

PDH-mediated metabolic flow is critical for skeletal muscle stem cell differentiation and myotube formation during regeneration in mice

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Short title: PDH drives skeletal muscle stem cell differentiation for regeneration

ABBREVIATIONS: PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PDC, pyruvate dehydrogenase complex; PDP, PDH phosphatase; TCA, tricarboxylic acid; OxPHOS, oxidative phosphorylation; ROS, reactive oxygen species; SMSCs, skeletal muscle satellite cells; RT-PCR, reverse transcription-polymerase chain reaction; TAM, tamoxifen; DMD, Duchenne Muscular Dystrophy; 1-AA, 1-aminoethylphosphonic acid; TMRE, tetramethylrhodamine ethyl ester perchlorate; TA, tibialis anterior; CTX, cardiotoxin; HIF, Hypoxia-inducible factor; CSA, cross section area; WT, wild-type

ABSTRACT: Skeletal muscle satellite cells (SMSCs), the major stem cells responsible for the regeneration of skeletal muscle, are normally cell cycle arrested but differentiate to generate myocytes upon muscle damage, forming new myofibers along with self-renewing stem cells in preparation for subsequent injury. In this study, we investigated which factors stimulate the proliferation and differentiation of SMSCs, and found that pyruvate, the end product of glycolysis, stimulates their differentiation. Pyruvate antagonizes the effects of hypoxia on preferential self-renewal of SMSCs through dephosphorylation/activation of pyruvate dehydrogenase (PDH), which mediates opening of the gateway from glycolysis to the tricarboxylic acid (TCA) cycle by producing acetyl CoA from pyruvate. Pyruvate dehydrogenase kinase 1 (PDK1) highly expressed under hypoxia is down-regulated under normoxic condition, leading to increase in dephosphorylated PDH. Conditional deletion of PDH in SMSCs affects cell divisions generating myocytes and subsequent myotube formation, inefficient skeletal muscle regeneration upon injury, and aggravated pathogenesis of a dystrophin-deficient mouse model of Duchenne Muscle Dystrophy (DMD). Thus, the flow from glycolysis to the TCA cycle mediated by PDH plays a pivotal role in differentiation of SMSCs, which is critical for the progression of skeletal muscle regeneration.

KEY WORDS: satellite cell, tissue regeneration, cell division, metabolic control

Much attention has recently been paid to the intimate relationship between metabolic control and tissue and organ development, homeostasis, and ageing (1, 2). Growing evidence suggests that metabolic control underlies stem cell maintenance and differentiation. Inner cell mass-derived embryonic stem cells exhibit high glycolytic activity similar to the Warburg effects of cancer cells, which is gradually substituted by respiration during organogenesis (3, 4). Undifferentiated paraxial mesodermal cell glycolysis is critical for their fate specification and axial elongation. These stem cells then gradually alter their mode of energy production to tricarboxylic acid (TCA) cycle/oxidative phosphorylation (OxPHOS) in mitochondria prior to somitogenesis (5).

Metabolic control also plays critical roles in adult hematopoietic stem cell functions. Changes in their state are associated with energy metabolism, glycolysis, and OxPHOS alterations (6–8). Whereas pyruvate dehydrogenase (PDH) is important for the metabolic switch from anaerobic glycolysis to the aerobic TCA cycle (9, 10), the activity of its regulator, pyruvate dehydrogenase kinase (PDK), causes predominant glycolysis, leading to maintenance of the undifferentiated hematopoietic stem cell state (6, 7). Induced pluripotent stem cell-derived neural progenitor cell differentiation into neurons is also accompanied by the glycolysis to TCA/OxPHOS transition (11). Moreover, mitochondrial reactive oxygen species (ROS) generation is required for mesenchymal stem cell differentiation into adipocytes (12). These studies suggest a general role of metabolic control in both embryonic and adult stem cell maintenance and differentiation.

To further clarify the nature and roles of metabolic control in stem cells, it is necessary to elucidate whether metabolic control also regulates injured tissue regeneration; i.e., the dynamic aspects of adult stem cells including the control of self-renewal, differentiation, and morphogenesis. Skeletal muscle is an ideal organ for investigating regeneration and stem cell behaviors. Skeletal muscle satellite cells (SMSCs), comprising stem cells that align with skeletal muscle fibers, underlie muscle fiber regeneration. Once muscle fibers suffer from injury or degenerate owing to diseases such as skeletal muscle dystrophy, Pax7-positive, cell cycle-arrested (quiescent) SMSCs rapidly arise, proliferate, differentiate, and fuse to form new muscle fibers that replace damaged ones. The transcription factors Myf5/MyoD and myogenin are essential for SMSC myogenic activation and differentiation during regeneration, respectively (13). Asymmetric cell divisions are involved in the simultaneous Myf5/MyoD-positive myoblast generation and Pax7-positive stem cell self-renewal.

These stem cells lose expression of Pax7 after differentiation (14–16); moreover, several mechanisms such as p38 MAPK activation and autophagy modulate their activation, cell fate choice, differentiation, and aging (17–22).

In this scenario, key questions include whether metabolic controls are involved in regulating SMSC self-renewal and differentiation and whether SMSC metabolic defects impact skeletal muscle regeneration. In this study, we demonstrate that the glycolysis to TCA cycle progression is critical for efficient SMSC differentiation into myocytes and regenerative myotube formation by analyzing mice and cultivated SMSCs in which PDH activity is genetically and conditionally deleted.

MATERIALS AND METHODS

Mice

B6.129P2-*Pdhal*^{tm1ptl}/J mice, in which two loxP sites are inserted into intron sequences flanking *Pdhal* exon 8, B6.Cg-*Pax7*^{tm1(cre/ERT2)Gata}/J mice, and B6.Cg-*Gt(ROSA)26Sor*^{tm14(CAG-tdTomato)Hze}/J mice were purchased from the Jackson Laboratory (stock number 017443, 017763, and 007914 respectively). Male adult mice (8–20 weeks old) were used. In timelapse imaging, female mice (20 days old) were used. *Pax7*^{CreERT2/+}; *Pdh*^{fllox8/-} and *Pax7*^{CreERT2/+}; *ROSA26*^{td-Tomato/+}; *Pdh*^{fllox8/-} mice were generated by crossing *Pdh*^{fllox8/-} and *ROSA26*^{td-Tomato/+} mice with *Pax7*^{CreERT2/+} mice to isolate PDH-deficient SMSCs. *DMD*-null mice (23), in which the dystrophin gene is entirely deleted, provided by Dr. K. Hanaoka, were crossed with *Pax7*^{CreERT2/+}; *Pdh*^{fllox8/-} mice. Tamoxifen (TAM; Sigma-Aldrich), 200 µl of 10 mg/ml in peanut oil (Sigma-Aldrich), was administered by intraperitoneal injection.

Culture of C2C12

We plated 1.0×10^4 C2C12 (obtained from American Type Culture Collection) cells per collagen type I-coated dishes with a diameter of 35 mm (Iwaki) in pyruvate-free or -added DMEM-high glucose (Nacalai Tesque) supplemented with 10% FBS (Moregate Biotech) and 1% penicillin-streptomycin (Nacalai Tesque)

and cultured them for 2 days. Cells were cultured with or without 10mM 1-aminoethylphosphonic acid (1-AA; Tokyo chemical industry). Culture medium was changed daily.

Primary culture

Mouse peritoneal muscles were dissected, digested with 0.2% collagenase type 2 (Worthington Biochemical) for 90 min at 37 °C, triturated, and washed to obtain mononucleated cell suspensions. We plated 1.0×10^5 cells per collagen type I-coated dishes with a diameter of 35 mm (Iwaki) in DMEM-high glucose (Nacalai Tesque) supplemented with 20% FBS (Moregate Biotech) and 1% penicillin-streptomycin (Nacalai Tesque) and cultured them for 4 days at 37 °C. Culture medium was changed daily.

TMRE and Mito Tracker staining

C2C12 cells and SMSCs digested from peritoneal muscle of mice plated on collagen type I-coated glass based dishes with a diameter of 35 mm (Iwaki) were incubated with 250 nM tetramethylrhodamine ethyl ester perchlorate (TMRE; T669, Invitrogen) and 50 nM Mito Tracker Deep Red (M22426, Invitrogen) for 30 min at 37 °C. Cells were observed using fluorescence microscopes (TCS SP8, Leica Microsystems). Evaluation of fluorescence intensity was carried out with ImageJ software.

Lactate measurement assay

Measurements of lactate concentration in cell culture medium were performed using Lactate Assay Kit-WST (Dojindo Molecular technologies) and microplate reader (GloMax Explorer System GM3500, Promega) according to the manufacturer's instructions.

Muscle injury

Injury of skeletal muscle was induced by injecting 50 µl of 10 µM cardiotoxin (CTX; Sigma-Aldrich) dissolved in PBS into the left tibialis anterior (TA) muscle with a 29 gauge needle. The right TA was injected 50 µl of PBS as a control. Mice were anesthetized by isoflurane (Mylan).

Histological analyses

Tibialis anterior (TA) muscles were isolated and frozen in liquid nitrogen-chilled isopentane (Nacalai Tesque). Frozen sections (10 μ m) were created using a cryostat (CM3050S, Leica Microsystems). These sections were stained with H&E staining. Cross section area (CSA) was evaluated on TA muscle sections after H&E staining using DP2 BSW software (Olympus). The number of muscle fibers was counted in any three areas of H&E stained sections. The weight of TA muscle was measured (BP301S, Sartorius) prior to making frozen sections.

Isolation and cell culture of SMSCs

Leg and peritoneal muscles of mice were dissected, digested with 0.2% collagenase type2 (Worthington Biochemical) for 90 min at 37 °C, triturated, and washed to obtain mononucleated cell suspensions. Dissociated cells were filtrated and labeled with SM/C-2.6 mouse IgG-biotin (1:100) (24), a gift from Dr. S. Fukada, CD45-PE (1:800, 30-F11, BD Pharmingen), CD31-PE (1:400, MEC 13.3, BD Pharmingen), SCA-1-PE (1:400, D7, Bio Legend) for 30 min. After centrifugation, cells were incubated with streptavidin-APC (1:400, Invitrogen) for 30 min in order to detect the SM/C-2.6 signal. Resuspended cells were filtered and stained with propidium iodide solution (PI; BioLegend) to exclude dead cells. Cell sorting and analysis were performed with FACS Aria II (BD). In culture experiments, we plated 1.0×10^4 sorted SMSCs per collagen I-coated dishes with a diameter of 35 mm (Iwaki) in pyruvate-free or -added DMEM-high glucose supplemented with 20% FBS and 1% penicillin-streptomycin and cultured them for 5 or 8 days at 37 °C. Culture medium was exchanged daily. In the differentiation induction experiment, the culture medium was changed to differentiation medium, DMEM-high glucose supplemented with 2% horse serum (HS) and 1% penicillin-streptomycin on day 4, and cultured for 2 days. In hypoxic culture, cell culture was performed under 2% oxygen concentration condition at 37 °C in a cell incubator (MCO-5MUV-PJ; Panasonic).

Immunofluorescence

Cultured cells were fixed with 4% paraformaldehyde at room temperature for 15 min, permeabilized with 0.5% Triton-X100/PBS at room temperature for 15 min, blocked with Blocking One (Nacalai Tesque) at room temperature for 30 min, and incubated with primary antibodies diluted in Blocking One at 4 °C overnight.

Primary antibodies were diluted as follows: Pax7 (1:200, Developmental Studies Hybridoma Bank), MyoD (1:200, Santa Cruz Biotechnology), myogenin (1:200, Santa Cruz Biotechnology), MF20 (1:200, Developmental Studies Hybridoma Bank), PDH-E1 α (1:200, Abcam), PDH-E1 α (pSer²³²) (1:200, Calbiochem), Caspase3 (1:200, 559565, BD Pharmingen), Ki67 (1:200, ab15580, Abcam). Alternatively, frozen sections were fixed 4% paraformaldehyde for 20 min, permeabilized with methanol at -20 °C for 6 min, blocked with 50 mM NH₄Cl, reacted with 0.01 M citric acid (pH 6.0) at 80 °C for 10 min for antigen retrieval, blocked using the M.O.M kit (Vector Laboratories) at room temperature for 1 h, and incubated with primary antibodies diluted in staining buffer (2% HS and 2% FBS /PBS) at 4 °C overnight. Primary antibodies were diluted as follows: Pax7 (1:100, Developmental Studies Hybridoma Bank), myogenin (1:500, Santa Cruz Biotechnology), PDH-E1 α (1:500, Abcam), collagen VI (1:500, Chemicon), and laminin α 2 (Santa Cruz Biotechnology). Cells or sections were washed with PBS and incubated with secondary antibodies conjugated with Alexa Fluor 488, 594, or 647 (1:500, Molecular Probes) at room temperature for 1 h. DAPI (1:5000, Molecular Probes) was used to stain nuclei acid. Fluorescence was observed using fluorescence microscopes (DMi8 AF7000 and TCS SP8, Leica Microsystems). Evaluation of fluorescence intensity was carried out with ImageJ software.

Live cell imaging

Leg and peritoneal muscles of *Pax7^{CreERT2/+};ROSA26^{td-Tomato/+}* mice were dissected and then digested in the same way as in the primary culture. We plated 1.0×10^5 cells per collagen type I-coated glass based dishes with a diameter of 35 mm (Iwaki) in DMEM-high glucose (Nacalai Tesque) supplemented with 20% FBS (Moregate Biotech) and 1% penicillin-streptomycin (Nacalai Tesque) and cultured them at 37 °C. Timelapse images were taken every 15 minutes. Images were observed using fluorescence microscopes (DMi8 AF7000, Leica Microsystems).

PCR

Genomic DNA from mouse tail or sorted SMSCs was isolated with 50 mM NaOH at 95 °C for 15 min and then 1 M tris-HCl (pH 8.0) was added. PCR was performed using KOD FX (Toyobo) according to the

manufacturer's instructions. The presence of *Pdhal* alleles was determined by PCR analysis of genomic DNA and specific sets of primers as follows: *Pdhal* (F; 5'-AGC AGC CAG CAC GGA CTA CT-3', R; 5'-GCA GCC AAA CAG ATT ACA CC-3') (25).

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cultured cells using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. For analysis of mRNA expression, cDNA was synthesized with Oligo dT primers (Life Technologies) and SuperScript III Reverse Transcriptase (Invitrogen). qRT-PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) on StepOnePlus (Applied Biosystems). The target gene expression was calculated relative to values from ribosomal protein (*Rpl13a*) as a control. Primer sequences are as follows: *Rpl13a* (F; 5'-GTG GTC CCT GCT GCT CTC AAG-3', R; 5'-CGA TAG TGC ATC TTG GCC TTT T-3'), *Pax7* (F; 5'-AGG CCT TCG AGA GGA CCC AC-3', R; 5'-CTG AAC CAG ACC TGG ACG CG-3'), *MyoD* (F; 5'-AGC ACT ACA GTG GCG ACT CA-3', R; 5'-GGC CGC TGT AAT CCA TCA T-3'), myogenin (F; 5'-CAA CCA GGA GGA GCG CGA TCT CCG-3', R; 5'-GGC GCT GTG GGA GTT GCA TTC ACT-3'), *Pdk1* (F; 5'-GAC TGT GAA GAT GAG TGA CCG-3', R; 5'-CAA TCC GTA ACC AAA CCC AG-3') (26), *Pdk2* (F; 5'-AAG AGA TCA ACC TGC TTC CTG-3', R; 5'-GCA TCT GTG AAC TGG CTT AGA G-3') (26), *Pdk3* (F; 5'-GCC AAA GCG CCA GAC AAA C-3', R; 5'-CAA CTG TCG CTC TCA TTG AGT-3'), and *Pdk4* (F; 5'-AGT GAC TCA AAG ACG GGA AAC-3', R; 5'-GTG TGA GGT TTA ATT CTG GCG-3') (26).

Statistical analyses

Statistical analyses were carried out with EZR (27) software using a paired t test or student's t test between two groups and one-way ANOVA with post hoc Tukey's test among more than three groups. Error bars represent standard error of the mean (s.e.m.). A *P*-value < 0.05 was considered statistically significant.

RESULTS

Pyruvate promotes SMSC proliferation and differentiation in culture

Exploration of culture media suitable for SMSC proliferation found that pyruvate significantly promotes SMSC proliferation (Fig. 1). Because SMSCs differentiate gradually in culture, we examined whether pyruvate also promotes their differentiation. Immunostaining of SMSCs cultured in pyruvate-free or -added medium for 5 days with anti-MyoD or anti-myogenin together with anti-Pax7 antibodies indicated that the Pax7⁻ myogenin⁺ but not the Pax7⁻ MyoD⁺ population was significantly larger in the latter medium (Fig. 2A, B). Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis verified that SMSCs cultured in pyruvate-added medium decreased Pax7 and increased myogenin expression (Fig. 2C). Thus, pyruvate promotes SMSC differentiation to myogenin⁺ myocytes.

Pyruvate antagonizes hypoxia effects on PDH phosphorylation

Because hypoxic conditions can suppress SMSC differentiation (28–32), we examined the effects of pyruvate on SMSC proliferation and differentiation under hypoxia. Comparison of SMSCs cultured under 2% (hypoxia) and 21% (normoxia) oxygen showed the former as preferential for Pax7⁺ myogenin⁻ stem cell proliferation and self-renewal. Pyruvate further stimulated proliferation (Fig. 3A) while increasing the Pax7⁻ myogenin⁺ population (Fig. 3B, D and Supplemental Fig. S1). Thus, pyruvate antagonized the effect of hypoxia on SMSC self-renewal and stimulated cell differentiation into myocytes.

Pyruvate, the end product of glycolysis, is also located at the entrance of the TCA cycle in the metabolic map. Pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase complex (PDC), which consists of PDH (E1), dihydrolipoyltransacetylase (E2), and dihydrolipoamide dehydrogenase (E3); the produced acetyl-CoA is incorporated into the TCA cycle for efficient ATP production via OxPHOS in mitochondria. Based on the competitive effects of pyruvate and hypoxia on SMSC self-renewal and differentiation, we examined whether hypoxia and pyruvate oppositely modulate the metabolic balance between glycolysis and the TCA cycle. PDC activity is controlled by phosphorylation/dephosphorylation of PDH-E1 α , the PDH α -subunit (33, 34), and PDK mediates PDH inactivation through its phosphorylation. QRT-PCR analysis revealed that expression of PDK1, among the four PDK isozymes (PDK1–4) (35), is significantly increased in SMSCs under hypoxic condition but not affected by the presence of pyruvate (Fig. 3C); PDK2, PDK3, and PDK4 expression did not

change under hypoxia (Supplemental Fig. S2). Stimulated PDK1 and attenuated PDH-E1 α expression resulted in increased PDH-E1 α phosphorylation at ser232 under hypoxia (Fig. 3E), which may explain how hypoxia efficiently blocked the glycolysis to TCA cycle switch. In contrast, pyruvate addition to the culture medium under hypoxia reduced its ser232 phosphorylated form (Fig. 3E), suggesting that pyruvate antagonizes the hypoxia effects and converts it to an enzymatically active form partially but significantly. Thus, pyruvate, the end product of glycolysis, likely induces SMSCs to initiate the TCA cycle by overriding the stimulatory effects of hypoxia on *PDK1* and changing the enzymatic balance from a PDK- to a PDH-dominant status.

PDH-deficient SMSCs exhibit defective terminal differentiation

Pyruvate stimulatory effects on SMSC differentiation and PDH-E1 α activation/dephosphorylation under hypoxia suggested that SMSC differentiation is dependent on energy production conversion from anaerobic glycolysis- to aerobic TCA cycle-driven mode through PDH activation. To confirm this concept, we genetically destroyed PDH/PDC activity to block the entry from glycolysis into the TCA cycle. PDH-E1 α , which contains the E1 active site, is encoded by *Pdha1* and *Pdha2*. *Pdha1*, herein abbreviated as *Pdh*, is located on chromosome X and expressed in somatic tissues, whereas *Pdha2* located on chromosome 19 is expressed only in the testis in mice (36–38). This indicates that PDH/PDC activity in SMSCs can be eliminated in *Pax7^{CreERT2/+};Pdh^{lox/Y}* male (herein annotated as *Pdh^{lox/-}*) mice. As *Pax7* function is not compromised in *Pax7^{CreERT2/+}* and *Pax7^{CreERT2/CreERT2}* mice (39), *Pax7^{+/+};Pdh^{lox/-}* mice were used as controls in in vivo experiments considering the influence of two loxP sites inserted into intron sequences flanking *Pdha1* exon 8 (25). By repetitive TAM (200 μ l of 10 mg/ml in peanut oil) administration prior to muscle isolation (Fig. 4A), PDH activity was efficiently eliminated in SMSCs and their derived myocytes (Supplemental Fig. S3). We also confirmed PDH deficiency specifically in regenerating muscle fibers in *Pax7^{CreERT2/+};Pdh^{lox/-}* (*Pdh^{cKO}*) mice albeit normal PDH expression and muscle regeneration in control mice (Supplemental Fig. S4A). Additionally, fluorescent protein td-Tomato was expressed only in regenerating muscle fibers and SMSCs after injury in *Pax7^{CreERT2/+};ROSA^{td-Tomato/+};Pdh^{lox/-}* mice (Supplemental Fig. S4B), indicating lack of cre leakage unless its activity in nuclei was induced with TAM.

PDH-deficient SMSCs isolated from Pdh^{cKO} mice differentiated similarly to those of wild-type (WT) in 5 days of culture (Fig. 4B). After 8 days, no significant difference was found in proliferation regardless of PDH activity, although $\text{Pax7}^- \text{myogenin}^+$ cell ratios significantly decreased in PDH-deficient SMSCs (Fig. 4B, C and D), suggesting that PDH activity in SMSCs is required for SMSC differentiation in a relatively later phase of the culture.

PDH is required for cell divisions to generate myocytes

Previous studies demonstrated asymmetric SMSC divisions, generating pairs of a self-renewing stem cell and a differentiated myoblast (14–16). Here, we addressed whether PDH activity regulates asymmetric SMSC divisions or is involved in expansions of myoblasts and/or myocytes essential for regenerating myotube formation. Live imaging of cultured SMSCs identified them as pairs of morphologically identical spherical cells clearly distinguishable from cells in other phases immediately after cell divisions (Supplemental Movie S1). Using these morphological features as signs of cells immediately post-division, we explored the types of cell divisions affected in PDH-deficient SMSCs. When a pair of $\text{Pax7}^+ \text{MyoD}^-$ (Pd) or $\text{Pax7}^+ \text{myogenin}^-$ (Pm) cells was observed, they were regarded as symmetric cell divisions for self-renewal and termed as Pd+Pd and Pm+Pm, respectively. Similarly, a pair of $\text{Pax7}^+ \text{MyoD}^+$ (PD), $\text{Pax7}^- \text{MyoD}^+$ (pD), $\text{Pax7}^+ \text{myogenin}^+$ (PM), and $\text{Pax7}^- \text{myogenin}^+$ (pM) cells (i.e., PD+PD, pD+pD, PM+PM, and pM+pM) were regarded as symmetric cell divisions of differentiated cells (Fig. 5A, B). Pd+pD, for example, was instead regarded as an asymmetric cell division. Results of the analyses after 8 days of culture are summarized in Fig. 5C; precise data are shown in Supplemental Fig. S5. SMSCs at this stage mainly divide to produce pairs of the same cell types regardless of PDH activity. However, clear difference was observed between WT and Pdh^{cKO} SMSCs; symmetric cell divisions producing stem cells, Pd+Pd and Pm+Pm, and those producing myogenin⁺ cells, PM+PM and pM+pM, significantly increased and decreased, respectively, in the absence of PDH activity. PDH-deficient SMSCs showed unchanged PD+PD and pD+pD (Fig. 5C) but fewer asymmetric Pd+pD and Pm+pM cell divisions, although this was not significant (Supplemental Fig. S5). These results suggested that PDH is required for efficient SMSC terminal differentiation into myocytes.

Because SMSC differentiation into myocytes is followed by their fusion to form myotubes, we further examined roles of PDH in SMSC myotube formation. SMSCs were cultured in growth medium for 4 days, then in differentiation medium for 2 days (Fig. 6A). Immunostaining showed that PDH-deficient SMSCs form fewer MF20⁺ myotubes than controls, which was further supported by the fusion index (Fig. 6B, C). Thus, PDH is dispensable for myocyte fusion but is involved in efficient SMSC myotube formation.

To examine whether cell divisions of myoblasts/SMSCs require PDH, we examined effects of PDH deficiency and pyruvate on the rate of Ki67-positive cells in cultured SMSCs or C2C12 cells. The ratio of Ki67-positive cells was much lower in PDH-deficient SMSCs than that in WT SMSCs. Addition of pyruvate to WT SMSCs further increased Ki67-positive cells (Fig. 7A). Ratios of Ki67-positive cells increased and decreased in C2C12 cells when they were treated with pyruvate and with 1-aminoethylphosphonic acid (1-AA), an analogue of pyruvate and PDH inhibitor (7, 40), respectively (Fig. 7B). On the other hand, we confirmed that enhanced growth of WT SMSCs with pyruvate and decreased growth of PDH-deficient SMSCs were not due to the decreased and increased cell death, respectively, detectable with caspase3 staining (data not shown). Thus, PDH is involved in cell proliferation of SMSCs and C2C12 cells.

PDH deficiency in SMSCs affects muscle regeneration and exacerbates skeletal muscle pathology in Duchenne Muscular Dystrophy (DMD) mice

Average cross section area of intact muscle of *Pax7*^{+/+}; *Pdh*^{lox/-} (control) and *Pdh*^{CKO} mice did not significantly differ (Supplemental Fig. S6A, B). To elucidate roles of PDH in skeletal muscle regeneration, we examined TA muscle regeneration induced by CTX injury (Fig. 8A). Muscle fibers with central nuclei were observed in both *Pdh*^{CKO} and control mice, indicating that muscle regeneration occurs in the absence of PDH (Fig. 8B). However, regenerating muscle fibers had significantly smaller diameter in *Pdh*^{CKO} than control mice at day 7, 14, and 30 after CTX injury (Fig. 8B, C). Moreover, TA muscle weight was significantly lighter in *Pdh*^{CKO} than control mice at day 30 after CTX injury although no significant difference was found in the TA cross section area or fiber number (Supplemental Fig. S7). It is because of the increased interstitial areas although muscle fibers themselves become thinner. Immunohistochemistry of TA muscle at day 7 after CTX injury using antibodies against myogenin and laminin showed that most myogenin⁺ cells were located inside the

regenerating muscle fibers surrounded by laminin⁺ basal lamina in control mice but were scattered in the interstitium outside the basal lamina in *Pdh*^{CKO} mice. In addition, collagen VI-rich interstitial areas were expanded in the latter group (Fig. 8D, E and, Supplemental Fig. S6C). These results suggested that PDH-deficiency in SMSCs affects formation of myotubes surrounded by the basal lamina during regeneration.

Skeletal muscle degeneration and regeneration repeatedly occurs in dystrophin knockout (*DMD-null*) model mice for Duchenne Muscular Dystrophy (DMD) (23). We examined whether the lack of PDH in SMSCs affected *DMD-null* mice disease development by comparing *Pax7*^{+/+}; *Pdh*^{fllox/-}; *DMD*^{-/-} and *Pax7*^{CreERT2/+}; *Pdh*^{fllox/-}; *DMD*^{-/-} mice. TAM injection exacerbated muscle degeneration in the latter after 1 month (Fig. 9), suggesting that defective myocyte production in PDH-deficient SMSCs is amplified in *DMD-null* mice because of repetitive myofiber regeneration in their muscles.

In order to prove that pyruvate and PDH regulate metabolic switch from glycolysis to TCA/OxPHOS, we evaluated mitochondrial activity of WT and PDH-deficient SMSCs by measuring mitochondrial transmembrane potential. A fluorescent dye tetramethylrhodamine ethyl ester (TMRE) detects polarized proton gradient of mitochondrial inner membrane used for ATP production in living cells (41). Mito Tracker labels both polarized and depolarized membranes, and thus, it can monitor the size of functional mitochondria. The measurement of fluorescence intensities of TMRE and Mito Tracker in individual cells revealed that both mitochondrial membrane potential and mass were lower in cultured PDH-deficient SMSCs than those in WT SMSCs (Fig. 10A, B). Similarly, both TMRE and Mito Tracker intensities were lowered in C2C12 cells cultured with 1-AA while their mitochondrial membrane potential was increased by pyruvate addition (Fig. 10C, D). These results show that both mitochondrial activity and mitochondrial mass are affected in PDH-deficient SMSCs. On the other hand, the effects of pyruvate and 1-AA on mitochondrial activity and those on lactate production were evaluated with C2C12 cells simultaneously so that we could compare glycolysis and mitochondrial activity in the same dishes. As a result, the level of lactate that accumulated in the culture medium increased and decreased by the addition of 1-AA and pyruvate, respectively (Fig. 10E). Thus, pyruvate enhances mitochondrial activity and attenuate lactate production whereas 1-AA increases lactate production and decreases mitochondrial activity. The results clearly show that pyruvate and PDH indeed regulate metabolic switch from glycolysis to TCA/OxPHOS.

DISCUSSION

In this study, we revealed that opening the gateway from glycolysis to the TCA cycle mediated by PDH is required for efficient SMSC differentiation and myogenesis. First, pyruvate stimulates SMSC terminal differentiation into myocytes by antagonizing effects of hypoxia on SMSC preferential self-renewal (31) through dephosphorylation/activation of PDH (Fig. 3*B, D* and *E*). Second, genetic, conditional PDH deletion in SMSCs results in inefficient SMSC differentiation into myocytes and myotube formation (Fig. 4, 5 and 6), affects muscle regeneration upon cardiotoxin-induced injury, and aggravates *DMD-null* mouse muscle degeneration (Fig. 8, 9).

Adult hematopoietic stem cell self-renewal relies on anaerobic glycolysis (7) whereas inhibiting mitochondrial respiration causes a defect in differentiation-associated divisions (42). Our study suggests the common metabolic regulation of adult stem cells; their differentiation requires more efficient ATP production pathways than self-renewal. Significance of the metabolic switch from glycolysis to the TCA/OxPHOS in adult tissue stem cells has previously been enigmatic. Tissue stem cell microenvironments contain different kinds of nutrients, e.g., fatty acids and amino acids as well as carbohydrates; thus, pyruvate as well as other pathways such as fatty acid beta-oxidation can promote the TCA cycle, in turn affecting regeneration (43). By deleting PDH activity specifically in SMSCs, our results demonstrate that entry into the TCA cycle plays a major role in SMSC terminal differentiation and regeneration, suggesting that terminal differentiation, such as myocyte expansion and myotube formation, requires a robust flow of energy production in SMSCs mediated by the TCA/OxPHOS. Alternatively, PDH-mediated entry into TCA cycle enhances production of acetyl-CoA and acetate, which might cause epigenetic activation of SMSCs differentiation. Such effects are observed in differentiation of pluripotent stem cells (4). Taking a previous finding that myogenic differentiation of C2C12 cells are transiently regulated by lactate (44) into consideration, increased lactate produced in PDH-deficient SMSCs could also contribute to their suppression of terminal differentiation to some extent. Because muscle regeneration upon injury still occurs to some extent in the absence of PDH

activity in SMSCs, other metabolic pathways contribute to myocyte production and likely to the regeneration process as well (45).

In contrast to proliferating tumor cells that tolerate PDH deficiency (46), organ-specific PDH deficiency causes abnormality in various tissues; e.g., structural and biochemical abnormality in the developing nervous system (47), and impaired pancreatic β cell insulin secretion (48). Male mice with heart/skeletal muscle PDH deficiency can survive on a high-fat diet but develop left ventricular hypertrophy and dysfunction (49). PDH requirements in adult stem cells such as SMSCs for differentiation and morphogenesis likely closely associate with organs and tissue function and morphological maintenance.

Our division pattern analyses suggest that altering the balance between stem cell self-renewal and myocyte differentiation highly depends on PDH-mediated entry into the TCA cycle. However, we found no evidence supporting such metabolic control in MyoD activation or the choice between symmetric or asymmetric stem cell division; although divisions generating Pd cell pairs increased upon PDH loss, the decreased asymmetric Pd+pD division pattern of PDH-deficient SMSCs was not statistically significant (Supplemental Fig. S5). Because PDH-deficient SMSCs differentiate properly during the initial phase of culture, we tentatively interpret the increased ratio of self-renewing cells as a secondary effect of their defective terminal differentiation. To clarify the role of PDH in SMSC asymmetric cell divisions, more precise analysis of cell division *in vivo* and gene expression patterns at single cell levels would be needed.

Our study also explored potential mechanisms linking glycolysis to the TCA cycle during SMSC differentiation. PDKs regulate PDH-E1 α phosphorylation at three serine residues (Ser232, Ser293, and Ser300) (26, 34). PDK1 is expressed mainly in the heart (35, 50) and efficiently phosphorylates Ser232 (26). We found that PDK1 in particular is significantly active under hypoxia in SMSCs (Fig. 3C). Despite the abundance of PDK2 and PDK4 in skeletal muscle, their gene expression levels did not change (Supplemental Fig. S2). Thus, PDK1 mediated PDH-E1 α phosphorylation at Ser232 in SMSCs under hypoxic condition (Fig. 3E), leading to their glycolysis-dominant status. This is consistent with previous reports showing that PDK1 is activated under hypoxic conditions and important for converting from aerobic to glycolytic metabolism (51–53).

Hypoxia-inducible factors HIF1 α and HIF2 α are involved in SMSC differentiation during muscle regeneration (54–57). SMSCs of HIF1 α /HIF2 α double knockout mice exhibit decreased self-renewal and increased differentiation, the converse of PDH-deficient SMSCs, owing to defective Notch signaling activation (56). The stem cells surrounding each tissue have different environments including oxygen concentration (58, 59). During skeletal muscle regeneration, SMSCs are exposed to much higher oxygen levels than usual through infiltration of inflammatory cells and serum into the injured sites. HIF-1 α and -2 α are oxygen-sensitive and destabilized by post-translational prolyl hydroxylation under exposure to high oxygen levels. Prolyl hydroxylation leads to von-Hippel-Lindau protein-dependent HIF-1 α ubiquitination and rapid proteasomal degradation (29, 58). PDK1 is an important target of HIF1 α (60). Our finding therefore indicates that a release from hypoxic environment stimulates dephosphorylation/activation of PDH through transcriptional downregulation of HIF-1 α -PDK1 pathway in SMSCs, which in turn stimulates their divisions generating myocytes and myotube formation.

Moreover, pyruvate is known to inhibit PDK as a substrate, and activates PDH (61). Thus, pyruvate addition dephosphorylates PDH-E1 α Ser 232 in SMSCs even under hypoxic conditions, likely by dampening the PDK1 activity. However, effects of pyruvate on dephosphorylation of PDH-E1 α Ser 232 in SMSCs cultured under hypoxia was incomplete compared to those under normoxia, suggesting existence of other mechanisms that contribute to the dephosphorylation of PDH in SMSCs upon exposure to high level of oxygen. In particular, we should note that phosphorylated PDH needs be activated by PDH phosphatases (PDPs), of which regulatory mechanisms have been less clarified than PDKs. Further studies are required to elucidate additional mechanisms by which high level of oxygen activates dephosphorylation of PDH in SMSCs.

In summary, this study uncovered critical roles of PDH- mediated entry from glycolysis to the TCA cycle in SMSC divisions generating myocytes and their myotube formation (Fig. 11). The PDH deficiency in SMSCs affects muscle regeneration after injury and aggravates *DMD-null* mouse pathogenesis probably due to unbalanced glycolysis and mitochondrial function in SMSCs. Fundamental questions to be solved in future include mechanisms by which PDH is activated when SMSCs are activated from quiescence, mechanisms how the PDH-mediated metabolic switch drives SMSC differentiation and myotube formation, and the relationship to known cellular and molecular mechanisms for differentiation, myogenesis, or aging of SMSCs

(16, 19) and other types of stem cells. Our findings provide clues for effective remedies for skeletal muscle dystrophy and related degenerative diseases, and may be applicable for developing improved culture protocols of SMSCs and other tissue stem cells.

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AUTHOR CONTRIBUTIONS

S. Hori and A. Sehara-Fujisawa designed research; S. Hori and Y. Hiramuki performed research; S. Hori, D. Nishimura, F. Sato, and A. Sehara-Fujisawa analyzed data; S. Hori and A. Sehara-Fujisawa wrote the paper.

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Figure Legends

Figure 1. Pyruvate promotes SMSC proliferation. *A)* Flow cytometry plots of CD31⁻/CD45⁻/Sca1⁻/SMC⁺ SMSCs. SMSCs are contained in the Q1 fraction. *B)* SMSCs were cultured in pyruvate-free or -added medium for 5 days. Scale bars = 200 μ m. *C)* Quantification of experiments shown in (*B*) (n = 3, each group). Error bars represent s.e.m. Paired t test, **P* < 0.05.

Figure 2. Pyruvate promotes SMSC differentiation. *A)* Ratios of SMSCs expressing Pax7 and/or MyoD (left) and Pax7 and/or myogenin (right). SMSCs were cultured in pyruvate-free or -added medium for 3 or 5 days. (n = 4, each group). Error bars represent s.e.m. Paired t test, **P* < 0.05. n s, not significant. *B)* Immunofluorescence staining using antibodies against Pax7 (red), myogenin (green), and DAPI (blue) of SMSCs cultured in pyruvate-free or -added medium for 5 days. Scale bars = 50 μ m. *C)* qRT-PCRs of transcripts for *Pax7*, *MyoD*, and myogenin genes expressed in SMSCs cultured in pyruvate-free or -added medium for 5 days. (n = 3, each group). Error bars represent s.e.m. Paired t test, **P* < 0.05. n s = not significant.

Figure 3. Pyruvate attenuates inhibitory effects of hypoxia on SMSC differentiation into myocytes and PDH phosphorylation. *A)* Quantification of the numbers of SMSCs cultured in pyruvate-free or -added medium under normoxic or hypoxic conditions for 5 days. (n = 4, each group). Error bars represent s.e.m. One-way ANOVA with post hoc Tukey's test, **P* < 0.05. n s, not significant. *B)* SMSCs cultured in pyruvate-free or -added medium under normoxic or hypoxic conditions for 5 days were immunostained with antibodies against Pax7, myogenin, and DAPI. Quantified ratios of SMSCs expressing Pax7 and/or myogenin are shown. (n = 9 dishes from 3 mice, each group). Error bars represent s.e.m. One-way ANOVA with post hoc Tukey's test, ***P* < 0.01. n s, not significant. *C)* qRT-PCR for *Pdk1* mRNA in SMSCs cultured in pyruvate-free or -added medium under normoxic or hypoxic conditions for 5 days. (n = 9 dishes from 3 mice, each group). Error bars represent s.e.m. One-way ANOVA with post hoc Tukey's test, **P* < 0.05. n s, not significant. *D)* Representative pictures of SMSCs cultured as described in (*B*). Pax7 (red), myogenin (green), and DAPI (blue). Scale bars = 50 μ m. *E)* Immunofluorescence staining of SMSCs using antibodies against PDH-E1 α (red), phosphorylated

S232 residue of PDH-E1 α (green), and DAPI (blue). SMSCs were cultured in pyruvate-free or -added medium for 5 days under normoxic or hypoxic conditions. Measurement of fluorescence intensity of PDH-E1 α and the ratio of fluorescence intensity (phosphorylated-PDH/PDH). (n = 20–31 cells). Scale bars = 50 μ m. AU: arbitrary unit. Error bars represent s.e.m. One-way ANOVA with post hoc Tukey's test, *** $P < 0.001$. n s, not significant.

Figure 4. PDH deficiency affects SMSC differentiation in long-term culture and yields defective myocyte differentiation. *A)* Schematic of the experimental procedure. TAM (200 μ l of 10 mg/ml in peanut oil) was administered intraperitoneally into WT and *Pax7^{CreERT2/+};Pdh^{lox/-}* (*Pdh^{cKO}*) mice daily for 5 days prior to SMSC isolation and plating. Growth and differentiation were evaluated after 5 or 8 days of culture. (n = 3, each group). *B)* Ratios of satellite cells expressing Pax7 and/or myogenin isolated from WT and *Pdh^{cKO}* mice. SMSCs were cultured in growth medium for 5 days (left) and 8 days (right). (n = 9 dishes from 3 mice per group). Error bars represent s.e.m. Student's t test, ** $P < 0.01$. # $P = 0.057$. *C)* Representative pictures of results in (*B*, right) are shown. Pax7 (red), myogenin (green), and DAPI (blue). Scale bars = 50 μ m. *D)* Quantification of the number of SMSCs cultured in growth medium for 8 days. (n = 3, each group). n s, not significant.

Figure 5. PDH deficiency affects SMSC division. *A)* Schematic SMSC division patterns. Pairs of morphologically identical, spherical cells immediately after WT and *Pax7^{CreERT2/+};Pdh^{lox/-}* (*Pdh^{cKO}*) SMSC division are characterized by dual immunostaining detecting Pax7 and MyoD or Pax7 and myogenin. *B)* Representative division patterns of SMSCs isolated from WT mice by dual immunostaining after cultured for 8 days: Pax7 (red), MyoD, myogenin (green) and DAPI (blue). “Pm+Pm” (arrow in upper panel), “pD+pD” (middle), and “pM+pM” (lower) are shown. Scale bars = 50 μ m. *C)* Division patterns of SMSCs isolated from WT and *Pdh^{cKO}* mice evaluated as indicated (Supplemental Figure S5) (n = 3, each group). Error bars represent s.e.m. Student's t test, # $P = 0.067$. * $P < 0.05$. *** $P < 0.001$. n s, not significant.

Figure 6. PDH deficiency in SMSCs affects myotube formation. *A)* Schematic of the experimental procedure. Tamoxifen (200 μ l of 10 mg/ml in peanut oil) was administered intraperitoneally daily for 5 days

prior to muscle isolation. SMSCs were cultured in growth medium for 4 days, then in differentiation medium for 2 days. *B*) Immunofluorescence staining of myotubes formed by SMSCs isolated from WT and *Pax7^{CreERT2/+};Pdh^{fllox/-}* (*Pdh^{cKO}*) mice using antibodies against MF20 (red). DAPI (blue). Scale bars = 50 μ m. *C*) The fusion index was calculated by dividing the number of nuclei in myotubes with three or more nuclei by the total number of nuclei. ($n = 3$, each group). Error bars represent s.e.m. Student's *t* test, $*P < 0.05$.

Figure 7. PDH deficiency attenuates SMSC proliferation. *A*) Ratios of Ki67-positive SMSCs, isolated from WT and *Pax7^{CreERT2/+};Pdh^{fllox/-}* (*Pdh^{cKO}*) mice, cultured with or without pyruvate for 4 days. ($n = 31$ –57 cells). Error bars represent s.e.m. One-way ANOVA with post hoc Tukey's test, $*P < 0.05$. $***P < 0.001$. *B*) Ratios of Ki67-positive C2C12 cells cultured with or without 1-AA and pyruvate for 2 days. ($n = 37$ –43 cells). Error bars represent s.e.m. One-way ANOVA with post hoc Tukey's test, $^{\#}P = 0.094$. $*P < 0.05$.

Figure 8. PDH deficiency in SMSCs affects muscle regeneration. *A*) Schematic of the experimental procedure. TAM (200 μ l of 10 mg/ml in peanut oil) was administered intraperitoneally daily for 5 days before and 3 days after CTX injection into TA muscle. TA muscles were isolated at day 7, 14, and 30 after CTX injection. *B*) H&E staining of *Pax7^{+/+};Pdh^{fllox/-}* (control) and *Pax7^{CreERT2/+}; Pdh^{fllox/-}* (*Pdh^{cKO}*) TA muscles at day 7, 14, and 30 after CTX injection. Scale bars = 100 μ m. *C*) Average cross section areas of TA muscle in control and *Pdh^{cKO}* mice at day 7, 14, and 30 after CTX injection ($n = 4$, each group). Error bars represent s.e.m. Student's *t* test. $*P < 0.05$. $**P < 0.001$. *D*, *E*) Immunostained sections of TA muscle isolated from control and *Pdh^{cKO}* mice at day 7 after CTX injury with antibodies against myogenin (red) (*D*) or collagen VI (red) (*E*), and laminin (white), with DAPI (blue). Scale bars = 50 μ m.

Figure 9. PDH deficiency in SMSCs in *DMD-null* mice aggravates their muscle degeneration. *A*) Schematic of the experimental procedure. Tamoxifen (200 μ l of 10 mg/ml in peanut oil) was administered intraperitoneally once a week for 5 weeks. TA muscles were isolated at day 30 after the final administration of tamoxifen. *B*) H&E staining of TA muscles in *Pax7^{+/+};Pdh^{fllox/-}; DMD^{-/-}* (left panels) and

Pax7^{CreERT2/+};Pdh^{flox/-}; DMD^{-/-} (right panels) mice. Note that the latter mice contain many degenerative myofibers. Scale bars = 100 μ m.

Figure 10. PDH deficiency attenuates the mitochondrial functions and increases the production of lactate. *A)* Immunofluorescence staining of SMSCs, isolated from WT and *Pax7^{CreERT2/+};Pdh^{flox/-}* (*Pdh^{cKO}*) mice, using TMRE (red) and Mito Tracker (white). SMSCs were cultured for 4 days. Scale bars = 50 μ m. *B)* Measurement of fluorescence intensity of TMRE (left) and Mito Tracker (right) in (*A*). (n = 71–73 cells). AU: arbitrary unit. Error bars represent s.e.m. One-way ANOVA with post hoc Tukey's test, ****P* < 0.001. *C)* Immunofluorescence staining of C2C12 cells using TMRE (red) and Mito Tracker (white). C2C12 cells were cultured with or without 1-AA and pyruvate for 2 days. Scale bars = 50 μ m. *D)* Measurement of fluorescence intensity of TMRE (left) and Mito Tracker (right) in (*C*). (n = 24–30 cells). AU: arbitrary unit. Error bars represent s.e.m. One-way ANOVA with post hoc Tukey's test, **P* < 0.05. ****P* < 0.001. n s, not significant. *E)* Measurement of lactate concentration in C2C12 cells culture medium. C2C12 cells were cultured with or without 1-AA and pyruvate for 24h. Error bars represent s.e.m. One-way ANOVA with post hoc Tukey's test, #*P* = 0.052. **P* < 0.05.

Figure 11. Critical roles of PDH in differentiation of SMSCs; PDH is a gatekeeper of the metabolic flow that activates cell divisions to generate myocytes. PDP: PDH phosphatase.

Figure 1.

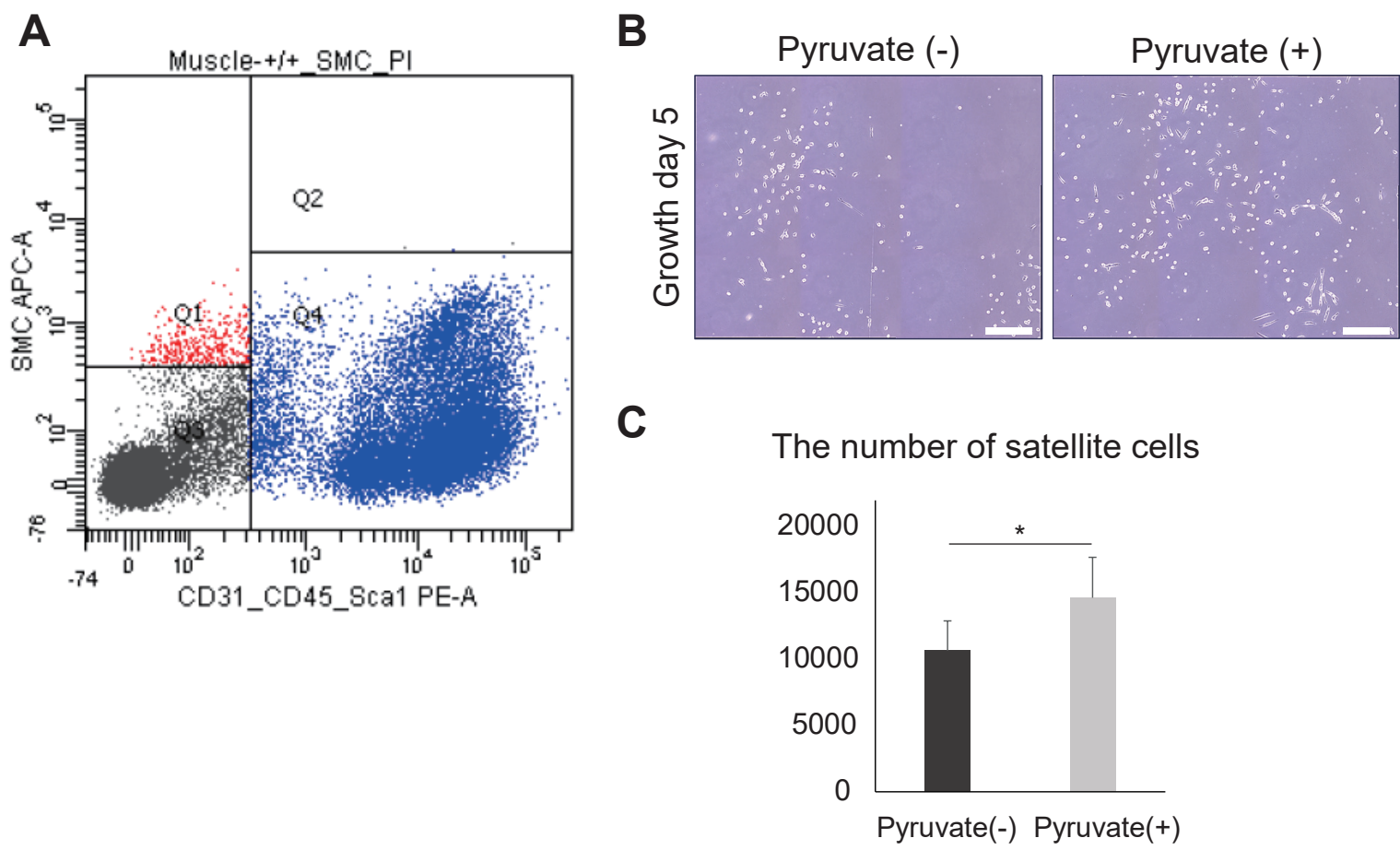


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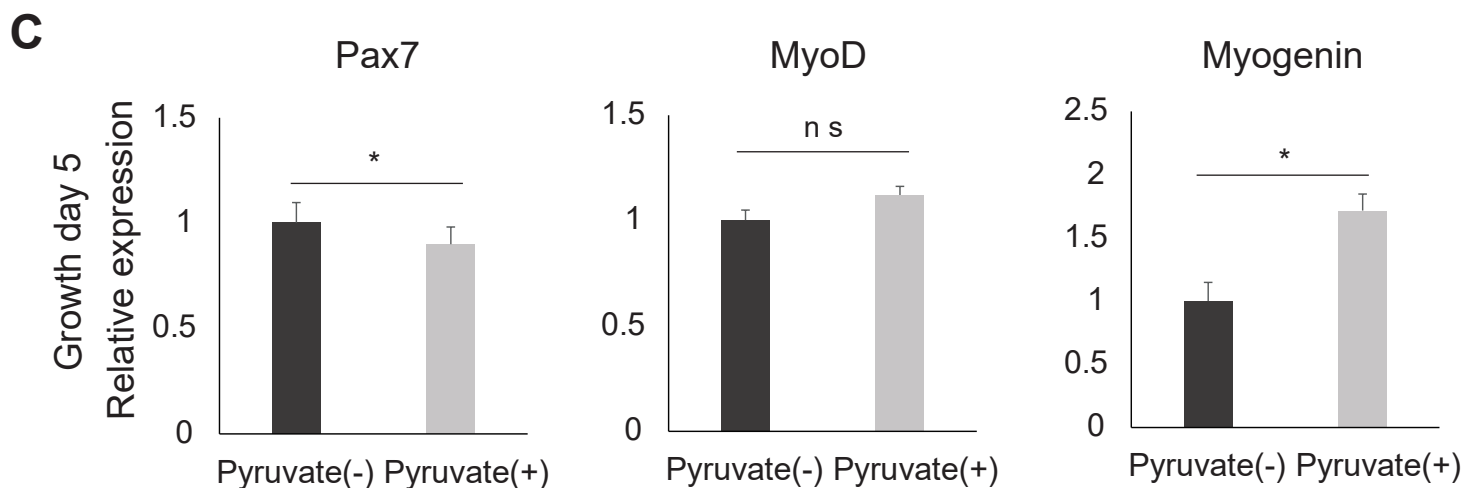
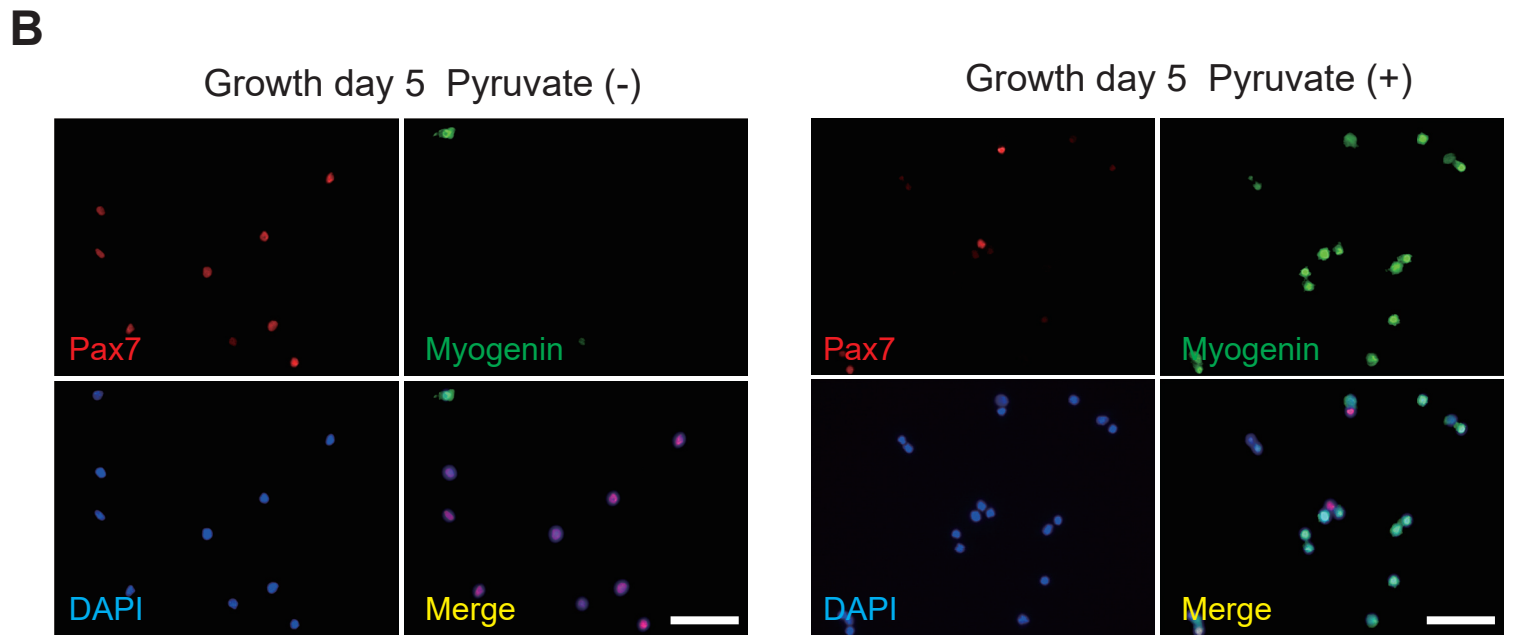
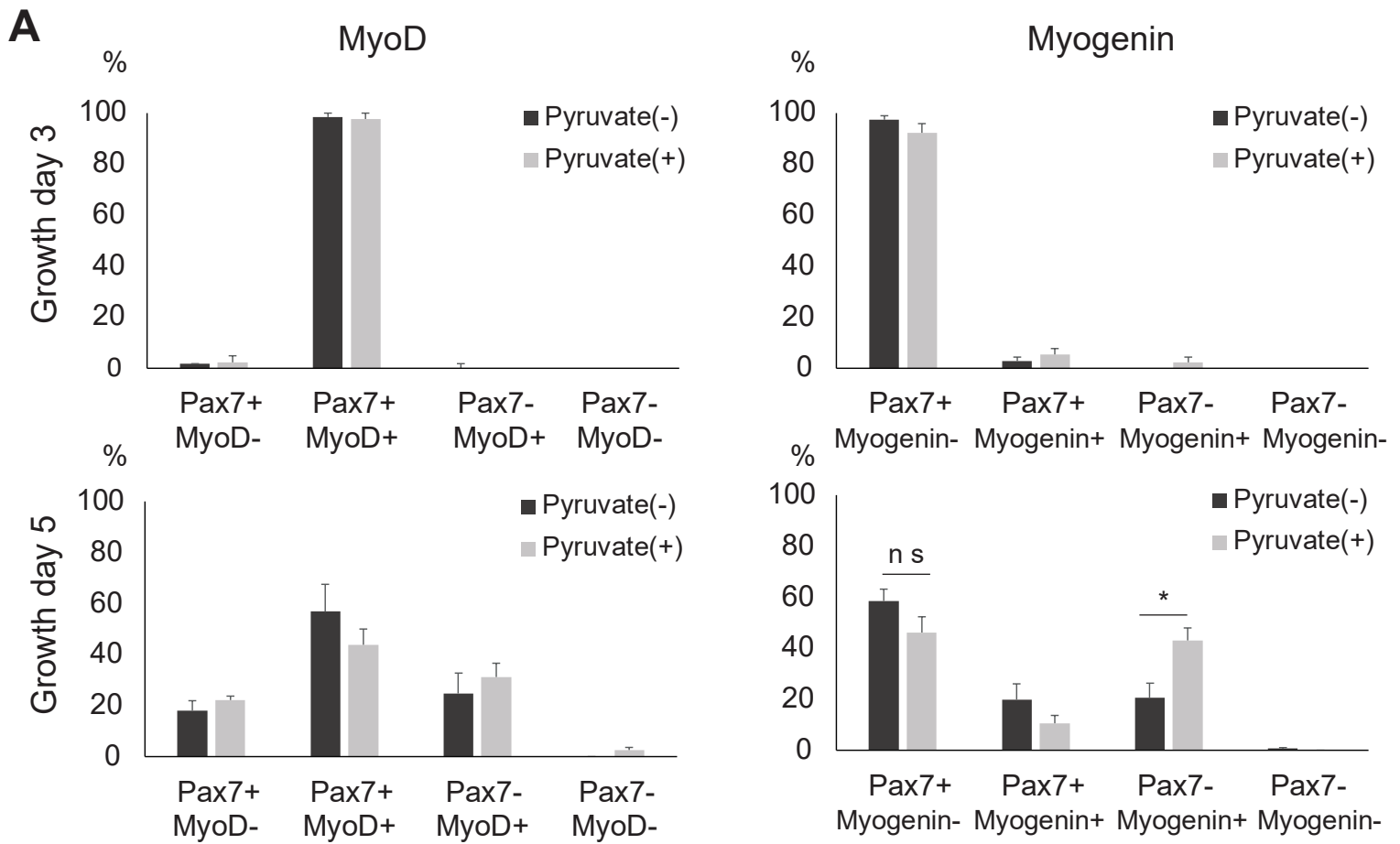


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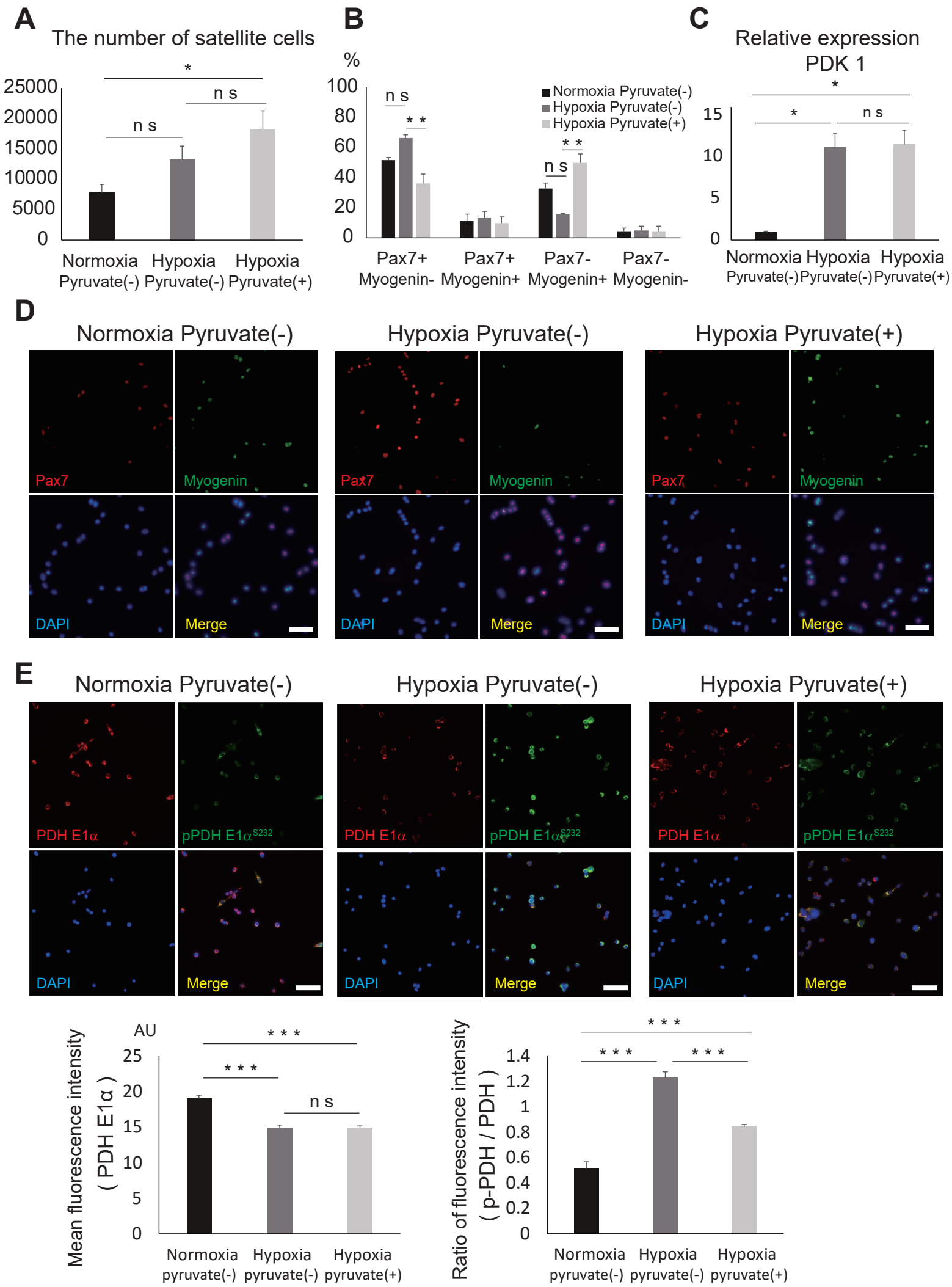
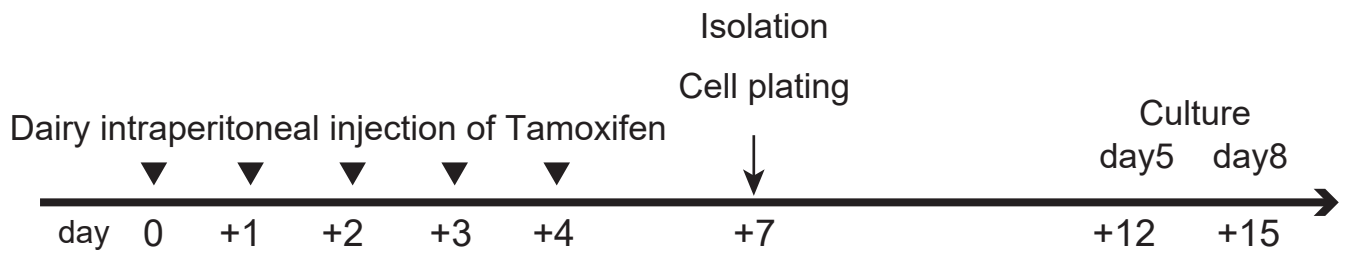
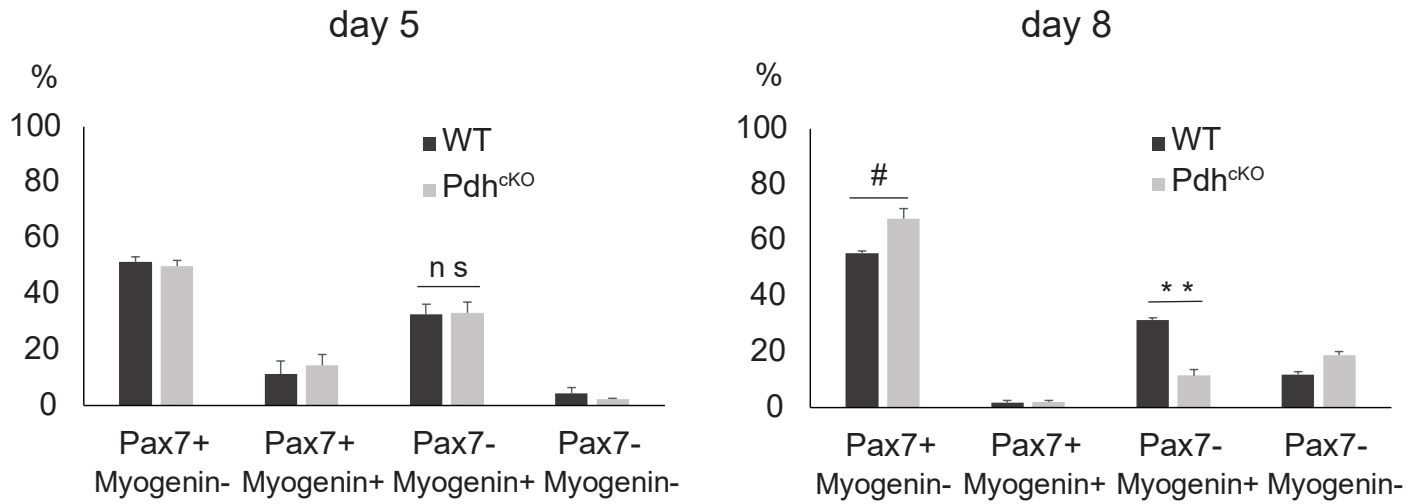


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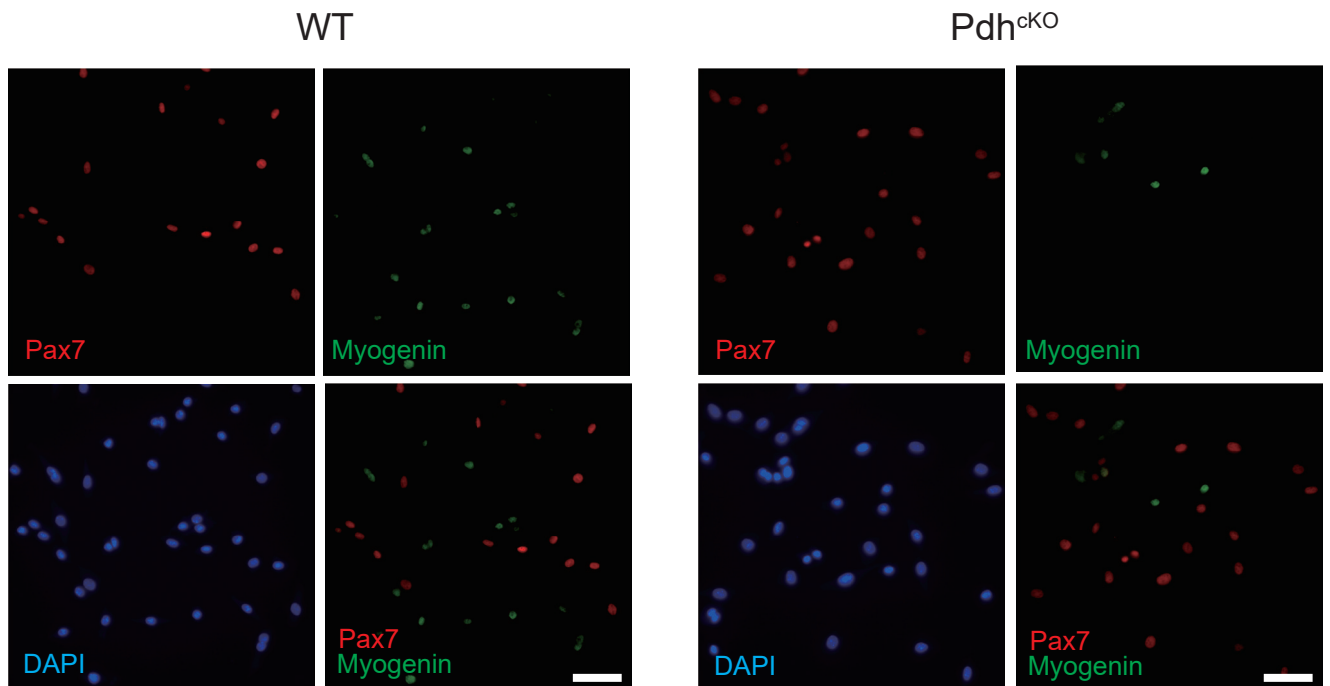
A



B

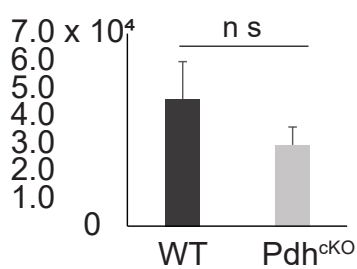


C



D

The number of satellite cells



A

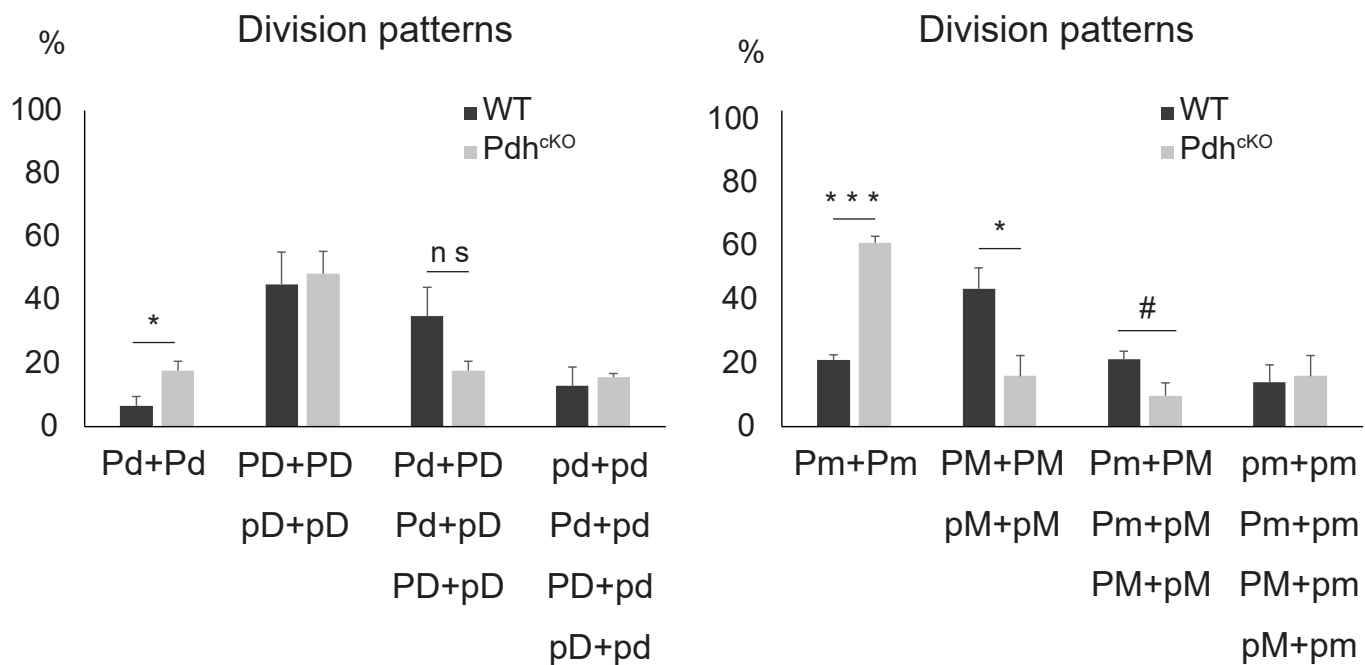


Figure 6.

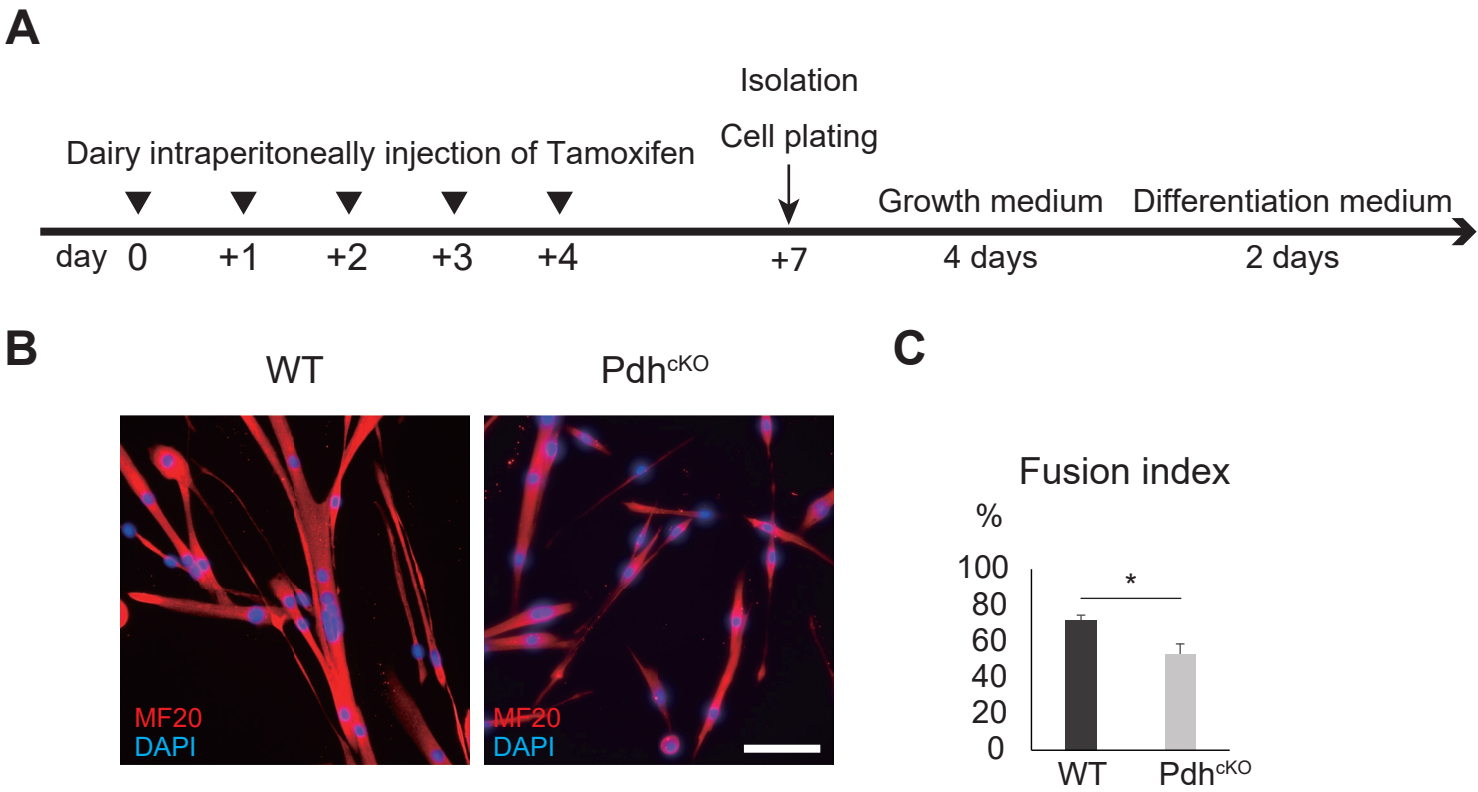


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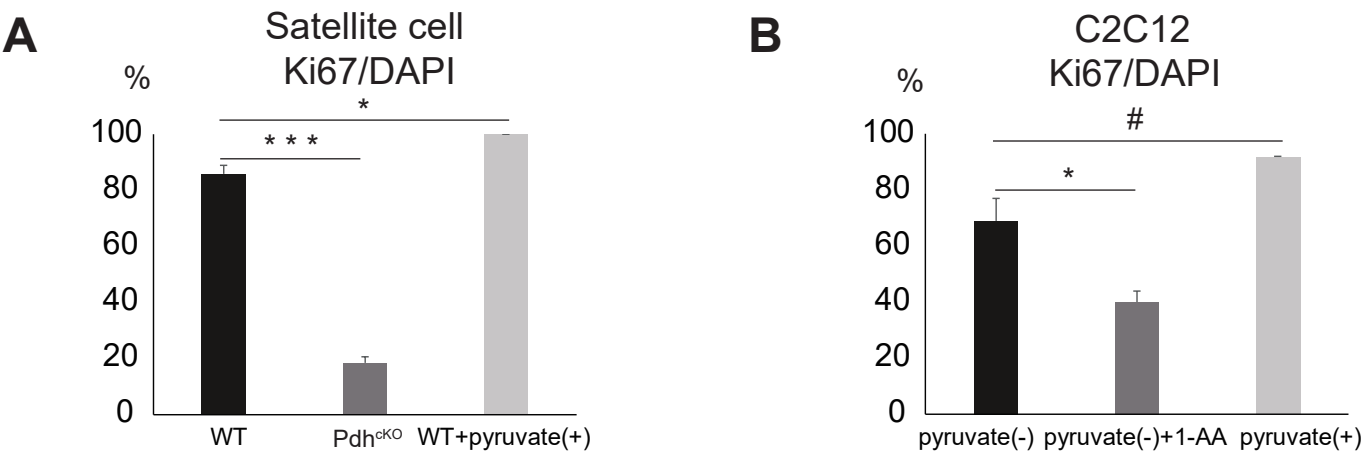


Figure 8.

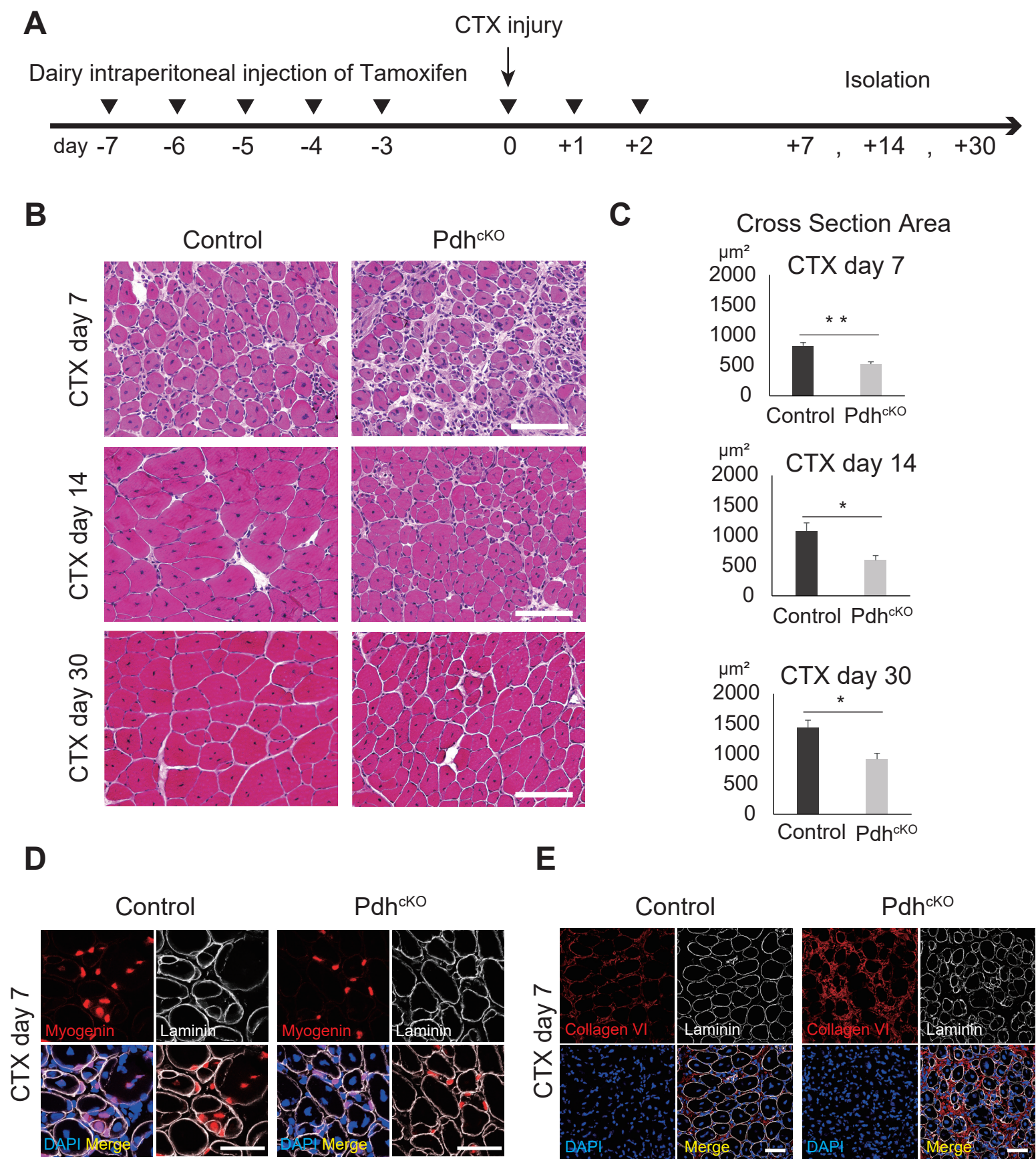
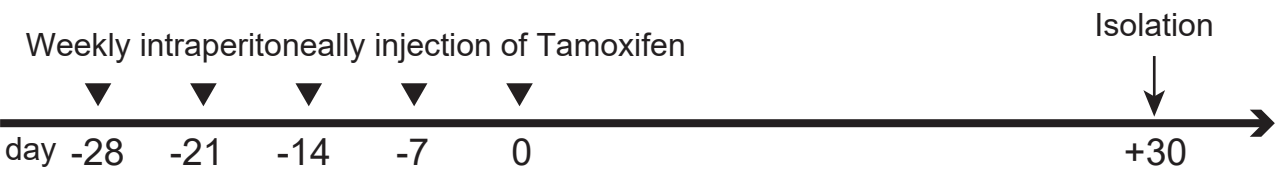


Figure 9.

A



B

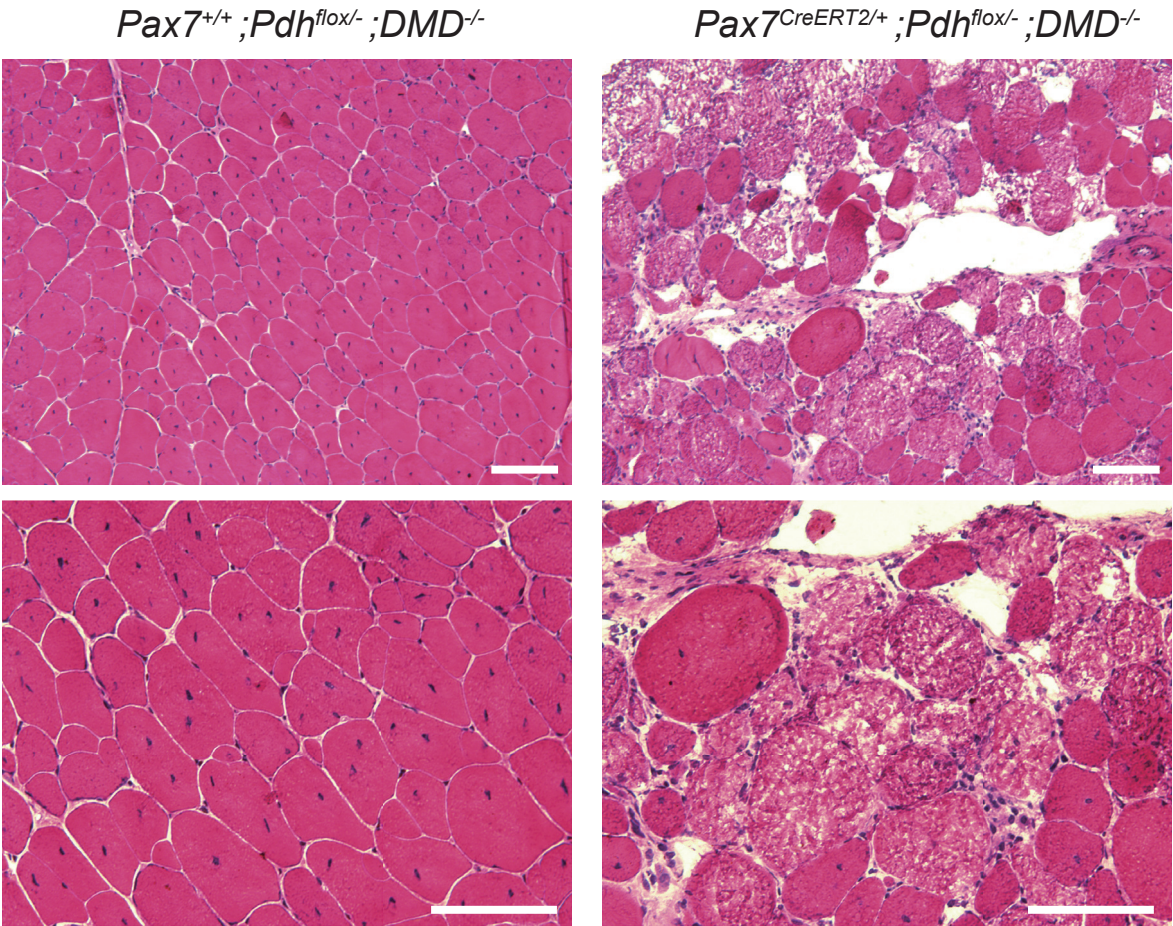


Figure 10.

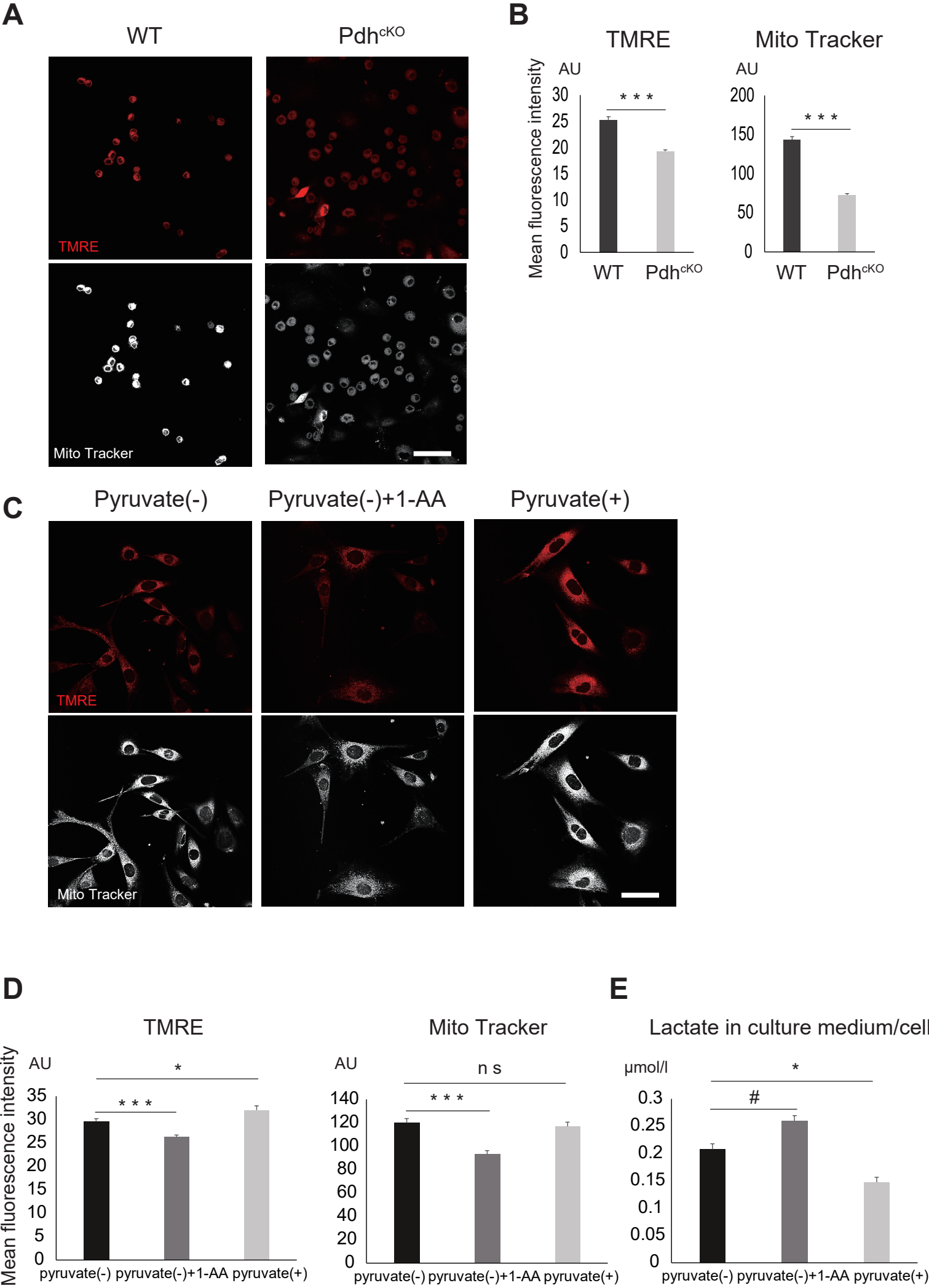


Figure 11.

