

# A role of tensin in skeletal-muscle regeneration

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Regeneration of skeletal muscle requires the activation, proliferation, differentiation and fusion of satellite cells to generate new muscle fibres. This study was designed to determine the role of tensin in this process. Cardiotoxin was used to induce regeneration in the anterior tibial muscles of tensin-knockout and wild-type mice. From histological analysis, we found that the regeneration process lasted longer in knockout than in wild-type mice. To investigate the mechanism involved in this delay, we examined each regeneration step in animals and cultured primary cells. We found fewer proliferating myogenic cells identified by bromodeoxyuridine and desmin double labelling in knockout mice on the first 2 days after injury. Expression of

myosin, paxillin, dystrophin and dystrophin-associated proteins were delayed in knockout mice. Withdrawal from the cell cycle was less efficient in isolated knockout myoblasts, and the fusion capacity was reduced in these cells as well. These defects in regeneration most likely contributed to the 9-fold increase of centrally nucleated fibres occurring in the non-injected knockout mice. Our results demonstrated clearly that tensin plays a role in skeletal-muscle regeneration.

**Key words:** actin binding, cardiotoxin, focal adhesion, SH2 domain.

## INTRODUCTION

Focal adhesions are specialized cell-substratum junctions that are present in cells attaching to extracellular matrix. They are formed around a transmembrane core of an  $\alpha$ - $\beta$  integrin heterodimer, which binds to a component of the extracellular matrix on its external surface, and interacts with the cytoskeletal network intracellularly. The association between the cytoskeleton and integrins is dynamic and influenced by the actions of oncogenes, growth factors, hormones and morphogens [1–4]. It has been postulated that the structure, functions, composition and dynamics of focal adhesions may influence the signal-transduction pathways associated with cytoskeletal reorganization and play key roles in many diverse biological processes and genesis of diseases. In muscle systems, dense plaques of smooth muscle, neuromuscular junctions and myotendinous junctions of skeletal muscle share structural characteristics with focal adhesions and contain the same repertoire of proteins as focal adhesions [1,5]. However, the precise role of focal adhesions in muscle is not well known. Disruption of genes by homologous recombination has allowed researchers to assess integrins' functions *in vivo*. Integrin  $\alpha 5$ - and  $\beta 1$ -deficient mice die at early embryonic stages [6,7], demonstrating a critical role in embryogenesis. Studies using  $\alpha 5$ - and  $\beta 1$ -null embryonic stem cells or chimaeric mice have shown that  $\alpha 5\beta 1$  integrin, similar to  $\alpha 7\beta 1$  integrin, is not essential for myogenesis, but is necessary for long-term integrity of myotubes [6,8–10]. On the other hand, it has been shown that integrins are involved in myoblast proliferation and differentiation in other experimental systems [11–13].

To regulate cell behaviour, integrins must function in association with other molecules in the focal adhesion as structural and signalling partners. Genetic ablations of such partners, including focal adhesion kinase, vinculin and talin ([14,15], and S. H. Lo and E. Fuchs, unpublished work), resulted in early

embryonic lethality, thereby preventing analysis of their functions in muscle cells. In contrast, tensin-null mice progressively develop cystic kidneys but are viable for several months, which provides us with an *in vivo* system to study the role of focal adhesions in skeletal-muscle regeneration.

Tensin binds to the barbed ends of actin filaments and is able to cross-link actin filaments [16,17]. Increased tyrosine phosphorylation of tensin was detected when cells were attached to extracellular matrix [18], or treated with platelet-derived growth factor [19], thrombin or angiotensin [20], as well as when transformed by oncogenes such as *v-src* or *bcr/abl* [21,22]. Tensin also contains a Src homology 2 (SH2) domain, which is able to interact with certain tyrosine-phosphorylated proteins such as phosphoinositide 3-kinase (PI 3-kinase) and p130Cas [22,23]. In addition, tensin also shares sequence homology with a tumour suppressor, PTEN/MMAC 1 (phosphatase and tensin homologue on chromosome 10/mutated in multiple advanced cancers) [24,25]. Integrin aggregation results in recruitment of tensin at focal-adhesion sites [26]. These findings suggest that tensin plays roles both as a structural protein and in signal transduction [27]. Like other focal-adhesion molecules, including talin, vinculin, paxillin and integrin  $\beta 1$ , tensin is localized at the dense plaques in smooth muscles [28]. It is also found in neuromuscular, myotendinous junctions and along the sarcolemma of skeletal muscles [29], implying that tensin may play a role in maintenance of muscle function. Although tensin is not required for myogenesis, since tensin-null mice have normal muscle development [30], the potential role of tensin in muscle regeneration has not been explored.

In the present study, we have used tensin-knockout (KO) mice as an animal model to examine the role of focal adhesions in skeletal-muscle regeneration induced by cardiotoxin, which provokes degeneration of muscle fibres while leaving the nerve and blood supply intact. We found the following: (i) after injury,

Abbreviations used: KO, knockout; WT, wild-type; HE, haematoxylin and eosin; DMEM, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; MyoD, myogenic-determination gene D; PI 3-kinase, phosphoinositide 3-kinase; BrdU, bromodeoxyuridine.

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skeletal muscle can regenerate successfully in tensin-KO mice; (ii) the regeneration process is delayed about 2-fold [from 4 weeks in wild-type (WT) to 8 weeks in KO mice]; (iii) the regeneration delay is a combination of defects in cell activation, proliferation, differentiation and fusion; and (iv) the regeneration delay probably contributes to the 9-fold increase in centrally nucleated fibres found in KO mice.

## MATERIALS AND METHODS

### Animals

Tensin-null mice were generated as described [30]. All regeneration experiments were done on 8-week-old KO mice with WT or heterozygous littermates as a control. All animals were handled in accordance with guidelines of the University of California-Davis, Sacramento, CA, U.S.A.

### Intramuscular administration of cardiotoxin and histochemistry

We injected 100  $\mu$ l (10 mM in 0.9% NaCl) of cardiotoxin (Sigma, St. Louis, MO, U.S.A.) into the anterior tibial muscle of 8-week-old tensin-KO mice and WT mice using a 27-gauge needle and a 1 ml syringe. The needle was inserted deep into the anterior tibial muscle longitudinally towards the knee from the ankle. The needle was held in place for a few seconds, and then slowly withdrawn along the long axis of the anterior tibial muscle with a little pressure to allow permeation of the cardiotoxin throughout the muscle. The anterior tibial muscles were isolated 0, 1, 2, 3, 5, 7, 14, 28 and 56 days after the injection and were frozen in liquid-nitrogen-cooled isopentane. There were three mice in each group. Serial transverse sections (10  $\mu$ m) were stained with haematoxylin and eosin (HE). The diameters of myofibres (750–900 in three sections of a mouse and three mice for each time point) were measured using Scion Image 1.62a. The Mann-Whitney U test was used for statistical analysis between KO and WT mice.

To determine the proliferating cells during muscle-fibre regeneration after cardiotoxin injection, bromodeoxyuridine (BrdU; 200 mg/kg) was administered intraperitoneally as a single pulse at 1, 3, 5 and 7 days after the cardiotoxin treatment. The muscles were dissected from the treated mice 1 h after the BrdU injection. The sections were then fixed with acetone for 10 min at  $-20^{\circ}\text{C}$ , treated with 1.5 M HCl for 30 min, washed with PBS and incubated with anti-BrdU antibody (Zymed, South San Francisco, CA, U.S.A.) for 16 h at  $4^{\circ}\text{C}$ , after blocking with 2% BSA in PBS. Subsequently, biotinylated secondary antibody and ABC reagent (Vector Laboratories, Burlingame, CA, U.S.A.) was applied to the sections for 30 min each, after washing with PBS. The sections were developed in diaminobenzidine (Vector Laboratories) and hydrogen peroxide.

### Immunofluorescence staining of frozen sections

For immunostaining, 8  $\mu$ m-thick frozen sections were fixed in cooled acetone ( $-20^{\circ}\text{C}$ ) for 10 min. The sections were then incubated in PBS for 10 min, treated with blocking solution for 30 min and incubated with primary antibody for 1 h. We used dystrophin (NCL-DY2, monoclonal antibody, 1:20; Novocastra, Burlingame, CA, U.S.A.) and desmin (polyclonal antibody, 1:20; Sigma). After washing with PBS, the sections were incubated with secondary antibody conjugated with Texas Red for 45 min. Samples were visualized with a Zeiss Axioplan microscope equipped with epifluorescent optics.

### Western blotting

Frozen tissues were homogenized in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA and 1% Nonidet P40), including protease inhibitors (1 mM PMSF, 10  $\mu$ g/ml leupeptin and 10  $\mu$ g/ml pepstatin). Samples were centrifuged at 14000 *g* in a microfuge at  $4^{\circ}\text{C}$  for 15 min. Protein concentration was measured using BioRad protein assay reagent. Protein (40  $\mu$ g) was subjected to SDS/PAGE on 8% gels. Proteins were then transferred on to nitrocellulose membranes that were stained with Ponceau S to verify equal loading and transfer. Membranes were incubated with blocking solution containing 5% skimmed milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl and 0.1% Tween 20) for 1 h at room temperature. The membrane was incubated with primary antibody in blocking solution at room temperature for 1 h. After washing with TBST for 10 min each, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG for 1 h at room temperature. After washing three times, the bound antibody was visualized using the ECL Western-blotting reagent (Amersham). Antibodies against paxillin (Transduction Laboratory),  $\alpha$ -syntrophin,  $\beta$ -dystroglycan, adhalin, myosin heavy chain (Novocastra) and pan-actin (Sigma) were used.

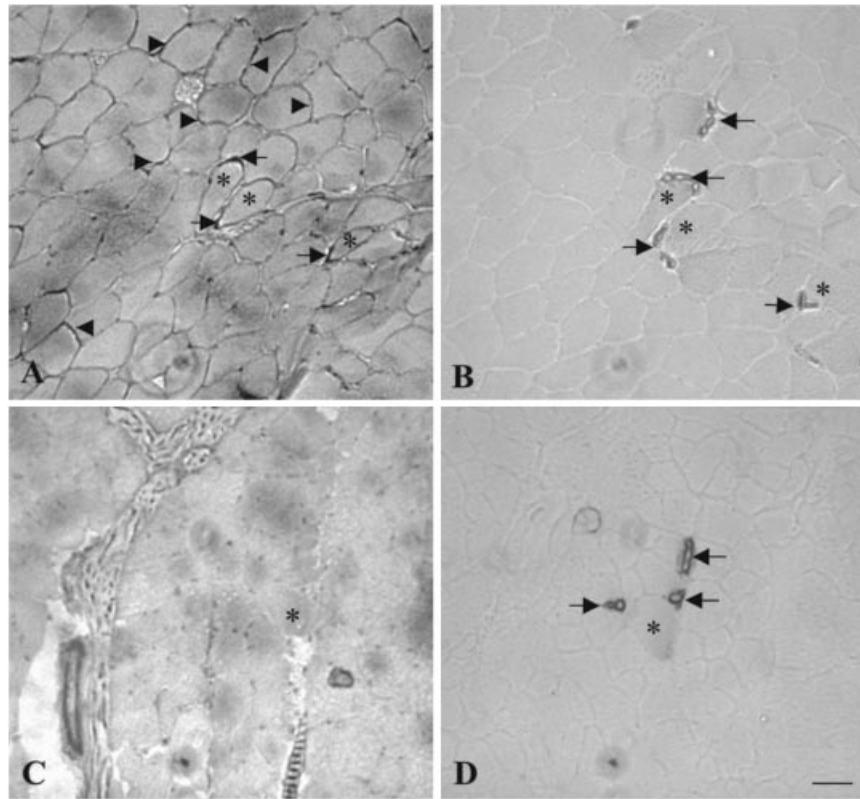
### Preparation of primary myogenic cells from muscles of WT and tensin-KO mice

Myofibres from tensin-KO and WT mice (8 weeks old) were isolated from anterior tibialis and extensor digitorum longus. Dissected muscles were treated with 0.54% collagenase type I (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) in Dulbecco's modified Eagle's medium (DMEM) at  $37^{\circ}\text{C}$  for 2 h. Muscle masses were dispersed and transferred to Matrigel (Becton Dickinson)-coated dishes using fire-polished Pasteur pipettes. Isolated cells were grown in DMEM supplemented with 20% fetal bovine serum (growth medium). Differentiation medium consisted of DMEM supplemented with 2% fetal bovine serum. All media contained penicillin G (200 units/ml), streptomycin (200 mg/ml) and fungizone (2.5 mg/ml), all from Gibco BRL. Cells were grown in a humidified incubator at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . Second and third passages of primary culture were used in this study.

### Assessment of growth and fusion indices of primary culture

To assess proliferative potential,  $1 \times 10^5$  cells were plated in 35 mm type-I-collagen-coated dishes in growth medium, and after 24 h the medium was replaced with low-serum (2%) medium. Control dishes were maintained in growth medium. The cells were cultured 1, 2, 3 or 5 days before the addition of 10 mM BrdU for 1 h. The dishes were then fixed with 3.7% formaldehyde for 5 min. BrdU staining was performed as described above.

To assess differentiation potential,  $5 \times 10^4$  cells were plated in 35 mm type-I-collagen-coated dishes with coverslips in growth medium. After 24 h, the growth medium was replaced by differentiation medium. The fusion index, measured on days 1, 2, 3, 5 and 7 after the medium was changed, was determined as the ratio of the number of nuclei in myotubes (cells with  $\geq 3$  nuclei) to the total number of nuclei [31]. Each fusion index was the mean count from three coverslips for each time point using three independently isolated primary cells.



**Figure 1** Localization of tensin in skeletal muscles

Frozen sections ( $8\ \mu\text{m}$ ) from WT (**A, B**) and tensin-KO (**C, D**) anterior tibial muscles were immunolabelled with anti-tensin antibody (**A, C**). Tensin was detected at the peripheries of muscle fibres (arrowheads) and neuromuscular junctions (arrows) in WT mice. The consecutive sections in (**B**) and (**D**) were stained with acetylthiocholine iodide, which detects acetylcholinesterase, to show neuromuscular junctions. Some of the muscle fibres that form neuromuscular junctions are designated with asterisks. There is no tensin staining in the KO muscle fibres (**C**). Scale bar,  $50\ \mu\text{m}$ .

## RESULTS

### Localization of tensin at the periphery of normal myofibres

Previously, we have detected tensin mRNA in mouse skeletal muscle [30]. To examine the localization of tensin in the skeletal muscle, anterior tibial sections from KO and WT mice were labelled with anti-tensin antibody. Tensin was detected at the peripheries of the normal myofibres, but not in KO muscle (Figures 1A and 1C). Consecutive sections were labelled for acetylcholinesterase to show neuromuscular junctions (Figures 1B and 1D), and it appeared that tensin was concentrated at the neuromuscular junctions.

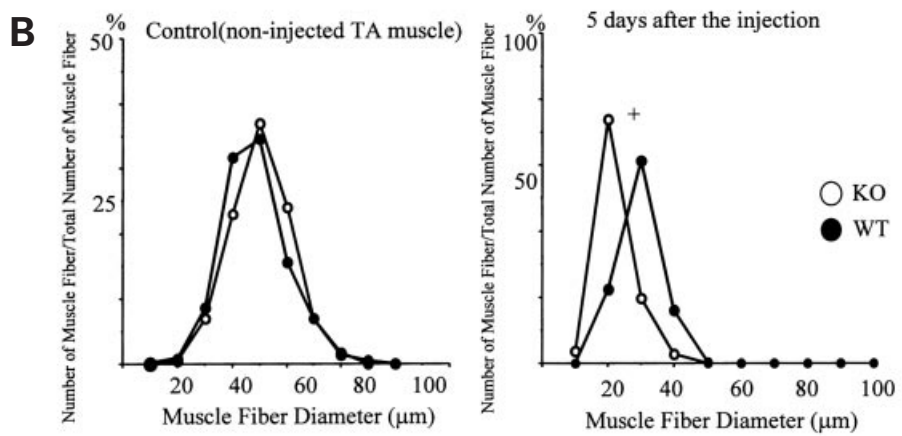
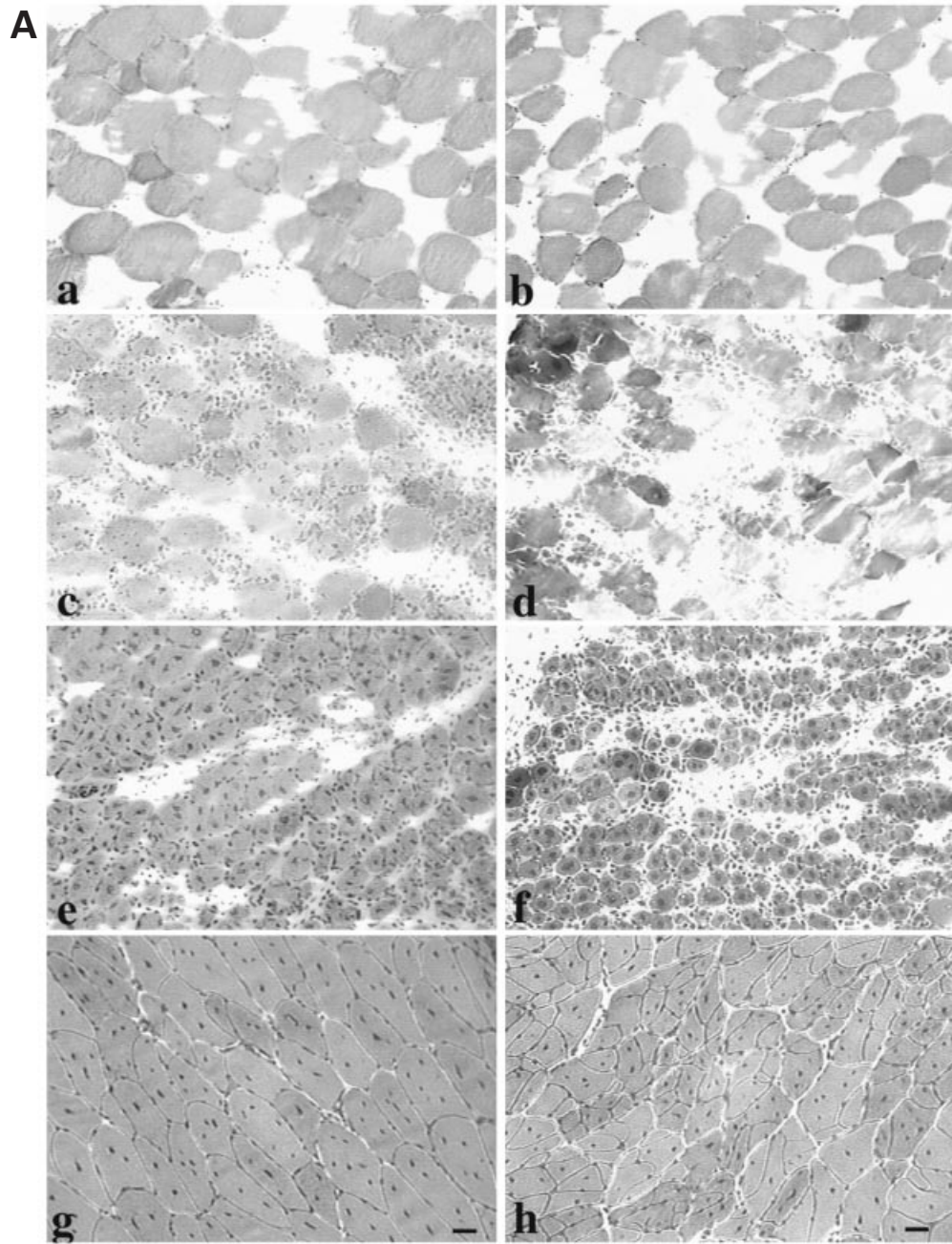
### Muscle-regeneration processes were delayed in tensin-null mice

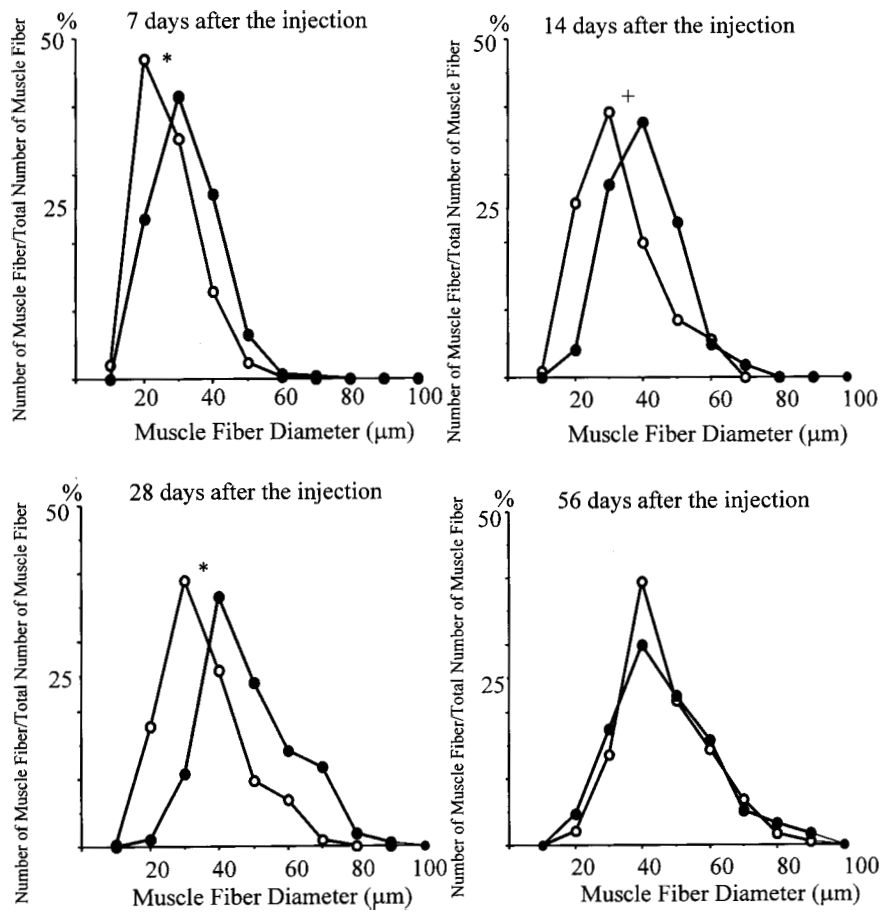
To test the hypothesis that tensin is involved in muscle regeneration, we have examined the regeneration process of experimentally damaged skeletal muscle in tensin-null mice. Cardiotoxin ( $100\ \mu\text{l}$ ) was injected into anterior tibial muscles of WT and tensin-KO mice. Necrosis and degeneration of the muscle fibres were observed in both WT and KO mice 1 day after injection (Figure 2A). Then, 3 days after injection, myoblasts and cellular infiltration, such as that of leucocytes and macrophages, were observed in WT and KO mice. Regenerating multinucleated myofibres were found 5 days after injection, and the diameters of myofibres of KO mice were smaller than those of

WT mice. The nuclei were located in the centre of WT and KO myofibres 28 days after injection, but the diameters of the KO myofibres were smaller than those of WT mice. These results were reproducible in three sets of mice. In one set we injected less cardiotoxin ( $80\ \mu\text{l}$ ) in KO mice and found similar results, indicating that the regeneration delay was not due to receiving more toxin in KO mice.

The diameters of 750–900 myofibres were assessed from each mouse for a total of three animals at each time point. The results were presented by the line graphs shown in Figure 2(B). The modal diameters (the diameters that occurred most frequently) of KO and WT myofibres were essentially the same prior to the injection. Following the injection (5–28 days), the modal diameter of regenerated KO myofibres was significantly lower than that of WT myofibres. At day 28, the modal diameter of the WT muscle fibres was around  $40\ \mu\text{m}$ , similar to the size of non-injected muscle fibres. Then, 56 days after injection, the modal diameter of the KO myofibres was approx.  $40\ \mu\text{m}$ , the same as those of WT and KO non-injected mice. Skeletal muscle of tensin-KO mice displayed a prominent regeneration delay that was apparent 5 days post-injection, as judged by histological analysis. These results demonstrated clearly that the regeneration process was much slower in tensin-null skeletal muscles than in WT muscle fibres.

We also examined the expression of dystrophin during this regeneration process. As shown in Figure 3(a), dystrophin was





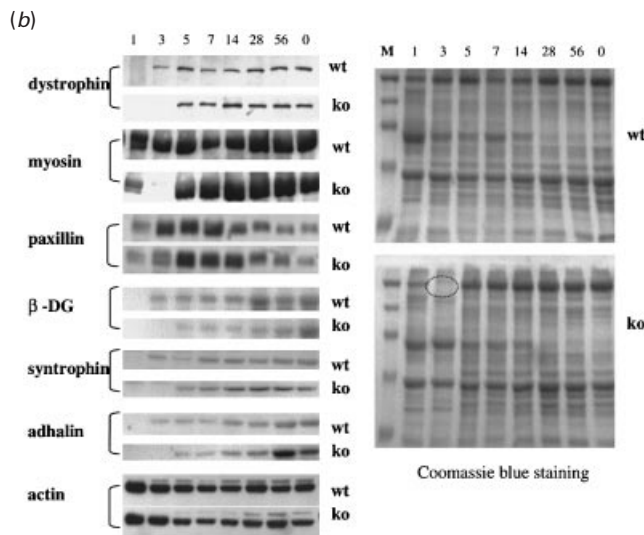
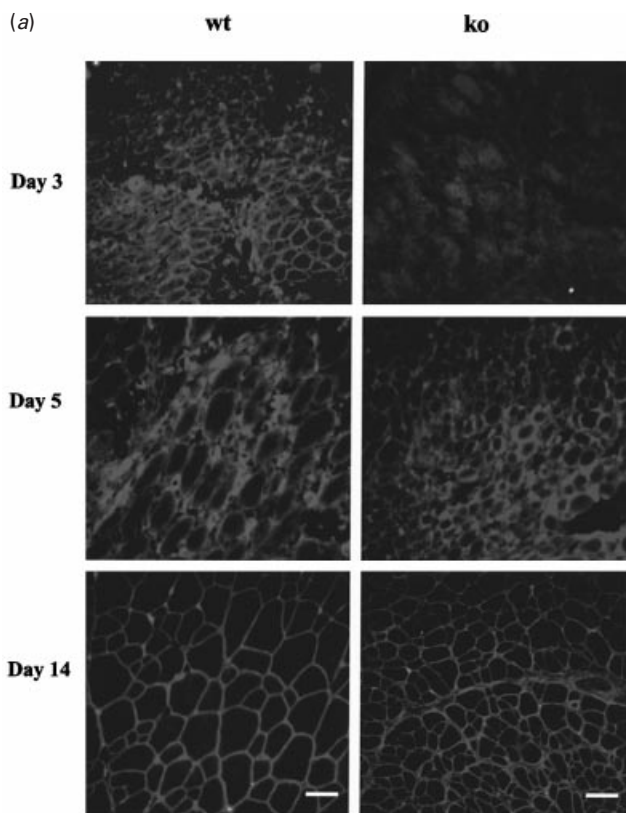
**Figure 2** Muscle regeneration process was delayed in tensin-null mice

(A) Histology of the regeneration process (HE staining) induced by cardiotoxin in WT mice (a, c, e, g) and tensin-KO mice (b, d, f, h). Muscle fibres were damaged by cardiotoxin in WT and tensin-KO mice 1 day after the injection (a, b). The necrotic fibres are pale and obscure after HE staining. Cell infiltrations, such as macrophages, were also found around the necrotic fibres. On day 3, many cells, recognized by their nuclei, accumulated at the wounded sites (c, d). New myofibres identified by central nuclei were formed on day 5 (e, f). The muscle fibres of tensin-KO mice are smaller than those of WT mice. Regenerated muscle fibres with central nuclei were seen in WT and KO mice 28 days after the injection (g, h). The diameters of muscle fibres in tensin-KO mice (h) are significantly lower than those in WT mice (g). Each photo is a representative sample from three animals. Scale bar, 50 μm. (B) The distributions of muscle-fibre diameters for WT (●) and tensin-KO (○) mice are shown for the control and for 5, 7, 14, 28 and 56 days after the cardiotoxin injection. There was no significant difference between WT and KO in non-cardiotoxin-injected control muscles. On day 5, the diameters of KO muscle fibres were significantly smaller than those of WT mice ( $^*P < 0.0002$ ). During growth of the muscle fibres, the differences remained significant. The diameters of the tensin-KO muscle fibres were significantly smaller than those of WT ones on days 7 ( $^*P = 0.0001$ ), 14 ( $^+P = 0.0002$ ) and 28 ( $^*P = 0.0001$ ) after the injection. On day 56 after the injection, the diameters of KO muscle fibres appeared to be the same as those of WT mice ( $P = 0.5518$ ). TA muscle, tibial anterior muscle.

detected in the regenerated muscle fibres of WT mice at day 3, whereas very weak and diffuse dystrophin staining, if any, was observed in KO muscle fibres. At day 5, dystrophin was detected at the peripheries of WT and KO myofibres. At day 14, both WT and KO myofibres showed the characteristic dystrophin staining pattern. Western-blot analysis using the anti-dystrophin antibody also detected a similar result (Figure 3b). On the Coomassie Brilliant Blue-stained gels, we found reduced expression of a 200 kDa protein in the day-3 KO sample. By Western blotting, we confirmed that this was the myosin heavy chain. In addition, the expression of paxillin appeared to be delayed too; the high-expression peaks shifted from days 3–5 in WT samples to days 5–14 in KO samples. Other dystrophin-complex proteins such as  $\beta$ -dystroglycan,  $\alpha$ -syntrophin and adhalin ( $\alpha$ -sarcoglycan) were also delayed. The delayed expression patterns of these proteins suggest a cell-differentiation defect in KO mice.

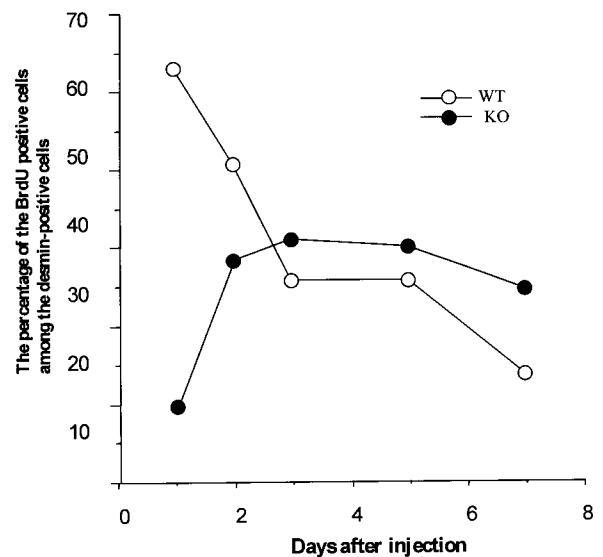
To assess the rate of cell proliferation and differentiation during the regeneration process, we injected BrdU into the

anterior tibial muscle 1 h prior to killing, and double labelled for BrdU incorporation and for desmin, a differentiation marker for myoblasts. In WT mice, 62% of cells were BrdU- and desmin-positive (proliferating myoblast) cells 1 day after injection (Figure 4). This number subsequently decreased rapidly when WT cells withdrew from the cell cycle. On day 7, the percentage of BrdU- and desmin-positive cells decreased to 17.5%, reflecting continuing differentiation at this stage since differentiated myoblasts do not proliferate. In contrast, only 14% proliferating myoblasts were found in KO mice 1 day after the injection, suggesting a delay in converting satellite cells (desmin-negative) to myogenic precursor cells (desmin-positive). On day 2, KO mice had 34% BrdU- and desmin-positive cells, and this number remained similar for the next few days. On day 7, 29.7% of cells were BrdU- and desmin-positive in KO mice, indicating that there was a higher proliferating myoblast population in the KO regenerating sites on day 7 (Figure 4). These findings indicate that the regeneration delay in KO mice starts at 1 day post-injection.



**Figure 3 Protein-expression profiles during muscle regeneration in WT and tensin-KO mice**

(a) The expression of dystrophin was detected around the muscle fibres 3 days after injection into WT muscles, but was diffuse in KO muscles. Dystrophin staining was seen around the regenerated muscle fibres 5 days after injection in WT and KO muscles. Dystrophin was expressed around the muscle fibre 14 days after injection in WT and KO muscles. Note that the muscle-fibre diameters of tensin-KO mice are significantly smaller than those of WT mice. Scale bar, 75  $\mu$ m. (b) Western-blot analysis of lysates (40  $\mu$ g) prepared from WT or KO anterior tibial muscles at different time points with anti-dystrophin, myosin heavy chain, paxillin,  $\beta$ -dystroglycan ( $\beta$ -DG),  $\alpha$ -syntrophin, adhalin and actin (against all isoforms) antibodies. Gels were stained with Coomassie Brilliant Blue to show the equal total-protein loading. Note that the reduced expression of a 200 kDa protein was detected in the 3-day KO sample (circle). This protein is most likely to be the myosin heavy chain. Protein markers (M), 212, 122, 83, 51 and 32 kDa.



**Figure 4 Myoblast proliferation during muscle regeneration in WT and KO mice**

Proliferating myoblasts were detected by BrdU and desmin double labelling. The percentage of BrdU-positive cells among desmin-positive cells decreased from 62% on day 1 to 17.5% on day 7 in WT muscles ( $\circ$ ). In contrast, it was 14% on day 1 and remained around 30% on days 2–7 in KO muscles ( $\bullet$ ).

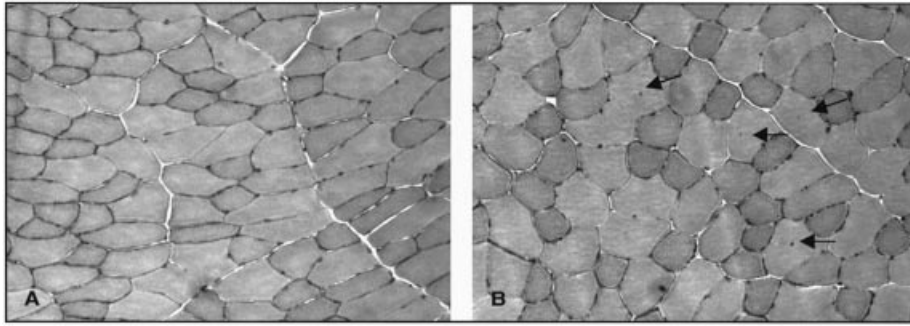
**Table 1 Reduced differentiation potential of tensin-KO primary muscle cells**

(a) The percentage of BrdU-positive cells among desmin-positive cells in WT and tensin-KO cells. There was no significant difference between BrdU-positive cells in WT and KO cells in the growth medium [20% fetal bovine serum (FBS) in DMEM]. The percentages of BrdU-positive cells decreased in the WT cells after changing from growth medium to differentiation medium (2% FBS in DMEM) for 24 h, whereas the percentages of BrdU-positive KO cells did not decrease as rapidly. There were significantly more BrdU-positive KO cells than WT cells in differentiation medium for days 1 and 2. However, there was no difference after 3 days in differentiation medium. (b) The fusion indices of WT and KO primary muscle cell cultures. The fusion index was determined as the ratio of the number of nuclei in myotubes (cells with three or more nuclei) over the total number of nuclei. This index was determined using cells 1, 2, 3, 5 and 7 days after changing from growth to differentiation medium. The fusion indices for the tensin-KO cells were significantly lower than those of the WT cells. All values are the means  $\pm$  S.D. from three separate experiments.

Conditions	Proliferative cells (%)	
	WT cells	KO cells
20% FBS in DMEM	19.3 $\pm$ 1.1	18.4 $\pm$ 2.3
2% FBS in DMEM		
Day 1	3.1 $\pm$ 0.4	8.6 $\pm$ 0.9
Day 2	2.0 $\pm$ 0.4	4.3 $\pm$ 1.4
Day 3	1.3 $\pm$ 0.4	2.0 $\pm$ 0.3
Day 5	0.9 $\pm$ 0.1	1.0 $\pm$ 0.1

Day	Fusion index	
	WT cells	KO cells
1	36.0 $\pm$ 4.6	19.3 $\pm$ 4.8
2	48.8 $\pm$ 5.4	27.5 $\pm$ 4.5
3	57.3 $\pm$ 4.4	31.9 $\pm$ 4.7
5	61.0 $\pm$ 7.7	41.5 $\pm$ 5.3
7	71.6 $\pm$ 7.9	45.0 $\pm$ 10.4



**Figure 5** Tensin-KO anterior tibial muscle contained more centrally nucleated fibres

Cryosections from anterior tibial muscle of 8-week-old WT (A) and tensin-KO (B) mice were stained with HE. No significant difference of muscle-fibre sizes between WT (A) and tensin-KO (B) muscles were found. No necrotic fibres were observed in either set of mice. However, more centrally nucleated fibres (arrows) were found in KO mice. Each photo is representative of three animals. Magnification,  $\times 70$ .

#### Inefficient cell-cycle withdrawal and terminal differentiation initiation in tensin-KO primary myoblasts

Satellite cells are quiescent in adult mice, but re-enter the cell cycle following injury to generate a population of proliferating myoblasts that again cease dividing, and subsequently fuse to form new myofibres [32]. Our *in vivo* results showed that although the proliferation rate was lower and there was an overall delay in the process, the regeneration process continued for longer in KO mice. To assess further the ability to withdraw from the cell cycle and to initiate differentiation, low-passage primary cultures isolated from skeletal muscles of 8-week-old WT and KO mice were used to measure the BrdU-positive myoblasts after replacing growth with differentiation medium for lengths of time (Table 1). Although the proliferating ratios were the same when cultured in growth medium ( $19.3 \pm 1.1\%$  versus  $17.4 \pm 2.3\%$  for WT and KO, respectively), about 80% of the proliferating cells withdrew from the cell cycle in WT myoblasts within 24 h, whereas only 50% withdrew in the KO cells (WT and KO,  $3.1 \pm 0.4\%$  versus  $8.6 \pm 0.9\%$ ). Even on the second day, there were more proliferating cells in the KO cells (WT and KO,  $2.0 \pm 0.4\%$  versus  $4.3 \pm 1.4\%$ ). However, there was no difference between WT and KO cells in terms of proliferation rate after 3–5 days.

To evaluate the fusion capacity, primary cultures were examined for the fusion index. These cells were maintained in growth medium and then shifted to differentiation medium, in which cells initiated terminal differentiation and fused into multi-nucleated myofibres. The fusion index represents the percentage of myoblasts that have fused into myofibres. The fusion indices increased from 36.0 to 71.6% in WT and 19.3 to 45.0% in KO cells within 7 days (Table 1). Our results clearly showed a defect in fusion capacity in KO primary cells.

#### Tensin-null skeletal muscle contains increased numbers of central nuclei

If normal muscles need to constantly refurbish regenerated fibres, and this process is much slower in tensin-KO mice, then we would expect to find more regenerated fibres in the KO mice. Although no fibre necrosis or phagocytosis was detected, more centrally nucleated fibres were detected by HE staining (Figure 5) in 8-week-old tensin-null mice than in their WT counterparts. There was an approx. 9-fold increase in the number of these

nuclei in the KO muscles ( $3.6 \pm 0.9\%$ ) compared with those of the WT muscles ( $0.4 \pm 0.2\%$ ). This ratio did not alter with age (12-month-old WT versus KO muscles,  $0.6 \pm 0.4\%$  and  $3.5 \pm 0.5\%$ ). Since the nuclei of mature skeletal-muscle fibres normally reside peripherally, the increase in centrally nucleated fibres indicates a higher population of premature muscle fibres.

#### DISCUSSION

Skeletal-muscle regeneration after injury is characterized by the activation and proliferation of muscle precursor cells, differentiation of these cells and their fusion with each other to form new multi-nucleated myofibres. Defects in any of these steps will impair the regeneration process. In our experimental system, the number of BrdU- and desmin-positive cells decreased from 61% on day 1 to 17.5% on day 7 in the WT mice. In contrast, KO mice had about 14% BrdU- and desmin-positive cells on day 1 and around 30% were found on days 2–7. This finding was apparently due to the defective satellite-cell activation in KO mice. However, the delay of satellite-cell activation cannot fully account for the characteristic muscle abnormality of mutant mice, since we should have detected around 60% BrdU- and desmin-positive cells on day 2 in KO mice. It is more likely that the satellite-cell activation and proliferation were both impaired. Why did the population of BrdU- and desmin-positive cells remain at about 30%? It is possible that either (i) a longer time was needed to generate sufficient myoblasts for fusing into myofibres in KO mice and/or (ii) proliferating tensin-null myoblasts were inefficient at cell-cycle withdrawal. Although we have detected a difference in the population of proliferating cells in animals, we have found no difference in cultured WT and KO myoblasts under the favoured conditions for proliferation, presumably because both cell types have adapted to the artificial environment. However, even with the same proliferative competence, cultured KO myoblasts appear to exit from the cell cycle, and initiate terminal differentiation upon mitogen removal less efficiently than their WT counterparts. Consequently, a reduction in fusion capacity was also observed in tensin-KO myoblasts. Our *in vitro* results do not exclude possibility (i), but clearly support the alternative hypothesis that cell-cycle withdrawal and myofibre fusion defects in the KO animals are not simply due to the delay and reduction in satellite-cell activation

and proliferation, but also contribute to the slower regeneration process observed in tensin-null skeletal muscles.

The detection of a 9-fold increase in the number of centrally nucleated fibres in the 8-week-old KO muscles strongly supports our finding that the regeneration process is defective without tensin. Although it is also possible that tensin-KO muscles are more fragile and degenerate faster, thereby requiring more regenerating fibres to compensate for their loss, we did not detect fibre necrosis, phagocytosis or increased endomysial connective-tissue production in the KO mice (results not shown). Also, the fact that the same percentage of centrally nucleated fibres was found in older (12 month) KO mice argues against the possibility that KO muscles are more fragile.

Myogenic-determination gene D (MyoD)-null and fibroblast growth factor (FGF)-6-null mice have normal myogenesis, but are severely deficient in their muscle-regeneration capacity. In the case of MyoD<sup>-/-</sup> mice, there was increased muscle stem-cell renewal and reduced satellite-cell differentiation after injury [33], and FGF-6<sup>-/-</sup> mice contained normal satellite cells but failed to activate for proliferation after trauma [34]. Tensin-null mice represent another type of regeneration defect. Tensin is neither a transcription factor like MyoD, nor a growth factor such as FGF-6. How could tensin function in the regeneration process? One possibility is through integrin-mediated signalling events. Recent studies with avian cells have demonstrated that integrins  $\alpha 5$  and  $\alpha 6$  indirectly regulated proliferative versus differentiative signals through  $\beta 1$  integrin [13]. Ectopic  $\alpha 5$  expression promoted proliferation, whereas ectopic  $\alpha 6$  expression promoted differentiation. Tensin is recruited to the focal-adhesion complex by  $\alpha 5\beta 1$  integrin aggregation [26] and always co-localizes with  $\alpha 5\beta 1$  [35]. The lack of a tensin- $\alpha 5\beta 1$  association may perturb the role of the integrin complex in promoting proliferation. Additionally, other pathways may be affected by tensin ablation, since cells could not efficiently exit from the cell cycle and promote differentiation. Another potential route is through PI 3-kinase. PI 3-kinase activity is associated with tensin upon growth-factor stimulation [23], and previous reports have shown that PI 3-kinase regulates proliferation and differentiation in muscle cells [36,37]. Perturbation of tensin-PI 3-kinase could potentially impair the downstream signalling events in proliferation, differentiation and/or the actin cytoskeleton [38]. These possibilities remain to be tested in the future.

Using tensin-null mice, we have revealed a role for tensin in skeletal-muscle regeneration. Furthermore, because the regeneration process is only delayed but not disrupted in tensin-null mice, these mice will be a useful system to study various aspects of muscle regeneration and perhaps open a new way for therapy.

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