

**Yutaka Takaoka, Mika Ohta, Akihiko Ito, Kunihiro Takamatsu, Aki Sugano,
Kotaro Funakoshi, Nobuo Takaoka, Nobuko Sato, Hiroshi Yokozaki, Naoki
Arizono, Shuji Goto and Eiichi Maeda**

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Electroacupuncture suppresses myostatin gene expression: cell proliferative reaction in mouse skeletal muscle

Yutaka Takaoka,^{1,2*} Mika Ohta,^{1,3*} Akihiko Ito,⁴ Kunihiko Takamatsu,³
Aki Sugano,^{1,3} Kotaro Funakoshi,¹ Nobuo Takaoka,⁵ Nobuko Sato,²
Hiroshi Yokozaki,⁴ Naoki Arizono,⁶ Shuji Goto,³ and Eiichi Maeda¹

¹Laboratory for Applied Genome Science, Clinical Genome Informatics Center, Kobe University Graduate School of Medicine, Kobe; ²Department of Biochemistry, Iwate Medical University School of Dentistry, Morioka; ³Department of Acupuncture Informatics, Goto College of Medical Arts and Sciences, Tokyo; ⁴Division of Surgical Pathology, Kobe University Graduate School of Medicine, Kobe; ⁵Faculty of Sciences, Kyushu University, Fukuoka; and ⁶Department of Medical Zoology, Kyoto Prefectural University of Medicine, Kyoto, Japan

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Takaoka Y, Ohta M, Ito A, Takamatsu K, Sugano A, Funakoshi K, Takaoka N, Sato N, Yokozaki H, Arizono N, Goto S, Maeda E. Electroacupuncture suppresses myostatin gene expression: cell proliferative reaction in mouse skeletal muscle. *Physiol Genomics* 30: 102–110, 2007. First published March 6, 2007; doi:10.1152/physiolgenomics.00057.2006.—Complementary and alternative medicine (CAM) may provide patients with an alternative to traditional medicine, but an assessment of its efficacy is required. One CAM method, electroacupuncture (EA) treatment, is a maneuver that utilizes stimulation of acupuncture needles with a low-frequency microcurrent. To study the effect of short-term EA, we evaluated the differential expression of genes induced by EA in mouse skeletal muscle for up to 24 h. We then used RT-PCR to confirm the expression patterns of six differentially expressed genes. Bioinformatic analysis of their transcription control regions showed that EA-inducible genes have numerous common binding motifs that are related to cell differentiation, cell proliferation, muscle repair, and hyperplasia. These results suggested that EA treatment may induce cell proliferation in skeletal muscle. To verify this possibility, we used EA to stimulate mouse skeletal muscle daily for up to 1 mo and examined the long-term effects. Immunohistochemical analysis showed that nuclei of muscle cells treated with EA for 1 mo, especially nuclei of satellite cells, reacted with anti-human PCNA. Also, expression of the gene encoding myostatin, which is a growth repressor in muscle satellite cells, was suppressed by daily EA treatment for 1 wk; EA treatment for 1 mo resulted in more marked suppression of the gene. These molecular findings constitute strong evidence that EA treatment suppresses myostatin expression, which leads to a satellite cell-related proliferative reaction and repair in skeletal muscle.

acupuncture; electrical current; satellite cell; muscle repair; regeneration

ACUPUNCTURE THERAPY, which is one constituent of complementary and alternative medicine (CAM), has long been believed to be a worthy component of routine clinical practice in eastern Asia, and its use for at least 2,500 yr can be verified. The usual theory of acupuncture practice has been based on a model of energy (qi) balance, which involves metaphysical cognition

and is quite different from the premise on which Western biomedical science is based; acupuncture may correct imbalances of qi flow at acupoints, which would then lead to disease cures and wound healing (16, 34).

In practice, acupuncture therapy is utilized to cure many diseases and to maintain health. The World Health Organization has listed more than 40 indications for acupuncture, and the National Institutes of Health has accepted the validity of acupuncture treatment (13). Major acupuncture techniques involve penetration of the skin by thin, solid metallic needles, which are manipulated manually or are stimulated electrically (27). Pain and other physical problems such as muscle exhaustion, including stiff shoulders in patients, as well as the conditioning of athletes, have benefited from acupuncture treatment.

Studies of acupuncture have reported the routes of acupuncture signal transmission in nerves, effects via the spinal reflex, and reactions in the brain (10, 40, 55). Figure 1 shows routes of acupuncture stimuli between acupoints and organs. In a previous investigation of acupuncture, only a neural mechanism of pain reduction was clear; anesthesia produced by acupuncture induced endogenous opioids (β -endorphin and enkephalin) (11). However, molecular mechanisms of other effects of acupuncture are as yet not defined (2).

Scientific evidence of efficacy is as important for CAM research as it is for research in Western medicine. Enhancement of blood flow in target organs by acupuncture treatment, which is a major reason for the effectiveness of acupuncture (41), cannot sufficiently explain the recovery of muscle from exhaustion, because it is not clear how the oxygen and nutrients supplied would be used during a cellular recovery process. Many cellular and physiological processes are regulated at the transcription level of gene expression. Identification of genes that are specifically modulated during the process of acupuncture would be an initial step toward elucidation of underlying mechanisms of this technique.

To search for differentially expressed transcripts that are induced by electroacupuncture (EA) before applying large-scale constitutive analysis such as DNA microarray, we used an mRNA fingerprinting method-differential display (DD) analysis, by which a number of samples can be compared in parallel, because of its ease of use, low cost, and ability to identify novel genes (32). The specific DD technique that we chose was amplified restriction fragment length polymorphism (AFLP) because it is one fingerprinting method that is not

* Y. Takaoka and M. Ohta contributed equally to this work.

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Address for reprint requests and other correspondence: Y. Takaoka, Laboratory for Applied Genome Science, Clinical Genome Informatics Ctr., Kobe Univ. Graduate School of Medicine, Kobe 650-0017, Japan (e-mail: ytakaoka@med.kobe-u.ac.jp).

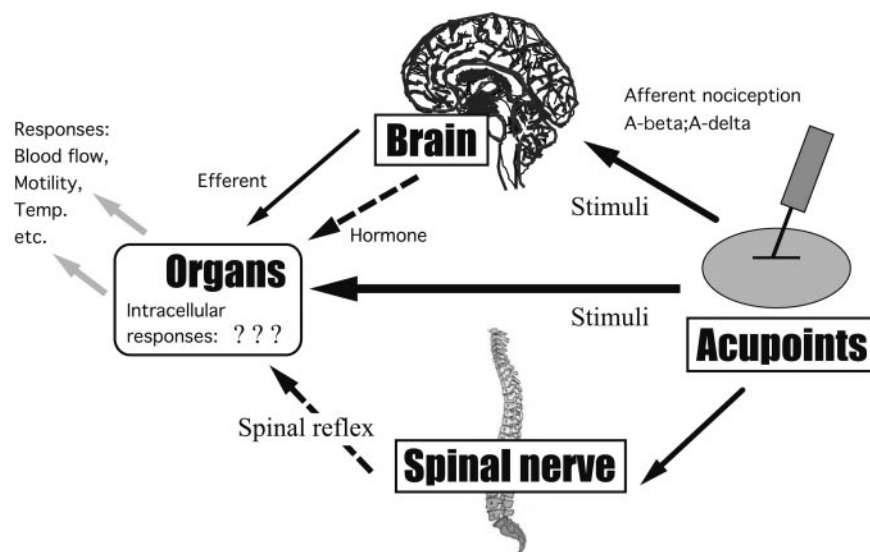


Fig. 1. Schematic diagram of transmission of acupuncture signals. Solid arrows indicate the nervous system; broken arrows, the extraneural system; gray arrows, organ responses. Modified from Cho et al. (10).

overly sensitive to reaction conditions or to the quality of the template DNA (38), so that the method has high reproducibility. For this analysis, bioinformatics may be an important bridge between the gene expression profile and the phenotype. We thus devised our own application that can extract shared characteristics from the differentially expressed transcripts by means of bioinformatics and comparative genomics. Finally, we used immunohistochemistry to verify these data obtained via bioinformatics.

The aim of this study was to identify the molecular characteristics of EA. In this report, we confirm the usefulness of molecular biological approaches utilizing bioinformatics for investigations of CAM methods such as acupuncture and show a proliferative effect on muscle satellite cells of long-term EA treatment.

MATERIALS AND METHODS

EA treatment conditions in animals and humans. We decided the conditions used for electrical stimulation by means of a digital oscilloscope (LS140; LeCroy, Chestnut Ridge, NY). We determined the EA treatment conditions as follows: we stimulated five inbred strain C57BL/6 male mice (Clea Japan, Tokyo, Japan), 8 wk old, in the hindleg muscle at points corresponding to BL 36 and BL 59 (for details of these acupoints, see our web site: <http://www.lifence.ac.jp/cs/adss/>). For comparison, we determined conditions used for electrical stimulation of human subjects at BL 55 and BL 58 ($n = 5$). We calculated the electrical current for mice on the basis of voltage across a 500-k Ω resistor and that for humans, a 1-k Ω resistor ($I = E/R$).

Stainless-steel acupuncture needles (40 mm long and 0.16 mm in diameter; Seirin, Shizuoka, Japan) were inserted into anesthetized mice at the acupoints, to a depth of 5–7 mm, and the needles were then stimulated with an electrical stimulator (Kyushu Ryodoraku, Fukuoka, Japan), with the muscle's constriction representing usual clinical conditions. All EA treatments were applied for 15 min with 1.2-Hz repetitions.

Sample collection and extraction of total RNA for study of short-term EA effects. Twenty mice (the same strain, sex, and age as described in the previous section) were stimulated under the treatment conditions (see Fig. 2A) at the same acupoints, with the same depth, and the same acupuncture needles. All EA treatments were applied for 15 min with 1.2-Hz repetitions. Immediately after (0 h) and then 1, 3, and 24 h after EA treatment ($n = 5$ for each time point), total RNA

was extracted from muscles (gastrocnemius, soleus, biceps femoris, and gluteal) by using a guanidine thiocyanate procedure (20). As a control group, five mice were similarly anesthetized but received no acupuncture or EA treatment. The control RNA samples were extracted from non-EA treated mice under the same procedure.

We also assessed the effect of using only acupuncture needles for stimulation (no electrical current), by placing the needles in five mice at BL 36 and BL 59 for 15 min. These RNA samples were obtained for the same time points by means of the same procedure as that used with EA-treated mice. All aliquots were stored at -80°C until used.

All mice in this research project were treated according to the Standards Relating to the Care and Management, etc. of Experimental Animals (Ministry of the Environment, Tokyo, Japan). This study was approved by the Committee for Safe Handling of Living Modified Organisms in Kobe University (permission number 17-21) and carried out according to the Guidelines of the Committee.

mRNA fingerprinting. For the DD analysis, 16 EA-treated mice were prepared by means of the same EA conditions as described in the previous section. We examined a mixed sample of individual muscles from four mice to exclude artifacts at each time point (0, 1, 3, and 24 h) and four control mice. RNA concentration was measured spectrophotometrically at 260 nm. Total RNA (2 μg) was reverse-transcribed into cDNA by using SuperScript RT (GIBCO/BRL SuperScript Preamplification System, Gaithersburg, MD), according to the company's instruction manual. AFLP-based mRNA fingerprinting was performed as described previously (17). The products of polymerase chain reaction (PCR) were heated for 3 min at 95°C and then quickly cooled on ice. These samples were separated on 5% (wt/vol) polyacrylamide/7.5 M urea sequencing gel.

Upregulated bands of interest in DD gels were excised by use of FluorImager SI (Molecular Dynamics, Sunnyvale, CA) after staining with SYBR green I (Molecular Probes, Eugene, OR). Bands were cut out and soaked in distilled H_2O on ice. These recovered fragments were reamplified with the same primers and then cloned into pBlue-script II KS(+) vector (Stratagene, Cedar Creek, TX) for DNA sequencing. DNA sequencing was performed with the T7 DNA polymerase sequencing kit (Amersham, Buckinghamshire, UK). BLAST programs [National Center for Biotechnology Information (NCBI), <http://www.ncbi.nlm.nih.gov/blast/>] were used with the mouse genome database to search for homologues of detected sequences of the cDNA fragments to obtain gene origins.

The expression level of each clone was compared by using RT-PCR analysis with gene-specific primers (Table 1), in a semiquantitative manner, to prove the reliability of the approach and to confirm

Table 1. *Effects of electroacupuncture on gene expression as determined by mRNA fingerprinting and RT-PCR*

Band Number in DD Gel	Gene Expression in DD Gel (Fig. 3A)	Size in Gel, bp	Identity	Pattern of Gene Expression by RT-PCR Primer Sequences (5'-3')
1	suppressed	255	unknown (dbEST accession no. AW400398)	Suppressed sense, GGAAAGCTGTGGCGTGATG antisense, GGTTGGCGTTAAACTCACAGT
2	induced	576	neuronatin	Induced sense, AATGAGGGAGCAAGGCAGGA antisense, AGCCCATCAACCAGCAGAATG
3	induced	564	NAD(H)-specific isocitrate dehydrogenase	Induced sense, TACTGCATACACATGGCCAT antisense, AACATCCTTCGCACCAGCCTA
4	induced	335	spindlin	Induced sense, GACTGTGCACGCTCTAAAGA antisense, TACAGAGAGCGCAGCATTCC
5	induced	271	ribosomal protein L23	Induced sense, CGGGCGAAATTCCGGATTT antisense, TTACCACTGCTGGATGTACC
6	induced	526	ubiquitin-specific protease UBP41	Induced sense, CGAAGACTGTAGAGCAGTAGC antisense, GGAACACGTGCTTCATGAACT
7	induced	484	unknown (Aig1, GenBank accession no. DQ167195)	Induced sense, ACTGGGATACACTCGTGAGC antisense, GACACAGGAAGGTCACCACCA
8	induced	315	spindlin	Induced sense, see sequence of gel band 4 antisense, see sequence of gel band 4
9	induced	526	ubiquitin-specific protease UBP41	Induced sense, see sequence of gel band 6 antisense, see sequence of gel band 6
10	induced	485	unknown (Aig1, GenBank accession no. DQ167195)	Induced sense, see sequence of gel band 7 antisense, see sequence of gel band 7
VEGF			VEGF/VPF	Induced sense, TGGCTTTACTGCTGTACCTC antisense, ACGCGAGTCTGTGTTTTTGC
Control			G3PDH	— sense, CATCTCACAGGTTACTTCAGA antisense, CTGTTGCTGTAGCCGTATTC

DD, differential display; VPF, vascular permeability factor; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

the expression pattern. PCR products processed from individual muscles ($n = 4$) were evaluated via an agarose gel. Additional PCR confirmation was done for other samples of individual muscles from mice treated with short-term EA ($n = 5$). We also used RT-PCR to determine the expression pattern of the gene encoding VEGF (VEGF/VPF, vascular endothelial growth factor/vascular permeability factor), which is a key molecule for reactions of electrically stimulated cells (35). Expression of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene served as an internal control (5, 21).

RT-PCR was performed with a reaction mixture (20 μ l) containing primers at 5 pmol, 0.2 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 1 \times PCR buffer, and 200 μ l/l dNTPs on a Thermal Cycler (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA). Thermal cycling parameters for PCR (23–27 cycles) were 45 s at 94°C for denaturation, 25 s at 55°C to 59°C for annealing, and 2 min at 72°C for extension.

Bioinformatics analysis of the transcription control region. To ascertain the shared transcription factor binding characteristics of the genes, we analyzed the transcription control region for each of the six genes by bioinformatics via comparative genomics, and we detected corresponding Refseq genes by means of the UCSC Genome Browser (<http://genome.ucsc.edu/>) (23). The RefSeq database of NCBI provides a nonredundant collection of sequences representing genomic data, transcripts, and proteins (45, 46). The information obtained from this database was used to determine the transcription starting site for the genes, except for the acupuncture-induced gene 1 (Aig1) gene. The full-length cDNA sequence of the Aig1 gene including the

transcription starting point was detected via cDNA cloning by use of the mouse brain cDNA library (Stratagene, La Jolla, CA) and via the cap site hunting method (52) by use of the cap site cDNA library (Nippon Gene, Tokyo, Japan) (Ohta M, unpublished observations).

The transcription control regions of these genes (up to 2,000 bp from the transcription starting point) were extracted from the mouse and human genome sequences. The transcription regulatory sequences in the promoter regions were analyzed by use of TRANSFAC (<http://motif.genome.jp/>) (58). Then, common transcription factors occurring in genes, mouse and human, were chosen from the search results. Finally, transcription factors that were conserved in the mouse and the human were selected as candidate factors. In this study, we used SHAFT (<https://ebraille.med.kobe-u.ac.jp/SHAFT/>), an automated application of the search and choice analyses for transcription factors that we had developed.

Immunohistochemical verification and RT-PCR analysis for the long-term EA efficacy study. Mice were placed in three groups of five animals each on the basis of treatment period: 1 day (1D), 1 wk (1W), and 1 mo (1M), except for the last group, which consisted of three mice because of two accidental deaths from anesthesia. Daily EA treatments were administered, similarly to the short-term EA study, to hindleg skeletal muscles. Mice under ether anesthesia were killed at 3 h after the final EA treatment of each treatment period. This experiment was done according to the Standards Relating to the Care and Management, etc. of Experimental Animals (Ministry of the Environment, Tokyo, Japan).

Tissue samples of EA-treated gastrocnemius muscle and biceps femoris muscle were fixed in 10% neutral buffered formalin and were embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (H&E). The same paraffin sections were analyzed by means of immunohistochemistry by using the avidin-biotin-peroxidase complex method (ABC kit; Vector, Burlingame, CA). The antibody used in this study was anti-human PCNA rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA). To assess the specificity of the immunostaining, normal rabbit serum was substituted for the specific primary antibodies. For negative controls, the same procedure was used but without the primary antibodies, as described previously (54).

The expression pattern of the myostatin gene was analyzed by means of RT-PCR with gene-specific primers as follows: sense: 5'-GACAAAACACGAGGTACTCC-3'; antisense: 5'-GATTCAGC-CCATCTTCTCC-3'. PCR was performed with a reaction mixture (20 μ l) containing primers at 5 pmol, 0.4 unit of AmpliTaq Gold DNA polymerase, 1 \times PCR buffer, and 200 μ l/dNTPs on a Thermal Cycler, in 26 cycles (1 cycle: denaturation at 94°C, annealing at 58°C, and extension 72°C, each for 60 s). The myostatin gene obtained from individual muscles ($n = 5$ for 1D and 1W groups; $n = 3$ for the 1M group) was evaluated via agarose gel electrophoresis. After electrophoresis, densitometric values of these PCR bands were determined by a gel-plotting macro of the NIH Image program (Wayne Rasband, National Institutes of Health, Bethesda, MD). Relative expression levels were then calculated by comparison with the G3PDH result as an internal control. These values are given as means \pm SD. All data are presented as percentages of controls (no treatment; anesthesia only). Student's *t*-test was used for statistical analysis, with *P* values <0.05 considered statistically significant.

RESULTS

Electroacupuncture condition. Figure 2 shows typical results in mice and human subjects. EA treatment at BL 36 and BL 59 in the mouse involved 2,521-mV impulses ($-1,021$ to $+1,500$ mV), as indicated by the oscilloscope, and the electrical current (-0.14 to $+0.30$ μ A) was calculated instantaneously only at pulse edges across a 500-k Ω resistor (-71.2 to $+150$ mV), as shown in Fig. 2A. Evaluation of EA in the human (at BL 55 and BL 58) utilized 11.5-V impulses (-5.5 to $+6.0$ V), as indicated by the oscilloscope, and the electrical current (-2.5 to $+3.8$ mA) was calculated instantaneously only at pulse edges across a 1-k Ω resistor (-2.5 to $+3.8$ V) (Fig. 2B). These results demonstrated that EA for the mouse consisted of weaker electrical stimulation than that for the human.

Differential gene expression in skeletal muscle after EA (short-term verification). Duplicate experiments revealed 37 differentially displayed bands among \sim 310 cDNA bands. We identified 10 of these 37 bands (Fig. 3A) as having specific expression patterns. The detected clones were nine induced genes (*bands 2 to 10*) and one suppressed species (*band 1*). Table 1 provides mRNA fingerprinting results from the search of the mouse genome database for homologies, which revealed that 7 of the 10 clones were derived from five known genes encoding the following: *band 2*, neuronatin (24); *band 3*, NAD(H)-specific isocitrate dehydrogenase (7); *bands 4 and 8*, spindlin (44); *band 5*, ribosomal protein L23 (51); and *bands 6 and 9*, ubiquitin-specific protease UBP41 (4). These five known genes that were identified function in cell-cycle activities, cell-cycle regulation, protein quality control, and/or signal transduction (4, 7, 24, 44). The patterns of transcripts were determined by RT-PCR analysis with gene-specific primers (Table 1).

One of the three unknown clones (*band 1*) was registered in dbEST (dbEST accession no. AW400398). Another unknown gene that was found, the full-length cDNA sequence of *bands 7 and 10*, was named Aig1 and was registered in a genome database (GenBank accession no. DQ167195). The transcription starting point of the Aig1 gene was determined by means of the cap site hunting method (dbEST accession nos. AB214884, AB214885, and AB231477).

VEGF/VPF (56), a key molecule related to electrical stimulation that is found in cultured endothelial cells (60), was expressed in muscle just after EA treatment (0 h) and then 1 h later, but expression returned to the prestimulation level after 3 h (Fig. 3B). This result suggests that VEGF may be a key molecule in EA-treated muscle for reactions of endothelial cells stimulated with electrical current.

Bioinformatics analysis of transcription control regions of EA regulatory genes. To elucidate the common factor in transcription control regions, we used the bioinformatics method via comparative analysis of mouse and human data. An interspecies sequence comparison is based on the simple premise that evolutionarily stable sequences are likely to be important. First we determined all mouse and human homologous genes, except the gene for mouse ribosomal protein L23, because no homologous gene was found in the human. Then, we extracted the transcription regulatory region (2 kb) of each

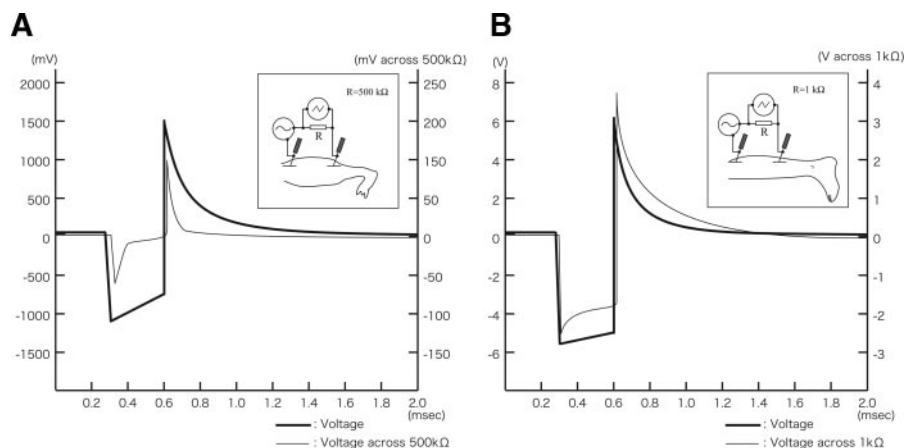
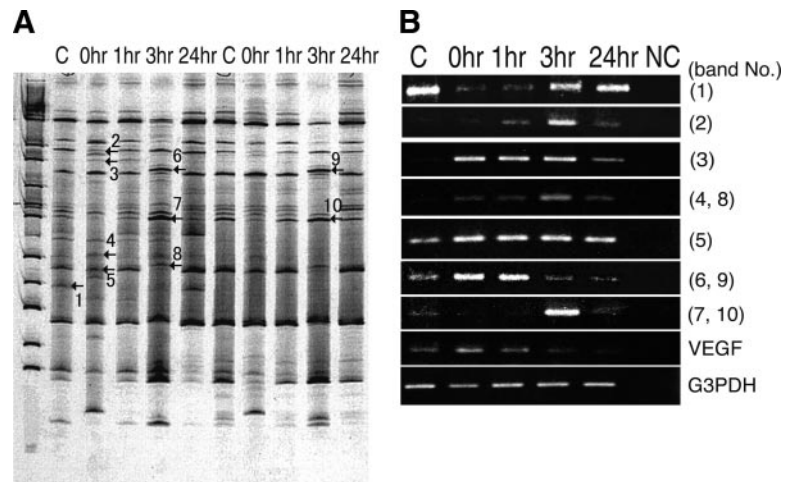


Fig. 2. Electrical voltage and current were measured with a digital oscilloscope (LS140; LeCroy, Chestnut Ridge, NY) connected to circuits as shown. Electrical current was calculated ($I = E/R$) on the basis of voltage across a 500-k Ω resistor in the case of the mouse (A) and a 1-k Ω resistor in the case of the human (B).

Fig. 3. Example of a typical pattern of gene expression for 10 transcripts identified by mRNA fingerprinting analysis. Samples of total RNA from mouse skeletal muscle without treatment (control) and 0, 1, 3, or 24 h after electroacupuncture (EA) treatment were subjected to mRNA fingerprinting by using an amplified restriction fragment length polymorphism (AFLP)-based method (A). Arrows indicate the bands (1 to 10) that were selected and sequenced for gene identification. Results of semiquantitative RT-PCR are also shown (B). Detailed information about these genes is given in Table 1. Lanes are as follows: *lane C*, no EA stimulation (control); *lane 0hr*, EA-treated sample just after stimulation; *lane 1hr*, EA-treated sample 1 h after stimulation; *lane 3hr*, EA-treated sample 3 h after stimulation; *lane 24hr*, EA-treated sample 24 h after stimulation; *lane NC*, negative PCR control containing no cDNA template in the PCR mixture; VEGF, vascular endothelial growth factor; G3PDH, glyceraldehyde-3-phosphate dehydrogenase (an internal control).



gene from the mouse genome database and the same region of each human homologous gene from the human genome database. Table 2 provides common transcription factors for the mouse and the human, as well as factors conserved in both species. Common transcription factors consisted of 15 factors in all mouse genes and 18 factors in human genes. Among these factors, AML-1a, AP-4, CdxA, c-Ets-1(p54), GATA-1, GATA-2, MyoD, Nkx-2.5, and SRY were conserved in both mouse and human (Table 2). These results suggest that EA treatment affected cell proliferation and repair of skeletal muscle.

Immunohistochemical verification and RT-PCR analysis of long-term EA efficacy. We performed histochemical and immunohistochemical analyses to verify long-term efficacy, that is, molecular findings related to muscle cell proliferation under the influence of daily EA treatment at 1 day, 1 wk, and 1 mo.

Figure 4 shows the results for EA-treated muscle after 1 mo of daily treatment. Nuclei of EA-treated muscle (Fig. 4, B and F) had clearly visible nucleoli but control muscle that had had no EA treatment (Fig. 4, A and E) did not. The presence of nucleoli confirmed DNA replication and synthesis of rRNA. EA-treated muscle showed a proliferative reaction, because PCNA antibody was prominent in nuclei in the S phase (48). Nuclei in cells of muscle treated daily with EA reacted with PCNA antibody (Fig. 4, D and H), whereas no reaction occurred in control muscle (Fig. 4, C and G). Tissues collected at the other time points, similar to the control samples, showed no PCNA antibody reactions (Table 3). Also, PCNA-positive nuclei (Fig. 4I) were found only in satellite cells that had clearly visible nucleoli (Fig. 4J) in the nuclei, which confirmed that EA caused a proliferative reaction in skeletal muscle.

To verify this finding, we used RT-PCR to examine expression of the myostatin gene, because myostatin functions as an

endogenous inhibitor of muscle growth and satellite cell-related muscle regeneration (50, 56). Indeed, in the study of long-term EA, expression of the myostatin gene was significantly suppressed by daily EA treatment after 1 day and 1 wk, and quite marked suppression of expression was observed after 1 mo (Fig. 5A). After only one EA treatment at 24 h, i.e., the study of the short-term EA, expression of the myostatin gene was at most 50% of control (Fig. 5B). These results suggested with some confidence that long-term EA treatment suppressed expression of the myostatin gene, which led to a satellite cell-related proliferative reaction and repair in skeletal muscle.

Acupuncture without electrical stimulation (Fig. 5C) did not cause significant myostatin gene suppression. This result compared with that achieved with short-term EA suggests that the mechanism of EA efficacy differs from that of acupuncture itself.

DISCUSSION

This study provides, by means of transcriptome analysis and bioinformatics, molecular evidence for a satellite cell-related proliferative reaction in skeletal muscle from EA-treated mice. We show that molecular biological analyses are effective approaches for experimental research in CAM areas such as acupuncture. The paper also supports the view that regulation of transcription by EA is key for understanding the efficacy of this treatment.

We first determined the characteristics of the electrical treatment in the EA-treated mouse model (at BL 36 and BL 59) and in humans (at BL 55 and BL 58), with muscle constriction, as found in usual clinical conditions (Fig. 2). These results suggested that the electrical dose per mouse skeletal muscle cell was approximately equal to or smaller than that for the

Table 2. *Bioinformatics analysis: Transcription factors conserved across species within the upstream 2000-bp region of the gene*

Expression Pattern	Present in All 6 Mouse Genes	Present in All 6 Human Homologous Genes
Present in the induced expression pattern	AML-1a, AP-4, c-Ets-1 (p54), CdxA, δ EF1, GATA-1, GATA-2, GATA-3, HSF2, Ik-2, Lmo2, MyoD, Nkx-2.5, Oct-1, SRY	AML-1a, AP-2, AP-4, CDP, CdxA, c-ETS-1(p54), c-Myb, GATA-1, GATA-2, GKLF, Lyf-1, MyoD, MZF1, NF-AT, Nkx-2.5, p53, Spl, SRY
Conserved in both mouse and human	AML-1a, AP-4, CdxA, c-Ets-1(p54), GATA-1, GATA-2, MyoD, Nkx-2.5, SRY	

human, because the total volume of muscles for the mice was much smaller than that for the human. Therefore, our mouse model served as a suitable experimental system for *in vivo* study of the molecular analysis of EA. In our current research, unlike other studies (10, 40, 55) that investigated transmission of the acupuncture signal, as shown in Fig. 1, we analyzed the direct effect of EA in treated organs.

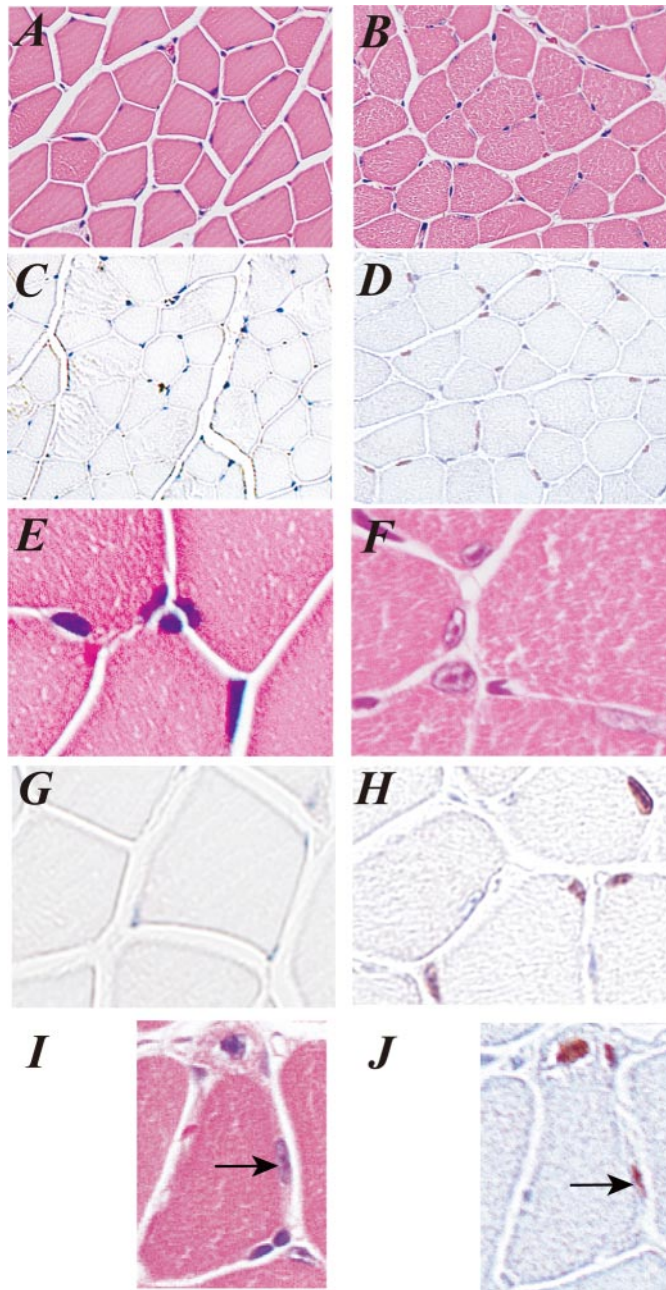


Fig. 4. Histochemical and immunohistochemical findings for EA-treated muscle (B, D, F, H, I, and J) and control (no treatment; anesthesia only) muscle (A, C, E, and G). Hematoxylin and eosin (H&E)-stained samples are A, B, E, F, and I; immunohistochemical samples are C, D, G, H and J. PCNA antibody-positive nuclei (stained in brown; D and H) and nucleoli (F) were clearly present in EA-treated muscle but not control muscle (C, E, and G). In serial sections, PCNA antibody reacted in satellite cells (J, indicated by the arrow), and nucleoli (I, indicated by the arrow) were found in the same nuclei by H&E staining. Original magnifications: $\times 40$ (A–D) and $\times 100$ (E–J).

Table 3. Reactivity of PCNA antibody in the nucleus of muscle cells and myostatin gene expression

Sample (n = 5 for each)	EA-treated Tissue: reaction with PCNA antibody (myostatin gene expression)	Tissue Treated With Acupuncture Only: reaction with PCNA antibody (myostatin gene expression)
Control	NR (expression)	NR (expression)
After 0 h	NR (slight suppression)	NR (expression)
After 1 h	NR (suppression)	NR (expression)
After 3 h	NR (suppression)	NR (expression)
After 24 h	NR (suppression)	NR (expression)
Daily treatment for 1D (after 3 h)	NR (slight suppression)	NT
Daily treatment for 1W (after 3 h)	NR (suppression)	NT
Daily treatment for 1M* (after 3 h)	PCNA reacted (marked suppression)	NT

1D, 1 day; 1W, 1 week; 1M, 1 month; EA, electroacupuncture; NR, not reacted with PCNA antibody; NT, not tested; *n = 3.

To examine the effect of EA on gene expression, we screened mouse muscles treated with short-term EA by use of an mRNA fingerprinting method, and we found 37 differentially expressed bands (Fig. 3A). Five known genes in these 37 bands (Table 1), with roles in cell-cycle activities, cell-cycle regulation, protein quality control, and/or signal transduction, were upregulated (4, 6, 7, 24, 44). In addition, we discovered two previously unknown genes and registered them in genome databases. One of these unknown genes was a full-length of

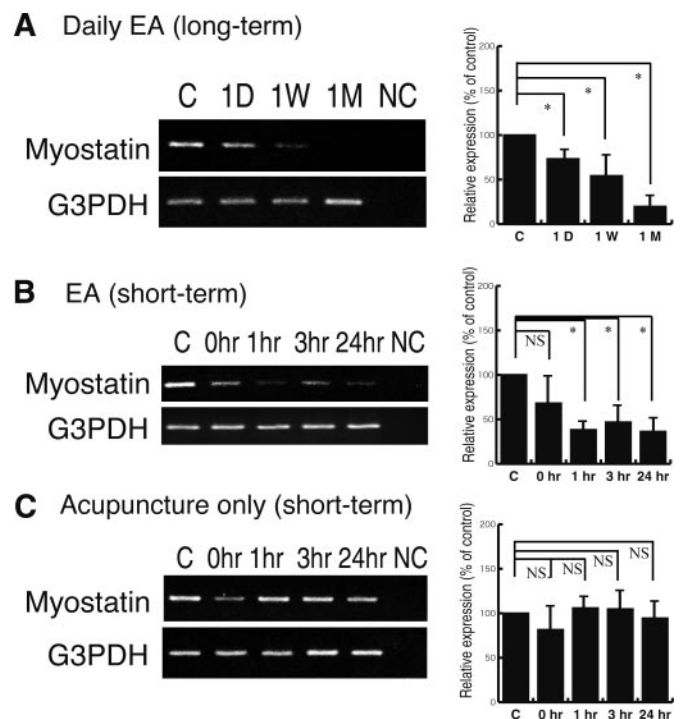


Fig. 5. RT-PCR analysis of myostatin gene expression after long-term EA (A), short-term EA (B), and acupuncture only (no electrical stimulation). Data were obtained from different individual samples (n = 5, except for the 1M group: n = 3). G3PDH was used as a loading control. Lanes are as follows: lane C, control; lane 1D, EA stimulation for 1 day; lane 1W, after daily EA stimulation for 1 wk; lane 1M, after daily EA stimulation for 1 mo. See Fig. 3 for additional lane designations. Relative transcript levels of myostatin are shown (means \pm SD). *P < 0.05; NS, not significant.

cDNA sequence, detected via the cap site hunting method (37), that we named *Aig1* (Ohta M, unpublished observations). All genes were classified as having either an inducible or a suppressible pattern after EA. DD gels revealed additional expression patterns in detail (Fig. 3A). These results indicated that EA caused a dynamic alteration of gene expression in a transcriptome. Although the known genes that were detected were relevant to cell functions, their expression did not clearly indicate an EA effect.

To elucidate EA-regulated gene expression in muscle, we used bioinformatics to analyze the 2-kb-long control regions of detected genes. In a search for common transcription factor binding motifs, nine conserved motifs-*AML-1a*, *AP-4*, *CdxA*, *c-Ets-1(p54)*, *GATA-1*, *GATA-2*, *MyoD*, *Nkx-2.5*, and *SRY*-were detected by RT-PCR in all mouse and human genes as candidate factors for EA-regulated gene expression. Among these transcription factors, only *MyoD* and *GATA2* showed differential gene expression, in that expression of *MyoD* was significantly suppressed and that of *GATA-2* was upregulated (data not shown). *MyoD* regulates muscle development, differentiation, and repair (12, 57), and it can block muscle proliferation independently of its transcriptional activity (26). In this study, we showed that EA inhibited *MyoD* transcription. This result suggests the possibility that EA induces *MyoD*-related gene expression, which would lead to muscle cell proliferation. Expression of the *GATA-2* gene was significantly induced except at 1 h after EA. *GATA-2* is a transcription factor for mast cell proliferation and development of hematopoietic stem cells (22, 33), and it, together with *NFAT*, also induces muscle growth (19, 49). These short-term EA effects led us to the proposal that EA treatment affected cell proliferation and repair of skeletal muscle.

To verify this hypothesis, we also examined long-term effects of EA by histochemical and immunohistochemical methods. EA-induced muscle cell proliferation after 1 mo of daily EA treatment was revealed by H&E staining (Fig. 4, A, B, E, F) and immunohistochemistry with PCNA antibody (Fig. 4, C, D, G, H). Only nuclei in EA-treated muscle showed reactions with PCNA antibody (Fig. 4, D and H), with nucleoli clearly visible (Fig. 4F). Nuclei in control cells did not react with PCNA antibody (Fig. 4, C and G), nor were nucleoli visible in these nuclei (Fig. 4E). As for other treatment periods, 1 day and 1 wk (Table 3), EA-treated muscles and controls did not react with PCNA antibody (data not shown). PCNA is a 36-kDa protein (3), which is also known as cyclin (59) or polymerase-associated protein (1). It is synthesized in early G₁ and S phases of the cell cycle and serves as an excellent marker for proliferating cells (48). PCNA reactions were observed only in nuclei whose nucleoli were revealed by H&E staining (nuclei in Fig. 4J and nucleoli in Fig. 4I). Figure 4, I and J, also indicates that the cells reacting with PCNA were satellite cells, which supports the EA effect on cell proliferation, thus leading to muscle repair and/or hypertrophy.

In addition, myostatin expression was significantly suppressed in EA-treated muscles (Fig. 5, A and B), which would lead to satellite cell proliferation and muscle regeneration. Indeed, although we found in our study here no suppression of myostatin without an electrical pulse (Fig. 5C), other studies report clinical effectiveness of treatment with EA for Guillain-Barré syndrome and chronic inflammatory demyelinating polyradiculoneuropathy (14, 43). That EA induced myostatin

gene suppression supports the idea that EA is involved in muscle repair, including recovery from damage. Our findings may also support the efficacy of EA for postoperative and myofascial pain. Thus, our results suggest that EA induces a satellite cell-dependent proliferative reaction in muscle.

EA induced the gene encoding VEGF (Table 1, Fig. 3B), and acupuncture alone (after 1 h) also induced mild VEGF gene expression (data not shown). The VEGF family comprises VEGF-A (also referred to as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, svVEGF, and PLGF; these factors can bind VEGF receptors (VEGFRs) including VEGFR-1, VEGFR-2, and VEGFR-3 so that endothelial cells, as well as vascular smooth muscle cells, monocytes, mononuclear phagocytes, polymorphonuclear cells, and others, can transmit several different signals (8, 15, 53). Zhao et al. (60) suggested that VEGF and VEGFR are key molecules for reactions of endothelial cells stimulated with electrical current because a VEGFR inhibitor abolished the endothelial cell reaction to electrical stimulation and because electrical stimulation markedly induced VEGF. VEGFR-2 is the major positive signal transducer that is related to migration, vascular permeability, survival, and proliferation of various cells (8, 53). The association of VEGF, VEGFR2, monocytes, and macrophages has been found especially for skeletal muscle regeneration and growth, which involve satellite cells (9, 47). Thus, certain reports support the possibility that VEGF and VEGFR signaling pathways may function in EA-dependent satellite cell proliferation. Our data suggest that VEGF may be a key factor in EA-stimulated muscle as well. We present our results here, that VEGF is induced by both EA and acupuncture alone, as novel findings.

VEGF is also an angiogenic cytokine that shows potential use in treatment of tissue ischemia (6). Indeed, a previous study reported that EA increased the survival rate of musculocutaneous flaps (42). In these tissue flaps, EA stimulation may activate the VEGFR signaling pathway for cell survival. However, EA stimulation may not induce angiogenesis because even gene therapy, with a VEGF gene transfer dose, for peripheral arterial disease did not produce marked vascularization (31).

Expression of the myostatin gene was different in EA-treated muscle and acupuncture-treated muscle (Fig. 5, B and C), which suggests that EA and acupuncture may not have the same effect on muscle regeneration.

Our preliminary experiment based on a forced swimming study (18) suggested that mice given 4 wk of daily EA treatment to the extremities could swim for long periods on average, but no significant difference compared with mice not treated with EA was found (data not shown). Further investigation of the physiological change in muscles after longer-term (e.g., 3 or 6 mo) daily EA is needed.

Our previous reports indicated that EA and moxibustion induce a stress protein [the heat shock protein (HSP)] in EA-treated muscle (29, 30). In our research, EA treatment also induced expression of the gene for HSF2 (data not shown), a transcription factor for the stress response (39), which was one of the common transcription factors derived from detected mouse genes (Table 2). These data suggest that EA-treated muscle reacts to stress caused by EA stimulation.

Certain research groups have used the DNA microarray approach for investigations of EA effects. Kim et al. (25)

reported that 30 min of EA applied every 16 h for 2 days induced a change in expression of NK cell activity-related genes in the spleen. Ko et al. (28) showed that gene expression of MAPK II, Fas-AP, and LIM increased in spinal cord after daily application of EA for 30 min for 3 wk. Both reports analyzed tissues collected only immediately after the final EA treatment. However, for our research, we collected samples for analysis of effects of both short-term EA (0 h, 1 h, 3 h, and 24 h after EA) and long-term EA (applied daily for 1 day, 1 wk, and 1 mo). Our sampling method should therefore allow a more systematic, large-scale screening of gene expression (e.g., via microarray expression profiling analysis or GeneChip technology), which would reveal the more diverse effects of acupuncture; such studies are now under way. Microarray analysis may yield additional new findings, because Mandaokar et al. (36) reported different transcript profiling results for microarray analysis and DD analysis even when they used the same samples.

In conclusion, in this study we show, by means of mRNA fingerprinting and bioinformatics approaches (including our SHAFT program), that EA has an effect on cell proliferation that leads to muscle repair. Bioinformatics approaches are important because they can dissect information from the gene expression profile. Additional molecular investigations are needed to elucidate the correlation between electrical stimulation (including EA) and cell reactions in genome-controlled functions and to provide molecular evidence for clinical results observed with acupuncture treatment.

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