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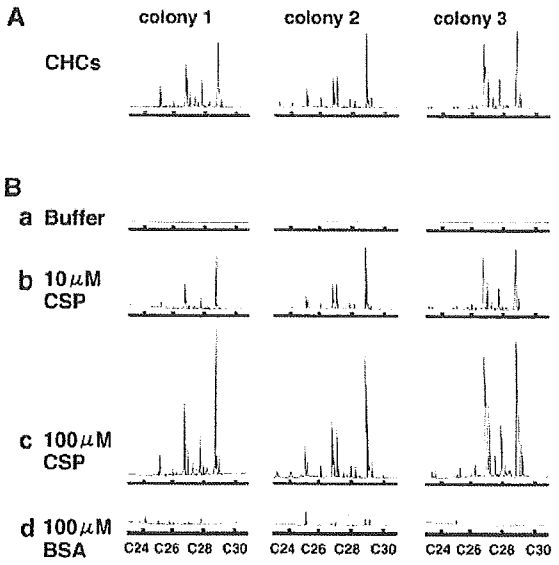

Fig. 4. CSP dissolves CHCs. (A) Gas chromatograms for the original CHC profiles from colonies 1 to 3. (B) (a, b, and c) CHC profiles dissolved in the buffer with 0, 10, and 100 μM CjapCSP, respectively; (d) CHC profiles dissolved in the buffer with 100 μM BSA as control. n = 10 for each assay using colony-specific CHC blend; the chromatograms are an average drawing of them.

sensory information from nestmate CHCs through other types of sensilla.

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Materials and Methods
Figs. S1 to S3
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Bone Marrow Stromal Cells Generate Muscle Cells and Repair Muscle Degeneration

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Bone marrow stromal cells (MSCs) have great potential as therapeutic agents. We report a method for inducing skeletal muscle lineage cells from human and rat general adherent MSCs with an efficiency of 89%. Induced cells differentiated into muscle fibers upon transplantation into degenerated muscles of rats and mdx-nude mice. The induced population contained Pax7-positive cells that contributed to subsequent regeneration of muscle upon repetitive damage without additional transplantation of cells. These MSCs represent a more ready supply of myogenic cells than do the rare myogenic stem cells normally found in muscle and bone marrow.

Cell transplantation therapy offers hope for the treatment of intractable muscle degenerative disorders. Embryonic stem (ES) cells and stem cells derived from muscle have been considered as candidates for transplantation therapy (*1*–*7*). Although they have great potential, they face limitations inherent in procurement from fetal tissue, including problems relating to histocompatibility and ethical concerns. Although muscle stem cells and satellite cells can be isolated from adult and prenatal tissues (*2*, *4*–*6*), the number of cells that can be harvested may be limited. Bone marrow is another source of myogenic stem cells (*3*, *8*); however, because the stem cell population is very small, the problem of inadequate tissue supply for therapeutic scale again arises.

Because bone marrow stromal cells (MSCs) are easy to isolate and expand rapidly from patients without leading to major ethical and technical problems, they have great potential as therapeutic agents. However, despite their potential for use in cell transplantation therapy, practical application to human muscle degenerative diseases depends on the ability to control their differentiation into functional skeletal muscle cells with high efficiency and purity. Recently we reported that efficient induction of neurons, without glial differentiation, from human and rat MSCs could be achieved by Notch1 intracellular domain (NICD) gene transfer and administration of certain trophic factors (*9*). Further addition of glial cell line–derived neurotrophic factor (GDNF) effectively induced dopamine-producing cells and resulted in functional recovery when those cells were grafted into the brains of Parkinson's disease model rats (*9*). Here we report a method to systematically and efficiently induce skeletal muscle lineage cells

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with high purity from a large population of adherent MSC's, rather than from a rare subpopulation of myogenic stem cells contained in the bone marrow. The induced population effectively differentiated into mature myotubes with some cells persisting as Pax7-positive satellite cells that continued to function in host muscle to restore degenerating muscles in the absence of repeated transplantations. Because our induction system uses a large population of adherent MSC's, which can be easily isolated and expanded, functional skeletal muscle cells including satellite cells can be obtained on a therapeutic scale in a short time period.

General adherent MSCs were established as described [(*10*), Note1]. After three passages, induction was initiated. The induction procedure and corresponding phase contrast images taken at each step are shown (Fig. 1, A and B). Human and rat MSC's plated at a set cell density ([(*10*), Note1] were treated with basic fibroblast growth factor (bFGF), forskolin (FSK; known to up-regulate intracellular cyclic adenosine 3′,5′-monophosphate), platelet-derived growth factor-AA (PDGF), and neuregulin for 3 days (cells at this stage are referred to as C-MSC's). The C-MSC's were then transfected with an NICD expression plasmid by lipofection followed by G418 selection and allowed to recover to 100% confluency (referred to as CN-MSC's). Although MyoD expression was detected in CN-MSC's (Fig. 2J), the frequency of spontaneous cell fusion (the fusion index) was very low ["percentage nuclei incorporated in myotubes (*11*)" was <0.1%] in both rat and human CN-MSCs 5 days after cells reached 100% confluency. To confirm the potential of CN-MSC's to differentiate into multinucleated myotubes, we supplied cells with either 2% horse serum or ITS (insulin-transferrin-selenite) serum-free medium, both of which promote differentiation of myoblasts to myotubes (*11*, *12*). The fusion index was ~24% at 5 days after administration of 2% horse serum or 12% by ITS serum-free medium (Fig. 1A). A much higher production of differentiated myotubes was observed based on the appearance of a muscle phenotype that mainly arose from the spontaneous differentiation of original MSC's (*13*). Because horse serum is not appropriate for clinical usage, and cell survival and myotube formation were unsatisfactory in ITS serum-free medium, we searched for alternative conditions. We found that the supernatant of the original MSC's was also an effective inducer, with a fusion index of about 20% at 5 days after administration and plateauing at ~40% 14 days after induction (Fig. 1C). In the following experiments, we used MSC supernatants for the fusion induction and refer to CN-MSC's treated with supernatant of MSC's as M-MSC's (muscle-MSC's). Rat CN-MSC's and M-MSC's displayed the same features as human MSC-derived cells. Some multinucleated cells in both rat and human M-MSC's exhibited spontaneous contraction in vitro. Furthermore, these multinucleated cells expressed MyoD, myogenin (Fig. 2, A and B), skeletal myosin (Fig. 2F), myosin heavy chain (MHC) (Fig. 2, A, B, and D), and troponin (Fig. 2E), exhibiting skeletal myotube characteristics (*11*). The multinucleated cells appeared postmitotic as determined by p21 immunostaining (Fig. 2C, arrows) and 5-bromo-2′-deoxyuridine (BrdU) incorporation (Fig. 2D) (*12*). In addition to multinucleated cells and MyoD-positive mononucleated cells, cells immunopositive for Pax7 (Fig. 2F, arrows) and c-MetR (Fig. 2E, arrows), both markers for muscle satellite cells (*14*, *15*), were detected. These data suggest that M-MSC's consist of skeletal muscle lineage cells.

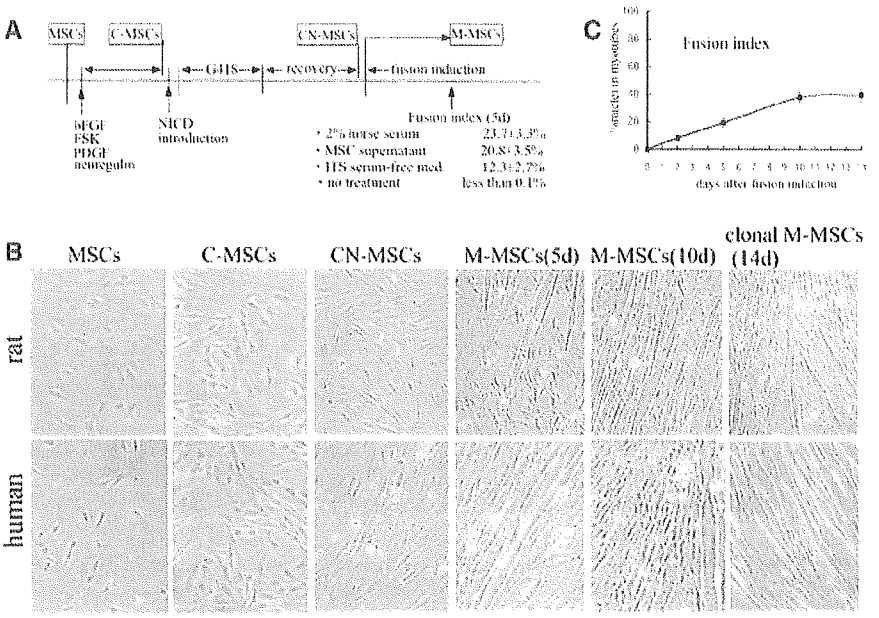
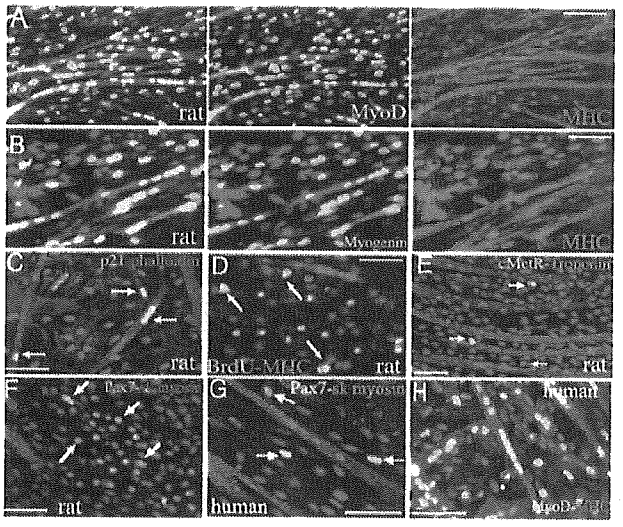
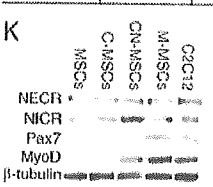


Fig. 1. Induction of skeletal muscle lineage cells. (A) Schematic diagram of the induction process. When human CN-MSCs reached 100% confluency, fusion induction was initiated. Fusion indexes were estimated after 5 days in human M-MSCs. For the cytokine treatment, omission of bFGF resulted in a major reduction of the fusion index in human M-MSCs (5 days; 0.5 ± 0.1%). Singular omission of Neuregulin, PDGF, or FSK singly resulted in fusion indexes of 1.8 ± 0.6%, 2.1 ± 0.4%, and 2.5 ± 0.7%, respectively. (B) Phase contrast microscopy of rat and human cells at each step and of clonal-M-MSCs (14 days). (C) Fusion indexes of human M-MSCs upon administration of human MSC supernatant.

To understand the induction events leading from MSC's to M-MSC's, we investigated the expression of genes related to myogenesis in these cells by means of reverse transcription polymerase chain reaction (RT-PCR) (Fig. 2J). In MSC's, Pax3, Six1, and Six4 were detected, whereas Pax7, MyoD, and myogenin were not. In C-MSC's, Pax3 was down-regulated, whereas Pax7 expression was detected [(*10*), Note 2], which persisted in CN-MSC's and M-MSC's. Expression of MyoD and myogenin was found in CN-MSC's and M-MSC's. These results were confirmed by Western blot analyses (Fig. 2K). Myf6/MRF4, a marker for mature skeletal muscle (*16*), was detectable only in M-MSC's (Fig. 2J). Whereas expression of Six1 and Six4 persisted in M-MSC's, another myogenic factor, myf5, was not detected in any MSC-derived cells (Fig. 2J). This induction process mimicked some aspects of conventional skeletal muscle development in that Pax3, Pax7, MyoD, Myogenin, and Myf6/MRF4, all of which are related to muscle development (*11*, *12*, *14*, *16*), could be detected in a sequential manner. However, because the characteristics of MSC's used in this induction system are different from those of the conventional myogenic progenitor cells, it is possible that some of the mechanisms might differ, especially in the initial step in which MSC's are converted to MyoD-positive CN-MSC's. For this initial step, cytokine pre



I	2 day	5 day
Pax7	17.9±1.7	3.3±0.8
MyoD	65.3±2.8	67.2±1.2
Myogenin	11.0±1.1	19.1±1.5



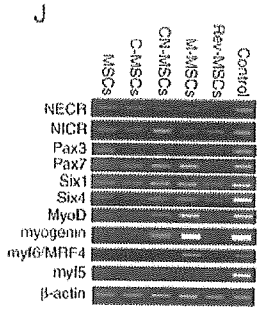


Fig. 2. Characterization of induced cells. (A to H) Immunocytochemical analysis of rat M-MSCs (10 days) (A to F) and human clonal-M-MSCs (14 days) (G and H). In (D), arrows indicate BrdU-incorporated mononucleated cells after 2 hours' incubation. Bars, 50 μm. (I) Ratios of Pax7-, MyoD-, and Myogenin-positive cells in rat clonal-M-MSCs. (J) RT-PCR and (K) Western blot of rat MSCs, C-MSCs, CN-MSCs and M-MSCs (5 days), and Rev-MSCs (5 days after fusion induction). In RT-PCR, the positive control (Control) is C2C12 cells, except for Pax3, which used ES cells. Notch extracellular region (NECR; corresponding to endogenous Notch) and intracellular region (NICR; corresponding to endogenous plus exogenous Notch) were detected in MSCs, suggesting that MSCs are endogenously expressing a small amount of Notch. After transfection with an NICD expression plasmid (CN-MSCs), NICR was up-regulated. The down-regulation of NECR in Rev-MSCs corresponds to the neuronal induction data in our previous report; when MSCs are first transfected with NICD, endogenous expression of Notch is down-regulated (*9*). β-tubulin was used as a loading control.

treatment and the subsequent NICD transfection are critical for MSC-derived cells to acquire competence for myogenic induction. Indeed, when we reversed the order of cytokine treatment and NICD transfection, muscle-lineage markers were not detected (Fig. 2J; Rev-MSCs), nor were multinucleated cells observed (*17*). The expression profiles of Notch and Hes genes during myogenic induction processes and effects of Notch/Hes signaling in the muscle induction system are described in (*10*), Note 3. Furthermore, we induced re-expression of NICD in CN-MSCs and estimated its effects on myogenic differentiation by analyzing the expression of MyoD and the fusion induction [(*10*), Note 3].

Bone marrow contains a small population of myogenic stem cells known to express c-Kit, CD45 and CD34 (*2*–*7*). However, the major population of MSCs is negative to these markers [(*10*), Note 1]. To exclude the possibility that the production of muscle-lineage cells was due to the vast proliferation of myogenic stem cells contained in MSCs, we isolated human MSCs negative for c-Kit, CD45, and CD34 by fluorescence-activated cell sorting (FACS) and subjected them to the induction process (Fig. 3A). We confirmed that isolated cells could also be driven to become muscle-lineage cells as efficiently as the unsorted MSCs. The data from rat MSCs were essentially identical to those from human MSCs. Thus, in our system, it appears that the major population of MSCs, rather than a small fraction of bone marrow derived myogenic stem cells, contributes to the production of muscle lineage cells.

We next tested the differentiation of clonal-M-MSCs in vivo by transplantation into animals. Human clonal-M-MSCs were labeled by means of a green fluorescent protein (GFP) encoding retrovirus and then transplanted by local injection (L.I.) into muscles or by intravenous injection (I.V.) into immunosuppressed rats whose gastrocnemius muscles were damaged with cardiotoxin pretreatment (*18*). Two weeks after transplantation, GFP-labeled clonal-M-MSCs incorporated into newly formed immature myofibers, and most of the GFP-positive myofibers exhibited centrally located nuclei in both L.I.- (*17*) and I.V.- (Fig. 3, B and D) treated animals. The incorporation ratios of human and rat GFP-positive cells at 2 weeks are indicated in (*10*), Note 4. Four weeks after transplantation, 60 to 70% of the GFP-positive myofibers exhibited mature characteristics with peripheral nuclei just beneath the plasma membrane (Fig. 3, F to G). Functional differentiation of grafted human clonal-M-MSCs was also confirmed by the detection of human dystrophin in GFP-labeled myofibers (Fig. 4A). In both L.I.- and I.V.-treated animals (4 weeks after injection), GFP-labeled human-derived cells were not detected in the host brain, heart, liver, kidney, and nondamaged muscles (*17*), suggesting that transplanted cells incorporate only into the damaged tissues. However, in the lung, a small number of rat and human GFP-positive cells were detected in the I.V.-treated animals (4 weeks), but not in the L.I.-treated animals. These findings indicate that clonal-M-MSCs are able to incorporate into damaged muscles and contribute to regenerating myofiber formation, regardless of the transplantation method.

In addition, some of the transplanted cells were observed between the plasma membrane and laminin-positive basal lamina that surround distinct myofibers (Fig. 3I). Because these cells expressed the satellite cell marker Pax7 (*14*) (Fig. 3H), they might be retained as satellite cells and/or developed into satellite cells in the host muscle. The ratios of transplanted Pax7/GFP-positive cells within total Pax7-positive satellite cells (transplanted and host satellite cells) are described in (*10*), Note 4. It is believed that muscle satellite cells contribute to regenerating myofiber formation upon muscle damage (*19*). We examined whether the transplanted satellite-like cells were able to function as satellite cells in vivo. Four weeks after transplantation of human clonal-M-MSCs (I.V.), cardiotoxin was readministered into the same muscles without additional transplantation just after the muscles were biopsied. The biopsies confirmed that 60 to 70% of GFP-positive myotubes displayed peripheral nuclei (Fig. 4A). Two weeks after the second cardiotoxin treatment (6 weeks after initial transplantation), we observed many regenerating GFP-positive myofibers with centrally located nuclei (Fig. 4B), and 16.5 ± 4.7% (mean ± SD; $n = 4$) of myofibers in the damaged area were GFP-positive. These results suggest that the Pax7-positive cells retained in the host muscle function as satellite cells, contributing to muscle repair. This implies that, upon transplantation of clonal-M-MSCs to muscles of patients, cells retained as satellite cells in clonal M-MSCs should be able to continue to contribute to future muscle regeneration. Similar characteristics were observed with rat clonal-M-MSCs (*17*).

Transplantation of muscle-lineage cells offers a potential therapeutic approach for the treatment of muscle degenerative disorders such as Duchenne muscular dystrophy. We therefore locally injected GFP-labeled human clonal-M-MSCs into cardiotoxin-pretreated

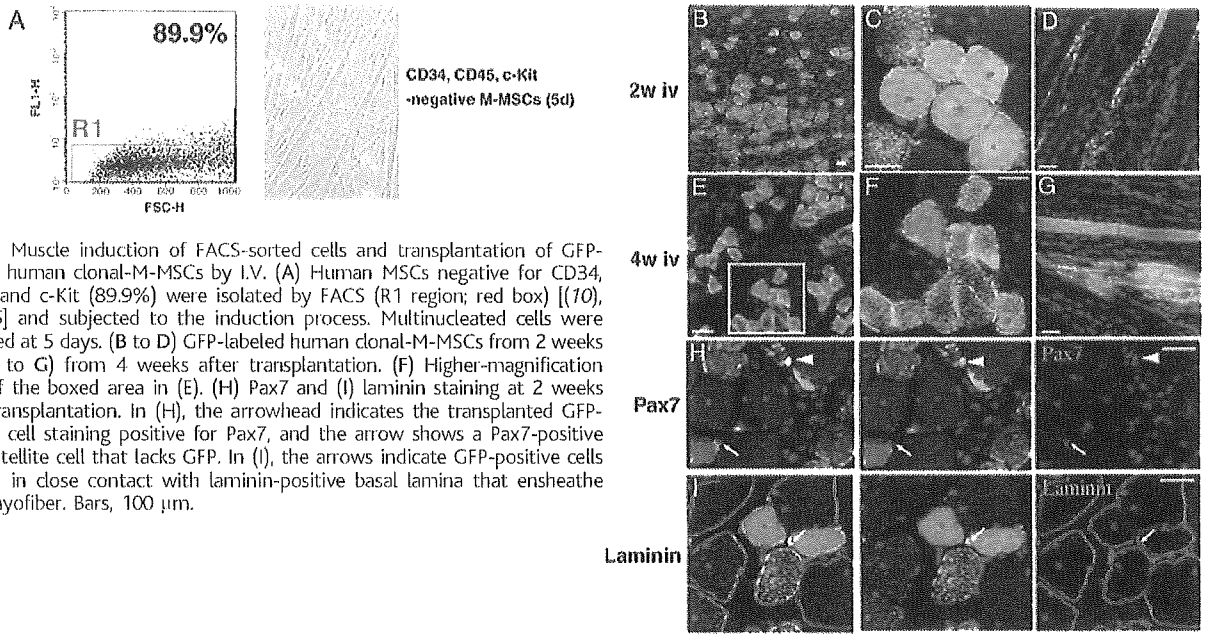


Fig. 3. Muscle induction of FACS-sorted cells and transplantation of GFP-labeled human clonal-M-MSCs by I.V. (A) Human MSCs negative for CD34, CD45, and c-Kit (89.9%) were isolated by FACS (R1 region; red box) [(*10*), Note 5] and subjected to the induction process. Multinucleated cells were observed at 5 days. (B to D) GFP-labeled human clonal-M-MSCs from 2 weeks and (E to G) from 4 weeks after transplantation. (F) Higher-magnification view of the boxed area in (E). (H) Pax7 and (I) laminin staining at 2 weeks after transplantation. In (H), the arrowhead indicates the transplanted GFP-labeled cell staining positive for Pax7, and the arrow shows a Pax7-positive host satellite cell that lacks GFP. In (I), the arrows indicate GFP-positive cells located in close contact with laminin-positive basal lamina that ensheathe each myofiber. Bars, 100 μm.

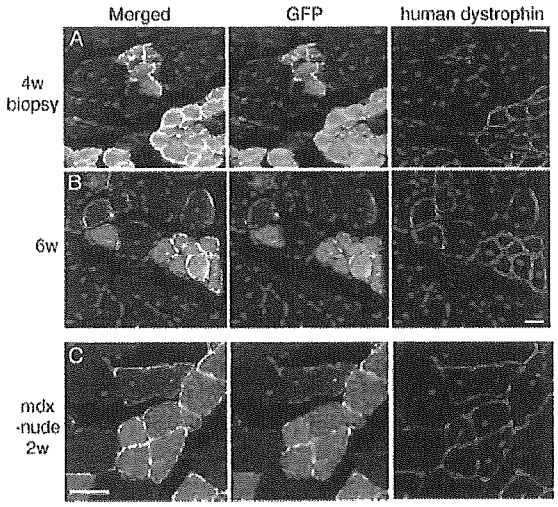


Fig. 4. Regeneration of human clonal-M-MSC transplanted rat (A and B) and mdx-nude mouse (C) gastrocnemius muscles after cardiotoxin treatment. (A) Specimen obtained from biopsy at 4 weeks by I.V. transplantation. Human dystrophin (red) is expressed by GFP-labeled transplanted cells. (B) After biopsy, cardiotoxin was administered and 2 weeks later (6 weeks after human clonal-M-MSC transplantation), gastrocnemius muscles were examined. Human dystrophin could be detected in GFP-labeled regenerating muscle fibers with centrally located nuclei. (C) Expression of human dystrophin in GFP-labeled cells in mdx-nude mouse gastrocnemius muscle after 2 weeks. Bars, 100 μm.

muscles of mdx-nude mice, which genetically lack dystrophin expression. Immunohistochemistry revealed the incorporation of transplanted cells into newly formed myofibers, which expressed human dystrophin 2 weeks after transplantation (Fig. 4C).

Compared to the various stem cell systems that have been reported (*1*, *20*–*22*), our MSCs offer several important advantages. First, MSCs can easily be obtained from patients or bone marrow banks and can be expanded efficiently in vitro. In the case of MSCs derived from inherited muscle dystrophy patients, genetic manipulation is possible after the isolation and expansion of MSCs. Second, transplantation of MSC-derived cells should encounter fewer ethical problems, because the use of these cells avoids the embryonic stem cell controversy and is in theory similar to bone marrow transplantation, which is currently in wide use for patients with leukemia, refractory anemia, etc. Third, autologous transplantation of MSC-derived muscle cells or transplantation of these cells with the same HLA (human leukocyte antigen) subtype from a healthy donor should minimize the risks of rejection. Because our induction system does not depend on a rare stem cell population, but can use the general population of adherent MSCs, which can be easily isolated and expanded, functional skeletal muscle cells can be obtained within a reasonable time on a therapeutic scale. At present, there are no effective therapeutic approaches for muscle dystrophy. Although the mechanism of muscle induction by NICD introduction remains to be clarified, we believe that our MSC differentiation system may contribute substantially to a major advance toward eventual cell-based therapies for muscle disease.

References and Notes

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Supporting Online Material

www.sciencemag.org/cgi/content/full/309/5732/314/DC1
Materials and Methods
Figs. S1 to S7
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28 January 2005; accepted 13 May 2005
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Supporting Online Material

Materials and Methods

MSC culture, GFP labeling, FACS analysis/sorting, transfection, single-cell clonal culture, immunostaining, BrdU-incorporation and Western blot were described previously (*1, 2*). The NICD cDNA encodes a transmembrane region that includes a small fragment of extracellular domain, followed by the entire intracellular domain of mouse Notch1 (initiating at amino acid 1703 and terminating at 3' untranslated sequence). This fragment was subcloned into a pCI-neo vector (Promega, WI) containing a neomycin resistance gene, and was transfected with MSCs followed by G418 selection as described (*2*). Measurement of fusion indexes were as reported(*3*), and 2000 cells were counted per measurement for 5 samples. Antibodies used; MyoD, myogenin, p21 (BD Bioscience), MHC, myf5, c-MetR (Santa Cruz), rat dystrophin, phalloidin, troponin, sk myosin (Sigma), laminin (Chemicon), human dystrophin (Vector) and Pax7 (University of Iowa, DSHB). RT-PCR primer sequences and conditions for NICR, NECR, Hes1, Hes5, Hes6, Pax3, Pax7, MyoD, myogenin, myf6/MRF4, myf5 are described elsewhere (*2, 4-5*). Primers for Six1: 5'-CCGCCTGCGATCACCT-3'(forward) and 5'-TCTCTTTGCCTCCGGTTCTTA-3'(reverse), and for Six4: 5'-ATGACTGTTGCCTGTCGTATT-3'(forward) and 5'-CGCCTTCTTTCTGAAATCA-3'(reverse). C2C12 for positive control cells were harvested for RT-PCR 5 days after incubation with differentiation media containing 2% horse serum.

Administration of bFGF (10 ng/ml), FSK (5 μM), neuregulin (200 ng/ml) and PDGF (5 ng/ml) was as described (*1, 2*). Supernatants from confluent rat and human MSCs

were collected and filtered (0.22 μm). For the horse serum administration, 2% horse serum in DMEM was used. For single-cell clonal culture, 192 clones were prepared and subjected to the analysis.

Gastrocnemius muscles of adult Wistar rat and mdx-nude mouse were injected with cardiotoxin as described (*6*). Rat and human M-MSCs were trypsinized and 10^6 cells (for rat) and 10^5 cells (for mdx-nude mouse) were transplanted by local injection (L.I.) or intravenous injection (I.V.) (tail vein) under anesthesia 2 days after cardiotoxin treatment. Immunosuppression for human cell transplantation was described previously (*2*). In brief, FK506 (1 mg/kg, Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) was injected subcutaneously every day.

Note1: The procedure for skeletal muscle lineage cell induction.

1. MSCs: Human MSCs obtained from healthy donors (informed consent obtained according to Guidelines of the Ethics Committee of the Kyoto University Graduate School and Faculty of Medicine) and adherent MSCs were established as described (*1, 2, 7*). Four human samples were used in this study.

In FACS analysis, human MSCs expressed CD29 (beta1-integrin), CD90 (Thy-1), CD54 (ICAM-1), CD44 (H-CAM) and CD71 (transferrin receptor), but not CD34 (a hematopoietic stem cell marker), CD11 (macrophages), CD45 (Leukocytes), CD117 (c-Kit), vWF (human endothelial cells) or CD3 (T cells) as shown in Fig.S1, in consistent with previous reports (*2, 7, 8*). Numbers in panels represent the mean fluorescent intensity of the cells expressing each marker.

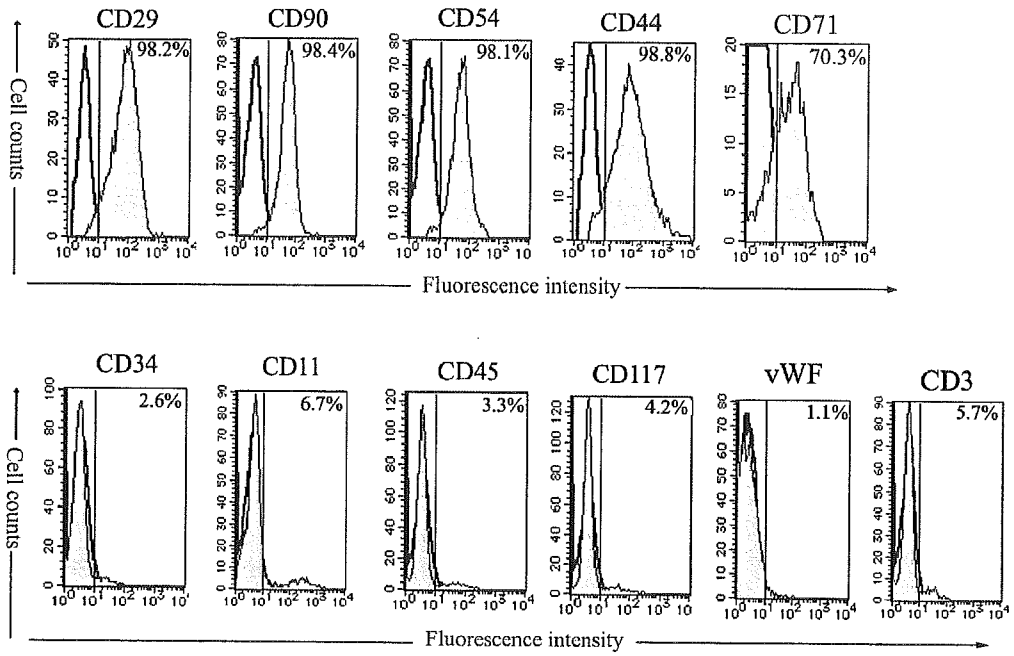

Figure S1. FACS analysis of human MSCs

All animal experiments were approved by the Animal Care and Experimentation Committee of Kyoto University Graduate School of Medicine. Adult rat (Wistar strain) MSCs were isolated and cultured as described in our previous report (*1*). In brief, tibias and femurs were prepared from adult male rats. The marrow was extruded with 10 ml of alpha-MEM (Sigma, St Louis, MO) and cultured in alpha-MEM containing 10% FBS, 2 mM L-glutamine and 100 mg/mL kanamycin, incubated at 37°C, humidity 95% and CO_2 5%. After 48hrs, the nonadherent cells were removed by replacing the medium. Adherent MSCs were subcultured and subjected to induction.

For characterization of rat MSCs, cell surface markers were assessed using F ACS as shown in Fig.S2. Rat MSCs were positive for CD29, CD90, CD54, CD44 and CD71, and were negative for CD34, CD11b/c, CD45, CD117, CD31 (endothelial marker) and

CD3.

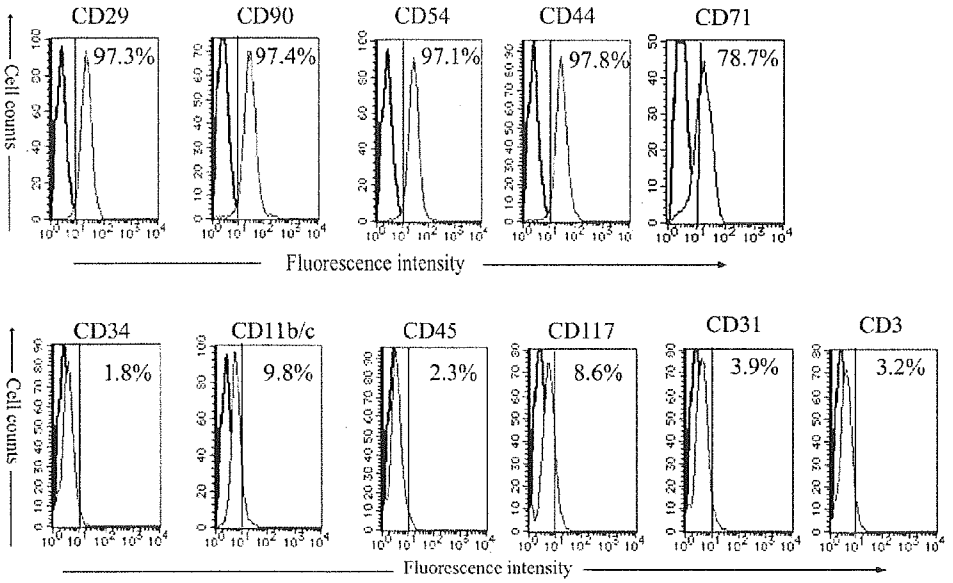

Figure S2. FACS analysis of rat MSCs.

Hematopoietic cells are generally non-adherent. Even though adherent MSCs after passages were brought to FACS analysis, small but discrete populations of cells are positive to hematopoietic lineage markers such as CD45, CD3 and CD11. Therefore, it is possible that MSCs are contaminated with hematopoietic cells. We performed immunocytochemical analysis of both human and rat MSCs. Immunopositive cells to CD45, CD3 or CD11 were mostly small-sized adherent cells. Accordingly, it appears unlikely that MSCs were contaminated with hematopoietic cells.

Adipogenic, chondrogenic and osteogenic differentiations of both human and rat MSCs were confirmed according to the method described by Pittenger et al. (*7*).

Human and rat MSCs were passaged three times, and then plated on plastic dishes at 1,700~1,900 cells/cm^2.

2. C-MSCs: 24 hrs later, MSCs were treated with bFGF (10ng/ml; Peprotech), FSK

(5μM; (Calbiochem,), neuregulin (200ng/ml; R&D Systems,) and PDGF (5ng/ml; R&D Systems) in alpha-MEM (Sigma) containing 10% FBS. After 3-days incubation, cells reached to 7,000~10,000 cells/cm^2.

In addition, we analyzed the minimum concentrations of four factors, and found that muscle lineage cells could not be induced when their concentrations were decreased to one tenth, suggesting that cytokine concentration should at least be above the certain level.

3. **CN-MSCs:** Cells were then transfected with NICD by using lipofectamine 2000 and selected by G418 for 11 days according to the manufacturer's protocols (Invitrogen, Carlsbad, CA, USA) (2). The efficiency of NICD transfection was assessed by lipofection of pNICD-IRES2-EGFP, a GFP-containing plasmid, showing that 99.0±0.8% of the cells were transfected with NICD after G418 selection. Cells were allowed to recover to 100% confluency (approximately 20,000~40,000 cells/cm^2) for 5~7 days after G418 selection.
4. **M-MSCs:** Cells were supplied with filtered supernatant of MSC culture media. For preparation of the supernatant, both rat and human MSCs were subcultured at 20,000 cells/cm^2, and the conditioned medium sample were collected when they reached 100% confluency and then filtered. For rat cells, 100% confluent rat MSC culture medium of 10% FBS in alpha-MEM was used for the rat MSC supernatant, and for human cells, human MSCs cultured with 10% human serum in alpha-MEM was used. Cells reached up to over 50,000 cells/cm^2 at 5 days.

Note2: Pax7 expression in C-MSCs.

As Pax7 was initially detectable in C-MSCs in RT-PCR, we analyzed its expression by

immunocytochemistry. A small fraction of C-MSCs (approximately 4%) showed positive immunostaining both in human and rat. However, Pax7 was undetectable in majority of cells (Fig.S3).

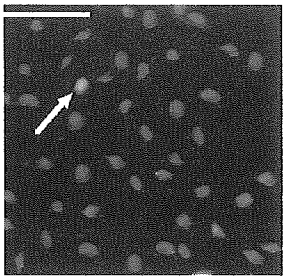

Figure S3. Pax7 immunocytochemistry in rat C-MSCs. An arrow indicates a Pax7 positive cell (green). Scale bar = 50 μm.

Note3: Effects of Notch/Hes signaling in muscle induction.

It is well established that Notch signaling inhibits myogenic differentiation; Delta1/Jagged1 inhibits MyoD expression, blocks the differentiation of myoblasts, and prevents the formation of myotubes (*9, 10*). Hes 1/5, downstream effectors of Notch, are reported unrelated to the inhibition of the myogenic pathway in C2C12 myoblasts (*11*), while others report that Hes1 up-regulation results in the prevention of myogenesis (*12*).

We examined the expression of Hes family members to judge whether conventional Notch pathway was activated in our induction process (*13-17*). The expression of Hes 1/5 was not significantly upregulated by NICD transfection (Fig.S4). The forced expression of Hes 1/5 in place of NICD failed to induce skeletal muscle lineage cells, suggesting that Hes 1/5 signaling is not involved in the muscle induction event in MSCs. Hes 6, another Hes family member, is known to induce the myogenic differentiation program (*13*). While Hes 6 was somewhat upregulated in our induction system (Fig.S4), muscle induction by the forced expression of Hes6 in place of NICD could barely elicit muscle lineage cells.

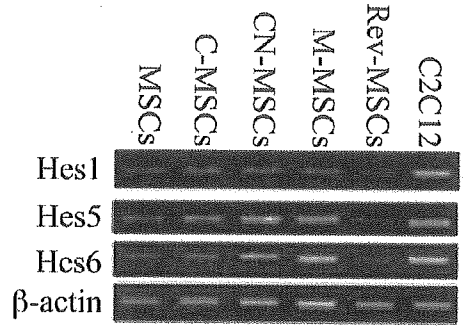

Figure S4. RT-PCR of rat MSCs, C-MSCs, CN-MSCs and M-MSCs (5 days), Rev-MSCs (reversed the order of cytokine treatment and NICD transfection; see main text) (5 days after fusion induction) and C2C12 cells.

In our induction system, NICD transfection lead to up-regulation of MyoD while it has been shown to inhibit myogenic differentiation in cultured muscle cells and in the embryo (*9, 10*). We re-expressed NICD in rat CN-MSCs and analyzed MyoD expression. CN-MSCs were transfected with pCI-neo-NICD by lipofection, followed by G418 selection as described in Materials and Method, and were brought to RT-PCR. NICD re-expression was confirmed by the up-regulation of NICR (Notch intracellular region) signals. The down-regulation of MyoD was recognized after re-expression of NICD in CN-MSCs (NICD-CN-MSCs) as well as in C2C12 cells (NICD-C2C12) (Fig. S5).

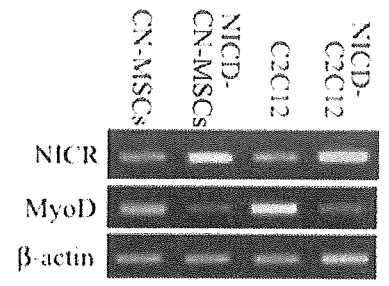

Figure S5. RT-PCR of rat CN-MSCs and C2C12 cells after NICD transfection

After the re-expression of NICD followed by G418 selection, cells were recovered to

100% confluency and myotube formation upon administration of differentiation medium containing 2% horse serum was analyzed. Usually, fusion index was approximately 20% five days after fusion induction (see, main text Fig. 1C). In contrast, the differentiation into multinucliated myotubes was significantly suppressed by re-expression of NICD both in rat and human CN-MSCs (less than 1%) as well as C2C12 cells. These results suggest that cellular response to NICD in MSCs is different from that of conventional myogenic progenitor cells, but once they differentiate into myogenic lineage cells, namely CN-MSCs, their response to NICD is similar to C2C12 cells (*9*, *10*).

Our results showing that NICD introduction accelerates the induction of skeletal muscle cells from MSCs are surprising from the viewpoint of conventional Notch signaling in myogenesis. Although our results appear inconsistent with previous work, they do not refute the known role of Notch-Hes signals in myogenesis. Rather, our results may illuminate the distinct cellular responses to Notch signals; for example, the repertoire of proteins, second messengers and other active factors may well be quite different between conventional myogenic progenitor cells and MSCs. Notably, in our previous report, we observed the induction of neuronal cells from MSCs by NICD introduction (*2*). A yet unknown signaling pathway downstream of Notch may be involved in these events. Further studies are nevertheless needed to identify the factor involved in this phenomenon.

Expression of NICR was very faint by RT-PCR in clonal-M-MSCs probably (Fig. S6) due to the diluting out of the transfected NICD plasmid, suggesting that NICD activity is required for C-MSCs to acquire myogenic potential to differentiate into muscle-lineage cells, but not for clonal-M-MSCs to maintain their characteristics.

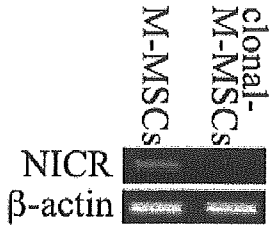

Figure S6. RT-PCR of NICR in rat M-MSCs and clonal-M-MSCs.

Note4: Incorporation of transplanted cells two weeks after transplantation.

Table S1: The ratio (%) of GFP(+) fibers in total fibers (1500 fibers with centrally located nuclei were counted for each sample)

Sample#	#1	#2	#3	#4	#5	#6	#7	#8	total (mean ± SD)
Rat L.I.	36.5	38.8	43.5	39.5	54.4	27.6	48.0	49.8	42.4±8.5
Rat I.V.	29.4	43.5	31.3	39.3	35.9	40.2	22.8	27.7	33.7±7.1
Human L.I.	45.8	29.8	46.8	27.7	44.0	42.8	19.8	39.7	37.1±9.9
Human I.V.	16.3	19.9	9.2	22.5	23.1	25.5	35.5	29.0	22.6±7.9

Table S2: The ratio (%) of Pax7/GFP(+) cells in total Pax7-positive cells (100 Pax7 positive cells in the damaged area were counted for each sample)

Sample#	#1	#2	#3	#4	#5	#6	#7	#8	total (mean ± SD)
Rat L.I.	14.2	13.4	20.8	17.3	26.9	12.9	24.6	23.0	19.1±5.4
Rat I.V.	6.4	13.6	9.4	10.2	5.6	12.0	4.9	5.8	8.4±3.2
Human L.I.	20.6	12.5	21.0	13.6	22.8	18.3	11.3	18.0	17.2±4.2
Human I.V.	3.2	6.1	2.7	4.1	7.0	5.3	11.1	8.3	5.9±2.8

Note5:. Human MSCs negative for CD34, CD45 and c-Kit.

Cells negative for all three CD34, CD45 and c-Kit (R1 region, red box) was isolated by FACS. Immediately after isolation, cells were resorted to confirm their negativity to these markers (Fig. S7).

-FACS sorting-

CD34, CD45, c-Kit negative cells

-resorting-

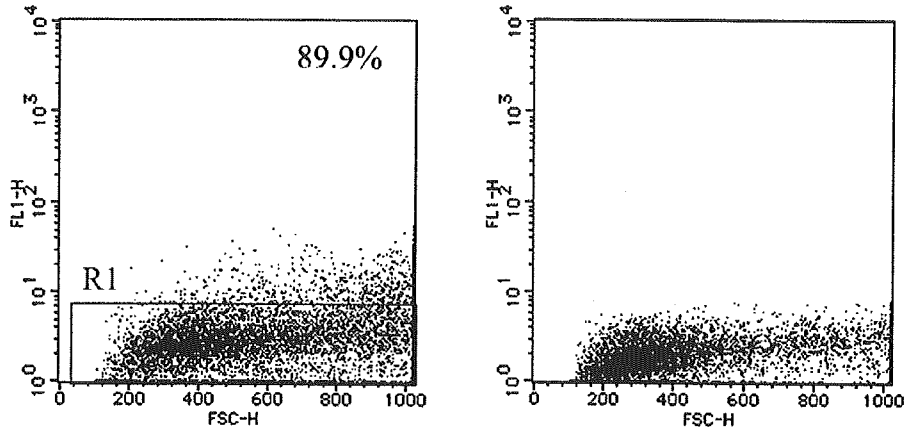

Figure S7. FACS sorting and resorting of human MSCs by CD34, CD45 and c-Kit

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