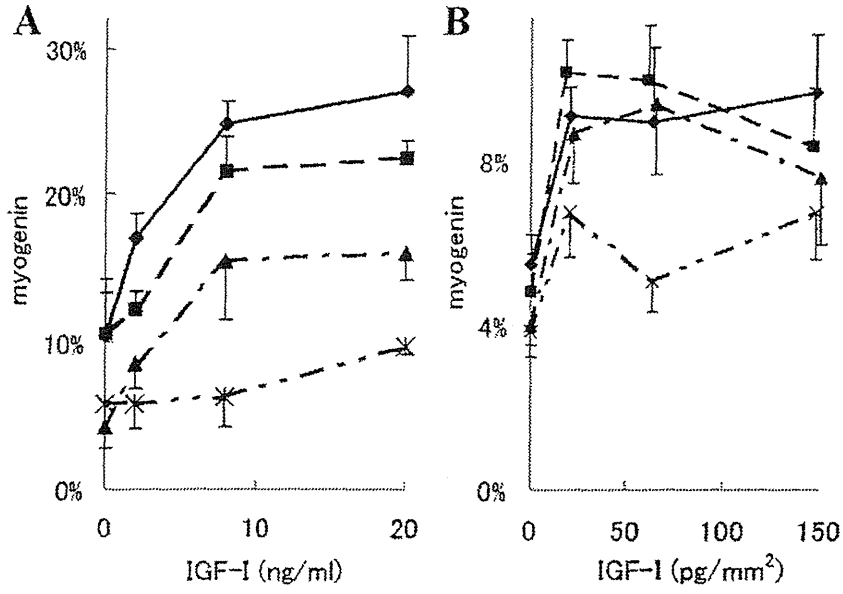

Fig. 12.3 Effect of IGF-I and FGF-2 on myogenin expression. (**a**) C2C12 cells were cultured with soluble IGF-I (0, 2, 8, 20 ng/mL) in combination with soluble FGF-2 (0(□), 2(■), 8(▲), 20(×) ng/mL). (**b**) C2C12 cells were cultured on growth factor arrays containing 16 sections consisting of IGF-I (0, 21, 64, 149 pg/mm^2) and FGF-2 (0(□), 41(■), 79(▲), 175(×) pg/mm^2)

expression was examined. When C2C12 cells were cultured on the growth factor arrays, the myogenin expression ratio varied among the different combinations of IGF-I and FGF-2 (Fig. 12.3). However, the myogenin expression pattern was different from that of the soluble growth factors. For soluble growth factors, the effect of IGF-I was remarkably high at 8 ng/mL and approximately 20 ng/mL of IGF-I appeared to be close to saturation. On the growth factor arrays, the ratio of the myogenin expression increased with 21 pg/mm^2 of IGF-I, though the higher quantity of IGF-I barely increased its stimulation. In contrast, the suppressive effect of FGF-2 was very clear for soluble FGF-2. However, the effect of the FGF-2 immobilized on the growth factor array seemed to be weaker. Only the concentration of 175 pg/mm^2 FGF-2 lowered the myogenin expression ratio.

12.3.2 Growth Factor Array with Surface Activated Substratum

12.3.2.1 Growth Factor Array with 3 Growth Factors

The immobilization efficiency of surface activated substratum was evaluated with BSA by ELIZA. The concentrations of growth factors used in this experiment did not exceed the immobilization capacity of activated surface. Hence, the concentrations of growth factors immobilized on the surface activated substratum were estimated from the printed quantity.

C2C12 cells were cultured on growth factor arrays fabricated with surface activated substratum. These arrays consisted of combinations of printed FGF-2, IGF-I and BMP-2 (0, 22, 61.8, 200 pg/mm^2, respectively) with 64 areas in all. Cell growth

or differentiation was significantly promoted on the areas where growth factors were immobilized.

To analyze the effects of growth factor combinations for myogenic differentiation, the relative fluorescent intensity of MyHC was obtained by standardizing with fluorescent intensity of TOTO-3 (Fig. 12.3). Without BMP-2, MyHC expression increased with IGF-I dose, but this effect was attenuated by FGF-2. However, when BMP-2 co-existed in the area, especially in high concentrations, the attenuation effect by FGF-2 was unstable.

To analyze the effects of growth factor combinations for osteogenic differentiation, the relative fluorescent intensity of ALP was obtained by standardizing fluorescent intensity of TOTO-3, but the relative fluorescent intensity of ALP was weak independent of the presence of BMP-2 (Data not shown).

12.3.2.2 Growth Factor Analysis in Liquid System with 3 Growth Factors

Growth factor arrays in liquid system were prepared to compare with the growth factor arrays fabricated with surface activated substratum. First, 3 growth factors, IGF-I (0, 4, and 40 ng/mL), FGF-2 (0, 4 and 40 ng/mL) and BMP-2 (0, 10 and 100 ng/mL) were used to prepare growth factor array in liquid system, resulting in 27 combinations (Fig. 12.2). After culturing C2C12 myoblast with this growth factor array in liquid system, TOTO-3, MyHC, and ALP were immunostained, and the fluorescent intensity was compared in the same way shown before. Relative fluorescent intensity of MyHC standardized by TOTO-3 fluorescent intensity is shown in Fig. 12.5. Without BMP-2, relative fluorescent intensity of MyHC increased as the concentration of IGF-I increase and this effect was attenuated by FGF-2. When BMP-2 coexists, expression of MyHC was suppressed and the relative fluorescent intensity decreased dose dependently. At high concentration (100 ng/mL) of BMP-2, the activity of IGF-I to promote differentiation to myoblasts was almost completely suppressed.

12.3.2.3 Evaluation of Immobilized BMP-2

Since the effect of immobilized BMP-2 was not clear in previous experiments, the effect of BMP-2 at higher concentrations was evaluated independently. BMP-2 was

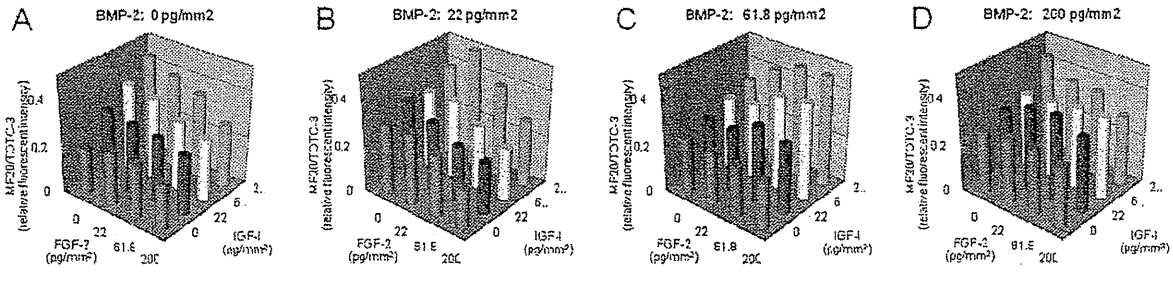

Fig. 12.4 Myogenic differentiation of C2C12 myoblast cultured on the growth factor array fabricated with surface activated substratum. The relative fluorescent intensity of MyHC was obtained by standardizing with fluorescent intensity of TOTO-3

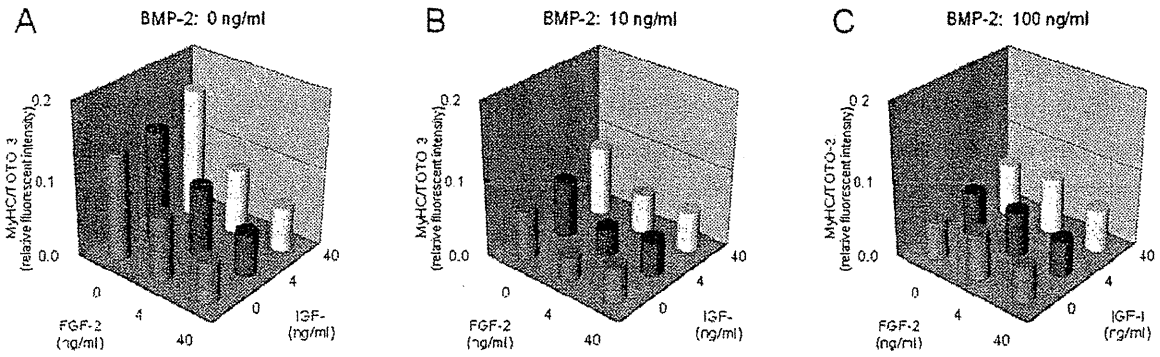

Fig. 12.5 Myogenic differentiation on growth factor array in liquid system consisted of 3 growth factors; IGF-I, FGF-2, and BMP-2. Relative fluorescent intensity of MyHC increased as the concentration of IGF-I increase and this effect was attenuated by FGF-2. When BMP-2 coexists, expression of MyHC was suppressed and the relative fluorescent intensity decreased with dosage

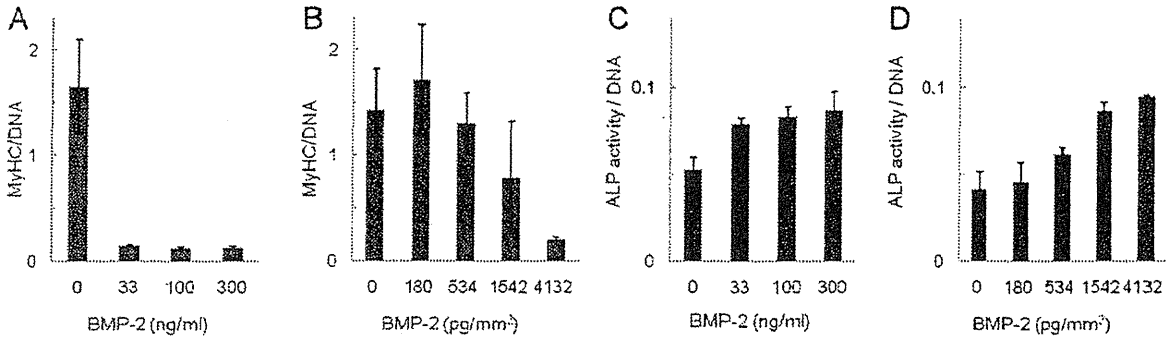

Fig. 12.6 The effect of myogenic differentiation by soluble BMP-2(**a**) and immobilized BMP-2 (**b**) and the effect of osteogenic differentiation by soluble BMP-2 (**c**) and immobilized BMP-2 (**d**)

immobilized on the surface-activated substratum and C2C12 was cultured on that substratum. In this experiments, higher concentration of BMP-2 works inhibitory for MyHC expression and enhanced ALP expression dose dependently, which correspond to the effect of soluble BMP-2 (Fig. 12.6). In this experiment, the highest concentration immobilized on the substratum was 4132 pg/mm^2, corresponding to 536 ng/ml in the soluble state, which is an enormously high concentration compared to the normal experimental condition in vivo or in vitro. At 1542 pg/mm^2, little effect for inhibition for myogenic differentiation was observed, whereas bone differentiation was close to the effect of soluble BMP-2 at 33 ng/ml. These results indicate that the activity of BMP-2 was lowered or changed through the printing and/or immobilization process to the substratum.

12.3.2.4 Growth Factor Array with 4 Growth Factors

Growth factor arrays composed of 4 growth factors were also prepared with surface activated substratum (Fig. 12.7a). This growth factor array consisted of 81 areas with combinations of EGF, FGF-2, IGF-I and PDGF, and the compound effects of these factors were examined. These proteins are known to promote the growth of C2C12 and FGF-2 is inhibitory for myogenic differentiation dose-dependently. C2C12

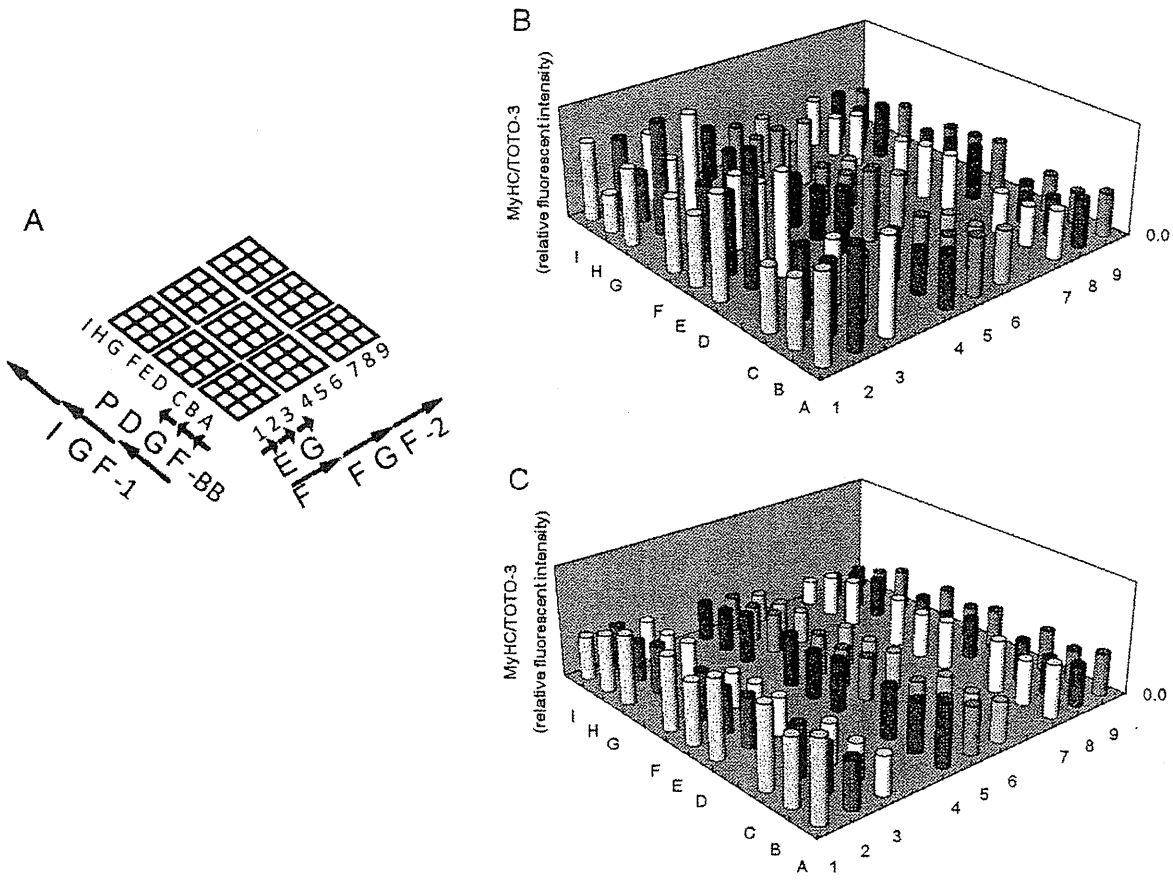

Fig. 12.7 Analysis of 4 growth factors. (**a**) The schematic diagram of growth factor array with 4 growth factors (EGF, PDGF-BB, FGF-2, IGF-I) fabricated with surface activated substratum and that in liquid system. (**b**) The effect of myogenic differentiation analyzed on the growth factor array with 4 growth factors fabricated with surface activated substratum. The concentrations of growth factors are as follows. EGF: 0, 67, 200 pg/mm^2, PDGF-BB: 0, 100, 300 pg/mm^2, FGF-2: 0, 67, 200 pg/mm^2, IGF-I: 0, 133, 400 pg/mm^2. (**c**) Myogenic differentiation on growth factor array in liquid system. The concentrations of growth factors are as follows. EGF: 0, 15, 44 ng/mL, PDGF-BB: 0, 22, 66 ng/mL, FGF-2: 0, 15, 44 ng/mL, IGF-I: 0, 30, 88 ng/mL

myoblasts were cultured on this growth factor array for 8 h in growth medium, then 72 hr in differentiation medium. After the culture, cell growth and myogenic differentiation was analyzed by staining TOTO-3 and MyHC antibody, respectively (Fig. 12.7b). The results show that cell growth was promoted by IGF-I, EGF, and FGF-2 but not by PDGF-BB. The compound effect for cell growth was similar to the sum of the effect of individual growth factors. Myogenic differentiation was inhibited by EGF but not by FGF-2 and PDGF-BB.

12.3.2.5 Growth Factor Array in Liquid System with 4 Growth Factors

Growth factor arrays in a liquid system composed of 4 soluble growth factors were also prepared in the same method. The growth factor pattern is the same as growth factor array in solid system (Fig. 12.7a). As a result, EGF, FGF-2, IGF-1 and PDGF-BB were promotive for growth of C2C12. FGF-2, EGF and PDGF-BB were inhibitory for myogensis (Fig. 12.7c). The inhibitory effect of FGF-2 and EGF was higher than that of immobilized growth factors.

12.3.3 Growth Factor Array with Slow Release System

12.3.3.1 Evaluation of Slow Release of Growth Factors

To evaluate the slow-release of growth factors, EGF was retained on the substrate with photoreactive gelatin with different photo-crosslink conditions. After immobilization of growth factors, the quantity of EGF released to PBS was measured with ELIZA (Fig. 12.8a). EGF release was detected within 24 h at all the conditions. However, when UV-irradiation condition was low (0.2 mJ/cm^2) or high (200 mJ/cm^2), the amount of EGF in PBS was constant while EGF amount increased at intermediate condition of UV irradiation (2 and 20 mJ/cm^2). After 7 days, EGF on the substratum was immunostained with anti-EGF antibody (Fig. 12.8b). Little EGF was detected with the substratum irradiated with 0.2 and 2 mJ/cm^2 UV, while retained EGF was still detected at the substratum irradiated with 20 and 200 mJ/cm^2. According to this result, the remaining experiments were operated with a 20 mJ/cm^2 UV-irradiation.

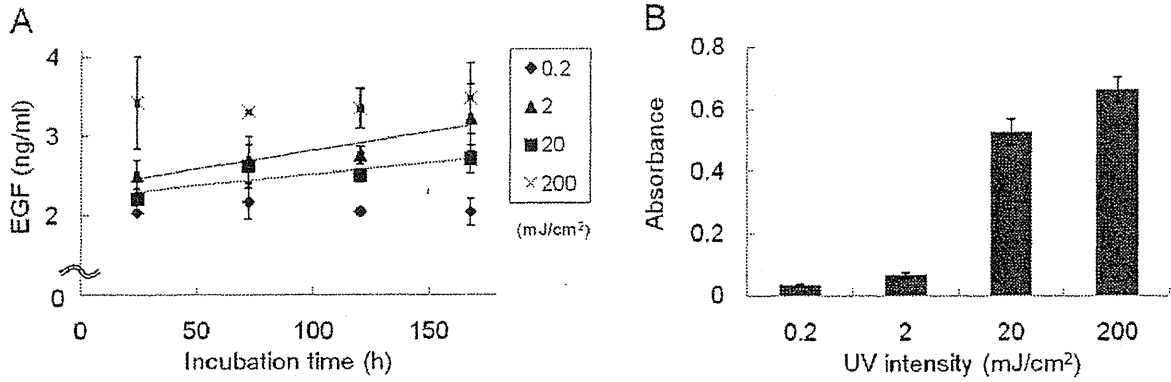

Fig. 12.8 Evaluation of slow-release of growth factors. (**a**) The quantity of released growth factor from the substratum was measured by detecting the released EGF in PBS by ELIZA. (**b**) Remaining EGF on the substratum after 7 days incubation in PBS detected by immunostaining the substratum with EGF antibody

12.3.3.2 Culture of C2C12 Myoblast with Growth Factor Array in Slow Release System

C2C12 myoblast was cultured on the growth factor arrays with slow release system consisting of IGF-I and BMP-2. After the culture, C2C12 was immunostained with TOTO-3 and either with anti-MyHC antibody as a myogenic marker or anti-ALP antibody as an osteogenic marker (Fig. 12.9). Cell growth was not significantly different among each area. However, myogenic differentiation was suppressed by BMP-2 in a dose-dependent manner. At the highest concentration of BMP-2 (450 pg/mm^2), even the effect of myogenic differentiation by IGF-I was completely suppressed. On the contrary, osteogenic markers were not significantly different among areas (Data not shown).

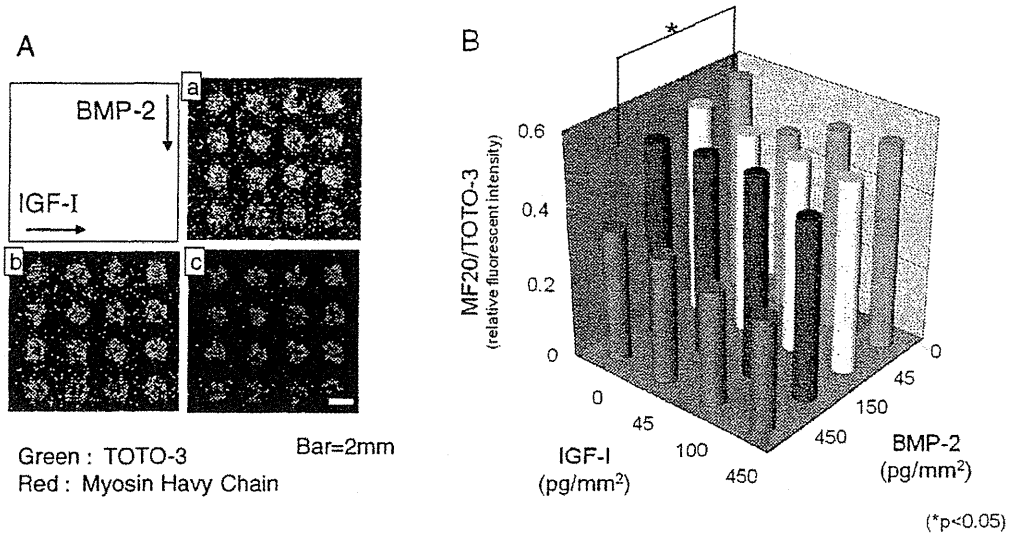

Fig. 12.9 Effect of BMP-2 and IGF-I on C2C12 culture in growth factor array with slow-release system. (**a**) C2C12 myoblast was cultured for 4 days on growth factor array in slow-release system. **a**: Merge, **b**: Fluorescence image of TOTO-3 staining. c: Fluorescence image indicating myogenic differentiation (scale bar: 2 mm). (**b**) Cells were immunostained with anti-MyHC antibody. Relative intensity of MF20 was measured. At the highest concentration of BMP-2(450 pg/mm^2), even the effect of myogenic differentiation by IGF-I was completely suppressed

12.3.3.3 MSC Culture on Growth Factor Array with Slow Release System

MSCs were cultured on the growth factor arrays with slow release system used in the previous experiment. MSCs were cultured for 14 days, and after the culture, cells were immunostained with osteogenic marker anti-ALP antibody (Fig. 12.10).

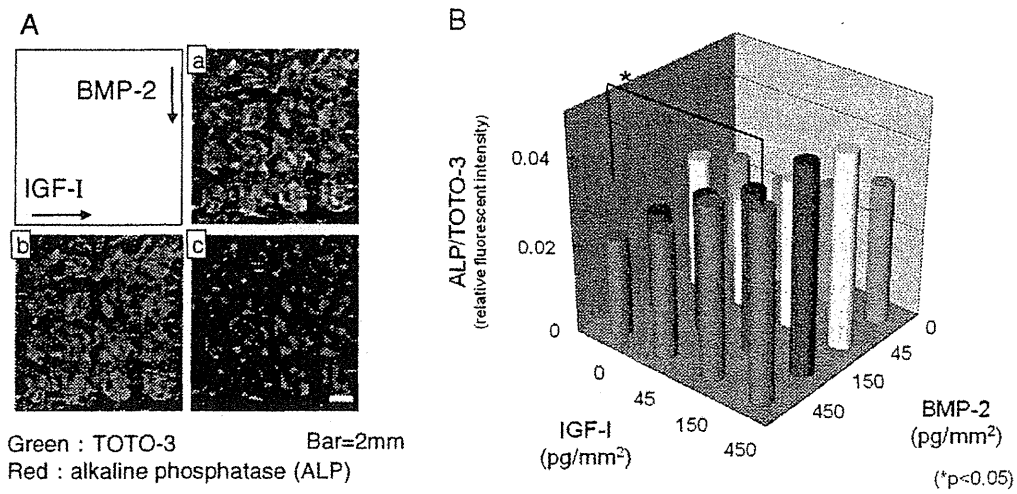

Fig. 12.10 MSC culture on growth factor array in slow-release system consisted of BMP-2 and IGF-I. (**a**) Fluorescent image of MSC cultured on growth factor array. a: Merge, b: Fluorescence image of TOTO-3 staining. C: Fluorescence image indicating osteogenic differentiation (bar: 2 mm). (**b**) ALP expression of MSC on growth factor array with slow release system (0, 45, 150, 450 pg/mm^2 of growth factors were combined) was measured by immunostaining with anti-ALP antibody. In the areas where BMP-2 was printed at concentrations of 150 and 450 pg/mm^2, osteogenic differentiation was promoted depended on IGF-I concentration

In this culture, the osteogenic differentiation was not promoted by BMP-2, but when IGF-I coexisted, osteogenic differentiation was promoted, depending on IGF-I concentration.

12.4 Discussion

12.4.1 Growth Factor Array Using Photoreactive Growth Factors

Immobilization efficiency of photoreactive growth factors was examined using [^{125}I] IGF-I. From this experiment, it was shown that the growth factors were printed with high accuracy. The immobilization efficiency decreased as the quantity of protein on the substratum increased. According to previously published work, growth factors immobilized with a phenyl-azido arm may form multi-layers on the substrata [28, 34]. This result suggests that when the quantity of the printed growth factors increases, these growth factor may form multi-layers, preventing a direct reaction with the substratum, and some of the protein may be washed away by rinsing with PBS.

To study the feasibility of the growth factor array, we first fabricated the arrays consisting of IGF-I and FGF-2 and cultured C2C12 myoblasts. We also confirmed the effect of soluble IGF-I and FGF-2 for myogenic differentiation. Several laboratories have shown that soluble IGF-I, at concentrations up to 20 ng/mL, promotes myogenic differentiation in a dose-dependent manner [35]. On the other hand, it has also been reported that FGF-2 inhibits expression of the myogenic transcription factor MyoD [8, 9, 36]. In the present work, FGF-2 inhibited the myogenin expression induced by IGF-I in a dose-dependent manner. Interestingly, soluble FGF-2 did not completely inhibit the myogenin expression. Approximately 5% of the cells remained myogenin-positive. According to the previous work, cultured muscle cells synthesize substantial amounts of IGF-I, which may cause the basal myogenin expression [37].

Compared to that of soluble growth factors, the effect of immobilized growth factors were lower. There are two possibilities for this discrepancy. The first possibility is a weakened activity of photoreactive growth factors from introducing the azido-phenyl group. In this method, the azido-phenyl group is supposed to be introduced to the amino groups of the N-terminus or to lysine residues. This chemical modification may cause conformational changes that can reduce the interaction activity with receptors. Another possibility is the restricted flexibility of immobilized growth factors due to the immobilization to the substratum via short arm, which may interfere the interaction with the receptor. Elongating the length of arm may improve the activity of immobilized growth factors.

12.4.2 Growth Factor Array Using Surface Activated Substratum

Although the use of photoreactive growth factors was one good way to immobilize growth factors on the substratum, a photoreactive arm needs to be introduced to

each of the growth factors. To make the fabrication process simpler, immobilization process without modifying growth factors will be necessary. We have prepared the surface activated substratum by treating polystyrene substratum with tresyl activated dextran and we have made the growth factor array with 3 and 4 growth factors.

From the results of growth factor arrays using 3 factors, it was confirmed that myogenic differentiation of C2C12 myoblast was promoted by IGF-I, and suppressed by FGF-2, which is similar to the result from growth factor array with photoreactive growth factors. In both cases, promotion of myogenic differentiation by higher concentration of IGF-I was independent of the concentrations of FGF-2 and BMP-2. Ito [38] reported that immobilized insulin bound to a receptor stimulates the cells continuously, resulting in a stronger effect than in dissolved insulin. In this experiment, the effect of IGF-I might be enhanced in the same manner.

BMP-2, on the other hand, neither suppressed myogenic differentiation nor promoted osteogenic differentiation, when the immobilized amount was relatively small, close to the concentrations of other factors. However, when higher concentration of immobilized BMP-2 (4,132 pg/mm^2) was used, osteogenic differentiation was promoted. The density of 4,132 pg/mm^2 in the immobilized state corresponds to 536 ng/mL in a soluble state, which is an extremely high concentration. At a concentration of 1,542 pg/mm^2 in immobilized system, suppression of myogenic differentiation was not significant, but promotion of osteogenic differentiation was observed at about the same extent as in the liquid system at a concentration of 33 ng/mL. This suggests that the activity of BMP-2 was not simply decreased by immobilization but rather changed.

Growth factor arrays composed of four factors (EGF, FGF-2, IGF-1, and PDGF-BB) were also examined. These factors were expected to exert synergic or combinatorial effects, because the downstream signal transduction pathways of these factors are slightly different. However, such effect was not detected. PDGF-BB showed lower activity compare to the native effect in liquid system. EGF and FGF-2, on the contrary, showed higher activity in the immobilized state. PDGF-BB at high concentration, however, showed suppression effects on growth and myogenic differentiation and also in the immobilized system, indicating that it is active in the immobilized system, though the activity is low. The low activity of PDGF-BB may be due to its binding mechanism to the receptors. PDGF-BB binds to the receptor as a dimer. It is known that one subunit of PDGF-BB binds to two receptors, whereas one receptor binds to two subunits. Because of this complicated binding, the affinity with the receptor may be lowered in immobilized system. As for EGF, it has been confirmed by previous work that the activity of EGF is higher in immobilized systems than in a liquid system, in agreement with this work [38]. The possible reason for elevated activity in the previously reported immobilized system is that the down regulation of the ligands does not occur in immobilized system, and thus activation of the receptors continues for a long period of time.

In some cases, the effect of growth factors in immobilized and liquid systems are different. For example, heparin-binding EGF-like growth factor (HB-EGF), which belongs to the EGF family, acts in two forms in vivo as secretory form (paracrine) and transmembrane form (juxtacrine) which exert different effects. Growth of hepatocyte strain EPI70.7, for instance, is promoted by juxtacrine of HB-EGF [39].

With paracrine, on the other hand, promotion of growth was not detected at all, although the ligand was considered to be bounded to the receptor. Therefore, it is likely that both the actions of soluble and immobilized systems have different functions in vivo. In fact, in this work, the activity of BMP-2 seemed to have changed in the immobilized system. At some concentrations, suppression of myogenic differentiation was weaker, and osteogenic differentiation was stronger, in comparison with those in soluble state. Many growth factors are down regulated by endocytosis after binding to the receptor. EGF and TGF-β underwent endocytosis, activating signal transduction pathways downstream in endosome [40]. Furthermore, EGF, like other growth factors, activates multiple signal transduction pathways downstream by binding to the receptors, and the degree of activation is different depending on the signal transduction either across the cell membrane or from inside the endosome [41, 42]. For immobilized growth factors, the signal is continuously transmitted on the cell membrane, whereas both paracrine and juxtacrine signals are transmitted in soluble growth factors. Considering this fact, the effect of growth factors in immobilized and liquid systems were different at least in terms of the signal transduction pathway.

12.4.3 Growth Factor Array in Slow Release System

As discussed above, immobilizing growth factors on the substratum may change the activity of growth factors. To examine the effect of growth factors more natively, we further constructed the slow-release system for growth factor arrays with photoreactive gelatin as the retaining material. In this slow release system, growth factors are retained to the substratum but not tightly immobilized.

According to the result from retaining growth factors on the substratum using photoreactive gelatin, the slow release depends on the intensity of UV-irradiation. When the intensity of UV-irradiation was low (0.2 mJ/cm^2), most of the EGF was lost after the first wash. On the other hand, when the UV intensity was high (200 mJ/cm^2), EGF was released in the supernatant for the first 24 h, but much less was released thereafter. Ito et al. reported that release of erythropoietin immobilized by photoreactive gelatin was not observed by treating with UV-irradiation at 160 mJ/cm^2. This implies that growth factors are firmly immobilized to the substratum by photoreactive gelatin with high UV intensity. On the contrary, when the UV intensity was 2 or 20 mJ/cm^2, slow release continued for 1 week. At these intensities, it is possible to minimize the loss of EGF in the first washing, yet keep the ability to release EGF later on.

Culture of C2C12 myoblasts in the slow release system resulted in effects similar to that in liquid system. The possible concern about the immobilization of growth factors using photoreactive growth factors or surface activated substratum is the decrease in activity and changes in action of the growth factors. Slow-release systems by photoreactive gelatin may solve these problems. However, the increased effect of growth factors in a slow release system, as reported by Bhang, was not

observed in our experiments [43]. It may be because the culture duration was too short (4 days) in our experiment.

For the growth factor arrays in the slow-release system consisting of BMP-2 and IGF-I, suppression of myogenic differentiation for C2C12 myoblast was confirmed with BMP-2 at the concentration of 450 pg/mm^2, independent of the concentration of IGF-I. We have also prepared the slow-release system in a 96-well plate (data not shown). Compared with the results of slow release culture on a 96-well plate, the amount of BMP-2 per unit area was larger in arrays but suppression effect of myogenic differentiation was lower in the growth factor array. This is most likely due to the released BMP-2 remaining in the 96-well plate, whereas in the growth factor array, it flows out to culture medium, resulting in the decreased effect of BMP-2 on the BMP-2 retained area. Promotion of osteogenic differentiation was not observed as we had expected, neither by BMP-2 alone nor by BMP-2 and IGF-I together. Katagiri et al. stated that, in culture of C2C12 myoblast, BMP-2 suppresses the myogenic differentiation at 100 ng/mL and the promotion of ALP expression can be observed at 300 ng/mL or higher [44]. One possible reason for observing the osteogenic differentiation but not the myogenic differentiation is that the BMP-2 concentration was high enough to suppress myogenic differentiation but not high enough as to promote osteogenic differentiation.

The previous work showed that osteogenic differentiation of MSC is not promoted by BMP-2 alone but promoted by BMP-2 and FGF-2 together [45]. In other works, it has been reported that IGF-I induces expression of BMP-2, and BMP-2 and IGF-I together promote osteogenic differentiation of C3H10T1/2 cells more effectively than BMP-2 alone [46, 47]. In this work, it was confirmed that BMP-2 and IGF-I together exert compound effect on osteogenic differentiation of MSC.

12.5 Conclusion

We have developed techniques to analyze compound effects of growth factors in various states, including immobilized, soluble, and slow release. Inkjet printer technology was highly effective for changing the density and the combinations of immobilized growth factors. Chen and Ito reported the patterning of EGF with concentration gradient using photo lithography by changing the intervals of thin lines so that the number of growth factor molecules touching the cells change [48]. An inkjet printer, however, allows much easier preparation of concentration gradient by simply multiply the printing times. Also, in vitro control of cell patterning such as muscle cell alignment can be achieved easily. By combining various immobilization techniques, inkjet technology makes it possible to construct the conditions similar to in vivo in various states such as soluble and immobilized. By further combining the patterning and various cytokines, growth factor arrays will be a powerful and essential tool for multivariable analysis for tissue engineering and regenerative medicine, especially for the optimization of differentiation conditions for pluripotent cells to differentiate to various types of cells.

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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

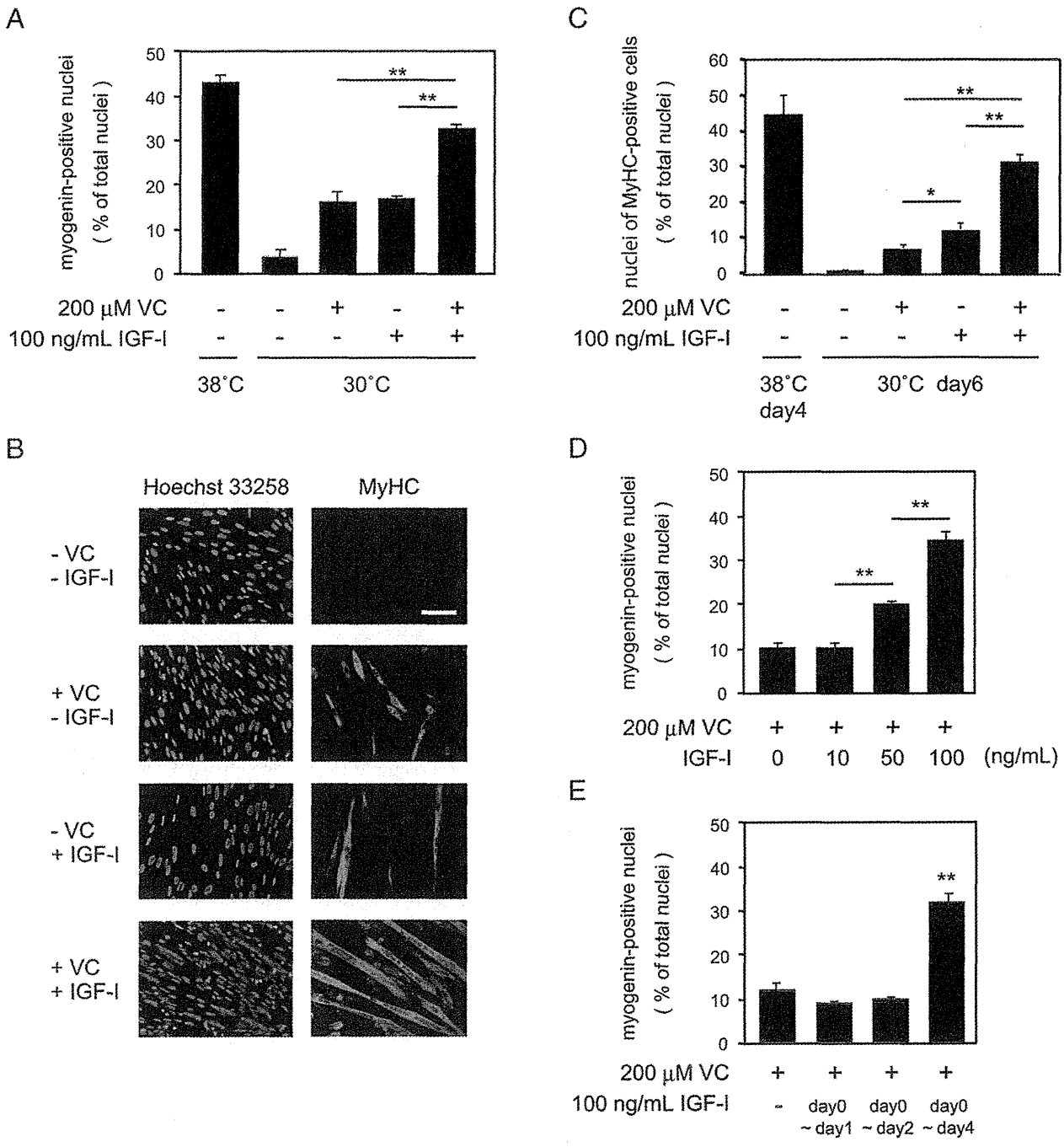

Fig. 1 – Effects of IGF-I and VC on myogenic differentiation of C2C12 cells at 30 °C. A. C2C12 cells were cultured in DM at either 38 or 30 °C and in DM containing 100 ng/ml IGF-I or 200 μM VC at 30 °C for 4 days prior to immunostaining for myogenin. Myogenin expression of the cells cultured in DM with 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.

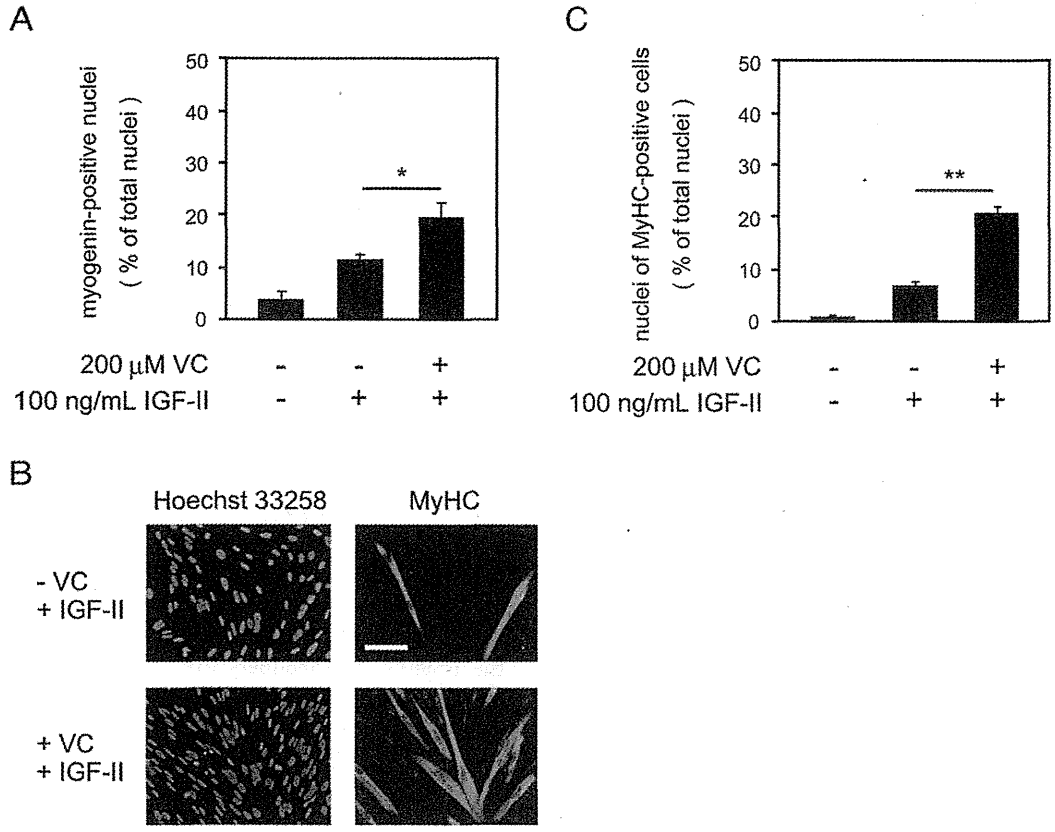

Fig. 2 – Effects of IGF-II and VC on myogenic differentiation of C2C12 cells at 30 °C. A. C2C12 cells were cultured in DM with or without 100 ng/ml IGF-II and 200 μM VC at 30 °C for 4 days and then immunostained for myogenin. Myogenin expression of the cells cultured in DM with both IGF-II and VC was 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

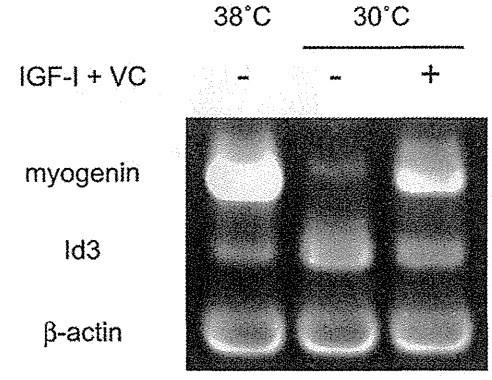

Fig. 3 – RT-PCR of myogenin and Id3 in C2C12 cells. Total RNA was isolated from C2C12 cells cultured in DM at 38 or 30 °C and in DM containing 100 ng/ml IGF-I and 200 μM VC at 30 °C for 4 days. IGF-I and VC increased myogenin expression and decreased Id3 expression at 30 °C compared with cells cultured 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).

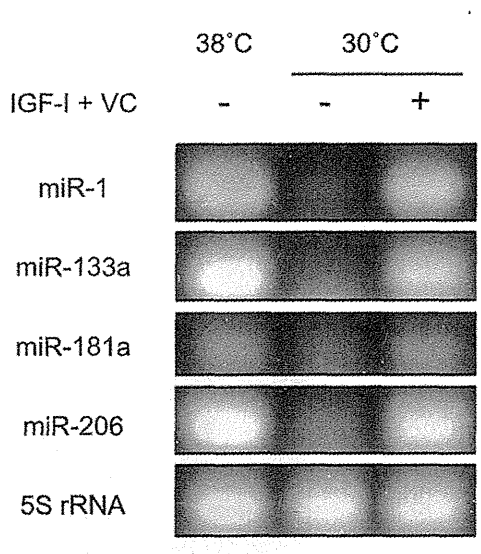

Fig. 4 – RT-PCR of muscle-specific miRNAs in C2C12 cells. Small RNAs were isolated from C2C12 cells cultured in DM at 38 or 30 °C and in DM with 100 ng/ml IGF-I and 200 μM VC at 30 °C for 4 days. RT-PCR for the muscle-specific miRNAs miR-1, -133a, -181a and -206 revealed that the cells cultured in DM 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).

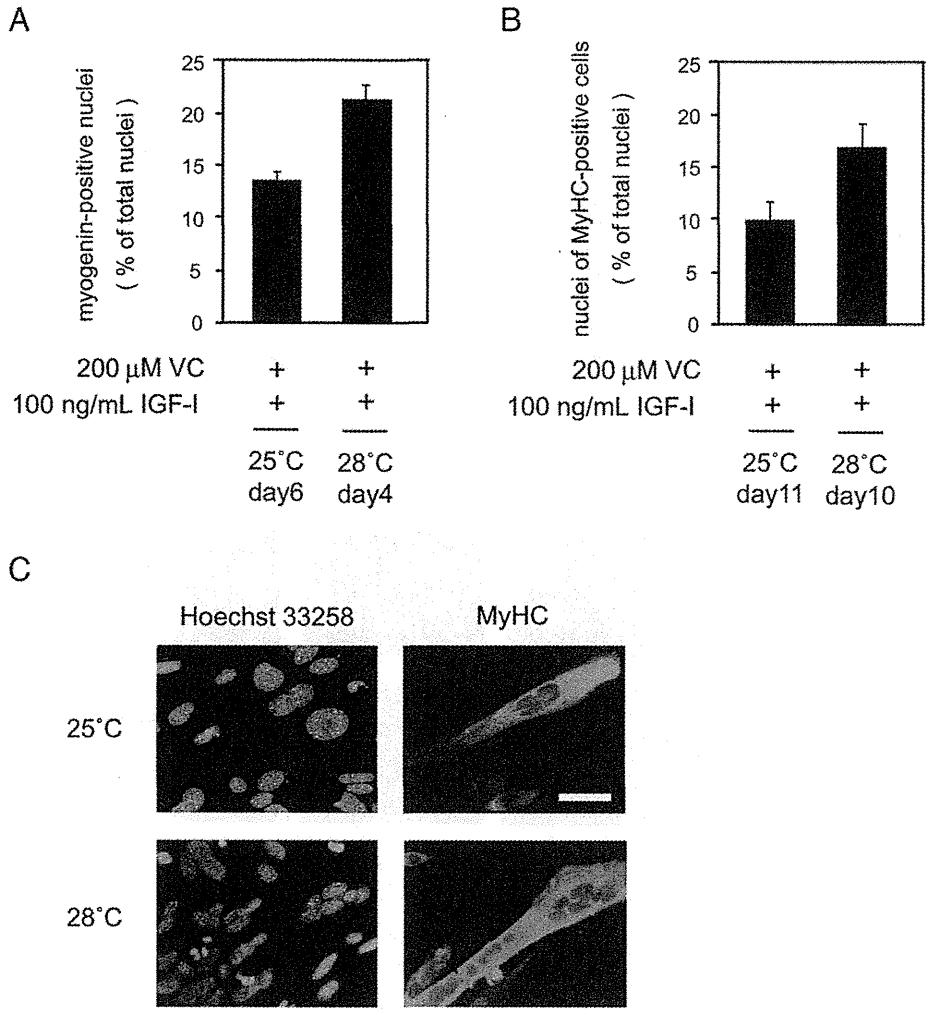

Fig. 5 – Effects of IGF-I and VC on myogenic differentiation of C2C12 cells at 25 and 28 °C. A. C2C12 cells were cultured in DM containing both 100 ng/ml IGF-I and 200 μM VC at 25 °C for 6 days or at 28 °C for 4 days and immunostained for myogenin. The percentages of myogenin-positive nuclei to the total 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