A Disintegrin and Metalloprotease 10 (ADAM10) Is Indispensable for Maintenance of the Muscle Satellite Cell Pool*

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Background: Satellite cells (SCs) are muscle-specific stem cells that are essential for muscle regeneration. Satellite cells (SCs) are muscle-specific stem cells that are essential for the regeneration of damaged muscles. Although SCs have a robust capacity to regenerate myofibers, the number of SCs decreases with aging, leading to insufficient recovery after muscle injury. We herein show that ADAM10 (a disintegrin and metalloprotease 10), a membrane-bound proteolytic enzyme with a critical role in Notch processing (S2 cleavage), is essential for the maintenance of SC quiescence. We generated mutant mice in which ADAM10 in SCs can be conditionally abrogated by tamoxifen injection. Tamoxifen-treated mutant mice did not show any apparent defects and grew normally under unchallenged conditions. However, these mice showed a nearly complete loss of muscle regeneration after chemically induced muscle injury. In situ hybridization and flow cytometric analyses revealed that the mutant mice had significantly less SCs compared with wild type controls. Of note, we found that inactivation of ADAM10 in SCs severely compromised Notch signaling and led to dysregulated myogenic differentiation, ultimately resulting in deprivation of the SC pool in vivo. Taken together, the present findings underscore the role of ADAM10 as an indispensable component of Notch signaling in SCs and for maintaining the SC pool.

Results: Conditional abrogation of a disintegrin and metalloprotease 10 (ADAM10) in SCs induces premature differentiation and results in depletion of SCs.

Conclusion: ADAM10 is indispensable to retain quiescence in SCs.

Significance: This study is the first to show the crucial role of ADAM10 as a gatekeeper of SC differentiation.

Satellite cells (SCs) are muscle-specific stem cells that reside between myofibers and the basement membrane with essential roles in repairing damaged muscle (1–3). Under normal conditions, the majority of SCs exist in a quiescent state or G0 phase. However, in response to injury, SCs rapidly proliferate and differentiate to form myofibers and repair damaged muscles. The number of SCs and their regenerative potency decrease with age, leading to insufficient recovery after muscle trauma. Because loss of muscle strength and plasticity can compromise activity of daily living, especially in the elderly, it is mandatory to learn more about the mechanisms underlying the maintenance of the number and the regenerative capacity of SCs.

Past studies have identified various molecules that are potentially involved in the maintenance and aging of SCs, including p38 mitogen-activated protein kinase (4, 5), Wnt (6), TGF-β (7), Jak-Stat (8), p16 (9), and Notch (10–12). Notch receptors and their ligands are highly conserved gene families and are critically involved in various cellular functions, including cell fate decision, cell growth, and differentiation (13, 14). There are four Notch receptors (Notch 1–4) and five ligands (Jagged 1 and 2 and DLL 1, 3, and 4) in mammals. Notch signaling is also involved in the maintenance of stem cells in certain type of tissues, and loss of Notch signaling in the stem cells in these tissues often results in dysregulated differentiation. Accordingly, studies in the past few years have shown that Notch signaling has a crucial role in the maintenance of SCs and that suppression of this signaling pathway results in dysregulated differentiation of SCs and a decrease in the SC population (10–12).

The Notch signaling pathway has a complex and unique mode of action in transmitting cell signaling. Because the receptors and ligands are all membrane-bound, Notch signaling is initiated through cell-cell contact between a receptor-expressing cell and a ligand-expressing cell. Upon activation by ligand binding, Notch receptors undergo sequential proteolytic cleavage by a disintegrin and metalloprotease (ADAM) and the γ-secretase complex (each responsible for S2 and S3 cleavage, respectively), releasing the intracellular domain. The intracellular domain translocates into the nucleus to form a complex with a transcriptional cofactor Rbpj-1 and functions as a transcriptional activator (14, 15).

There are >20 ADAM genes in mammals. Among these, ADAM10 and ADAM17 (also known as TNFα-converting enzyme or TACE) are probably best characterized as enzymes...
involved in the proteolytic release of membrane-bound proteins, a process also referred to as ectodomain shedding (16, 17). These two genes are closely related to one another and have distinct and overlapping substrates. The identity of the ADAM protease responsible for S2 cleavage of the Notch receptor remains somewhat controversial. Several studies have suggested the potential involvement of ADAM17 in S2 cleavage (18, 19). However, results from studies of ADAM10 mutant mice, which often exhibit Notch-related defects (20–23), suggest that ADAM10 is the principal enzyme responsible for S2 cleavage in vivo.

In the present study we aimed to clarify the potential roles of ADAM10 in SCs and muscle regeneration. We generated a mutant mouse line in which Adam10 can be inactivated specifically in SCs upon tamoxifen injection. The mutant mice did not exhibit any apparent defects under unchallenged conditions; however, these mice almost completely lacked the capacity for muscle generation after muscle injury. Most importantly, we found that the mutant mice are nearly devoid of SCs in skeletal muscle due to defective Notch signaling. Collectively, our data show that ADAM10 is indispensable for maintaining the SC population and for Notch signaling in SCs and further consolidate the notion that ADAM10 as the major sheddase for Notch in vivo.

**Experimental Procedures**

**Mice**—The generation of Adam10flox/flox mice was previously described (20). Adam10flox/flox mice were crossed with Pax7tm2.1(cre/ERT2)Fan/J transgenic mice (24) to specifically abrogate the Adam10 allele from SCs (henceforth referred to as Adam10flox7 mice). Conditional excision of the floxed allele was achieved by intraperitoneal injection of tamoxifen (75 μg/kg; Toronto Research Chemicals, Toronto, Canada) dissolved in corn oil (20 mg/ml). For fate-mapping experiments, we crossed Adam10flox7 mice with CAG-CAT-EGFP reporter mice (25) (referred to as Adam10flox7:EGFP mice), by which deletion of the floxed Adam10 allele and transcriptional activation of EGFP in SCs can be simultaneously achieved. As a control, we also generated mice hemizygous for both the Pax7-Cre and EGFP transgenes by crossing Pax7tm2.1(cre/ERT2)Fan/J transgenic mice and CAG-CAT-EGFP reporter mice (henceforth referred to as WT:EGFP mice). SC-specific expression of Cre recombinase was confirmed by analyzing the expression of EGFP and immunostaining for Pax7 in the muscle fibers collected from the WT:EGFP mice (data not shown). The Adam10flox7/flox mice exhibited no apparent defects and were used as control animals in the present study (henceforth referred to as Ctrl mice) (20). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Keio University School of Medicine.

**Reagents and Antibodies**—All siRNAs were purchased from Sigma. The following antibodies were used: anti-PAX7 (1:100, ab34360; Abcam, Cambridge, England), anti-activated Notch1 (1:400, ab8925; Abcam), anti-MyoD (1:50, sc-32758; Santa Cruz, Dallas, TX), anti-GFP (1:500, GF090R; Nacalai Tesque, Kyoto, Japan), anti-perilipin (1:500, D1D8; Cell Signaling Technology), anti-ADAM10 (1:2000, 422751; EMD Millipore, Germany), and anti-GAPDH (1:5000, G9545; Sigma).

**Flow Cytometry**—Skeletal muscle from 8–12-week-old mice was harvested for flow cytometric analysis. After visible fat tissues, vessels, nerves, and tendons were removed, the muscles were thoroughly chopped and digested in a mixture of collagenase (Wako Pure Chemical Industries, Osaka, Japan), dispase (Life Technologies), and CaCl2. Digested samples were filtered through cell strainers to remove debris, and red blood cells were removed using Red Blood Cell Lysis Buffer (Roche Diagnostics) before antibody application. The following fluorochrome-conjugated monoclonal antibodies were used: anti-Scal (1:200, D7), anti-CD31 (1:80, MEC13.3), and anti-CD45 (1:1333, 30-F11). These antibodies were purchased from Biolegend. The biotinylated-SM/C2.6 monoclonal antibody was generously provided by Dr. S. Fukada (26). The flow cytometric analysis was performed using a Gallios Flow Cytometer (Beckman Coulter, Brea, CA).

**In Situ Hybridization**—Paraffin-embedded sections of cardio-tox treated tibialis anterior (TA) muscles were used for in situ hybridization. The sections were deparaffinized and then probed for Pax7 transcripts using an RNAscope Fluorescent Multiplex Reagent kit (Probe-Mm-Pax7; 314181; Advanced Cell Diagnostics, Hayward, CA) as instructed by the manufacturer. The sections were counterstained with Mayer’s hematoxylin.

**Muscle-injury Model**—The mice were anesthetized with a peritoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Muscle injury was induced with an intramuscular injection of 50 μl cardiotoxin/PBS (10 μM) in the TA muscle. The mice were closely monitored until fully recovered from the anesthesia and treatment.

**Isolation, Culture, and Staining of SCs**—SCs were isolated from myofibers of the extensor digitorum longus muscles digested with type 1 collagenase (Worthington, Lakewood, NJ). The isolated fibers were cultured in DMEM, high glucose, Gluta-Max supplement (Life Technologies), 20% FBS, 1% chicken embryonic extract (USBiological, Pittsburgh, PA), and antibiotics on dishes coated with Matrigel (Corning, Corning, NY). When necessary, 4-hydroxytamoxifen (4-OHT, Sigma) diluted in ethanol (10 μM) was added to the medium. The SCs were detached from the fibers and were cultured for 3–4 days. The cells that were fixed with 4% paraformaldehyde, permeated with 0.2% Triton X/PBS, and immunostained for analysis. Images were collected using an Olympus FSX100 fluorescence microscope (Olympus, Tokyo, Japan) and Olympus FSX-BSW software, and processed using Adobe Photoshop CS6.

**C2C12 Cell Culture**—C2C12 cells were maintained in DMEM high glucose (Nacalai Tesque) supplemented with 20% FBS and antibiotics. To induce myogenic differentiation, the medium was replaced with DMEM supplemented with 2% horse serum and antibiotics. Transfection of siRNAs was performed using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) as instructed by the manufacturer. For the induction of Notch signaling, cells were plated on culture dishes coated with recombinant human soluble DLL4 (1.5 μg/ml; PeproTech, Rocky Hill, NJ).

**Quantitative RT-PCR**—Total RNA was extracted from freshly isolated muscles and cultured cells using Sepasol-RNA I Super G (Nacalai Tesque) and reverse-transcribed using Rever-
Tra Ace reverse transcriptase (Toyobo Life Science, Osaka, Japan). PCR amplification and quantification were performed using Thunderbird qPCR Mix (Toyobo) and a Light Cycler II (Roche Diagnostics). Gene transcript levels were normalized to the expression levels of Gapdh transcripts. Muscle tissues were collected 3 and 5 days after injury.

Statistical Analysis—GraphPad Prism software (Version 6.05; La Jolla, CA) was used for the statistical analyses. Student’s t test (for two samples, assuming equal variances) was used to calculate the p values (two-tailed), and p < 0.05 was considered statistically significant.

Results

Abrogation of ADAM10 in SCs Did Not Lead to Any Noticeable Defects during Postnatal Growth—To investigate the potential roles of ADAM10 in SCs, we generated a mutant mouse line in which ADAM10 can be specifically abrogated in SCs by tamoxifen treatment (Adam10Pax7 mice) (20, 24). We first isolated SCs from Adam10Pax7 and Ctrl mice and cultured the cells in the presence of 4-OHT for 6 days. As shown in Fig. 1A, we found that Ctrl SCs expressed ADAM10 and that the expression of ADAM10 was markedly reduced in the cells derived from Adam10Pax7 mice, indicating that the floxed allele in Adam10Pax7 mice was successfully removed upon 4-OHT treatment.

3-Week-old Adam10Pax7 and Ctrl littermate mice were treated with tamoxifen consecutively for 5 days and once a week afterward as outlined in Fig. 1B. The Adam10Pax7 mice grew normally and did not exhibit any apparent systemic defects at the time of analysis (9-week-old) compared with their Ctrl littermates (data not shown). Cross-sections of the TA muscle and the evaluation of the myofiber cross-sectional area (CSA) showed no noticeable differences between Ctrl and Adam10Pax7 mice (Fig. 1C and D). These observations indicate that, at least under the current unchallenged experimental settings, ADAM10 in SCs is not essential for the postnatal development or homeostasis of skeletal muscle.

Loss of Muscle Regeneration Capacity in Adam10Pax7 Mice—Because SCs play essential roles in muscle regeneration, we next asked how the abrogation of ADAM10 in SCs affects the capacity for regeneration in skeletal muscle after injury. Cardiotoxin was injected into the TA muscle of 9-week-old Adam10Pax7 mice and Ctrl mice to induce extensive muscle injury. The mice were euthanized 7 or 28 days post-injury (DPI) for analysis (Fig. 2A). The size of the cross-sections of the TA muscle, which reflects the degree of regeneration, was significantly smaller in Adam10Pax7 mice compared with Ctrl mice even at 7 DPI (Fig. 2, B and C). A large number of regenerating myofibers, characterized by central nuclei, were found in the TA muscle sections from the Ctrl mice (Fig. 2D). However, these regenerating myofibers were almost completely missing from the TA muscle of the Adam10Pax7 mice, as the damaged region was predominantly filled with immature adipocytes, fibrous tissue, and infiltrating immune cells (Fig. 2E). Consistent with these findings, immunostaining for the adipocyte marker perilipin revealed positive staining in the damaged muscle of the Adam10Pax7 mice but not in that of the Ctrl mice (Fig. 2, F and G). Masson Trichrome staining showed dense collagen fibers surrounding the vessels and very few myofibers.
in Adam10Pax7 mice (Fig. 2, H and I). At 28 DPI, the damaged muscle was almost fully regenerated in the Ctrl mice with a few myofibers exhibiting central nuclei (Fig. 2, J and L). However, in the Adam10Pax7 mice, the damaged myofibers were replaced by adipocytes with no regenerating myofibers (Fig. 2, K and M). Immunostaining for perilipin clearly demonstrated infiltration of mature adipocytes in the Adam10Pax7 mice but almost none in Ctrl mice (Fig. 2, N and O). In addition, the vessels were densely surrounded with fibroblasts and collagenous fibers in the Adam10Pax7 mice (Fig. 2, P and Q). These observations show that the abrogation of ADAM10 in SCs results in nearly complete loss of the capacity for muscle regeneration after injury and in muscle degeneration, which is accompanied by fat cell infiltration and accumulation of collagen fibers.

**Marked Decrease in the Expression of Myogenic Markers in Adam10Pax7 Mice after Injury**—To further investigate the defects in Adam10Pax7 mice, we next examined the gene expression profiles of the muscles of Adam10Pax7 mice and Ctrl mice after injury. Because the genes that are involved in muscle regeneration are robustly induced within the first week after injury, we collected muscle tissues 3 and 5 days after cardiotoxin treatment. Consistent with the histology, there was a significant decrease in the transcript levels of myogenic markers, including Pax7, Myh3 (encodes embryonic myosin heavy-chain), Myod1, and Myf5, in the injured TA muscle of Adam10Pax7 mice compared with Ctrl mice (Fig. 3). On the other hand, there was a transient increase in the adipogenic markers Pparg (encodes PPARγ (peroxisome proliferator-acti-
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vated receptor γ) and Lep (encodes leptin) after injury in the Ctrl mice; however, these transcripts decreased nearly to basal levels by 5 DPI. By contrast, these adipogenic markers remained highly expressed in the Adam10Pax7 mice, reflecting the adipogenic changes revealed by the histological assays (Fig. 2). In addition, we also found an increase in the expression of Pdgfra, a marker for fibro/adipogenic progenitors (FAPs) or PDGFRα+ mesenchymal cells, which serve as a source for fibroblasts and adipogenic cells in muscle (27–29), in Adam10Pax7 mice compared with Ctrl mice. Taken together, the gene expression analyses suggest that myogenic differentiation was severely hampered in the Adam10Pax7 mice, whereas adipogenic and fibroblastic differentiation were enhanced.

SCs Are Depleted in Adam10Pax7 Mice—The decreased expression of myogenic-related genes in Adam10Pax7 mice suggests that SCs lacking ADAM10 failed to differentiate into myogenic lineage or that the number of SCs was decreased in Adam10Pax7 mice. To explore these possibilities, we performed in situ hybridization using a cRNA probe against Pax7 transcripts. As shown in Fig. 4A, there were numerous Pax7-positive cells in the injured muscle sections of Ctrl mice; however, very few cells were positive for Pax7 transcripts in the sections of Adam10Pax7 mice. This result suggests that the SC population was depleted or that the proliferation of SCs was severely hampered in Adam10Pax7 mice. To test these hypotheses, we next performed flow cytometric analysis using a single-cell suspension prepared from uninjured muscles to evaluate the size of the SC pool under unchallenged conditions. We found that the SC population (defined as SM/C2.6+ Sca1− CD45− CD31− cells) (26) was significantly reduced in Adam10Pax7 mice com-

![Figure 3](image_url)

**FIGURE 3.** Expression of myogenic transcripts is significantly reduced in Adam10Pax7 mice. Relative expression of Pax7, Myh3, Myod1, Myf5, Pparg, Lep, and Pdgfra in uninjured TA muscles (UI) or in muscles at 3 DPI and 5 DPI collected from Ctrl and Adam10Pax7 mice. n = 4–10 for each genotype. Error bars represent S.D. **p < 0.005.

![Figure 4](image_url)

**FIGURE 4.** Depletion of SCs in Adam10Pax7 mice. A, sections of the TA muscle taken at 7 DPI from Ctrl and Adam10Pax7 mice probed for Pax7 transcripts and counterstained with Mayer’s hematoxylin. The arrowheads indicate Pax7-positive cells. Bar, 10 μm. B, flow cytometric analysis of the SC population using a single-cell suspension prepared from the muscles of uninjured Ctrl and Adam10Pax7 mice (boxed areas). C, percentage of SM/C2.6+ Sca1− cells in the CD45− CD31− gated population of a single-cell suspension collected from the muscles of Ctrl and Adam10Pax7 mice. n = 4 mice for each genotype. Error bars represent S.D. **p < 0.005.
pared with Ctrl mice (Fig. 4, B and C). Although we cannot fully exclude the possibility that abrogation of ADAM10 also exhibits negative effects on myogenic differentiation or proliferation of SCs during muscle regeneration, these observations lean toward the idea that ADAM10 is involved in the maintenance of the SC pool in vivo. We thus concluded that Adam10Pax7 mice are incapable of regenerating damaged muscles most likely due to the loss of SCs.

Myogenic Differentiation Is Accelerated in SCs in the Absence of ADAM10—Depletion of the SC population can be explained by either increased apoptosis or a dysregulation of myogenic differentiation in the SCs. We collected myofibers from tamoxifen-treated Adam10Pax7 mice and co-immunostained them with anti-PAX7 and anti-MYOD1 antibodies (the schedule for the tamoxifen treatment is shown in Fig. 5A). Cells that were positive for PAX7 and/or MYOD1 were counted, and the numbers were statistically analyzed. There was a significant reduction in the number of PAX7+/MYOD1− cells (quiescent SCs) and an increase in PAX7+/MYOD1+ cells (committed myoblasts) in Adam10Pax7 mice compared with Ctrl mice (Fig. 5B). On the other hand, no significant difference was found in the number of caspase-3-positive apoptotic cells between Adam10Pax7 and Ctrl mice (data not shown), suggesting that ADAM10 in SCs is not essential for cell survival. These observations indicate that SCs lacking ADAM10 cannot efficiently maintain their quiescent state and consequently undergo myogenic differentiation.

SCs Lacking ADAM10 Are Defective in Notch Signaling—The phenotype of Adam10Pax7 mice observed in the current study resembles those described for Notch signaling-defective mice and SC-depleted mutant mice (30–34). Because a disruption of Notch signaling results in enhanced differentiation of SCs along the myogenic lineage and because ADAM10 is potentially the most important sheddase for Notch, the defects observed in Adam10Pax7 mice were assumed to arise from aberrant Notch processing. We performed fate-mapping analysis of SCs in Adam10Pax7 mice by crossing these mice with CAG-CAT-EGFP reporter mice (25) (Adam10Pax7-EGFP mice). As a control, we used mice hemizygous for both the Pax7-Cre and EGFP transgenes (WT Pax7-EGFP mice). Cells were isolated from the myofibers of these mice and immunostained using a Notch1 intracellular domain (N1ICD)-specific antibody. We found that Adam10Pax7-EGFP cells were positive for EGFP but negative for N1ICD represent activated or differentiated SCs. D, percentage of N1ICD+/EGFP+ cells harvested from the myofibers of WT Pax7-EGFP and Adam10Pax7-EGFP mice. n = 4 mice per genotype. **, p < 0.005. E and F, C2C12 cells transfected with control siRNA or siRNA against Adam10 were incubated on recombinant soluble DLL4 (sDDL4)-coated wells for 4 days. The formation of myotubes and the expression levels of Myog transcripts were evaluated by microscopy (E) and quantitative PCR (F), respectively. Bar, 100 μm. n = 3. **, p < 0.005.
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**Discussion**

In the present study we showed that abrogation of ADAM10 in SCs results in depletion of the SC pool and loss of the muscle regenerative capacity after injury. The decrease in the SC population was not due to increased apoptosis but due to a dysregulated and accelerated differentiation of SCs toward the myogenic lineage. We also found that SCs lacking ADAM10 are defective in Notch signaling, a critical regulator for maintaining the quiescent state in SCs (10–12). In agreement, suppression of ADAM10, SCs lose the ability to sustain Notch signaling and consequently differentiate along the myogenic lineage.

To test the hypothesis that ADAM10 mediates Notch-dependent inhibition of myogenic differentiation, we next used the myoblast-like cell line C2C12. C2C12 cells differentiate into myoblast-like cells and form myotubes in low serum conditions. In agreement with past studies, we found that the formation of myotubes was significantly inhibited when the cells were plated on Notch ligand DLL4-coated dishes (Fig. 5E) (35). However, the Notch-mediated suppression of the myogenic differentiation and expression of Myog were effectively rescued by gene-silencing of Adam10 (Fig. 5, E and F). Gene silencing and reduced expression of ADAM10 by siRNA were confirmed by quantitative PCR and Western blot (Fig. 5G). These observations support the idea that ADAM10 functions as a negative regulator of SC differentiation.

Interestingly, gene expression analysis revealed a transient increase in the transcripts for Pdgfra, a marker for the aforementioned FAPs/PDGFRA⁺ mesenchymal cells, and adipocyte-related genes (Pparγ and Lep) after injury in Ctrl mice, indicating that adipocyte differentiation is induced after muscle injury. However, the induction toward adipocyte differentiation subsides in Ctrl mice as the myofibers start to regenerate. On the contrary, the expression of these genes was not suppressed in Adam10⁰⁺⁺ mice, resulting in severe infiltration of fat tissue in damaged muscles. These observations suggest that both SCs and FAPs/PDGFRA⁺ mesenchymal cells are activated upon muscle injury; however, the activation of FAPs/PDGFRA⁺ mesenchymal cells are effectively suppressed as the SCs cells differentiate and regenerate damaged muscle fibers. Given these observations, it is tempting to speculate that there is a potential interaction between these two cell types, by which the balance of myogenesis and adipocyte/fibroblast differentiation is determined.

![FIGURE 6. The schematic model proposed by the present study.](https://example.com/figure6)

3 S. Mizuno and K. Horiuchi, unpublished observation.
In summary, our data show that conditional ablation of ADAM10 in Pax7-expressing cells results in a depletion of the SC population and failure to regenerate damaged muscles. Notch processing was hampered in SCs lacking ADAM10, further corroborating the notion that ADAM10 is the major sheddase for Notch. Conclusively, the present study demonstrates that ADAM10 functions as a gatekeeper of SCs by preventing premature differentiation through processing and activation of Notch (Fig. 6).

Author Contributions—S. M. designed, performed, and analyzed most of the experiments. M. Y. designed, performed, and analyzed the experiments shown in Fig. 4, B and C, and provided technical assistance. M. S. and Y. O. performed the histological analysis shown in Fig. 2. T. T., S. T., Y. T., M. N., and M. M. provided technical assistance and contributed to the preparation of the figures. K. H. conceived and coordinated the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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