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Embryonic deregulation of muscle stress signaling pathways leads to altered postnatal stem cell behavior and a failure in postnatal muscle growth

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Abstract

PW1 is a mediator of p53 and TNFα signaling pathways previously identified in a screen to isolate muscle stem cell regulators. We generated transgenic mice carrying a C-terminal deleted form of PW1 (ΔPW1) which blocks p53-mediated cell death and TNFα-mediated NFκB activation fused to the myogenin promoter. Embryonic/fetal muscle development appears normal during transgene expression, however, postnatal transgenic pups display severe phenotypes including runtism, reduced muscle mass and fiber diameters resembling atrophy. Atrogin-1, a marker of skeletal muscle atrophy, is expressed postnatally in transgenic mice. Electron microscopic analyses of transgenic muscle reveal a marked decrease in quiescent muscle satellite cells suggesting a deregulation of postnatal stem cells. Furthermore, transgenic primary myoblasts show a resistance to the effects of TNFα upon differentiation. Taken together, our data support a role for PW1 and related stress pathways in mediating skeletal muscle stem cell behavior which in turn is critical for postnatal muscle growth and homeostasis. In addition, these data reveal that postnatal stem cell behavior is likely specified during early muscle development.

Keywords: Satellite cells; Stem cells; Skeletal muscle; Development; Mouse; PW1/Peg3; p53; TNF; Atrophy

Introduction

Vertebrate trunk and limb musculature originates from founder cells that become committed to the skeletal muscle lineage in the somites (Cinnamon et al., 1999; Ordahl and Le Douarin, 1992). Successive waves of myogenesis occur during fetal and postnatal development leading to the formation of adult muscle fibers (Stockdale, 1992). While the origin of embryonic myoblasts has been well defined (Gross et al., 2000; Tajbakhsh and Cossu, 1997; Tajbakhsh et al., 1996, 1997), the origin(s) of secondary (fetal) myoblasts and satellites cells (postnatal) remains unclear and may have non-somitic origin. Genetic data reveal that Pax7 plays a critical role in the establishment and/or maintenance of satellite cells. Specifically, Pax7 mutant mice are born with normal musculature but display severely compromised postnatal growth and lack muscle satellite cells (Seale et al., 2000). Curiously, these mice show a compensatory increase in the number of hematopoietic stem cells residing in the skeletal muscle tissue (Seale et al., 2000). While these data reveal that Pax7 is required for the establishment and/or maintenance of satellite cells, it remains unclear if satellite cells are derived from a resident population of pluripotent stem cells in postnatal muscle or if they are derived from the initial population of embryonic and/or fetal myoblasts. While regeneration of adult skeletal muscle recapitulates many aspects of embryonic/fetal myogenesis, results from MyoD-deficient mice reveal a specific role for MyoD during regeneration (Kablar et al., 1997; White et al., 2000) suggesting that the postnatal myogenic

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program is distinct from the embryonic/fetal program (Parker et al., 2003; Sabourin and Rudnicki, 2000, for review). Stimuli that lead to recruitment of muscle stem cells include mechanical damage, denervation and inflammatory cytokines all of which are normally part of postnatal life and not prenatal development (Darr and Schultz, 1987; Fong et al., 1989; Hill et al., 2003; Li, 2003; Musaro et al., 2004; Rodrigues Ade et al., 2002).

In an effort to identify genes expressed during early mesoderm-to-skeletal muscle commitment, we used a differential screening approach which resulted in the isolation of a gene called PW1/Peg3 (Relaix et al., 1996). PW1 encodes for a large protein (~210 kD) containing 12 zinc fingers and multiple proline-rich repeat motifs. The pattern of PW1 expression during mouse development is complex, however expression initiates in the primitive streak (mesoderm) and is subsequently downregulated in mesodermally derived tissues as they differentiate with the exception of skeletal muscle which maintains moderate levels perinatally which later decline in the adult (Relaix et al., 1996). Levels of PW1 expression are high in most myogenic cell lines as well as in primary myoblasts obtained from postnatal muscle (Coletti et al., 2002; Relaix et al., 1996). Subsequent studies have revealed that PW1 participates in the TNFα–NFκB signaling cascade through direct association with TRAF2 (Relaix et al., 1998). In addition, PW1 has been identified as a p53-induced gene in fibroblasts induced to undergo cell death (Relaix et al., 2000). In both these contexts, a C-terminal deleted form of PW1 (ΔPW1) is a potent blocker of TNFα-mediated NFκB activation and p53-mediated cell death (Relaix et al., 1998, 2000). TNFα is one of several potent inflammatory cytokines that is responsible for muscle wasting (cachexia) (Argiles and Lopez-Soriano, 1999; Fong et al., 1989; Tracey et al., 1988) and is also capable of blocking terminal differentiation of myogenic cells in vitro (Coletti et al., 2002). We have recently demonstrated that PW1 is required to mediate the TNFα block of differentiation in myoblasts through the recruitment of p53 downstream effectors including bax and caspase 9 (Coletti et al., 2002). A role for PW1 in muscle stem cells in vivo comes from a recent study in which PW1 expression was found to be almost absent in MyoD mutant myoblasts compared to the high levels found in wildtype myoblasts (Seale et al., 2004a). As these myoblasts are deficient in participating in normal muscle regeneration, these data suggest that PW1 may participate in a regulatory process controlling stem cell maintenance and stress responses.

In an effort to determine the in vivo function of PW1 as well as the potential role of PW1-mediated pathways during muscle development, we generated transgenic mice carrying ΔPW1 under the control of the myogenin promoter (Myo1565) (Cheng et al., 1992; Edmondson et al., 1992). We show that a transient expression of ΔPW1 during embryonic development does not interfere with embryonic and fetal muscle development, however transgenic mice display a profound failure in postnatal growth similar to the phenotype seen in Pax7 mutant mice (Seale et al., 2000). In contrast to Pax7 mutant mice which show either an absence or a marked decrease in satellite cells (Oustanina et al., 2004; Seale et al., 2000), we observe that satellite cells are formed in transgenic mice but they are not quiescent. We show that satellite cells and differentiating myoblasts are found in clusters with PW1 expressing cells found outside of the basal lamina suggesting a common origin for these stem cells. Furthermore, transgenic muscle shows aberrant high levels of Atrogin-1 expression consistent with the atrophic muscle phenotype observed. Whereas wildtype primary cultures of myoblasts are inhibited to differentiate in the presence of TNFα, primary transgenic myoblasts are resistant to exogenous TNFα suggesting that the transgene induces a permanent change in stem cell behavior. Taken together, this study demonstrates that PW1-mediated cell stress response pathways are critical for early postnatal stem cell behavior and postnatal muscle growth and homeostasis. Our data also indicate that the postnatal muscle stem cell population is likely specified during somitogenesis.

Materials and methods

Transgenic construct

Transgenic mice carrying a myogenin promoter fragment-ΔPW1-IRESLacZ fusion gene were generated by pronuclear injection. The ΔPW1 sequence corresponding to the N-terminal portion of the PW1 protein (aa residues 1 to 592) (Relaix et al., 1998, 2000) was cloned under control of a regulatory sequence derived from nucleotides −1565 to +18 (relative to the transcription start site) of the mouse myogenin promoter (Myo1565) (Cheng et al., 1992; Edmondson et al., 1992) (gift from Dr. E.N. Olson, University of Texas, Houston). Additional sequences were added to optimize expression in vivo (Fig. 1) including the rabbit β-globin intron linked to the IRES-LacZ-SV40 polyadenylation signal sequence (from plasmid 601DW58, gift from Dr. T. Lufkin, Mount Sinai School of Medicine, New York) which allows for efficient translation of β-galactosidase concomitant with transgene expression (Li et al., 1997). A GTI-2 promoter linked to the neomycin gene and a SV40 polyadenylation signal were also cloned downstream of the entire construct for potential selection (a gift from Dr. T. Lufkin, Mount Sinai School of Medicine, New York).

Transfection procedures, cell culture and TNFα treatment

In vitro analysis of transgene expression was performed by transfection of the C2C12 myogenic line with the construct or the control backbone (without ΔPW1) using FuGene 6 reagent (Roche Molecular Biochemicals-Boehringer Mannheim, Indianapolis, IN) according to the

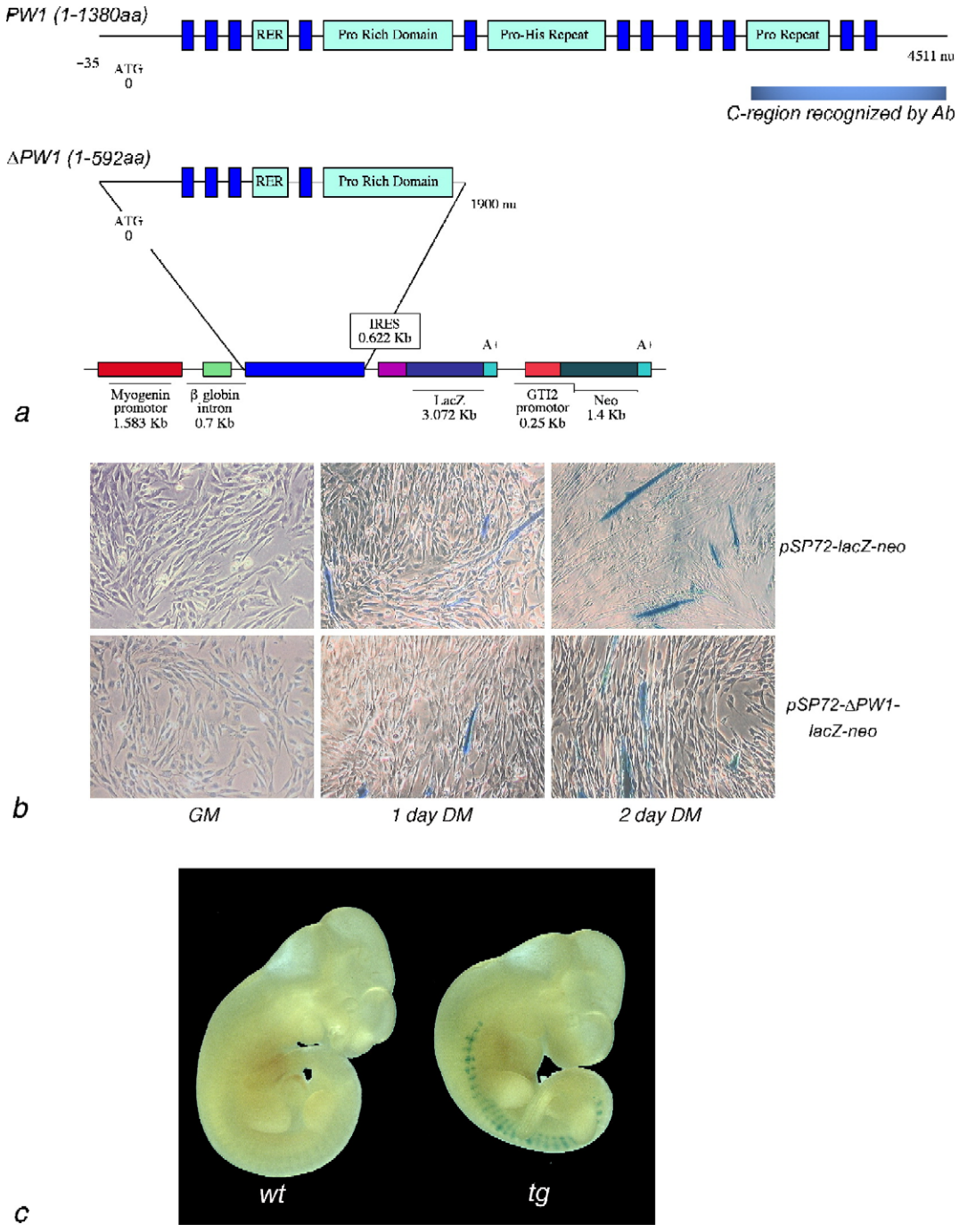

Fig. 1. Expression of the myog-ΔPW1 transgene in vitro and in vivo. (a) Diagram of full length coding portion of the PW1 cDNA and ΔPW1 cDNA. The zinc fingers are represented as dark blue boxes, the RER and proline-rich sequences are indicated as light blue boxes. Schematic diagram of the transgenic construct (distances are not to scale) in which nt −35 to +1900 (aa 1–592) was placed downstream of the myogenin promoter (myo 1565) in order to obtain muscle-specific expression. A β-globin intron region and an IRES-LacZ-polyA sequence, β-galactosidase (LacZ) and additional sequences were placed into the transgene as described in the Materials and methods. (b) C2C12 cells were transfected with myog-ΔPW1-LacZ construct or myog-LacZ and β-galactosidase activity was measured under growth (GM) and differentiation (DM) conditions. As expected, transgene expression was detected by 1 day in DM and increased by 2 days. We did not see any obvious difference between levels and numbers of cells that expressed ΔPW1 (myog-ΔPW1-LacZ) or the construct carrying the IRES *lac*Z alone (or myog-LacZ) suggesting that ΔPW1 expression has no obvious deleterious effect under these conditions. (c) Wildtype (wt) and transgenic (tg) embryos at embryonic day 10.5 (ED10.5). β-galactosidase positive cells were detected in the somite, however the number of cells was lower than expected and declined steadily during myotome differentiation. We note that we either observed strong muscle-specific staining or a very weak specific staining in transgenic embyros.

manufacturer's guidelines. C2C12 cells were grown and differentiated as previously described (Coletti et al., 2002). The β-galactosidase activity was determined as described below.

Primary myoblasts were isolated as previously described (Coletti et al., 2002), from fore- and hindlimb muscles of 6 days postnatal founder pups. Primary cultures were maintained on collagen-coated dishes in Ham's F10 (Invitrogen, Carlsbad, CA) supplemented with 20% FCS, 200 U/ml penicillin and 200 mg/ml streptomycin (GM). Fresh bFGF (Boehringer) (2.5 ng/ml) was added every other day. All experiments were done using cultures passaged less than 10 times. For immunohistochemistry, cells were replicate plated onto 6 different collagen-coated coverslips at the concentration of 5000 cells/500 μl for wt and 2500 cells/500 μl for transgenic primary myoblasts. Primary myoblasts were differentiated in DMEM supplemented with 5% horse serum and antibiotics (DM).

Cells were treated with 50 ng/ml of recombinant mouse TNFα (Roche Molecular Biochemicals-Boehringer Mannheim, Indianapolis, IN) and analyzed after 3 days in DM.

Myosin was detected using the MF20 antibody (Coletti et al., 2002). Quantitative analysis of differentiation was determined by counting the number of nuclei in MF20-positive cells and expressing this as a percentile of the total number of nuclei from at least 5 fields per experiment (>50 nuclei/field). Each experiment was performed in triplicate on 3 independent primary cultures obtained from three different transgenic pups showing a phenotype at 6 days after birth.

Generation of transgenic mice and genotype

Transgenic mice were generated by standard pronuclear injection procedures using the Mouse Genetics Shared Research Facility at the Mount Sinai School of Medicine, New York. The transgene DNA construct (Fig. 1) was released from the plasmid by digestion with *Sal*I. The 10 Kb fragment was gel purified using a Qiagen agarose gel extraction kit according to the manufacturer's instructions (Qiagen, Valencia, CA), eluted with the injection buffer (10mM Tris, pH 7.4, 0.2mM EDTA) and filtered twice through a 0.22 μm filter (Millex-GV4, Millipore). The DNA was then injected at a final concentration of 2 ng/μl in the pronuclei of fertilized F2 (CBAxC57/Bl6) mouse eggs according to the protocol of Hogan et al. (1994). Injected embryos were reimplanted into the oviducts of pseudopregnant Swiss Webster females and assigned a gestational age of 0.5 days. Transgenic animals were identified by Southern blot of genomic DNA extracted from yolk sac of transient embryos or from tail-biopsies of the pups. 10 μg of genomic DNA was digested overnight at 37°C in a 50 μl solution with the restriction enzyme *Bam*HI and the appropriate buffer from New England Biolabs, Beverly, MA. DNA was transferred to a nylon membrane Hybond-N+(Amersham, Piscataway, NJ) and hybridized with a random-primed ^{32}PdCTP-labeled LacZ *Bam*HI fragment.

Detection of β-galactosidase activity

Transgenic and wildtype embryos or hindlimb muscles were retrieved in ice-cold 1× PBS and fixed for 5–10 min at room temperature in a formaldehyde–glutaraldehyde solution (2% formaldehyde, 0.2% glutaraldehyde, 0.02% Nonidet P-40 (NP-40) and 0.01% sodium deoxycholate in PBS). For determination of the β-galactosidase activity on C2C12 cells, the medium was gently removed and the cells were washed in PBS at room temperature before fixation for 2 min. After fixation, tissues and cells were rinsed briefly and stained using procedures described previously (Degenhardt et al., 2002; Rentschler et al., 2001).

Histology, electron microscopy and immunofluorescence

For analysis by TEM (transmission electron microscopy), hindlimb muscles were removed, immediately placed into cold 1× PBS and fixed overnight in 2% glutaraldehyde solution at 4°C. Tissues were post-fixed in osmium tetroxide and embedded in epon resin. Semithin sections (1–2 μm thickness) were collected onto subbed slides and stained with Toluidine Blue for standard light microscopic analysis. Ultrathin sections of 70–90 nm were collected onto copper grids and examined by electron microscopy (JOEL instruments). A minimum of 10 different fields from at least 3 sections were analyzed from 3 different wildtype and transgenic 6 days postnatal hindlimbs. We then counted all the nuclei in each field and classified them according to the categories of myonuclei, sublamina non-myonuclei (presumably satellite cells) and interstitial cells. We did not include vascular cells in these counts. We then normalized our counts to the total number of fibers in each field and present the data as a percentage or proportion of this population. The classification of 'activated' versus quiescent satellite cell was made based upon criteria outlined previously (Mauro, 1970). In brief, quiescent satellite cells are sublamina but are separated from the myofiber by a distinct plasma membrane, have condensed heterochromatic nuclei, few to no extensions and little cytoplasm with few organelles. If a sublaminal cell did not have at least 2 of these characteristics, we classified the cell as activated.

Hindlimb muscles from 6 days pups were fixed overnight in freshly prepared 4% paraformaldehyde in 1× PBS and embedded in paraffin following procedures described elsewhere (Sassoon and Rosenthal, 1993). Cross-sections of 8 μm were prepared using a Leica RM2125 microtome at the midanterior–posterior level of the hindlimb, collected on gelatin subbed slides and stained with hematoxylin and eosin.

Fore and hindlimb muscles from 6 days postnatal pups were snap frozen in isopentane cooled liquid nitrogen. Five

to seven micron sections were collected with a Bright Instruments Cryostat (Bright, UK) onto gelatin subbed slides, fixed in 4% paraformaldehyde and permeabilized in methanol (6 min at −20°C). A citric acid antigen retrieval protocol was used to facilitate Pax7 immunostaining. Slides were heated in a 90°C solution of 0.01 M citric acid (pH 6) two times for 5 min in a microwave oven, followed by cooling in the same solution for 10 min. After washing with PBS, sections were blocked in 2% BSA in PBS (block). Anti-PW1 antibody polysera (Coletti et al., 2002; Relaix et al., 1996) was diluted 1/3000 in block. Anti-pax7 monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa) and anti-laminin polysera (Sigma, St. Louis MO) were diluted 1/20 and 1/50 respectively in Roche Blocking reagent (cat 1096176 Roche Molecular Biochemicals-Boehringer Mannheim, Indianapolis, IN). Antibody binding was visualized by using goat anti-rabbit Alexa 488 and goat anti-mouse IgG1 Alexa 568 (Molecular probes) and CY5-conjugated goat anti-rabbit IgG (Jackson Lab). The slides were mounted and observed with a Leica TCS-SP(UV) confocal microscope at the MSSM-Microscopy Shared Resource Facility.

Northern blotting

For Northern blot analysis, total RNA was isolated from hindlimb muscles (ages indicated on the blot) by using TRIzol (Life Technologies, Grand Island, NY) reagent, following the manufacturer's instructions. 10 μg of total RNA samples was separated by electrophoresis on a denaturing 1.2% agarose/1× MOPS/6% formaldehyde gel, transferred to a nylon membrane Hybond-N+(Amersham, Piscataway, NJ) and UV cross-linked. Northern blots were hybridized with 6×10^6 d.p.m./ml of a random-primed ^{32}P-dCTP-labeled gel-purified *Kpn*I/*Xba*I fragment of the Atrogin-1 coding sequence (bp 1500–2368) (Gomes et al., 2001). Blots were stripped and rehybridized with the total GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA as standard control of RNA loading. Prehybridization for 2 h and an overnight hybridization were performed at 42°C in Hybrisol I (Chemicon International, Temecula, CA) solution containing 50% formamide, 6 × SSC. Washes were done twice at 42°C in 2 × SSC/0.1% SDS for 5 min, one time in 0.2 × SSC/0.1% SDS for 15 min and in 0.1 × SSC/0.1% SDS for 15 min at 60°C. Northern blots were then exposed at −20°C in the presence of a Biomax intensifying screen (Kodak, New Haven, CT).

Results

Transgenic construct

PW1 encodes a large protein (1380 aa residues) containing 12 Krüpple-like zinc fingers organized into three clusters (Relaix et al., 1996 and Fig. 1). The N-terminal portion of PW1 (spanning residues 1–592) contains an arginine-rich motif (RER) and a proline-rich domain as well as a cluster of 3 zinc fingers and one isolated zinc finger (Fig. 1). Whereas PW1 can activate NFκB and mediate p53-mediated cell death, ΔPW1 blocks these pathways (Relaix et al., 1998, 2000). In order to generate transgenic mice expressing ΔPW1 directed specifically to the muscle lineage, we placed a cDNA encoding residues 1–592 of PW1 in an expression vector carrying the myogenin promoter (Myo1565; Cheng et al., 1992) as shown in Fig. 1. We chose the myogenin promoter since PW1 and myogenin are both expressed in the early myogenic compartments of the embryo (Cheng et al., 1992; Relaix et al., 1996) and the specific regulatory element used had been shown to drive MRF4 transgene expression in a similar setting (Zhu and Miller, 1997). In order to follow expression of the transgene, we cloned the β-galactosidase gene downstream of an internal ribosome entry sequence (IRES) derived from the encephalomyocarditis virus to generate a bicistronic mRNA encoding both cDNAs. This system has been shown to allow an efficient translation of the β-galactosidase gene concomitant to the transgene expression (Li et al., 1997).

ΔPW1 expression does not affect myogenesis in vitro

We transiently transfected C2C12 myoblasts with myogenin-ΔPW1 and compared with cells transfected with the empty vector (no ΔPW1) driving IRES *lac*Z under the same regulatory elements. As expected, both constructs do not show detectable β-galactosidase expression in proliferating myoblasts maintained in growth conditions (GM), however 1 and 2 days after cells are induced to differentiate (DM), blue myotubes are easily detected with both constructs (Fig. 1). No obvious difference was seen in the differentiation capacity of myoblasts expressing ΔPW1 as compared to the empty vector suggesting that ectopic expression of ΔPW1 does not interfere with normal myogenesis. Similar results have been obtained when ΔPW1 is driven by the CMV promoter (data not shown).

ΔPW1 is transiently expressed during the embryonic myogenic program

To verify proper expression of myogenin-ΔPW1 in vivo and to test whether embryonic transgene expression results in overt muscle phenotypes, we generated founder transgenic embryos by pronuclear injection and the expression pattern of the transgene was followed by detection of β-galactosidase activity. At embryonic day 10.5 (ED10.5), β-galactosidase expressing cells were readily detected in the somite (Fig. 1). We observed however that β-galactosidase activity declined during myotome differentiation (Fig. 1) and at later stages, only weak expression could be detected in pre-muscle masses

in the developing limbs and other processes (data not shown). While the mechanism underlying the loss of transgene expression at later stages of development is unclear, we note that no overt malformation of muscle masses could be observed in these founder embryos. Histological analyses of the stained cells in the somite region showed no obvious abnormalities (data not shown) although we were unable to follow the fate of the cells expressing the transgene. A similar transient transgene expression pattern has been reported in a study in which MRF4 was placed under the control of the myogenin promoter (Zhu and Miller, 1997).

Postnatal growth is compromised in ΔPW1 transgenic mice

Since myogenin-ΔPW1 founder embryos appeared normal, we set out to generate stable transgenic lines using our transgene construct. We noted that few transgenic mice survived past weaning suggesting that transgene expression results in perinatal lethality. We thus genotyped pups soon after birth and found that founder transgenic pups were present in higher numbers at birth and failed to survive past 2–3 weeks with the peak of mortality around 1 week after birth. In contrast, few non-transgenic pups were lost after birth. Furthermore, as each transgenic pup results from an independent pronuclear injection and thus independent integration events, we conclude that the perinatal lethality is due solely to transgene expression. As we could obtain transgenic founders with relatively high frequency, we investigated the effects of the transgene further by setting up multiple rounds of pronuclear injections and analyzing early postnatal stages. This approach was feasible since we could obtain transgenic pups with over 30% efficiency (20/65) of which 50% of the transgenic pups (10/20) showed a profound postnatal phenotype (Fig. 2). Specifically, we note that by 5–7 days after birth, transgenic mice fail to show significant body growth and are only 60% as large as their wildtype siblings (Fig. 2). We note that transgenic pups undergo modest growth, are able to feed and grow body hair although their movements are spastic and uncoordinated. Only a small number of transgenic mice survive past 1 week, however the surviving transgenic pups continue to grow but do not catch up in size and ultimately die.

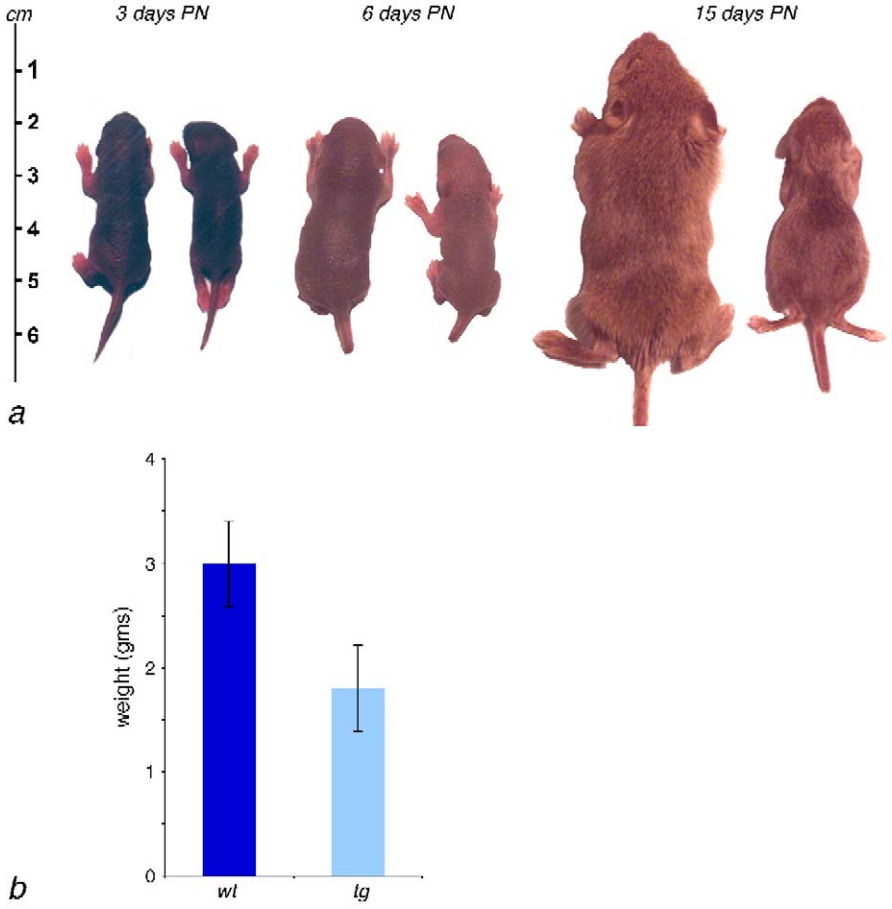

Fig. 2. Postnatal growth defect of the transgenic mice and penetrance of the phenotype. (a) 3 days, 6 days and 15 days postnatal wildtype (left) and transgenic (right) pups showing postnatal growth deficit as compared to wildtype littermates. (b) Statistical analyses of 6 days postnatal pups ($n = 3$) show a significant difference ($P < 0.1$) in body weights. Note that pups do grow and develop hair and other characteristics typical of postnatal maturation.

Postnatal muscle organization is disorganized in transgenic mice

We performed a more detailed analysis of hindlimb muscles at 6 days after birth using founder analysis to assure an adequate number of transgenic pups for our study. At 6 days postnatal, transgenic pups nurse adequately and still appear healthy although they begin to show a clear difference in body size (Fig. 2). Cross-sections at the midlevel of the hindlimbs revealed that, while overall bone diameters were normal, the muscle masses were notably smaller (Fig. 3). We note that all muscle groups are present and correctly organized although the soleus appeared less affected as compared to the tibialis (Fig. 3). Counts of fiber number revealed no significant change (data not shown) however measurements of fiber size (area) revealed a 60% decrease as compared to wildtype siblings (Fig. 3). A similar decrease in fiber size was also observed in other muscles including dorsal musculature and the intercostal muscles as well as a severe reduction of the diaphragm (data not shown) which may ultimately account for the postnatal mortality. We noted that cells in a satellite cell position (closely associated with myofibers) with condensed heterochromatic nuclei could be easily detected in wildtype muscles (Fig. 3). Cells in a similar anatomical location ('satellite') could be readily observed in the transgenic muscle samples although the nuclei were enlarged and euchromatic (Fig. 3). As satellite cells can only be identified rigorously based upon specific morphological criteria which require higher resolution than possible in our semithin plastic sections (Mauro, 1961; Oustanina et al., 2004; Seale et al., 2000), we used electron microscopy to examine sublaminar nuclei in wildtype and transgenic 6 days postnatal hindlimb muscles (Fig. 4). At this early postnatal stage, satellite cells are more numerous as compared to later postnatal stages (Oustanina et al., 2004) presumably reflecting perinatal secondary or tertiary myofiber formation and maturation including the addition of more myonuclei which also occurs under the basal lamina (McLennan, 1983; Ontell, 1986; Ontell and Kozeka, 1984a,b; Ontell et al., 1982, 1988; Zhang and McLennan, 1995, 1998). Nonetheless, we can detect morphologically normal satellite cells in wildtype 6 days postnatal muscle (Fig. 4). We therefore counted the number of cells in the satellite cell position and

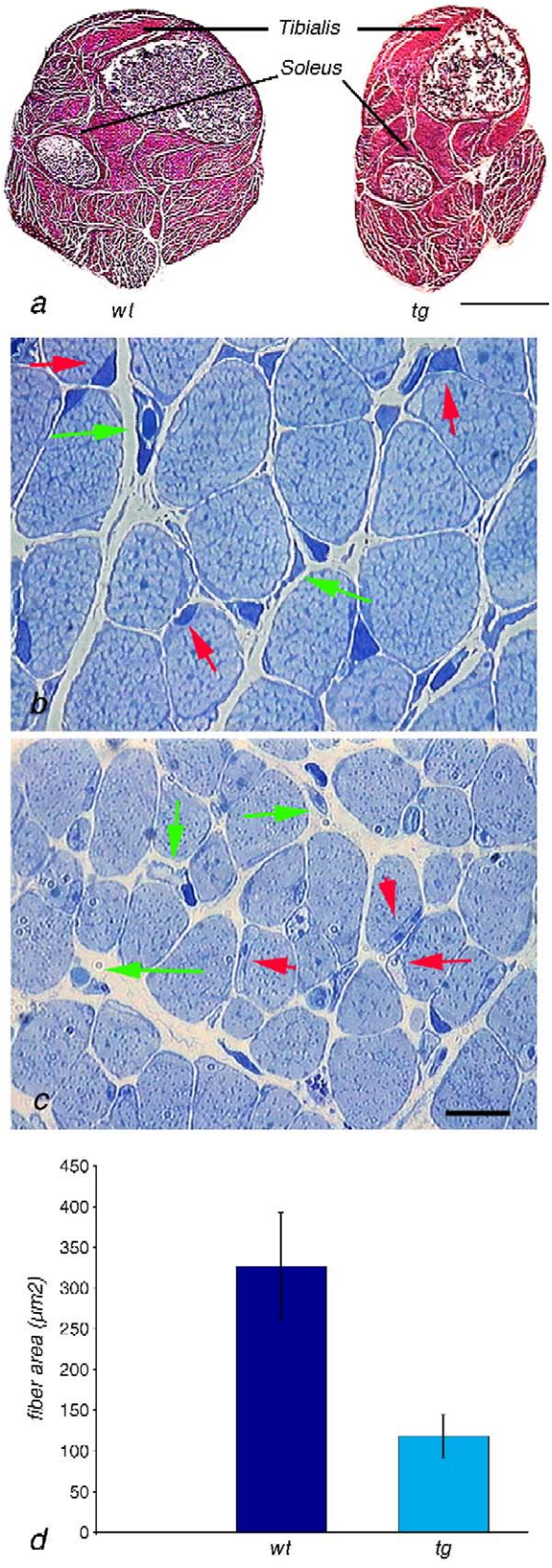

Fig. 3. Skeletal muscle fibers are smaller and possess a decreased number of quiescent satellite cells. (a) Hematoxylin–eosin stained cross-sections of hindlimbs at the midanterior–posterior level of 6 days postnatal pups (scale bar = 3 mm). All muscle groups are formed and correctly organized; however muscle masses show a reduced size. The soleus appeared less affected as compared to the tibialis. (b) Semithin plastic sections of 6 days postnatal hindlimb cross-section of a wildtype (b) and transgenic (c) pup at the midanterior–posterior level (scale bar = 10 μm). Presumptive satellite cells located peripherally to the myofiber with condensed heterochromatic nuclei are easily detected in wildtype muscles (red arrows, b), while numerous cells in a satellite cell position with euchromatic nuclei are observed in the transgenic muscles (red arrows, c). Green arrows in panels b and c indicate interstitial cells. (d) Measurements of the muscle fiber area reveal a 60% decrease in the mean fiber area of transgenic (tg) muscles as compared to wildtype (wt) siblings at 6 days postnatal ($P < 0.1$).

normalized it to the number of muscle fibers from 3 wildtype and 3 transgenic gastrocnemius muscles which were cross-sectioned at the midlevel of the muscle length. The counting was performed on at least 10 different fields per ultrathin section which was analyzed and photographed using TEM. Sublaminal nuclei which were separated by a plasma membrane from the adjacent myofibers were counted and normalized to the number of fibers. As we do not see a change in fiber number but only in fiber size, this allowed us to determine if the overall number of satellite cells was different between wildtype and transgenic muscles. We also counted interstitial cells which were outside of the myofiber basal lamina as well as the number of myonuclei which were also normalized to the total number of myofibers. No significant differences were observed between wildtype and transgenic muscles with regard to the number of myonuclei or interstitial cells (Fig. 4). However, we found that, while the wildtype and transgenic muscles had the same number of cells under the basal lamina and thus in a satellite cell position, the transgenic muscle had a high percentage of these cells that displayed euchromatic nuclei as well as more cytoplasm, cellular extensions and organelles as compared to wildtype tissue suggesting that satellite cells either fail to enter cell cycle arrest or are activated myocytes as described by others (Bischoff, 1975, 1986a,b, 1990). Thus, in order to quantify our data, we considered sublaminar nuclei of either bonafide satellite cells and/or satellite-like cells in order to assess if the satellite cell population was changed. We found that, while the total number of satellite cells normalized to fiber number was unchanged, there was a striking decrease in the percentage of quiescent satellite cells in the transgenic muscles ($P < 0.05$). We further note that these cells are often present as clusters of 3–5 cells (Figs. 4 and 5). Thus, while transgenic muscle shows an apparent increase in 'activated' myocytes, there does not seem to be an overall change in the number of satellite and satellite-like cells suggesting that the transgenic muscle is not undergoing stem cell recruitment typical of regenerating muscle and that the smaller muscle mass in the transgenic mice may reflect perturbations in other aspects of stem cell behavior such as participating in postnatal maturation.

Pax7 is a reliable molecular marker of satellite cells in postnatal muscle (Polesskaya et al., 2003; Seale et al., 2000, 2004b). We note that the Pax7 mutant mice display a similar

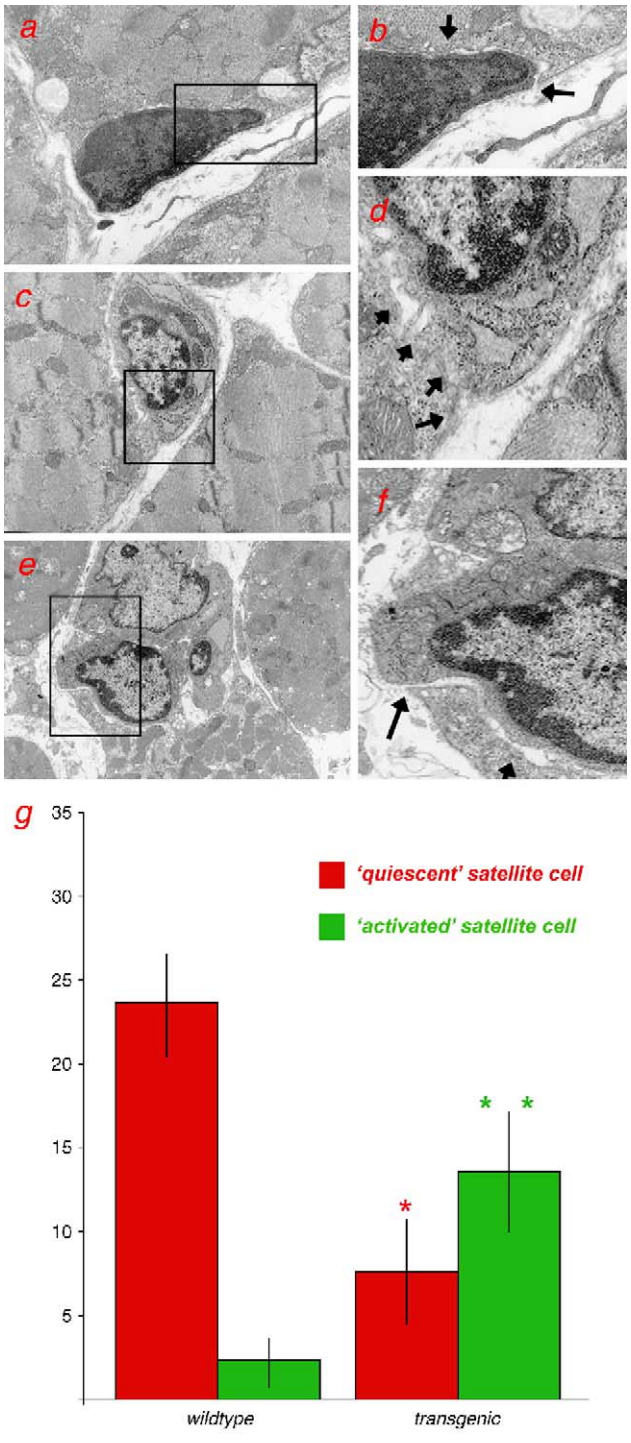

Fig. 4. Transgenic muscles display a change in satellite cell morphologies. (a–f) Transmission electron micrographs of 6 days postnatal hindlimb cross-sections obtained from midanterior–posterior hindlimb levels. (a) Typical satellite cell with condensed heterochromatic nucleus is in wildtype muscle. (b) Inset from panel a shows the paucity of cytoplasm and membrane boundary between the satellite cell and adjacent myofiber (arrows). (c, e) Cross-sections from hindlimb muscles taken from 2 different transgenic mice with corresponding insets shown at higher resolution (d, f). Sublaminal cells with euchromatic (uncondensed) nuclei can be detected containing more cytoplasm and organelles as compared to wildtype satellite cells (compare panels c, e with a). Arrows in panels d and f point to a clear cytoplasmic separation between the satellite cell and adjacent myofiber although fusion may be occurring in panel d. Panels e and corresponding inset (f) show a typical cell cluster found in the satellite cell position in the transgenic muscles which are rarely observed in the wildtype muscle. (g) Counts of quiescent (heterochromatic) cells normalized to number of muscle fibers reveal a marked and significant decrease in quiescent satellite cells in the transgenic muscles as compared to wildtype muscles coincident with a marked increase in activated sublaminal cells (*, **$P < 0.05$). All counts were normalized to the number of myofibers (see Materials and methods for details). No change was observed in myonuclei nor interstitial cells nor was a change in the overall proportion of sublaminal satellite cells.

phenotype to the ΔPW1 mice generated in this study (Seale et al., 2000). One potential cause of the reduced muscle mass in the Pax7 mutant mice is either a major reduction in the number of satellite cells in postnatal muscle that participate in postnatal muscle growth (Seale et al., 2000) or the ability of these cells to undergo proper renewal and propagation (Oustanina et al., 2004). Given the critical role of Pax7 in normal muscle stem cell behavior, we used triple immunofluorescence confocal microscopy to examine the expression of Pax7 and PW1 in combination with an antibody to laminin to define the myofiber basal lamina (Fig. 5). Previous studies revealed that PW1 protein is localized primarily to the nuclei of myoblasts and nascent myotubes in vitro as well as during early development (Coletti et al., 2002; Relaix et al., 1996). Ongoing studies examining PW1 expression in postnatal muscle reveal that PW1 localizes to both satellite and a subset of interstitial cells closely associated with the myofibers and levels rise sharply in response to damage (manuscript in preparation and results shown here). We note that PW1 is not expressed in the interstitial cells of non-muscle tissues examined suggesting that these cells are not simply connective tissue components but may represent a non-satellite cell stem cell compartment. Consistent with these studies, we find that wildtype muscle shows clear PW1 and Pax7 expression and that a subset of cells express both markers (Fig. 5). While some Pax7 and PW1 positive cells are present in small 'clusters' in wildtype muscle, the majority of cells appear as single cells that either express PW1, Pax7 or both (Fig. 5). In marked contrast, transgenic muscle displays clusters of cells expressing PW1 and/or Pax7 and they are present in clusters of multiple cells (Fig. 5). The antibody we had previously generated to PW1 is directed to the C-terminal part of the protein (Relaix et al., 1996), therefore the staining observed in the transgenic muscle corresponds only to endogenous PW1. While the staining appears to represent an increase in cells that stain for Pax7 and or PW1, we note that our counts of satellite and interstitial cells indicate that this is simply due to the overall smaller fiber size which gives rise to an apparent increase in nuclear density per field. We note that many clusters of labeled cells contain Pax7/PW1+ cells as well as heterogeneous clusters of cells. This result suggests that resident stem cells divide in situ and generate both Pax7 and PW1 expressing cells and that transgenic muscle contains an actively dividing and increased stem cell population. Additionally, no expression of the transgene was observed by PCR on postnatal muscle (data not shown and see Zhu and Miller, 1997).

The decreased muscle mass in the ΔPW1 mice is not only reminiscent of the Pax7 mutant mice but also may result from a more general muscle wasting or atrophy. Such

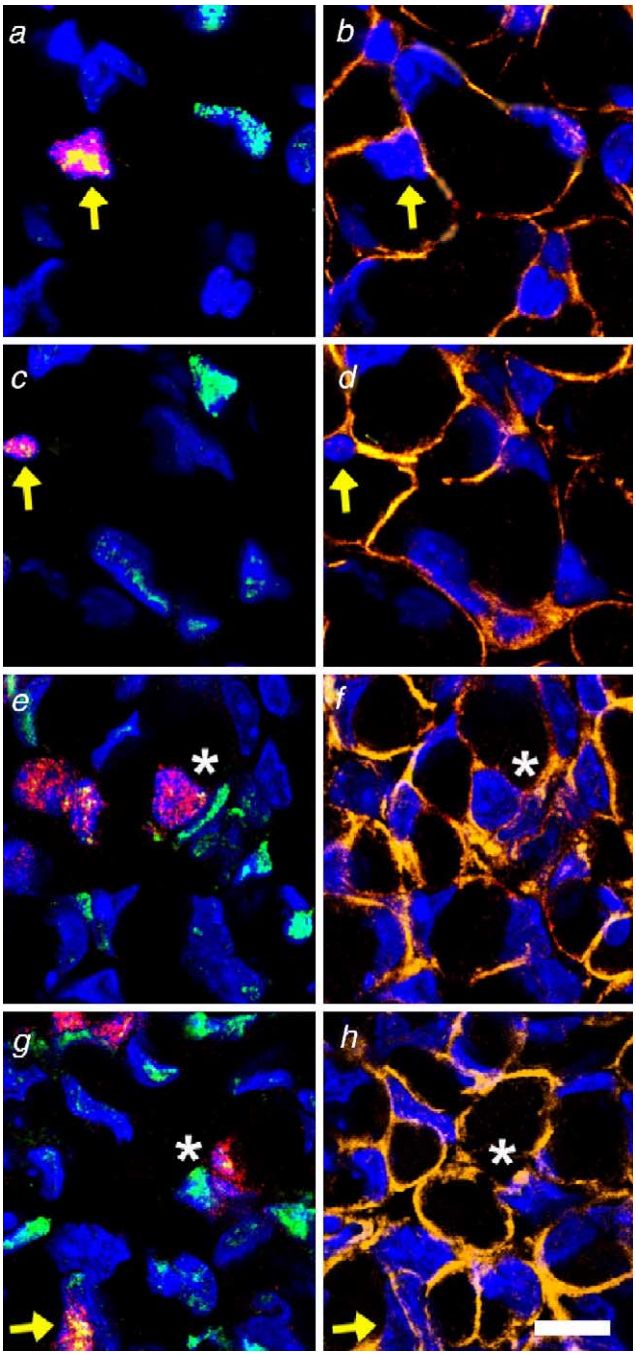

Fig. 5. Expression of Pax7 (a satellite cell marker) and PW1 is expressed in transgenic muscle cell 'clusters'. 6 days postnatal muscle sections processed for dapi (blue) to visualize nuclei and stained using immunofluorescence for Pax7 (red) and PW1 (green) and laminin (orange-yellow). Images were captured using a confocal microscope at 100×. Left column of panels a, c, e and g show DAPI, Pax7 and PW1 only whereas the right column (b, d, f, h) show laminin and dapi to orient cells with relation to the basal lamina. (a–d) Representative sections from wildtype muscle at 6 days showing typical colocalization of PW1 with Pax7 (yellow staining) whereas other cells are stained for PW1 alone. Comparing the labeled cells in panels a and c with panels b and d reveal a clear sublaminar localization of the nuclei which express both Pax7 and PW1 or PW1 only. (e–h) Representative sections from transgenic muscles at 6 days showing clusters of cells (marked by *) expressing PW1 and/or Pax7. Yellow arrow in panel g shows cell co-expressing both PW1 and Pax7 which is in a sublaminal position. We note that the staining for laminin is less clear in the transgenics as compared to wildtype and that many of the cells appear to traverse the lamina. Scale bar = 4 μm.

atrophy or cachexia has been demonstrated recently to involve the specific induction of the E3 ligase, Atrogin-1/MAFbx (Bodine et al., 2001; Gomes et al., 2001; Lecker, 2003). Given that PW1 participates in inflammatory cytokine pathways such as TNFα-mediated NFκB activation (Relaix et al., 1998) and as chronic elevated levels of inflammatory cytokines lead to cachexia (Fong et al., 1989; Tisdale, 2001; Tracey and Cerami, 1992; Tracey et al., 1988), we performed Northern analyses on mRNA obtained from wildtype and transgenic muscles at 3, 5, 6 and 8 days postnatal development. We observe that there is an elevated expression of Atrogin-1 in the transgenic muscles however by 6 days, we note some variation such that some transgenic mice do not show elevated levels and by 8 days, levels appear indistinguishable from wildtype muscles (Fig. 6). We did not detect elevated levels of TNFα mRNA in the transgenic muscle (data not shown), but we note that a large array of inflammatory cytokines as well as non-cytokine stimuli may lead to atrogin-1 upregulation (Gomes et al., 2001; Sandri et al., 2004; Spate and Schulze, 2004). Our previous studies revealed that PW1 plays a key role in mediating the responses of myoblasts to TNFα (Coletti et al., 2002). Specifically, when myogenic cell lines or primary myoblasts are grown in the presence of exogenous TNFα, differentiation is blocked and this response depends upon PW1 and specific p53 downstream effectors including bax and caspase 9 (Coletti et al., 2002). We therefore obtained primary myoblasts from 3 independent wildtype and transgenic 6 days postnatal limb muscles and tested early passage cells for their response to TNFα. As reported previously (Coletti et al., 2002), wildtype cells showed robust differentiation in standard differentiation media conditions (DM) but differentiation was reduced when cells were placed in DM supplemented with TNFα (Fig. 6). In contrast, myoblasts obtained from transgenic mice showed normal differentiation in both the absence and presence of exogenous TNFα (Fig. 6).

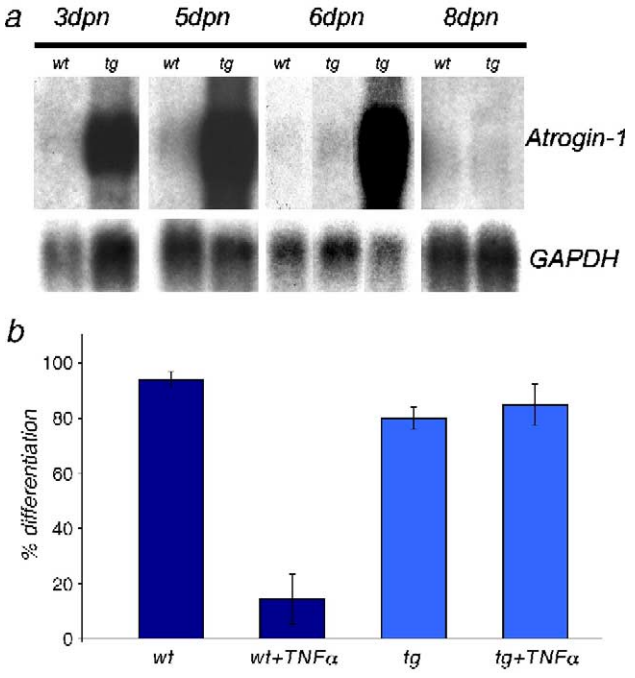

Fig. 6. Atrogin-1 levels are transiently elevated in transgenic muscles and myoblasts display altered sensitivity to TNFα. (a) Detection of Atrogin-1 mRNA, a specific marker of muscle wasting by Northern blot. Atrogin-1 mRNA is highly expressed in transgenic hindlimb muscles at 3, 5 and 6 days postnatal. Levels are high in 3 and 5 days postnatal muscles whereas we note variation by 6 days and low levels by 8 days postnatal development. None of the wildtype muscles showed high levels of Atrogin at any stage. Blots were hybridized with GAPDH to verify the homogeneity of the mRNA loading. (b) Quantitative analysis of myogenic differentiation reveals that both wildtype and transgenic myoblasts show robust differentiation under normal conditions, only wildtype myoblasts show a strong inhibition of differentiation in response to exogenous TNFα. Primary cells from 3 different transgenic and wildtype mice were established from 6 day postnatal muscles and tested for differentiation in the presence or absence of TNFα. Multiple fields were counted for total nuclei and number of nuclei in myosin positive myotubes. Data are presented as percentage of nuclei in biochemically differentiated myotubes.

Discussion

Postnatal growth is affected by overexpression of ΔPW1 during embryogenesis

In this study, we show that ectopic expression of ΔPW1 during embryonic development affects postnatal growth. A similar phenotype has been described for the Pax7 mutant mice which fail to grow normally after birth (Seale et al., 2000). In Pax7 mutant mice, satellite cell formation is impaired but secondary fibers form correctly. In contrast, we see that cells in a satellite cell position (sublaminal but distinct from the myofiber) which express Pax7 are easily detected in transgenic muscles, but the number of cells which show a typical satellite cell morphology including a condensed heterochromatic nuclei and low cytoplasmic and organelle content is greatly reduced. In contrast, there is a compensatory increase in sublaminal cells that appear as activated myocytes and are present in clusters of 2–4 cells in the transgenic muscle. This pattern of expression in the transgenic muscle, combined with the observation that, in both wildtype and transgenic muscles, some cells express both markers, suggests that PW1 plays a role in governing stem cell numbers and behavior. We note that in a recent study examining explanted muscle fibers, similar ‘clusters’ of muscle stem cells have been described following damage which contain cells that either express MyoD, Pax7 or both (Zammit et al., 2004). These authors propose that the different patterns of expression of these genes reflect an asymmetric cell fate that determines if stem cells return to a quiescent state or are determined to terminally differentiate into muscle fibers (Zammit et al., 2004). Deregulation of muscle stem cell behavior has also been observed in mice lacking the forkhead/winged helix protein, MNF and like ΔPW1 transgenic mice in our studies and Pax7 mutant mice, MNF mutant mice are runted at birth (Garry et al., 2000).

Lastly, MyoD mutant mice which show a postnatal defect in satellite cell behavior are otherwise normal with the exception that mutant mice are often runted and fail to achieve normal postnatal body size (Rudnicki et al., 1992; White et al., 2000; Yablonka-Reuveni et al., 1999). We note that, for each genotype, the overall body growth is compromised even though each mouse shows a different severity of the phenotype. Taken together, we conclude that postnatal muscle stem cells are critical in the postnatal growth of muscle and in turn can control overall body growth.

PW1 expression is associated with stem cell differentiation

Recent studies indicate that bone-marrow-derived muscle stem cells (BMDSCs) participate in the formation of satellite cells and muscle fibers (Asakura et al., 2002; Dreyfus et al., 2004; Ferrari et al., 1998; Musaro et al., 2004; Polesskaya et al., 2003; Rudnicki, 2003; Seale et al., 2003) as well as cells originating from embryonic dorsal aorta (De Angelis et al., 1999; Minasi et al., 2002). While the involvement of BMDSCs has been well documented during regeneration, their involvement in postnatal growth has not been demonstrated to date. We note however that Pax7 mutant postnatal muscle possesses a population of cells which express identical cellular markers seen in BMDSCs and that this population of cells display an increased potential to differentiate along the hematopoietic lineage (Seale et al., 2000). In addition, it has also been demonstrated that pluripotent cells can originate from vascular tissue (mesoangioblasts) that have strong myogenic potential (De Angelis et al., 1999). Ongoing studies in our laboratory reveal that mesoangioblasts as well as cells localized in vascular tissue in muscle express PW1 (study in preparation). Thus, the localization of PW1 expression to satellite cells (Pax7+), interstitial cells and vascular cells is highly consistent with a role in regulating some aspect of pluripotent stem cell behavior.

ΔPW1 induced postnatal muscle atrophy

We observe that transgenic muscle appears atrophic and has smaller fiber diameters. Consistent with this morphological observation, we find that transgenic muscle expresses high levels of Atrogin-1 mRNA, a specific marker of muscle atrophy. The molecular mechanisms responsible for muscle atrophy are just beginning to be understood. While a significant number of studies support the model that various factors such as inflammatory cytokines (TNF, IL), starvations or denervation lead to an increase in protein catabolism in existing muscle fibers (Tisdale, 2001; Tracey and Cerami, 1992; Tracey et al., 1988), it is possible that these same factors also impede muscle regeneration or the ability of myogenic stem cells to fuse to pre-existing myofibers which may ultimately contribute to the atrophic outcome. Indeed, we and others have demonstrated that TNFα is a potent blocker of myogenic differentiation (Coletti et al., 2002; Grounds and Torrisi, 2004; Li, 2003; Li and Reid, 2000). In this study, we find that myogenic cells obtained from transgenic pups display robust terminal differentiation in the presence of TNFα as compared to wildtype which suggests that a modification of transgenic muscle stem cell behavior contributes to the muscle phenotype.

Our finding that the transgenic mice have atrophic muscle and an increase proportion of the muscle stem cell population in an activated state would seem paradoxical. However, it was observed that denervation, which also leads to muscle atrophy, is followed immediately by a transient wave of satellite cell proliferation however no new muscle is formed nor satellite cell nuclei enter into myofibers already present (McLennan, 1983). In addition, we see no increase in the proportion of sublaminal cells in the transgenic muscles which suggests that this population is not expanding as it would during secondary fiber formation or regeneration. Indeed, whereas satellite cells proliferate transiently after denervation in normal muscle, their number sharply declines in long-term denervated muscle (Rodrigues Ade et al., 2002) suggesting that satellite cell activation can precede eventual satellite cell loss. We found that neither MyoD transcripts nor protein levels are high in transgenic muscles (data not shown) consistent with a mechanism whereby satellite cells are activated but do not participate in further myogenesis.

We note that the satellite cells are activated under pathological conditions in which the muscle is degenerating which triggers a regenerative response. For example, in the case of the mdx mouse carrying a mutation corresponding to the human muscular dystrophy Duchenne gene, over time the muscle also consists of small myotubes with activated satellite cells. This would suggest that the transgenic muscle is not atrophic but merely undergoing successive rounds of regeneration. We believe this not to be the case however since mdx muscle does not express abnormally high levels of Atrogin-1 (personal communication D. Glass). It is of interest however that exposure of mdx muscle to antibody reagents that block endogenous TNFα appears to protect myofiber integrity (Grounds and Torrisi, 2004).

Transient expression of ΔPW1 during early development affects satellite cell formation

The myogenin promoter used for this study has been shown to be expressed in myogenic precursors in the myotome and limb buds. At later stages however, only few fibers show expression for this promoter construct despite the fact that endogenous myogenin expression is much higher (Cheng et al., 1995). Similarly, it was demonstrated that transgenic mice in which MRF4 was placed under the control of the same myogenin regulatory elements used for our study (Zhu and Miller, 1997), MRF4 expression declined during fetal development. Since we find that the myog-ΔPW1 transgenic mice show a similar spatial and

temporal pattern of expression, we conclude that the decline in transgene expression reflects the regulatory elements used and is not influenced by ΔPW1 expression. Since we see such robust and clear expression of the transgene at 10.5 days of embryonic development at the level of the somite which later declines, we propose that this transient expression underlies the phenotypic effects that become apparent only after birth although verification of this awaits the generation of conditional alleles. Our analyses of the transgenic mice reveal a distinct change in the myogenic cells in situ. Whether or not postnatal muscle stem cells are specified at the level of the somite when the ΔPW1 transgene is expressed remains to be determined. We note that, while stem cells are present in the transgenic muscle, they appear to fail to enter growth arrest and do not respond to exogenous TNFα once isolated in culture suggesting that these cells are either permanently changed or represent a different population due to events during early somitogenesis. While it is unclear how this change in cytokine responsivity contributes to the overall muscle phenotype observed in the transgenic mice, these observations reveal that the behavior of postnatal myogenic cells is altered due to prior transgene expression in the embryo. These data suggest that components of the TNFα–NFκB and/or p53 pathway may play a critical role during early development in specifying the identify and/or behavior of postnatal stem cells. Furthermore, these studies raise the interesting hypothesis that deregulation of normal stem cell behavior can trigger muscle atrophy.

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