Chronic p53 activity leads to skeletal muscle atrophy and muscle stem cell perturbation

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Abstract

p53 tumor suppressor activity has been proposed to regulate the rate of ageing in part by suppressing postnatal stem cell numbers. Severe and rapid skeletal muscle atrophy is a hallmark of cachexia whereas muscle atrophy that occurs during aging (sarcopenia) has a slower rate of progression. Despite these differences, these two forms of muscle atrophy share many features although it remains unclear whether these processes are regulated by the same key regulatory factors. We demonstrated a requirement for p53 function in mediating severe and rapid cachexia induced by tumor load. Furthermore, the p53 target gene, PW1, activates p53 providing a potential positive feedback loop whereby a stress response is amplified in muscle cells. In the presence of TNF, a p53/PW1-dependent pathway mediates the block of myogenic differentiation in vitro and in vivo. To further characterize how p53 and PW1 mediate muscle atrophy, we analyzed a mouse model in which chronic p53 hyperactivation leads to early onset aging. We demonstrate that p53 hyperactivity is sufficient to induce muscle atrophy consistent with sarcopenia in ageing muscle. We observe that this process is accompanied by alterations in the distribution, but not in the number of muscle stem cells. Finally, we demonstrate that p53 upregulates PW1 expression in muscle in vitro and in vivo. Taken together, our data demonstrate that p53 and PW1 activities are required in promoting muscle atrophy induced by cytokines.

Key Words: Muscle Wasting Disease, muscle atrophy; skeletal muscle homeostasis; p53; PW1/Peg3; TNF, stem cells catabolism

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Skeletal muscle cachexia arises from an imbalance between protein synthesis and proteasome-mediated degradation [20,47]. Two muscle-specific E3 ubiquitin-protein ligases, atrogin-1/MAFbx and Murf are specifically expressed in skeletal muscle during atrophy [1,15]. Atrogin-1 expression is dependent upon the activation of the Foxo transcription factors [39], which also upregulate autophagocytosis in atrophying skeletal fibers [23]. Inflammatory cytokines, including TNF [3], are elevated in cachexia, and act as inhibitors of muscle regeneration [5] and differentiation in vitro [6,16,26,44] raising the hypothesis that deregulation of muscle stem cell function also contributes to muscle atrophy. Myogenic stem cells play a central role during postnatal growth as well as in muscle homeostasis and deregulation of myogenic stem cell function leads to growth failure and muscle atrophy [13,29,41]. Satellite cells are mitotically quiescent myogenic cells, which are activated in response to muscle damage or stress [27]. The paired homeobox gene, Pax7, is crucial for maintaining the satellite cell population during postnatal life and its expression in postnatal skeletal muscle is confined to the satellite cell compartment [41,30,31,56]. In addition to Pax7, we reported recently that satellite cells also express the p53 target gene, PW1 [29]. However, PW1 expression is also found in an additional population of postnatal cells, located in the muscle interstitium, which have myogenic potential in vitro [29,45]. Overexpression of a dominant-negative form of PW1 in myogenic cells results in

postnatal growth defects and muscle atrophy [29]. TNF inhibits myogenesis by activating p53-mediated apoptotic pathways in a PW1-dependent manner [6]. Several studies demonstrate a central role for PW1 in the p53-mediated cell death pathway: PW1 associates with two other p53 targets, SIAH-1 and bax and participate as a complex in mediating cell death [8,36]. In addition, PW1 participates in the TNF-NFκB signaling pathway through its direct association with TRAF2 [37]. p53 is a tumor suppressor gene which responds to a variety of stress signals, including DNA damage, resulting in cell cycle arrest, apoptosis, or senescence [50,51,52]. The role of p53 in muscle differentiation and homeostasis is less known although several studies reveal a role for p53 during skeletal muscle differentiation [33,43,46]. Myogenic cells permanently exit the cell cycle upon differentiation in a process that involves the upregulation of the cyclin-dependent kinase inhibitor p21 and dephosphorylation of retinoblastoma protein (pRb) [30]. p53 is activated and cooperates with MyoD during myogenic differentiation [46,54], whereas genotoxic stress suppresses the differentiation program in cell lines and primary myoblasts [18,19,34]. It is noteworthy that p53 is not essential for muscle development and regeneration: p53 -/- mice develop normal muscle [10] and show normal muscle regeneration upon injury [55]. TNF-mediated inhibition of muscle differentiation requires the p53 cell death effectors PW1, bax and caspases [6,28]. We have recently reported that p53 is required for the TNF-mediated inhibition of differentiation in vitro and that p53 and PW1 are involved in a regulatory loop that underlies postnatal stem cell number and mediates tumor-load mediated muscle atrophy [45]. In this study, we investigated a mutant mouse model for p53 in which an N-terminal truncated recombined p53 allele product (termed mt) interacts with wildtype p53 leading to increased p53 stability and chronic activation of p53 during the lifespan of these animals [14]. Although p53+/mt mice display an increased cancer resistance, they exhibit a 23% reduction in median longevity compared to the wt counterparts [48]. Furthermore, p53+/mt mice develop an early onset ageing phenotype including reduced body weight, lordokyphosis, osteoporosis, and a decreased tolerance to stress [48]. Here we demonstrate that overactivity of p53 alone (p53 +/mt) results in muscle atrophy in vivo, consistent with a pivotal role for p53 in muscle homeostasis. p53 is expressed in muscle stem cells and its expression level is critical for muscle stem cell number.

Our data reveal a novel role for p53 and its effector PW1 in mediating stem cell balance and muscle atrophy.

Materials and Methods

Cell culture procedure and differentiation

Primary myoblasts were isolated from 8-days old mouse hindlimbs and maintained in culture in high-serum medium (GM) as described previously [6,29,25]. For differentiation experiments cells were plated on collagen-coated cover slips and incubated overnight in growth medium. Myogenic differentiation was obtained by culture in differentiation medium (DM) for 3 days, as previously described [6,29].

Immunofluorescence of cultured cells

Cells grown on collagen coated coverslips were fixed in 4% paraformaldehyde. Cells were double stained with antibodies against Myosin Heavy Chain (MF20, Developmental Hybridoma Bank of the University of Iowa) and PW1 [35], followed by incubation with AlexaFluor568-conjugated anti-mouse and AlexaFluor488-conjugated anti-rabbit antibodies as previously described [6,45]. Nuclei were counterstained with DAPI. Photomicrographs were obtained using a Zeiss Axiophot microscope fitted with a SPORT RT Slider camera (Diagnostic Instruments).

Transmission electron microscopy

The Tibialis anteriors (TA) of 14-d-old pups were removed and immediately placed into 1x PBS, and fixed overnight in 2% glutaraldehyde solution at 4oC. Tissues were post-fixed as described earlier [29]. Ultrathin sections of 70-90 nm were collected onto copper grids and examined by electron microscopy (JOEL instruments).

NADH-staining and Immunolocalization on cryosections

Muscle cryosections of Tibialis anterior (TA) were stained for NADH-transferase as previously described [7]. Photomicrographs were taken with a Zeiss Axiophot microscope fitted with a SPORT RT Slider camera (Diagnostic Instruments). At least four random fields (corresponding to 300-500 cross-sectioned fibers) were photomicrographed for each muscle and all Type IIb fast fibers were analyzed using ImageJ, NIH. All data are expressed as mean +/- SEM; statistical analysis was performed by Student's t-test.

For immunofluorescence hindlimb muscles from 14-d-old postnatal pups were snap frozen in isopentane-cooled liquid nitrogen. Cryosections were post-fixed with 4% paraformaldehyde and immunostained for PW1 and Pax7 (Developmental Hybridoma Bank of the University of Iowa) and laminin (Sigma) as previously described [6,29,45]. The primary antibodies were detected by using Alexa-488-conjugated goat-anti-rabbit IgG (Molecular Probes), biotin-conjugated goat anti-mouse IfG1 specific followed by Cy3-conjugated streptavidin (Jackson Immunoresearch), and Cy5-conjugated goat anti-rabbit IgG (Jackson

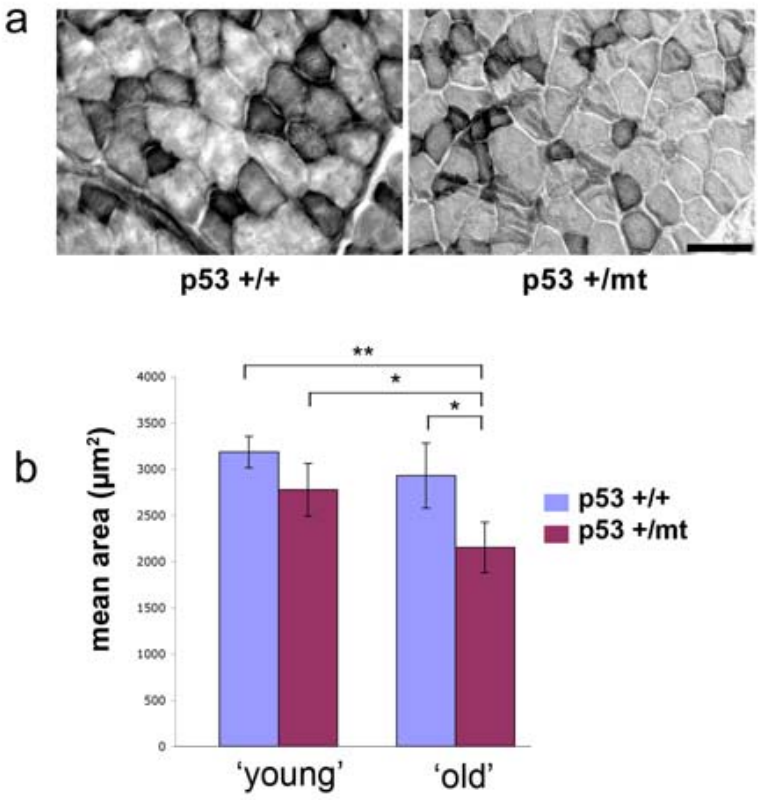

Fig. 1. *Chronic p53-activtation promotes skeletal muscle atrophy*
(a) Representative photomicrographs of cross-sections from the Tibialis anterior (TA) of old (18 – 22 months) wildtype (p53 +/+) and p53+/mt mice. Sections were stained for NADH activity to identify fast glycolytic (light staining) and slow (dark staining) fibers. Scale bar= 50 microns
*(b) Changes in fast glycolytic fiber size with age in 2 months (young) and 18-22 months old (old) wildtype (p53 +/+) and p53+/mt mice. "Young" p53+/mt mice show no statistically significant decrease of fiber diameter as compared to control young wildtype. In contrast, "Old" p53+/mt mice display a statistically significant reduction in fiber size as compared to control old wildtype as well as to the young wildtype and p53+/mt mice. The values are the mean +/- SD of 3-4 animals. * =p<0.05, **=p<0.01 by Student's t test.*

Immunoresearch). Nuclei were visualizes by DAPI staining. Photomicrographs were captured using a Leica TCS-SP (UV) confocal microscope at the MSSM-Microscopy Shared Resource Facility.

Results

Chronic p53-activation causes muscle atrophy in vivo

We demonstrated previously that p53 function is required for muscle atrophy progression in response to tumor load in vivo [45]. To determine whether chronic p53 activation is sufficient to induce muscle wasting, we examined the p53+/mt mouse which shows constitutively increased p53 activity [48]. Morphometric analyses of muscle fiber size in p53+/mt mice demonstrate a significant decrease in fiber size as compared to wildtype siblings (Figure 1). We note that the onset of this phenotype is gradual and becomes evident by 18 months of age (Figure 1). Despite the overt muscle atrophy of p53+/mt, we detect only low levels of atrogin-1 (data not shown) suggesting the involvement of other factors. We conclude that chronic activation of p53 leads to muscle fiber size reduction typical of aging.

p53 regulates myogenic stem cell number

We observe that ~10% of the p53+/mt mice show delayed and perturbed postnatal growth. Previous studies by us and others have demonstrated that muscle mass growth depends on the maintenance of muscle stem cell populations and that a failure in muscle growth resulting from defective stem cell behavior leads to a decrease in overall body size [13,29,41]. Expression of Pax7 and PW1 define postnatal muscle stem cells [6,29,45]. Therefore we examined the

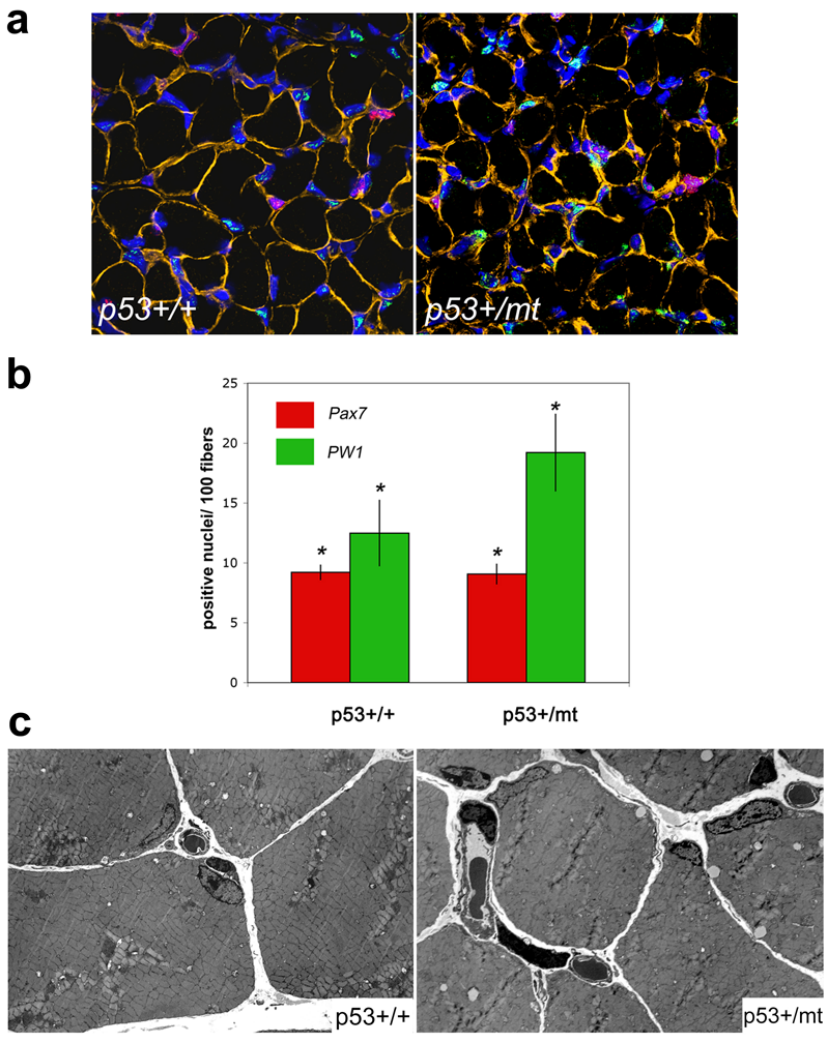

Fig. 2. *p53 regulates myogenic stem cell number*
(a) 14 days old hind limbs of wildtype (p53 +/+) and p53m/+ muscle sections processed for Dapi (blue) to visualize nuclei and stained using immunofluorescence for PW1 (green), Pax 7 (red) and laminin (orange). Images were captured using a confocal microscope at 63x. The p53m/+ shows an increase in PW1 positive cells while the number of Pax7 positive cells appears unchanged as compared to the wildetype.

expression of Pax7 and PW1 in hindlimb muscle sections obtained from p53+/mt and wt littermates mice at 2 weeks after birth. Sections were also stained for laminin and counts were normalized for muscle fiber number. We find that p53+/mt muscle does not show any changes in the number of Pax7-positive cells, however, we notice a marked increase in PW1 expressing cells in p53 +/mt muscle as compared to the wt littermates (Fig. 2a and b). Transmission electron microscopic analysis of the TA muscle obtained from 2 weeks old mice reveals an accumulation of cell clusters in the interstitial cell position in the p53+/mt muscle. Interstitial cells are more enriched in euchromatin and contain more cytoplasm and organelles than in the corresponding wt muscle (Fig 2c).

p53 regulates PW1 expression in differentiated myotubes

Primary myoblasts and most myogenic cell lines express PW1 [6,29,45,35]. We showed that PW1 expression is prematurely downregulated during postnatal muscle development in p53 mutant mice and that PW1 also activates p53 [45]. To further characterize PW1 regulation by p53, we isolated primary myoblasts from 8 days old wt, p53+/mt and p53-/- pups to analyze their myogenic differentiation capacity as well as the expression of PW1. p53+/mt myoblasts and p53-/- myoblasts are capable of myogenic differentiation in vitro (Fig. 3). Accordingly, p53+/mt as well as p53-/- mice develop normal differentiated fibers in vivo [45]. However, while wt

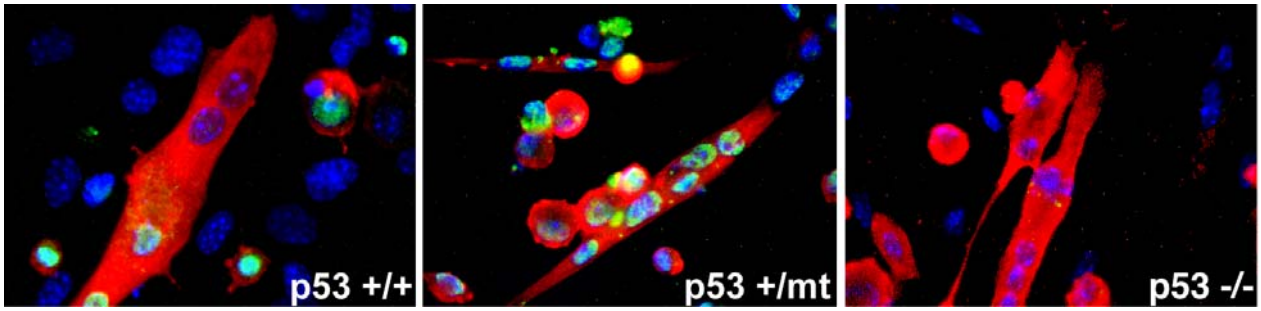

Fig. 3. *p53 regulates PW1 expression in differentiated primary cultures. Differentiated myoblasts from wildtype (p53 +/+), p53 +/mt and p53 -/- were immunostained for MHC as a marker for myogenic differentiation (red). Nuclei were visualized with DAPI (blue). PW1 expression (green) is modest in differentiated wt myotubes, absent in p53 -/- myotubes, whereas p53 +/+ myotubes maintain a high level of PW1 expression.*

myotubes show low levels of PW1 expression, we note a marked increase of PW1 expression in p53+/mt muscle (Fig. 3).

Discussion

Severe skeletal muscle atrophy is the hallmark of cachexia, which represents a primary cause of morbidity and mortality in chronically ill patients [17]. Cachexia involves multiple cellular mechanism, including increased muscle protein breakdown via up-regulation of muscle-specific ubiquitin E3-ligases as well as an induction of the autophagy pathway [39,23]). An additional contribution to cachexia can arise from impaired myogenic stem cell function (reviewed in [40]). TNF exposure triggers cachexia and stimulates the expression of atrogin-1 [5]. In addition, we and others have shown that TNF exposure inhibits myogenic differentiation in vitro and in vivo via an activation of p53 effectors including PW1, bax, and caspases [5,6]. Similarly, increased levels of inflammatory cytokines occur during aging concomitant with the onset of muscle fiber atrophy and reduction of satellite cell myogenic potential [38,9,32,4].

Recent studies suggest a fundamental role for p53 in organismal senescence. Several mouse models that display chronic p53-activation or chronic cell stress pathway activation display premature ageing associated with pronounced tissue atrophy [48,24,49]. In particular, p53+/mt mice are resistant to cancer but exhibit an accelerated ageing and a shortened lifespan [48]. p53+/mt mice initially have ample functional stem cell reserves to maintain organ homeostasis, but with age the stem cell reserve decline more rapidly as compared to wt mice [14,11]. Indeed, p53+/mt hematopoietic stem cells are reduced in number, even though they exhibit similar proliferative capacity as compared to wildtype [12]. Therefore, increased p53 activity inhibits stem cell and/or progenitor cell proliferation and/or differentiation. Our observations that p53 deficient mice are resistant to cachexia [45] whereas p53+/mt mice show spontaneous muscle atrophy provide genetic evidence that p53 is a key mediator of muscle wasting and homeostasis in vivo.

Several studies suggest that p53 activity plays a role in regulating muscle homeostasis. For instance, p53 is up-regulated in immobilized muscle [21], during unloading-induced muscle atrophy [42] , and in ageing skeletal muscle [2]. Specifically, the p53/p21 pathway is involved in age-associated loss of satellite cell proliferative capacity [22]. We have reported a decrease in satellite cell number in p53 deficient mice [45], however satellite cell number is not affected in p53+/mt mice, indicating that minimal p53 activity is necessary to sustain satellite cells but satellite cell number is not affected by gene dosage. In contrast, we see an increase in PW1 expressing cells in p53+/mt mice. PW1 is expressed in both satellite cells and a subpopulation of interstitial cells with myogenic potential [28]. p53 and PW1 are co-expressed in primary myoblasts and in regenerating myofibers and myogenic stem cells in vivo [45]. PW1 and p53 are downregulated upon differentiation, but chronic activation of p53, as in the p53+/mt primary cultures, leads to an increased expression of PW1 in differentiated myotubes in vitro, confirming the positive feedback loop of p53 and PW1 [45]. In this study, we report that PW1 expression is increased in response to chronic p53-activation: in muscle stem cells PW1 may inhibit cell expansion and/or maintenance as well as their capacity to properly contribute to the myogenic program. This would explain the atrophic phenotype that we observe in the p53+/mt muscle. We conclude that p53 has a pivotal role in skeletal muscle homeostasis and that p53-mediated upregulation of PW1 expression in muscle underlies an imbalance of myogenic stem cell number or function which leads to muscle atrophy.

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