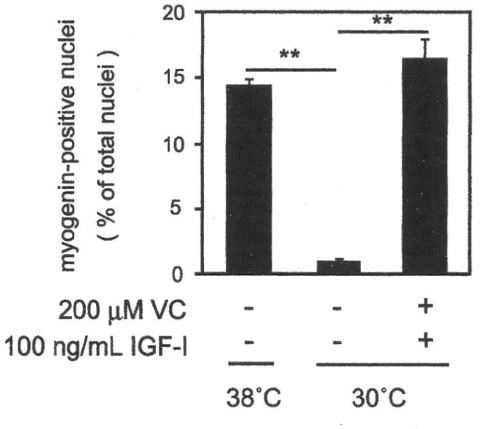

Fig. 8 – Myogenin expression in human skeletal muscle cells cultured with IGF-I and VC at 30 °C. Human skeletal muscle cells were cultured in DM at 38 or 30 °C and in DM containing 100 ng/ml IGF-I and 200 μM VC at 30 °C for 6 days and then immunostained for myogenin. Myogenin expression in the human 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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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

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 4×10^5 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 3.6×10^5 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

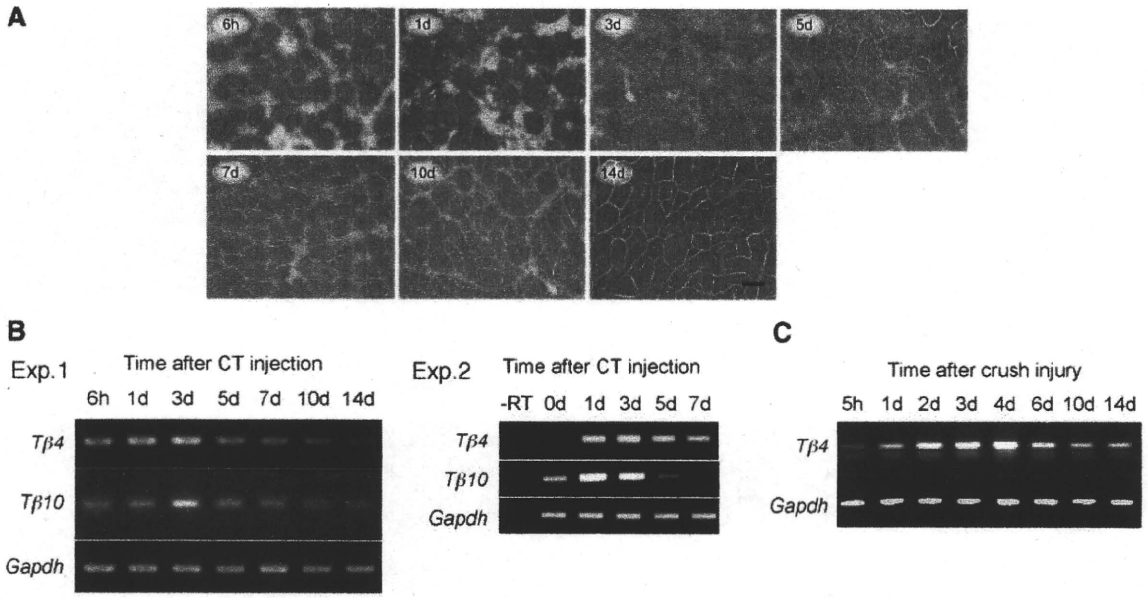

Fig. 1 Up-regulation of 'Tβ4' and 'Tβ10' mRNAs during skeletal muscle regeneration. (A) Histological appearance of skeletal muscle during the course of regeneration after CT injection. TA muscles were recovered from CT-injected mice at indicated time points (6 h to 14 days). Their frozen sections were 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.

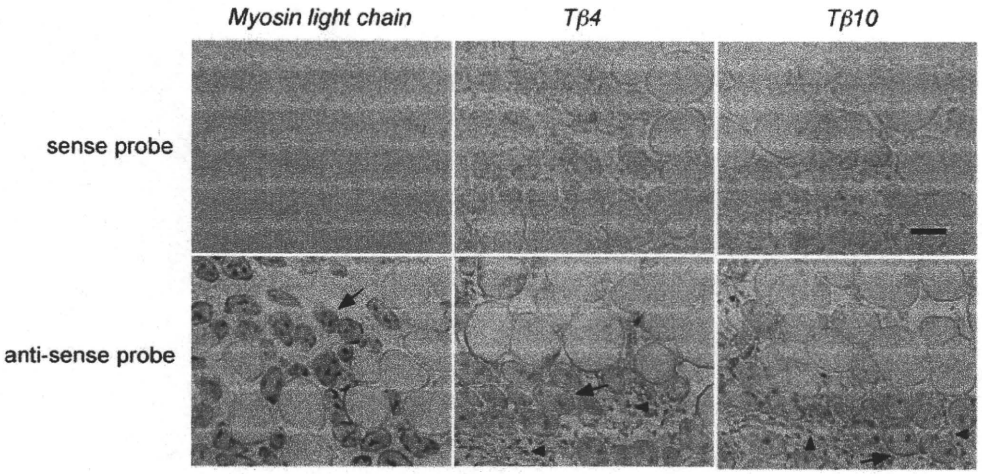

Fig. 2 Location of 'Tβ4' and 'Tβ10' mRNAs in the regenerating skeletal muscle. Transverse cryosections of TA muscles were harvested 5 days after CT injection and were hybridized with DIG-labelled sense (upper panels) or anti-sense (lower panels) cRNA probes for 'Myosin light chain', 'Tβ4' and 'Tβ10', 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.

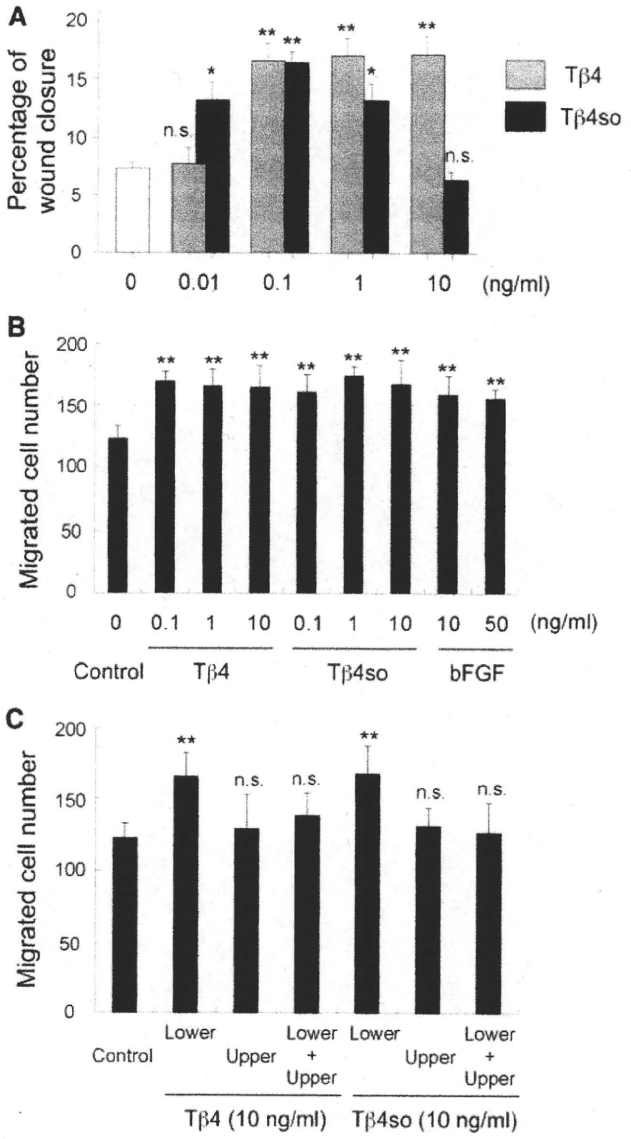

Fig. 3 Wound closure and chemotactic responses of C2C12 cells by exogenous Tβ4 and Tβ4so. (A) Recovery ratio of scratched wounded areas of C2C12 monolayer was measured in the presence or absence of indicated concentrations of Tβ4 or Tβ4so. (B) Chemotactic responses of C2C12 cells to various concentrations 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

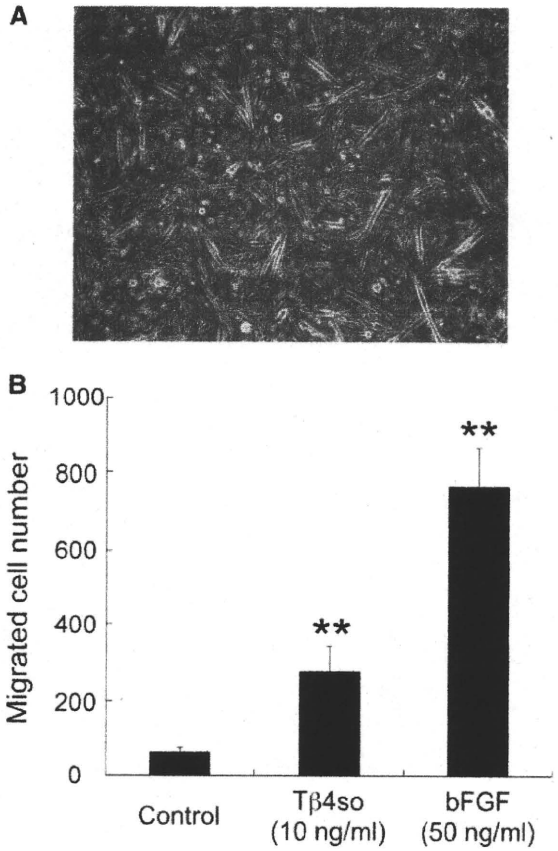

Fig. 4 Chemotaxis of satellite cells-derived myoblasts/myocytes to exogenous Tβ4so. (A) Morphological appearance of primary myoblasts and myocytes derived from muscle satellite cells of adult mice. (B) Chemotactic responses of primary myoblasts/myocytes to Tβ4so and bFGF. Cells and indicated reagents 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

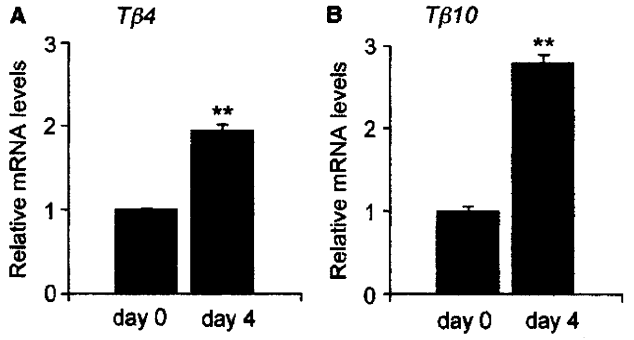

Fig. 5 Increase of 'Tβ4' and 'Tβ10' mRNAs during myotube differentiation of C2C12 cells. (A) C2C12 cells were cultured in the differentiation media to induce myotube-like structures. Expression levels of 'Tβ4' (A) and 'Tβ10' (B) mRNAs before (Day 0) and after (Day 4) the myotube differentiation were 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

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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

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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, 5×10^4 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).