Lactoferrin promotes murine C2C12 myoblast proliferation and differentiation and myotube hypertrophy

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Abstract. Lactoferrin (Lf) is a multifunctional glycoprotein, which promotes the proliferation of murine C2C12 myoblasts. In the present study, it was investigated how Lf promotes myoblast proliferation and whether Lf promotes myoblast differentiation or induces myotube hypertrophy. Lf promoted the proliferation of myoblasts in a dose-dependent manner. Myoblast proliferation increased on day 3 when myoblasts were cultured in the presence of Lf for three days and also when myoblasts were cultured in the presence of Lf for the first day and in the absence of Lf for the subsequent two days. In addition, Lf induced the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 in myoblasts. The mitogen-activated protein kinase kinase 1/2 inhibitor U0126 inhibited Lf-induced ERK1/2 phosphorylation and repressed Lf-promoted myoblast proliferation. C2C12 myoblasts, myotubes and skeletal muscle expressed low-density lipoprotein receptor-related protein (LRP)1 mRNA and Lf-promoted myoblast proliferation was attenuated by an LRP1 antagonist or LRP1 gene silencing. The knockdown of LRP1 repressed Lf-induced phosphorylation of ERK1/2. Furthermore, when myoblasts were induced to differentiate, Lf increased the expression of the myotube-specific structural protein, myosin heavy chain (MyHC) and promoted myotube formation. Knockdown of LRP1 repressed Lf-induced MyHC expression. Lf also increased myotube size following differentiation. These results indicate that Lf promotes myoblast proliferation and differentiation, at least partially through LRP1 and also stimulates myotube hypertrophy.

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Introduction

Skeletal muscle, the most abundant tissue in the body, contributes not only to mobility and movement but also to glucose and lipid metabolism. The loss of skeletal muscle mass causes decreased locomotion and decreased energy expenditure, resulting in a higher risk of metabolic diseases such as obesity and type 2 diabetes (1,2). When skeletal muscle is damaged by intense resistance training or traumatic injury, regeneration of muscle cells occurs by highly orchestrated processes (3). The myogenic precursor satellite cells proliferate and then differentiate into myoblasts. Subsequently, the mononucleated myoblasts proliferate and differentiate, and then fuse with each other and pre-existing myofibers, resulting in the formation of multinucleated myotubes and myofibers. Likewise, the proliferation and differentiation of muscle cells occurs during developmental and postnatal myogenesis (4). The activation of myogenic regulatory factors such as MyoD and myogenin regulates the expression of myosin heavy chain (MyHC), which is a myotube-specific structural protein (5). On the other hand, an increase in the size of individual myotubes and myofibers, called hypertrophy, causes an increase in skeletal muscle mass (4). Therefore, promotion of myoblast proliferation and differentiation and an induction of myotube hypertrophy should be beneficial for muscle regeneration and muscle mass regulation.

Lactoferrin (Lf) is a multifunctional non-heme iron-binding glycoprotein, which is present in exocrine fluids such as saliva, tears, and bile (6). Neutrophils release Lf into circulation during inflammation (7), and it exerts antioxidant (8), anti-infective (9), anti-inflammatory (10), and anti-cancer effects (11,12). Serum Lf levels are in the range of 2-7 and 1-60 μg/ml in healthy subjects and patients with rheumatoid arthritis, respectively (7). Lf levels in synovial fluid are in the range of 1-100 μg/ml in osteoarthritic patients, suggesting that local Lf levels are higher during inflammation. Furthermore, the serum Lf level increases immediately after running exercise (13). Several receptors for Lf have been identified on the surface of various cells, and Lf binds to intelectin (14),

low-density lipoprotein receptor-related protein (LRP)1 (15), nucleolin (16), and Toll-like receptor (TLR)4 (17).

Lf is known to promote the proliferation of murine C2C12 myoblasts (18), but its underlying mechanism remains unclear. Furthermore, Lf induces the conversion of murine C2C12 myoblasts into cells that proceed along the osteoblastic and chondroblastic lineages (18). Given that chondrogenic, osteogenic, adipogenic, and myogenic lineages can originate from common progenitor cells (19), it is of interest whether Lf has any effect on myoblast differentiation. Here, we provide evidence that Lf promotes myoblast proliferation by activating the extracellular signal-regulated kinase (ERK)1/2 signaling pathway, at least partially through LRP1, and induces the differentiation of myoblasts into myotubes. Moreover, we found that Lf promotes myotube hypertrophy.

Materials and methods

Animals. All animals were cared for in accordance with the guidelines of the Animal Care and Use Committee of Osaka Prefecture University, which also provided ethical approval for the present study (approval no. 28-185). Male Kwl:ddY mice were obtained from Kiwa Laboratory Animals (Wakayama, Japan) and had free access to water and a rodent diet. The mice were housed under controlled temperature (23±2˚C), humidity (60±10%), and light (a 12 h light-dark cycle starting at 08:00 a.m.) conditions.

Cell culture. Murine myoblast C2C12 cells were obtained from RIKEN Cell Bank (Tsukuba, Japan) and were maintained as described previously (20). In brief, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (growth medium) at 37°C in 5% CO_2 and 95% air at 100% humidity. For proliferation assays, cells were cultured in DMEM supplemented with 2% FBS and the antibiotics mentioned above; this medium was termed the proliferation medium and allowed the myoblasts to proliferate but not differentiate, in the present study. For the induction of differentiation, confluent cells were cultured in DMEM supplemented with 2% horse serum and the antibiotics mentioned above (differentiation medium).

alamarBlue cell viability assay. Cell viability was determined using the alamarBlue cell viability reagent (Trek Diagnostic Systems, Cleveland, OH, USA) (21). For growth curve experiments, myoblasts were seeded onto 48-well plates at a density of $0.5x10^3$ cells/cm^2 and cultured for six days. For the determination of cell viability, myoblasts were seeded onto 48-well plates at a density of $2.0x10^3$ cells/cm^2 and cultured for three days. After one day, this time point was denoted as day 0. Cells were cultured in fresh proliferation medium supplemented with Lf (iron saturated; approx. 20%) (Morinaga Milk Industry Co., Ltd., Tokyo, Japan) in the presence or absence of the mitogen-activated protein kinase kinase (MEK)1/2 inhibitor U0126 (2.5 μM) or the LRP1 inhibitor receptor-associated protein (RAP, 50 nM) for three days. Before determination, cells were incubated in phenol red-free proliferation medium supplemented with Lf for 1 h, followed by incubation in the above medium containing 5% alamarBlue reagent for 3 h. The fluorescence intensity of the medium was determined by FluoroSkan Ascent FL (Labsystems, Helsinki, Finland) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Data are expressed as relative values (fluorescence intensity of the experimental group divided by fluorescence intensity of the vehicle group at day 0 or at the same time point).

Western blot analysis. For the determination of ERK1/2 signaling, myoblasts were cultured in serum-free DMEM for one day and incubated with Lf for the indicated time periods. For the determination of MyHC expression, myoblasts were cultured in the differentiation medium. Cells were sonicated in cell lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 20,000 x g for 15 min. The supernatants were subjected to SDS-polyacrylamide gel electrophoresis, followed by western blot analysis with the following primary antibodies: rabbit polyclonal anti-p-ERK1/2 (Thr202/Thr204; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-ERK1/2 (Cell Signaling Technology, Inc.), rabbit monoclonal anti-LRP1 (Abcam, Cambridge, UK), mouse monoclonal anti-MyHC (clone MF20; Developmental Studies Hybridoma Bank, University of Iowa, Iowa city, IA, USA), and anti-β-actin (Abgent, San Diego, CA, USA). The primary antibodies were detected using the suitable horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse or goat anti-rabbit) and the Immobilon Western Chemiluminescent HRP substrate (EMD Millipore, Billerica, MA, USA), and exposed to a luminescent image analyzer (LAS-4000 IR multicolor; Fujifilm Life Sciences, Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from C2C12 myoblasts and myotubes, murine fresh skeletal muscle (gastrocnemius, soleus, and extensor digitorum longus), and small intestinal mucosa, and cDNA was synthesized. The resulting cDNA was amplified by PCR using the following specific primers: Primers for *Itln1* (forward 5'-TGACAATGGCCCAGCATTACC-3' and reverse 5'-TGACAATGGCCCAGCATTACC-3'); for *Lrp1* (forward 5'-ACTATGGATGCCCCTAAAACTTG-3' and reverse 5'-GCAATCTCTTTCACCGTCACA-3'); for *nucleolin* (forward 5'-GCACTTGGAGTGGTGAATCAAA-3' and reverse 5'-AAATGCATACCCTTTAGGTTTGCC-3'); for *Tlr4* (forward 5'-GCAGAAAATGCCAGGATGATG-3' and reverse 5'-AACTACCTCTATGCAGGGATTCAAG-3'); for *Myod* (forward 5'-TGGGATATGGAGCTTCTATCGC-3' and reverse 5'-GGTGAGTCGAAACACGGATCAT-3'); for *myogenin* (forward 5'-CATCCAGTACATTGAGCGCCTA-3' and reverse 5'-GAGCAAATGATCTCCTGGGTTG-3'); and for *Actb* (forward 5'-GTGGGCCGCCCTAGGCACCA-3' and reverse 5'-CTCTTTGATGTCACGCACGATTTC-3').

siRNA-mediated knockdown. The siRNA duplexes targeting murine LRP1 (siLRP1) and control siRNA (MISSION siRNA Universal Negative Control) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The siLRP1 sequence

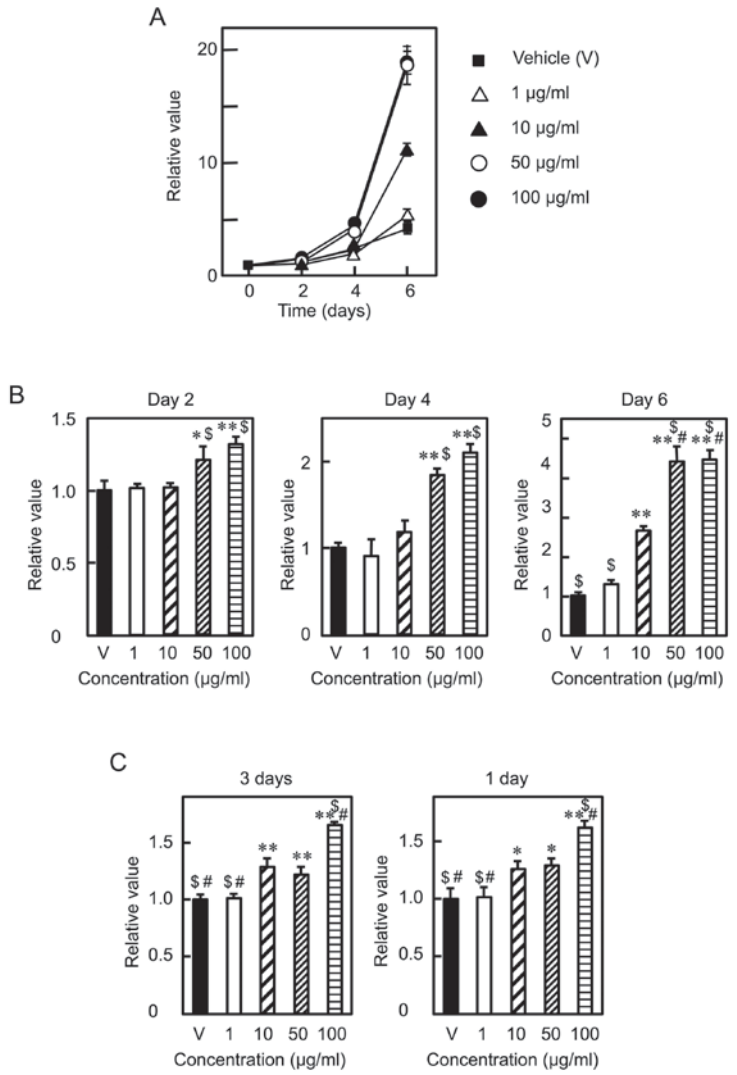

Figure 1. Effects of Lf on the proliferation of C2C12 myoblasts. (A) After attachment, myoblasts were cultured in the presence of the vehicle (V) or Lf (1, 10, 50, and 100 μg/ml) for the indicated times. Cell viability was determined using the alamarBlue fluorescent dye. Data are expressed as relative values (fluorescence intensity (FI) of the experimental group divided by FI of the vehicle group at day 0). Values are indicated as the mean ± SD (n=3). (B) Data are expressed as relative values (FI of the experimental group divided by FI of the vehicle group at the same time point). (C) (left panel) After attachment, myoblasts were cultured with Lf at various concentrations for three days. (right panel) After attachment, myoblasts were cultured in the presence of Lf for one day and in the absence of Lf for the next two days. Data are expressed as relative values (FI of the experimental group divided by FI of the vehicle group). Values are indicated as the mean ± SD (n=4). Statistically significant differences were determined by one-way ANOVA and Tukey's post-hoc test. *P<0.01, **P<0.001 vs. the V group. $^{\$}$P<0.001 vs. Lf (10 μg/ml). $^{\#}$P<0.001 vs. Lf (50 μg/ml). Each result is representative of three or more independent experiments. Lf, Lactoferrin; FI, fluorescence intensity; ANOVA, analysis of variance; SD, standard deviation; V, vehicle.

was as follows: 5'-CCAUGUUUGUGACCCGAAUdTdT-3'. The siRNA duplexes (20 nM) were transfected into myoblasts using Lipofectamine RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 6 h, according to the manufacturer's transfection protocol.

Immunofluorescence microscopy. Myotubes were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), as described previously (22). Fixed cells were permeabilized with 0.1% Triton X-100 in PBS, blocked with blocking solution (10% FBS and 5% bovine serum albumin in PBS), and incubated with mouse monoclonal anti-MyHC antibodies. This was followed by further incubation with Alexa 488-conjugated anti-mouse IgG. The nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS for 10 min. Fluorescent images were analyzed by a BIOREVO BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). The fusion index was calculated as follows: The number of nuclei within MyHC-positive myotubes containing two or more nuclei was divided by the total number of nuclei in five random fields, and the resulting number was termed the fusion ratio. The fusion ratio in Lf-treated cells was normalized to the fusion ratio in vehicle-treated cells, resulting in the fusion index. The diameters of the short axis of myotubes were measured in five random fields. The mean myotube diameter was determined from more than 20 myotubes for each sample.

Statistical analysis. Data were analyzed by one-way or two-way ANOVA followed by Tukey's post-hoc test for multiple comparison analysis. Statistical analysis was performed using JMP statistical software, version 8.0.1 (SAS Institute, Cary, NC, USA). Data are expressed as the mean ± standard

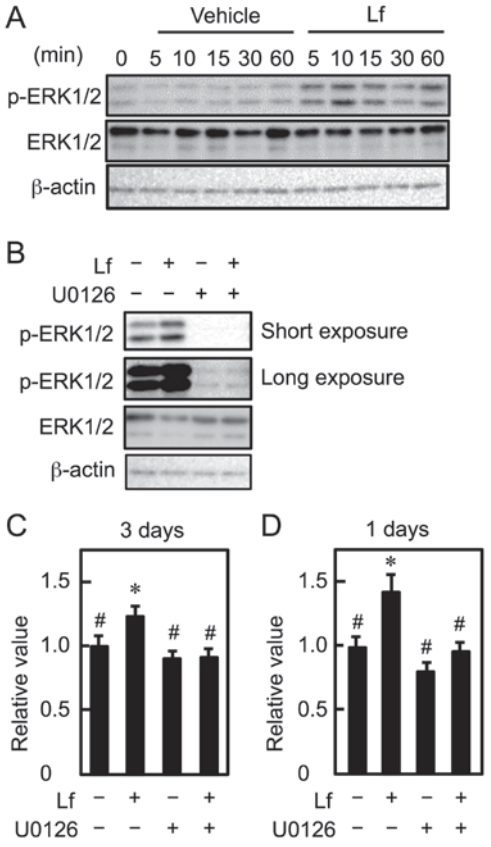

Figure 2. Involvement of ERK1/2 in the Lf-induced proliferation of myoblasts. (A) Myoblasts were incubated with Lf (100 μg/ml) for the indicated times. The expression of ERK1/2 and phosphorylated ERK1/2 (p-ERK1/2) was analyzed by western blots with anti-ERK1/2 and anti-p-ERK1/2 antibodies. The expression of β-actin was analyzed as a loading control. (B) Myoblasts were incubated with Lf in the presence of U0126 for 10 min. The expression of ERK1/2 and p-ERK1/2 was analyzed by western blots. The expression of β-actin was analyzed as a loading control. The data for p-ERK1/2 expression level were obtained as digitized images for short (upper panel) or long (middle panel) exposure times. (C) Myoblasts were cultured with Lf (10 μg/ml) in the presence of U0126 for three days. (D) Myoblasts were cultured with U0126 in the presence of Lf (10 μg/ml) for the first day and in the absence of Lf for the next two days. (C and D) Cell viability was determined by the alamarBlue assay. Data are expressed as relative values (FI of the experimental group divided by FI of the vehicle group (-Lf, -U0126). Values are indicated as the mean ± standard deviation (n=4). Statistically significant differences were determined by one-way ANOVA and Tukey's post-hoc test. *P<0.001 vs. vehicle group (-Lf, -U0126). $^{\#}$P<0.001 vs. Lf group (+Lf, -U0126). Each result is representative of three independent experiments (C) or two independent experiments (D). ERK, extracellular signal-regulated kinase; FI, fluorescence intensity; Lf, lactoferrin; p-, phosphorylated.

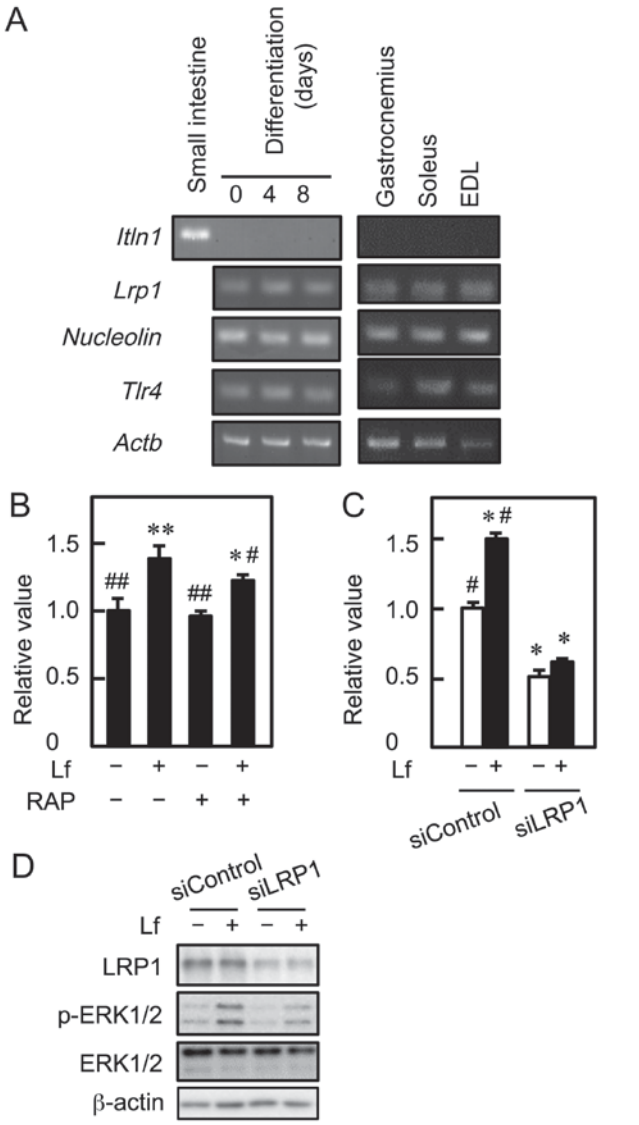

Figure 3. Involvement of LRP1 in the Lf-promoted proliferation of myoblasts. (A) cDNA was synthesized using total RNA from C2C12 cells, skeletal muscle tissue (gastrocnemius, soleus, and extensor digitorum longus, and small intestine, and was amplified by PCR. (B) Myoblasts were cultured with Lf (25 μg/ml) in the presence of RAP for three days. Cell viability was determined by the alamarBlue assay. Data are expressed as relative values (FI of the experimental group divided by FI of the vehicle group (-Lf, -RAP). Values are indicated as the mean ± SD (n=4). Statistically significant differences were determined by one-way ANOVA and Tukey's post-hoc test. *P<0.01, **P<0.001 vs. vehicle group (-Lf, -U0126). $^{\#}$P<0.05, $^{\#\#}$P<0.001 vs. Lf group (+Lf, -RAP). (C) Myoblasts were transfected with control siRNA (siControl) or LRP1 siRNA (siLRP1), followed by culture with Lf for three days. Cell viability was determined by the alamarBlue assay. Data are expressed as relative values (FI of the experimental group divided by FI of the vehicle group (-Lf, siControl)). Values are indicated as the mean ± SD (n=4). Statistically significant differences were determined by two-way ANOVA and Tukey's post-hoc test. *P<0.001 vs. siControl group (-Lf, siControl). $^{\#}$P<0.001 vs. siLRP1 group (-Lf, siLRP1). (D) LRP1 knockdown cells were incubated with Lf for 10 min. The expression of LRP1, ERK1/2, and phosphorylated ERK1/2 (p-ERK1/2) was analyzed by western blotting. Each result is representative of three independent experiments. EDL, extensor digitorum longus; ERK, extracellular signal-regulated kinase; Itln1, intelectin 1; Lf, lactoferrin; Lrp1, low-density lipoprotein receptor-related protein 1; RAP, receptor-associated protein; siRNA, small interfering RNA; Tlr4, Toll-like receptor 4; SD, standard deviation; ANOVA, analysis of variance.

deviation (SD), and P<0.05 was considered to indicate a statistically significant difference.

Results

Involvement of ERK1/2 signaling in Lf-promoted proliferation of C2C12 cells. C2C12 myoblasts were cultured in the presence of Lf at various concentrations (0, 1, 10, 50, and 100 μg/ml) for six days. Lf promoted cell proliferation in a dose- and time-dependent manner (Fig. 1A), consistent with previous results (18). When cell proliferation was determined at the same time points, Lf increased cell proliferation at day 2 and 4 at concentrations of 50 μg/ml or higher and at day 6 at concentrations of 10 μg/ml or higher (Fig. 1B). Lf stimulated cell proliferation at concentrations of 10 μg/ml or higher when cells were cultured in the presence of Lf at various

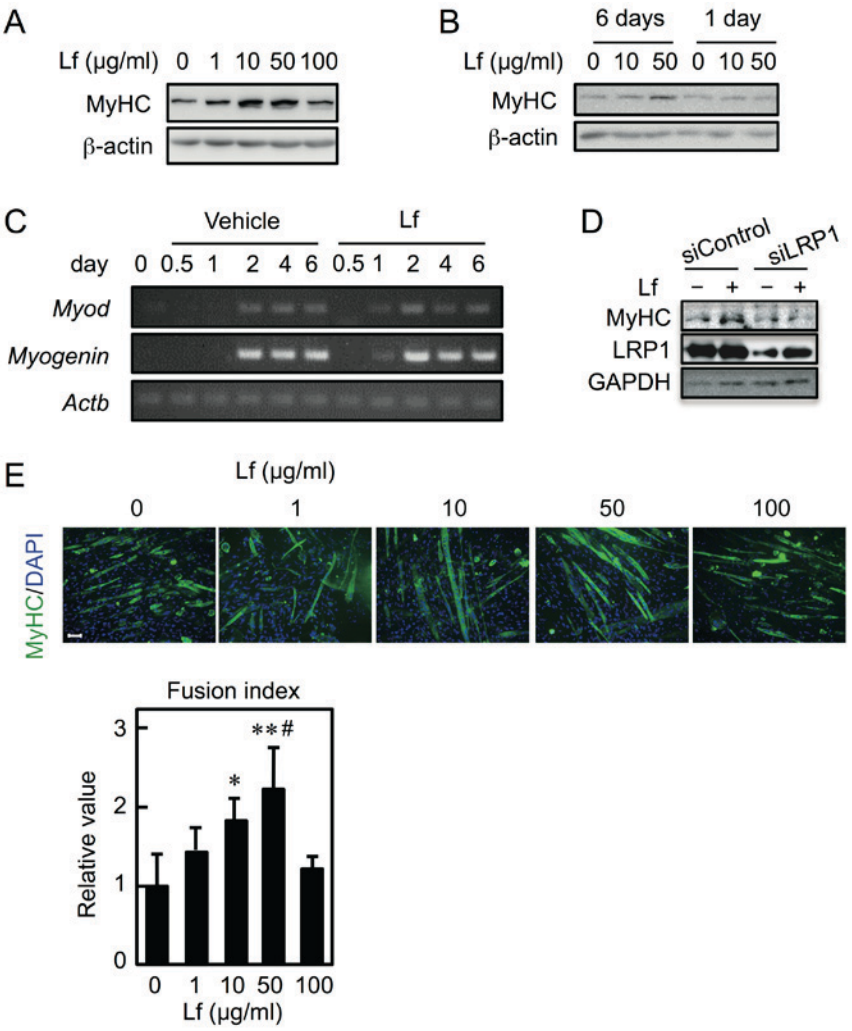

Figure 4. Effect of Lf on myoblast differentiation. (A) Myoblasts were cultured in differentiation medium containing Lf at various concentrations for six days. (B) Myoblasts were cultured in differentiation medium in the presence of Lf for six days (6 days) or in differentiation medium in the presence of Lf for one day and in the absence of Lf for the next 5 days (1 day). (A and B) The expression of MyHC and β-actin was analyzed by western blots. (C) Myoblasts were cultured in differentiation medium containing Lf (50 μg/ml). cDNA was synthesized and genes were amplified by polymerase chain reaction. (D) Myoblasts were transfected with control siRNA (siControl) or LRP1 siRNA (siLRP1) and were differentiated in the presence of Lf (50 μg/ml) for three days. The expression of MyHC, LRP1, and GAPDH was analyzed by western blotting. (E) (Upper panel) Fixed cells were reacted with anti-MyHC antibody and a fluorescence-labeled secondary antibody (green). The nuclei were stained with DAPI (blue). (Lower panel) The fusion index was calculated. Statistically significant differences were determined by one-way ANOVA and Tukey's post-hoc test. *P<0.05, **P<0.001 vs. Lf (0 μg/ml) group. $^{\#}$P<0.05 vs. Lf (1 μg/ml) group. Each result is representative of three (A, B, C, and E) or two independent experiments (D). Scale bar, 100 μM for all images. Lf, lactoferrin; LRP1, low-density lipoprotein receptor-related protein 1; MyHC, myosin heavy chain; siRNA, small interfering RNA.

concentrations for three days (Fig. 1C, left panel). Likewise, Lf enhanced cell proliferation at concentrations of 10 μg/ml or higher when cells were cultured in the presence of Lf for the first day and in the absence of Lf for the next two days (Fig. 1C, right panel). These results indicate that Lf promotes myoblast proliferation and that Lf stimulation only in the early period (i.e., the first day) is sufficient to promote cell proliferation. Next, we assessed what signaling is required for Lf-promoted myoblast proliferation. Activation of MEK1/2 causes the phosphorylation of 44 kDa ERK1 and 42 kDa ERK2, which are homologous isoforms. Lf increased ERK1/2 phosphorylation within 5 min in C2C12 myoblasts (Fig. 2A). Furthermore, to determine whether the ERK1/2 signaling pathway is involved in Lf-stimulated myoblast proliferation, myoblasts were cultured with Lf in the presence of U0126 (2.5 μM). Lf-stimulated ERK1/2 activation was inhibited by U0126, and U0126 decreased basal phosphorylation levels of ERK1/2 (Fig. 2B). Next, we determined the effects of U0126 on myoblast proliferation when cells were cultured in the presence of Lf for three days. The proliferation-promoting effect of Lf on myoblasts was abolished following U0126 treatment, regardless of the length of the incubation time with Lf (Fig. 2C and D). These results suggest that Lf increases myoblast proliferation by activating the MEK1/2-ERK1/2 signaling pathway.

LRP1 is involved in Lf-stimulated proliferation of C2C12 myoblasts. We next determined the receptors through which Lf promotes proliferation and activates ERK1/2 signaling in myoblasts. To assess whether mRNA for Lf receptors is expressed in C2C12 cells and murine skeletal muscle, RT-PCR was performed. Myoblasts expressed *Lrp1*, *Nucleolin*, and *Tlr4* mRNA before and after differentiation (Fig. 3A). These mRNA were also detected in skeletal muscle tissue

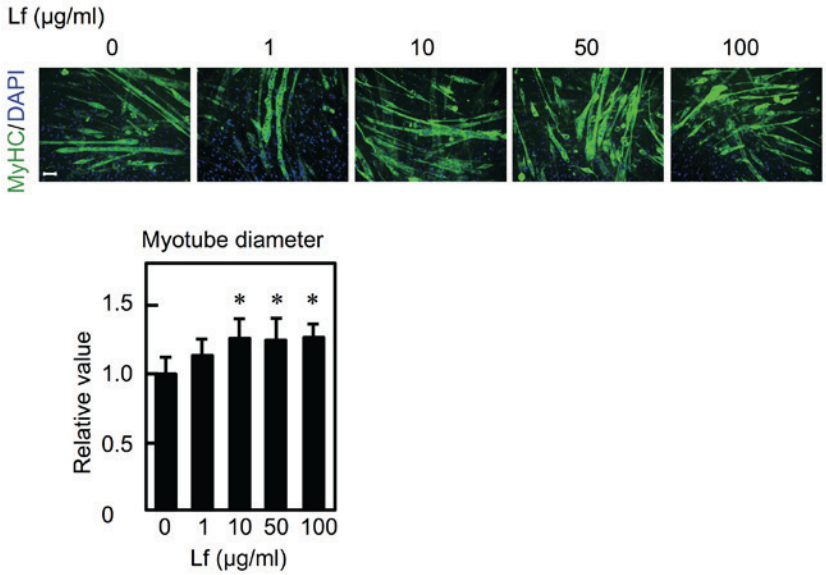

Figure 5. Effect of Lf on myotube hypertrophy. (Upper panel) Fixed cells were visualized with anti-MyHC antibody and a fluorescence-labeled secondary antibody (green). The nuclei were stained with DAPI (blue). (Lower panel) The diameter of the short axis of myotubes was measured. Scale bar, 100 μM for all images. Each result is representative of three independent experiments. Statistically significant differences were determined by one-way ANOVA and Tukey's post-hoc test. *P<0.05 vs. Lf (0 μg/ml) group. Lf, lactoferrin; MyHC, myosin heavy chain.

of the gastrocnemius, soleus, and extensor digitorum longus. However, *Itln1* mRNA was not detected in C2C12 cells and skeletal muscle, although it was detected in the small intestine. To determine whether LRP1 was involved in Lf-stimulated myoblast proliferation, C2C12 cells were cultured with Lf in the presence of RAP, an LRP1 antagonist, and cell viability was determined. RAP attenuated Lf-induced myoblast proliferation (Fig. 3B). Furthermore, C2C12 cells were transfected with LRP1 siRNA, followed by culture with Lf. Knockdown of LRP1 inhibited Lf-induced myoblast proliferation and decreased the basal growth of myoblasts (Fig. 3C). Depletion of LRP1 by approximately 70% did not completely inhibit, but attenuated Lf-activated phosphorylation of ERK1/2 (Fig. 3D). These results indicate that LRP1 is involved in Lf-induced myoblast proliferation and Lf-stimulated ERK1/2 activation.

Lf induces differentiation of myoblasts into myotubes. To determine the effect of Lf on myoblast differentiation, C2C12 cells were cultured in differentiation medium in the presence of Lf for six days. Expression of MyHC increased at concentrations ranging from 10-50 μg/ml but not at 100 μg/ml (Fig. 4A). Furthermore, to determine whether Lf-mediated stimulation only occurs in the early period (i.e., the first day) and is sufficient to promote cell differentiation, myoblasts were differentiated into myotubes in the presence of Lf for the first day and in the absence of Lf for the next five days. Lf had no influence on myoblast differentiation (Fig. 4B). The mRNA expression of *MyoD* and *myogenin* was detected earlier when cells were differentiated in the presence of Lf for six days (Fig. 4C). Furthermore, to determine whether LRP1 is involved in Lf-induced myoblast differentiation, C2C12 cells were transfected with LRP1 siRNA and were allowed to differentiate in the presence of Lf for three days. Knockdown of LRP1 repressed the Lf-induced increase in MyHC expression (Fig. 4D). Next, to determine the fusion index, myoblasts were cultured in differentiation medium in the presence of Lf for six days. The myotubes were reacted with an anti-MyHC antibody and probed using a fluorescence-tagged secondary antibody; their nuclei were then counted (Fig. 4E, upper panel). The fusion index showed that Lf promoted the fusion of myoblasts into myotubes at 10 and 50 μg/ml but not at 100 μg/ml (Fig. 4E, lower panel). Furthermore, the effect of Lf on the diameter of the short axis of myotubes was determined. After myoblasts were differentiated into myotubes for six days, myotubes were cultured in the presence of Lf for four days. The myotubes were labeled with an anti-MyHC antibody and subjected to immunofluorescent analysis, and the nuclei were stained (Fig. 5, upper panel). Lf increased the diameter of the short axis of myotubes at a concentration of 10 μg/ml or higher (Fig. 5, lower panel). These results indicate that Lf stimulates myoblast differentiation at concentrations of 10 and 50 μg/ml, perhaps through LRP1, and increases myotube size at concentrations of 10 μg/ml or higher.

Discussion

Lf promotes the proliferation of C2C12 myoblasts and induces osteoblastic and chondroblastic differentiation of C2C12 myoblasts (18). The present study demonstrates the mechanism by which Lf promotes myoblast proliferation and provides information about the roles of Lf in myoblast differentiation and myotube hypertrophy.

Lf promoted the proliferation of C2C12 myoblasts, which is consistent with a previous report on the biological effect of Lf on C2C12 myoblast proliferation (18). In this study, we found that Lf led to increased cell proliferation when C2C12 myoblasts were exposed to Lf for only the first day. Thus, stimulation by Lf only in the early period was sufficient to promote myoblast proliferation. When skeletal muscle is injured, myoblasts proliferate and then differentiate to repair and regenerate the injured muscle. Skeletal muscle regeneration is regulated by the interplay between skeletal muscle stem

cells (satellite cells or myoblasts) and the immune system (23). With respect to the latter, injury to skeletal muscle induces an inflammatory response, resulting in infiltration of inflammatory cells into the local sites of the injured muscle (24). Neutrophils are the first inflammatory cells that infiltrate the injured muscle within 2 h of muscle damage, and their levels peak 1-3 days post-injury before returning to basal levels (25). The first-day stimulation by Lf to promote cell growth is consistent with the timing of neutrophil infiltration into the injured area during regeneration. These results indicate that Lf plays a critical role in myoblast proliferation and suggest that Lf may function as a critical player in muscle regeneration.

The proliferation of muscle cells is regulated by several regulatory signaling pathways, including the ERK1/2 signaling pathway (26). Lf induced the phosphorylation of ERK1/2 in myoblasts, and U0126 treatment inhibited Lf-stimulated cell proliferation. U0126 is a selective inhibitor of MEK1 and MEK2, which inhibit ERK1/2 activation. Activation of ERK1/2 is required for myoblast proliferation (27). Yagi *et al* (18) reported that C2C12 cells express LRP1, but the role of LRP1 in C2C12 cells remains unclear. The present study demonstrated that the administration of RAP, an LRP1 antagonist, and depletion of LRP1 attenuate Lf-stimulated cell growth and ERK1/2 phosphorylation. Furthermore, knockdown of LRP1 repressed Lf-induced MyHC expression. Given that mesenchymal stem cells are capable of differentiating into multiple lineages such as chondrogenic, osteogenic, adipogenic, and myogenic lineages (19), their lineages may have common Lf receptors and signal transduction pathways. For example, Lf promotes the proliferation and osteogenic differentiation of human adipose-derived stem cells (28), although the underlying mechanisms remain unclear. Grey *et al* (29) reported that RAP and U0126 inhibited Lf-induced mitogenesis in osteoblasts and suggested that Lf promoted osteoblastic cell growth by activating the ERK1/2 signaling pathway through LRP1. In human chondrocytes, Lf promotes proliferation and activates ERK1/2, although it remains unclear whether ERK1/2 has any influence on cell proliferation (30). In contrast, Lf promotes the differentiation of osteoblasts independently of LRP1, although ERK1/2 is activated through LRP1 (31). Although there are still contradictory results with regard to the mechanism by which Lf promotes proliferation of mesenchymal stem cell-derived lineages, our results indicate that Lf promotes myoblast proliferation by activating the ERK1/2 signaling pathway, at least partially through LRP1, and that LRP1 is involved in Lf-promoted myoblast differentiation.

LRP1 is a member of the low-density lipoprotein receptor family and exerts two different biological functions: i) it acts as a scavenger receptor that contributes to the endocytosis of various ligands (at least 40) and ii) it acts as a signaling receptor that regulates different cellular processes (32). The conventional LRP1 knockout in mice is lethal, indicating the indispensability of LRP1 in cellular physiology (33). Taken together with the fact that the knockdown of LRP1 decreased the basal growth of myoblasts (Fig. 3C), LRP1 may function as a receptor for certain ligands that promote the proliferation of myoblasts in the absence of Lf.

U0126 completely inhibited Lf-stimulated ERK1/2 activation and cell proliferation. However, knockdown of LRP1 by approximately 70% did not result in complete inhibition of Lf-stimulated ERK1/2 activation. Lf promotes proliferation by activating ERK1/2 signaling through intelectin 1 in intestinal epithelial cells (34), but no intelectin 1 was detected in C2C12 myoblasts or skeletal muscle. On the other hand, knockdown of nucleolin decreased the expression levels of phosphorylated ERK1/2 in hepatocellular carcinoma (35) and repressed epidermal growth factor- or stromal cell-derived factor 1-induced ERK1/2 activation in esophageal squamous cell carcinoma (36). Furthermore, knockdown of TLR4 inhibits the 60-kDa heat shock chaperonin protein-induced ERK1/2 activation in A7r5 vascular smooth muscle cells (37). Future studies will look to determine if Lf is able to stimulate cell growth via the ERK1/2 signaling cascade through nucleolin and/or TLR4 in myoblasts.

Lf increased the expression of MyHC and the fusion index. These results indicate that Lf induces myoblast differentiation and myotube formation. When C2C12 myoblasts are exposed to differentiation medium (DMEM supplemented with 2% horse serum), they withdraw from the cell cycle and differentiate into myotubes. In the present study, C2C12 myoblasts were induced to differentiate by culturing in the differentiation medium, and Lf promoted the expression of *Myod* and *myogenin*. In contrast, Yagi *et al* (18) suggested that Lf repressed C2C12 myoblast differentiation, because MyoD expression was suppressed when myoblasts were cultured in the low-mitogen differentiation medium (DMEM supplemented with 5% FBS) in the presence of Lf. However, in this study, myoblasts proliferated when cultured in DMEM supplemented with 2% FBS, but did not differentiate into myotubes. At this time, we have no suitable explanation for the discrepancy in the effect of Lf on C2C12 myoblast differentiation. The effect of Lf on the differentiation of skeletal muscle stem cells (satellite cells) may resolve this discrepancy.

Lf promoted the differentiation of myoblasts at concentrations of 10 and 50 μg/ml but not at a concentration of 100 μg/ml. Thus, the promotion of myoblast differentiation by Lf was exerted in a limited dose range rather than in a dose-dependent manner, which is contrary to the observation that Lf promotes myoblast proliferation in a dose-dependent manner. In contrast, in other cells derived from mesenchymal stem cells, Lf stimulates osteogenic differentiation at 100 μg/ml (28) and represses adipogenic differentiation at concentrations higher than 10 μg/ml (38). Given these seemingly contradictory results, it is possible that myoblasts express at least two types of Lf receptors that regulate differentiation. For instance, one receptor might promote myogenic differentiation and the other might repress myogenic differentiation; however, further research is needed to determine if this is indeed the case.

Increased skeletal muscle mass is due to the expanded cross-sectional area of individual myofibers. In this study, Lf increased myotube size at concentrations higher than 10 μg/ml. Thus, Lf has the potential to function in both myoblasts and myotubes, which express common Lf receptors such as LRP1, nucleolin, and TLR4. We are now attempting to study how Lf acts on myoblast differentiation and myotube hypertrophy.

Lf has two iron-binding sites and the conformation of Lf varies depending on whether it is iron-free (apo-Lf) or iron-saturated (holo-Lf) (39). Lf. Apo- and holo-Lf exhibit different physiological functions. For example, apo-Lf represses the proliferation of human intestinal epithelial

Caco-2 cells, whereas holo-Lf enhances it (40). In contrast, apo-Lf promotes osteoblast proliferation to the same degree as holo-Lf (41). In addition, holo-Lf, but not apo-Lf, enhances tropoelastin expression through LRP1 in human dermal fibroblasts (42). The Lf used in the present study was approximately 20% iron-saturated bovine Lf. Therefore, it is of interest to determine which Lf promotes myoblast proliferation and differentiation and myotube hypertrophy.

In conclusion, this study demonstrates that Lf stimulation for one day promotes myoblast proliferation and that Lf seems to stimulate cell proliferation by activating ERK1/2 signaling pathway, at least partially through LRP1. Furthermore, we found that Lf induced myoblast differentiation and myotube hypertrophy. This study reveals that Lf may affect skeletal muscle repair and regeneration, as well as developmental and postnatal myogenesis.

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