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Platelet releasate normalises the compromised muscle regeneration in a mouse model of hyperlipidaemia

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Title: Platelet releasate normalises the compromised muscle regeneration in a mouse model of hyperlipidaemia

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Running Title: Skeletal muscle stem cells function and regeneration in ApoE null mice

Abstract: Muscle satellite cells are important stem cells for skeletal muscle regeneration and repair after injury. ApoE deficient mice, an established mouse model of hyperlipidaemia and atherosclerosis, show evidence of oxidative stress-induced lessions and fat infiltration in skeletal muscle followed by impaired repair after injury. However, the mechanisms underpinning attenuated muscle regeneration remain to be fully defined. Key to addressing the latter is to understand the properties of muscle stem cells from ApoE deficient mice and their myogenic potential. Muscle stem cells

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from ApoE deficient mice were cultured both ex vivo (on single fibres) and in vitro (primary myoblasts) and their myogenic capacity was determined. Skeletal muscle regeneration was studied on days 5 and 10 after cardiotoxin injury. ApoE deficient muscle stem cells showed delayed activation and differentiation on single muscle fibres ex vivo. Impaired proliferation and differentiation profiles were also evident on isolated primary muscle stem cells in culture. ApoE deficient mice displayed impaired skeletal muscle regeneration after acute injury in vivo. Administration of platelet releasate in ApoE deficient mice reversed the deficits of muscle regeneration after acute injury to wild-type levels. These findings indicate that muscle stem cell myogenic potential is perturbed in skeletal muscle of a mouse model of hyperlipidaemia. We propose that platelet-releasate could be a therapeutic intervention for conditions with associated myopathy such as peripheral arterial disease.

New Findings: The impact of obesity-independent hyperlipidaemia on skeletal muscle stem cell function of ApoE deficient (ApoE-/-) mice remains unknown. Using ex vivo, in vitro and in vivo approaches, we report that compromised muscle stem cell function accounts for the impaired muscle regeneration in hyperlipidaemic ApoE-/- mice. Importantly, impaired muscle regeneration is normalized by administration of platelet-releasate.

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Research article

Platelet releasate normalises the compromised muscle regeneration in a mouse model of hyperlipidaemia

Running title: Skeletal muscle stem cell function and regeneration in ApoE knockout mice

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Key words: Skeletal muscle stem cells, ApoE deficiency, hyperlipidaemia, myogenesis, platelet releasate

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New Findings

- What is the central question of this study?

  The impact of obesity-independent hyperlipidaemia on skeletal muscle stem cell function of ApoE deficient ($ApoE^{-/-}$) mice remains unknown.

- What is the main finding and its importance?

  Using *ex vivo, in vitro* and *in vivo* approaches, we report that compromised muscle stem cell function accounts for the impaired muscle regeneration in hyperlipidaemic $ApoE^{-/-}$ mice. Importantly, impaired muscle regeneration is normalized by administration of platelet-releasate.

Abstract

Muscle satellite cells are important stem cells for skeletal muscle regeneration and repair after injury. ApoE deficient mice, an established mouse model of hyperlipidaemia and atherosclerosis, show evidence of oxidative stress-induced lessions and fat infiltration in skeletal muscle followed by impaired repair after injury. However, the mechanisms underpinning attenuated muscle regeneration remain to be fully defined. Key to addressing the latter is to understand the properties of muscle stem cells from ApoE deficient mice and their myogenic potential. Muscle stem cells from ApoE deficient mice were cultured both *ex vivo* (on single fibres) and *in vitro* (primary myoblasts) and their myogenic capacity was determined. Skeletal muscle regeneration was studied on days 5 and 10 after cardiotoxin injury. ApoE deficient muscle stem cells showed delayed activation and differentiation on single muscle fibres *ex vivo.* Impaired proliferation and differentiation profiles were also evident on isolated primary muscle stem cells in culture. ApoE deficient mice displayed impaired skeletal muscle regeneration after acute injury *in vivo*. Administration of platelet releasate in ApoE deficient mice reversed the deficits of muscle regeneration after acute injury to wild-type levels. These findings indicate that muscle stem cell myogenic potential is perturbed in skeletal muscle of a mouse model of hyperlipidaemia. We propose that platelet-releasate could be a therapeutic intervention for conditions with associated myopathy such as peripheral arterial disease.

Introduction

Muscle stem cells, also known as satellite cells, are muscle progenitor cells that reside in niches between the sarcolemma and the basal membrane of the myofibres (Relaix & Zammit, 2012; Yin *et al.*, 2013). Abundant evidence suggests that muscle stem cells are an integral part of skeletal muscle growth and regeneration in response to injury (Relaix & Zammit, 2012). In undamaged adult skeletal muscle, stem cells generally persist in a mitotically quiescent state. They are activated when myofibre damage or hypertrophy occurs, during which muscle stem cells differentiate into myoblasts and fuse together for *de novo* myotube formation, or fuse to damaged myofibres for replacement of myonuclei (Relaix & Zammit, 2012; Yin *et al.*, 2013). In several pathological conditions, such as muscular dystrophy and chronic obstructive pulmonary disease, muscle stem cells have impaired capacity for activation and differentiation (Girgenrath *et al.*, 2005; Morgan & Zammit, 2010; Pomies *et al.*, 2015). In metabolic diseases, such as obesity, muscle stem cells have been reported to exhibit decreased myogenic capacity after skeletal muscle injury (Fu *et al.*, 2016; Xu *et al.*, 2018). However, skeletal muscle stem cell function in the context of systemic hyperlipidaemia has only recently begun to unravel (Verpoorten *et al.*, 2020).

ApoE deficient ($ApoE^{-/-}$) mice are a well-established model of hyperlipidaemia and atherosclerosis exhibiting primarily elevated VLDL/IDL and secondary LDL plasma levels (Bolanos-Garcia & Miguel, 2003; Schreyer *et al.*, 2003; Ishida *et al.*, 2004; Hofmann *et al.*, 2008; Bartelt *et al.*, 2011; Lee *et al.*, 2011; Raman *et al.*, 2011; Li *et al.*, 2013; Ulasova *et al.*, 2013; Sfyri & Matsakas, 2017). We have shown that obesity-independent hyperlipidaemia induced intramuscular lipid accumulation and skeletal muscle oxidative stress in $ApoE^{-/-}$ mice (Sfyri *et al.*, 2018). $ApoE^{-/-}$ mice have delayed skeletal muscle regeneration and wound healing after injury (Kang *et al.*, 2008; Crawford *et al.*, 2013; Arnold *et al.*, 2015) (Hiebert *et al.*, 2013);. This delay in skeletal muscle regeneration was attributed mainly to perturbed macrophage infiltration and phagocytosis due to ApoE deficiency (Kang *et al.*, 2008; Arnold *et al.*, 2015). Specifically, Kang et al. showed that proinflammatory cytokines remained increased in $ApoE^{-/-}$ injured

muscle even after 14 days of injury (Kang *et al.*, 2008). Moreover, Arnold et al. reported that ApoE deficiency impacts negatively on macrophage phagocytic activity and is at least partially responsible for the impairment of skeletal muscle regeneration (Arnold *et al.*, 2015). However, the skeletal muscle stem cell myogenic capacity and function in ApoE$^{-/-}$ mice and their role in muscle regeneration remain largely unknown so far. Moreover, the effect of growth factor-rich biomaterials such as platelet secretome on the regenerative capacity of the ApoE$^{-/-}$ mouse remains to be determined. Therefore, in this study we aimed to determine whether hyperlipidaemia in skeletal muscle of ApoE$^{-/-}$ mice would affect muscle stem cell myogenic progression, independently of the proinflammatory environment identified previously. We hypothesised that muscle stem cell function is perturbed in atherosclerotic mice with systemic hyperlipidaemia and skeletal muscle oxidative stress, and this may contribute to impaired muscle regeneration *in vivo*. We further hypothesised that platelet releasate would boost muscle regeneration in ApoE$^{-/-}$ mice.

To achieve this, ApoE$^{-/-}$ muscle stem cell myogenesis was evaluated in three different experimental settings, i.e. on single muscle fibres, isolated muscle stem cells and injured hindlimb muscle. Firstly, myofibres from ApoE$^{-/-}$ mice were isolated and cultured *ex vivo* to investigate the myogenic progression and self-renewal of muscle stem cells. Secondly, muscle stem cells were removed from their microenvironment, i.e. myofibres, and their myogenic potential was studied in cultures *in vitro*. Thirdly, tibialis anterior (TA) muscles were injured with cardiotoxin, and muscle regeneration was studied at day 5 of recovery. Most importantly, we provide evidence that treatment of ApoE$^{-/-}$ mice with platelet releasate normalises skeletal muscle regeneration after injury to wild-type levels. These data provide novel findings on the role of muscle stem cells in the regeneration of the ApoE deficient mouse and set the ground for the development of novel therapies for skeletal myopathy as seen in patients with peripheral arterial disease.

Materials and Methods

Ethical approval. All experiments were carried out according to the guidelines laid down by their institution's animal welfare committee and a project license (PFD1DF7E8) from the United Kingdom Home Office in agreement with the revised Animals (Scientific Procedures) Act 1986. Animals were humanely sacrificed via Schedule 1 killing (i.e. cervical dislocation) under terminal anaesthesia with graded introduction of carbon dioxide. Blood sampling from healthy human volunteers was performed with their written informed consent and was approved by the University's Ethics Committee and conducted according to the Declaration of Helsinki. All experiments were carried out according to the principles and regulations of the Journal (Grundy, 2015).

Animal Maintenance*.* Thirty-week-old male C57Bl/6J (wild-type, WT) and ApoE deficient ($ApoE^{-/-}$) mice on the same genetic background were housed at the University of Hull under standard environmental conditions (20–22 °C, 12–12 h light–dark cycle) and were provided standard chow (normal diet, ND) and water *ad libitum*.

Single myofibre isolation and culture. Murine C57Bl/6J single myofibres were isolated from the extensor digitorum longus (EDL) muscle as previously described (Omairi *et al.*, 2016; Scully *et al.*, 2018b). Briefly, EDL muscles were dissected and chemically digested with collagenase (0.2%; Sigma Aldrich; cat. C2674) for 3-4 hours at 37 °C and 5% CO_2. Isolated myofibres were cultured for 24, 48 and 72 hours in single fibre media containing 10% Horse Serum, 0.5% chick embryo extract and 1% penicillin-streptomycin. Myofibres were immunostained with mouse monoclonal anti-Pax7 (1:200, Santa Cruz; cat. sc-81648, RRID:AB_2159836), rabbit polyclonal anti-MyoD (1:200; Santa Cruz; cat. sc-760, RRID:AB_2148870) and rabbit polyclonal anti-Myogenin (Santa Cruz; cat. sc-576, RRID:AB_2148908). Secondary antibodies used are AlexaFluor 488 goat-anti-mouse (Invitrogen; cat. A11029, RRID:AB_138404) and Alexa Fluor 594 goat-anti-rabbit (Invitrogen; cat A11037, RRID:AB_2534095). Relative expression of the above factors is shown as percentage per myofibre.

Primary muscle stem cell isolation from single myofibres and culture. Primary muscle stem cells were isolated from the EDL of wild-type and ApoE$^{-/-}$ mice as previously described (Scully *et al.*, 2018b). Briefly, muscles were dissected and subjected to 0.2% collagenase digestion for 4 hours at 37°C. Muscle stem cells were collected from isolated myofibres by trypsinisation in 0.125% trypsin-EDTA solution and seeded in Matrigel (1mg mL-1; Corning Matrigel; cat. 354234)-coated 24-well cell culture plates, in muscle stem proliferation media (30% FBS, 1.5% chick embryo extract and 1% penicillin-streptomycin). Differentiation of muscle stem cells was achieved through culturing for 3 days in proliferation medium before switching to differentiation medium (5% Horse Serum, 0.5% chick embryo extract, 1% penicillin-streptomycin and 0.1% amphotericin B) for a further 5 days. Isolated primary muscle stem cells were seeded on coverslips in 1mL of media in 24-well plates (Corning® Costar® TC-Treated 24-Well Plates) and stained with rabbit polyclonal anti-Myogenin (Santa Cruz; cat. sc-576, RRID:AB_2148908) as previously described (Scully *et al.*, 2018b). The fusion index (i.e. number of myogenin positive nuclei within myotubes divided by total number of nuclei), and morphometric analysis of myotubes was performed on an AxioImager fluorescence microscope equipped with an Axiocam digital camera using the ZEN imaging software (Zeiss, Germany).

***In Vivo* cardiotoxin-induced muscle injury**. On day 1, wild-type and ApoE$^{-/-}$ mice were injected with a total of 30µL, 50µM *Naja pallida* cardiotoxin (CTX; Latoxan, Valence France; cat. L8102) into the TA muscle of one limb (n=6 per group) under general anaesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally. Post-cardiotoxin analgesia (buprenorphine at 0.1 mg/kg) was administered on day 1 and 2. At day 5 (D5) and day 10 (D10) after injury, mice were sacrificed, the TA muscles were collected, immediately frozen and 12µm cryo-sections were processed for immunohistochemistry.

Immunohistochemistry of injured muscle sections. Primary antibodies for anti-myosin heavy chain 3 (i.e. embryonic myosin) and F4/80 (Santa Cruz; cat.

sc-53091 RRID:AB_670121, and R&D Biosystems; cat MBA3249 respectively) were used to evaluate regenerating fibres, and macrophage infiltration respectively. Primary antibodies for MyoD (1:200; Santa Cruz; cat. sc-760, RRID:AB_2148870) and Myogenin (Santa Cruz; cat. sc-576, RRID:AB_2148908) were used to evaluate skeletal muscle stem cell activation and differentiation in injured muscle. Injury was detected via haematoxylin & eosin staining. IgG staining on injured muscle sections was used to identify necrotic fibres due to altered cell membrane permeability (Straub *et al.*, 1997; Begam & Roche, 2018). For morphological analyses, muscles were stained using hematoxylin-eosin (H&E).

Dihydroethidium (DHE) staining: TA muscle tissue sections were incubated with 10μM of dihydroethidium (DHE) in PBS, for 30 min at 37 °C and were subsequently washed in PBS three times with each wash lasting 5 minutes. The sections were mounted in fluorescent mounting medium, using DAPI to counterstain cell nuclei.

Preparation of platelet releasate. Platelet releasate was prepared as described previously (Scully *et al.*, 2018b). In brief, whole human blood from healthy adult and medication-free volunteers was collected in acid citrate dextrose (ACD) at a ratio of 1:5 ACD to blood. Blood was centrifuged at 190 *g* for 15 minutes, platelet rich plasma was collected, and platelets inactivated using prostaglandin I_2 (534 nmol/L; Cayman Chemical, cat. 18220). The platelet rich plasma was then centrifuged in a swing-out rotor at 800 *g* for 12 minutes and the supernatant platelet-poor plasma was removed. The platelet pellet was resuspended in modified Tyrode's buffer (NaCl, HEPES, NaH_2PO_4, $NaHCO_3$, KCl, $MgCl_2$ and D-Glucose) to a concentration of 1×10^9 platelets mL^{-1} using a cell counter (Beckman Coulter; Z1 - Series Coulter® Particle Counter). The platelet preparation was activated using a PAR1 agonist (TRAP6; 20μmol/L; AnaSpec; cat. AS - 60679, Cambridge Bioscience, Cambridge, UK), in an aggregometer (CHRONO - LOG® Model 490 4 + 4 Optical AggregationSystem, USA). Platelets were centrifuged at 9500 *g* for 10 minutes, and the supernatant

releasate was collected for further use. Platelet releasate (100 μL/mouse) was administered on days 0, 1 and 3 post-injury by intraperitoneal injections.

Muscle stem cell proliferation and viability analysis. Muscle stem cell proliferation was evaluated by the pyrimidine analogue EdU incorporation assay using the fluorescent Click-iT® EdU Cell Proliferation Assay (Invitrogen, Life Technologies, Grand Island, NY, USA) as described previously (Scully *et al.*, 2018b). Proliferating cells were measured as a percentage of EdU positive divided by DAPI (4',6-diamidino-2-phenylindol-stained (Dako))-stained nuclei.

RNA extraction and real-time PCR analysis. Quantitative PCR was performed as described previously (Matsakas *et al.*, 2012). Briefly, muscle primary stem cells were proliferated in 6-well plates and total RNA was isolated on day 4 of differentiation using the EZNA Total RNA Kit I (Omega Biotek, USA). Total RNA (1.5 μg) was reverse-transcribed to cDNA and analysed by quantitative real-time RT-PCR on a StepOne Plus cycler (Applied Biosystems, UK). Primers were designed using the software Primer Express 3.0 (Applied Biosystems, UK). mRNA levels of *MyoD*, Scribble planar cell polarity protein (*Scrib1*), Myogenin, transmembrane protein 8c (*Tmem8c*), brain expressed X-linked 1 (*Bex1*), Serum response factor (*Srf*), myosin heavy chain 1 (*Mhc1*) and actin alpha 1 (*Acta1*), were measured/determined for wild-type and ApoE$^{-/-}$ myotubes. Relative expression was calculated using the ΔΔCt method with normalisation to the reference genes encoding cyclophilin-B (*Cyp*) and hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) (Sfyri *et al.*, 2018). Primers can be provided upon request.

Statistical analysis. Data are reported as mean±SD for both the *in vitro* muscle stem cell experiments and the *ex vivo* muscle stem cell myogenic progression. Cell culture experiments were conducted with n=6-9 technical replicates and n=3 independent experiments from n=3 mice/group, as indicated in figure legends. Statistical differences between experimental groups were determined by the Mann-Whitney U test and were considered as significant for $p<0.05$.

In vivo experiments were conducted with n=6 mice/group. Statistical analysis was performed by the Mann-Whitney U, Chi square test, and Kruskal-Wallis test as indicated in figure legends. Data are mean±SD. Statistical analysis was performed with the SPSS software (IBM SPSS Statistics version 24).

Results

Ex vivo proliferation and differentiation profiles of muscle stem cells from ApoE$^{-/-}$ and WT single fibres

It has been recently shown that ApoE$^{-/-}$ mice have increased myofibre cross-sectional area (Sfyri *et al.*, 2018). Therefore, we initially sought to determine the myonuclear domain, defined as the cytoplasmic area of a myofibre controlled by a single myonucleus using the single fibre model in EDL muscle (Allen *et al.*, 1999). We found no significant differences in total myonuclear number per fibre between genotypes. However, the relative myofibre cross sectional area (CSA) was significantly higher by 63% in ApoE$^{-/-}$ compared to WT EDL myofibres (**Figure 1A**). Consequently, the relative myonuclear density was significantly lower in ApoE$^{-/-}$ myofibres compared to WT myofibres (64.20%±4.97 vs. 100%±6.52, respectively, **Figure 1A**). We next took advantage of the differential expression patterns of transcription factors and myogenic regulatory factors as follows: in adult skeletal muscle, quiescent stem cells express Pax7, activated stem cells switch on MyoD expression, proliferating stem cells co-express Pax7 and MyoD, stem cells committed to differentiation are Pax7-negative and MyoD-positive, whereas differentiated stem cells give rise to new myonuclei that express myogenin (Fuchtbauer & Westphal, 1992; Grounds *et al.*, 1992; Yablonka-Reuveni & Rivera, 1994; Yablonka-Reuveni *et al.*, 1999; Zammit *et al.*, 2002; Zammit *et al.*, 2004). We determined the proliferation and differentiation profiles of muscle stem cells during myogenic progression. Muscle stem cells retained in their niche on single EDL myofibres were immunostained for Pax7 at baseline (T0), Pax7 and MyoD at 24 and 48 hours (T24, T48), and Pax7 and Myogenin at 72 hours of culture (T72) (**Figure 1B**). We found no significant differences in total muscle stem cell numbers between ApoE$^{-/-}$ and WT mice at any time point (**Figure 1C**). ApoE$^{-/-}$ muscle stem cells exhibited the same proliferation patterns as in WT EDL myofibres at 24 and 48 hours, despite a transient delay in MyoD activation (i.e. Pax7^{+}/MyoD^{-} staining) at 24 hours (**Figure 1C**). Specifically, at T24 the majority of muscle stem cells had become activated in both genotypes (80%±1.6 in WT and 72%±2.0 in ApoE$^{-/-}$ muscle stem cells) as shown by the expression of MyoD and co-

expression of Pax7/MyoD (**Figure 1C**). Similarly, at T48, 94.82%±2.1 of WT and 93.79%±1.9 of ApoE$^{-/-}$ muscle stem cells were activated. However, at T72 ApoE$^{-/-}$ muscle stem cells had a significantly lower expression of myogenin by 17% compared to WT (**Figure 1C**). Moreover, Pax7 expression was significantly increased by 25% in ApoE$^{-/-}$ compared to WT fibres (**Figure 1C**). This data indicates that despite the absence of significant differences in muscle stem cell proliferation between ApoE$^{-/-}$ and WT muscle stem cells, there appears to be compromised differentiation of muscle stem cells in ApoE$^{-/-}$ EDL fibres.

Compromised proliferative capacity of primary muscle stem cells from ApoE$^{-/-}$ mice

Given the compromised muscle stem cell differentiation in EDL at T72 on single myofibres *ex vivo*, we next aimed to determine *in vitro* the proliferation patterns of primary muscle stem cells from ApoE$^{-/-}$ and WT EDL fibres. Primary muscle stem cells were cultured for 24 hours and cell proliferation was measured by the EdU incorporation assay (**Figure 2A**). Muscle stem cells from the EDL of ApoE$^{-/-}$ mice showed significantly reduced proliferation by 27% compared to WT muscle stem cells (i.e. 48.6%±10.7 vs. 35.5%±11.04, **Figure 2B**). These findings indicate that ApoE deficiency impacts on the proliferative capacity of primary muscle stem cells when the local niche of the myofibre is eliminated.

Impaired differentiation and fusion of primary muscle stem cells from ApoE$^{-/-}$ mice

Given the *ex vivo* finding that a lower proportion of ApoE$^{-/-}$ muscle stem cells express myogenin, we hypothesised that this may impair the myogenic differentiation *in vitro*. Therefore, we sought to determine whether there were any deficits in the capacity of primary muscle stem cells from the ApoE$^{-/-}$ mice to fuse and form myotubes as compared to wild-type muscle stem cells after 4

days of differentiation. To this aim, differences in total nuclear number, fusion index (defined as Myogenin^{+ve} nuclei in myotubes/ DAPI as a percentage), overall number of myotubes, myotube area and length were determined in differentiated muscle stem cells (**Figure 3**). There was a significantly lower total nuclear number by 35% in ApoE$^{-/-}$ EDL cultures as compared to WT. The fusion index – an indicator of the ability of muscle stem cells to form myotubes, was significantly decreased by 46% in the ApoE$^{-/-}$ myotubes as compared to WT (**Figure 3B**). Most importantly, we found that ApoE$^{-/-}$ muscle stem cells from the EDL muscle, formed significantly fewer myotubes by 45% per viewing field normalized to myonuclear number (i.e. DAPI; **Figure 3B**). We also found that the myotubes formed had a lower area by 68%, and length was significantly smaller by 12% in ApoE$^{-/-}$ compared to WT cultures. These findings indicate that primary muscle stem cells from ApoE$^{-/-}$ mice exhibit impaired differentiation and fusion in culture.

Muscle stem cells from ApoE$^{-/-}$ mice exhibit reduced expression of genes regulating muscle stem cell fate, fusion and contractile proteins

Primary ApoE$^{-/-}$ muscle stem cells from the EDL exhibited significantly reduced capacity to form myotubes *in vitro*. Thus, we next sought to determine whether this decrease was at least partially due to impaired myoblast differentiation. To this aim, primary muscle stem cells were differentiated for 4 days to form myotubes and mRNA levels of genes involved in muscle stem cell fate (i.e. *MyoD*, *Scrib1* and *Myogenin*), myoblast fusion (i.e. *Bex1*, *Tmem8c* and *Srf*) and genes coding for contractile proteins (i.e. *Mhc1, Acta1*) were measured. We found significantly lower mRNA levels of all studied genes in ApoE$^{-/-}$ myotubes (**Figure 4**). These findings indicate that both differentiation and fusion are impaired at the transcriptional level in ApoE$^{-/-}$ primary muscle stem cells.

Impaired skeletal muscle regeneration of ApoE$^{-/-}$ mice 5 days post injury

Having shown that ApoE deficiency had an impact on function of skeletal muscle stem cells, TA muscles were injured with cardiotoxin, and regeneration was examined after 5 days of recovery in wild-type and ApoE$^{-/-}$ mice. We firstly examined the quality of muscle sections using H&E staining which showed that the tissue integrity had been preserved. TA muscles of ApoE$^{-/-}$ mice displayed significantly more necrotic fibres (identified by IgG staining) by 200% in the ApoE$^{-/-}$ vs. wild-type mice (**Figure 5B,E**). Furthermore, ApoE$^{-/-}$ TA muscles had significantly smaller regenerating fibres (i.e. eMHC^{+} fibres) compared to wild-type (**Figure 5C,E**). The ApoE$^{-/-}$ mouse also displayed more macrophage infiltration as seen by F4/80 staining, indicating an increased inflammatory response (**Figure 5D,E**). Taken together, these findings indicate impaired regeneration of the ApoE$^{-/-}$ mouse after cardiotoxin injury.

Skeletal muscle regeneration deficit in ApoE$^{-/-}$ mice is rescued by treatment with platelet-releasate

ApoE deficiency resulted in delayed regeneration of skeletal muscle after cardiotoxin injury. Therefore, we sought to determine whether this deficit could be mitigated via treatment with platelet releasate. ApoE$^{-/-}$ mice were administered platelet releasate on days 0, 1 and 3 post-injury by intraperitoneal injections. We found that TA muscles of ApoE$^{-/-}$ mice treated with releasate had improved regeneration 5 days post-injury compared to control ApoE$^{-/-}$ mice (**Figure 5A-E**). The number of necrotic fibres per muscle were significantly reduced in ApoE$^{-/-}$ mice by treatment with releasate compared to control ApoE$^{-/-}$ mice (**Figure 5B,E**). Interestingly, no significant difference in necrotic fibres was detected between treated ApoE$^{-/-}$ muscles and untreated muscle from WT mice (**Figure 5B,E**). Releasate-treated ApoE$^{-/-}$ mice displayed a significantly higher CSA of regenerating fibres by 27% compared to control ApoE$^{-/-}$ mice, reaching the average size of regenerating fibres from WT mice (**Figure 5C,E**). Furthermore, the platelet releasate treatment significantly decreased macrophage infiltration by 27% 5 days post-injury in the ApoE$^{-/-}$ mouse muscle, although this was significantly higher than that seen in the WT muscle (**Figure**

5D,E). Taken together, these findings suggest that platelet releasate accelerates skeletal muscle regeneration after injury in ApoE$^{-/-}$ mice.

Skeletal muscle stem cell deficit in activation and differentiation is found in injured ApoE$^{-/-}$ TA muscle and can be alleviated by platelet releasate treatment

Having established a deficit in skeletal muscle regeneration after cardiotoxin injury in ApoE deficient mice, we sought to determine the impact on activation and differentiation of skeletal muscle stem cells in injured TA muscles *in vivo*. MyoD and myogenin expression remains elevated in regenerating muscle for several days post-injury (Kang *et al.*, 2008; Tian *et al.*, 2016). We found that TA muscles of ApoE$^{-/-}$ mice expressed MyoD in a lower proportion of cells compared to wild type by 42%. Importantly, platelet releasate treatment normalised MyoD expression in the injured TA to WT muscles (**Figure 6A,B**). Furthermore, the differentiation of skeletal muscle stem cells post-injury *in vivo* followed a similar pattern, with ApoE deficient mice expressing 82% fewer myogenin positive cells than WT. Again, releasate treatment of ApoE$^{-/-}$ mice normalised myogenin expression to the WT levels (**Figure 6A,B**). Taken together, these findings indicate that skeletal muscle stem cells of ApoE deficient mice display impaired activation and differentiation, which may explain the deficits in post-injury muscle regeneration. Furthermore, platelet releasate restores skeletal muscle stem cell function in ApoE deficient mice.

Delayed skeletal muscle regeneration of ApoE$^{-/-}$ mice 10 days post injury is rescued by platelet releasate

Having shown that ApoE deficiency had an impact on skeletal muscle regeneration 5 days after cardiotoxin injury, we conducted morphometric analysis of injured muscles after 10 days post-injury. For this reason, we measured the cross sectional area of regenerating fibres with centrally located

nuclei. TA muscles of ApoE$^{-/-}$ mice displayed smaller regenerating fibres by 43% compared to WT mice. Platelet releasate treatment normalised the size of regenerating fibres to WT levels (**Figure 7A,B**). These findings indicate that impaired muscle regeneration of the ApoE$^{-/-}$ mouse after cardiotoxin injury is maintained for at least 10 days. The fact that the ApoE$^{-/-}$ group has largely dissimilar fibres in size, many smaller diameter fibres with central nuclei and lower fibre density indicates a slower muscle regeneration.

Oxidative stress in injured muscle of ApoE$^{-/-}$ mice

Given that skeletal muscle oxidative stress is increased in ApoE$^{-/-}$ mice and that reactive oxygen species (ROS) impair myogenic differentiation by inhibiting MyoD (Ardite *et al.*, 2004; Catani *et al.*, 2004; Barbieri & Sestili, 2012; Sfyri *et al.*, 2018) we also sought to determine the levels of ROS in the injured TA (**Figure 8**). While the injured WT mouse TA had increased ROS compared to the uninjured, the levels of ROS were vastly increased (by 395%) in the injured TA muscle of ApoE$^{-/-}$ mice independent of platelet releasate treatment. These data suggest that the increased oxidative stress seen in ApoE$^{-/-}$ mice may contribute to the impaired myogenic differentiation reported here and possibly account for decreased MyoD and myogenin expression in muscle stem cells from ApoE deficient mice.

Discussion

Skeletal muscle has a remarkable intrinsic regenerative capacity mainly brought about by skeletal muscle stem cells; these are muscle-specific progenitor cells essential for skeletal muscle maintenance and regeneration (Relaix & Zammit, 2012). Recent evidence suggests that muscle stem cell function may be abnormal or impaired in the context of obesity both after skeletal muscle injury as well as various experimental conditions *in vitro* and *in vivo* (Hu *et al.*, 2010; Nguyen *et al.*, 2011; Akhmedov & Berdeaux, 2013; D'Souza *et al.*, 2015; Fu *et al.*, 2016; Verpoorten *et al.*, 2020). Recently we found that CD36-deficient mice - another diet-independent mouse model of systemic hyperlipidaemia - have impaired muscle stem cell function and delayed muscle regeneration (Verpoorten *et al.*, 2020). However, the function of muscle satellite cells in ApoE deficient mice has not received much attention so far.

Hyperlipidaemia is a major risk factor for atherosclerosis and cardiovascular disease as well as an independent risk factor for peripheral arterial disease (PAD). PAD is a chronic disease characterised by restriction and finally blockage of the arteries of the lower extremities (Varu *et al.*, 2010; Haas *et al.*, 2012). PAD is a risk factor of cardiovascular morbidity and mortality (Varu *et al.*, 2010; Haas *et al.*, 2012). Patients with PAD exhibit skeletal myopathy that is characterised by altered myofibre morphology, increased oxidative damage, inflammation, mitochondriopathy and poor muscle regeneration impacting on the patients' quality of life (Fu *et al.*, 2008; Weiss *et al.*, 2013; Koutakis *et al.*, 2014; Koutakis *et al.*, 2015). Although our understanding about the function of muscle stem cells in obesity and diabetes is still evolving, the obesity-independent impact of systemic hyperlipidaemia followed by increased oxidative stress on muscle stem cell myogenic progression and self-renewal is largely unknown (Sfyri *et al.*, 2018). Therefore, this study determined the muscle stem cell myogenic proliferation and differentiation profiles in ApoE$^{-/-}$ mice, an established mouse model of obesity-independent hyperlipidaemia and atherosclerosis. Moreover, we took advantage of cutting-edge platelet-based applications as biomaterials in order to deliver a cocktail of growth factors in

order to boost the regenerative capacity of the skeletal muscle from ApoE deficient mice after injury (Scully *et al.*, 2018a; Scully *et al.*, 2018b; Scully *et al.*, 2020).

In the present study, we found that the muscle stem cell progeny numbers were similar between genotypes for all timepoints, indicating that muscle stem proliferation is normal in ApoE$^{-/-}$ mice. We also report a transient impairment of muscle stem cell myogenic progression (i.e. activation) at T24 shown by decreased expression of Pax7/MyoD co-expression. More strikingly, we report here impaired myogenin expression at T72 in the stem cells of EDL myofibres from ApoE$^{-/-}$ mice *ex vivo*. This finding is in line with the decreased expression of myogenin found in the gastrocnemius of ApoE$^{-/-}$ mice after hindlimb ischemia (Kang *et al.*, 2008). Taken together, these findings indicate that ApoE deficiency may delay *ex vivo* myogenic progression of muscle stem cells and perturb myogenic differentiation.

Beyond hyperlipidaemia, ApoE$^{-/-}$ mice have elevated intramuscular reactive oxygen species (ROS) production and oxidative stress (Sfyri & Matsakas, 2017; Sfyri *et al.*, 2018). We reported here excessive levels of ROS in the injured TA muscle of ApoE$^{-/-}$ mice independent of the presence of platelet releasate. Studies have reported that elevated ROS production impairs myogenic differentiation *in vitro* through inhibition of MyoD and MyoD-dependent transcription (Ardite *et al.*, 2004; Catani *et al.*, 2004; Barbieri & Sestili, 2012). Evidence suggests that the major pathways that may lead to reduction of MyoD levels, are through the increase of NF-κB activity or TNFα expression (Guttridge *et al.*, 2000). Thus, the delayed myogenic progression in our study may be attributed to the impact of increased ROS from the myofibre environment that is possibly due to elevated intramuscular lipid content (Sfyri *et al.*, 2018). However, the role of apolipoprotein E on muscle stem cell myogenic progression is not well established. Although it has been shown that apolipoprotein E protein is expressed in differentiating human muscle stem cells, its function has yet to be investigated (Le Bihan *et al.*, 2015). Hence, another possible explanation for the delayed muscle stem cell differentiation

could be that apolipoprotein E plays a role in muscle stem cell activation and differentiation.

To further investigate the above findings, we isolated muscle stem cells from single fibres and subjected them to cell culture experiments *in vitro* as described previously (Omairi *et al.*, 2016; Scully *et al.*, 2018b; Verpoorten *et al.*, 2020). Interestingly, ApoE$^{-/-}$ muscle stem cells stripped from their niche and myofibre environment exhibited impaired proliferation compared to wild-type. The impaired proliferation and differentiation could be attributed to oxidative stress altering their ability to self-renew and differentiate (Chen *et al.*, 2017; Sriram *et al.*, 2019). Furthermore, it has been shown that satellite cells from diet-induced obese mice have impaired proliferation *in vitro* (D'Souza *et al.*, 2015). The above finding suggests that muscle stem cells have perturbed activation that is more prominent in the *in vitro* environment, highlighting the role of myofibre stem cell niche (Takemoto *et al.*, 2019). We report here novel findings that muscle stem cells from ApoE$^{-/-}$ mice exhibit compromised myogenic differentiation as shown by severely impaired myotube formation, fusion index and myotube length *in vitro*.

To verify that the impairment in differentiation and fusion was not a consequence of the fewer number of myotubes, expression of genes involved in the above process were determined. Indeed, we found decreased mRNA levels of genes involved in muscle stem cell fate (*MyoD, Scrib1* and *Myogenin*) fusion (i.e. *Bex1*, *Tmem8c* and *Srf)* as well as late myogenesis (i.e. *Mhc1 and Acta1*), suggesting that this impairment was due to muscle stem cell functional capacity. Studies have shown that oxidative stress as well as lipotoxicity lead to reduced C2C12 - an immortalised myoblast line - proliferation and differentiation *in vitro* (Bosutti & Degens, 2015; Pomies *et al.*, 2015; Grabiec *et al.*, 2016; Lee *et al.*, 2017). Thus, the hyperlipidaemic environment of the myofibres may have rendered muscle stem cells susceptible to impaired proliferation and differentiation. We recently showed that ApoE$^{-/-}$ mice have ectopic fat deposition in skeletal muscle leading to elevated intramuscular triacylglycerol contents followed by evidence of perturbed antioxidant capacity and increased oxidative

stress (Sfyri *et al.*, 2018). Taken together, these findings suggest that ApoE$^{-/-}$ muscle stem cells have perturbed myogenic progression in two different experimental settings (i.e. *ex vivo* and *in vitro*) and this may be attributed to impaired muscle stem cell functional capacity, probably secondary to oxidative stress and increased lipid accumulation or ApoE deficiency *per se.* However, further research is needed to determine the impact of hyperlipidaemia on potential satellite cell epigenetic modifications.

To further explore the effect of impaired skeletal muscle stem cell function on muscle regeneration, ApoE$^{-/-}$ and wild-type mice were subjected to an acute injury protocol with cardiotoxin *in vivo*. We report here impaired muscle regenerative capacity in the injured ApoE$^{-/-}$ mice on days -5 and -10 post injury as evidenced by eMHC expression and IgG infiltration (i.e. indicating fibre necrosis); and the size of regenerating fibres respectively compared to wild-type. These findings are in line with previously published data showing deficits in muscle regeneration in ApoE$^{-/-}$ mice 14 days post ischaemic injury (Kang *et al.*, 2008). Furthermore, intramuscular macrophage infiltration in injured TA muscle was significantly higher in the ApoE$^{-/-}$ mice, indicating a delay in the temporal sequence of inflammatory and regerative events following muscle injury (Ciciliot & Schiaffino, 2010). Most importantly, the muscle stem cell myogenic deficits in the *in vitro* and *ex vivo* settings of this study were validated *in vivo* by showing a deficit in muscle stem cell activation and differentiation in injured ApoE$^{-/-}$ muscle due to impaired myoD and myogenin expression respectively. Taken together, the present study links for the first time the delayed skeletal muscle regeneration of the ApoE$^{-/-}$ mouse to impaired muscle stem cell function.

Platelet-based therapies have been used for almost 50 years since the first developed of platelet rich plasma (PRP) (Andia & Abate, 2013). A huge number of commercial enterprises have arisen that each market PRP products to treat a spectrum of human medical conditions ranging from osteoarthritis to central nervous system pain management (Centeno *et al.*, 2017; Le *et al.*, 2018). The use of PRP has a number of disadvantages most based on the notion that

preparations containing platelets may induce aggregation and potentially occlude blood vessels (Wybier, 2008). This and other issues have led in part to the development of platelet lysates, rich in growth factors but devoid of cellular material. Indeed, these have been shown to have therapeutic value especially in the treatment of pain and orthopaedic injuries (Centeno *et al.*, 2017). Recently we and others have refined platelet-based therapies by developed secretomes that produced in a cell-regulated manner (rather than lysis of platelets) using specific mediators of degranulation. Platelet releasate is rich in numerous growth factors, cytokines and myokines constituting a powerful biomaterial for regenerative medicine (Sassoli *et al.*, 2018; Scully *et al.*, 2018a; Scully *et al.*, 2018b; Tsai *et al.*, 2018; Scully & Matsakas, 2019; Scully *et al.*, 2020). Given that, platelet releasate increases the myogenic potential and promotes differentiation of myoblasts *in vitro* and *ex vivo* (Scully *et al.*, 2018a; Scully *et al.*, 2020), we sought to determine whether platelet releasate mitigates the impaired skeletal muscle regeneration of the ApoE$^{-/-}$ mice *in vivo*. We report here for the first time, that treatment with platelet releasate normalised regeneration of ApoE$^{-/-}$ skeletal muscle to the levels of wild-type as judged by increased CSA of regenerating fibres (i.e. eMHC expressing fibres), reduced number of necrotic fibres (i.e. IgG infiltrated fibres), restored muscle stem cell myogenic deficits and tapered inflammatory response. It is suggested that the improved regenerative response to injury with platelet releasate has been brought about by improved function of skeletal muscle stem cells (Scully *et al.*, 2018b). Human platelet releasate contains several growth factors including, but not limited, to pdgfs, vegfs, fgfs, egfs, hgf, tgfb etc. Evidence suggests that human releasate also contains ApoE precursor and ApoE (Piersma *et al.*, 2009; Parsons *et al.*, 2018; Szklanna *et al.*, 2019). Therefore, it cannot be ruled out that some of the beneficial effects of the releasate treatment on the muscle regeneration of the ApoE deficient mice may have been brought about by ApoE. Further research is needed to pinpoint the role of individual factors of the platelet releasate on skeletal muscle regeneration.

Conclusions

This study does not differentiate the potential individual input of skeletal muscle oxidative stress, systemic hyperlipidaemia or ApoE deficiency *per se* on the impaired myogenic progression. However, this is the first study to investigate the impact of ApoE deficiency on skeletal muscle stem cell myogenic progression. We provide evidence that muscle stem cells on single fibres from ApoE$^{-/-}$ mice have decreased differentiation *ex vivo*. Similarly, isolated muscle stem cells have an impaired capacity to proliferate and differentiate when cultured *in vitro*. This has deleterious effects in the myogenic potential of ApoE$^{-/-}$ stem cells backed up by impaired expression of gene involved in muscle stem cell fate, myoblast fusion and contractile proteins as well as impaired muscle stem cell activation and differentiation after injury *in vivo*. These results establish a link between obesity-independent hyperlipidaemia followed by skeletal muscle oxidative stress and perturbed muscle stem cell function with implications for skeletal muscle regeneration. Most importantly, we provide evidence that the impaired muscle regeneration after injury in the ApoE deficient mouse can be rescued by the administration of platelet-derived releasate. These findings may have strong implications in the treatment of myopathy for patients with peripheral arterial disease and systemic hyperlipidaemia. Future research will focus to further establish the molecular mechanisms of muscle stem cell impairment and skeletal muscle function *in vivo*.

Additional information

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Author Contributions

The study was performed at Hull York Medical School, University of Hull. JB, PS and AM designed the work. JB, PS, RM, SV, DS, CA, PP, KP and AM acquired, analysed and interpreted data of the work. JB, PS and AM drafted the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Figure Legends

Figure 1

Impaired differentiation of muscle stem cells of EDL myofibres from ApoE$^{-/-}$ mice ex vivo.

(**A**) Baseline differences in total nuclear number per myofibre, relative cross sectional area (CSA), (i.e. normalised to the CSA of the WT myofibres) and Nuclear/CSA ratio in isolated EDL myofibres from WT and ApoE$^{-/-}$ mice. (**B**) Single myofibres were isolated from the EDL muscle and studied at different time points (T0, T24, T48 and T72 hours) to determine quiescent, proliferating and differentiating satellite cells, by means of Pax7, MyoD and Myogenin staining. Representative images of immunofluorescent detection of Pax7 (T0), Pax 7 and MyoD (T24 & T48) and Pax7 and Myogenin (T72, x40 magnification, scale bar=50µm, inset images depict enlarged satellite cell clusters). (**C**) Average number of satellite cells per myofibre and relative quantification of expression of Pax7, MyoD and Myogenin. Data are mean±SD (n=70-85 myofibres from n=4 mice/group). Statistical analysis was performed by Mann-Whitney U test, *p<0.05 and ***p<0.001 ApoE$^{-/-}$ vs WT.

Figure 2

Impaired proliferation and myotube formation of isolated primary muscle stem cells from ApoE$^{-/-}$ mice in vitro.

Proliferation of primary muscle stem cells isolated from EDL of wild-type and ApoE$^{-/-}$ mice was detected by EdU staining. (**A**) Representative images of proliferating myoblasts stained for DAPI (blue) and EdU (green) from the EDL myofibres (x5 magnification, scale bar 200µm). (**B**) Quantification of the percentage of proliferating cells (EdU positive) per total number of nuclei from EDL. Data are mean±SD (40=images per group, n=9 technical replicates (i.e. cultures) from 3 independent experiments from n=3 mice/group). Statistical analysis was performed by Mann-Whitney U, ***p<0.001 vs WT.

Figure 3

Impaired myotube formation of isolated primary muscle stem cells from ApoE$^{-/-}$ mice *in vitro*.

Myotube number and morphology from differentiated muscle stem cells isolated from the EDL of wild-type (WT) and ApoE$^{-/-}$ mice. (A) Representative immunofluorescence images for nuclei (DAPI) and myogenin in the EDL (x10 magnification, scale bar 100µm). Myotubes are shown with bright field images. (**B**) Quantification of total number of nuclei, fusion index (number of myogenin positive nuclei within myotubes divided by total number of nuclei), the number of myotubes per field normalised to number of nuclei, myotube area, and myotube length in the EDL of WT and ApoE$^{-/-}$ mice. Data are mean±SD (n=9 technical replicates (i.e. cultures) from 3 independent experiments from n=3 mice/group). Statistical analysis was performed by Mann-Whitney U test, ***p<0.001 vs WT.

Figure 4. Gene expression patterns in differentiating myotubes derived from cultured EDL primary muscle stem cells from ApoE$^{-/-}$and WT mice.

mRNA levels of genes involved in differentiation and muscle stem cell fate (*MyoD, Scrib1 and Myogenin), myoblast* fusion (*Bex1, Tmem8c* and *Srf*) and contractile proteins (*Mhc1* and *Acta1*) were assessed in differentiating primary muscle stem cells from the EDL of WT and ApoE$^{-/-}$ mice by qPCR. Data are shown as mean±SD (n=6 technical replicates from 3 independent experiments from n=3 mice/group). Statistical analysis was performed by Mann-Whitney U tests with *p<0.05, **p<0.01 vs WT.

Figure 5. Impaired skeletal muscle regeneration of ApoE$^{-/-}$ mice 5 days after injury is rescued by platelet releasate. Representative images of TA sections for the identification of (**A**) regenerating fibres (identified by centrally located nuclei, x20) stained with H&E. (**B**) Necrotic fibres (identified by IgG staining inside muscle fibres, x20), (**C**) the identification of regenerating muscle fibres (through the expression of embryonic myosin heavy chain (eMHC), x20. Scale bar: 50µm), (**D**) Macrophages (identified through F4/80 staining, x40) on day 5 after cardiotoxin injury and platelet releasate treatment, (**E**) Quantification of necrotic fibres, regenerating fibres and macrophages compared to wild-type

TA, Scale bar: 20μm at x40. Data are mean±SD (n=6 mice/group). Statistical analysis was performed by Kruskal-Wallis test with Bonferroni post hoc test. Differences are **p<0.01, ***p<0.001 vs WT, $^{\#\#}$p<0.01 vs ApoE$^{-/-}$.

Figure 6. Activation and differentiation of skeletal muscle stem cells is impaired in ApoE$^{-/-}$ mice and can be alleviated by treatment with platelet releasate. (**A**) Representative images of TA sections for myoD and myogenin staining from wild-type, ApoE$^{-/-}$ and ApoE$^{-/-}$ mice treated with platelet releasate on day 5 after cardiotoxin injury. (**B**) Quantification of MyoD and Myogenin as a percentage of total nuclei. Scale bar: 50μm, magnification: x20. Data are mean ±SD (n=6 mice/group). Statistical analysis was performed by Kruskal-Wallis test with Bonferroni post hoc test. Differences are ***p<0.001 vs all other groups.

Figure 7. Impaired skeletal muscle regeneration of ApoE$^{-/-}$ mice 10 days after injury is rescued by platelet releasate. Representative images of TA sections of regenerating injured muscle fibres via H&E stain (containing centrally located nuclei) on day 10 after cardiotoxin injury and platelet releasate treatment, (**B**) and quantification of regenerating fibre CSA. Scale bar: 50μm; magnification: x20. Data are mean±SD (n=6 mice/group). Statistical analysis was performed by Kruskal-Wallis test with Bonferroni post hoc test. Differences are ***p<0.001 vs all other groups.

Figure 8. ApoE$^{-/-}$ mice have increased ROS levels in injured TA muscle independent of platelet releasate treatment. TA muscles of injured WT, ApoE$^{-/-}$ and ApoE$^{-/-}$ treated with releasate, as well as uninjured WT mice were stained with DHE to visualise ROS levels. ROS levels were quantified by fluorescence intensity of DHE positive nuclei. Scale bar: 50μm; magnification: x20. Data are mean±SD (n=6 mice/group). Statistical analysis was performed

by Kruskal-Wallis test with Bonferroni post hoc test. Differences are *p<0.05 vs. WT uninjured, $^{###}$p<0.001 vs. WT injured.

References

Akhmedov D & Berdeaux R (2013). The effects of obesity on skeletal muscle regeneration. *Front Physiol* **4,** 371.

Allen DL, Roy RR & Edgerton VR (1999). Myonuclear domains in muscle adaptation and disease. *Muscle Nerve* **22,** 1350-1360.

Andia I & Abate M (2013). Platelet-rich plasma: underlying biology and clinical correlates. *Regen Med* **8,** 645-658.

Ardite E, Barbera JA, Roca J & Fernandez-Checa JC (2004). Glutathione depletion impairs myogenic differentiation of murine skeletal muscle C2C12 cells through sustained NF-kappaB activation. *Am J Pathol* **165,** 719-728.

Arnold L, Perrin H, de Chanville CB, Saclier M, Hermand P, Poupel L, Guyon E, Licata F, Carpentier W, Vilar J, Mounier R, Chazaud B, Benhabiles N, Boissonnas A, Combadiere B & Combadiere C (2015). CX3CR1 deficiency promotes muscle repair and regeneration by enhancing macrophage ApoE production. *Nat Commun* **6,** 8972.

Barbieri E & Sestili P (2012). Reactive oxygen species in skeletal muscle signaling. *J Signal Transduct* **2012,** 982794.

Bartelt A, Orlando P, Mele C, Ligresti A, Toedter K, Scheja L, Heeren J & Di Marzo V (2011). Altered endocannabinoid signalling after a high-fat diet in Apoe(-/-) mice: relevance to adipose tissue inflammation, hepatic steatosis and insulin resistance. *Diabetologia* **54,** 2900-2910.

Begam M & Roche JA (2018). Damaged muscle fibers might masquerade as hybrid fibers - a cautionary note on immunophenotyping mouse muscle with mouse monoclonal antibodies. *Eur J Histochem* **62**.

Bolanos-Garcia VM & Miguel RN (2003). On the structure and function of apolipoproteins: more than a family of lipid-binding proteins. *Prog Biophys Mol Biol* **83,** 47-68.

Bosutti A & Degens H (2015). The impact of resveratrol and hydrogen peroxide on muscle cell plasticity shows a dose-dependent interaction. *Sci Rep* **5,** 8093.

Catani MV, Savini I, Duranti G, Caporossi D, Ceci R, Sabatini S & Avigliano L (2004). Nuclear factor kappaB and activating protein 1 are involved in differentiation-related resistance to oxidative stress in skeletal muscle cells. *Free Radic Biol Med* **37,** 1024-1036.

Centeno C, Markle J, Dodson E, Stemper I, Hyzy M, Williams C & Freeman M (2017). The use of lumbar epidural injection of platelet lysate for treatment of radicular pain. *J Exp Orthop* **4,** 38.

Chen F, Liu Y, Wong NK, Xiao J & So KF (2017). Oxidative Stress in Stem Cell Aging. *Cell Transplant* **26,** 1483-1495.

Ciciliot S & Schiaffino S (2010). Regeneration of mammalian skeletal muscle. Basic mechanisms and clinical implications. *Curr Pharm Des* **16,** 906-914.

Crawford RS, Albadawi H, Robaldo A, Peck MA, Abularrage CJ, Yoo HJ, Lamuraglia GM & Watkins MT (2013). Divergent systemic and local inflammatory response to hind limb demand ischemia in wild-type and ApoE-/- mice. *J Surg Res* **183,** 952-962.

D'Souza DM, Trajcevski KE, Al-Sajee D, Wang DC, Thomas M, Anderson JE & Hawke TJ (2015). Diet-induced obesity impairs muscle satellite cell activation and muscle repair through alterations in hepatocyte growth factor signaling. *Physiol Rep* **3**.

Fu S, Zhao H, Shi J, Abzhanov A, Crawford K, Ohno-Machado L, Zhou J, Du Y, Kuo WP, Zhang J, Jiang M & Jin JG (2008). Peripheral arterial occlusive disease: global gene expression analyses suggest a major role for immune and inflammatory responses. *BMC Genomics* **9,** 369.

Fu X, Zhu M, Zhang S, Foretz M, Viollet B & Du M (2016). Obesity Impairs Skeletal Muscle Regeneration Through Inhibition of AMPK. *Diabetes* **65,** 188-200.

Fuchtbauer EM & Westphal H (1992). MyoD and myogenin are coexpressed in regenerating skeletal muscle of the mouse. *Dev Dyn* **193,** 34-39.

Girgenrath M, Kostek CA & Miller JB (2005). Diseased muscles that lack dystrophin or laminin-alpha2 have altered compositions and proliferation of mononuclear cell populations. *BMC Neurol* **5,** 7.

Grabiec K, Majewska A, Wicik Z, Milewska M, Blaszczyk M & Grzelkowska-Kowalczyk K (2016). The effect of palmitate supplementation on gene expression profile in proliferating myoblasts. *Cell Biol Toxicol* **32,** 185-198.

Grounds MD, Garrett KL, Lai MC, Wright WE & Beilharz MW (1992). Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes. *Cell Tissue Res* **267,** 99-104.

Grundy D (2015). Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology. *Exp Physiol* **100,** 755-758.

Guttridge DC, Mayo MW, Madrid LV, Wang CY & Baldwin AS, Jr. (2000). NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. *Science* **289,** 2363-2366.

Haas TL, Lloyd PG, Yang HT & Terjung RL (2012). Exercise training and peripheral arterial disease. *Compr Physiol* **2,** 2933-3017.

Hiebert PR, Wu D & Granville DJ (2013). Granzyme B degrades extracellular matrix and contributes to delayed wound closure in apolipoprotein E knockout mice. *Cell Death Differ* **20,** 1404-1414.

Hofmann SM, Perez-Tilve D, Greer TM, Coburn BA, Grant E, Basford JE, Tschöp MH & Hui DY (2008). Defective lipid delivery modulates glucose tolerance and metabolic response to diet in apolipoprotein E-deficient mice. *Diabetes* **57,** 5-12.

Hu Z, Wang H, Lee IH, Modi S, Wang X, Du J & Mitch WE (2010). PTEN inhibition improves muscle regeneration in mice fed a high-fat diet. *Diabetes* **59,** 1312-1320.

Ishida T, Choi SY, Kundu RK, Spin J, Yamashita T, Hirata K, Kojima Y, Yokoyama M, Cooper AD & Quertermous T (2004). Endothelial lipase modulates susceptibility to atherosclerosis in apolipoprotein-E-deficient mice. *J Biol Chem* **279,** 45085-45092.

Kang J, Albadawi H, Patel VI, Abbruzzese TA, Yoo JH, Austen WG, Jr. & Watkins MT (2008). Apolipoprotein E-/- mice have delayed skeletal

muscle healing after hind limb ischemia-reperfusion. *J Vasc Surg* **48,** 701-708.

Koutakis P, Miserlis D, Myers SA, Kim JK, Zhu Z, Papoutsi E, Swanson SA, Haynatzki G, Ha DM, Carpenter LA, McComb RD, Johanning JM, Casale GP & Pipinos, II (2015). Abnormal accumulation of desmin in gastrocnemius myofibers of patients with peripheral artery disease: associations with altered myofiber morphology and density, mitochondrial dysfunction and impaired limb function. *J Histochem Cytochem* **63,** 256-269.

Koutakis P, Weiss DJ, Miserlis D, Shostrom VK, Papoutsi E, Ha DM, Carpenter LA, McComb RD, Casale GP & Pipinos, II (2014). Oxidative damage in the gastrocnemius of patients with peripheral artery disease is myofiber type selective. *Redox Biol* **2,** 921-928.

Le ADK, Enweze L, DeBaun MR & Dragoo JL (2018). Current Clinical Recommendations for Use of Platelet-Rich Plasma. *Curr Rev Musculoskelet Med* **11,** 624-634.

Le Bihan M-C, Barrio-Hernandez I, Mortensen TP, Henningsen J, Jensen SS, Bigot A, Blagoev B, Butler-Browne G & Kratchmarova I (2015). Cellular Proteome Dynamics during Differentiation of Human Primary Myoblasts. *Journal of Proteome Research* **14,** 3348-3361.

Lee H, Lim JY & Choi SJ (2017). Oleate Prevents Palmitate-Induced Atrophy via Modulation of Mitochondrial ROS Production in Skeletal Myotubes. *Oxid Med Cell Longev* **2017,** 2739721.

Lee MY, Li H, Xiao Y, Zhou Z, Xu A & Vanhoutte PM (2011). Chronic administration of BMS309403 improves endothelial function in apolipoprotein E-deficient mice and in cultured human endothelial cells. *Br J Pharmacol* **162,** 1564-1576.

Li MW, Mian MO, Barhoumi T, Rehman A, Mann K, Paradis P & Schiffrin EL (2013). Endothelin-1 overexpression exacerbates atherosclerosis and induces aortic aneurysms in apolipoprotein E knockout mice. *Arterioscler Thromb Vasc Biol* **33,** 2306-2315.

Matsakas A, Yadav V, Lorca S, Evans RM & Narkar VA (2012). Revascularization of ischemic skeletal muscle by estrogen-related receptor-gamma. *Circ Res* **110,** 1087-1096.

Morgan JE & Zammit PS (2010). Direct effects of the pathogenic mutation on satellite cell function in muscular dystrophy. *Exp Cell Res* **316,** 3100-3108.

Nguyen MH, Cheng M & Koh TJ (2011). Impaired muscle regeneration in ob/ob and db/db mice. *ScientificWorldJournal* **11,** 1525-1535.

Omairi S, Matsakas A, Degens H, Kretz O, Hansson KA, Solbra AV, Bruusgaard JC, Joch B, Sartori R, Giallourou N, Mitchell R, Collins-Hooper H, Foster K, Pasternack A, Ritvos O, Sandri M, Narkar V, Swann JR, Huber TB & Patel K (2016). Enhanced exercise and regenerative capacity in a mouse model that violates size constraints of oxidative muscle fibres. *Elife* **5**.

Parsons MEM, Szklanna PB, Guerrero JA, Wynne K, Dervin F, O'Connell K, Allen S, Egan K, Bennett C, McGuigan C, Gheveart C, F NÁ & Maguire PB (2018). Platelet Releasate Proteome Profiling Reveals a Core Set of Proteins with Low Variance between Healthy Adults. *Proteomics* **18,** e1800219.

Piersma SR, Broxterman HJ, Kapci M, de Haas RR, Hoekman K, Verheul HM & Jiménez CR (2009). Proteomics of the TRAP-induced platelet releasate. *J Proteomics* **72,** 91-109.

Pomies P, Rodriguez J, Blaquiere M, Sedraoui S, Gouzi F, Carnac G, Laoudj-Chenivesse D, Mercier J, Prefaut C & Hayot M (2015). Reduced myotube diameter, atrophic signalling and elevated oxidative stress in cultured satellite cells from COPD patients. *J Cell Mol Med* **19,** 175-186.

Raman KG, Gandley RE, Rohland J, Zenati MS & Tzeng E (2011). Early hypercholesterolemia contributes to vasomotor dysfunction and injury associated atherogenesis that can be inhibited by nitric oxide. *J Vasc Surg* **53,** 754-763.

Relaix F & Zammit PS (2012). Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. *Development* **139,** 2845-2856.

Sassoli C, Vallone L, Tani A, Chellini F, Nosi D & Zecchi-Orlandini S (2018). Combined use of bone marrow-derived mesenchymal stromal cells (BM-MSCs) and platelet rich plasma (PRP) stimulates proliferation and

differentiation of myoblasts in vitro: new therapeutic perspectives for skeletal muscle repair/regeneration. *Cell and Tissue Research* **372,** 549-570.

Schreyer SA, Lystig TC, Vick CM & LeBoeuf RC (2003). Mice deficient in apolipoprotein E but not LDL receptors are resistant to accelerated atherosclerosis associated with obesity. *Atherosclerosis* **171,** 49-55.

Scully D & Matsakas A (2019). Current Insights into the Potential Misuse of Platelet-based Applications for Doping in Sports. *Int J Sports Med* **40,** 427-433.

Scully D, Naseem KM & Matsakas A (2018a). Platelet biology in regenerative medicine of skeletal muscle. *Acta Physiol (Oxf)* **223,** e13071.

Scully D, Sfyri P, Verpoorten S, Papadopoulos P, Munoz-Turrillas MC, Mitchell R, Aburima A, Patel K, Gutierrez L, Naseem KM & Matsakas A (2018b). Platelet releasate promotes skeletal myogenesis by increasing muscle stem cell commitment to differentiation and accelerates muscle regeneration following acute injury. *Acta Physiol (Oxf)***,** e13207.

Scully D, Sfyri P, Wilkinson HN, Acebes-Huerta A, Verpoorten S, Muñoz-Turrillas MC, Parnell A, Patel K, Hardman MJ, Gutiérrez L & Matsakas A (2020). Optimising platelet secretomes to deliver robust tissue-specific regeneration. *J Tissue Eng Regen Med* **14,** 82-98.

Sfyri P & Matsakas A (2017). Crossroads between peripheral atherosclerosis, western-type diet and skeletal muscle pathophysiology: emphasis on apolipoprotein E deficiency and peripheral arterial disease. *Journal of biomedical science* **24,** 42-42.

Sfyri PP, Yuldasheva NY, Tzimou A, Giallourou N, Crispi V, Aburima A, Beltran-Alvarez P, Patel K, Mougios V, Swann JR, Kearney MT & Matsakas A (2018). Attenuation of oxidative stress-induced lesions in skeletal muscle in a mouse model of obesity-independent hyperlipidaemia and atherosclerosis through the inhibition of Nox2 activity. *Free Radic Biol Med* **129,** 504-519.

Sriram S, Yuan C, Chakraborty S, Tay W, Park M, Shabbir A, Toh SA, Han W & Sugii S (2019). Oxidative stress mediates depot-specific functional differences of human adipose-derived stem cells. *Stem Cell Res Ther* **10,** 141.

Straub V, Rafael JA, Chamberlain JS & Campbell KP (1997). Animal models for muscular dystrophy show different patterns of sarcolemmal disruption. *J Cell Biol* **139,** 375-385.

Szklanna PB, Parsons ME, Wynne K, O'Connor H, Egan K, Allen S, F NÁ & Maguire PB (2019). The Platelet Releasate is Altered in Human Pregnancy. *Proteomics Clin Appl* **13,** e1800162.

Takemoto Y, Inaba S, Zhang L, Tsujikawa K, Uezumi A & Fukada SI (2019). Implication of basal lamina dependency in survival of Nrf2-null muscle stem cells via an antioxidative-independent mechanism. *J Cell Physiol* **234,** 1689-1698.

Tian ZL, Jiang SK, Zhang M, Wang M, Li JY, Zhao R, Wang LL, Li SS, Liu M, Zhang MZ & Guan DW (2016). Detection of satellite cells during skeletal muscle wound healing in rats: time-dependent expressions of Pax7 and MyoD in relation to wound age. *Int J Legal Med* **130,** 163-172.

Tsai WC, Yu TY, Chang GJ, Lin LP, Lin MS & Pang JS (2018). Platelet-Rich Plasma Releasate Promotes Regeneration and Decreases Inflammation and Apoptosis of Injured Skeletal Muscle. *Am J Sports Med* **46,** 1980-1986.

Ulasova E, Perez J, Hill BG, Bradley WE, Garber DW, Landar A, Barnes S, Prasain J, Parks DA, Dell'Italia LJ & Darley-Usmar VM (2013). Quercetin prevents left ventricular hypertrophy in the Apo E knockout mouse. *Redox Biol* **1,** 381-386.

Varu VN, Hogg ME & Kibbe MR (2010). Critical limb ischemia. *J Vasc Surg* **51,** 230-241.

Verpoorten S, Sfyri P, Scully D, Mitchell R, Tzimou A, Mougios V, Patel K & Matsakas A (2020). Loss of CD36 protects against diet-induced obesity but results in impaired muscle stem cell function, delayed muscle regeneration and hepatic steatosis. *Acta Physiologica* **228,** e13395.

Weiss DJ, Casale GP, Koutakis P, Nella AA, Swanson SA, Zhu Z, Miserlis D, Johanning JM & Pipinos, II (2013). Oxidative damage and myofiber degeneration in the gastrocnemius of patients with peripheral arterial disease. *J Transl Med* **11,** 230.

Wybier M (2008). Transforaminal epidural corticosteroid injections and spinal cord infarction. *Joint Bone Spine* **75,** 523-525.

Xu P, Werner JU, Milerski S, Hamp CM, Kuzenko T, Jahnert M, Gottmann P, de Roy L, Warnecke D, Abaei A, Palmer A, Huber-Lang M, Durselen L, Rasche V, Schurmann A, Wabitsch M & Knippschild U (2018). Diet-Induced Obesity Affects Muscle Regeneration After Murine Blunt Muscle Trauma-A Broad Spectrum Analysis. *Front Physiol* **9,** 674.

Yablonka-Reuveni Z & Rivera AJ (1994). Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Dev Biol* **164,** 588-603.

Yablonka-Reuveni Z, Seger R & Rivera AJ (1999). Fibroblast growth factor promotes recruitment of skeletal muscle satellite cells in young and old rats. *J Histochem Cytochem* **47,** 23-42.

Yin H, Price F & Rudnicki MA (2013). Satellite cells and the muscle stem cell niche. *Physiol Rev* **93,** 23-67.

Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA & Beauchamp JR (2004). Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? *J Cell Biol* **166,** 347-357.

Zammit PS, Heslop L, Hudon V, Rosenblatt JD, Tajbakhsh S, Buckingham ME, Beauchamp JR & Partridge TA (2002). Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. *Exp Cell Res* **281,** 39-49.

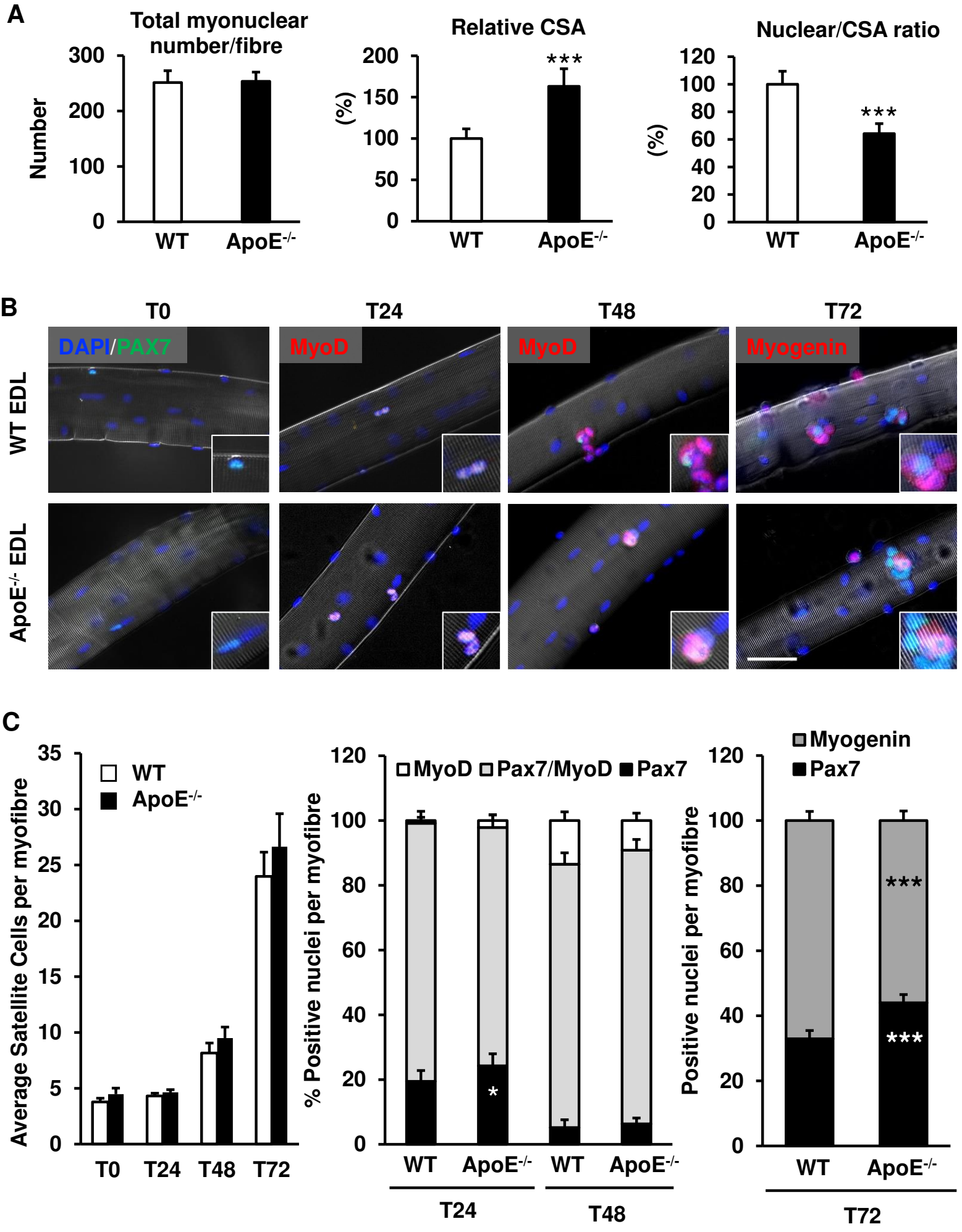
A
Total myonuclear number/fibre
Number
300
200
100
0
WT
ApoE-/-
Relative CSA
(%)
200
150
100
50
0
***
WT
ApoE-/-
Nuclear/CSA ratio
(%)
120
100
80
60
40
20
0
***
WT
ApoE-/-
B
T0
T24
T48
T72
DAPI/PAX7
MyoD
MyoD
Myogenin
WT EDL
ApoE-/- EDL
C
Average Satellite Cells per myofibre
35
30
25
20
15
10
5
0
WT
ApoE-/-
T0
T24
T48
T72
% Positive nuclei per myofibre
120
100
80
60
40
20
0
MyoD
Pax7/MyoD
Pax7
*
WT
ApoE-/-
WT
ApoE-/-
T24
T48
Positive nuclei per myofibre
120
100
80
60
40
20
0
Myogenin
Pax7
***
***
WT
ApoE-/-
T72

Fig. 1

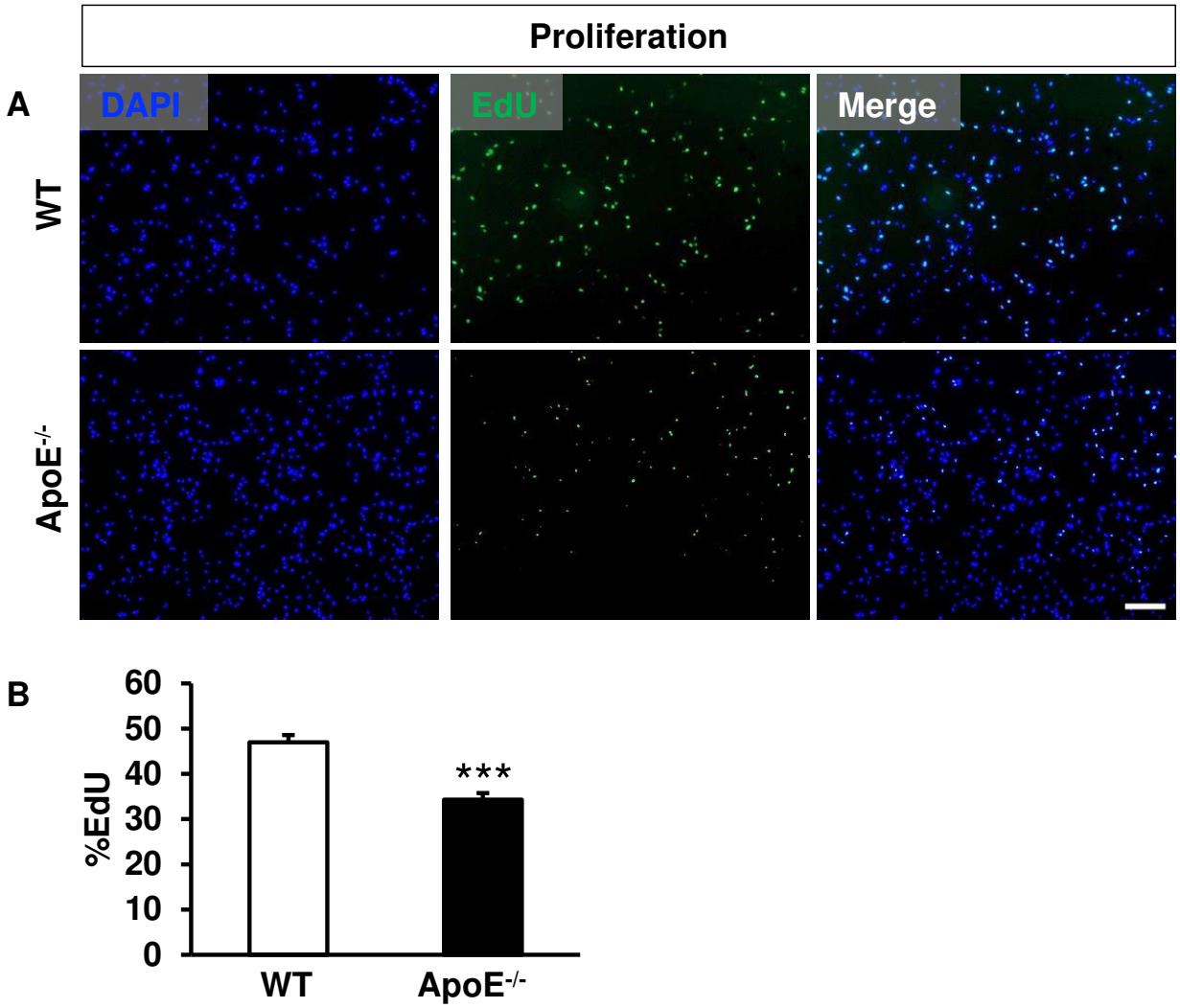
Proliferation
A
DAPI
EdU
Merge
WT
ApoE-/-
B
%EdU
60
50
40
30
20
10
0
***
WT
ApoE-/-

Fig. 2

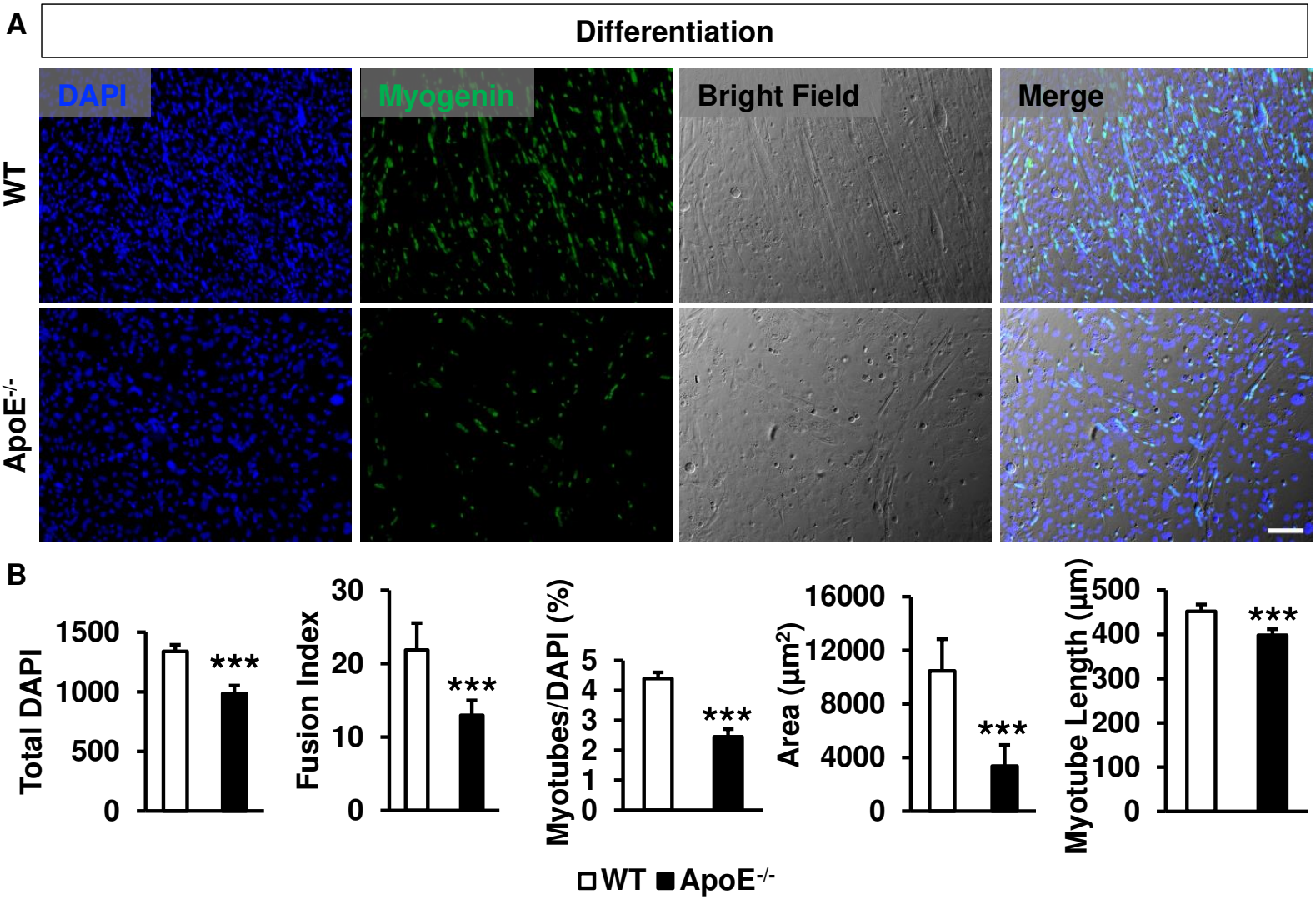
A
Differentiation
DAPI
Myogenin
Bright Field
Merge
WT
ApoE-/-
B
Total DAPI
1500
1000
500
0
***
Fusion Index
30
20
10
0
***
Myotubes/DAPI (%)
5
4
3
2
1
0
***
Area (μm2)
16000
12000
8000
4000
0
***
Myotube Length (μm)
500
400
300
200
100
0
***
WT
ApoE-/-

Fig. 3

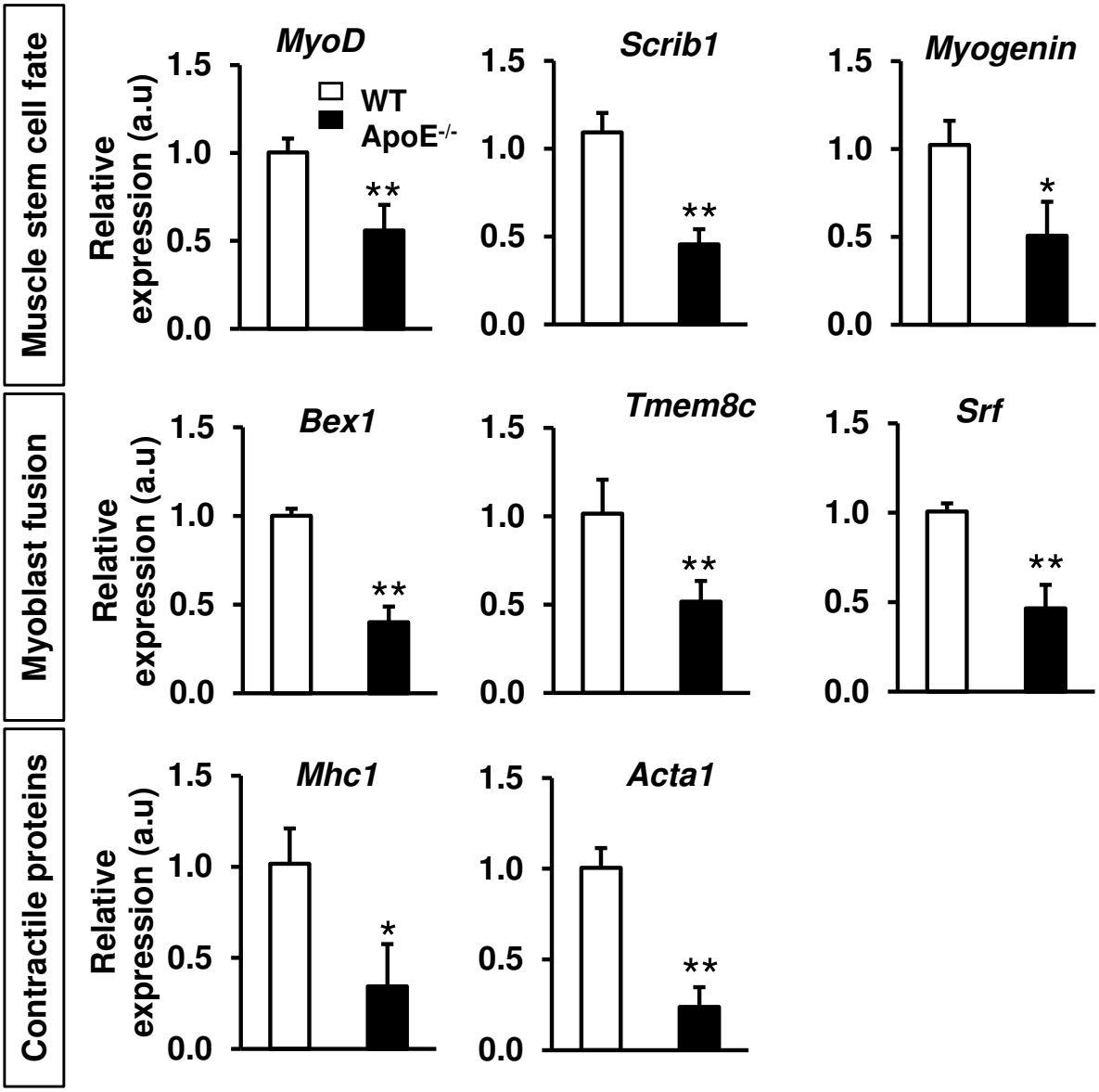
Muscle stem cell fate
Myoblast fusion
Contractile proteins
Relative expression (a.u)
WT
ApoE-/-
MyoD
Scrib1
Myogenin
Bex1
Tmem8c
Srf
Mhc1
Acta1
1.5
1.0
0.5
0.0
*
**

Fig. 4

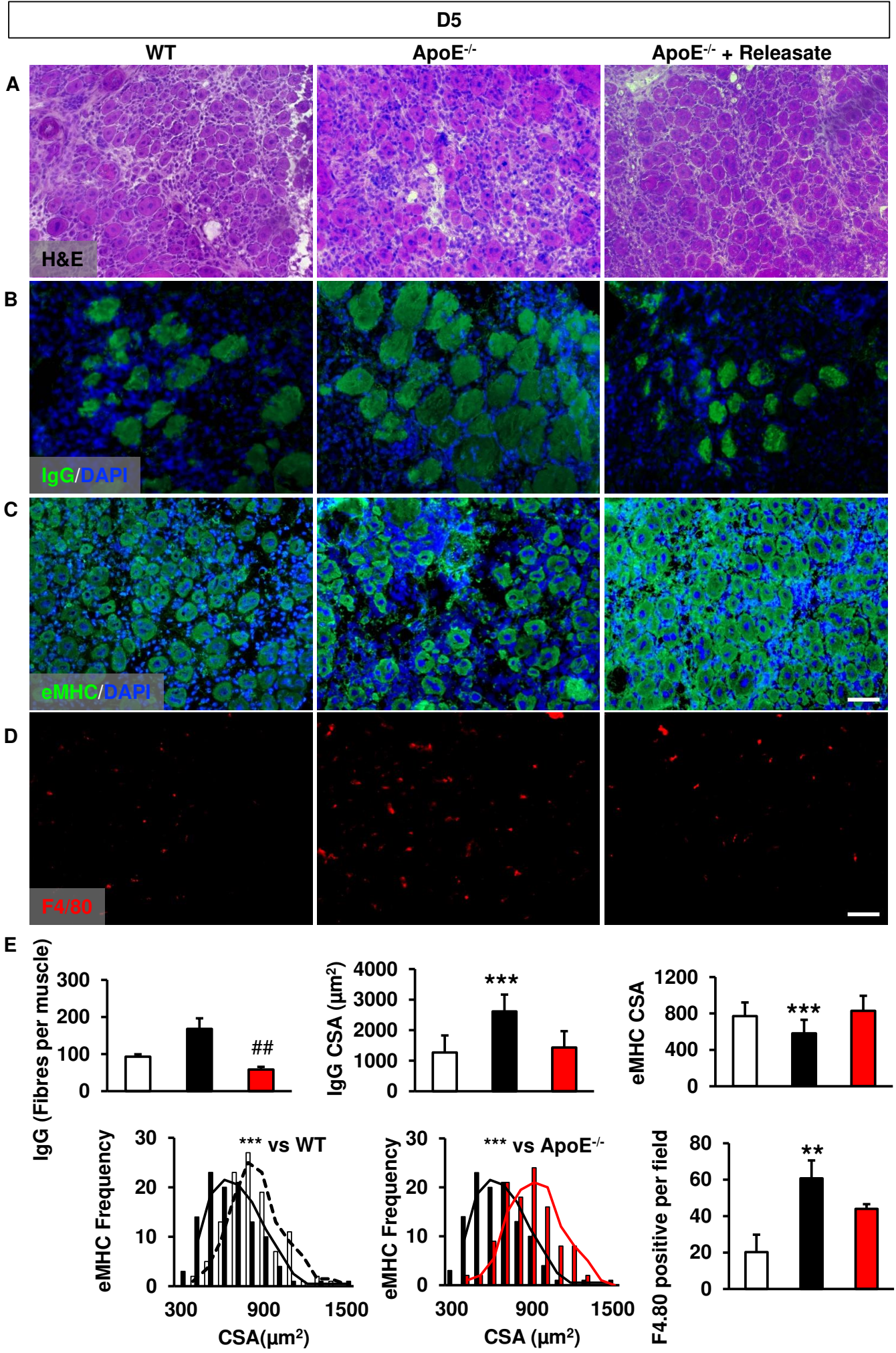

Fig. 5 -- WT — ApoE-/- — ApoE-/- + Releasate □ WT ■ ApoE-/- ■ ApoE-/- + Releasate

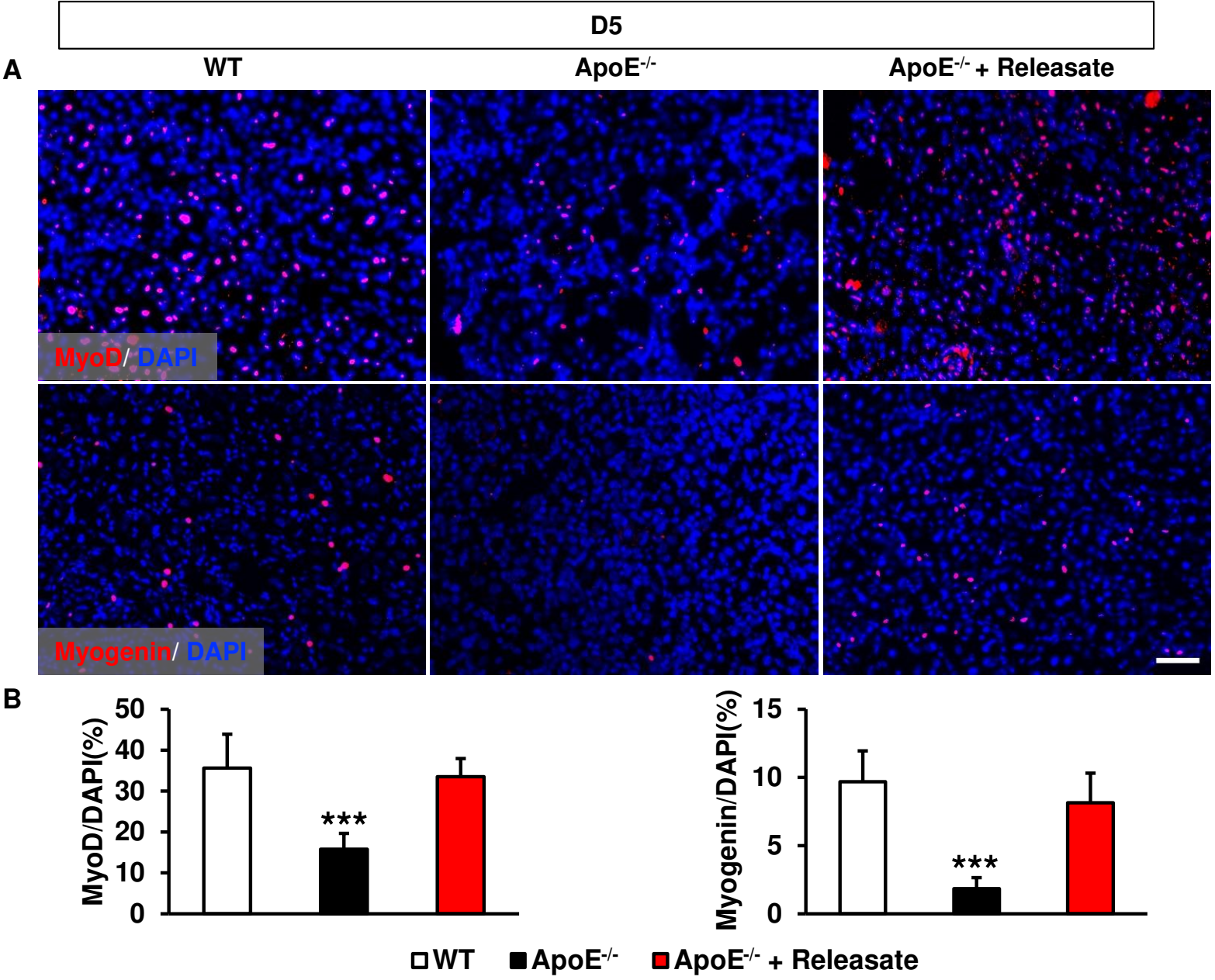
D5
A
WT
ApoE-/-
ApoE-/- + Releasate
MyoD/ DAPI
Myogenin/ DAPI
B
MyoD/DAPI(%)
50
40
30
20
10
0
***
Myogenin/DAPI(%)
15
10
5
0
***
WT
ApoE-/-
ApoE-/- + Releasate

Fig. 6

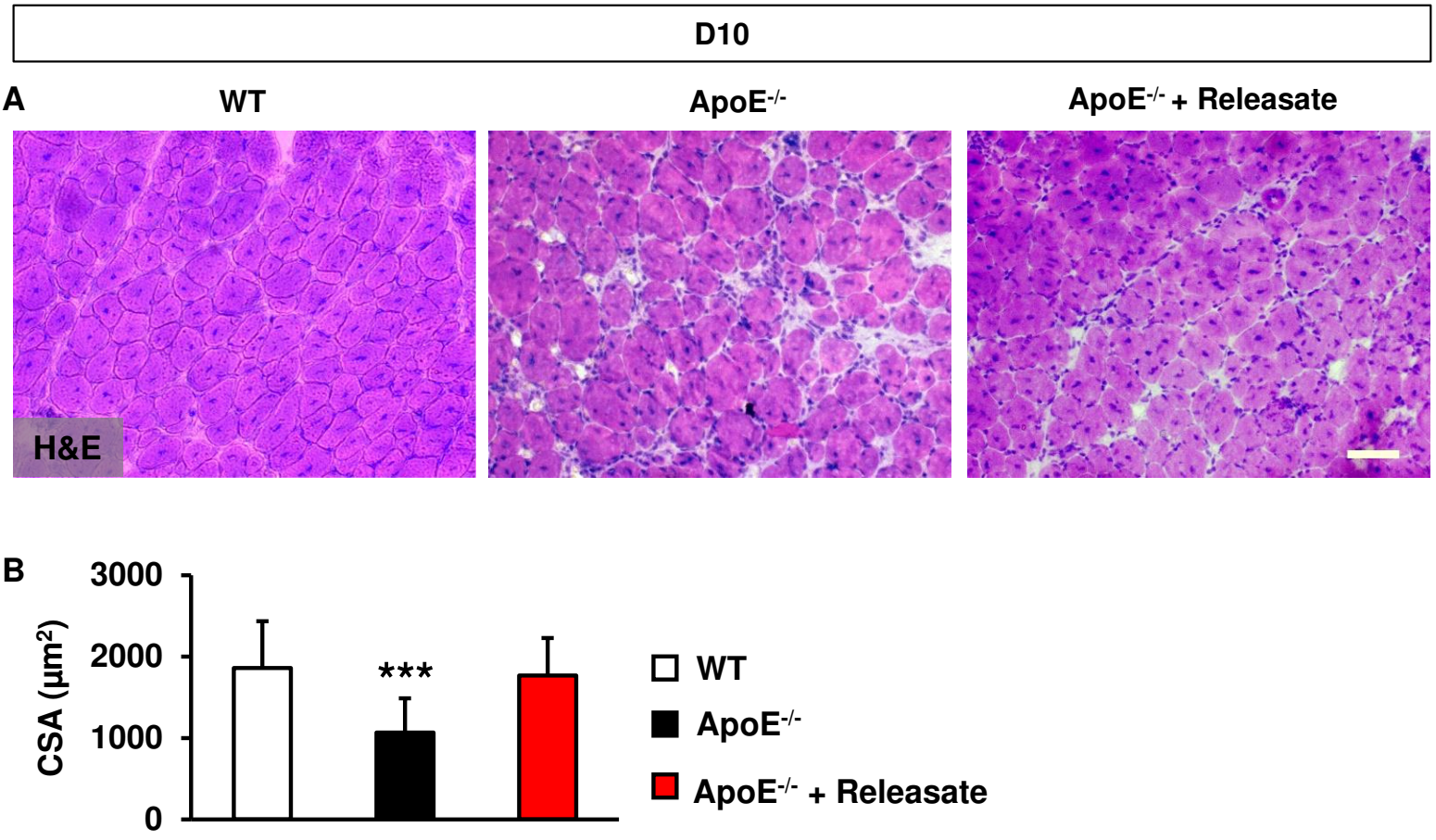
D10
A
WT
ApoE-/-
ApoE-/- + Releasate
H&E
B
3000
2000
1000
0
CSA (µm2)
***
WT
ApoE-/-
ApoE-/- + Releasate

Fig. 7

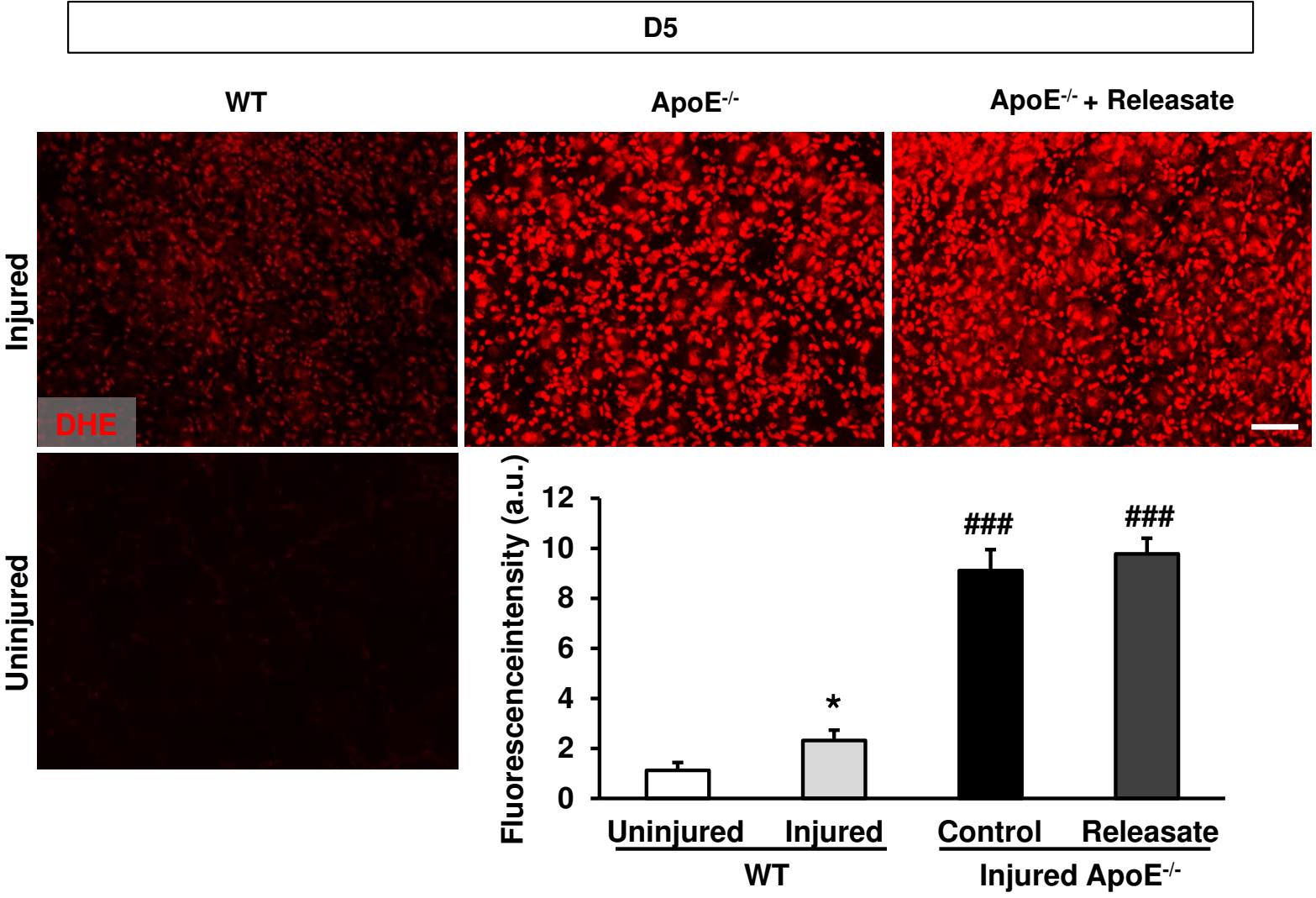
D5
WT
ApoE-/-
ApoE-/- + Releasate
Injured
Uninjured
DHE
Fluorescenceintensity (a.u.)
12
10
8
6
4
2
0
*
###
###
Uninjured
Injured
Control
Releasate
WT
Injured ApoE-/-

Fig. 8