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The role of p53 *in vivo* during skeletal muscle post-natal development and regeneration: studies in p53 knockout mice

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ABSTRACT The tumour suppressor gene *p53* is recognised as a central regulator of the cell cycle and apoptosis. Post-natally, *p53* mutations are associated with many cancers and mice lacking *p53* are prone to spontaneous tumour formation. The present study examines skeletal muscle formation in post-natal mice lacking *p53* using two different models of skeletal muscle regeneration. The level of endogenous myogenic cell proliferation in mature skeletal muscle was examined and the time course of muscle regeneration after whole muscle transplantation or crush injury were compared in *p53* (-/-) and control C57Bl/6J adult mice, using desmin and proliferating cell nuclear antigen (PCNA) immunohistochemistry and histological analysis. The pattern of inflammation, myoblast proliferation and myotube formation in regenerating *p53* (-/-) skeletal muscles appears normal and similar to those in control C57Bl/6J muscle. These data indicate that p53 is not required for the regulation of myoblast proliferation, differentiation and myotube formation *in vivo* during myogenesis of adult skeletal muscle.

KEY WORDS: *p53, knockout mouse, skeletal muscle, regeneration, whole muscle graft, fusion, proliferation*

Introduction

Skeletal muscle has an exceptional ability to regenerate after tissue breakdown following mechanical or chemical injury and pathological insult. The regeneration of damaged skeletal muscle follows a series of clearly defined events beginning with myofibre necrosis (Grounds, 1991). This results in inflammatory cells such as polymorphonuclear leucocytes and macrophages being attracted to the site of damage where they phagocytose myofibre debris and also produce many factors that stimulate myogenesis. In response to muscle damage, quiescent muscle precursor cells on the surface of myofibres (called satellite cells) are activated and these are widely referred to as myoblasts. The myoblasts proliferate, exit the cell cycle, differentiate, and fuse together to form multinucleated myotubes; these then fuse with the 'sealed' ends of the damaged myofibres to repair the myofibres (Roberston *et al.*, 1993). Re-innervation completes the process of new muscle formation.

The differentiation of myoblasts is characterised by permanent withdrawal from the cell cycle, activation of muscle specific gene expression, and fusion into multinucleated myotubes. The activation, proliferation, differentiation and fusion of myoblasts to form myotubes, in the absence of p53, has been studied *in vitro* where myotube formation is severely impaired (Porrello *et al.*, 2000). The tumour suppressor gene p53 is a transcription factor commonly considered to be the "gatekeeper for cell replication", being important for maintaining the quiescent state of cells (Levine *et al.*, 1991).

The development of a viable p53 knockout (-/-) mouse demonstrates that the p53 molecule is not critical during development. The p53 (-/-) mouse is however susceptible to spontaneous tumour formation after birth (Donehower *et al.*, 1992) indicating a basic requirement for the p53 gene post-natally, possibly with particular relevance to preventing accumulation of mutations within the genome (Levine, 1997). In addition, hepatocytes from adult livers of p53(-/-) mice divide at over 2.5 times the rate of normal mice and show altered morphology *in vivo* (Dumble *et al.*, 2001) supporting the proposal that p53 may play an important role post-natally in some cell types.

This study examines the role of p53 in skeletal muscle of adult mice. It specifically tests the hypothesis that the absence of p53 will result in extended myoblast proliferation, delayed differentiation and subsequently delayed myotube formation, during skeletal muscle regeneration in p53(-/-), compared to wild type control mice.

Abbreviations used in this paper: PCNA, proliferating cell nuclear antigen; TA, tibialis anterior.

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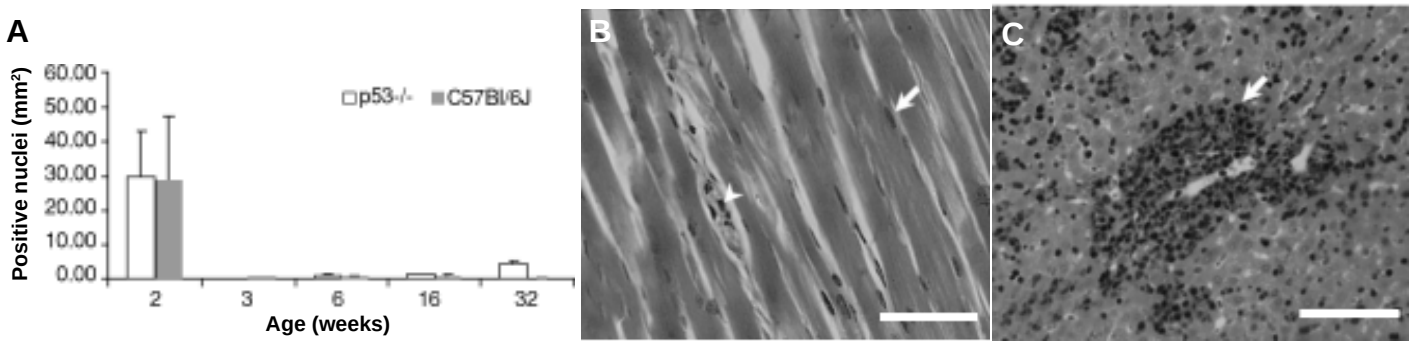

Fig. 1. Cell proliferation in post-natal tissues. *PCNA immunohistochemistry was used to evaluate cell replication in post-natal TA muscle and liver of p53(-/-) and control C57Bl/6J mice aged 2 to 32 weeks of age.* **(A)** *Quantification of cell proliferation in uninjured TA muscles. The number of PCNA positive nuclei was counted in 10 randomly selected non-contiguous fields of view (at 200x magnification) and the mean numbers of positive nuclei are expressed per mm².* **(B)** *In longitudinal sections of p53(-/-) muscle, PCNA positive nuclei are associated with myofibres (arrows) and blood vessels (arrowheads).* **(C)** *The livers from p53(-/-) mice show many PCNA positive cells around the peri-portal regions. The severity of the liver pathology in p53(-/-) mice ranged from mild, to moderate (shown here), to severe. Scale bar, 50 μm.*

Results

Uninjured Post-Natal Skeletal Muscle and Liver

Cell Proliferation. Cell proliferation was examined by PCNA immunohistochemistry in uninjured adult skeletal muscle of p53(-/-) and control mice aged 2, 3, 6, 16 and 32 weeks of age (Fig. 1). The numbers of PCNA positive nuclei per unit area in control and p53(-/-) skeletal muscle are summarised in Fig. 1A. Although PCNA positive cells were also present in areas of connective tissue and blood vessels, these were not included in the quantitative analysis; only nuclei lying within myofibres or intimately associated with the surface of myofibres were counted (Fig. 1B). At 2 weeks of age significant cell proliferation was present in p53(-/-) and control skeletal muscles, but by 3 weeks cell proliferation was considerably reduced in muscles of both strains. In control skeletal muscle this low (background) level of cell proliferation was maintained out to 32 weeks of age. In contrast, in the oldest p53(-/-) skeletal muscle examined (at 32 weeks) a significant increase in cell proliferation was noted (Fig. 1A). We were unable to obtain p53(-/-) skeletal muscle from older mice due to the increasing tumour load and poor health of the animals after this age.

In contrast to skeletal muscle, many proliferating cells were present in the liver of the p53(-/-) mice at all ages, and this was most pronounced in peri-portal regions (Fig. 1C). This pattern of cell proliferation was never observed in livers from control mice. There was marked heterogeneity in the liver histopathology between mice and this did not seem to be age related. However, the pathology was consistent throughout each liver. A detailed analysis of cell replication in the livers of p53(-/-) mice was not done, but these observations accord closely with those of Dumble *et al.* (2001). The number of PCNA positive cells observed in skeletal muscle was not correlated with the severity of the liver histopathology in individual p53(-/-) mice.

Studies of Regenerating Skeletal Muscle

Whole Muscle Autografts. The morphological changes occurring during regeneration in whole skeletal muscle autografts are extensively described elsewhere (Roberts *et al.*, 1997; White *et al.*, 2000). The main features of this process are the presence of two distinct zones within a transverse section of the EDL graft: (i) the central necrotic zone and; (ii) the peripheral regenerative zone

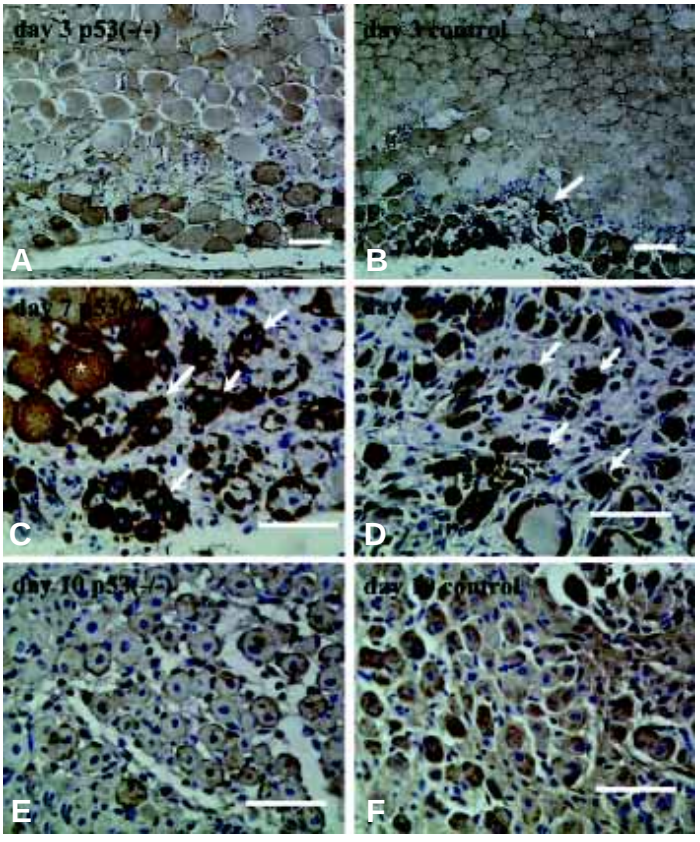

Fig. 2. Desmin immunostaining of regenerating whole muscle autografts. *Desmin immunohistochemistry on transverse sections of p53(-/-) (A,C) and control C57Bl10/6J (B,D) whole muscle autografts. At day 3, myotubes (arrows) are scarce and small in both p53(-/-)* **(A)** *and C57Bl/6J* **(B)** *autografts. At day 7, many desmin positive myotubes (arrows) are present and similar numbers of myotubes are seen in both p53(-/-)* **(C)** *and control* **(D)** *autografts. In all grafts some surviving myofibres (*) which have not undergone necrosis are present near the interface of the graft and the underlying TA muscle. At day 10, myotube formation is essentially complete with myotubes covering the entire area of the p53(-/-)* **(E)** *and control* **(F)** *grafts. Scale bar, 50 μm.*

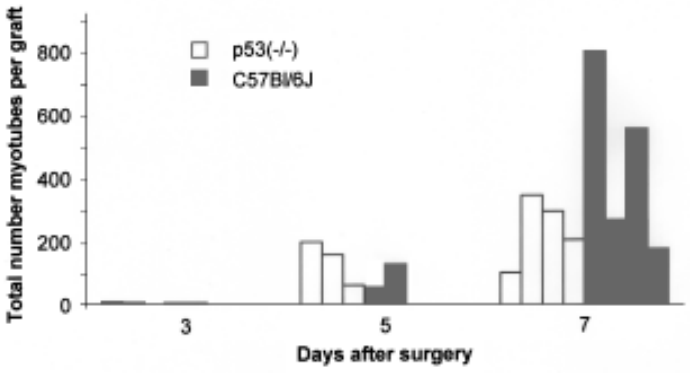

Fig. 3. Quantitation of myotube formation in whole muscle autografts. *Numbers of desmin positive myotubes in transverse sections of p53(-/-) and C57Bl/6J autografts at 3, 5 and 7 days after transplantation. The total numbers of myotubes were counted in a single transverse section of each graft. Each vertical column represents a single graft. There was no significant difference in myotube formation between the two strains at any time.*

(inflammatory cells, myoblasts and myotubes), which starts at the very edge of the graft and progressively moves inwards to replace the necrotic tissue.

At 3 days after surgery, very few myotubes were present in any of the grafts examined and there was no significant difference in the number of myotubes in p53(-/-) compared to C57BL/6J grafts. In three of the four p53(-/-) grafts at 3 days, no myotubes were apparent, and in the remaining graft only a single myotube was observed (Fig. 2A). In C57BL/6J mice at day 3, a single graft had no myotubes and the remaining three had isolated myotubes (Fig. 2B). At day 5, desmin immunostaining was pronounced in myoblasts and in "cuffing cells" (activated satellite cells) lining the contour of the persisting basement membrane of necrotic myofibres. Many plump desmin positive myotubes were present in the regenerating zone at the periphery of grafts in both p53(-/-) and control C57Bl/6J mice. At day 7, myotube formation had progressed and the regenerative zone was approaching the centre of the grafts in both strains (Fig. 2 C,D). At day 10, regeneration was essentially complete and myotubes filled the entire graft in both p53(-/-) and control C57Bl/6J mice (Fig. 2 E,F). The numbers of myotubes were counted in a single transverse section of each graft at days 3, 5 and 7 and the data are summarised in Fig. 3. At all times there was no significant difference in the extent of myotube formation between p53(-/-) and control autografts. The numbers of myotubes in day 10 and 14 grafts was not quantified, as myotube formation was essentially complete with myotubes covering the entire area of the grafts at these times.

Crush Injured Muscles. The regenerative process is more difficult to quantify after severe crush injury but the pattern of regeneration in p53(-/-) skeletal muscle after crush injury was indistinguishable from control C57Bl/6J muscles in the few samples studied. In both strains, equivalent myotube formation was seen at day 7 and 14 (Fig. 4).

Discussion

Cell Replication in Post-Natal p53 *(-/-) Skeletal Muscle is Normal*

Using PCNA immunohistochemistry, no difference in the extent of cell replication in post-natal skeletal muscle was observed between p53(-/-) and control mice up to the age of 32 weeks. In contrast, in the livers of p53(-/-) mice many proliferating blast-like cells were present; these were not seen in control livers. The numbers of proliferating cells in p53(-/-) livers did not seem to be age related and the severity of the pathology was variable. Even in younger mice that exhibited a severe liver phenotype, cell replication in the skeletal muscle was the same as control muscle. The striking difference in pathology between various tissues in post-natal p53(-/-) mice is the subject of a separate investigation.

The significant increase in cell proliferation in skeletal muscle of 32 week old p53(-/-) mice suggests that even skeletal muscle may be susceptible to pathology over time. We were unable to analyse muscle from older mice as most died beyond 32 weeks of age. While the change in old skeletal muscle might reflect an age-related instability of the myofibres lacking p53, it might well be an indirect effect resulting from the declining health of the animals, due to significant tumour formation by this time.

Regeneration is Unaffected in p53 *(-/-) Skeletal Muscle*

Skeletal muscle regeneration was examined in p53(-/-) mice to test the hypothesis that sustained myoblast proliferation and/or delayed differentiation would result in delayed myotube formation. Two models of skeletal muscle regeneration were used. In whole autografts of EDL muscles, the onset and extent of myotube formation, examined between 3 to 14 days after transplantation, was the same in p53(-/-) and control C57Bl/6J grafts at all times. Significant numbers of myotubes were first observed at day 5. This accords with the onset of myotube formation seen in other strains of mice such as BALB/c and SJL/J (Roberts *et al.*, 1997; Smythe *et al.*, 2001; White *et al.*, 2000). The pattern of cellular events during regeneration was indistinguishable between p53(-/-) and control muscle autografts. Similarly, after crush injury where there was severe disruption to muscle architecture, myotube

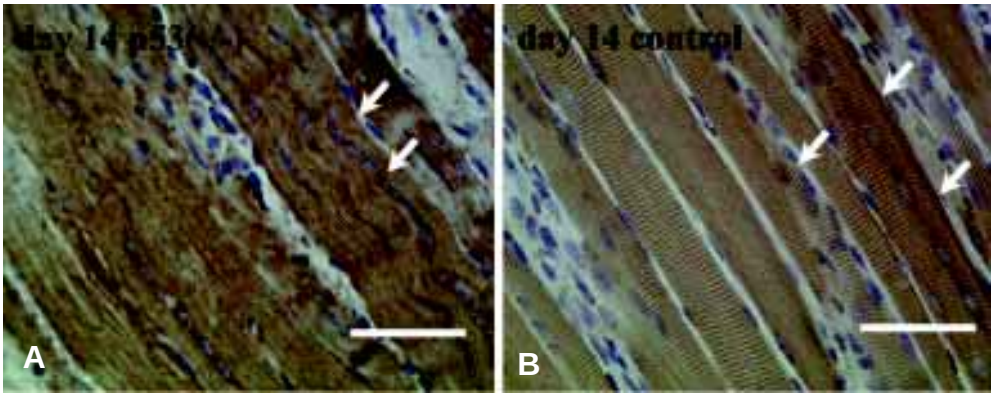

Fig. 4. Myotube formation after crush injury in *p53* (-/-) and control skeletal muscle. *Desmin immunohistochemistry on muscles from p53(-/-)* **(A)** *and control C57Bl/6J* **(B)** *at 14 days after crush injury. Large myotubes (arrows) with striations are apparent in injured muscle from both strains. Scale bar, 50 μm.*

formation was essentially the same at 7 days in p53(-/-) and control mice and was complete at 14 days in both strains. There are some fundamental differences between the dynamics of skeletal muscle regeneration in these two models of injury. Regeneration occurs in whole muscles grafts within the persisting architecture of the basement membrane, but the grafted muscle is removed from both vascular and nervous supply. This is in stark contrast to crush injury where there is significant disruption to the muscle structure, but the blood and nervous supply remains relatively intact (Mitchell *et al.*, 1992). While regeneration can occur in the absence of a basement membrane (Caldwell *et al.*, 1990), the presence of this extracellular matrix scaffold facilitates regeneration. However, the pattern of myotube formation was not affected in either the whole muscle graft or crush injury models in the absence of p53.

The two events of cell proliferation and differentiation are mutually exclusive since myoblasts exit the cell cycle prior to differentiation and subsequent myotube formation. We have previously shown using the whole muscle graft model, that in MyoD(-/-) muscle where myoblast proliferation is sustained for 2-3 days (compared with controls) this results in a 2-3 day delay in myotube formation (White *et al.*, 2000). In regenerating whole muscle grafts of desmin(-/-) mice myotube formation was delayed by 1 day (Smythe *et al.*, 2001). However, no such delay (or enhancement) in myotube formation was seen in regenerating p53(-/-) muscles. These observations provide strong evidence that there is no defect in the regulation *in vivo* of myoblast proliferation and fusion in the absence of p53.

The behaviour of myogenic cells during myogenesis of developing skeletal muscle appears to be essentially the same as in regenerating adult muscle. The most striking difference between myogenesis during embryogenesis and in post-natal muscle is the presence of inflammatory cells during regeneration (Grounds, 1991). The apparently normal development of skeletal muscle in p53(-/-) mice (Donehower *et al.*, 1992) and the 'normal' regeneration shown here in post-natal p53(-/-) muscle indicates that p53 is either not a significant regulator of muscle cells during development or regeneration, or that some other factor is compensating for the absence of p53 in these null mice. Two members of the p53 family, p63 and p73, show weak but transcriptionally active heterotypic interactions (Davison *et al.*, 1999; Di Como *et al.*, 1999). The p63 and p73 share homology with p53 within the activation, DNA binding and oligomerisation domains. Furthermore, when over-expressed, p63 and p73 are able to transactivate some p53 target genes (Kaelin, 1999; Oren, 1997). Mice lacking p63 or p73 have more marked developmental defects than those seen in p53(-/-) mice (Mills *et al.*, 1999; Yang *et al.*, 1999; Yang *et al.*, 2000). However, these papers do not describe embryonic skeletal muscle development in p63 and p73 null mice. Thus it is not unreasonable to predict that *in vivo*, p63 or p73 may compensate for the lack of p53. While *in vitro* evidence suggests that p63 and p73 cannot successfully substitute for the role of p53 in muscle differentiation (Porrello *et al.*, 2000), this does not exclude the possibility of such compensatory mechanisms in the more complex *in vivo* situation.

TABLE 1

SUMMARY OF THE NUMBER OF SAMPLES TAKEN AT VARIOUS TIMES AFTER WHOLE MUSCLE AUTOGRAFTING (WMG) AND CRUSH INJURY (CRUSH) IN *P53* (-/-) AND C57BL/6J MICE

All samples were fixed in 4% paraformaldehyde and processed for paraffin embedding

Time [days]	3	5	7		10	14	
	Wmg	wmg	wmg	crush	wmg	wmg	crush
p53(-/-)	4	3	5	1	2	1	1
C57Bl/6J	4	2	3	1	2	1	1

A Possible Role for p53 in the Molecular Regulation of Muscle Differentiation

Cell cycle withdrawal is associated with increased expression of the cdk inhibitor p21 (a p53 inducible gene) which in turn inhibits the phosphorylation of retinoblastoma protein (pRb) and further promotes cell cycle withdrawal. In cultures of primary skeletal muscle and myogenic cells lines it has been shown that pRb co-operates with the skeletal muscle specific transcription factor MyoD to promote the expression of late markers of differentiation (Chen *et al.*, 1996; Gu *et al.*, 1993; Novitch *et al.*, 1996) through activation of MEF2 (Novitch *et al.*, 1999). Active pRb is required for proper functional activity of MyoD during skeletal muscle differentiation (Gu *et al.*, 1993; Novitch *et al.*, 1996; Novitch *et al.*, 1999). Within the promoter region of pRb there are p53 binding sites (Osifchin *et al.*, 1994) and p53 binds the Rb promoter in skeletal muscle cells (Porrello *et al.*, 2000), indicating that such transcriptional control mechanisms may play a role during cessation of replication and the onset of differentiation in myoblasts. Expression of p53 increases during differentiation and fusion of skeletal muscle cells *in vitro* (Halevy *et al.*, 1995; Soddu *et al.*, 1996; Tamir and Bengal, 1998), and p53 is thought to co-operate with MyoD in the transcriptional induction of muscle creatine kinase (MCK), a gene that is induced during muscle differentiation. While these tissue culture studies using both primary p53(-/-) skeletal muscle cells and dominant negative p53 myogenic cells lines strongly support a role for p53 in myogenesis this is clearly not manifested in the *in vivo* environment.

In stark contrast to the excellent myotube formation *in vivo* observed in regenerating adult p53(-/-) muscle in the present study, myotube formation *in vitro* is impaired in myogenic C2C12 cells expressing a dominant negative p53 protein and in primary myoblasts derived from p53(-/-) muscle. After growth factor withdrawal *in vitro,* these myoblasts exit the cell cycle but fail to effectively form myotubes (Porrello *et al.*, 2000). These p53 negative myoblasts do not up-regulate pRb and have a reduced MyoD activity.

In the above *in vitro* studies differentiation was induced by growth factor withdrawal. Differentiation of skeletal muscle cells can alternatively result from increasing cell-cell contact which occurs as cultures reach confluence (Martelli *et al.*, 1994). It has been proposed that induction of cell cycle (cdk) inhibitors, either by cell-cell contact (involving p27) or growth factor withdrawal (involving p21), maintains the activity of MyoD in the differentiated myotube (Martelli *et al.*, 1994; Porrello *et al.*, 2000). Interestingly, Porrello *et al.,* (2000) report as 'unpublished observations' that confluent cultures of both primary p53(-/-) myoblasts and dominant negative p53 C2C12's up-regulate Rb in a p53 independent manner. There is intimate cell-cell contact *in vivo* in developing and regenerating p53(-/-) muscle where effective myotube formation is observed. Therefore, Rb may be upregulated independent of p53 *in vivo* and the critical role of cell contact may possibly account for the differences between *in vitro* and *in vivo* results.

This study emphasises the importance of following up initial *in vitro* observations with carefully considered *in vivo* studies to test overall biological significance. A striking difference between the

responses of liver and skeletal muscle to the absence of p53 is evident *in vivo*. The complex regulation of biological processes in the *in vivo* environment, including the intimate contact between many cell types and inherent flexibility and redundancy of many signalling networks, can quite frequently compensate for the absence of a single factor considered in isolation. For these reasons, the present study clearly demonstrates no essential role for p53 during both development and regeneration of skeletal muscle *in vivo*.

Materials and Methods

Animals

p53(-/-) and C57Bl/6J mice were obtained from specific pathogen free colonies held at the Animal Resources Centre, Murdoch, Western Australia. These colonies were established from mice generously supplied by Prof. Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA, USA). Female p53 (+/-) mice were crossed with male p53(-/-) mice to generate p53(-/-) and p53(+/-) offspring. These mice are difficult to breed and thus the supply of experimental mice was restricted and only limited numbers of p53(-/-) mice were available for the present study. The p53 gene status was assessed individually using PCR (Jacks *et al*., 1994). Experiments were conducted in strict accordance with guidelines of the Animal Ethics Committee of The University of Western Australia and the National Health and Medical Research Council, Australia. All mice were housed in individual cages under a 12-hour day/night cycle and allowed access to food and water *ad libitum*.

Surgical Procedures

Autografts of whole muscles were made in both legs of 15 p53(-/-) and 10 C57Bl/6J mice. The transplantation procedure for whole muscle grafts has been described in detail previously (Roberts *et al*., 1997; White *et al*., 2000). In brief, mice were anaesthetised with a gaseous mixture of halothane, N_2O and O_2 and the extensor digitorum longus (EDL) muscles were removed from both hind legs of each mouse and were relocated over the tibialis anterior (TA) muscles of the same leg. Each EDL autograft was sutured proximally to the distal tendon of the quadriceps femoris muscle and distally to the distal tendon of the TA and the skin closed with 6.0 braided silk sutures. Grafts were left for 2 to 14 days before sample collection and tissue processing. Regeneration was also studied in two p53(-/-) and two C57Bl/6J muscle after crush injury, as described previously (Mitchell *et al*., 1992).

Tissue Collection and Processing

All mice were killed by cervical dislocation. For studies on regenerating muscles, samples were taken at 3, 5, 7, 10 and 14 days after grafting and at 7 and 14 days after crush injury (Table 1). For grafted muscles, both the EDL graft with the underlying (undamaged) TA was dissected and for crush injuries the TA was taken. In addition, for p53(-/-) and control mice at various ages (2, 6, 8, 16 and 32 weeks) the TA and liver were removed: at least two mice were analysed at each age. All tissues were fixed immediately in 4%(w/v) paraformaldehyde in phosphate buffered saline (PBS) for 60 minutes before transfer to 70% ethanol and processing for routine paraffin sections.

Immunohistochemistry

Antibodies. The primary antibodies used were a polyclonal rabbit anti-desmin (DAKO Corporation, Carpinteria, CA, USA), and rabbit polyclonal anti-PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The biotinylated secondary antibody used was a donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and the biotin conjugate was detected with horseradish peroxidase conjugated avidin D (Vector Laboratories, Burlingame, CA, USA).

Immunohistochemical Staining. The cytoskeletal protein desmin is an excellent marker for identifying activated muscle precursor cells and early myotubes *in vivo* (Lawson-Smith and McGeachie, 1998). Desmin is an intermediate filament that is rapidly up-regulated in activated myoblasts and in mature striated muscle desmin is associated with the Z disks (Smythe *et al*., 2001). Desmin was used to identify myoblasts and myotubes within the muscle grafts. Desmin immunohistochemistry in paraffin sections required high temperature antigen retrieval in citrate buffer pH 6.0 (Lawson-Smith and McGeachie, 1998) and the procedure was the same as (described by White *et al*., 2000). Replicating cells in grafts were stained in paraffin sections using PCNA immunohistochemistry as for desmin staining except that antigen retrieval was not required for PCNA detection (White *et al*., 2000).

Analysis of Tissues

Histological analysis and counts of desmin and PCNA positive cells were performed using Image Pro Plus 4.0 (Media Cybernetics, Silver Spring, ML, USA). The pattern of regeneration in transverse sections of whole muscle grafts is uniform throughout the length of the graft (White *et al*., 2000), thus all analyses were based on a single representative transverse sections from the mid region of each graft. For desmin stained sections the number of positive myotubes within an entire transverse section were counted and tagged to avoid double counting. The number of myotubes in each graft was corrected for the area of regenerating tissue. The level of significance between p53(-/-) and control grafts at each time was tested with a Student's t-test. A result was deemed to be significantly different if $P<0.05$.

For PCNA stained sections of uninjured TA in the developmental study (for mice aged 2 to 32 weeks), 10 randomly selected non-contiguous fields of view were selected using an automated XY stage and Stage Pro controller software. The numbers of positively stained nuclei were counted in each of the 10 fields of view. The average number of PCNA positive nuclei were calculated and expressed per unit area (mm^2). The level of significance between p53(-/-) and control grafts at each time was tested with a Student's t-test. A result was deemed to be significantly different if $P<0.05$.

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