

Exercise-related novel gene is involved in myoblast differentiation

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(Received 4 February 2005; and accepted 12 February 2005)

ABSTRACT

In this study we tried to identify new genes or proteins in skeletal muscle induced by exercise. We analyzed alterations of protein expression in mouse gastrocnemius muscles induced by swim-exercise using two dimensional gel electrophoresis and mass spectrometry. Nine spots were significantly altered between control and swim groups. One of the four protein spots whose expression was decreased was identified as functionally unknown “expressed sequence AI854635” gene. The AI854635 gene has C2H2 type zinc finger motif, and is considered to be a transcription factor. The mRNA of AI854635 gene was expressed in skeletal muscle, brain, kidney, and thymus. To elucidate the function of the AI854635 gene we analyzed mRNA expression levels during C2C12 myoblast differentiation. C2C12 myoblast began to form myotube around 20 h after the initiation of differentiation. The mRNA expression levels of AI854635 gene was significantly induced around 6 h and increased till 48 h, indicating a pivotal role in myoblast differentiation. Although the significance of decreased expression of AI854635 gene induced by swim-exercise is not clear, we found that this gene is involved in myoblast differentiation.

The formation of skeletal muscle during embryogenesis depends on multifactorial processes (9, 10, 13, 19). The myogenic regulatory factors (MRFs) are essential for myogenesis. The MRFs consist of MyoD, Myf-5, myogenin and MRF4. MyoD and Myf-5 play pivotal roles in myogenic determination, whereas myogenin and MRF4 are important for terminal differentiation (4, 10, 11, 13, 15, 18, 19, 25). The MRF proteins belong to a superfamily of basic helix-loop-helix (bHLH) transcription factors (12, 21). The molecular mechanism of myoblast differentiation is not fully understood. C2C12 cultured cells are skeletal muscle precursor cells which have proven an excellent system to elucidate myogenic differentiation (1, 4, 26).

Resistance or eccentric exercise activates satellite cells in mature skeletal muscle cells and induces

differentiation leading to muscle cell hypertrophy (2, 6, 17, 23). Exercise induces several muscle gene expression including MRF protein expression (2, 17).

Proteomic technologies using two dimensional gel electrophoresis and mass spectrometry provide the tool to discover and identify novel genes or proteins (3, 7, 14, 24).

MATERIALS AND METHODS

Animals and exercise. C57BL/6J male mice (4 week old) were purchased from Japan SLC Co. Ltd. (Hamamatsu, Japan), and grouped to 10 control and 10 swim-exercised mice. According to Zhou's swimming protocol (28), control mice were kept sedentary and swim-exercised mice were exercised by swimming in plastic barrel (40 × 60 × 30 cm) filled with warm water (35°C). Mice swam for 30 min periods for up to 150 min interrupted by 10 min rest periods. At 150 min, 10 mice of each group were sacrificed by cervical dislocation and gastrocnemius

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muscles were isolated for protein and total RNA extraction. Three independent experiments were performed and we confirmed the data. For proteomics analysis 10 gastrocnemius muscles (at 150 min) were pooled. This study was performed according to the guidelines of The University of Tokyo and conformed with the national law for animal rights.

Protein and RNA extraction. The pooled skeletal muscles (0.4 g) were homogenized and extracted in 1 ml lysis buffer [9.5 M urea, 1% dithiothreitol, 2% 3-[(3-cholamidopropyl) dimethylammonio] 1-propanesulfonate (CHAPS), 0.8% Pharmalyte pH3–10 (Amersham Biosciences, Tokyo, Japan), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), 10 µg/ml leupeptin, 10 µg/ml aprotinin]. The protein concentrations were measured using Bradford method (Bio-Rad, Hercules, CA, USA). Total RNA was extracted using a QuickPrep Total RNA Extraction kit (Amersham Biosciences).

Proteomics analysis. Using 400 µg of muscle homogenate two dimensional gel electrophoresis was performed. First, isoelectric focusing was performed using the Immobiline™ DryStrip pH3-10NL, 13 cm (Amersham Biosciences). Then, SDS (10%) polyacrylamide gel electrophoresis was performed. The gels were silver-stained using PlusOne Silver Staining kit (Amersham Biosciences). Samples for matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) analysis were excised from the gels. In-gel digestion of the individual protein spots was done using 12.5 µg/ml trypsin (Wako Pure Chemical Industry, Osaka, Japan) as described previously (3, 7, 14, 24). The peptide solutions were vacuum-concentrated until the volume was decreased to 10 µl, and desalted using ZipTip™ C18 column (Millipore, Bedford, MA, USA) according to the manufacturer's

protocol. AXIMA-CFR Shimadzu model MALDI-TOF mass spectrometer (Shimadzu, Kyoto, Japan) was used for mass analysis for tryptic peptide mixtures.

Cell culture. C2C12 myoblast was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured with Dulbecco's modified Eagle's media (DMEM) containing 10% fetal calf serum. Differentiation of C2C12 cells were induced by DMEM containing 2% horse serum (4). Three independent experiments were performed.

RT-PCR. RT-PCR was performed using a TAKARA RT-PCR kit (55°C 30 min for RT, 94°C 30 s, 60°C 30 s, and 72°C 90 s for PCR). The sequences of RT-PCR primers and the number of PCR cycles are shown in Table 1. The PCR products were electrophoresed on 2% agarose gels and 100-bp ladder markers were used to compare the size of PCR products. After the gels were stained with ethidium bromide, the images were acquired using the Chemi-Doc XRS image analyzer (Bio-Rad). Using the installed applications the quantity of the PCR products was measured and expressed as pixel counts.

Statistical analysis. Results obtained from triplicate experiments were shown as the mean ± standard deviation. Significance was analyzed by Student's t-test. P < 0.05 was considered to be significant.

RESULTS

Identification of protein spots on two dimensional gels

Fig. 1A and 1B shows silver-stained gels derived from gastrocnemius muscles of controls and of swim-exercised mice (150 min), respectively. Nine spots were remarkably and differentially expressed

Table 1 RT-PCR Primers and PCR Cycles

| Target gene | Direction | Sequence | PCR cycles | | Target size (bp) |
|-------------|-----------|---|------------|-----------|------------------|
| | | | for muscle | for C2C12 | |
| AI854635 | forward | 5'-gaa aaa gga ggg act tcc caa gga aga a-3' | 28 | 28 | 424 |
| | reverse | 5'-aaa agc ttt ccc gca ctg tta aca cgt g-3' | | | |
| Myf-5 | forward | 5'-aag agt agc agc ctt egg agc aca caa a-3' | 30 | 25 | 488 |
| | reverse | 5'-ctg cag cac atg cat ttg ata cat cag g-3' | | | |
| myogenin | forward | 5'-aag gtg tgt aag agg aag tct gtg tgc g-3' | 25 | 25 | 406 |
| | reverse | 5'-acg atg gac gta agg gag tgc aga ttg t-3' | | | |
| GAPDH | forward | 5'-acc aca gtc cat gcc atc ac-3' | 22 | 22 | 452 |
| | reverse | 5'-tcc acc acc ctg ttg ctg ta-3' | | | |

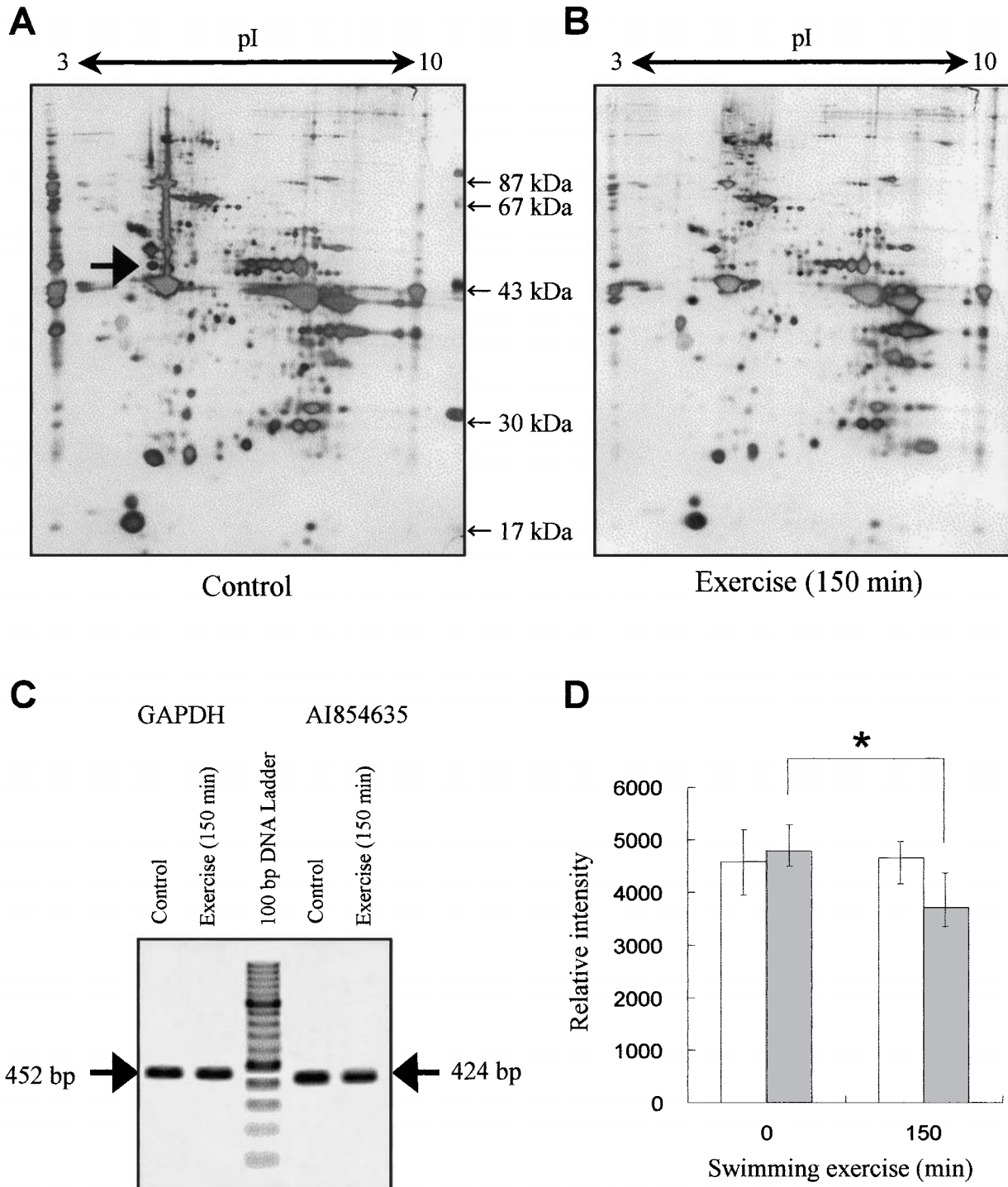


Fig. 1 Silver-stained two dimensional gels derived from gastrocnemius muscles. Gastrocnemius muscles of control mice and of swim-exercised mice (150 min) were analyzed by using two dimensional gels. Fig. 1A and 1B show silver-stained gels derived from gastrocnemius muscles of control mice and of swim-exercised mice, respectively. The arrow shows a spot whose protein expression (AI854635) was remarkably decreased. To confirm the results of two dimensional gel experiment, RT-PCR was performed using total RNA derived from control and swim-exercised (150 min) mice. The mRNA expression data of the AI854635 and GAPDH (control) are shown in Fig. 1C, and the quantitative data are shown in Fig. 1D (mean ± SD, n = 3). Statistical significance was obtained between swim-exercised and control mice (*P < 0.05). 100 bp DNA ladder was used as a DNA marker.

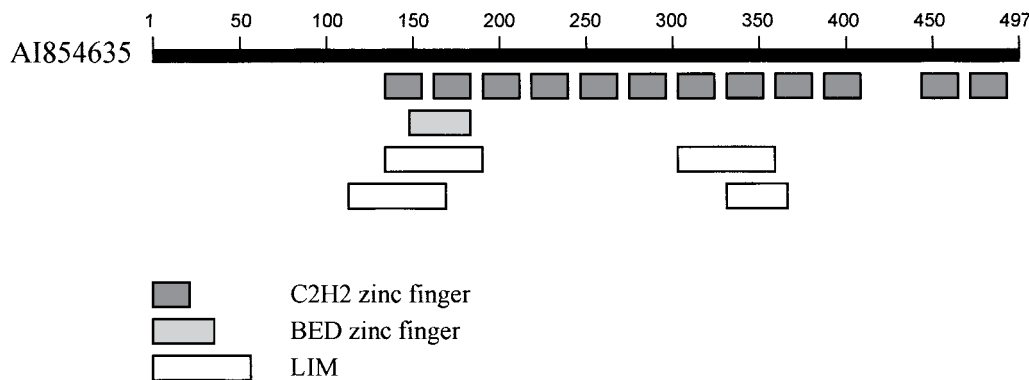


Fig. 2 Domain search of AI854635. Domain search was performed using the NCBI Conserved Domain Search Program. A putative amino acid sequence of the AI854635 (497 amino acids) was applied to the program. The AI854635 has 12 C2H2 type zinc finger domains, 1 BED zinc finger domain and 4 LIM domains.

between controls and exercised mice. Although we analyzed 9 spots using the mass spectrometry as described in Materials and Methods, only two spots could be identified. One of the five spots whose expression was increased was identified as cytoplasmic dynein. One of the four spots whose expression was decreased was identified as functionally unknown “expressed sequence AI854635 (NM_177565)” gene (as indicated by an arrow in Fig. 1A) (20, 22). To confirm the result of two dimensional gel electrophoresis, we performed RT-PCR using total RNA derived from control and swim-exercised mice. As shown in Fig. 1C and 1D, the AI854635 mRNA expression of swim-exercised mice was significantly decreased, compared to control mice.

Domain search of AI854635 gene

We next performed domain search using the NCBI (National Center for Biotechnology Information) Conserved Domain Search Program. A putative amino acid sequence of the AI854635 (497 amino acids) was applied to the program. As shown in Fig. 2, the AI854635 has 12 C2H2 type zinc finger domains, 1 BED zinc finger domain and 4 LIM domains. Thus, the AI854635 is considered to be a transcription factor. Several transcription factors such as ZT2, ZT3, KRAB, and MDZF proteins have C2H2 zinc finger domains (5, 8, 16, 27).

mRNA expression levels of AI854635 gene in various tissues

The mRNA expression levels of AI854635 gene in various tissues of mice were analyzed using semi-quantitative RT-PCR. The conditions for RT-PCR are shown in Table 1. The tissues analyzed were gastrocnemius muscle, soleus muscle, heart, thymus,

brain, kidney, spleen, and liver. As shown in Fig. 3, the mRNA expression levels of AI854635 gene were relatively high in skeletal muscle, brain, kidney and thymus. We next studied whether it is involved in myoblast differentiation.

mRNA Expression of AI854635 gene during C2C12 myoblast differentiation

To elucidate the function of the AI854635 gene we studied mRNA expression levels during C2C12 myoblast differentiation. C2C12 cells started differentiation upon a reduction of the serum content in culture media, as has been reported (4). Fig. 4 shows morphological alteration during C2C12 myoblast differentiation under a phase-contrast microscope. C2C12 began to form myotube around 20 h after the initiation of differentiation upon a reduction of serum by switching from DMEM containing 10% fetal calf serum to DMEM containing 2% horse serum.

Figs. 5 A, B, C and D show a time course (from 0 h to 48 h) of mRNA expression levels of GAPDH, myogenin, Myf-5 and AI854635 genes during myoblast differentiation, respectively. The GAPDH gene expression was used as a control. The mRNA expression levels of AI854635 gene was significantly induced around 6 h and increased till 48 h, indicating a pivotal role in myoblast differentiation. The mRNA expression levels of myogenin was significantly induced from 20 h to 48 h. The Myf-5 expression levels were unchanged from 0 h to 36 h. The MyoD expression levels were low from 0 h to 36 h (data not shown). The data suggest that AI854635 gene expression was induced prior to myogenin induction, and was involved in C2C12 myoblast differentiation, especially during earlier

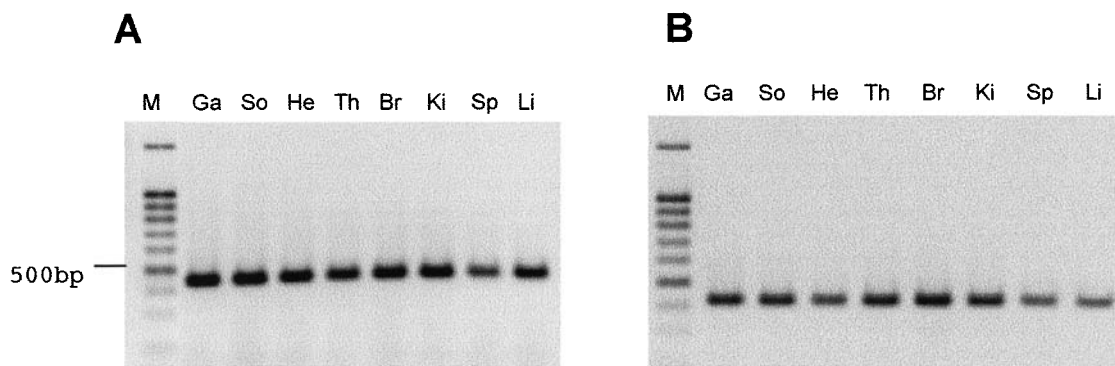


Fig. 3 The expression level of AI854635 in various tissues. The mRNA expression of AI854635 gene was analyzed using semi-quantitative RT-PCR. The PCR products were analyzed using 2% agarose gels. The tissues used were gastrocnemius muscle (Ga), soleus muscle (So), heart (He), thymus (Th), brain (Br), kidney (Ki), spleen (Sp), and liver (Li). A 100 base-pairs DNA ladder was used as a DNA size-marker. A. GAPDH mRNA expression, B. AI854635 mRNA expression

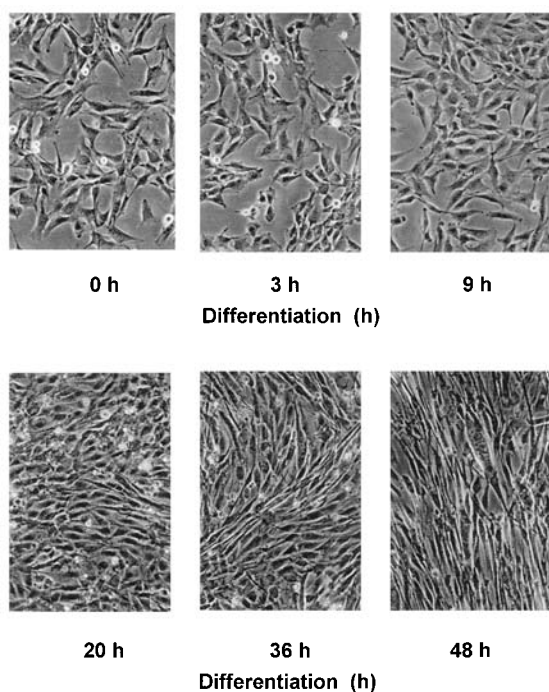


Fig. 4 Morphological alteration during C2C12 myoblast differentiation. Morphological alteration during C2C12 myoblast differentiation was visualized under a phase-contrast microscope ($\times 100$). C2C12 began to form myotube around 20 h after the initiation of differentiation upon a reduction of serum.

step of differentiation.

DISCUSSION

In the present study we analyzed alterations of protein expression in mouse gastrocnemius muscles in-

duced by exercise (150 min swimming exercise) using proteomics techniques. One of the four decreased protein spots was identified as functionally unknown AI854635 gene (20, 22). A putative amino acid sequence of the AI854635 (497 amino acids) was applied to the domain search program, and we found that the AI854635 has 12 C2H2 type zinc finger domains, 1 BED zinc finger domain and 4 LIM domains. Several transcription factors such as ZT2, ZT3, KRAB, and MDZF proteins have C2H2 zinc finger domains (5, 8, 16, 27). Thus, the AI854635 is considered to be a transcription factor. In addition, the AI854635 is abundantly expressed in muscle. Therefore, we tested whether this gene is involved in myogenic differentiation using myoblast C2C12 cell differentiation system (4). We found that the mRNA expression of the AI854635 gene was significantly induced around 6 h prior to the induction of myogenin mRNA expression (from 20 h), suggesting a pivotal role during earlier step of myoblast differentiation.

There are several reports that exercise activates satellite cells in mature skeletal muscle cells and induces their differentiation leading to skeletal muscle cell hypertrophy (2, 6, 17). Exercise also increases myogenin protein expression (17), which indicates a close link between exercise and skeletal muscle differentiation. In contrast to myogenin, the mRNA expression of the AI854635 gene was decreased after swimming exercise. Although the reason for the difference between myogenin and AI854635 gene expression is not clear, there are two possibilities. One is that decreased expression of the AI854635 gene induced by exercise is irrelevant to skeletal muscle cell hypertrophy *in vivo*. Another one is that de-

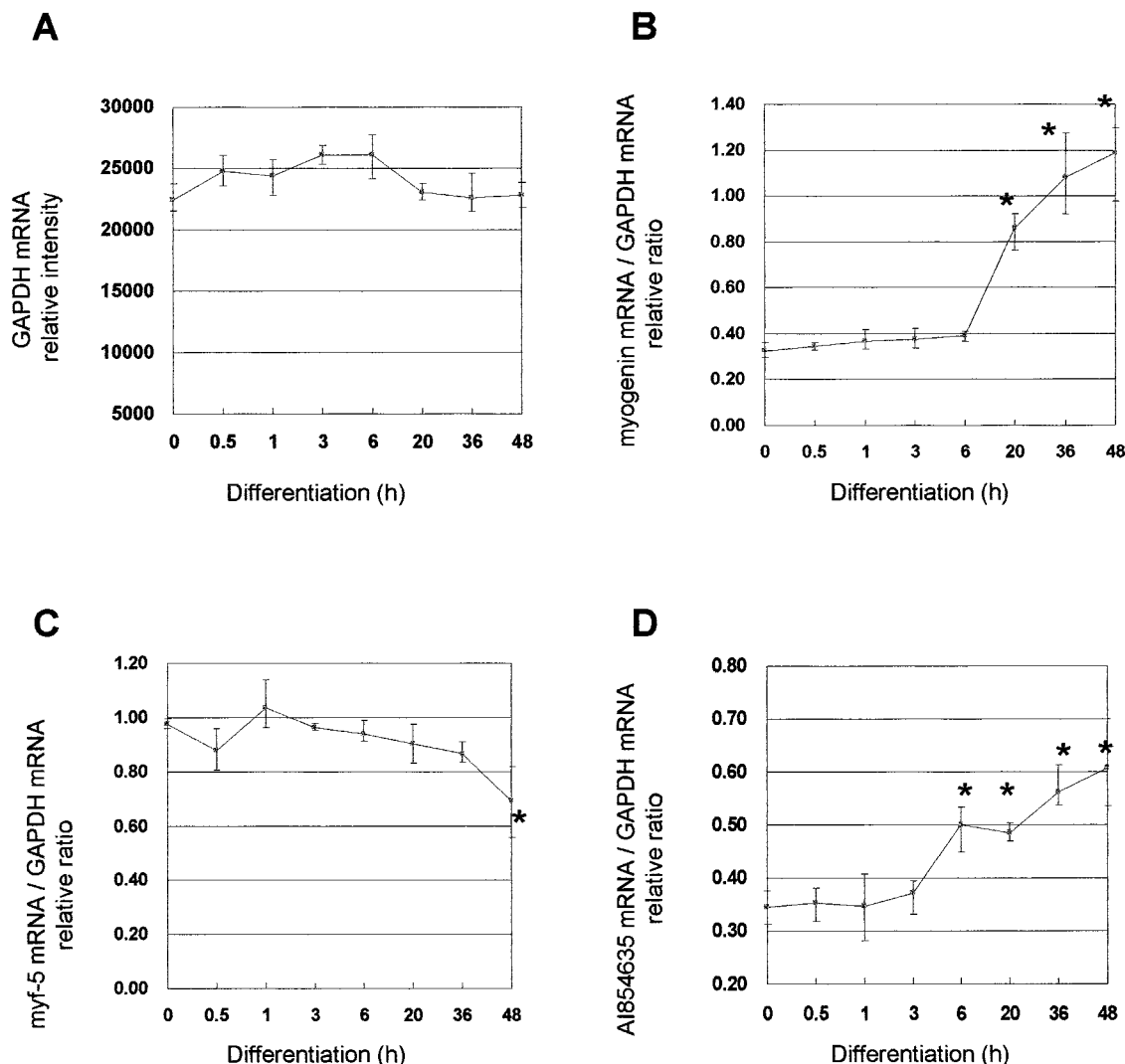


Fig. 5 mRNA expression levels of the AI854635 during myoblast differentiation. Time course (from 0 h to 48 h) of mRNA expression levels of the myogenin, Myf-5 and AI854635 genes during myoblast differentiation was studied using C2C12 myoblast differentiation system *in vitro* ($n = 3$). The levels of mRNA expression of these genes were analyzed using RT-PCR. Using ChemiDoc installed applications the quantity of the PCR products were measured and expressed as pixel counts as described in Materials and Methods. The expression levels were corrected using the levels of GAPDH mRNA expression. Significance of mRNA expression levels compared to 0 h was evaluated using Student's t test. * $P < 0.01$

creased expression of the AI854635 gene may indirectly lead to skeletal muscle cell hypertrophy *in vivo*. In the near future we plan to investigate to elucidate the mechanism for explaining the latter possibility. In summary, we found that the AI854635 gene is involved in myoblast differentiation using cultured C2C12 myoblast in the present study, although the significance of decreased expression of AI854635 gene induced by swim-exercise is unknown.

Acknowledgements

This study was supported by a grant under the Ministry of Education, Science, Sports, and Culture, Japan.

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