Promethazine hydrochloride inhibits ectopic fat cell formation in skeletal muscle

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Running title: Drug that inhibits adipogenesis in muscle

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Abstract

Fatty degeneration of skeletal muscle leads to muscle weakness and loss of function. Preventing fatty degeneration in skeletal muscle is important, but no drug has been used clinically. In this study, we performed drug repositioning using human platelet-derived growth factor receptor α (PDGFRα)-positive mesenchymal progenitors that have been proved to be an origin of ectopic adipocytes in skeletal muscle. We found that promethazine hydrochloride (PH) inhibits adipogenesis in a dose-dependent manner without cell toxicity. PH inhibited expression of adipogenic markers and also suppressed phosphorylation of cAMP response element binding protein (CREB), which was reported to be a primary regulator of adipogenesis. We established a mouse model of tendon rupture with intramuscular fat deposition and confirmed that emerged ectopic adipocytes are derived from PDGFRα$^+$ cells using lineage tracing mice. When these injured mice were treated with PH, formation of ectopic adipocytes was significantly suppressed. Our results demonstrated that PH inhibits PDGFRα$^+$ mesenchymal progenitor-dependent ectopic adipogenesis in skeletal muscle and suggest that treatment with PH can be a promising approach to prevent fatty degeneration of skeletal muscle.

Introduction

Skeletal muscle is responsible for exercise and physical activity, and therefore is a vital organ for healthy life. But in some pathological conditions such as Duchenne muscle dystrophy[1], aging[2] and trauma[3], ectopic fat cells emerge in skeletal muscle. Fatty degeneration of skeletal muscle leads to muscle weakness[4] and reduction in quality of life[3]. Thus, preventing ectopic fat cell formation in skeletal muscle is an important task in the clinical setting.

Although origin of ectopic adipocytes in skeletal muscle had been unclear for a long time, we and others identified platelet-derived growth factor receptor α (PDGFRα)-positive mesenchymal progenitors in murine muscle interstitium and demonstrated that these cells are the origin of ectopic fat cells[5, 6]. Subsequently, we further demonstrated that the same is true in human[7]. Therefore, mesenchymal progenitors can be an ideal target to prevent fatty degeneration of skeletal muscle. Recently, we optimized isolation and culture conditions, which enable us to obtain large number of mesenchymal progenitors derived from human skeletal muscle[8, 9]. Thus, it is now possible to perform drug screening by using human mesenchymal progenitors as target cells.

Discovering and developing new drugs require enormous amounts of time and money. Despite that, recent successful case is reported to be only 4%[10]. Therefore, more economical and efficient strategy would be required. Drug repositioning (DR), which is a method to discover new application of drugs that had been used for certain disease, is receiving attention in late years. Because DR uses existing drugs, it can reduce time and cost for drug development, and shorten the transition time to clinical usage[11].

In this study, we performed DR using human PDGFRα$^+$ mesenchymal progenitors to find drugs that inhibit fatty degeneration of skeletal muscle. This screening identified promethazine hydrochloride (PH) as a candidate compound. PH inhibited adipogenic differentiation of human PDGFRα$^+$ mesenchymal progenitors almost completely and suppressed ectopic fat cell formation in mouse model of tendon rupture with intramuscular fat deposition. Our results suggest that PH can be a promising drug for the treatment of

fatty degeneration of skeletal muscle.

Materials and Methods

Cell preparation from human skeletal muscle

Human skeletal muscle tissue was obtained from gluteus medius muscles of patients who underwent total hip arthroplasty. Written informed consent was obtained from all patients. Experiments using human samples were approved by the Ethical Review Board for Clinical Studies at Fujita Health University. Human skeletal muscles were transferred to PBS. Muscles were minced by scissors and digested with 0.2% collagenase type II (Worthington, Lakewood, NJ) at 37°C for 30 min. Digested muscles were passed through an 18 G needle several times and digested again at 37°C for 15 min. The digested slurry was filtered through a 100 μm cell strainer, and then through a 40 μm cell strainer (BD Biosciences, Franklin Lakes, NJ). Erythrocytes were eliminated by treating the cells with Tris-buffered 0.83% NH_4Cl. Cells were cultured on 60 mm collagen I-coated dishes (Iwaki, Tokyo, Japan) in the growth medium (GM) consisting of DMEM with 20% FBS, 1% penicillin–streptomycin, and 2.5 ng/ml bFGF (Katayama Chemical, Osaka, Japan) at 37°C in 3% O_2 and 5% CO_2. Cells were trypsinized, and suspended in washing buffer consisting of PBS with 2.5% FBS. Then, cells were stained with PE-conjugated anti-CD56 (1:20; Miltenyi Biotec, Bergisch Gladbach, Germany, #130-104-944) and biotinylated anti-PDGFRα (2.5 μg/ml; R&D, Minneapolis, MN, #BAF322) at 4°C for 30 min, and then stained with streptavidin-PE/Cy5 (1:200; BD Pharmingen, Franklin Lakes, NJ, #554062) at 4°C for 30 min in the dark. $CD56^-PDGFR\alpha^+$ cells were sorted by FACSVantage SE (BD Biosciences) or MoFlo Astrios (Beckman Coulter, Brea, CA), and cultured on 100 mm collagen I-coated dishes (Iwaki) in GM at 37°C in 3% O_2 and 5% CO_2.

Screening of FDA approved compounds

An FDA-approved chemical compound library (Prestwick Chemical, Illkirch, France) consisting of 1,186

compounds was used. We excluded 579 compounds due to inappropriateness for chronic treatment and 287 compounds due to unavailability in Japan. The remaining 320 compounds were screened. PDGFRα$^+$ cells were seeded in wells of 96-well plates coated with Matrigel (BD Bioscience) at a density of 5 x 10^3 cells/well in GM and incubated at 37°C in 3% O_2 and 5% CO_2. When cells reached 90% confluency, GM was changed to adipogenic induction medium (IM) consisting of DMEM with 10% FBS, 0.5 mM IBMX (Sigma-Aldrich, St. Louis, MO), 0.25 μM dexamethasone (Sigma-Aldrich), and 10 μg/ml insulin (Sigma-Aldrich) for 3 d, and then cultured in adipogenic maintenance medium (MM) consisting of DMEM with 10% FBS and 10 μg/ml insulin for 1 d at 37°C in 21% O_2 and 5% CO_2. These treatments were repeated three times. Compounds were added at the concentration of 10 μM in triplicate through the differentiation process. To assess the degree of adipogenic differentiation, cells were fixed with 10% neutral buffered formalin solution (Wako, Osaka, Japan) and then stained with Hoechst 33258 for nuclei and Bodipy® for lipid droplets using an Adipocyte Fluorescent Staining Kit (Cosmo Bio, Tokyo, Japan). Numbers of nuclei and Bodipy-stained areas were measured using ArrayScan VTI-HCS (Thermo Fisher Scientific, Waltham, MA). The degree of adipogenic differentiation was determined by dividing the Bodipy-stained area by the number of nuclei.

Cell viability assay

PDGFRα$^+$ cells were cultured on Matrigel-coated 6-well or 96-well plates in GM as described above. Then cells were cultured in DMEM containing 10% FBS and 1.25, 2.5, 5, or 10 μM PH for 3 d at 37°C in 21% O_2 and 5% CO_2. For Live/Dead assay, cells were trypsinized and detached from 6-well plates, and then stained using LIVE/DEAD viability/cytotoxicity assay kit (Thermo Fisher Scientific). Percentage of live cells was analyzed using FACS Verse (BD Biosciences). For WST-8 assay, 10 μl of WST-8 reagent (Cell Counting Kit-8, Dojindo Molecular Technoligies, Kumamoto, Japan) was added to each well of 96-well plates and incubated for an additional 2 h. Absorbance at 450 nm was measured by Multiskan JX (Thermo Fisher

Scientific). These experiments were repeated three times using PDGFRα$^+$ cells obtained from three independent preparations.

RNA extraction and real-time RT-PCR

Ten thousand PDGFRα$^+$ cells were cultured on Matrigel-coated 48-well plates and subjected to adipogenic differentiation as described above with or without 10 μM PH. Total RNA was extracted on day 0, 4, 8, and 12 of adipogenic differentiation using an RNeasy Micro Kit (Qiagen, Hilden, Germany), and equal amounts of RNA were reverse transcribed into cDNA using a QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real time PCR was performed using a Thermal Cycler Dice Real Time System (Takara, Shiga, Japan) and SYBR® Premix Ex tag II (Takara Bio). Primer sequences were 5'-TGAAGCTGAACCACCCTGAG-3' and 5'-TGCAGTAGCTGCACGTGTTC-3' for *PPARG*, 5'-CCTGGTGAGAAGGGTGAGAA-3' and 5'- TCTCCTTTCCTGCCTTGGA-3' for *ADIPOQ*, 5'-CGTCAAAGGTGAAGCAGGAC-3' and 5'-CTCCAGTGCCCGTAGATCAG-3' for *NDUFA13*, and 5'-TATCATGGCAGCCTTCATCC-3' and 5'-GGGTAGATGAGGGGGTTCAG-3' for *HRH1*. *NDUFA13* was used as an internal control gene. These experiments were repeated three times using PDGFRα$^+$ cells obtained from three independent preparations.

Immunofluorescent staining

Cells were rinsed with PBS, and fixed with 4% paraformaldehyde (PFA) for 5 minutes. Frozen muscle sections were cut by cryostat and fixed with 4% PFA. Specimens were blocked with protein block serum-free reagent (Dako, Glostrup, Denmark) for 5 min and incubated with primary antibodies at 4°C overnight, followed by secondary staining. Primary and secondary antibodies used were anti-PPARγ antibody (1:100; Cell Signaling, Danvers, MA, #2435), anti-Perilipin (1:250; Sigma-Aldrich, #P1873), anti-GFP (1:200; Acris, Herford, Germany, #AB0020), anti-Laminin α2 (1:400; Santa Cruz, #sc-59854),

Alexa Fluor 488 anti-rabbit IgG antibody (1:1000; Molecular Probes, Eugene, OR, #A21206), Alexa Fluor 488 anti-goat IgG (1:1000; Molecular Probes, #A11055), Alexa Fluor 594 anti-rabbit IgG (1:1000; Jackson ImmunoResearch, #111-585-144), and Alexa Fluor 647 anti-rat IgG (1:1000; Molecular Probes, #A21472). Stained samples were counterstained with DAPI (Invitrogen, Waltham, MA) and mounted with SlowFade Gold anti-fade reagent (Invitrogen). Stained images were obtained using Opera Phenix (Perkin Elmer, Waltham, MA), a confocal laser scanning microscope system LSM700 (Carl Zeiss, Oberkochen, Germany), and an inverted fluorescence microscope BZ-9000 (Keyence, Osaka, Japan). The percentage of PPARγ-positive cells in cultured cells was analyzed by Opera Phenix (Perkin Elmer), and the fat-occupied area in muscle sections was analyzed by BZ-II analyzer software (Keyence). In some cases, stained sections were subsequently subjected to hematoxylin and eosin (HE) staining as described previously[5].

Western blotting

PDGFRα$^+$ cells were cultured on Matrigel-coated 6-well plates at a density of 1 x 10^5 cells/well and subjected to adipogenic differentiation as described above with or without 10 μM PH. Cells were extracted on day 0, 4, 8, or 12 of adipogenic differentiation. Cells were lysed in lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM NaF, 1% NP-40, and a protease inhibitor cocktail (Roche, Basel, Switzerland). For detection of the phosphorylated proteins, a phosphatase inhibitor cocktail (Roche) was added. Ten μg of protein was separated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane, followed by immunoblotting with anti-CREB (1:1000; Cell Signaling, #9197), anti-phospho-CREB (1:1000; Cell Signaling, #9198), and anti-GAPDH (1:1000; Cell Signaling, #2118) antibodies. After incubation with a horseradish peroxidase-conjugated secondary antibody (1:10000; Cell Signaling, #70745) and chemiluminescence reactions, images of the immunoblots were obtained using a Light-Capture imaging system (ATTO, Tokyo, Japan). Intensity of the immunoblots was quantified using Win ROOF software (Mitani, Fukui, Japan). Phosphorylated CREB was normalized for CREB. These

experiments were repeated three times using PDGFRα$^+$ cells obtained from three independent preparations.

Mouse experiments

Experiments using mice were approved by the Institutional Animal Care and Use Committee at Fujita Health University. C57BL/6 female mice were purchased from Japan SLC at 8 weeks old. One Achilles tendon was resected and the skin was sutured, while a sham operation was performed by only skin incision. Four weeks after operation, the mice were sacrificed, and gastrocnemius muscles were excised and frozen rapidly. Seven-μm-thick sections were cut at the position 2.5 mm from the proximal side and subjected to HE staining. To assess the effects of oral administration of PH, mice received PH in drinking water (0.05 mg/ml or 0.1 mg/ml) after the operation. Oral administration doses were calculated by measurement of the amount of water drunk and the concentration of PH in the drinking water. The water was changed twice a week. Four weeks after operation, mice were sacrificed, and gastrocnemius muscle sections were cut as described above and subjected to immunofluorescent staining. PDGFRα-CreER mice and R26R-EYFP mice were purchased from Jackson Laboratory and MyoD-iCre mice were kindly provided by Dr. Goldhamer[12]. PDGFRα-CreER mice or MyoD-iCre mice were crossed with R26R-EYFP mice. Four mg of tamoxifen was injected intraperitoneally for five consecutive days into 10-week-old PDGFRα-CreER/R26R-EYFP mice to induce EYFP expression. Achilles tendons of generated mice were excised as described above. Four weeks after operation, these mice were sacrificed, and gastrocnemius muscles were excised and fixed with 4% PFA for 1 h. Fixed muscles were suspended in 10% sucrose solution at 4°C overnight and then in 20% sucrose solution at 4°C overnight. Muscles were frozen and 7-μm-thick sections were cut at the position of 2.5 mm from the proximal side and subjected to immunofluorescent staining.

Statistical Analysis

Data were presented as the mean ± SEM. The Jonckheere-Terpstra trend test was used to assess dose-dependency. One-way ANOVA followed by Dunnet's or Tukey's post-hoc test was used to assess cell toxicity and fat area proportion and muscle weight. Two-way ANOVA was used to assess effects of the compound in quantitative real-time PCR, immunofluorescent staining, and immunoblotting. P values less than 0.05 were considered significant. The statistical analyses were performed with SPSS statistics 21 (IBM, Armonk, NY, USA).

Results

To identify drugs that suppress ectopic adipocyte formation in skeletal muscle, we searched for compounds that inhibit adipogenic differentiation of human PDGFRα$^+$ mesenchymal progenitors using FDA-approved compound library. We isolated PDGFRα$^+$ cells from human skeletal muscle and expanded them according to the previously established procedure[8, 9], and cultured in adipogenic medium, which contains adipogenic inducers, with or without compounds followed by high-throughput fluorescent imaging of lipid droplets (Figure 1A). Among the compounds examined, we chose PH and used it for further experiments because PH inhibited adipocyte formation strongly and consistently compared with compound-free control (Figure 1B). We next examined dose-dependency of PH and confirmed dose-dependent inhibition of adipocyte formation (Figure 1C). To exclude the possibility that inhibitory effect of PH on adipogenesis is simply due to toxic effect on PDGFRα$^+$ cells, we examined number of cells revealed by nuclear staining. PH did not affect cell number even at highest concentration we used (Figure 1C). We also examined cell viability by LIVE/DEAD assay and WST-8 assay and confirmed that PH does not have any toxicity on PDGFRα$^+$ cells (Figure 1D and E).

To investigate that PH indeed inhibits adipogenic differentiation, not merely inhibits accumulation of lipid droplets, we assessed expression of adipogenic markers. Quantitative real-time PCR revealed that PH downregulates expression of peroxisome proliferator activated receptor γ (*PPARG*), a master regulator of

adipogenic differentiation, and almost completely prevents induction of adiponectin (*ADIPOQ*) expression, a mature adipocyte marker (Figure 2A). Immunofluorescent staining against PPARγ further showed that treatment with PH leads to significant reduction of PPARγ$^+$ cells (Figure 2B). We next investigated more upstream event of adipogenesis. Activation of cAMP response element binding protein (CREB) was shown to be sufficient for initiation of adipogenesis[13], and therefore, CREB is known as a primary regulator of adipogenesis. Importantly, we observed consistent reduction in phosphorylation level of CREB during adipogenic differentiation by PH treatment (Figure 2C). These results suggest that PH efficiently inhibits adipogenic differentiation of human PDGFRα$^+$ mesenchymal progenitors by disturbing early phase of adipogenesis.

To assess the anti-adipogenic effect of PH in vivo, we first established mouse model of tendon rupture by excising Achilles tendons, which we aim to mimic rotator cuff tear, a common disorder accompanied by intramuscular fat deposition in the field of orthopedics. In contrast to muscles of sham operation side that showed normal histology, gastrocnemius muscles with tendon excision had increased interstitial cells at 2 weeks of post-operation and developed ectopic adipocytes at 4 weeks after operation (Figure 3A and Supplemental Figure 1). We and others reported that only PDGFRα$^+$ mesenchymal progenitors can generate ectopic adipocytes in skeletal muscle by transplanting labeled PDGFRα$^+$ cells into glycerol-injured muscle[5, 6]. To demonstrate that endogenous PDGFRα$^+$ cells contribute to ectopic fat cell formation in more pathologically relevant condition, we applied tendon rupture model to lineage tracing mice that are generated by crossing PDGFRα-CreER mice[14] or MyoD-iCre mice[12] with R26R-EYFP mice[15]. Before operation, we injected tamoxifen into PDGFRα-CreER/R26R-EYFP mice to permanently label PDGFRα$^+$ cells. We confirmed that only PDGFRα$^+$ cells became positive for EYFP and labeling efficiency was approximately 70% (data not shown). Four weeks after tenotomy, 79.5±17.5% of emerged ectopic adipocytes expressed EYFP (Figure 3B and C), indicating that almost all ectopic adipocytes are derived from endogenous PDGFRα$^+$ cells. On the other hand, no ectopic adipocytes were labeled with EYFP in

MyoD-iCre/R26R-EYFP mice, confirming that myogenic cells never contribute to fatty degeneration (Figure 3B and C). Thus, established model provides pathological features relevant to a common orthopedic disorder and is suitable for investigating fatty degeneration of skeletal muscle that is dependent on endogenous PDGFRα$^+$ mesenchymal progenitors. Using this model, we assessed the efficacy of PH in vivo. After tenotomy, mice were given drinking water supplemented with PH for 4 weeks. Immunofluorescent imaging of whole muscle section revealed that PH significantly suppressed ectopic adipocyte formation (Figure 3D). Although lower concentration of PH was less effective, significant reduction in ectopic adipose content was evident even in this group (Figure 3E). Intriguingly, higher concentration of PH suppressed ectopic adipose content to the level comparable to sham operation group (Figure 3E). Although there was clear inhibition of ectopic fat development, PH did not have any effect on muscle weight loss (Figure 3F). These results indicate that PH possesses inhibitory effect on ectopic fat cell formation in skeletal muscle but not on muscle atrophy evoked by Achilles tendon resection.

Discussion

Fatty degeneration of skeletal muscle leads to muscle weakness and loss of function, which further deteriorate total health. Previous studies including ours demonstrated that fatty degeneration of skeletal muscle occurs through differentiation of PDGFRα$^+$ mesenchymal progenitors into ectopic adipocytes[5, 6]. Importance of PDGFRα$^+$ cells in fatty degeneration is also suggested by recent study demonstrating that inhibition of PDGFRα signaling attenuated fat infiltration in mouse model of rotator cuff tear[16]. This notion is further supported by the present study in which lineage tracing experiments revealed endogenous PDGFRα$^+$ cells as a source of ectopic fat cells in tendon rupture model. In our previous study, we established culturing method of human PDGFRα$^+$ mesenchymal progenitors in large scale[9]. This provides valuable opportunity to search drugs that would control pathological behavior of PDGFRα$^+$ mesenchymal progenitors. Using this culture system, we performed DR and found that PH has a strong inhibitory effect on

PDGFRα$^+$ cell-dependent adipogenesis both in vitro and in vivo. PH is a first-generation antihistamine drug and has been used mainly to treat allergic rhinitis. Histamine H1 receptor signaling was reported to stimulate adipogenic differentiation of 3T3-L1 preadipocyte cell line[17] and PDGFRα$^+$ mesenchymal progenitors strongly express histamine H1 receptor (Supplemental Figure 2), suggesting that this signaling pathway represent an important regulator of fatty degeneration of skeletal muscle. However, therapeutic value of antihistamine drug on fatty degeneration has not been documented. Although it is needed to test dose of PH in human carefully, it could be used in a short time in the clinical setting because adverse effects and contraindications are already established. To elucidate mechanism whereby PH exerts anti-adipogenic action on PDGFRα$^+$ mesenchymal progenitors, we focused on CREB because it was shown to be activated by histamine H1 receptor in non-adipose cells[18] and is known as a primary regulator of adipogenesis[13]. We found that PH inhibits phosphorylation of CREB during adipogenic differentiation of PDGFRα$^+$ cells. Thus, strong inhibiting effect of PH could be explained by its action on early and critical regulator of adipogenesis. Although PH suppressed PPARγ expression at both the mRNA and protein levels, inhibitory effect was greater in protein level than in mRNA level. Because PPARγ protein is known to be regulated post-translationally through ubiquitin-proteasome-dependent degradation and Hsp90-mediated stabilization in adipogenic cells[19, 20, 21], such post-translational regulation of PPARγ protein may account for the different inhibition seen in mRNA and protein. Ectopic fat cell formation is usually accompanied by muscle atrophy, and muscle atrophy is also major cause of loss of strength and function. We showed that PH inhibited ectopic adipocyte formation but did not exert any effect on muscle atrophy in tendon rupture model. Although PH could not prevent muscle atrophy, inhibiting fatty degeneration should be still beneficial because accumulated ectopic adipocytes can hinder a recovery from atrophy and therefore further deteriorate muscle function.

In conclusion, we identified PH as the candidate drug for the treatment of fatty degeneration of skeletal muscle. Because PH is already in clinical use, it could be applied to muscle disorders in a short time.

Use of PH will be effective means for preventing decline of muscle function and improving effect of rehabilitation.

Acknowledgments

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Figure Legends

Figure 1. DR utilizing human PDGFRα$^+$ cells identifies PH as an adipogenesis-inhibiting drug.

(A) Schema of DR. Human skeletal muscle tissue was subjected to enzymatic digestion and isolated cells were cultured. PDGFRα$^+$ cells were purified by FACS and expanded. Purified PDGFRα$^+$ cells were then induced to differentiate into adipocytes with or without FDA-approved compounds in 96-well plates.

(B) Representative image of PH-treated and control wells. Lipid droplets were visualized with Bodipy® (green) and nuclei were stained with DAPI. Adipogenic differentiation degree was shown as means ± SEM of triplicate wells. PH inhibited adipocyte formation strongly and consistently in two independent screening. **P<0.01

(C) PH inhibited adipocyte formation in a dose-dependent manner. Adipogenic differentiation degree and number of cells were shown as means ± SEM of three independent preparations. Inset shows magnified image. Scale bar: 100 μm. *P<0.05

(D) Cell viability was analyzed by LIVE/DEAD assay. Boxed regions represent live cells. Percentage of live cells was quantified and shown as means ± SEM of three independent preparations.

(E) Cell viability was analyzed by using WST-8 reagent and shown as means ± SEM of three independent preparations.

Figure 2. PH downregulates adipogenic markers.

(A) Relative *PPARG* and *ADIPOQ* expression during adipogenic differentiation of PDGFRα$^+$ cells were analyzed by quantitative real time PCR and presented as means ± SEM of three independent preparations. *NDUFA13* was used as internal control gene. *P<0.05.

(B) PH-treated and control cells were stained with anti-PPARγ antibody (green). PPARγ positive ratio was quantified and shown as means ± SEM of three independent preparations. Scale bar: 100 μm. ***P<0.001.

(C) Phosphorylated CREB and total CREB are assessed by immunoblotting. GAPDH is shown as loading

control. Ratio of phosphorylated CREB to total CREB was quantified and shown as means ± SEM of three independent preparations. ***P<0.001.

Figure 3. PH inhibits ectopic adipocyte formation in skeletal muscle in vivo.

(A) Representative images of muscles of tendon rupture model. Four weeks after Achilles tendon resection, gastrocnemius muscles were subjected to histological analysis. Sham-operated legs were used as control. Scale bar: 100 μm.

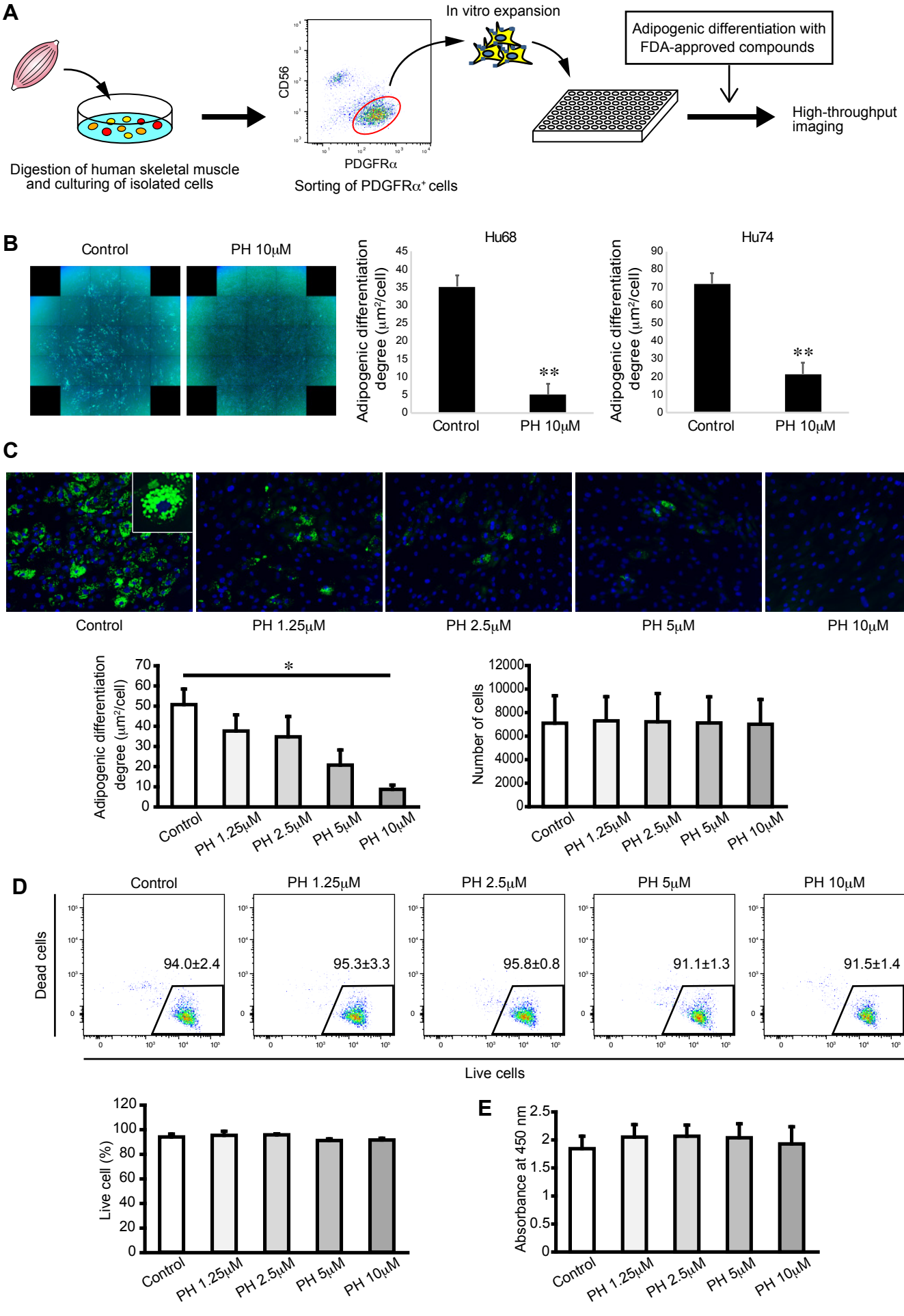
(B) Muscle sections of PDGFRα-CreER/R26R-EYFP and MyoD-iCre/R26R-EYFP mice were stained with antibodies against YFP (green), perillipin (red), and laminin α2 (white). The percentages of YFP positive adipocytes are shown in the panels as means ± SEM, n=9 randomly selected fields from 3 mice. Scale bar: 100 μm.

(C) Magnified images of boxed areas in B. Scale bar: 20 μm.

(D) Perilipin immunofluorescent images of whole gastrocnemius muscle sections from sham operation, vehicle-treated, and PH-treated groups (upper panels). Higher magnification images are shown in middle panels and HE images of corresponding areas are shown in lower panels. Scale bars: 200 μm in upper panels and 50 μm in lower panels.

(E) Percentage of area occupied by ectopic fat in whole section was quantified and shown as means ± SEM, n=6. *P<0.05 and ***P<0.001.

(F) Weight of gastrocnemius muscle was measured and shown as means ± SEM, n=6. ***P<0.001.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High-throughput imaging
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PDGFRα
Digestion of human skeletal muscle and culturing of isolated cells
Sorting of PDGFRα+ cells
B
Control
PH 10μM
Hu68
Hu74
Adipogenic differentiation degree (μm²/cell)
Control
PH 10μM
C
Control
PH 1.25μM
PH 2.5μM
PH 5μM
PH 10μM
Adipogenic differentiation degree (μm²/cell)
Number of cells
D
Dead cells
Live cells
94.0±2.4
95.3±3.3
95.8±0.8
91.1±1.3
91.5±1.4
Live cell (%)
E
Absorbance at 450 nm

Figure 1

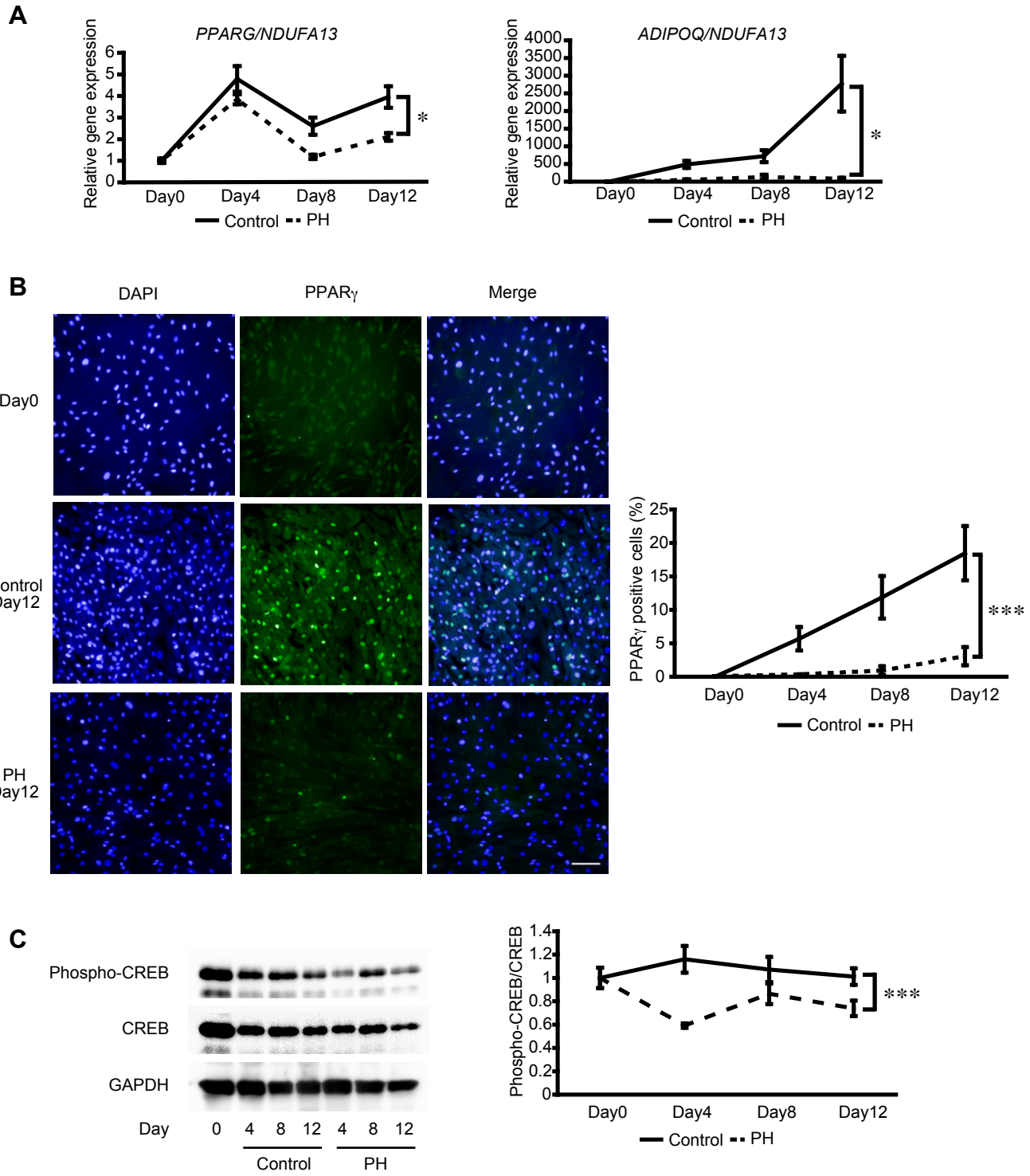

A
PPARG/NDUFA13
Relative gene expression
Day0
Day4
Day8
Day12
Control
PH
*
ADIPOQ/NDUFA13
Relative gene expression
Day0
Day4
Day8
Day12
Control
PH
*
B
DAPI
PPARγ
Merge
Day0
Control Day12
PH Day12
PPARγ positive cells (%)
Day0
Day4
Day8
Day12
Control
PH
***
C
Phospho-CREB
CREB
GAPDH
Day 0 4 8 12 4 8 12
Control
PH
Phospho-CREB/CREB
Day0
Day4
Day8
Day12
Control
PH
***

Figure 2

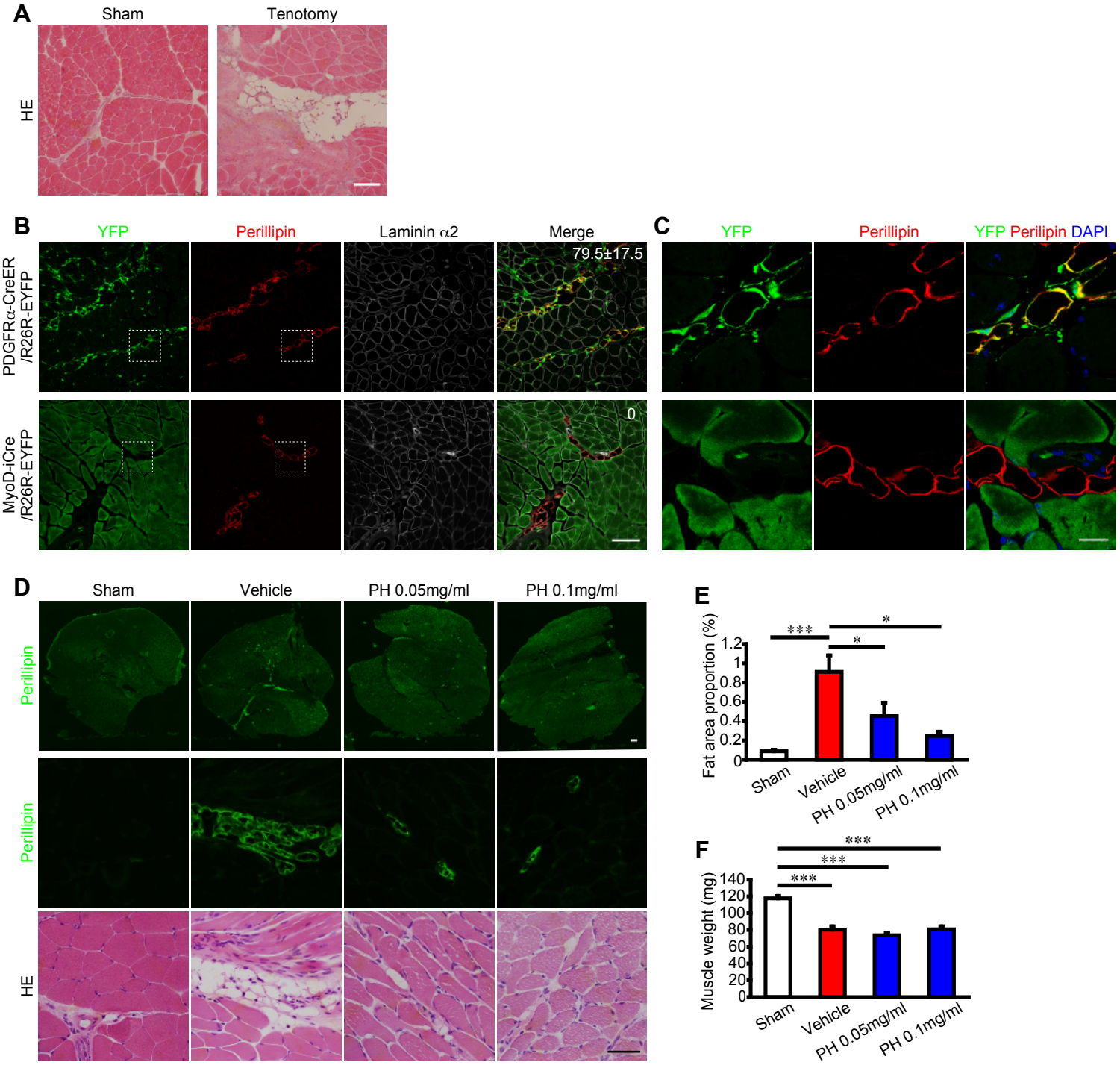
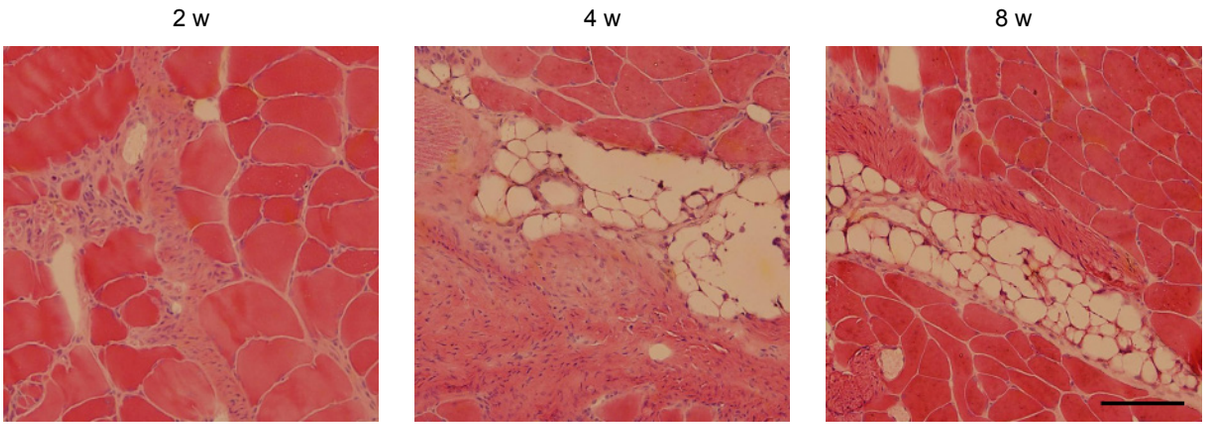
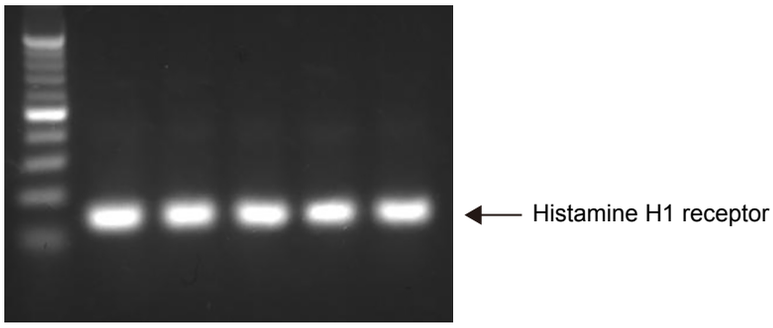

A
Sham
Tenotomy
HE
B
YFP
Perillipin
Laminin α2
Merge
79.5±17.5
PDGFRα-CreER /R26R-EYFP
MyoD-iCre /R26R-EYFP
0
C
YFP
Perillipin
YFP Perilipin DAPI
D
Sham
Vehicle
PH 0.05mg/ml
PH 0.1mg/ml
Perillipin
Perillipin
HE
E
Fat area proportion (%)
1.2
1
0.8
0.6
0.4
0.2
0
***
*
*
Sham
Vehicle
PH 0.05mg/ml
PH 0.1mg/ml
F
Muscle weight (mg)
140
120
100
80
60
40
20
0
***
***
***
Sham
Vehicle
PH 0.05mg/ml
PH 0.1mg/ml

Figure 3



Supplemental Figure 1



Supplemental Figure 2