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PPARdelta activation enhances skeletal muscle regeneration

A dissertation submitted in partial satisfaction of the requirements for the degree of
Doctor of Philosophy

in

Biology

by

Emi Kanakubo Embler

Committee in charge:

>   Professor Ronald M. Evans, Chair
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2012

The dissertation of Emi Kanakubo Embler is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

UNIVERSITY OF CALIFORNIA, SAN DIEGO

2012

DEDICATION

いつでも、いつまでも、私をサポートしてくれる両親に
この卒業論文を捧げます。

パパとお母さんの協力と後押しがあったから、大きな壁を
乗り越えられました。心から感謝しています。

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

PPARδ…………… Peroxisome Proliferator-Activated Receptor delta
TG……………... Transgenic
WT……………….. Wildtype
KO………………... Knock-out
TA………………... Tibialis Anterior
Gastroc…………... Gastrocnemius
Sol………………... Soleus
uninj……………... Uninjured
inj………………… Injured
GW………………. GW501516
Ctrl………………. Control
Veh………………. Vehicle
DMSO…………… Dimethyl Sulfoxide
EBD……………… Evans Blue Dye
IP………………… Intrapreitoneal
H&E……………... Hematoxylin and Eosin

LIST OF FIGURES AND TABLES

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PUBLICATIONS

Narkar V.A., Downes M., Yu R.T., **Embler E**., Wang Y.X., Banayo E., Mihaylova M.M., Nelson M.C., Zou Y., Juguilon H., Kang H., Shaw R.J., and R.M. Evans. "AMPK and PPARdelta agonists are exercise mimetics." *Cell.* 134(3): 405-15, 2008.

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ABSTRACT OF THE DISSERTATION

PPARdelta activation enhances skeletal muscle regeneration

by

Emi Kanakubo Embler

Doctor of Philosophy in Biology

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Professor Ronald M. Evans, Chair

Skeletal muscle relies mostly on its resident progenitor cells, the satellite cells, for postnatal growth and regeneration. Therefore, maintaining proper function and healthy population of satellite cells are critical for the ability of muscle to control damage. Endurance exercise elicits a myriad of adaptive responses in the muscle, including an increase in the satellite cell number. Targeted expression of constitutively active Peroxisome Proliferator-Activated Receptor delta (VP16-PPARδ) in the skeletal muscle mimics endurance training induced fiber type remodeling,

including, glycolytic to oxidative fiber type switch and concomitant increase in the running capacity. Currently, however, it is unknown whether transcriptionally directed "endurance exercise training" adaptations by PPARδ in the muscle is sufficient to affect satellite cell homeostasis and function. We herein present that PPARδ activation promotes acceleration of regenerative process after an acute injury. We found that the skeletal muscle specific over-expression of PPARδ induces increase in the satellite cell population. Furthermore, we observed an increase in the number of proliferating cells after injury, leading to an increase in the number of nascent regenerating fibers. Gene expression analyses showed an earlier resolution of inflammatory response and induction of myogenic markers, suggesting that PPARδ actuates temporal shift of the regenerative process. Interestingly, PPARδ promotes myoblast proliferation immediately after the injury, which correlates with strong induction of Notch signaling pathway by PPARδ. Additionally, acute pharmacological activation of PPARδ also promoted efficient restoration of fiber integrity. Collectively, our results demonstrate a new role for PPARδ in skeletal muscle regeneration. Our findings allude to the therapeutic potential of PPARδ, not only for the treatment of an acute injury, but also in Notch-dependent aging associated loss of regenerative capacity of the muscle.

Chapter 1:

Introduction

Skeletal muscle fiber is a multinucleated syncytia and constitutes a single cell unit of the muscle. Diversity of fiber types in their metabolic and contractile properties allows for the plasticity of skeletal muscles to adapt to the changing demands of the body. Myofibers can be characterized by their expression of myosin heavy chain (MHC) isoforms. Myofibers express either type I or II MHC isoforms, where type II is further subdivided into a, b and x based on their metabolic properties, where type IIa fibers are fast oxidative and type IIb are fast glycolytic and type IIx lying in bewteen. Type I fibers have slow peak to tension time and utilizes oxidative metabolism, whereas type II fibers have fast peak to tension time and metabolically glycolytic (Zierath and Hawley, 2004). The heterogeneity of fiber types allows for a wide range of metabolic and mechanical output.

Skeletal muscles are adaptable to meet the demands of the body. For example, exercise induces fiber type transitions in skeletal muscles (Bassel-Duby, 2006). Exercise can largely be classified into two major subtypes: resistance and endurance. Both types of exercise elicit profound changes in skeletal muscle, but through different molecular mechanisms (Baar, 2006). Resistance exercise has been shown to induce hypertrophy via increased protein synthesis (MacDougall, 1995). Correspondingly, hallmarks of endurance exercise are increased mitochondrial mass, oxidative enzymes, and slow twitch proteins, decreased glycolytic proteins and hypotrophy in fast twitch fibers (Baar, 2006). Resistance exercise has been shown to inflict damage to the muscle by permeabilization of tissue and subsequent hyperemia (Vierck, 2000; Darr, 1987). This enables the influx of soluble factors, presumably leading to the activation of satellite cells. In rats, endurance training has also been shown to increase satellite

cell number (Umnova, 1991). Also in rats, fast to slow fiber type switch induced by chronic low frequency electric stimulation, is inhibited by ablation of satellite cells by γ irradiation (Martins, 2006). These findings demonstrate that the skeletal muscle remodeling in adult animals are mediated by satellite cells. It is unclear whether in managing exercise induced adaptations in skeletal muscles, contributions from satellite cells are solicited in the absence of damage to the fibers.

Alexander Mauro first described satellite cells based on their unique habitat within a myofiber: they are "wedged" between the basement membrane and the plasma membrane of a myofiber (Mauro, 1961). Seminal findings came in the 1970s, which brought satellite cells into the limelight. In 1971, Moss and Leblond demonstrated proliferation and fusion capacity of satellite cells using single-injection ^{3}H-thymidine labeling in the tibialis anterior muscle of growing (young) rats (Moss, 1970). A few years later, Schultz et al. used continuous infusion of ^{3}H-thymidine to show that the majority of satellite cells in non-growing (adult) mice are mitotically quiescent (Schultz, 1978). Around the same time, M.H. Snow showed that transplantation of minced ^{3}H-thymidine labeled triceps muscle into extirpated triceps of unlabeled littermate is able to reconstitute injured host myofibers (Snow, 1978). Collectively, these findings steered toward the view that the satellite cells are a distinct population of cells residing on the periphery of myofibers and contributing to the growth and regeneration of skeletal muscles. Over 40 years after the initial observations by Mauro, the "stemness," origin and identity of satellite cells have continuously been scrutinized. Presently, satellite cells are best classified as myogenic progenitors. Their self-renewal and differentiation capacity have alluded to the

archetypic "stemness," but their fate seems largely committed (Sinanan,2006;

Beauchamp, 2000).

Developmentally, satellite cells originate from the somites (Schienda, 2005;

Gros, 2005). In vertebrates, paraxial mesoderm undergoes segmentation to form

somites, which then compartmentalize into scleratome and dermomyotome (Christ,

1995). Muscle progenitor cells delaminate from hypaxial dermomyotome and migrate

and proliferate to form muscle masses in putative limbs (Relaix, 2004). These

developmental processes, propagated by the interplay between mesenchymal cells and

their surroundings, are mediated by various regulatory factors. Pax3, and its target

gene c-met, are critical during early stages of myogenesis (Shi, 2006). Mice with

homozygous mutation in Pax3 gene are embryonic lethal and show significant defect

in neural tube and neural crest cell formation as well as somitic defects, resulting in a

complete lack of limb muscles (Bober, 1994). Expression of Pax3 is first detected in

presomitic mesoderm, and persists through to the somitic epithelium of the

dermomyotome (Relaix, 2005; Goulding, 1991). Pax7 is detected in central

dermamyotome (Relaix, 2004). Subsequently in the myotome, about 87% of the cells

are Pax3+Pax7+ (Relaix, 2005). Subpopulation of these Pax3+Pax7+ cells are

believed to persist through development without entering myogenic program, and later

exhibit the properties of satellite cells (Schienda, 2005; Gros, 2005; Relaix, 2005).

Pax7, a paralogue of Pax3, has been shown to be indispensable for a complete

myogenesis. Initially, Pax7-/- mice were reported to suffer from cephalic neural crest

defects, exhibiting malformations of cranial structures and succumbing to premature

death at a mere 2 weeks of age (Mansouri, 1996). Notably, the authors of this report

observed no obvious phenotype in skeletal muscle, such that embryonic and fetal myogenesis produced architecturally sound skeletal muscles (Mansouri, 1996). Beyond the obvious, however, Pax7-/- mice are completely devoid of satellite cells, thus unable to sustain postnatal growth of musculature, exhibiting growth retardation (Seale, 2000).

Aside from their classical definition based on their sublaminar positioning, efforts to identify satellite cells by a unique set of markers have led to the idea of phenotypic and functional heterogeneity amongst the cells residing in the satellite cell compartment (Mauro, 1961; Sinanan, 2006; Wagers, 2005; Peault, 2007; Dhawan. 2005). Physical infiltration of the satellite cell compartment by hematopoetic cells in response to damage has been documented (Wagers, 2005). Albeit at low frequency, skeletal muscle harbors a variety of cell types, including side population (SP) cells, "muscle-derived stem cells" (MDSC) or "multipotent adult progenitor cells" (MAPC), mesenchymal stem cells (MSC), and mesoangioblasts (Sinanan, 2006; Wagers, 2005; Dellavalle, 2007; Peault, 2007). They are known to possess multipotency, however, their contributions in muscle regeneration are considered insignificant compared to that of satellite cells (Collins, 2005; Sherwood, 2004). Functionally, a small portion of satellite cells has been shown to exhibit a distinctively slower rate of proliferation (Schultz, 1996). Additionally, dynamics of their cellular states may contribute to the appearance of heterogeneity of the satellite cell population. Commonly, satellite cells are extracted from tissue by enzymatic digestion and enriched by cell sorting based on cell-surface markers. However, satellite cells are highly prone to activation upon extraction from their niche – hence, change in cellular states. Currently, most

quiescent satellite cells are identified by the expressions of Pax7, M-Cadherin, CD34, and Myf5 (Beauchamp, 2000; Seale, 2000; Irintchev, 1994). It has also been reported that a novel antibody, SM/C-2.6, detects a unique epitope expressed by the quiescent satellite cells (Fukuda, 2004).

Perixisome Proliferator-Activated Receptors (PPAR) are ligand-dependent nuclear transcription factors that belong to group C in superfamily of nuclear hormone receptors (Nuclear Receptor Nomenclature Committee, 1999). To date, there are 48 members of the nuclear hormone receptor superfamily in humans and 49 members in mice (Figure 1.1) (Bookout, 2006). The PPARs were named based on the discovery of PPARα, which was shown to be activated by hepatocarcinogens that cause proliferation of peroxisomes (Issemann, 1990). Shortly thereafter, two more isoforms, γ, and β/δ, were found, and together, they came to be referred to as the PPAR family (Laudet, 2002). PPARs form obligatory heterodimers with retinoid X receptor (RXR) and bind to peroxisome proliferator response elements (PPREs) of their target genes to initiate transcription upon ligand binding (Kliewer, 1992). PPRE is a characteristic DR-1 motif, where two direct repeats of consensus sequence AGGTCA is separated by a single nucleotide (Figure 1.2) (Kliewer, 1992). PPARs have the typical modular structure of nuclear receptors (Figure 1.3) The least conserved domain between the three PPAR isoforms is the N-terminal ligand-independent transactivation domain, followed by the most similar DNA binding domain. The hinge region is critical for structural characteristics of the nuclear receptors and also accepts interactions with cofactors (Dowell, 1997; Iwata, 2001; Tomaru, 2006). Ligand binding domain serves

as a platform for heterodimerization with RXR and interactions with coactivators (Nolte, 1998; Schulman, 1998). The LBD of PPARs is hydrophobic, large Y-shaped cavity with a flexible entrance, which allows for accepting a variety of endogenous and synthetic lipophilic ligands (Ehrenborg, 2009).

PPARα is predominantly expressed in the liver, PPARγ I abundant in adipose tissues and PPARδ is ubiquitously expressed albeit at low levels in the liver (Chawla, 1994; Kliewer, 1994; Issemann, 1990). They serve as lipid sensors of the body and as such, bind to and are activated by dietary fatty acids and their derivatives in order to transcriptionally regulate lipid homeostasis in the body (Barish, 2006; Evans, 2004). PPARs α, γ are already being exploited as pharmacological targets for treatment of obesity and metabolic diseases that ensue, including dyslipidemia, hypertension, atherosclerosis and type 2 diabetes – physiological disorders that besiege an alarming number of adults in the recent years (Evans, 2004). While PPARδ has yet to serve the masses as a clinical target, evidence for their potential as drug target for the treatment of metabolic diseases are emerging. Adipose specific over-expression of constitutively active PPARδ, via VP16-PPARδ construct driven by aP2 promoter, confers protection from diet-induced obesity, steatosis and hyperlipidemia (Wang, 2003). Complimentarily, pharmacological activation of PPARδ via its synthetic ligand, GW501516, has been demonstrated to enhance fatty acid oxidation in skeletal muscles and confer protection from high fat diet-induced obesity and insulin resistance (Tanaka, 2003). Molecularly, key genes responsible for fatty acid transport and

subsequent oxidation in myocytes have been shown to be PPARδ targets (Ehrenborg, 2009).

The youngest member of the PPAR family, PPARδ, is unique in that in addition to its ability to ameliorate signatures of the metabolic syndrome, it is implicated in a variety of physiological processes, including inflammation, neuronal differentiation, mitochondrial respiration, thermogenesis, keratinocyte differentiation, wound healing and skeletal muscle remodeling (Ehrenborg, 2009). In the skeletal muscle, PPARδ is expressed at a higher level in the oxidative type I muscles than glycolytic type II muscles (Wang, 2004). Moreover, PPARδ activity seems to play an important role in fiber type determination. Skeletal muscle specific over-expression of PPARδ induces glycolytic to oxidative fiber type switch (Wang, 2004; Luquet, 2003). Conversely, muscle specific deletion of PPARδ reduces the proportion of type I fibers (Schuler, 2006). Additionally, increased expression of its coactivator PGC1α or the reduction of its corepressor RIP140 increases the proportion of type I fibers in muscle (Lin, 2002; Seth, 2007). Furthermore, molecular characteristics of oxidative fibers, such as higher myoglobin and mitochondrial content, observed in the transgenic models are translatable to a physiological output. Transgenic mice over-expressing constitutively active form of the receptor are able to run 1.75 times longer and twice the distance compared to their wild-type littermates (Wang, 2004).

In this report, we demonstrate that both genetic and pharmacological activation of PPARδ promote regeneration after acute thermal injury. PPARδ activation during

regeneration expedites resolution of inflammatory response and restoration of contractile proteins. Most interestingly, PPARδ activation leads to a transient up-regulation of Notch pathway during early phase of regeneration. Altogether, we offer a novel role of PPARδ during adult muscle regeneration and its potential as a therapeutic target to enhance regenerative capacity of skeletal muscle.
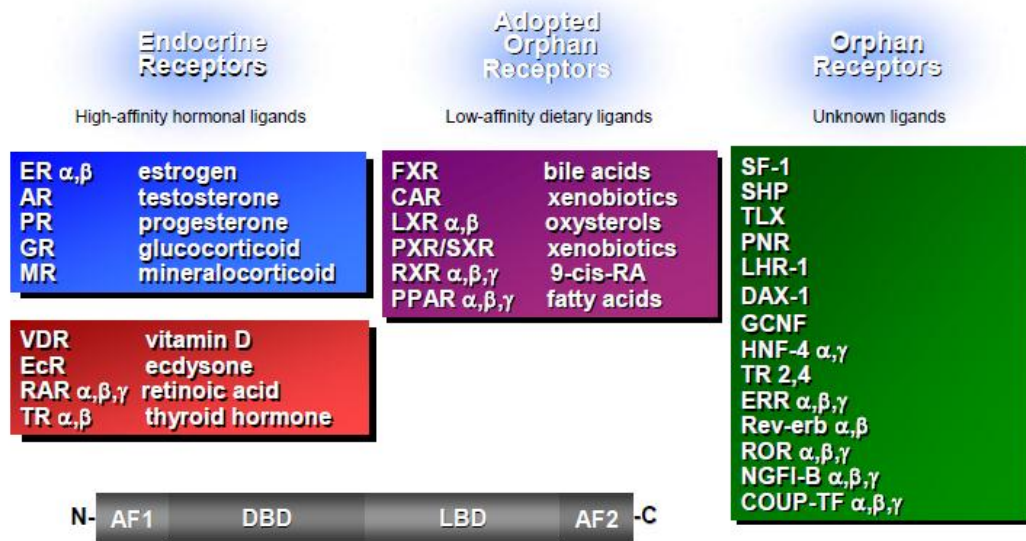
Figure 1.1: Nuclear receptor superfamily. NR family members are classified by their ligand of activation

Figure 1.2: Nuclear receptor binding DNA motifs.
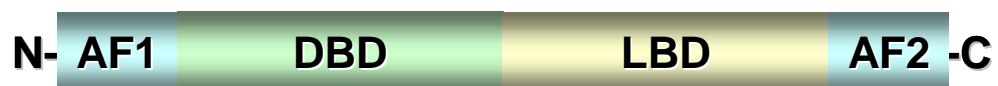
Figure 1.3: Schematic representation of the functional domains of PPARdelta. From the N-terminal: ligand-independent transcription activation (TA) domain, DNA binding domain (DBD), the hinge region (H) and the ligand binding domain (LBD).

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Chapter 2:

Effects of muscle specific activation of PPARdelta during skeletal muscle regeneration after acute injury.

Abstract

Muscle specific over-expression of constitutively active PPARδ induces fiber type remodeling and concomitant changes in metabolic and functional capacity of the muscle (Wang, 2004). We sought to test whether PPARδ mediated muscle remodeling also alters the regenerative capacity of the muscle. We employed the transgenic mouse model bearing skeletal muscle specific expression of constitutively active PPARδ (VP16-PPARδ) to examine the regenerative response to acute thermal injury. PPARδ activation promoted restoration of fiber integrity and nascent fiber formation. Furthermore, PPARδ activation potentiated resolution of inflammatory response and induction of myogenic markers. Additionally, we observed a 40-60% higher number of proliferating cells after the injury, which coincided with elevated expression of Notch signaling pathway components during the early phase of the regenerative process. Finally, VP16-PPARδ animals exhibited ~6 fold increase in the number of resident satellite cells. Collectively, we demonstrate that muscle specific activation of PPARδ is sufficient to confer regenerative advantage after an acute muscle injury.

Introduction

Skeletal muscle is a mechanically and energetically active organ, supporting vital functions such as breathing, locomotion, and glucose and lipid metabolism. Therefore, maintaining proper function is critical for the success of an individual. Inevitably, skeletal muscle suffers damage through a variety of stimuli: acute injury by physical, mechanical or chemical insults, chronic state of pathology and simply, aging. While skeletal muscle does not undergo constant turn-over under normal conditions, upon receipt of damage, it is capable of executing robust regenerative response through mobilization of its resident progenitor cells (Moss, 1970; Schultz, 1978; Snow, 1978).

Upon injury, skeletal muscle promptly responds to damage in three distinct but overlapping phases: degeneration, regeneration and finally remodeling phase (Charge, 2004). Immediately following the injury, inflammatory cells such as neutrophils and macrophages are recruited to the injury site via release of cytokines, such as HGF, IL-6, -15 and LIF, from the compromised myofibers (Tidball, 200; McLennan, 1996; Pimorady-Esfahani, 1997; Vierck, 2000). Myeloid cells promote tissue necrosis by cell lysis and debris clearance by phagocytosis, but also actively facilitate the regenerative process (Arnold, 2007). The regenerative process is characterized by mobilization of satellite cells. Through various *in vivo* and *in vitro* studies, monocytes and macrophages have been shown to interact - via chemotaxis – with satellite cells to promote proliferation and thus myogenesis (Charge, 2004; Robertson, 1993; Cantini, 1995a; Chazaud , 2003; Tidball, 2007). In culture, monocytes promote autocrine secretion of IL-6 by satellite cells, which in turn stimulate their proliferation (Cantini, 1995b). Satellite cells comprise less than 5% of total nuclei on an adult myofiber,

nevertheless, based on their proliferation kinetics and capacity, they are sufficient to regenerate an entire muscle (Schmalbruch, 1977; Kelly, 1978; Gibson, 1982; Bischoff, 1994; Zammit, 2002). Additionally, proliferation and differentiation of the satellite cells are tightly controlled by the signaling tag team of Notch and Wnt pathways during the regeneration phase (Brack, 2008). Temporal and spatial regulation Notch signaling is necessary for expansion and replenishment of the satellite cell pool (Conboy, 2002). Forced activation of Notch-1 rejuvenates muscle regeneration in aged mice while inhibition interferes with muscle regeneration on young mice (Conboy, 2003). Lastly, remodeling phase concludes the regeneration process by reassembling contractile proteins to restore muscle function.

Inherently, slow and fast muscles can respond differently to various conditions leading to muscle damage. In age-induced muscle loss, preferential atrophy occurs in type II fibers, along with preferential loss of satellite cells from type II fibers (Larsson, 1978; Lexell, 1988; Lexell and Downham, 1992; Verdijk, 2007). In ischemia-reperfusion injury model, predominantly slow-fiber consisting muscle, i.e. soleus, suffers less damage than predominantly fast-fiber consisting muscles, i.e. EDL, plantaris, TA (Walters, 2008; Gardner, 1984; Jennische, 1985; Woitaske, 1998; Chan, 2004). Additionally, soleus muscles show accelerated structural and functional recovery after ischemic injury, albeit, soleus muscle suffer inefficient and aberrant regeneration after injuries causing nerve damage (Woitaske, 1998; Chan, 2004; Carvalho, 1995; Kalhovde, 2005; Zimowska, 2001).

Freeze burn injury has been widely used to simulate acute injury to the muscle (Schiaffino and Partridge, 2008). It elicits standard course of regenerative response from the

muscle, including satellite cell activation. However, it is not sever enough to completely incapacitate the animal. The damaged fibers regain their cross sectional area by 21 days after the injury. Additionally, since the injury is directly applied to the surface of the muscle, it is highly localized and reproducible.

We and others have shown that the activation of peroxisome proliferators activated receptor δ (PPARδ) induces fiber type switch toward oxidative phenotype, altering both metabolic and functional output of the muscle (Wang, 2003, Luquet, 2003). Recently, endurance exercise alone has been shown to improve ageing induced decrease in satellite cell number and their myogenic capacity (Shefer, 2010). Therefore, we sought to determine if transcriptionally modulated endurance phenotype by PPARδ activation confers regenerative advantage after acute muscle injury.

In this report, we demonstrate that the genetic activation of PPARδ promote muscle regeneration after acute thermal injury. Muscle specific PPARδ activation during regeneration expedites resolution of inflammatory response and restoration of contractile proteins. Most interestingly, PPARδ activation leads to a transient up-regulation of Notch pathway during early phase of regeneration. Altogether, we offer a novel role of PPARδ during adult muscle regeneration and its potential as a therapeutic target to enhance regenerative capacity of skeletal muscle.

Materials and Methods

Animals

VP16-PPARδ mice (Wang, 2004) were bred to CB6F1 strain (Jackson Laboratories) and used as heterozygotes in experiments. The wildtype littermates served as controls. All experiments were performed when animals were 8 weeks of age, unless otherwise noted. Skeletal muscle specific PPARδ knock-out mice were generated by crossing animals carrying *loxP* sites around exon 4 of PPARδ gene (Barak, 2002) and MCK-Cre, which restricts expression of Cre recombinase to mature muscles (Bruning, 1998). For experiments, animals that are homozygous for *loxP* PPARδ allele with or without the concurrent expression of Cre recombinase were used. Nestin-GFP mice (Mignone, 2004) were kindly provided by Dr. Fred Gage at the Salk Institute for Biological Studies. They were bred with VP16-PPARδ animals to generate double transgenic animals. Single transgenic animals positive for GFP expression were used as controls.

Fiber typing gel

The proportion of each fiber type in a muscle group was calculated by separating myosin heavy chain isoforms on PAGE according to a published protocol with a few modifications (Mizunoya, 2008). Muscle tissues were harvested and roughly minced by a razor blade. Minced tissue was placed in Homogenization Buffer (10% SDS, 40mM DTT, 5mM EDTA, 0.1M Trus-HCl, pH 8.0, and protease inhibitor cocktail (Roche)) and homogenized with Dremel hand-held homogenizer at the setting of "15" for 10 seconds or until no residual tissue pieces were visible. Samples were

boiled at 100°C for 5 minutes, then diluted (Soleus: 1/400; TA: 1/20,000) with

Homogenization Buffer and mixed with 2X Loading Buffer (1% β-Mercaptoethanol,

4% SDS, 0.16M Tris-HCl, pH 6.8, 43% glycerol and 0.2% Bromophenol Blue).

Prepare 0.75mm thick gels (Separating gel: 8% x/v Acrylamide-Bis (99:1), 0.2M Tris-

HCl, pH 8.8, 0.1M Glycine, 0.4% w/v SDS, 0.1% w/v APS, 35% glycerol, 0.05%

TEMED; Stacking gel: 4% w/v Acrylamide-Bis (50:1), 70mM Tris-HCl, pH 6.7,

4mM EDTA, 0.4% w/v SDS, 0.1% APS, 30% glycerol, 0.05% TEMED) and load

10ul per well per sample and run at constant 40mA through the stacking gel and

constant 140V thereafter for 22 hours in the cold room. MHC isoforms were

visualized using SilverSNAP Stain Kit II (Pierce) by following the kit's instructions.

Gels were air dried for several days in between cellophane sheets.

Freeze burn injury

Tibialis anterior muscles were injured according to previously published

methods with a few modifications (Brack, 2007). The animals were anesthetized by

Isofurane inhalation during the procedure. The skin was shaved over the TA muscles

on both legs and the area was cleaned with 100% isopropynol. A small incision was

made on the skin to expose the TA muscle. On the control leg, the incision was

mended without further treatment. On the experimental leg, a stainless-steel 1g

weight equilibrated to the temperature of dry ice was placed directly on the exposed

TA for 10 seconds. Following the thermal injury, incision was closed using VetBond.

All injury procedures were performed on the left leg, and the right leg was used as

control. The injured portion of the muscle was carefully removed for gene expression analyses.

Histology

Animals were perfused with 15 ml of ice-cold PBS followed immediately by 20 ml of 10% saline buffered formalin (Fisher). Tibialis anterior muscles were excised and immersed in 4% paraformaldehyde (EMB) for at least 48 hours at 4°C. Tissues were washed thrice in PBS for 10 minutes each, at room temperature with gentle rocking. Tissues were sequentially dehydrated in the following order: 25%, 50%, 75%, 95% and 100% ethanol, 20 minutes each with two changes of solution at each concentration for the total of 40 minutes in each concentration. Finally, tissues were cleared in three changes of xylene at 10 minutes each. Xylene was replaced by paraffin for a few hours at 60°C then changed to fresh paraffin to let it permeate through the tissues over night. Tissues were then embedded in plastic molds.

Paraffin embedded tissue blocks were sectioned at 7 um thick on Leica Jung 2500 Microtome. 3-5 sections were acquired per step, 2 slides per step and each step was separated by 200 um. One slide from each step was H&E stained , on average 10 slides per animal, where "step" with the largest injury area was deemed as the center of the injury and used for analyses. For H&E staining, sections were de-paraffinized and rehydrated by submerging in the following: 3x xylene, 3 minutes each; 2x 100%, 90%, 75% ethanol for 2 minutes each; 50% ethanol for 2 minutes and ddH$_2$O for 2 minutes. Slides were then stained in 50/50 Gill's No2 Hematoxylin/H$_2$O mixture. After 5 minutes, slides were placed under running tap water for 5 minutes and

dehydrated in the following sequence: 50%, 75% and 95% ethanol for 2 minutes each.

Slides were counter stained in 1% eosin (1% eosin Y in 95% ethanol with 500ul

glacial acetic acid) solution for 2 minutes. Slides then underwent two changes of

100% EtOH for 2 minutes each. Finally, they were cleared in three changes of xylene

at 5 minutes each. Slides were dried under the fume hood and mounted with Entellen

mounting media.

Three random non-overlapping fields were photographed for analysis.

Regenerating fiber number was measured by counting the number of discernible

muscle fibers with centralized myonuclei (Ge, 2009). Regenerating fiber cross

sectional area (CSA) was measured using Image J software.

BrdU staining

50mg/kg body weight of BrdU (Sigma) was injected intraperitoneally as

solution of 10mg/ml BrdU in saline, either at 12, 24 or 48 hours after the freeze burn

injury. TA muscles were harvested at 7 days after injury and processed for paraffin

sections as described above. BrdU incorporation was visualized using the BrdU

Labeling and Detection Kit I (Roche) and the slides were mounted with Vectashield

with DAPI (Vector Labs).

Evans Blue dye staining

Injured animals were injected with Evans Blue dye according published

protocol (Hamer et al., 2002). Sterile 1% w/v Evans Blue dye in PBS was

intraperitoneally injected at 1% volume relative to the animal's body mass. 7 hours

after the injection, injured TA muscles were harvested. TA muscles were placed on a

mound formed by mixing gum tragacanth with O.C.T. compound and snap-frozen by isopentane quenching in liquid nitrogen. The frozen tissues were stored at -80°C. Frozen sections were cut in 10μm thickness and dried at room temperature for at least 1 hour. Sections were then fixed in ice-cold acetone for 2 minutes, air dried and quickly dipped in xylene. Dehydrated sections were mounted under coverslips with DPX. Sections were analyzed with the Rhodamine channel setting of the Leica fluorescent microscope. Amount of staining was measured by ImageJ software.

Isolation and culture of satellite cells

Satellite cells were harvested from TA, gastrocnemeus and soleus of 8 weeks old animals according to published protocols with some modifications (Day, 2007). Muscles were removed and washed briefly in DMEM on ice. They were then minced to fine slurry with razor blade on 60mm culture dish over ice. Minced muscles were transferred to one well of a 6-well plate containing 5 ml of 450KPU/ml pronase in DMEM with 10% FBS. The tissues were digested at 37°C/5% CO_2 for 60 minutes. Tissues were vigorously triturated 20 times through 10ml serological pipet. Digested tissues were filtered through 40 micron cell strainer and washed with equal volume of DMEM supplemented with 20% horse serum. Cells were spun down at 1000rpm and resuspended in either sorting buffer (DMEM with 10% FBS) or growth media (DMEM with 20% FBS, 10% HS, 1% CEE and 100U penicillin and 100ug/ml streptomycin). Cells were plated on culture dishes coated with 10ug/ml of laminin (Blanco-Bose, 2001). During subsequent splittings, in order to eliminate contaminating fibroblasts and enrich for the myoblasts, cells were pre-plated on tissue

culture plates for 15-60 minutes. The medium containing the cells that did not adhere during pre-plating were then plated and maintained on fresh laminin coated plates.

RT-QPCR

Whole or partial tissues were homogenized by Polytron probe homogenizer in Trizol reagent (Invitrogen). 3ml of Trizol was used for up to 50mg of tissue and the homogenizer was set at level 5 and applied to the samples for 7 seconds. Total RNA was extracted from the homogenates according to the manufacturer's protocol with a few alterations: after the phases were separated, aqueous phase containing RNA was loaded onto the RNeasy Mini kit (Qiagen) to clean and isolate RNA according to the kit instructions. RNA concentrations were measured spectrophotometrically on NanoDrop 8000 (Thermo Scientific). 1ug of total RNA was reverse transcribed (RT) using Superscript II (Invitrogen) or iScript Reverse Transcription Supermix (BioRad) according to the respective manufacturer's instructions. cDNAs were diluted 1/40 with ddH$_2$O and used as templates in RT-QPCR reactions with SYBRGreenER qPCR SuperMix detection system. qPCR reactions were performed on 7900HT Fast Real-Time PCR system (Applied Biosystems). Data were processed and analyzed using the SDS Software v2.4 (Applied Biosystems).

After isolation, satellite cells were FACS sorted for their GFP expression on BD FACSAria flow cytometer. Sorted cells were processed as above to isolate RNA, except RNeady Micro kit (Qiagen) was used to efficiently procure small amount of RNA. Isolated RNA was amplified using the WT Ovation RNA Amplification System (NuGEN) according to the manufacturer's protocol.

Primers

Q-PCR primers were constructed by "Pick Primers" function on NCBI website

for each gene. PPARδ F:GCCTCGGGCTTCCACTAC, R: AGATCCGATCGCACT

TCTCA; VP16 F: CGCTAGACGATTTCGATCTGGAC, R: CGGTAAACATCTGC

TCAAACTCG; PDK4: 3'GCCATTATAAAGAAAGCAACTAAGCA, 5' CCAGAG

ACGCGTATTTCTACCA; CPT1β: 3'GGGTCCCAAAGTGGCCA, 5' CATGGGAC

TGGTCGATTGC; catalase F:AGCGACCAGATGAAGCAGTG, R: TCCGCTCTCT

GTCAAAGTGTG ; MCP1 F: GGCTCAGCCAGATGCAGTTAA, R: CCTACTCAT

TGGGATCATCTTGCT; MyoD F: GCCGCCTGAGCAAAGTGAATG, R: CAGCG

GTCCAGGTGCGTAGAAG; MYH8 F: CGTACGGTGTATCATTCCCAATG, R:TG

GTGCAGGACCAGTTCGT; Notch1: 3' GAG CCCTCGTTGCAGGGGTT, 5' CTG

CGAATGTCCGCATGGGC; Hey1: 3' GCGAAGGGCTGGGGGTAGTC, 5' CTTTC

GGCATGAAGCGGCCC; HES1 F: GGAGAAGAGGCGAAGGGCAAGA, R: TGC

AGGTTCCGGAGGTGCTT; HEY1 F: CTTTCGGCATGAAGCGGCCC, R: GCGA

AGGGCTGGGGG TAGTC; Pax7 F: CTCAGTGAGTTCGATTAGCCG, R: AGAC

GGTTCCCTTTGT CGC; Tuj1 F: CCAGACCGAACACTGTCCA, R: CCCAGCGG

CAACTATGTAGG

Myofiber isolation

Individual myofibers were isolated according to published protocol with slight

modifications (Rosenblatt, 1995). Gastrocnemius muscles were dissected and placed

in glass scintillation vials or 5ml glass test tubes. Muscles were digested in 0.2%

(w/v) collagease I in DMEM for 1 hour at 35 °C with gentle shaking in a water bath

(Sigma). Loosely liberated myofibers were mechanically individualized by repeated trituration using wide-mouthed, fire-polished-tip Pasteur pipets in PBS supplemented with 10% FBS. Isolated myofibers were washed thrice with PBS with 10% FBS and immediately mounted on glass slide with Vectashield or fixed in 4% paraformaldehyde.

Cryosection and immunofluorescence staining

Frozen muscle tissues were transversely sectioned at 7-10 um thickness on Leica CM1850 cryostat. Sections were dried on slides at room temperature for at least 1 hour prior to storage at -80 °C.

For IF, slides were allowed to equilibrate to room temperature for at 15 minutes. If the sections were mounted in O.C.T., slides were washed twice in PBS for 3 minutes each to remove the mounting media. Sections were then fixed in pre-cooled 50/50 acetone/methanol mixture for 20 minutes at -20 °C. Slides were washed thrice in PBS at room temperature for 2 minutes each to remove the fixative. Tissues were blocked in 10% goat serum (GS) for 1 hour at room temperature. Afterwards, tissues were incubated in primary antibody over night at 4 °C in humidified chamber. Excess antibody and loosely bound non-specific interactions were removed by three washes in 0.01% Tween-20/PBS at 5 minutes each. Sections were then incubated in appropriate secondary antibody at room temperature for 30 minutes. Slides were washed thrice in 0.01% Tween-20/PBS at 5 minutes each and mounted under coverslips with Vectashield with DAPI. Where appropriate, M.O.M. Fluorescein staining kit (Vector

Labs) was used according to the manufacturer's instructions. Antibody dilutions:

Pax7 – 1:2500, CD31 – 1:400.

Results

Muscle specific activation of PPARδ confers regenerative advantage

We tested the regenerative capacity of VP16-PPARδ using the freeze burn injury (Conboy, 2003). Majority of the metabolic genes are down regulated in response to freeze burn injury (Summan, 2003). Curiously, PPARδ expression was induced over 2 fold at 2 days after the injury, eventually returning to the basal level by day 7 (Figure 2.1A). Injury dependent up-regulation of PPARδ suggests a positive role for PPARδ during the early part of the regenerative process. First, we grossly assessed the fiber integrity 5 days after thermal injury using Evans Blue uptake (Figure 2.1B). Evans Blue dye penetration was used as a marker of myofiber damage to assess regeneration (Hamer, 2002). Injected dye permeates into the syncytia of non-intact fibers, while being excluded from the intact fibers. By comparing the proportion of stained fibers within cross sections of the injured muscle, we determined the permeability, thus degree of damage. At 5 days after the injury, VP16-PPARδ animals show significant improvement over the WT animals. While WT animals show staining of 14% of the total CSA, i.e. the proportion of non-intact fiber area, only 5% of VP16-PPARδ muscle was stained by the dye ($p=0.001$) (Figure 2.1C). At 12 and 36 hours after the thermal injury, both WT and VP16-PPARδ animals showed similar proportions of stained area (50.6% and 47.4% ($p=0.67$), and 38.5% and 43.3% ($p=0.228$), respectively) (Figure 2.1D). This suggests that both WT and VP16-PPARδ animals initially sustain similar degree of damage due to the thermal injury.

The reduction in the stained area suggests that the muscle specific PPARδ activation is sufficient to facilitate accelerated progression through the regenerative program.

We then examined other markers of regeneration, namely the morphological hallmarks of regenerating fibers. H&E stained transverse sections through the injured area were examined at 3, 5 and 7 days post injury. At 3 days after the injury, both wildtype and VP16-PPARδ mice show similar degrees of destruction – necrosing fibers surrounded by infiltrating monocytes (Figure 2.2A). No regenerating fibers, characterized by small, round shape and centralized nuclei, are discernible at this time point in wildtype animals, but a notable few were seen in TG animals (arrows, Figure 2.2A). By day 5 after the injury, obvious differences begin to emerge. In wildtype animals, small regenerating fibers are visible but necrosing fibers and monocytes are still prevalent at the site of the injury (arrowheads, Figure 2.2A). While in VP16-PPARδ animals, the injury site seems relatively sound, with prominence of regenerating fibers. By day 7 post injury, the injury site appears similar between the two groups; where both show a field of regenerating fibers with centralized nuclei, most of the infiltrating cells have retreated and damaged fibers appear to have been properly managed. However, quantification of regenerating fiber number and CSA reveals that by 5 days post injury, the regenerative advantage of VP16- PPARδ is clearly discernible. Both CSA of the regenerating fibers and the number of regenerating fibers were 43.5% (n=5 or 6; p<0.03) and 33.0% (n=11 or 12; p<0.001), respectively, greater for VP16- PPARδ animals (Figures 2.2B&C). At 21 days after the injury, both WT and VP16- PPARδ animals have restored their fiber size and

number to that of the uninjured level (Figure 2.2D). These data demonstrate that the

muscle specific activation of PPARδ sufficiently bestows regenerative advantage over

their wildtype counterparts.

PPARδ activation leads to temporal shift, thus increased efficiency, of the
regenerative process

Skeletal muscle regeneration is an intricately orchestrated process involving a

variety of cell types. Therefore, we then decided to examine the effects of PPARδ

activation in the non-myogenic arm of the regenerative process in our injury model.

Immune cells, both neutrophils and macrophages, are necessary for the proper

progression of regenerative process (Zacks, 1982; Grounds, 1987; Teixeira, 2003;

Summan 2006; Contreras-Shannon, 2007; Segawa, 2008). Additionally, various

cytokines are necessary to promote chemotaxis of monocytes and also to directly

regulate the activities of myogenic cells (Warren, 2004; Yahiaoui, 2008; Chazaud,

2003).

We first identified a global, injury specific gene expression changes in VP16-

PPARδ animals. Comparing the gene expression profiles of injured to TG to WT,

there were total of 3257 genes that changed expression, of those, 1375 of them were

down regulated and 1882 were up regulated. Gene ontology classification of up-

regulated genes revealed that cellular processes (i.e. organization, localization,

signaling), metabolic processes (i.e. fatty acid oxidation) and response to stimuli (i.e.

response to stress, defense response) were the top regulated processes by the activation

of PPARδ at 3 days after the injury (Figure 2.3). Interestingly, genes involved in

myogenesis were robustly up-regulated by PPARδ activation (Figure 2.3).

Additionally, genes involved in developmental processes, angiogenesis and anti-

apoptotic processes emerged from the analysis (Figure 2.3). Collectively, PPARδ

activation appears to control network of genes involved directly in myogenesis and

also in remodeling and repair process after injury.

In order to validate and temporally expand microarray data, expression of

select inflammatory (TNFα and CD68) and myogenic (MyoD) markers were

measured by QPCR (Figure 2.4A&B) In short, we observed a temporal shift in the

expression patterns of regenerative markers for VP16-PPARδ animals compared to

their wildtype littermates. Specifically, VP16-PPARδ animals showed earlier

induction of inflammatory genes whose expressions peaked sooner and were

subsequently down regulated earlier than in the wildtype animals. Interestingly,

inflammatory markers studied here peaked at similar levels between the two genotypes,

which suggests that VP16-PPARδ animals do not completely suppress their

inflammatory responses. Instead, it appears that VP16-PPARδ animals respond and

resolve their inflammatory responses more efficiently. Furthermore, VP16-PPARδ

animals show higher expression of perinatal myosin heavy chain gene, Myh8, a

regeneration marker (Figure 2.4C).

PPARδ activation positively regulates quiescent satellite cell number

In order to determine cellular basis for the regenerative advantage conferred by

PPARδ activation, we sought to quantify satellite cells number in uninjured VP16-

PPARδ animals using nestin as a marker (Day, 2007). We thus created nestin-

GFP;VP16-PPARδ double transgenic animals to genetically mark quiescent satellite

cells in vivo (Mignone, 2004). Gastrocnemius muscles were enzymatically digested to

liberate individual fibers, then mounted for quantification of the number of GFP+ SCs.

While double transgenic animals averaged 1.01 SCs per mm of fiber length, GFP+

animals only had 0.15 SCs per mm, a 6.48 fold higher SC content on VP16- PPARδ

muscle fiber (Figure 2.5A&B). In order to confirm functionality of the increased

satellite cell number, we measured satellite cell activity as myoblast proliferation

elicited by the freeze burn injury in vivo. After the freeze burn injury, BrdU was

intraperitoneally injected at 12 hrs, 24 hrs and 2 days after the injury and the muscles

were harvested 7 days after the injury to calculate the ratio of BrdU+ to total nuclei.

TG animals showed 40-60% increase in the number of BrdU+ proliferating cells at all

three injection times (Figure 2.6A). Notch signaling is activated and necessary during

the early phase of regeneration to support myoblast proliferation (Brack, 2008). Most

interestingly, gene expression analysis revealed injury induced Notch1 and its ligand,

Delta1, up-regulation is transiently amplified in TG animals (Fig 2.6B&E).

Concomitantly, Notch1 target gene, Hey1 and Hes1, is also induced at a higher level,

demonstrating that PPARδ activation during injury enhances Notch signaling to

promote myoblast proliferation (Figure 2.6C&D).

Skeletal muscle remodeling conferred by PPARδ activation exerts satellite cell non-autonomous effects to facilitate regenerative advantage.

The use of human skeletal α-actin promoter limits, in theory, the expression of

the transgene to the myonuclei in mature myofibers. We confirmed the expression of

the transgene to be absent in freshly isolated quiescent satellite cells (Figure 2.7A&B).

This suggests that the regenerative advantage conferred by PPARδ activation is not

satellite cell autonomous. We sought to determine an adaptive response bestowed by

PPARδ activation in the muscle which may be contributing to the observed beneficial

effects on regeneration. Increased vasculature is one of the hallmarks of oxidative

myofibers, which is known to facilitate introduction of immune cells and also supports

increased number of satellite cells. Immunostaining transverse sections of uninjured

TA from WT and TG animals revealed 36% increase in the number of CD31+

capillaries per field by PPARδ activation (Figure 2.8A&B). Furthermore, microarray

analysis shows significant increases in angiogenic genes, VEGFa, VEGFb and FGF1,

by PPARδ activation (Figure 2.8C). Microarray data for the expression of VEGFa

was confirmed by QPCR (Figure 2.8D). While it is possible that there is some signal

being transmitted from the myofiber to the satellite cells and other types of cells

involved in regenerative process to accelerate the process, increased vascularity could

at least contribute to this phenomenon.

Discussion

We herein report that PPARδ activation expedites skeletal muscle regeneration following an acute thermal injury. VP16- PPARδ transgenic animals showed increased satellite cell proliferation at the early phase of the regenerative process, which subsequently translated into increased CSA and the number of nascent regenerating fibers. Most interestingly, we have learned that the PPARδ activation augments transient Notch signaling during the early phase of regeneration, which maybe driving the increased proliferation in these animals after the injury. Additionally, muscle specific over expression of PPARδ seems to increase the resident satellite cell pool. Increased satellite cell population on a muscle fiber seems to, at least in part, contribute to the accelerated resolution of the injury. These findings unveil a novel role for PPARδ in the maintenance of skeletal muscle; as a potential therapeutic target for accelerated restoration of muscle mass after an acute injury and other atrophic conditions.

Notably, PPARδ activation seems to promote rapid emergence of nascent fibers after the injury. There being no evidence of hyperplasia at 21 days after the injury when the regenerative process is essentially complete, we conclude that the additional nascent fibers efficiently fuse with each other to restore mature fibers (Karpati, 2008). While IGF-1 and myostatin seem to rely on fiber hypertrophy to augment regenerative progress, PPARδ seems to employ a unique way to promote regeneration (Menetrey, 2000; Wagner, 2002; Bogdanovich, 2002). Underlying this difference may be the increased number of quiescent satellite cells. Higher number of

progenitor cells leads to the increase in post injury proliferating cells and consequent increase in the number of nascent fibers. While various growth factors and chemokines, including IGF-1 and myostatin, have been shown to enhance proliferation of satellite cells and promote regeneration, it is unclear whether any of them positively regulate the number of quiescent satellite cells (Husmann, 1996; McCroskery, 2003; Musaro, 2001; Amthor, 2009). Our findings strongly suggest a novel role of PPARδ as a positive regulator of satellite cell pool. Notably, the direct role of PPARδ in satellite cell homeostasis was recently reported using Myf5-Cre conditional knockout model (Angione, 2011). Interestingly, since we did not observe constant cell proliferation under normal condition, PPARδ mediated satellite cell expansion is transient and tightly regulated, most likely elicited by external stimuli, such as signals for postnatal growth and injury. Moreover, in VP16-PPARδ model, transgene is over-expressed only in the mature fiber, thereby suggesting indirect effects on satellite cell homeostasis through soluble factors or solicitation of other changes in the muscle. Nevertheless, in an adult muscle, satellite cell number is finite, diminishing detrimentally in disease state and aging. It is of great therapeutic benefit if PPARδ activation can bestow infinite abundance of satellite cell population throughout the life of an organism.

Chapter 2, in part, is currently being prepared for submission for publication. Embler, EK; Atkins, A; Downes, M; Evans RM. The dissertation author was the primary investigator and author of this material.
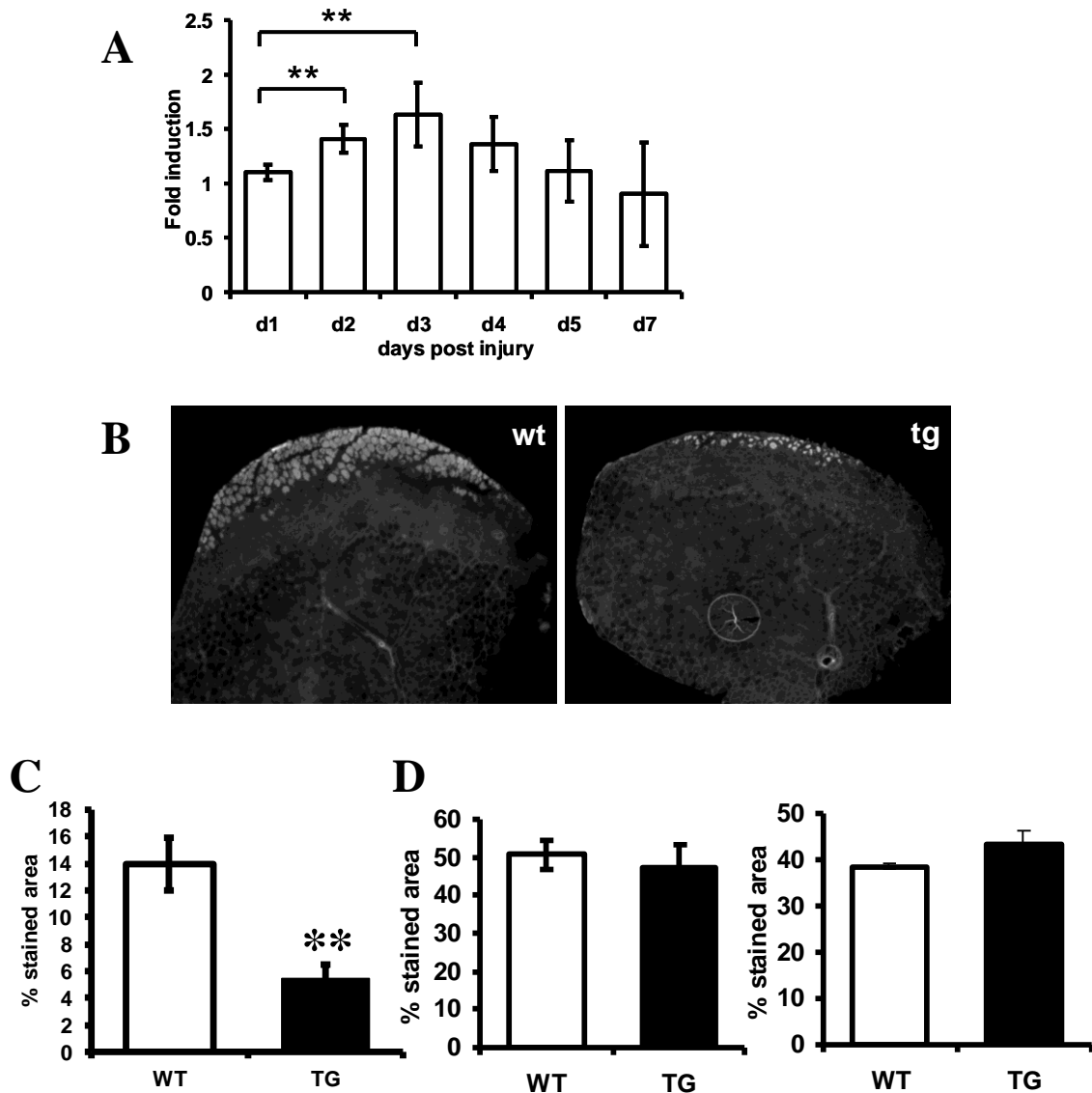
Figure 2.1: VP16-PPARdelta animals show increased efficiency in restoration of fiber integrity after injury. A) thermal injury was administered to the TA of 8 week-old wildtype animals and the injured portion was removed for expression of PPARδ by QPCR. B) 8 week-old VP16-PPARδ animals and their littermate controls were injured then allowed to recover for 5 days (n=8 for WT; n=5 for TG). Evans Blue dye was injected intraperitoneally to detect fiber permeability. C) Proportions of damaged area were calculated as percentage of stained area over total cross sectional area of TA. D) 8 week-old VP16-PPARδ animals were injured as above, and fiber integrity was measured at either 12 or 36 hours post injury by Evans Blue dye injection (n=3, each genotype).
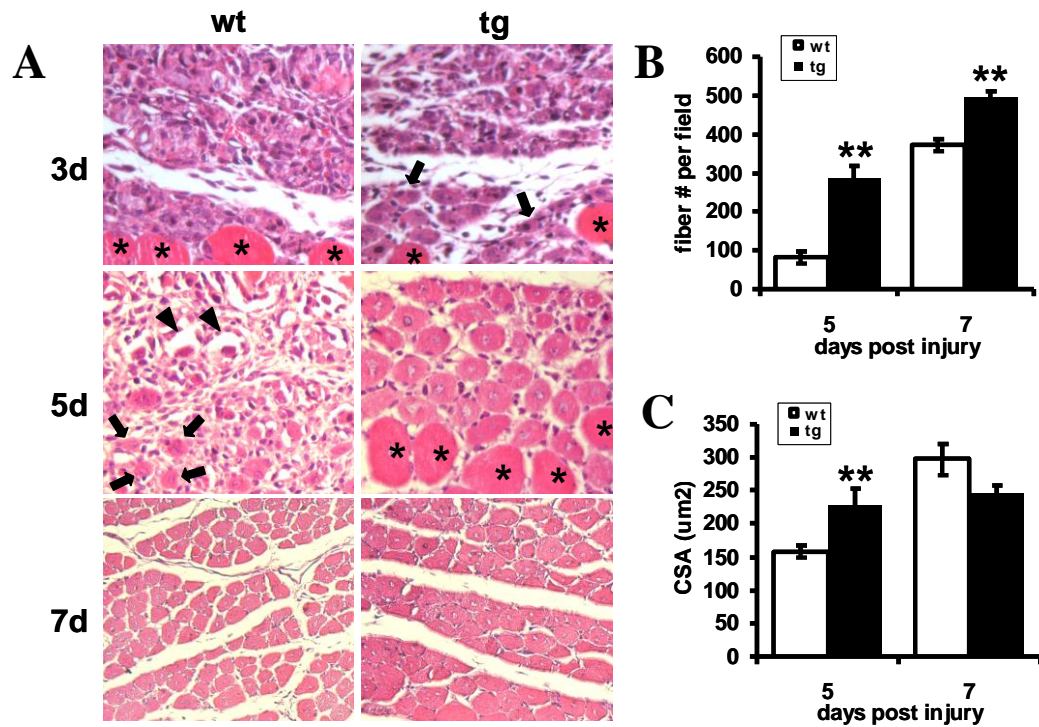
Figure 2.2: Skeletal muscle specific activation of PPARdelta expedites regenerative process after acute injury. A) H&E stained transverse sections of injured TA from wild-type (WT) and VP16- PPARδ (TG) animals were morphometrically analyzed (n=6 to 12 each genotype). TAs were harvested at 3, 5 and 7 days after injury. Regenerating fibers with centralized nuclei are marked by arrows. Arrowheads denote hollowed remains of basal lamina. Uninjured fibers are marked by asterisks. B) Quantification of the number of regenerating fibers per field. C) CSA of regenerating fibers measured by Image J.
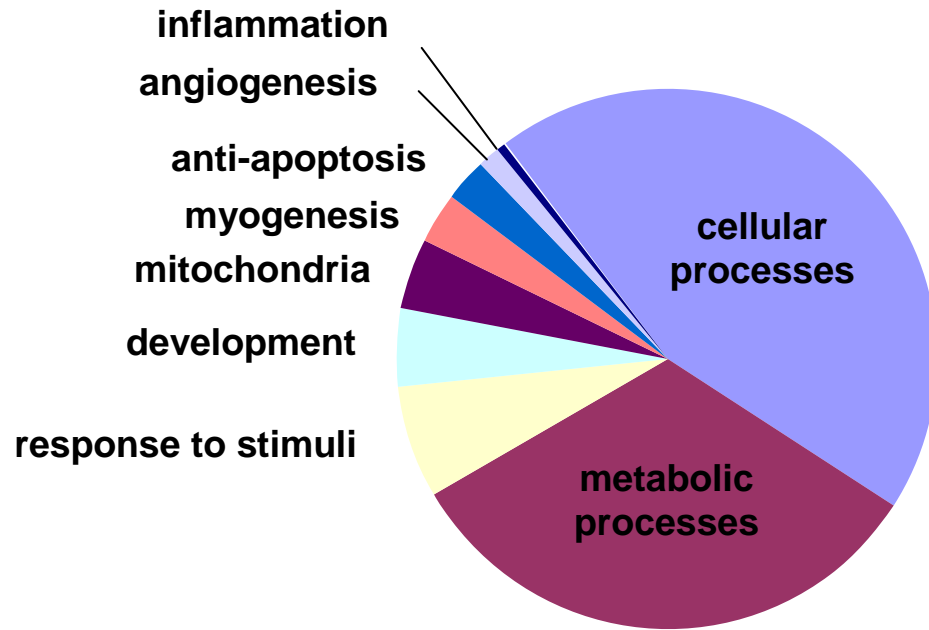
Figure 2.3: GO analysis of up-regulated genes in TG compared to WT in response to acute thermal injury.

Table 2.1: List of notable up-regulated genes in injured TG compared to WT from microarray analyses of 3 days post injury TA.

| Locus	Name/description	Fold change
Inflammation/chemotaxis		
CCL2	Chemokine (C-C motif) ligand 2	3.425
IL10	Interleukin 10	1.408
IL1β	Interleukin 1β	4.42
TNFα	Tumor necrosis factor	1.679
CD68	CD68 antigen	16.799
Oxidative stress defense		
Txn1	Thioredoxin 1	2.44
MT1	Metallothionein 1	2.352
MT2	Metallothionein 2	1.431
Extracellular matrix		
MMP2	Matrix metallopeptidase 2	1.58
MMP9	Matrix metallopeptidase 9	1.778
Myogenic factors		
Myh8	Myosin, heavy peptide, perinatal	17.766
MyoD	Myogenic differentiation	8.641
Myog	Myogenin	12.807
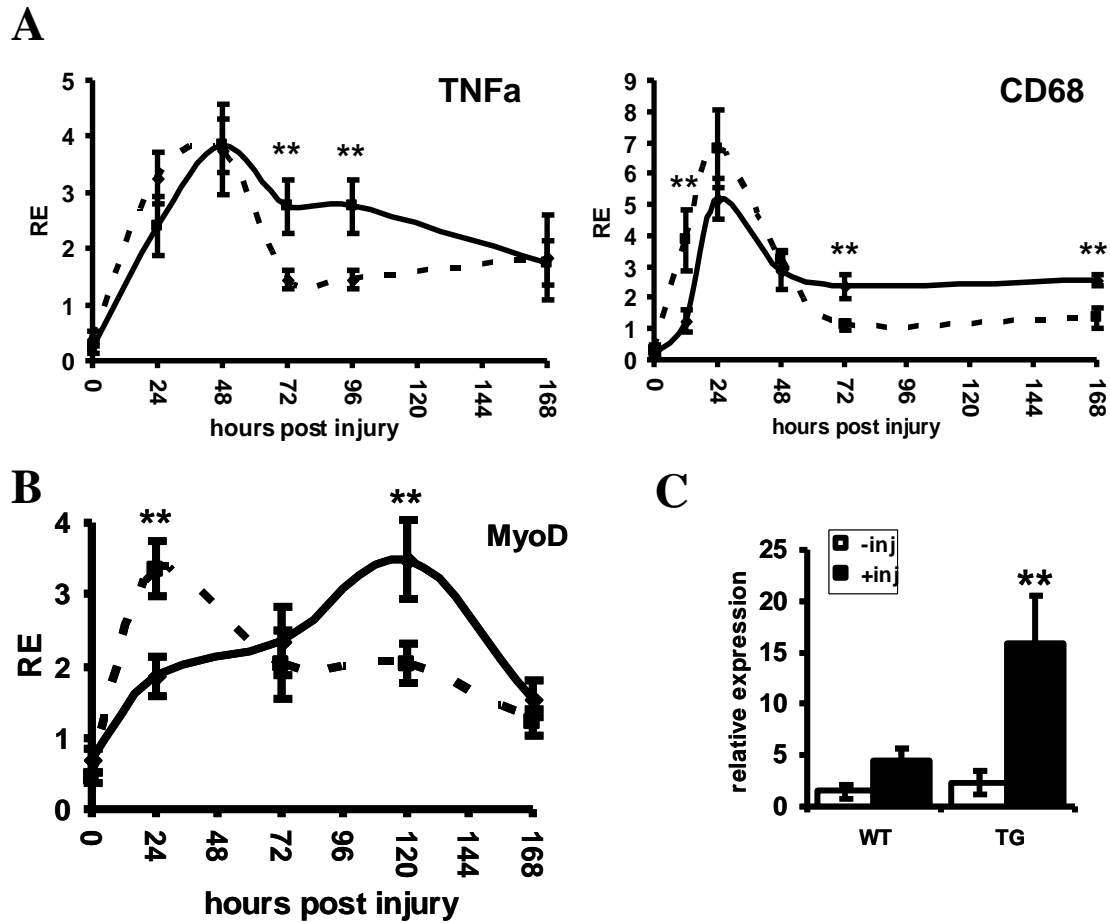
A

B

C

Figure 2.4: PPARδ activation leads to temporal shift, thus increased efficiency, of the regenerative process. A) Temporal gene expression profiles of inflammatory (TNFα and CD68) and B) myogenic (MyoD) markers in response to injury measured by Q-PCR. C) Myh8 mRNA level 7 days post injury.
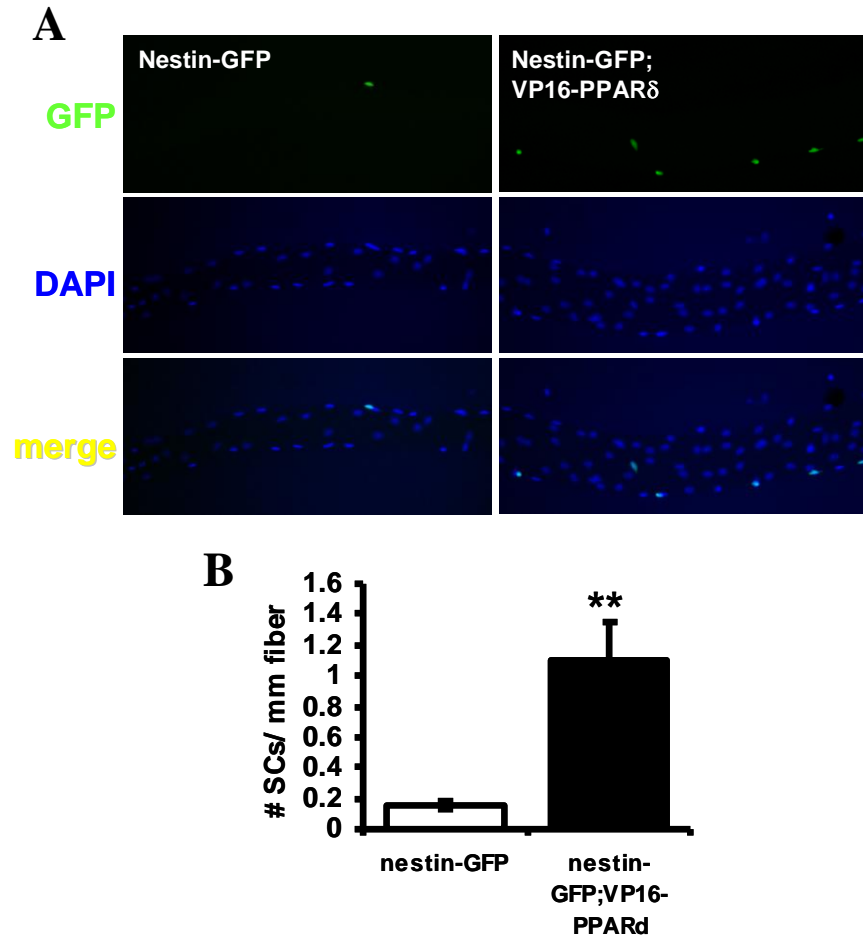
Figure 2.5: Muscle specific activation of PPARδ increases quiescent satellite cell population. A) Visualization of quiescent satellite cells (+GFP) on isolated myofibers from lateral gastrocnemius of 8 wk old male mice. B) Quantification of GFP+ satellite cells per unit length of myofiber. C) QPCR on isolated nestin positive satellite cells from TG and WT animals.
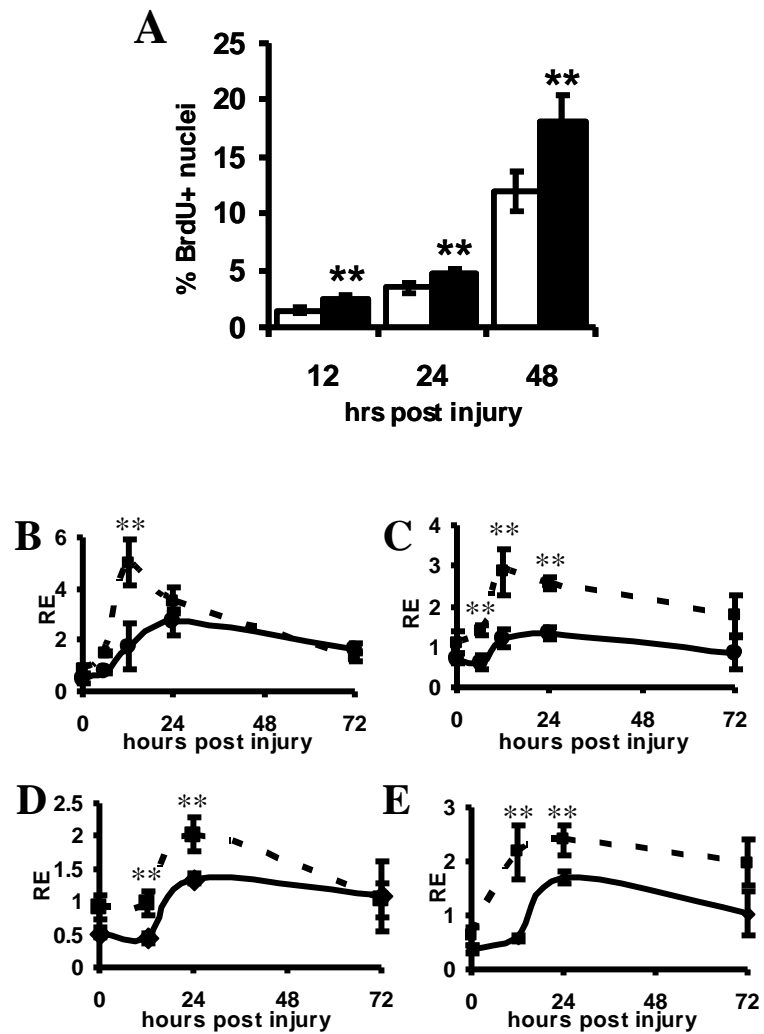
Figure 2.6: PPARδ activation augments myoblast proliferation after the injury. A) Quantification of myogenic progenitor cell proliferation by BrdU incorporation after the injury. BrdU was injected once at 12, 24 or 48 hours after injury. TA was harvested 7 days after injury and BrdU staining was assessed against DAPI nuclear staining to calculate percentage of BrdU positive cells in total myonuclei per field. Temporal expression profile of Notch signaling pathway genes after the injury: B) Notch1 C) Hey1 D) Hes1 and E) Dll1.
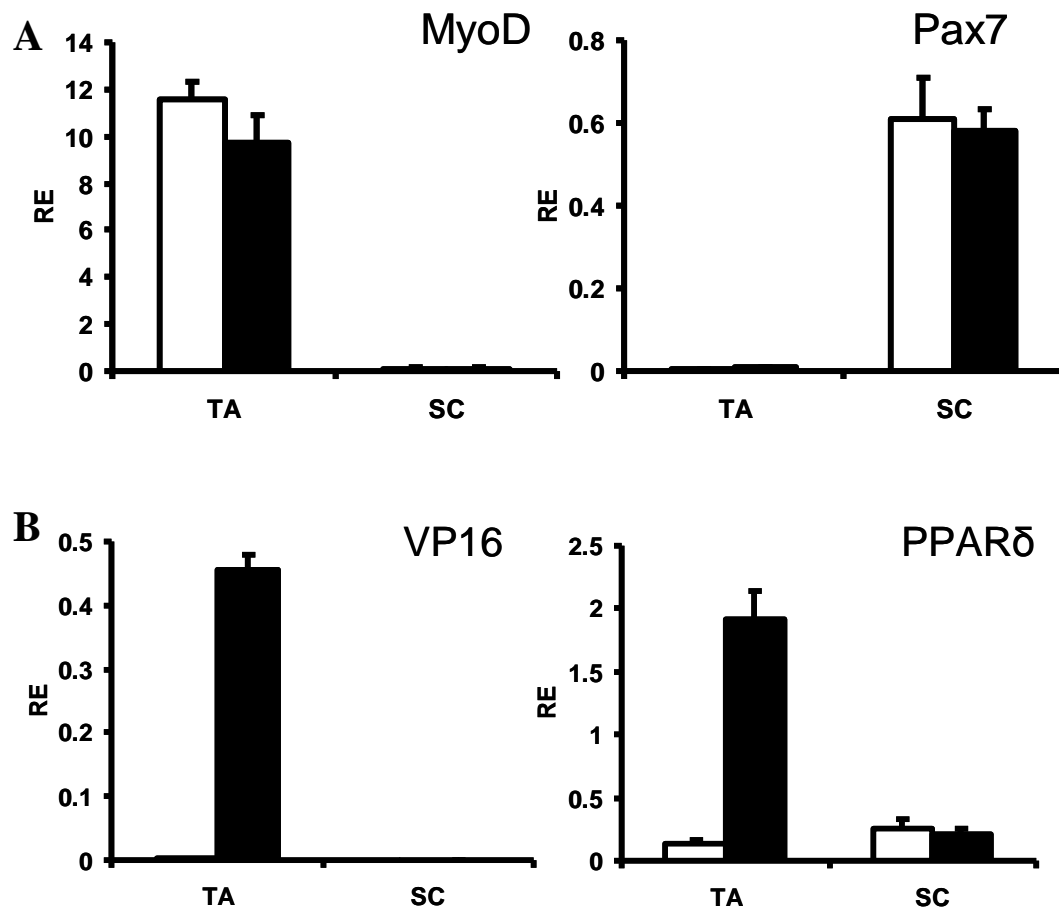
Figure 2.7: Transgene is not expressed in the satellite cells. Quiescent satellite cells were isolated and FACS sorted from nestin-GFP;VP16-PPARδ double transgenic animals and gene expression was measured by QPCR. A) Isolated cells show absence of myogenic marker (MyoD) and presence of quiescent satellite cell marker (Pax7). B) The VP16 activation domain is absent, as a result, PPARδ is not over-expressed in satellite cells.

A

B

C

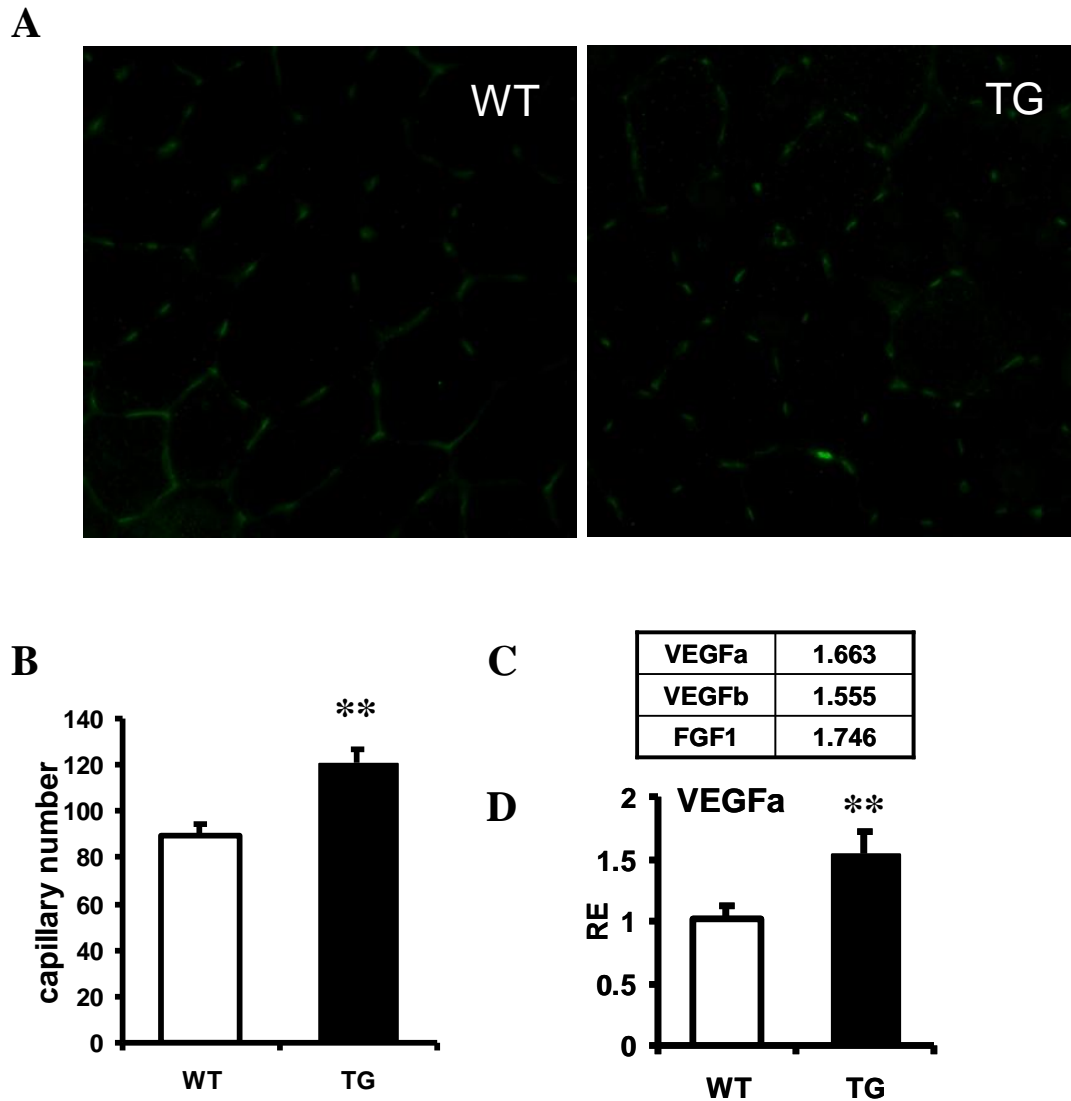|	
VEGFa	**1.663**
VEGFb	**1.555**
FGF1	**1.746**

D

Figure 2.8: PPARδ activation induces increase in vascularity. A) Immunostaining of transverse sections of TA from WT and TG animals for CD31. B) Quantification of CD31+ capillary number per field of view. C) Fold induction values of angiogenic genes from microarray comparing TG to WT. D) QPCR validation of VEGFa expression in TA.

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Chapter 3:

Effects of pharmacological activation of PPARdelta during skeletal muscle regeneration after acute injury

Abstract

Commercially available synthetic ligands make PPARδ an ideal target for pharmacological manipulation. Activation by oral administration of PPARδ specific ligand, GW501516, has been shown to mimic genetic activation of the receptor in many ways (Narkar, 2008; Tanaka, 2003). Therefore, we wondered if acute activation of PPARδ by orally administered GW501516 can also confer benefits during skeletal muscle regeneration after acute thermal injury. We found that acute treatment with GW501516 is sufficient to promote efficient restoration of fiber integrity after the injury. Acute activation of PPARδ reduces expression of inflammatory genes and increases BrdU positive cells 48 hours after the injury. Collectively, we demonstrate PPARδ as a possible pharmacological target in treatment of acute muscle injury.

Introduction

Potent PPARδ specific agonist, GW501516, was developed by GlaxoSmithKline through combinatorial chemistry and structure based drug design (Figure 3.1). It is highly selective for human and also murine PPARδ, 125 times against murine PPARα and 50 times against murine PPARγ (Sznaidman, 2003). Its physiological activity was confirmed in vivo by dosing insulin-resistant middle-aged obese rhesus monkeys. GW501516 caused dose-dependent rise in serum HDL cholesterol while lowering LDL, fasting triglycerides, and fasting insulin (Oliver, 2001).

In skeletal muscle, administration of GW501516 ameliorated diet-induced weight gain and insulin resistance, specifically characterized by an enhanced metabolic rate and fatty acid beta-oxidation, mitochondria biogenesis and a reduction of intramuscular lipid droplets (Tanaka, 2003). Combined with exercise training regimen, GW501516 treatment increased oxidative fibers in gastrocnemius and enhanced endurance running capacity (Narkar, 2008).

We herein present PPARδ as a possible therapeutic target to promote muscle regeneration after an acute injury. We found that the pharmacological activation of PPARδ promotes restoration of fiber integrity. Acute PPARδ activation facilitates suppression of inflammatory genes in the muscle shortly after the injury. Additionally, PPARδ ligand treatment supports proliferation of myoblasts after the injury.

Collectively, we show that acute pharmacological activation of PPARδ is sufficient to confer beneficial effects on regenerative process after an acute injury.

Materials and Methods

Animals

GW1516 was administered either by oral gavage or feed to 8 week old male C57BL6J mice (Jackson Laboratories). 5mg per kg of body weight per day of GW1516 was dissolved in DMSO and delivered in total volume of 200 ul of 0.5% carboxymethyl cellulose to each animal. Vehicle treated animals were given equal volume of DMSO and 0.5% carboxymethyl cellulose without the drug. Alternatively, animals were ad lib fed with regular rodent chow infused with 40mg/kg GW1516 or drug-free control chow (Harlan).

Freeze burn injury

Tibialis anterior muscles were injured according to previously published methods with a few modifications (Brack, 2007). The animals were anesthetized by Isofurane inhalation during the procedure. The skin was shaved over the TA muscles on both legs and the area was cleaned with 100% isopropynol. A small incision was made on the skin to expose the TA muscle. On the control leg, the incision was mended without further treatment. On the experimental leg, a stainless-steel 1g weight equilibrated to the temperature of dry ice was placed directly on the exposed TA for 10 seconds. Following the thermal injury, incision was closed using VetBond. All injury procedures were performed on the left leg, and the right leg was used as control. The injured portion of the muscle was carefully removed for gene expression analyses.

Histology

Animals were perfused with 15 ml of ice-cold PBS followed immediately by 20 ml of 10% saline buffered formalin (Fisher). Tibialis anterior muscles were excised and immersed in 4% paraformaldehyde (EMB) for at least 48 hours at 4°C. Tissues were washed thrice in PBS for 10 minutes each, at room temperature with gentle rocking. Tissues were sequentially dehydrated in the following order: 25%, 50%, 75%, 95% and 100% ethanol, 20 minutes each with two changes of solution at each concentration for the total of 40 minutes in each concentration. Finally, tissues were cleared in three changes of xylene at 10 minutes each. Xylene was replaced by paraffin for a few hours at 60°C then changed to fresh paraffin to let it permeate through the tissues over night. Tissues were then embedded in plastic molds.

Paraffin embedded tissue blocks were sectioned at 7 um thick on Leica Jung 2500 Microtome. 3-5 sections were acquired per step, 2 slides per step and each step was separated by 200 um. One slide from each step was H&E stained , on average 10 slides per animal, where "step" with the largest injury area was deemed as the center of the injury and used for analyses. For H&E staining, sections were de-paraffinized and rehydrated by submerging in the following: 3x xylene, 3 minutes each; 2x 100%, 90%, 75% ethanol for 2 minutes each; 50% ethanol for 2 minutes and ddH$_2$O for 2 minutes. Slides were then stained in 50/50 Gill's No2 Hematoxylin/H$_2$O mixture. After 5 minutes, slides were placed under running tap water for 5 minutes and dehydrated in the following sequence: 50%, 75% and 95% ethanol for 2 minutes each. Slides were counter stained in 1% eosin (1% eosin Y in 95% ethanol with 500ul

glacial acetic acid) solution for 2 minutes. Slides then underwent two changes of 100% EtOH for 2 minutes each. Finally, they were cleared in three changes of xylene at 5 minutes each. Slides were dried under the fume hood and mounted with Entellen mounting media.

Three random non-overlapping fields were photographed for analysis. Regenerating fiber number was measured by counting the number of discernible muscle fibers with centralized myonuclei (Ge, 2009). Regenerating fiber cross sectional area (CSA) was measured using Image J software.

BrdU staining

50mg/kg body weight of BrdU (Sigma) was injected intraperitoneally as solution of 10mg/ml BrdU in saline, either at 12, 24 or 48 hours after the freeze burn injury. TA muscles were harvested at 7 days after injury and processed for paraffin sections as described above. BrdU incorporation was visualized using the BrdU Labeling and Detection Kit I (Roche) and the slides were mounted with Vectashield with DAPI (Vector Labs).

Evans Blue dye staining

Injured animals were injected with Evans Blue dye according published protocol (Hamer, 2002). Sterile 1% w/v Evans Blue dye in PBS was intraperitoneally injected at 1% volume relative to the animal's body mass. 7 hours after the injection, injured TA muscles were harvested. TA muscles were placed on a mound formed by mixing gum tragacanth with O.C.T. compound and snap-frozen by isopentane quenching in liquid nitrogen. The frozen tissues were stored at -80°C. Frozen

sections were cut in 10μm thickness and dried at room temperature for at least 1 hour. Sections were then fixed in ice-cold acetone for 2 minutes, air dried and quickly dipped in xylene. Dehydrated sections were mounted under coverslips with DPX. Sections were analyzed with the Rhodamine channel setting of the Leica fluorescent microscope. Amount of staining was measured by ImageJ software.

RT-QPCR

Whole or partial tissues were homogenized by Polytron probe homogenizer in Trizol reagent (Invitrogen). 3ml of Trizol was used for up to 50mg of tissue and the homogenizer was set at level 5 and applied to the samples for 7 seconds. Total RNA was extracted from the homogenates according to the manufacturer's protocol with a few alterations: after the phases were separated, aqueous phase containing RNA was loaded onto the RNeasy Mini kit (Qiagen) to clean and isolate RNA according to the kit instructions. RNA concentrations were measured spectrophotometrically on NanoDrop 8000 (Thermo Scientific). 1ug of total RNA was reverse transcribed (RT) using Superscript II (Invitrogen) or iScript Reverse Transcription Supermix (BioRad) according to the respective manufacturer's instructions. cDNAs were diluted 1/40 with ddH$_2$O and used as templates in RT-QPCR reactions with SYBRGreenER qPCR SuperMix detection system. qPCR reactions were performed on 7900HT Fast Real-Time PCR system (Applied Biosystems). Data were processed and analyzed using the SDS Software v2.4 (Applied Biosystems).

After isolation, satellite cells were FACS sorted for their GFP expression on BD FACSAria flow cytometer. Sorted cells were processed as above to isolate RNA,

except RNeady Micro kit (Qiagen) was used to efficiently procure small amount of

RNA. Isolated RNA was amplified using the WT Ovation RNA Amplification

System (NuGEN) according to the manufacturer's protocol.

Primers

Q-PCR primers were constructed by "Pick Primers" function on NCBI website

for each gene. PPARδ F:GCCTCGGGCTTCCACTAC, R: AGATCCGATCGCACT

TCTCA; VP16 F: CGCTAGACGATTTCGATCTGGAC, R: CGGTAAACATCTGC

TCAAACTCG; PDK4: 3'GCCATTATAAAGAAAGCAACTAAGCA, 5'

CCAGAGACGCGTATTTCTACCA; CPT1β: 3'GGGTCCCAAAGTGGCCA, 5'

CATGGGACTGGTCGATTGC; catalase F:AGCGACCAGATGAAGCAGTG, R:

TCCGCTCTCTGTCAAAGTGTG; MYH8 F: CGTACGGTGTATCATTCCCAATG,

R:TG GTGCAGGACCAGTTCGT; MCP1 F: GGCTCAGCCAGATGCAGTTAA, R:

CCTACTCAT TGGGATCATCTTGCT.

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Cryosection and immunofluorescence staining

Frozen muscle tissues were transversely sectioned at 7-10 um thickness on

Leica CM1850 cryostat. Sections were dried on slides at room temperature for at least

1 hour prior to storage at -80 °C.

For IF, slides were allowed to equilibrate to room temperature for at 15

minutes. If the sections were mounted in O.C.T., slides were washed twice in PBS for

3 minutes each to remove the mounting media. Sections were then fixed in pre-cooled

50/50 acetone/methanol mixture for 20 minutes at -20 °C. Slides were washed thrice

in PBS at room temperature for 2 minutes each to remove the fixative. Tissues were blocked in 10% goat serum (GS) for 1 hour at room temperature. Afterwards, tissues were incubated in primary antibody over night at 4 °C in humidified chamber. Excess antibody and loosely bound non-specific interactions were removed by three washes in 0.01% Tween-20/PBS at 5 minutes each. Sections were then incubated in appropriate secondary antibody at room temperature for 30 minutes. Slides were washed thrice in 0.01% Tween-20/PBS at 5 minutes each and mounted under coverslips with Vectashield with DAPI. Where appropriate, M.O.M. Fluorescein staining kit (Vector Labs) was used according to the manufacturer's instructions.

inflammatory marker genes at 12 and 24 hours after the injury. While the initial

inflammatory responses are similarly generated with or without the PPARδ ligand

treatment at 12 hours after the injury, by 48 hours after the injury, the expressions of

inflammatory marker genes were significantly reduced by the PPARδ agonist

treatment (Figure 3.3).

GW 501516 has been shown to induce cell proliferation in vitro (Angione,

2011). Therefore, we sought to determine whether pharmacological activation of

PPARδ in vivo can elicit similar responses during regeneration. BrdU injection at 48

hours after the injury revealed that PPARδ activation does promote myoblast

proliferation after the injury (Figure 3.4). Lastly, we determined the activation of

Notch signaling pathway in response to PPARδ activation in uninjured Quadriceps

after 9 days of ligand treatment. Interestingly, while Notch1 and its target genes, Hes1

and Foxo1, were induced by PPARδ ligand treatment, Notch ligand, Delta1 was not

induced. It is possible that endogenous level of Delta1 may be sufficient to up-

regulate the signaling pathway if the Notch1 receptor level is induced (Figure 3.5).

Curiously, activation of Notch signaling pathway was confirmed at 3 days after the

injury in drug treated animals (data not shown). It is possible that Notch signaling had

already been down regulated at the end of proliferative phase by day 3. Further

analysis on time points immediately following the injury is necessary.

Discussion

We herein present a novel role for pharmacological activation of PPARδ by its synthetic agonist, GW501516, in muscle regeneration. The agonist treatment facilitates restoration of fiber integrity after an acute thermal injury. Molecularly, GW501516 reduces expression of inflammatory genes 48 hours after the injury. Strikingly, ligand treatment also increases the number of BrdU positive proliferating cells at 48 hours after the injury. Additionally, ligand treatment induces activation of Notch signaling pathway in the absence of injury, while same was not observed 3 days after the injury.

In this study, PPARδ ligand was administered orally and delivered systemically for effects targeted to multiple cell types. Specifically, suppression of inflammatory genes after the injury may be directed by the PPARδ activation in the macrophages (Barish, 2008). It has been shown that acute PPARδ activation can rapidly induce angiogenesis in muscle, which may establish key infrastructure to support critical immune response after the injury (Gaudel, 2008). Moreover, GW501516 may be directly activating the receptor in satellite cells to enhance proliferative kinetics (Angione, 2011). Nonetheless, as a potential treatment for a complex, multi-phasic regenerative process, involving a variety of cell types, it may be necessary to target multiple components of the program to achieve beneficial effects.

Activation of Notch signaling pathway by the PPARδ ligand may also be exploited for conditions in which decline in Notch signaling capacity leads to loss of muscle mass, such as in aging. Simply increasing the circulating Notch level is known

to rejuvenate satellite cell function in older animals (Conboy, 2003). It is of interest to administer PPARδ ligand to older animals to observe the effects on regenerative capacity of the muscle. Of note, 4 weeks treatment with the GW ligand failed to induce an increase in the quiescent satellite cell number (data not shown). Since satellite cells do not undergo rapid turnover, length of ligand treatment may have been too short. Moreover, it is possible that an additional activation cue in the form of external stimuli may be required to initiate their exit from quiescence to proliferate. Nonetheless, GW treatment appears to promote myoblast proliferation in vivo after the injury, therefore, increased number of satellite cells is not required to expedite the regenerative process, but rather increased proliferative kinetics is sufficient.

Chapter 3, in parts, is currently being prepared for submission for publication. Embler, EK; Atkins, A; Downes, M; Evans RM. The dissertation author was the primary investigator and author of this material.
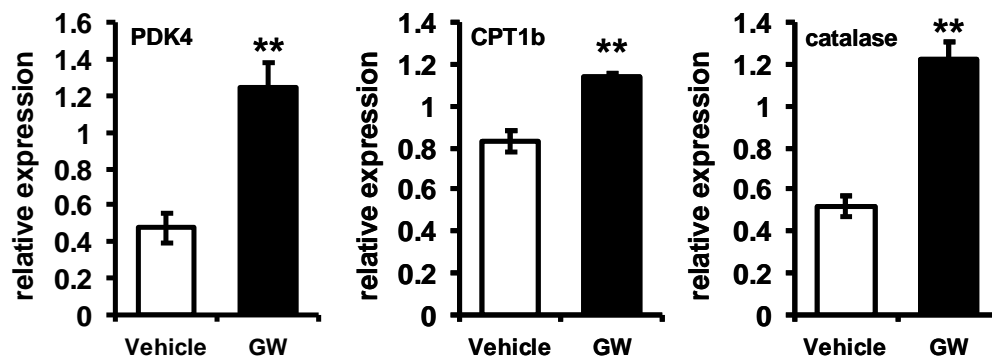
Figure 3.1: Oral administration of GW501516 induces expression of PPARδ target genes in quadriceps. After 9 days of GW501516 treatment, RNA was harvested from quadriceps, reverse transcribed and subjected to RT-QPCR (+GW n=10; Vehicle n=6).
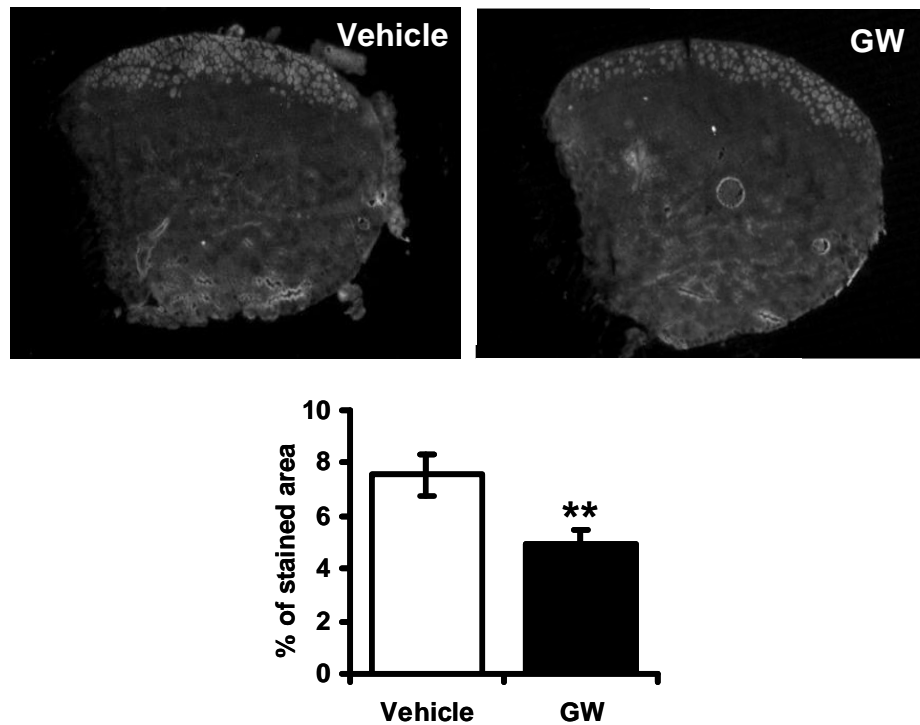
Figure 3.2: GW501516 treatment expedites restoration of fiber integrity after thermal injury. 8 week-old male C57BL6J animals were treated with GW501516 or vehicle only for 4 days prior to and 5 days after the injury (GW n=10; Vehicle n=6). 5 days after the injury, animals were injected intraperitoneally with Evans Blue dye. Proportions of stained area were calculated as percentage of stained to total cross sectional are of TA.
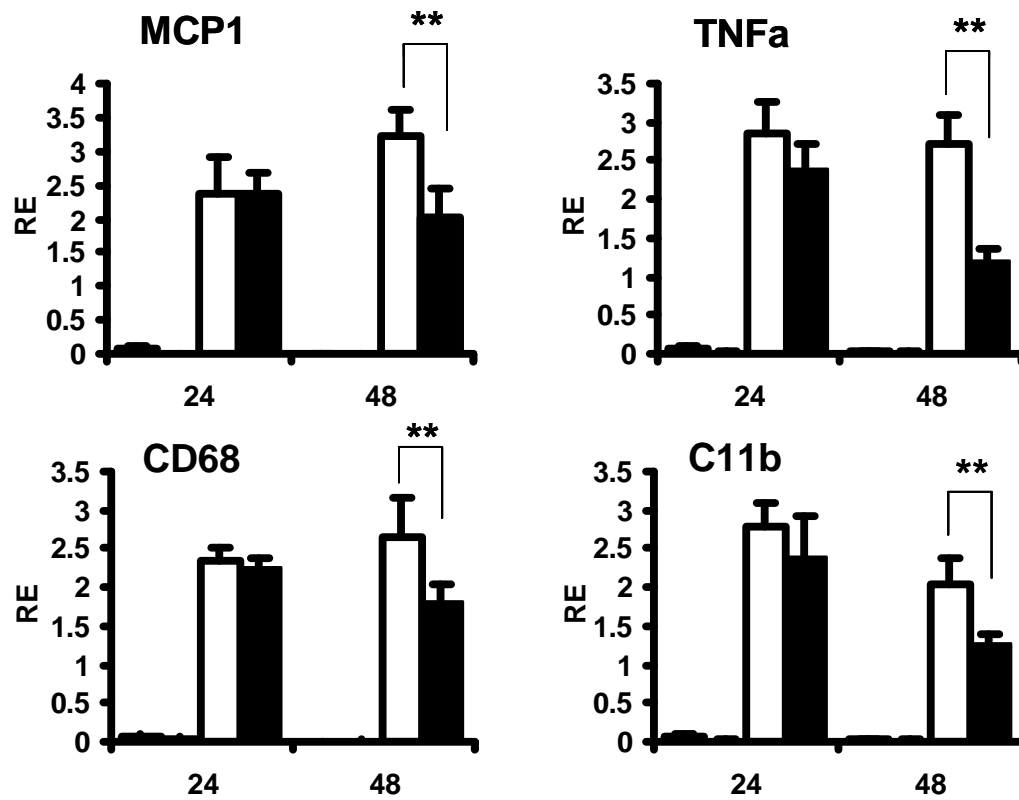
Figure 3.3: GW501516 treatment promotes resolution of inflammatory response. QPCR analysis for the expression of inflammatory markers, MCP1, TNFα, CD68 and CD11b at 24 and 48 hours after the injury.
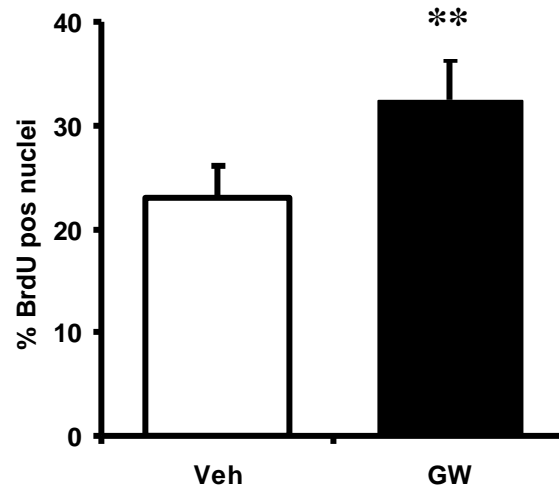
Figure 3.4: GW501516 treatment enhances proliferation of myoblasts after the injury. Proliferating cells were marked by incorporation of BrdU and weighted against total nuclei in a field. 2-3 random fields were chosen per animal, with n=4 for each genotype, p<0.05.
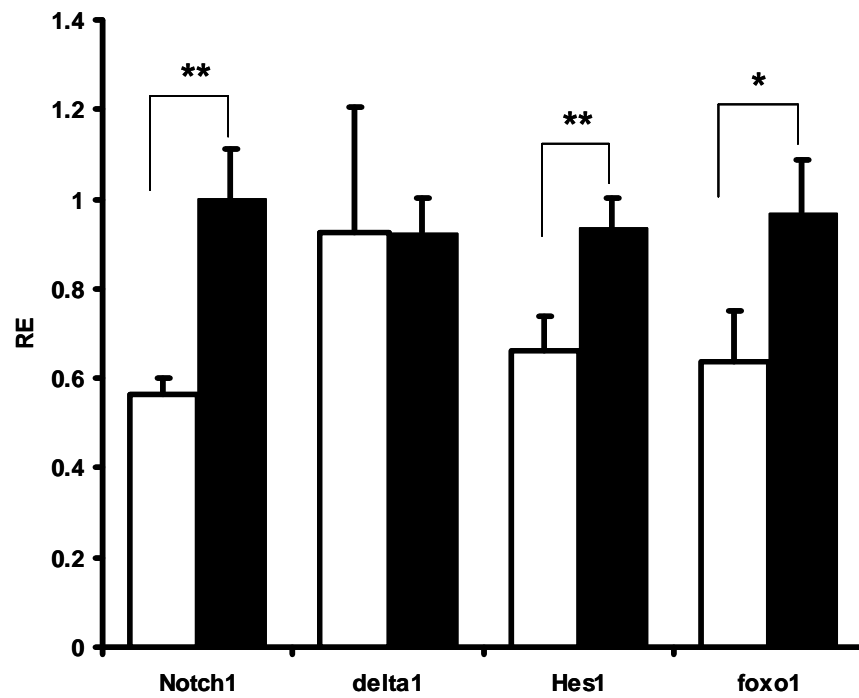
Figure 3.5: GW501516 treatment activates Notch1 signaling pathway. TA was harvested from C57BL6J animals treated with GW501516 for 9 days and processed for gene expression analysis by QPCR. White bar = vehicle treated; black bar = GW treated.

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Chapter 4:

Conclusions

In this study we report that both genetic and pharmacological activation of PPARδ accelerates the muscle regenerative process after an acute injury. PPARδ transgenic mice demonstrated both earlier resolution of the inflammatory response and earlier induction of myogenic markers, suggesting that PPARδ actuates a temporal shift of the regenerative process. In the uninjured muscle, we found that constitutive PPARδ activation lead to over 6 fold increase in the number of quiescent satellite cells. Consistently, we observed an increase in the number of proliferating cells after injury, and the accelerated formation of nascent regenerating fibers in PPARδ transgenic mice. Importantly, the regenerative benefits of genetic over-expression of constitutively active PPARδ was reproduced by acute administration of the PPARδ specific ligand, GW501516, which also promotes efficient restoration of fiber integrity, resolution of inflammatory response and proliferation of myoblasts after injury. Collectively, our findings allude to the therapeutic potential of PPARδ, to accelerate the recovery from acute muscle injury.

While regenerative advantage was achieved in both genetic and pharmacological models of PPARδ activation, it is important to acknowledge the inherent differences in the modes of receptor activation. In the genetic mouse model, the transgene is driven by human skeletal α-actin promoter, whose expression is limited to the mature myofiber. Moreover, endogenous skeletal α-actin protein is detectable at 9 d.p.c., and as such, it is possible that the ectopic expression of PPARδ may be exerting its effects during embryogenesis (Miniou, 1999). In the synthetic ligand model, the drug is ingested orally, thereby activating endogenous PPARδ in a

variety of tissues and cell types. Therefore, it is possible that these two models are achieving the same physiological output via different mechanisms.

Interestingly, both genetic and pharmacological activation of PPARδ seem to affect similar aspects of the regenerative program. Both models promoted resolution of inflammatory response. In the transgenic model, the effects originate from the muscle itself, possibly attributable to the increase in the production of myokine or other factors. Moreover, we have shown that PPARδ activation leads to adaptive changes in the vasculature, which is known to facilitate introduction of non-myogenic cells to the muscle and transport of soluble factors. While in the GW treated animals, the effects could be directly attributable to the PPARδ receptor activation on macrophages and other immune cells, as well as the muscle itself. Furthermore, PPARδ mediated increase in the quiescent satellite cell pool, which was not observed in the acute ligand model, may be attributable to the effects of the transgene during embryogenic satellite cell specification process. Proliferation kinetics of the satellite cells harvested from the transgenic animals was not determined. However, it is of interest to see if and when the transgene is turned on, and at which point how it may affect the cell properties, including proliferation and differentiation kinetics and self-renewal potential. We can not exclude the possibility that the transgene expression in the mature fibers may be inducing some soluble factors upon injury to promote myoblast proliferation. Conversely, because we did not observe an increase in the satellite cell pool due to the ligand treatment, increased myoblast proliferation is likely due to the direct drug effect on those cells.

Lastly, activation of Notch signaling pathway is of great interest. Notch signaling has been shown to control cell proliferation and fates in various tissues (Artvanis-Tsakonas, 1999; Fre, 2005; Blanpain, 2006). Specifically in the muscle, Notch signaling confers anti-myogenic properties, where abrogation of the signaling pathway during development and regeneration leads to premature differentiation (Schuster-Gossler, 2007; Vasyutina, 2007; Coboy 2002). In aging muscle, decline in regenerative potential is attributed to loss of Notch signaling (Conboy, 2003; Conboy, 2005). In light of our findings, PPARδ activation may be beneficial not only in expediting regenerative process in young adults but also in ameliorating aging induced decline in satellite cell function.

In this study, we have expanded our previous understanding of the role of PPARδ in muscle physiology. We have shown that PPARδ not only controls running endurance and metabolic parameters in the muscle, but also its regenerative program. PPARδ activation affects multiple facets of the regenerative program, exerting comprehensive but transient effects to expedite the progress. Our findings suggest that PPARδ should serve as a potential pharmacological target to enhance regenerative capacity of the muscle in injury and other degenerative conditions where satellite cell function is compromised. Subsequently, it is of great interest to test the efficacy of the drug in aging or disease models.

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