Hesr1 and Hesr3 are essential to generate undifferentiated quiescent satellite cells and to maintain satellite cell numbers

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SUMMARY
Satellite cells, which are skeletal muscle stem cells, divide to provide new myonuclei to growing muscle fibers during postnatal development, and then are maintained in an undifferentiated quiescent state in adult skeletal muscle. This state is considered to be essential for the maintenance of satellite cells, but their molecular regulation is unknown. We show that Hesr1 (Hey1) and Hesr3 (Heyl) (which are known Notch target genes) are expressed simultaneously in skeletal muscle only in satellite cells. In Hesr1 and Hesr3 single-knockout mice, no obvious abnormalities of satellite cells or muscle regenerative potentials are observed. However, the generation of undifferentiated quiescent satellite cells is impaired during postnatal development in Hesr1/3 double-knockout mice. As a result, myogenic (MyoD and myogenin) and proliferative (Ki67) proteins are expressed in adult satellite cells. Consistent with the in vivo results, Hesr1/3-null myoblasts generate very few Pax7+ MyoD− undifferentiated cells in vitro. Furthermore, the satellite cell number gradually decreases in Hesr1/3 double-knockout mice even after it has stabilized in control mice, and an age-dependent regeneration defect is observed. In vivo results suggest that premature differentiation, but not cell death, is the reason for the reduced number of satellite cells in Hesr1/3 double-knockout mice. These results indicate that Hesr1 and Hesr3 are essential for the generation of adult satellite cells and for the maintenance of skeletal muscle homeostasis.

KEY WORDS: Satellite cells, Undifferentiated quiescent state, Hesr1 (Hey1), Hesr3 (Heyl), Mouse

INTRODUCTION
Satellite cells, which are muscle-specific stem cells, are anatomically identified as mononuclear cells that reside external to the myofiber plasma membrane and beneath the basal lamina (Mauro, 1961). During postnatal development, satellite cells divide to provide new myonuclei to growing muscle fibers (Moss and Leblond, 1971), and then change to an undifferentiated quiescent state in adult skeletal muscle (Schultz et al., 1978). This state is considered essential for sustaining the satellite cell compartment. Skeletal muscle regeneration also depends on satellite cells. When muscles are damaged, satellite cells exit from quiescence and start to proliferate. Proliferating satellite cells, called myoblasts, then differentiate, fuse with each other or with injured myofibers, and eventually regenerate mature myofibers (Charge and Rudnicki, 2004). The activation, proliferation and differentiation of satellite cells are mainly controlled by the myogenic regulatory factors, a group of skeletal muscle-specific basic helix-loop-helix (bHLH) transcription factors comprising MyoD (also known as Myod1), Myf5, myogenin and Mrf4 (also known as Myf6). Myogenin and Mrf4 are crucial for the late stages of myogenic differentiation (Sabourin and Rudnicki, 2000). The Myf5 locus is active in most quiescent satellite cells (Beauchamp et al., 2000). By contrast, quiescent satellite cells do not express MyoD, but start to express it when they are activated and start to proliferate (Zammit et al., 2004). During regeneration, MyoD is essential for the proliferation of satellite cell-derived myoblasts (Megeney et al., 1996). A recent study showed that entry of quiescent myogenic cells into the S phase of the cell cycle requires MyoD expression (Zhang et al., 2010). Therefore, the molecular mechanism that upregulates MyoD expression is key to satellite cell activation, whereas MyoD suppression in quiescent satellite cells seems to be essential for maintaining the satellite cell pool in an undifferentiated quiescent state.

The Notch signaling pathway is an evolutionarily conserved intercellular signaling system that has multiple essential roles in cell fate decisions and in patterning events (Lai, 2004). When Notch is activated, the intracellular domain of Notch is cleaved by γ-secretase and translocates to the nucleus where it activates the transcription of target genes through interaction with Rbpj (also known as Cbf1). It is well known that the Hes (Hairy and enhancer of split) and Hesr (Hes-related, also known as Hey/Herp/Hrt/Gridlock/Chf) families of bHLH transcriptional repressor genes are the primary target of Notch signaling and play important roles in nerve, heart and vascular development, among others (Fischer and Gessler, 2007). In the case of myogenic cells, it has been reported that the activation of Notch signaling by delta-like 1 (Dll1), one of the Notch ligands, inhibits MyoD expression in C2C12 cells (a myogenic cell line) (Kuroda et al., 1999) and in

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avian myotomal cells (Hirsinger et al., 2001). Furthermore, Kuang et al. reported that, in vitro, the number of Pax7+ MyoD– quiescent satellite cells was significantly decreased when proliferating satellite cells were treated with DAPT, an inhibitor of γ-secretase (Kuang et al., 2007). These results indicate the important roles of Notch signaling in myogenic cells, but the roles of the transcriptional targets of Notch signaling in myogenic cells, including satellite cells, remain largely unknown.

In this study, we show that Hesr1 and Hesr3 are important regulators that are responsible for the generation of undifferentiated quiescent satellite cells and for the maintenance of satellite cell numbers. Our findings have implications for the roles of Hes family genes in the maintenance of tissue homeostasis and facilitate investigation of the molecular regulation of satellite cells.

MATERIALS AND METHODS

Mice

Hesr1−/− mice were described previously (Kokubo et al., 2005b). Hesr3−/− mice were generated by H.K. (unpublished). To generate Hesr1−/− Hesr3−/− mice, Hesr1+/− Hesr3−/− and Hesr1+/− Hesr3+/− mice were crossed. All procedures for experimental animals were approved by the Experimental Animal Care and Use Committee at Osaka University.

Muscle injury

Muscle injury was induced by injecting 2.5 μl 10 μM (in saline) cardiotoxin (Wako Pure Chemical Industries, Tokyo, Japan) per gram mouse body weight into the tibialis anterior (TA) muscle.

RT-PCR

Total RNA was extracted from sorted or cultured cells using the RNeasy Mini Kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany) and then reverse-transcribed into cDNA using TaqMan reverse transcription reagents (Roche Diagnostics, Mannheim, Germany). PCR was performed with the cDNA using the primers listed in Table S1 in the supplementary material.

Real-time PCR was performed using SYBR Premix Ex Taq (Takara, Shiga, Japan) in a final reaction volume of 10 μl. Specific forward and reverse primers for optimal amplification in real-time PCR of reverse-transcribed cDNAs were used (see Table S1 in the supplementary material). Real-time PCR and data analyses were performed on a LightCycler Quick System 350S using LightCycler Software (Roche Diagnostics). Samples were amplified and the relative gene expression levels were calculated using standard curves generated by serial dilutions of the cDNA.

Histological analyses

TA muscles were isolated and frozen in liquid nitrogen-cooled isopentane (Wako Pure Chemical Industries). Transverse cryosections (10 μm) were stained with Hematoxylin and Eosin (H&E), Oil Red O (Sigma-Aldrich, St Louis, MO, USA), or Sirius Red (Sigma-Aldrich).

Immunocytochemistry and immunohistochemistry

For immunohistochemical examinations, transverse cryosections (6 μm) were fixed with 4% PFA for 10 minutes. For eMyHC staining, the sections were fixed with cooled acetone for 10 minutes at −20°C. After blocking with 5% skimmed milk, sections were stained with primary antibodies. Antibodies used are listed in Table 1.

For Pax7 and eMyHC staining, an M.O.M. Kit (Vector Laboratories, Burlingame, CA, USA) was used to block endogenous mouse IgG. After the first staining at 4°C overnight, sections were incubated with secondary antibodies conjugated with Alexa Fluor 488, 568 or 647 (Molecular Probes, Eugene, OR, USA). For EdU detection, the Click chemical reaction was performed after primary and secondary staining according to the manufacturer’s instructions using a Click-iT EdU Imaging Kit (Invitrogen, Carlsbad, CA, USA). Coverslips were mounted using Vectashield (Vector Laboratories).

Cultured cells were fixed with 4% PFA for 10 minutes and then permeabilized with 0.1% Triton X-100 in PBS for 20 minutes. After blocking with 5% skimmed milk, the cells were stained as described above. The signals were recorded photographically using a confocal laser-scanning microscope system (TCS-SP5, Leica, Heerbrugg, Switzerland) or a fluorescence microscope (BX51, Olympus, Tokyo, Japan) equipped with a DP70 CCD camera (Olympus).

Preparation and FACS analyses of skeletal muscle-derived mononuclear cells

Mononuclear cells from uninjured limb muscles were prepared using 0.2% collagenase type II ( Worthington Biochemical, Lakewood, NJ, USA) as previously described (Uezumi et al., 2006).

Table 1. Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Ig type</th>
<th>Conjugate</th>
<th>Supplier</th>
<th>Application and dilution</th>
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<tr>
<td>CD31</td>
<td>390</td>
<td>Rat IgG2a, κ</td>
<td>FITC</td>
<td>BD Pharmingen</td>
<td>FACS, 1:400</td>
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<tr>
<td>CD45</td>
<td>30-F11</td>
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<tr>
<td>ScaI</td>
<td>D7</td>
<td>Rat IgG2a, κ</td>
<td>PE</td>
<td>BD Pharmingen</td>
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</tr>
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<td>Satellite cells</td>
<td>SM/C-2.6</td>
<td>Rat IgG2a</td>
<td>Biotin</td>
<td>Fukada et al., 2004</td>
<td>FACS, 1:200</td>
</tr>
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<td>laminin α2</td>
<td>4H8-2</td>
<td>Rat IgG1</td>
<td>–</td>
<td>Alexis</td>
<td>IHC, 1:200</td>
</tr>
<tr>
<td>Pax7</td>
<td>PA7X</td>
<td>Mouse IgG1, κ</td>
<td>–</td>
<td>DSHB</td>
<td>IHC, 1:2 (supernatant)</td>
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<td>M-cadherin</td>
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<td>Rabbit IgG</td>
<td>–</td>
<td>S.T.</td>
<td>IHC, 1:1000</td>
</tr>
<tr>
<td>calcium receptor</td>
<td>Polyclonal</td>
<td>Rabbit IgG</td>
<td>–</td>
<td>AbD Serotec</td>
<td>IHC, 1:100</td>
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<tr>
<td>MyoD</td>
<td>Polyclonal</td>
<td>Rabbit IgG</td>
<td>–</td>
<td>Santa Cruz</td>
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<tr>
<td>MyoD</td>
<td>5.8A</td>
<td>Mouse IgG1, κ</td>
<td>–</td>
<td>BD Pharmingen</td>
<td>IHC, 1:200</td>
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<td>myogenin</td>
<td>FSD</td>
<td>Mouse IgG1, κ</td>
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<td>–</td>
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<td>Rabbit IgG</td>
<td>–</td>
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<td>IHC, 1:100</td>
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<td>Sarcomeric α-actinin</td>
<td>EA-53</td>
<td>Mouse IgG1</td>
<td>–</td>
<td>Sigma</td>
<td>ICC, 1:100</td>
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<td>eMyHC</td>
<td>F1.652</td>
<td>Mouse IgG</td>
<td>–</td>
<td>DSHB</td>
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<tr>
<td>Hesr3</td>
<td>Polyclonal</td>
<td>Rabbit IgG</td>
<td>–</td>
<td>Fukada et al., 2007</td>
<td>IHC, 1:200</td>
</tr>
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Secondary antibodies

| Rat IgG | – | Goat | Alexa Fluor 568 | Molecular Probes | IHC, 1:1000 |
| Mouse IgG | – | Goat | Alexa Fluor 568 | Molecular Probes | IHC, 1:1000; ICC, 1:1000 |
| Mouse IgG | – | Donkey | Alexa Fluor 488 | Molecular Probes | IHC, 1:1000 |
| Rabbit IgG | – | Goat | Alexa Fluor 488 | Molecular Probes | IHC, 1:1000 |
| Rat IgG | – | Chicken | Alexa Fluor 647 | Molecular Probes | IHC, 1:1000 |

DSHB, Developmental Studies Hybridoma Bank; FACS, fluorescence-activated cell sorting; IHC, immunohistochemistry; ICC, immunocytochemistry.
Mononuclear cells derived from skeletal muscle were stained with FITC-conjugated anti-CD31 (Pecam1 – Mouse Genome Informatics), anti-CD45 (Ptprc – Mouse Genome Informatics), phycoerythrin-conjugated anti-Sca1 (Ly6a – Mouse Genome Informatics) and biotinylated SM/C-2.6 (Fukada et al., 2004) antibodies. Cells were then incubated with 1:400 streptavidin-allophycocyanin (APC) (BD Biosciences, San Diego, CA, USA) on ice for 30 minutes, and resuspended in PBS containing 2% FCS and 2 μg/ml propidium iodide (PI). Cell sorting was performed using a FACS Aria II flow cytometer (BD Immunocytometry Systems, Mountain View, CA, USA). Debris and dead cells were excluded by forward scatter, side scatter and PI gating. Data were collected using FACS Diva software (BD Biosciences). Myogenic cells from the regenerating muscle were also highly enriched in the SM/C-2.6+ CD31– CD45– Sca1– cell fraction (Segawa et al., 2008).

### Satellite cell culture

Freshly isolated satellite cells were cultured in a growth medium (GM) of high-glucose Dulbecco’s modified Eagle’s medium (DMEM-HG; Sigma-Aldrich) containing 20% FCS (Trace Biosciences, N.S.W., Australia), 2.5 ng/ml bFGF (FGF2) (PeproTech, London, UK), and penicillin (100 U/ml)-streptomycin (100 μg/ml) (Gibco BRL, Gaithersburg, MD, USA) on culture dishes coated with Matrigel (BD Biosciences). Differentiation was induced in differentiation medium (DM) containing DMEM-HG, 5% horse serum and penicillin-streptomycin for 3-4 days.

### Co-culture with CHO-Dll1

Satellite cells were isolated from C57BL/6 mice and 1×10⁵ cells were plated in six-well plates. Three days later, CHO cells stably transfected with mouse Dll1 or empty vector (Dyczynska et al., 2007) were added (5×10⁵ cells/well) and incubated in GM without G418. After 24 hours, total RNA was extracted from the cultured cells.

### Detection of apoptotic cells

Freshly isolated satellite cells were cultured on eight-well Lab-Tek chamber slides (Nunc, Rochester, NY, USA) in GM. After culturing for 24 hours, apoptotic cells were detected by Rhodamine fluorescence using the ApopTag Red In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, USA).

### In vitro cell proliferation assay

Isolated satellite cells were cultured on eight-well Lab-Tek chamber slides in GM for 2-3 days, and then EdU (final concentration 10 μM; Invitrogen) was added. After additional culture for 12 hours, cells were fixed and stained following the protocol supplied by the manufacturer (Invitrogen). Three to six random fields per sample were counted.

### Clonal analysis

Single cell sorting was performed using the FACS Aria II. Sorted cells were grown in GM on Matrigel-coated 96-well dishes. During 7 days of culture, cell number and frequency of colony formation from a single satellite cell were quantified.

### In vivo EdU-uptake assay

EdU was dissolved in PBS at 0.5 mg/ml and injected intraperitoneally at 0.1 mg per 20 g body weight at the time points indicated.

### Measurement of myofiber area

ImageJ software was used to measure myofiber size and eMyHC-positive, Oil Red O-positive and Sirius Red-positive areas per section. To calculate the TA myofiber area, 80-145 myofibers per mouse were stained following the protocol supplied by the manufacturer (Invitrogen). Graphs representing myofiber areas were generated and analyzed by ANOVA.

### Statistics

Values are expressed as mean ± s.d. Statistical significance was assessed by Student’s t-test. In comparisons of more than two groups, non-repeated measures analysis of variance (ANOVA) followed by the Bonferroni test (versus control) or the Student-Newman-Keuls (SNK) test (multiple comparisons) was used. P<0.05 or P<0.01 was considered statistically significant.

### RESULTS

#### Expression of Hesr family genes in muscle satellite cells

The Hesr family comprises three members: Hesr1 (Hey1/Hey1/Hey2), Hesr2 (Hey2/Hey2/Herp1) and Hesr3 (Hey3/Hey3/Hey3). In a previous study (Fukada et al., 2007), we identified the most highly expressed genes in adult quiescent satellite cells by comparing them with activated/proliferating satellite cells and non-myogenic cells. Hesr3, a known Notch effector gene, is one of these genes, being highly expressed in quiescent satellite cells but not in myofibers. Hesr3 protein expression was also confirmed in quiescent satellite cells, but not in cultured activated/proliferating satellite cells (Fig. 1A, see Fig. S1A in the supplementary material). As shown in Fig. 1B, Hesr1 is also expressed in quiescent satellite cells. However, in contrast to Hesr3, a weak Hesr1 signal was detected in activated/proliferating satellite cells cultured for 3 days (myoblasts). Hesr2 was expressed in neither quiescent satellite cells nor myoblasts.

To reveal the expression of Hesr1 and Hesr3 in adult skeletal muscle, mononuclear cells derived from uninjured skeletal muscle were separated into four fractions using CD31 as an endothelial marker, SM/C-2.6 as a satellite cell marker (Fukada et al., 2004), and Sca1 as a fibro/adipogenic cell or mesenchymal progenitor marker (Joe et al., 2010; Uezumi et al., 2010). The expression of Pax7 and Myf5 in the isolated CD31– Sca1+ SM/C-2.6+ cells confirmed the purity of the satellite cell population (Fig. 1C). Consistent with our previous study, Hesr3 was expressed exclusively in muscle satellite cells in normal skeletal muscle, whereas both satellite cells and endothelial cells expressed Hesr1. Hesr3 was identified as an adult quiescent satellite cell gene. However, neonatal proliferating satellite cells expressed Hesr1 and Hesr3 (see Fig. S1B,C in the supplementary material). In vivo proliferating satellite cells showed similar results to cultured myoblasts (see Fig. S1B,C in the supplementary material). Therefore, in some aspects, neonatal satellite cells are different from regenerating satellite cells, as previously described (Pallafacchina et al., 2010).

#### Hesr1 or Hesr3 single-knockout mice show no obvious skeletal muscle phenotype

To determine the function of Hesr genes in skeletal muscle and satellite cells, we first investigated Hesr1 and Hesr3 single-knockout mice. It was previously reported that Hesr1 (1KO) and Hesr3 (3KO) knockout mice show no abnormalities in fertility or life expectancy, or any major developmental defect (Fischer et al., 2004; Fischer et al., 2007; Kokubo et al., 2005a). Consistent with these studies, skeletal muscle weight as a proportion of total body weight was similar in 1KO and 3KO mice to that of littermate wild-type (WT) mice (Fig. 1D). No obvious defects in satellite cell number or regeneration ability were observed in either single-knockout mouse (Fig. 1E,F). Because restricted co-expression of Hesr1 and Hesr3 was observed in muscle satellite cells, we generated double-knockout (dKO) mice to examine the roles of Hesr1/3 in satellite cells.

#### Hesr1/3 double-knockout mice exhibit decreased body and skeletal muscle weights

Fischer et al. reported that the survival of Hesr1/3 dKO mice depends on the genetic background (Fischer et al., 2007). In the F2 and F9 backcross generation into C57BL/6 mice, ~90% and less than 5%, respectively, of the dKO mice survived the first 3 weeks.
after birth. To obtain an adequate number of adult dKO mice for our analyses, we crossed F7 Hesr+/– Hesr3–/– mice and obtained ~40% dKO mice in the expected Mendelian ratio at 4 weeks of age. As littermate control mice, we used 3KO mice because they showed no apparent phenotype.

In contrast to the 1KO and 3KO results, compared with littermate control mice the dKO mice showed a slight decrease in body size and a significant decrease in body weight regardless of gender (see Fig. S2A in the supplementary material). Furthermore, 56- to 90-day-old dKO and 3KO mice exhibited a significant difference in muscle weight (see Fig. S2B in the supplementary material). The loss of muscle weight did not simply reflect the decreased body weight because there was also a significant difference in muscle weight as a proportion of body weight between dKO and littermate 3KO mice (Fig. 2A).

To assess the cause of muscle weight loss in dKO mice, the number and size of myofibers were quantified. As shown in Fig. 2B,C, compared with 3KO mice, a decrease in both myofiber number and size was observed in dKO mice. Because satellite cells contribute physiologically to muscle growth, these results imply that there are defects in the dKO satellite cells.

### Decreased number of satellite cells in dKO mice

To examine the effects of the Hesr1/3 deficiency on satellite cells, transverse sections of 8-week-old male mouse skeletal muscles were stained with three different satellite cell-specific antibodies: anti-Pax7, anti-M-cadherin (cadherin 15) and anti-calcitonin receptor (Fukada et al., 2007). As shown in Fig. 2D,E,F, there were significantly fewer satellite cells in dKO than in 3KO muscles. The y-axis shows the number of Pax7+ satellite cells per 100 cross-sectional myofibers with s.d. (F) A TA muscle of each 8-week-old mouse was injected with cardiotoxin and the muscles were fixed 2 weeks after the injection. The number of mice used in each study is indicated. Scale bars: 25 μm in A; 100 μm in F.
The frequency and cell size of the non-satellite cell population, including endothelial, hematopoietic and Sca1+ CD31– CD45– cells, were not affected by the absence of both Hesr1 and Hesr3 (Fig. 2G). Upon activation, the organelles and volume of cytoplasm in satellite cells increase and the cells become mitotically active as they transit from the G0 to G1 cell cycle phase (Kadi et al., 2005; Fukada et al., 2007). These results suggest that the satellite cells in dKO mice were not in a quiescent state, but in a more differentiated and/or activated state.
Increased expression of differentiation/ proliferation markers in dKO satellite cells

To elucidate the expression of proliferation and myogenic differentiation markers in dKO satellite cells, transverse sections of 10-week-old female mouse tibialis anterior (TA) muscles were stained with anti-Ki67, anti-MyoD and anti-myogenin antibodies. As shown in Fig. 3, ~20% of Pax7+ cells expressed Ki67, a proliferative marker that is not expressed in G0 cells. Intriguingly, in uninjured dKO skeletal muscle, ~70% of Pax7+ satellite cells expressed MyoD and ~6% of M-cadherin+ satellite cells expressed myogenin. In contrast to dKO satellite cells, almost all WT, 1KO and 3KO satellite cells did not express these proliferative and myogenic markers. These results indicate that most of the dKO satellite cells were not in an undifferentiated quiescent state in adult skeletal muscle.

dKO satellite cells do not enter the undifferentiated quiescent state

The dKO satellite cells exhibited an impairment in their normally undifferentiated quiescent state in adult skeletal muscle. To determine whether this unusual state of dKO satellite cells results from a failure of entry into, or an impairment in the maintenance of, the undifferentiated quiescent state, we examined the frequency and number of undifferentiated quiescent satellite cells (Pax7+ MyoD, Ki67- cells) from postnatal day 7 (P7) to P56. In P7 muscle, most Pax7+ cells were not undifferentiated quiescent satellite cells, as described previously (Fig. 4A,B) (Pallafacchina et al., 2010), but the frequency and number of undifferentiated quiescent satellite cells increased in 4- to 8-week-old 3KO mice (Fig. 4C). However, such an increase in undifferentiated quiescent satellite cells was not observed in dKO mice. Examination of the frequency of Pax7+ MyoD and of Pax7+ Ki67- cells showed similar results (Fig. 4D). These results indicate that dKO mice do not successfully generate undifferentiated quiescent satellite cells during postnatal development.

Hesr1 and Hesr3 are essential for the generation of reserve cells in vitro

To elucidate the characteristics of dKO satellite cells, satellite cells were isolated from 6- to 13-week-old 3KO and dKO mice. Even though the same numbers of cells were plated on culture dishes, a significant decrease in the number of dKO satellite cells was observed after being cultured in a growth medium (GM) for 3 days. To examine the proliferative potential of dKO satellite cells, an EdU-uptake assay was performed. As shown in Fig. 5A, the frequency of EdU+ myoblasts was similar in dKO and 3KO cultures. To examine the survival of dKO satellite cells, freshly isolated satellite cells were cultured in GM for 24 hours and then a TUNEL assay was performed. As shown in Fig. 5B, a significantly increased number of TUNEL+ cells was detected in dKO satellite cells.

We next investigated the survival of dKO satellite cells by clonal analysis. After isolating satellite cells from limb muscles of 6- to 13-week-old WT, 1KO, 3KO and dKO mice, satellite cells were cultured for 1 week. dKO satellite cell survival was lower than that of WT, 1KO and 3KO satellite cells (Fig. 5C). Low survival of dKO satellite cells was restricted to the period just after isolation from skeletal muscle, as dKO satellite cells that survived proliferated normally (Fig. 5D). These results suggest that Hesr1 and Hesr3 are not necessary for satellite cell proliferation in vitro.

To examine the roles of Hesr1 and Hesr3 in the generation of reserve cells (an in vitro model of in vivo undifferentiated quiescent satellite cells), 1×10^4 satellite cells derived from 3KO and dKO mice were cultured and allowed to differentiate in differentiation medium (DM) for 3.5 days. As shown in Fig. 5E, the fusion index of dKO satellite cells was slightly higher than that of 3KO satellite cells. By contrast, the frequency of reserve cells in dKO was substantially lower than that in WT, 1KO and 3KO satellite cell cultures (Fig. 5F). In dKO satellite cell cultures, the number of Pax7+ cells was already reduced in DM by 1.5-2 days (Fig. 5G). Because almost all Pax7+ cells expressed MyoD in both 3KO and dKO in GM (see Fig. S4 in the supplementary material), the loss of the Pax7+ MyoD population in dKO satellite cells was unlikely to affect this result. In addition, re-expression of Hesr3 was detected in Pax7+ cells in DM (Fig. 5H), and an increase in the number of apoptotic cells was not observed in dKO satellite cells in DM (data not shown). Therefore, these results indicate that Hesr1 and Hesr3 are necessary for the generation of undifferentiated satellite cells.

Gradual decrease in the number of dKO satellite cells in adult skeletal muscle

Since dKO satellite cells were not maintained in an undifferentiated quiescent state, we investigated the change in satellite cell numbers with age. As shown in Fig. 6A, a significant decrease in the number of Pax7+ satellite cells (80% of control) was observed in P7 dKO compared with 3KO mice. There is the possibility that the diminished satellite cell number at the neonatal stage leads to the
loss of muscle weight in dKO mice (Fig. 2A, see Fig. S2B in the supplementary material). Similar to previous reports (White et al., 2010), the number of satellite cells in littermate 3KO mice was constant after P28 (Fig. 6A). By contrast, the number of satellite cells in dKO mice decreased even after P28. To follow the decline of the satellite cell pool in dKO mice further, we stained satellite cells using anti-M-cadherin antibody. As shown in Fig. 6B, we obtained a result similar to that of the Pax7-staining experiment. In addition, the satellite cell frequency observed in the flow cytometry experiment indicated that the satellite cell number continued to gradually decrease even after the age of 4 weeks (Fig. 6C). These results suggest that the number of satellite cells in dKO mice was continuously decreasing even beyond the age of 4 weeks, by which time the satellite cell number has stabilized in 3KO mice.

There are two possible mechanisms for the diminution of satellite cell numbers in dKO muscle: cell death or accelerated cell fusion. We did not detect TUNEL+ satellite cells in dKO muscle in vivo, although more than 4000 M-cadherin+ cells were counted at P7, P28, P56 and P80 (data not shown). We next examined the...
possibility of accelerated cell fusion. Because many dKO satellite cells exhibited Ki67 expression in 6- to 8-week-old mice, 6- to 8-week-old dKO mice were injected with EdU three times, and 2-3 weeks later, the muscles were fixed and observed. EdU+ myonuclei, which located beneath the dystrophin, were detected in dKO myofibers (Fig. 6D). The average number of EdU+ myonuclei per section was 0.13 and 0.33 in 3KO and dKO, respectively (n=3).

Because, in general, DNA synthesis is not observed in myonuclei (Moss and Leblond, 1971), these results suggest that dKO satellite cells fused with the myofibers.

A remarkable regeneration defect in dKO mice with age
In dKO mice, immunohistological and flow cytometry studies showed satellite cell number decreases estimated to be 30-60%, 20-40% and less than 20% relative to satellite cell numbers in 4-, 8- and 20-week-old control mice, respectively (Fig. 7A). To investigate the relationship between the regenerative potential and the age of dKO mice, we injected cardiotoxin (CTX) into the TA muscle of 4-, 8- and 20-week-old mice (Fig. 7A). Three days after CTX injection, many M-cadherin+ cells were observed in the regenerating TA muscle of both 4- and 20-week-old dKO mice. However, the number of M-cadherin+ cells was significantly lower in 8-week-old than in 4-week-old dKO mice (Fig. 7B). Furthermore, 4 days after CTX injection, 8-week-old dKO mice showed a greater decrease in the embryonic myosin heavy chain (eMyHC+) myotube area than 4-week-old mice (Fig. 7C). Similar to the finding for M-cadherin+ cells, there was no significant difference in the eMyHC+ area of 4- and 8-week-old 3KO mice. Similar to the situation for satellite cell numbers, the eMyHC+ area of 4- and 8-week-old dKO mice was 30-60% and 10-30% of that of control mice, respectively. Consistent with the in vitro results, an in vivo EdU-uptake assay also indicated that the proliferative potentials of dKO and 3KO satellite cells were similar (Fig. 7D). These results suggested that the substantial decrease in M-cadherin+ cells and eMyHC+ area with age are the result of a diminished satellite cell pool in dKO mice.

We also examined the regenerative potential of dKO mice at a later stage, 14 days after injury. As shown in Fig. 7E, the injured muscle weight of dKO mice showed a tendency to decrease when mice were injected with CTX at 4 weeks of age. Consistent with the trend in satellite cell number, dKO mice injected with CTX at 8 and 20 weeks of age exhibited a remarkable loss of muscle weight (Fig. 7E). By contrast, WT, 1KO and 3KO mice did not show such a decrease in muscle weight after CTX injection.

The histological results also confirmed that there was a decrease in regeneration potential in dKO mice with age. In 3KO mice, normal regenerative potential was observed regardless of age (Fig. 7F,G). By contrast, 4-week-old dKO mice showed a slight loss of myofiber number without any fat or fibrosis accumulation (Fig. 7F,G, see Fig. S5 in the supplementary material). One of the 8-week-old dKO mice examined showed increased fat and fibrosis (3.57% and 19.7%, respectively), whereas the rest did not. In contrast to the 8-week-old dKO mice, 20-week-old mice showed significant fat and fibrosis accumulation, with a decrease in the number and area of myofibers compared with 4-week-old dKO and littermate control mice (Fig. 7F,G, see Fig. S5 in the supplementary material). These results indicate that the satellite cell number decreases with age and that this underlies the impaired skeletal muscle regeneration in dKO mice.
DISCUSSION

Notch signaling regulates stem cells in many different tissues, including the nervous system, hematopoietic system, skin and intestine. Several studies indicate that it plays essential roles in skeletal muscle as well. Vasyutina et al. reported that deletion of Rbpj in the myogenic cells of Pax3-Cre or Lbx1-Cre transgenic mice leads to premature differentiation of the myogenic progenitor pool (Vasyutina et al., 2007). As a result, the complete loss of satellite cells in E18.5 distal limb muscle and severe skeletal muscle hypotrophy with a decreased number of myonuclei were observed in these mice. Schuster-Gossler et al. showed similar results using Dll1 mutant mice (Schuster-Gossler et al., 2007).

Hes1, the major downstream target of Notch signaling, regulates the differentiation of multiple cell types (Kageyama et al., 2000). However, there is no apparent skeletal muscle phenotype in Hes1-null mice (Kageyama et al., 2000). In addition, Hesr1 and Hesr3
are predominantly induced by stimulation of C2C12 cells with Dll4, which is one of the Notch ligands, whereas induction of \( \text{Hesr}_2 \) and Hes family genes was not observed (Biau et al., 2009). We also observed \( \text{Hesr}_1 \) and \( \text{Hesr}_3 \), but not \( \text{Hesr}_2 \) and \( \text{Hesr}_1 \), expression in primary myoblasts stimulated with Dll1 (see Fig. S6A in the supplementary material).

In the present study, we showed \( \text{Hesr}_1 \) and \( \text{Hesr}_3 \) expression in neonatal and adult satellite cells and examined the physiological roles of \( \text{Hesr}_1 \) and \( \text{Hesr}_3 \) in skeletal muscle. Furthermore, upregulation of \( \text{Hesr}_1 \) and \( \text{Hesr}_2 \) was not observed in \( \text{dKO} \) satellite cells (see Fig. S6B in the supplementary material). However, the phenotype of \( \text{Rbpj} \) mutant mice is different from that of the \( \text{Hesr}_1/3 \) dKO. In contrast to \( \text{Rbpj} \) mutant mice, \( \text{Hesr}_1/3 \) dKO mice show slight losses of the satellite cell pool and decreases in myofiber number at P7 (data not shown). Therefore, although \( \text{Hesr}_1 \) and \( \text{Hesr}_3 \) might play some role, they are not essential effector genes of Notch signaling in embryogenesis. Conboy et al. indicated that downregulation of Notch signaling leads to impaired regeneration in adult skeletal muscle (Conboy et al., 2003). In addition, they showed that Notch signaling was necessary for the proliferation of satellite cells. However, in this study, \( \text{Hesr}_1/3 \) dKO satellite cells exhibited normal proliferation in vitro and in vivo. These results suggest that Notch-mediated proliferation of satellite cells is not dependant on \( \text{Hesr}_1 \) and \( \text{Hesr}_3 \).

Each Hes family gene is specifically expressed in particular tissues (Leimister et al., 1999; Nakagawa et al., 1999). Their important roles in the heart and vascular system during development have been revealed using knockout mice. In contrast to \( \text{Hesr}_2 \) knockout mice, \( \text{Hesr}_1 \) and \( \text{Hesr}_3 \) knockout mice exhibit no obvious phenotype. However, a combined defect of \( \text{Hesr}_1 \) and \( \text{Hesr}_3 \) leads to ventricular septum defects with impairment of the mesenchymal-epithelial transition in the heart (Fischer et al., 2007). It has been reported that \( \text{Hesr}_1 \) and \( \text{Hesr}_2 \) functions overlap in cardiovascular development (Fischer et al., 2004; Kokubo et al., 2005b). Redundancy among Hes family members has also been reported (Fischer and Gessler, 2007). Thus, the effect of redundancy among Hesr or Hes family members is observed in multiple tissues and organs. In this report, we showed a new complementary effect of \( \text{Hesr}_1 \) and \( \text{Hesr}_3 \) in skeletal muscle physiology, especially in muscle satellite cells. In addition, during \( \text{Hesr}_1/3 \) dKO heart development, the incidence of ventricular septum defects was not 100% (but 21% in F2 and 82% in F9). However, all of our F7 dKO mice showed decreased satellite cell numbers, demonstrating the significance of \( \text{Hesr}_1/3 \) in muscle satellite cells. Combinations of Hesr family and/or Hes family genes might also play central roles in the regulation of other stem cells to maintain diverse tissue homeostasis.

All tissue stem cells are maintained in an undifferentiated and quiescent state, and this state is considered to be essential for their long-term maintenance. Imayoshi et al. showed that adult neural stem cells differentiate into neurons via transit-amplifying cells that eventually result in complete neural stem cell depletion in \( \text{Rbpj} \) conditional knockout mice crossed with tamoxifen-inducible \( \text{Nestin-CreERT2} \) mice (Imayoshi et al., 2010). This indicates that the undifferentiated quiescent state is essential to sustain stem cell pools and that the lack of these mechanisms leads to loss of the stem cell compartment via premature differentiation. As shown here, most dKO satellite cells do not enter into the undifferentiated quiescent state during postnatal development. MyoD and myogenin are essential for myogenic differentiation. Therefore, the unusual MyoD and myogenin expression in dKO satellite cells leads to a loss of the satellite cell pool due to fusion with myofibers. Asakura et al. observed that MyoD expression is related to cell survival and that Myod-null myoblasts possess remarkable resistance to apoptosis compared with WT myoblasts in vitro (Asakura et al., 2007). In vitro, dKO satellite cells exhibit low survival in the period just after starting the culture. However, we observed no evidence for increased apoptosis in dKO mice in vivo and others have reported no evidence of TUNEL+ cells in the premature myogenic progenitor cells of \( \text{Rbpj} \) or Dll1 mutant mice during embryonic myogenesis (Schuster-Gossler et al., 2007; Vasyuta et al., 2007). These results suggest that, in vivo, dKO satellite cells undergo differentiation rather than cell death, although we cannot exclude the possibility that a low-level increase in programmed cell death contributes to the decrease in satellite cell number. Although dKO satellite cells exhibit premature differentiation but not cell death, we did not observe transient hypertrophy in dKO mice. This result suggests that: (1) a deficiency of \( \text{Hesr}_1/3 \) does not accelerate cell cycle progression, but prolongs the entrance of satellite cells into quiescence; and/or (2) a slightly lower number of satellite cells in postnatal dKO mice obscured any transient hypertrophy.

\( \text{Pax7} \)-deficient mice exhibit severe loss of satellite cells and regeneration potential (Kuang et al., 2006; Seale et al., 2000). However, a recent study using mice with inducible and conditional \( \text{Pax7} \) inactivation indicated that adult satellite cells do not require \( \text{Pax7} \) for their function and maintenance, but that satellite cells do require \( \text{Pax7} \) for 3 weeks after birth (Lepper et al., 2009). It is possible that \( \text{Hesr}_1/3 \) are also necessary for postnatal development, but not for the maintenance of the satellite cell pool in adult skeletal muscle, similar to \( \text{Pax7} \). Therefore, analyses of conditional and satellite cell-specific \( \text{Hesr}_1/3 \) mutant mice will be necessary to examine the roles of \( \text{Hesr}_1/3 \) in established adult satellite cells.

In conclusion, \( \text{Hesr}_1 \) and \( \text{Hesr}_3 \) play crucial roles in skeletal muscle homeostasis by regulating the undifferentiated quiescent state of satellite cells. Investigations of \( \text{Hesr}_1/3 \) will help to elucidate the molecular regulation of satellite cells in both physiological and pathological conditions.

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