Inactivation of PPAR β/δ adversely affects satellite cells and reduces postnatal myogenesis

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Author's contribution
RK, CM and MS designed the study. PC, RM, XG and SB performed the experiments. PC wrote the draft of the manuscript. RK, CM, WW and MS supervised the study and RK, CM, WW, MS and PC edited the manuscript. All authors read and approved the final manuscript.

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Abstract

PPAR β/δ is a ubiquitously expressed gene with higher levels observed in skeletal muscle. Recently, we showed that PPAR β/δ modulates Myostatin activity to induce myogenesis in skeletal muscle. In the present study, we show that PPAR β/δ-null mice display reduced body weight, skeletal muscle weight and myofibre atrophy during postnatal development. In addition, a significant reduction in satellite cell number was observed in PPAR β/δ-null mice, suggesting a role for PPAR β/δ in muscle regeneration. In order to investigate this, Tibialis Anterior muscles were injured with notexin and muscle regeneration was monitored on days 3, 5, 7 and 28-post injury. Immunohistochemical analysis revealed an increased inflammatory response and reduced myoblast proliferation in regenerating muscle from PPAR β/δ-null mice. Histological analysis confirmed that the regenerated muscle fibers of PPAR β/δ-null mice maintained an atrophy phenotype with reduced numbers of centrally placed nuclei. Even though satellite cell numbers were reduced prior to injury, satellite cell self-renewal was found to be unaffected in PPAR β/δ-null mice after regeneration. Previously we had showed that inactivation of PPAR β/δ increases myostatin signaling and inhibits myogenesis. Our results here indeed confirm that inactivation of myostatin signaling rescues the atrophy phenotype and improves muscle fiber cross-sectional area in both uninjured and regenerated TA muscle from PPAR β/δ-null mice. Taken together, these data suggest that absence of PPAR β/δ leads to loss of satellite cells, impaired skeletal muscle regeneration and postnatal myogenesis. Furthermore, our results also demonstrate that functional antagonism of myostatin has utility in rescuing these effects.

Key words: PPAR β/δ, myostatin, skeletal muscle regeneration
**Introduction**

The peroxisome proliferator-activated receptor (PPAR) ligand-inducible nuclear hormone receptor subfamily consists of three members, PPAR α, PPAR β/δ and PPAR γ. The conformational change induced upon interaction of PPARs with their ligands activates the heterodimeric complexes they form with retinoid-X receptors (RXR) (8, 12, 16). These activated PPAR-RXR complexes are then able to bind to peroxisome proliferator response elements (PPREs) to activate gene transcription (6, 31). Classically, each of the three PPAR isotypes have been identified in species ranging from *Xenopus* to humans and are expressed in distinct tissue-specific patterns. PPAR α (NR1C1) is involved in fatty acid catabolism and amino acid metabolism; and is primarily responsible for body energy homeostasis. PPAR α is highly expressed in liver, heart, skeletal muscle, intestinal mucosa and brown adipose tissue and is metabolically very active (14). PPAR γ (NR1C3), highly expressed in white and brown adipose tissue, colon and spleen, is mainly involved in adipocyte differentiation and lipid storage in adipose tissue, and also in enhancing body insulin sensitivity (36). In contrast to PPAR α and PPAR γ, which are highly expressed in a few tissues, PPAR β/δ (NR1C2) is expressed ubiquitously, but with varying levels in different tissues (3, 40). PPAR β/δ has been shown to be involved in lipid metabolism, energy homeostasis, and wound healing (41).

PPAR-α, β/δ and γ also play important roles in epidermal maturation and tissue repair processes (28). While PPAR β/δ is pro- or anti-carcinogenic is still debated, both effects have been reported in various tumor types, which might reflect different genetic defects within the tumor (27, 33). In addition, PPAR β/δ has been implicated in increasing the β-oxidation of fatty acids in skeletal muscle and adipose tissue (9). Furthermore, PPAR β/δ plays a pivotal role in regulating hepatic gene expression, which modulates glucose homeostasis in liver (17).
Overexpression of PPAR β/δ has been shown to influence muscle metabolism. Previous studies have established that the mRNA expression of PPAR δ is higher in oxidative soleus muscle when compared to the predominantly glycolytic extensor digitorum longus (EDL) muscle (19). It was further noted that oxidative muscles also display the highest levels of PPAR-β/δ protein. PPAR β/δ overexpression promotes hyperplasia of skeletal muscle, specifically resulting in an increase in oxidative muscle fibres and capillary density. In agreement with this, overexpression of a constitutively active PPAR β/δ in mouse skeletal muscle was found to induce differentiation of mitochondria-rich, oxidative type-I muscle fibres (42). In addition, muscle-specific overexpression of PPAR-β/δ has been shown to promote myonuclear density (13). PPAR β/δ also plays an important role in the adaptation of skeletal muscle to environmental changes such as long-term fasting, by increasing the number of oxidative fibres (10). In line with these observations, muscle-specific ablation of PPAR β/δ causes a functional switch of the skeletal muscle fiber type toward lower oxidative capacity, which is followed by the appearance of age-dependent obesity and type 2 diabetes (38).

Furthermore, PPAR β/δ ligands may upregulate fatty acid transport and oxidation, thereby reducing fatty acid-induced inflammation and insulin resistance in skeletal muscle (5, 20). A recent report suggests that activation of PPAR β/δ, through addition of specific agonist, enhances myogenesis in C2C12 myoblasts by increasing both myoblast proliferation and differentiation (2). Consistent with this, loss of PPAR β/δ resulted in reduced proliferation of primary myoblasts and defective differentiation. In addition, a recent study reported that skeletal muscle-specific knock out of PPAR δ in mice (PPAR δ-cKO) leads to reduced satellite cell number and impaired skeletal muscle regeneration upon cardiotoxin-mediated injury (1). Recent results have revealed that PPAR β/δ positively regulates postnatal
myogenesis through transcriptional activation of Gasp-1, leading to reduced activity of its
downstream target Myostatin (2).

Here, using a germ line deleted $PPAR_{\beta/\delta}$ knockout mouse model ($PPAR_{\beta/\delta}$-null), we have
further characterized the role of $PPAR_{\beta/\delta}$ in postnatal skeletal muscle growth and
regeneration. Given the increased Myostatin activity in $PPAR_{\beta/\delta}$-null muscle (2) we
hypothesize that postnatal myogenesis is impaired in $PPAR_{\beta/\delta}$-null mice. Consistent with
this, $PPAR_{\beta/\delta}$-null mice showed impaired muscle regeneration, evidenced by reduced
muscle weight and decline in regenerated myofibre size and reduced centrally located
myonuclei number, 28 days post notexin administration. We also observed increased
inflammatory response with reduced myoblast proliferation during later phase of regeneration
in $PPAR_{\beta/\delta}$-null regenerating TA muscle. On the other hand, antagonism of Myostatin in
$PPAR_{\beta/\delta}$-null mice resulted in increased body and muscle weight and significant
hypertrophy of muscle fibres. Subsequent blockade of Myostatin using the Myostatin
inhibitor sActRIIB improved skeletal muscle regeneration by increasing the number of
centrally formed nuclei and promoting hypertrophy of regenerated muscle fibres. Therefore,
we conclude that $PPAR_{\beta/\delta}$ positively regulates postnatal skeletal muscle myogenesis through
reducing Myostatin activity in mice.
Materials and Methods

Animals

PPAR β/δ-null mice were gifted from Prof. Walter Wahli (University of Lausanne, Lausanne, Switzerland). Animals were housed and maintained at a constant temperature (20 °C) with a 12-h light-dark cycle with ad libitum access to water at the Nanyang Technological University animal facility. Wild type (WT) mice (C57BL/6) were purchased from the Center for Animal Resources, National University of Singapore (NUS-CARE), Singapore. All animal procedures were reviewed and approved by the Institute Animal Ethics Committee, Singapore.

Muscle injury model

Six-week-old male WT and PPAR β/δ-null mice were anesthetized by intraperitoneal injection of a mixture of 10mg/ml ketamine and 1mg/ml xylazine at 0.1ml/10g body weight. Fifteen microlitres of notexin (10ug/ml in 0.9% saline; Latoxan, Rosans, France) was injected into the M. Tibialis anterior (TA) muscle of the left leg using a 28-gauge syringe (Hamilton Co., Whittier, California, USA). The TA muscle from the contralateral limb was used as the uninjured control. TA muscles were harvested on days 3, 5, 7 and 28 after notexin-induced injury. For the sActRIIB Myostatin antagonist trial, mice were anesthetized with ketamine and xylazine as stated above. Fifteen microlitres of notexin (10ug/ml) was injected into the TA muscle of left leg with a 28-gauge syringe and the contralateral limb was used as an uninjured control. The mice were injected intraperitoneally with sActRIIB (43) (4ug/g body weight) 3 times per week for a period of 4weeks. TA muscles were harvested on day 28 after notexin-induced injury for further histological and molecular analysis.
Muscle tissues were embedded in OCT (Optimal Cutting Temperature, Tissue-Tek, Sakura Fine TEK, Torrance, CA, USA) compound and then frozen in liquid nitrogen cooled isopentane. Serial cross sections (10μm) were cut from the mid-belly of the muscle and mounted on cryoslides for histological, immunochemical and metabolic staining. Haematoxylin and Eosin (H&E) and Van Gieson's staining were performed according to the manufacturer's instructions [Catalog No. 115973-1 (Solution A), 115973-2 (Solution B) Merck Millipore, Singapore]. For H&E staining, the cryosections were stained with Haematoxylin for 1 minute and rinsed with tap water. Following 2 x 1 minute washes in Scott’s tap water the sections were stained with Eosin for 2 minute, rinsed 3 times in 50% ethanol and then in 70% ethanol. Sections were further rinsed for 2 minutes in 95% ethanol, washed 2 x 2 minutes each in 100 % ethanol, followed by 2 x 5 minutes in Xylene. The stained sections were mounted with DPX mounting reagent and allowed to dry overnight. Images were captured using the Leica CTR 6500 microscope equipped with the Leica DFC 310 FX camera (Leica, Singapore). ImagePro Plus software (Media Cybernetics, Bethesda, MD, USA) and ImageJ software (National Institute of Health, USA) were used for image analysis. H&E stained images were used for Cross Sectional Area (CSA) measurement and fibrotic tissue was assessed through Van Gieson's stain. The area of interstitial fibrosis was quantitatively assessed using Van Gieson's stained images and was measured according to Luz et al. (21). 5 images (magnification, 10x) around the lesion area from each sample were used for quantification, according to Xiaojia et al. (11) and calculated as the ratio of fibrotic area to cross sectional area (CSA) (%) of the images. For the contralateral uninjured control, the CSA from 1000 myofibres from randomly selected fields were counted. For regenerated myofibres, the CSA of centrally nucleated myofibres within the field were measured.
**Immunohistochemical staining for Mac-1**

Frozen muscle sections (10 μm) were stained for macrophages according to the previously established protocol (11). Briefly, sections were fixed in 2% paraformaldehyde (PFA) for 5 minutes and then permeabilized with 0.3% TritonX 100 in PBS for 5-10 minutes. The sections were then blocked with 10% normal goat serum (NGS) in TBS for 1 hour at room temperature and then incubated with primary rat anti-mouse Mac-1 antibody (BD Pharmingen, San Diego, CA, USA; 1:100) in TBS overnight at 4°C. Following incubation with goat anti rat IgG biotinylated (1:400) secondary antibody, in 5 % NGS in TBS for 1 hour at room temperature, the sections were then incubated with streptavidin conjugated Alexa Fluor 488 (Invitrogen; 1:400) tertiary antibody in 5 % NGS in TBS for 30 minutes. Nuclei were counterstained with 4’6-diamidino-2-phenylindole (DAPI) (1:1000; Invitrogen Molecular Probes, Singapore) and mounted with ProLong Gold Antifade mounting reagent (Invitrogen, Singapore). Mac-1 positive cells were counted and expressed as a percentage of DAPI positive nuclei.

**Immunohistochemical staining for Pax7 and MyoD**

TA muscle sections (10 μm) were fixed in 4% PFA for 5 minutes and then permeabilised with 0.2% PBS-Tween 20. The sections were then blocked with a solution containing 6% mouse IgG blocking reagent (MOM Immunodetection kit; Vector laboratories, Inc, CA, USA) and 3% bovine serum albumin (BSA) in PBS for 1hr, followed by blocking again with MOM protein diluent with 1.5% BSA in PBS, as per the manufacturer's instruction. Muscle sections were then incubated with either mouse monoclonal anti-Pax7 (Developmental Studies Hybridoma Bank; DSHB, Iowa city, IA, USA; 1:1000) or anti-MyoD (1:25; BD pharmingen, San Diego, CA, USA) primary antibody in 1.5% BSA in PBS overnight at 4°C. Following incubation with biotinylated horse anti-mouse IgG (Vector laboratories, Inc.,CA, USA;
1:500), rabbit polyclonal anti-Laminin (Sigma-Aldrich, Singapore; 1:1000) and rabbit polyclonal anti-MyoD (Santa Cruz, USA; 1:40) antibodies for 3 hours, the sections were then washed and incubated with Streptavidin conjugated Alexa Fluor 488 (Invitrogen; 1:1000) and Goat anti-rabbit Alexa Fluor 594 (Invitrogen; 1:1000) for 30 minutes. Nuclei were counterstained with DAPI (Invitrogen Molecular Probes, Singapore; 1:5000) and mounted with ProLong Gold Antifade mounting medium (Invitrogen, Singapore). Pax7+/MyoD- cells were quantified in the stained sections and expressed as number of Pax7+/MyoD- cells per 100 myofibre.

Succinate dehydrogenase (SDH) staining

SDH activity used to determine the overall oxidative capacity of skeletal muscles was measured using a colorimetric assay, as described by Masuda et al. (22). Muscle sections were air-dried for 30 min and incubated in pre-warmed PBS buffer containing 50 mM sodium succinate and 0.6 mM Nitro Blue Tetrazolium (NBT) (Sigma-Aldrich, Singapore) for 25 min at 37°C. The reaction was terminated by rinsing the sections thoroughly with distilled water. After dehydration and clearance by Xylene, the sections were mounted using DPX (Sigma-Aldrich, Singapore). Images were captured using the Leica CTR 6500 microscope, equipped with the Leica DFC 310 FX camera and ImagePro Plus software (Media Cybernetics, Bethesda, USA). SDH activity was quantified using ImageJ software (National Institute of Health, USA) by converting the image to grey scale and measuring the grey intensity of the muscle section. The mean of the three sections was used to determine SDH activity.
Statistical Analysis

Statistical differences between groups was determined using unpaired two-tailed Student’s $t$-test and the results were considered significant at $P<0.05$ (*), $P<0.01$ (**), or $P<0.001$ (***)

Data are expressed as mean ± S.E.M (standard error of the mean).
**Results**

**PPAR β/δ-null mice exhibit pronounced skeletal muscle atrophy**

We observed reduced body weight and a significant decrease in *M. tibialis anterior* (TA), *M. gastrocnemius* muscle (Gas), *M. extensor digitorum longus* (EDL) and *M. soleus* (Sol) muscle weights in *PPAR β/δ*-null mice, when compared to Wild-type (WT) (Fig. 1A, B, C). Although, no significant difference in *M. quadriceps* (Quad) muscle weights was noted, there was a trend towards reduced muscle weight in *PPAR β/δ*-null mice (Fig.1B). Consistent with the reduced muscle weights, a significant reduction in TA muscle fiber CSA was observed in *PPAR β/δ*-null mice, compared to WT mice (Fig. 1D), although no significant difference was noted in myofibre number (*data not shown*). Satellite cells play crucial roles in postnatal muscle growth and maintenance (32). As such, we next quantified the number of Pax7⁺/MyoD⁻ satellite cells in muscle fiber sections through immunohistochemical analysis. Results revealed a significant reduction in the number of Pax7⁺/MyoD⁻ satellite cells in resting muscle fibers of *PPAR β/δ*-null mice, compared to WT controls (Fig. 1E).

**Histological analysis of skeletal muscle during early regeneration**

To further study the effect of loss of *PPAR β/δ* on postnatal myogenesis, skeletal muscle regeneration was assessed in WT and *PPAR β/δ*-null mice injected with notexin. The uninjured TA muscles from both *PPAR β/δ*-null and WT mice are shown in Fig. 2A, and upon injury both the genotypes showed complete and extensive muscle degeneration 3 days post notexin-induced injury (Fig. 2A). However, more extensive necrosis was seen on day 3-post notexin injection in *PPAR β/δ*-null mice, compared to WT mice (Fig. 2A). At day 5-post injury a mixed population of hyperstained necrotic myofibres and newly developing myofibres were observed in TA muscle from both *PPAR β/δ*-null mice and WT controls (Fig. 2A). At 7 days post-injury nascent myofibres, characterized by centrally located nuclei and
small fibre size, were abundant in TA muscle from both PPAR β/δ-null mice and WT controls; however, visibly smaller myofibres were observed in PPAR β/δ-null mice when compared to WT controls (Fig. 2A). Immunohistochemical analysis with Mac-1 antibodies revealed increased infiltration of macrophages in PPAR β/δ-null mice at day 3 post-notexin injection (Fig. 2B). However, no significant difference in the percentage of Mac-1 positive cells was noted between PPAR β/δ-null mice and WT controls 28 days following notexin injection (Fig. 2C). Collectively these data suggest that loss of PPAR β/δ affects the early stages of muscle regeneration.

Reduced numbers of proliferating myoblasts in PPAR β/δ-null regenerating muscle

We next performed MyoD immunohistochemical analysis on notexin-injured TA muscle sections to assess whether or not loss of PPAR β/δ affects satellite cell (SC) activation. Results revealed a significant reduction in MyoD-positive myoblasts from 3 days post-notexin injection; with greater than 50% reduction in MyoD-positive cells noted at day 3, ~38% reduction at day 7 and ~50% reduction at day 28 post injury in PPAR β/δ-null TA muscle, compared to respective WT controls (Fig. 2D). These data suggest that loss of PPAR β/δ significantly reduces the pool of proliferating myoblasts present in injured and regenerating skeletal muscle.

We further assessed SC self-renewal in PPAR β/δ-null muscle through MyoD and Pax7 immunohistochemical analysis on regenerated (day 28-post notexin injection) TA muscle sections. After injury and regeneration we still observe a significant reduction in SC number in PPAR β/δ-null mice in comparison to WT mice (Fig. 2E). Upon further analysis we did not find any significant difference in SC number between uninjured and regenerated TA muscle in both the genotypes (comparison not shown). These data suggest that the self-renewal capacity of SCs remains unaffected in PPAR β/δ-null mice.
Loss of PPAR β/δ results in reduced centrally placed myonuclei in regenerated muscle

Histological analysis was performed to quantitatively examine muscle regeneration 28-days post notexin-induced injury (Fig. 3A). Morphologically we noted an observable reduction in myofibre size in regenerated TA muscle from PPAR β/δ-null, when compared to WT controls (Fig. 3A). Subsequent analysis of myofibre CSA confirmed the reduced myofibre size in regenerated TA muscle from PPAR β/δ-null mice (Fig. 3B), which was consistent with the atrophy phenotype noted in PPAR β/δ-null mice prior to injury (Fig. 1D). Consistent with this, the weight of regenerated TA muscle was significantly lower in PPAR β/δ-null mice when compared to WT controls (Fig. 3C). As regenerated myofibres are characterized by the presence of centrally located myonuclei (34), we next assessed the number of central myonuclei per myofibre in regenerated TA muscle from WT and PPAR β/δ-null mice. As can be seen in Fig. 3D, loss of PPAR β/δ led to a significant increase in myofibers with 1 centrally placed nuclei, concomitant with a significant reduction in the percentage of myofibres with 2 and ≥3 centrally placed nuclei, when compared to WT controls (Fig.3D). Specifically, the number of myofibres with 1 centrally placed nuclei were found to be 52.99 ± 2.01% in WT and 66.76 ± 1.78% in PPAR β/δ-null mice. Whereas the number of myofibres with 2 centrally placed nuclei were found to be 33.07 ± 0.7% in WT and 26.03 ±1% in PPAR β/δ-null, and greater than 3 centrally placed nuclei was found to be 13.9 ± 1.53% in WT and 7.2 ± 0.87% in PPAR β/δ-null mice (P<0.01, P<0.001) (Fig. 3D).
Loss of PPAR β/δ does not significantly alter scar tissue formation or metabolic properties of regenerated muscle

The final phase of muscle regeneration is usually characterized by fibrotic tissue formation, which contributes to incomplete functional recovery (15). To examine the extent of fibrotic tissue formation in regenerated TA muscle of WT and PPAR β/δ-null mice, muscle sections were stained with Van Gieson's stain and the extent of fibrotic tissue was quantified. Subsequent quantification of fibrotic tissue formation revealed that there was no significant difference in the area of fibrotic tissue in regenerated TA muscle sections between WT and PPAR β/δ-null mice (Fig. 4A).

Skeletal muscle regeneration has been shown to increase the number of oxidative muscle fibers (4). Hence, we further assessed the proportion of oxidative muscle fibers in regenerated (day 28-post notexin injection) TA muscles through assessing Succinate dehydrogenase (SDH) activity (22). As expected there was an increase in SDH activity, as measured through increased SDH staining, in regenerated TA muscles from both WT and PPAR β/δ-null mice, when compared to respective uninjured controls. However, it is noteworthy to mention that SDH staining, and thus activity, was comparable between uninjured TA muscle from WT and PPAR β/δ-null mice and between regenerated TA muscle from WT and PPAR β/δ-null mice (Fig. 4B, C). These results indicate that loss of PPAR β/δ does not lead to a change in oxidative capacity in injured skeletal muscle tissue.

Blockade of Myostatin leads to skeletal muscle hypertrophy in PPAR β/δ-null mice pre- and post-injury

Myostatin has been established to bind with high affinity to sActRIIB (18), inhibiting myostatin signaling and contributing to increased musculature. Hence, we tested the effect of myostatin inhibition in PPAR β/δ-null mice using sActRIIB. Increased body weight was
observed in PPAR β/δ-null mice administered sActRIIB (4μg/g body weight), compared to dialysis buffer (DB) injected control mice (Fig. 5A). Similarly, we also observed a slight increase in muscle weights of PPAR β/δ-null mice treated with sActRIIB, compared to control treated mice, although this increase was not statistically significant (Fig. 5B, 5C). We further noted an increase in muscle fiber CSA in sActRIIB injected PPAR β/δ-null mice (Fig. 5D) when compared to control treated mice, which is consistent with skeletal muscle hypertrophy and loss of Myostatin activity. These data suggest that the reduction in body weight, muscle weights and muscle CSA observed in PPAR β/δ-null mice may be due to enhanced activity of Myostatin.

We further tested the effect of sActRIIB injection on skeletal muscle regeneration in PPAR β/δ-null mice. We observed no significant change in regenerated (day 28-post notexin-induced injury) muscle weights of PPAR β/δ-null mice treated with sActRIIB, when compared to respective DB treated control (data not shown). Despite this, we did find a significant increase in muscle fiber CSA, consistent with hypertrophy, in regenerated TA muscle sections of sActRIIB treated PPAR β/δ-null mice, when compared to respective DB treated control (Fig. 5E). A significant increase in the number of centrally formed nuclei was also observed in regenerated TA muscle sections from PPAR β/δ-null mice treated with sActRIIB, when compared to respective DB treated control (Fig. 5F).

Taken together, these data suggest that sActRIIB-mediated blockade of Myostatin is able to improve skeletal muscle regeneration in PPAR β/δ-null mice in response to injury.
Discussion

Overexpression of constitutively active PPAR β/δ in mouse skeletal muscle promotes muscle development and enhances myogenesis by increasing both myoblast proliferation and differentiation in C2C12 myoblasts (2, 13, 42). In this study, we used notexin-induced muscle injury to analyze the regeneration capacity of PPAR β/δ-null muscle and therefore understand the role of PPAR β/δ in postnatal myogenesis.

During postnatal myogenesis PPAR β/δ-null mice show reduced body weight, reduced Tibialis anterior, Gastrocnemius, Extensor digitorum Longus and Soleus muscle weight (Fig.1A, 1B and 1C). However, in contrast to the results presented here, Angione et al. have shown no significant change in body weight and muscle weights in skeletal muscle-specific PPAR β/δ knock-out mice (PPAR δ-cKO) (1). It is quite possible that the differences noted in body and muscle weights between Angione et al., and the current study could be due to the different transgenic mouse models used. In particular, the Angione et al. study made use of muscle-specific PPAR β/δ knock-out mice, whereas we have used germ line deleted PPAR β/δ-null mouse model. However, it is important to highlight that Angione et al. assessed for differences in body weight between female WT and PPAR δ-cKO mice, whereas we have assessed differences in body and muscle weights between male WT and PPAR β/δ-null mice. Thus the variation in body weights and muscle weights observed between the study by Angione et al. and our current study could be due to sexual dimorphism as previous reports indicate that hormones such as estrogen influence muscle growth (7, 24). However, further research will need to be performed to ascertain whether PPAR β/δ influences estrogen levels and thereby affects muscle mass. We further noted decreased muscle fibre size (atrophy) in PPAR β/δ-null mice when compared to WT mice (Fig.1D) with no appreciable change in fibre number (data not shown). Such atrophy could be due to reduced satellite cell number and impaired satellite cell function. Previous work from our lab has also demonstrated that
Myostatin is a potent negative regulator of satellite cell activation and function (25) and excess Myostatin levels have been shown to promote muscle wasting (26). Since \textit{PPAR} \(\beta/\delta\)-null mice have been previously shown to have increased Myostatin activity (2) it is quite possible that the observed atrophy may be due to increased Myostatin activity. In agreement with this, Pax7 immunostaining on muscle sections confirmed there was a significant reduction in satellite cell number in TA muscles of 10-week-old \textit{PPAR} \(\beta/\delta\)-null mice. These data are consistent with the Angione \textit{et al.} study that reported a reduction of 40% in the number of Pax7 positive satellite cells in \textit{PPAR} \(\delta\)-cKO mice. Given that the number of Pax7\(^+/\)MyoD\(^-\) quiescent Satellite cells (SCs) between resting muscle and regenerated muscle (day 28-post notexin injection) in \textit{PPAR} \(\beta/\delta\)-null mice are comparable, we suggest that SCs from \textit{PPAR} \(\beta/\delta\)-null mice are able to self-renew their population normally during postnatal growth (Fig. 2E). Given that SC self-renewal may not be impaired in \textit{PPAR} \(\beta/\delta\)-null mice, we predict that the reduction in SCs is due to alterations in the specification of SCs during embryonic/fetal development (30) in \textit{PPAR} \(\beta/\delta\)-null mice. It was previously shown that a subset of Pax3/Pax7 expressing myoblasts in dermomyotome of embryonic day E16.5 to E18.5 mature into satellite cells (35). Perhaps lack of \textit{PPAR} \(\beta/\delta\) is responsible for either specification of these myoblasts in the dermomyotome or for their replication. Much more extensive investigations are required to investigate the precise mechanism of \textit{PPAR} \(\beta/\delta\) in regulating satellite cell specification during fetal growth. Interestingly, in Xenopus development, \textit{PPAR} \(\beta/\delta\) is essential for muscle and neural differentiation as early as in gastrulation when \textit{PPAR} \(\beta/\delta\) governs a massive wave of transcriptional modifications affecting the later differentiation of muscle and brain (37). SCs are proven muscle stem cells that give rise to new muscle fibers during skeletal muscle regeneration (32). Therefore, we next assessed the myogenic function of \textit{PPAR} \(\beta/\delta\)-null SCs by studying muscle regeneration. During the early phase of muscle regeneration, we found
significantly increased macrophage infiltration in PPAR β/δ-null injured muscle after 3 days of notexin-induced injury (Fig. 2A-B). In agreement with the data presented here, reduced macrophage infiltration has been reported in diabetic nephropathy mice treated with PPAR β/δ agonist and activation of PPAR β/δ has been further shown to inhibit macrophage recruitment and vascular inflammatory gene expression (23, 39). We also observed reduced numbers of MyoD positive activated SCs at day 3 in regenerating PPAR β/δ-null TA skeletal muscle (Fig. 2D). Since PPAR β/δ-null mice have reduced SCs to begin with, which have previously been shown to proliferate slower (2), we speculate that both these factors contribute to the reduced number of MyoD positive myoblasts observed at day 3 of regeneration. In addition, previously published results from our lab have revealed increased Myostatin activity in PPAR β/δ-null mice, which leads to reduced proliferation and differentiation of primary myoblasts (2). Thus, it is quite possible that the reduced numbers of MyoD positive myoblasts in PPAR β/δ-null mice during regeneration is due to increased expression/activity of Myostatin observed in these mice. It is noteworthy to mention that despite increased macrophage infiltration and impaired SC activation in PPAR β/δ-null mice, we observed no difference in fibrotic tissue formation in regenerated muscle (day 28-post notexin injection) between WT and PPAR β/δ-null mice (Fig. 4A).

In agreement with Angione et al., we also observed a reduced differentiation potential of myoblasts as the histology on PPAR β/δ-null regenerated skeletal muscle at day 28 confirmed a severely atrophied regenerated muscle fibers with lower number of centrally formed nuclei (Fig. 3B, D). Several factors have been shown to affect satellite cell activation, proliferation and differentiation (1, 29). Previously we have shown that myostatin is a potent negative regulator of satellite cell activation and myoblast proliferation (25). Therefore, lack of myostatin improved muscle healing by increasing both myoblast proliferation and differentiation. Since the PPAR β/δ-null mice used in this study have increased myostatin
activity (2), it is quite possible that the impaired SCs function and reduced myogenesis observed in these mice could be due to increased Myostatin. In order to confirm the role of myostatin in PPAR β/δ-null mice during regeneration, we induced muscle injury via notexin injection and blocked excess myostatin signaling via treatment with a dominant negative soluble receptor decoy (sActRIIB) (44). The results confirmed that blockade of excess myostatin signaling in PPAR β/δ-null mice resulted in increased muscle hypertrophy of not only resting muscle but also regenerated (day 28-post notexin injection) muscle. Therefore we propose that increased myostatin activity is at least in part responsible for the atrophy phenotype observed in PPAR β/δ-null mice.

In summary, PPAR β/δ plays a critical role in muscle regeneration from early inflammatory response, primary myogenesis and formation of completely regenerated myofibres. Based on the results from this study, we propose that PPAR β/δ regulates SC number, function and myoblast differentiation via regulation of Myostatin.
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Disclosures

The authors declare no conflict of interest.


**Figure captions**

**Fig. 1 Enhanced skeletal muscle atrophy in PPAR β/δ-null mice.**

(A) Body weight (g) of Wild type (WT) and PPAR β/δ-null mice at 10 weeks of age. Error bars represent mean ± s.e.m, n=3 for PPAR β/δ-null mice and n=4 for WT mice. (B) Graph showing combined average weight of left and right TA, Gas and Quad muscles, normalized to total body weight, of WT and PPAR β/δ-null mice at 10 weeks of age. Error bars represent mean ± s.e.m, n=3 for PPAR β/δ-null mice and n=4 for WT mice. (C) Graph showing combined average weight of left and right EDL and Sol muscles, normalized to total body weight of WT and PPAR β/δ-null mice at 10 weeks of age. Error bars represent mean ± s.e.m, n=3 for PPAR β/δ-null mice and n=4 for WT mice. (D) Frequency distribution of TA muscle fiber cross sectional area (CSA; μm²) in resting muscle from WT and PPAR β/δ-null mice at 10 weeks of age. (E) Number of quiescent SCs (Pax7+/MyoD-) per 100 myofibre, in resting TA muscle from 10-week-old WT and PPAR β/δ-null mice. Error bars represent mean ± s.e.m, n=3 per group. Statistical differences are indicated in relevant panels, P<0.05 (*), P<0.01 (**) P<0.001 (***)

**Fig. 2 PPAR β/δ deficiency leads to increased inflammatory response and reduced myoblast activation following notexin-induced injury.**

(A) Representative images of H&E stained uninjured TA muscle cross sections and TA muscle sections at day 3-, 5-, and 7-post notexin injection. Infiltration of inflammatory cells (I), necrotic fibers (N) and newly formed myofibres (*) are marked on the images. Scale bars represent 100 μm. (B) Quantification of Mac-1 positive cells, as assessed through immunohistochemistry, in TA muscle cross sections from WT and PPAR β/δ-null mice at day 3- post notexin injection. Mac-1 positive cells were expressed as a percentage of the total nuclei (DAPI positive). (C) Quantification of Mac-1 positive cells, as assessed through
immunohistochemistry, in TA muscle cross sections from WT and PPAR β/δ-null mice at day 28- post notexin injection. Mac-1 positive cells were expressed as a percentage of the total nuclei (DAPI positive). (D) Quantification of MyoD positive cells, as assessed through immunohistochemistry, in TA muscle cross sections from WT and PPAR β/δ-null mice at day 3-, 7- and 28-post notexin injection. MyoD positive cells were expressed as a percentage of total nuclei (DAPI positive). (E) Quantification of Pax7+/MyoD- cells, as assessed through immunohistochemistry, in regenerated (day 28-post notexin-induced injury) TA muscles cross sections harvested from WT and PPAR β/δ-null mice. Pax7+/MyoD- cells were expressed as number of Pax7+/MyoD- cells per 100 myofibre. Error bars represent mean ± s.e.m, n=3 per group. Statistical differences are indicated in relevant panels, P<0.05 (*), P<0.01 (**) and P<0.001 (***)

**Fig. 3 Impaired muscle regeneration in PPAR β/δ-null mice.**

(A) Representative images of H&E stained TA muscle cross sections at day 28-post notexin injection. Newly formed myofibres (*) are marked on the images. Scale bars represent 100 µm. (B) Frequency distribution of WT and PPAR β/δ-null mice TA muscle fiber CSA (µm²) at day 28-post notexin injection. (C) TA muscle weights, normalized to total body weight, of WT and PPAR β/δ-null mice at day 28-post notexin injection. (D) Quantification of centrally formed myonuclei within regenerated (day 28-post notexin-induced injury) TA myofibres from WT and PPAR β/δ-null mice. Graph displays the percentage of myofibres with 1, 2 or ≥3 centrally formed myonuclei. Error bars represent mean ± s.e.m, n=3 in each group. Statistical differences are indicated in relevant panels, P<0.05 (*), P<0.01 (**) and P<0.001 (***)
Fig. 4 Scar tissue and muscle oxidative capacity remained unaltered between WT and PPAR β/δ-null during regeneration.

(A) Quantification of interstitial fibrotic tissue in TA muscle cross sections at day 28-post notexin injection, as assessed through Van Geison’s staining. The percentage of fibrosis in each sample was calculated as the ratio of the fibrotic area to cross sectional area (CSA) (%) of the images. (B) Representative images of succinate dehydrogenase (SDH) staining of WT and PPAR β/δ- null TA muscle cross sections at day 28-post notexin injection. Black boxes in the lower panels indicate the regions in the magnified image in the upper panel; Scale bars in the upper panel represent 100 μm in higher-magnification image. (C) Quantitative analysis of SDH activity in uninjured and regenerated (day 28-post notexin-induced injury) TA muscles cross sections harvested from WT and PPAR β/δ-null mice. Error bars represent mean ± s.e.m, n=3 in each group. Statistical differences are indicated in relevant panels, P<0.05 (*).

Fig. 5 Functional antagonism of Myostatin results in skeletal muscle hypertrophy pre and post notexin-induced injury.

(A) Increase in body weight (g) of PPAR β/δ-null mice injected with either vehicle control (Dialysis buffer; DB) or sActRIIB (4μg/g body weight) Myostatin antagonist for a period of 4 weeks. (B) Graph showing TA, Gas and Quad muscle weights from the right hindlimb, normalized to total body weight, of PPAR β/δ-null mice 4 weeks post-injection of either DB or sActRIIB (4μg/g body weight) Myostatin antagonist. (C) Graph showing EDL and Sol muscle weights from the right hindlimb, normalized to total body weight of PPAR β/δ-null mice 4 weeks post-injection of either DB or sActRIIB (4μg/g body weight) Myostatin antagonist. (D) Frequency distribution of TA muscle fiber cross sectional area (CSA; μm²) in PPAR β/δ-null mice 4 weeks post-injection of either DB or sActRIIB (4μg/g body weight) Myostatin antagonist. (E) Frequency distribution of muscle fiber cross sectional area (CSA;
μm²) in regenerated (day 28-post notexin-induced injury) TA muscle from PPAR β/δ-null mice injected with either DB (day 28-DB) or sActRIIB (4μg/g body weight) Myostatin antagonist (day 28-sActRIIB). (F) Quantification of centrally formed myonuclei within regenerated (day 28-post notexin-induced injury) TA myofibres from PPAR β/δ-null mice injected with either DB (day 28-DB) or sActRIIB (4μg/g body weight) Myostatin antagonist (day 28-sActRIIB). Error bars represent mean ± s.e.m, n=3 in each group. Statistical differences are indicated in relevant panels, P<0.05 (*), P<0.01 (**) and P<0.001 (***).
Fig. 2

A

WT

Uninjured

Day 3

Day 5

Day 7

PPAR β/δ-null

B

Percentage of Mac-1 positive nuclei

Day 3

C

Percentage of Mac-1 positive nuclei

Day 28

D

Percentage of MyoD positive nuclei/
total nuclei

Day 3

Day 7

Day 28

E

Pax7+/MyoD- cells/100 myofibres

WT

PPAR β/δ-null

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Fig. 3

Muscle weight/ body weight (%)

WT PPARγ/δ-null

Percentage of myofibres with centrally formed nuclei

Number of central myonuclei

Fibre number

Fibre cross-sectional area (ȝm²)

Day 28

WT PPARγ/δ-null
Fig. 4

A

Fibrotic area (Percentage)

WT  PPAR β/δ-null

B

WT  PPAR β/δ-null

SDH activity (Arbitrary units)/ muscle section

Uninjured  Regenerated

C

SDH activity (Arbitrary units)/ muscle section

WT  PPAR β/δ-null

* Uninjured  * Regenerated
Fig. 5