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Absence of α7 integrin in dystrophin-deficient mice causes a myopathy similar to Duchenne muscular dystrophy

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Both the dystrophin–glycoprotein complex and α7β1 integrin have critical roles in the maintenance of muscle integrity via the provision of mechanical links between muscle fibres and the basement membrane. Absence of either dystrophin or α7 integrin results in a muscular dystrophy. To clarify the role of α7 integrin and dystrophin in muscle development and function, we generated integrin α7/dystrophin double-mutant knockout (DKO) mice. Surprisingly, DKO mice survived post-natally and were indistinguishable from wild-type, integrin α7-deficient and *mdx* mice at birth, but died within 24–28 days. Histological analysis revealed a severe muscular dystrophy in DKO mice with endomysial fibrosis and ectopic calcification. Weight loss was correlated with the loss of muscle fibres, indicating that progressive muscle wasting in the double mutant was most likely due to inadequate muscle regeneration. The data further support that premature death of DKO mice is due to cardiac and/or respiratory failure. The integrin α7/dystrophin-deficient mouse model, therefore, resembles the pathological changes seen in Duchenne muscular dystrophy and suggests that the different clinical severity of dystrophin deficiency in human and mouse may be due to a fine-tuned difference in expression of dystrophin and integrin α7 in both species. Together, these findings indicate an essential role for integrin α7 in the maintenance of dystrophin-deficient muscles.

INTRODUCTION

Muscular dystrophies are a group of heterogeneous genetic disorders. The disturbance of the molecular link between the extracellular matrix and the cytoskeleton underlies the pathologies associated with various types of muscular dystrophy. The absence of dystrophin results in Duchenne muscular dystrophy (DMD), the most common progressive muscle-wasting disease in humans (1,2). Within the dystrophin–glycoprotein complex (DGC), dystrophin is involved in the anchorage of muscle cells to the extracellular laminin network (3–5). The DGC is thought to protect the sarcolemma against the local stresses during muscle contraction and therefore is critical for the integrity of skeletal muscle fibres (6). The absence of dystrophin in DMD patients causes muscle instability and subsequently muscle fibre damage. The gradual loss of muscle fibres contributes to a progressive clinical muscular weakness, which ultimately leads to death post-puberty as a result of either respiratory or heart failure in early adulthood (7,8). *mdx* mice, in which a nonsense mutation in the dystrophin gene results in the absence of the protein (9,10), have been extensively used as an animal mode for DMD. Although the murine equivalent of DMD shares similar pathological features to human patients, *mdx* mice have a normal life span and

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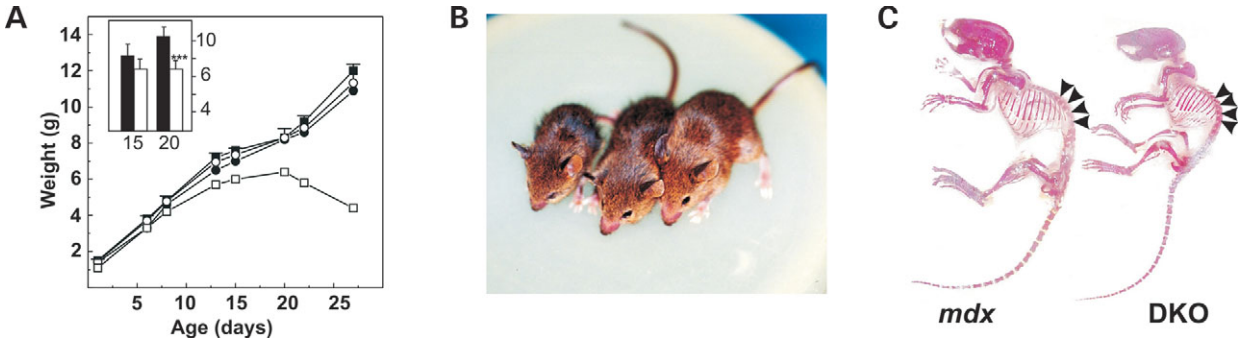

Figure 1. Post-natal development of integrin α7 and dystrophin double-mutant mice. (**A**) Representative graph of gain of weight of mice of one litter. Weight gain is normal until 15 days after birth, after which it is reduced in male and female DKO mice when compared with controls (closed square, female mdx mice; open and closed circles, male and female mdx mice, heterozygous for integrin α7, respectively; open square, female DKO mice). *Inset*: Histogram showing the weight of *mdx* and DKO mice at P15 ($n = 5$) and P20 ($n = 15$). DKO mice are similar in weight to *mdx* mice at P15, but weigh 30–40% less at P20. $^{***}P < 0.0001$. (**B**) DKO mice (left) are viable at P20 but are smaller in size than *mdx* littermates (middle and right). (**C**) Skeleton preparation of 18 days old *mdx* (left) and DKO (right) mice, showing a stronger curvature (arrowheads) of the spine in the double mutant.

hindlimb muscles do not experience the apparent permanent loss of muscle fibres (6). An exception for the mild phenotype is the diaphragm, in which the pathology is comparable to that of DMD limb muscles (11). Differences in the progression of dystrophin-null disease in human and mouse suggest that the *mdx* mouse is not an ideal model for DMD.

Integrins are heterodimeric transmembrane receptors that are linked to the cytoskeleton and mediate cell–cell and cell–matrix interaction (12). Integrins provide thus a similar mechanical link between muscle fibres and the basement membrane as does the DGC (13). We have previously shown that mice carrying an inactivated integrin α7 gene develop a mild but progressive muscular dystrophy (14). Human patients suffering from a congenital myopathy with delayed motor milestones have subsequently been identified due to primary integrin α7 deficiency, which results from mutations in their *ITGA-7* gene (15). The phenotypes displayed both in the mouse model and human patients are highly similar, and de- and regeneration of muscle fibres are not a predominant pathological feature.

Previous data suggest that the DGC and α7β1 integrin are independently controlled laminin receptors. The expression profile of components of the DGC remained unchanged in the absence of α7 integrin in murine and human muscle (14–16). Furthermore, the DGC maintains the sarcolemmal membrane at sites where lateral force transmission occurs in muscle fibres, whereas integrin α7β1 is essential for the integrity of myotendinous junctions (MTJs), where longitudinal force is finally transmitted to the tendon (17,18).

However, previous studies have raised the possibility that α7β1 integrin may functionally compensate for the loss of the DGC. Increased staining intensity for α7β1 integrin has been observed in DMD patients and *mdx* mice (16,19,20). In addition, overexpression of α7 integrin has been shown to improve mobility and increase life span in dystrophin/utrophin double-mutant mice (21,22), supporting a compensatory role for α7β1 integrin in restoring muscle integrity.

In view of the critical roles of both the DGC and α7β1 integrin in the maintenance of muscle integrity, the question has been raised as to the consequences of both complexes being absent. We have therefore interbred integrin α7-deficient mice with *mdx* mice. Double-mutant knockout (DKO) mice develop a severe muscular dystrophy with an early onset of muscle pathology, loss of muscle fibres and premature death post-puberty. DKO mice resemble thus closely the clinical signs of DMD and the data suggest that α7 integrin is essential for the maintenance of dystrophin-deficient muscle in mice. More importantly, our results provide novel evidence that dystrophin and α7 integrin act in concert to maintain muscle regeneration.

RESULTS

DKO mice show early lethality

Integrin α7/dystrophin double-mutant mice were generated by crossing *mdx* mice with mice heterozygous for integrin α7 deficiency. Surprisingly, DKO mice survived post-natally and were indistinguishable from wild-type, integrin α7 null and *mdx* pups at birth. From post-natal (P) day 15, however, DKO mice gained less body weight than their littermate controls (Fig. 1A). By P20, DKO mice displayed a slack posture and appeared smaller than their controls (Fig. 1B). Skeleton analysis revealed a severe curvature of the spine (Fig. 1C), whereas tibia length of the double mutants was comparable to controls, implying that development of the skeleton was unaffected. The severity of symptoms progressed and DKO mice died rapidly 24–28 days after birth with a body weight of only about one-third of *mdx* controls.

Integrin α7/dystrophin double-mutant mice develop a severe muscular dystrophy

In agreement with the reduced weight of DKO mice, transverse sections through the lower hindlimbs were thinner at P20 when compared with control genotypes (data not shown). An overall muscle pathology was apparent in DKO mice, yet the severity differed between muscle types. The flexor muscles including the deeply located flexor hallucis longus and flexor digitorum (FD) longus and plantaris (PA) muscles were affected more severely than the extensors, implying that DKO muscles are more vulnerable to the

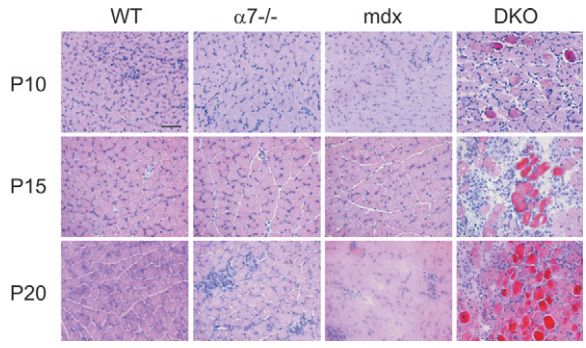

Figure 2. Progressive muscular dystrophy in mice lacking both integrin α7 and dystrophin. H&E staining of transverse sections of flexor hallucis longus and FD longus muscles. Eosinophilic fibres are seen as early as P10 in DKO mice and de- and regeneration are more evident afterwards. *mdx* and integrin α7-deficient mice show only little muscle pathology at P20. Bar: 50 μm.

mechanical tension of plantar flexion. The extensor digitorium longus (EDL) was the least affected muscle in DKO mice. In contrast, obvious de- and regeneration were only detected in the soleus muscle of integrin α7-deficient mice, as previously reported (14), and signs of muscle degeneration were only found in some local areas in *mdx* lower hindlimb muscles, notably in the tibialis anterior and gastrocnemius (GC).

Histological analysis revealed that DKO mice developed a progressive severe muscular dystrophy (Fig. 2). No signs of muscle degeneration were found at P5 (data not shown). At P10, some eosinophilic fibres and centrally located nuclei were found only in hindlimb muscles of DKO mice. Five days later, increased numbers of eosinophilic fibres, fibre size variation and endomysial fibrosis were observed (Fig. 2). From P15 onwards, a pervasive muscular dystrophy was apparent in DKO mice.

Expression of the laminin α2 chain remains unchanged in DKO muscles

Immunofluorescence analysis further confirmed that DKO muscles were null for the expression of dystrophin and integrin α7 (Fig. 3). Utrophin, a dystrophin homologue, is expressed in blood vessels and nerves and normally confined to NMJs and MTJs in skeletal muscle structures (23). In *mdx* mice, utrophin is upregulated and is uniformly found at the sarcolemma (24). In line with these data, utrophin was restricted to blood vessels and NMJs in wild-type and integrin α7-deficient controls and only seen at the sarcolemma in *mdx* and DKO muscles, with a higher staining intensity in the latter because of its regeneration-associated expression (25) (Fig. 3). As previously reported (26), α-dystroglycan and α-sarcoglycan, components of the DGC, were detected at similar levels in *mdx* and DKO muscle, but drastically reduced as demonstrated by staining (Fig. 3) and immunoblotting (data not shown) when compared with controls and staining intensities correlated with utrophin upregulation in the absence of dystrophin (data not shown). From these data, we conclude that dystrophin-associated proteins and utrophin are

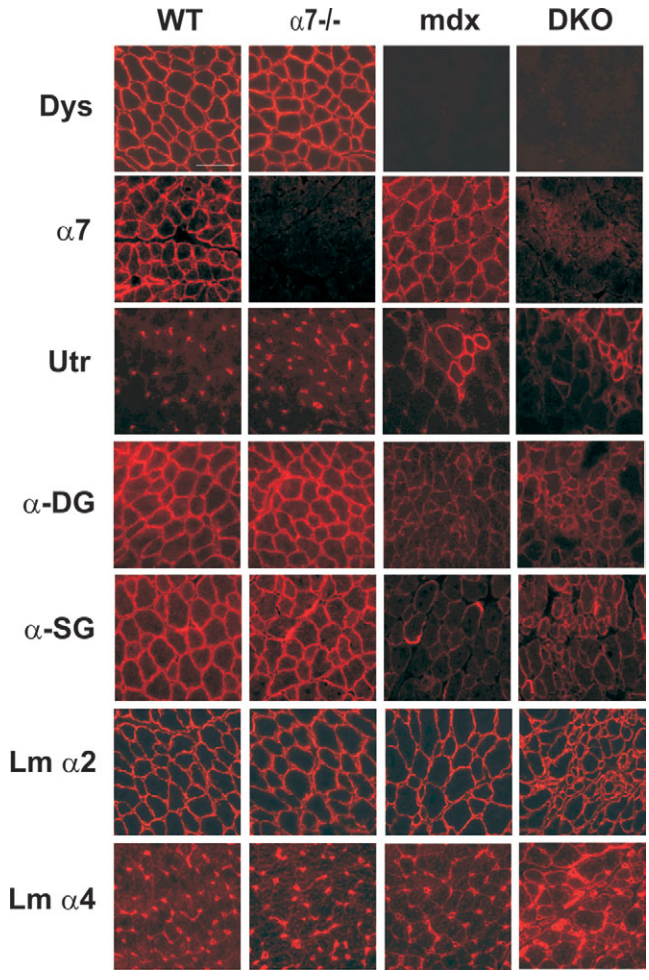

Figure 3. Immunohistochemical characterization of transverse sections of the lower hindlimb at P20. As expected, dystrophin (Dys) and integrin α7 are absent in DKO mice and in mice lacking either of the two individually. Utrophin (Utr) is upregulated in the absence of dystrophin in *mdx* and DKO mice and is uniformly found at the sarcolemma. A further increase in staining for utrophin is seen in regenerating areas. In contrast, utrophin is confined to blood vessels and NMJs in wild-type (WT) and integrin α7-deficient mice. The expression of α-dystroglycan (α-DG) and α-sarcoglycan (α-SG) is greatly diminished in DKO and *mdx* mice when compared with normal. Staining for the laminin (Lm) α2 is unaltered in the four genoptypes. In wild-type and integrin α7-deficient mice, staining for the laminin α4 chain is seen exclusively in blood vessels, whereas it is weakly detectable at the sarcolemma in mdx mice and strongly upregulated in regenerating areas in DKO mice. Bar: 50 μm.

similarly regulated in the absence of both integrin α7β1 and dystrophin as in dystrophin deficiency only, but that compensation by utrophin is not sufficient to protect skeletal muscle integrity.

Integrin α7β1 and the DGC are the main laminin receptors in skeletal muscle. We therefore tested whether the concomitant loss of DGC and integrin α7β1 affected the expression of laminin α chains in the musculature. We did not detect the laminin α1 chain nor was the staining pattern of the laminin α5 chain altered in DKO mice when compared with wild-type controls (data not shown). The laminin α4 chain was only evident in *mdx* and DKO muscle structures. Interestingly,

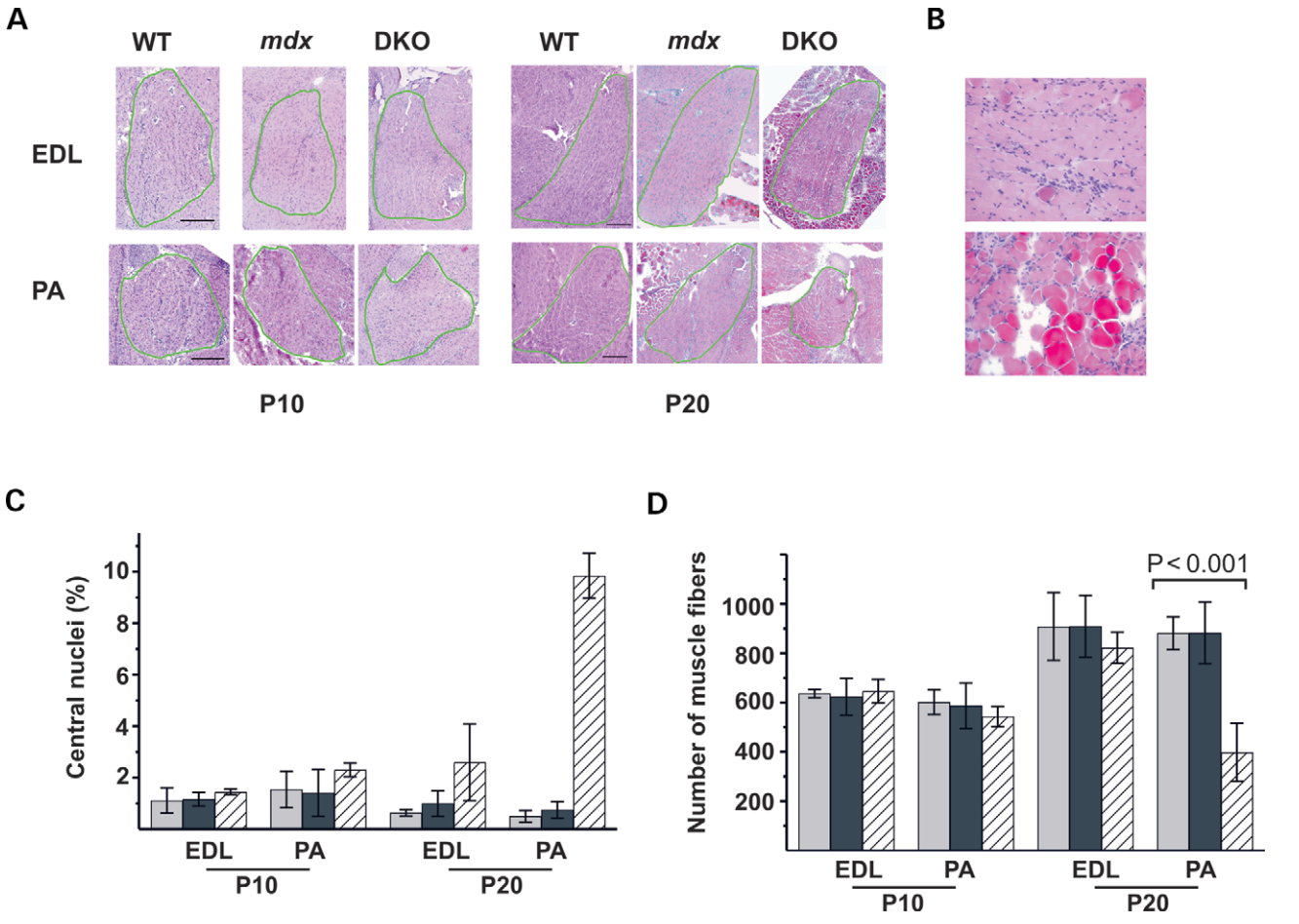

Figure 4. Impaired muscle regeneration in mice lacking both integrin α7 and dystrophin. (**A**) Sections of EDL and PA muscles from the middle part of the lower hindlimb were stained with H&E. The EDL muscle of DKO mice is similar in size to wild-type and *mdx* controls at P10 and P20 (circled areas). In contrast, muscle size is significantly reduced in the PA muscle of P20 DKO mice. Bar: 200 μm. (**B**) PA (bottom) and EDL (top) muscle sections of 20 days old DKO mice. The EDL muscle is only mildly affected, whereas the PA muscle shows many eosinophilic fibres. (**C**) Histogram showing the percentage of centrally located nuclei in the EDL and PA muscles of wild-type (light grey), *mdx* (dark grey) and DKO (hatched) mice at P10 and P20. A low percentage of central nuclei are observed both in EDL and PA muscles of DKO mice. (**D**) Histogram showing the number of muscle fibres in the EDL and PA muscles of wild-type (light grey), *mdx* (dark grey) and DKO (hatched) mice at P10 and P20. Muscle fibre numbers in the DKO EDL at P10 and P20 are comparable to controls, whereas the number of muscle fibres in P20 DKO PA muscle is reduced by 50% ($P < 0.001$).

when compared with *mdx* controls, sarcolemmal staining of the laminin α4 chain was more uniformly distributed in DKO mice and at a higher level (Fig. 3). The laminin α2 chain is the only laminin α chain present post-natally and in the adult in the normal mouse. In addition, secondary loss of integrin α7β1 has been reported in laminin α2 chain deficient muscles from patients and dy/dy mice (16,19,20). Surprisingly, the laminin α2 chain was indistinguishable between the genotypes (Fig. 3) and detectable staining disappeared at the same antibody dilution in DKO mice as in controls. Together, these data suggest that synthesis and assembly of laminin isoforms in skeletal muscle are independent of its receptors.

Muscle regeneration is impaired in DKO mice

DKO mice fail to gain weight 15 days after birth (Fig. 1A) and have significantly smaller hindlimbs than controls. We considered three possibilities leading to the progressive muscle wasting: (i) muscle fibre loss and muscle atrophy caused by insufficient ingestion, (ii) muscle growth arrest caused by developmental defects or (iii) substantial muscle fibre loss independent of ingestion and growth. However, signs of only mild muscle degeneration in striated muscle of the oesophagus (data not shown) did not support the first hypothesis. To assess the other two possibilities, we examined two lower hindlimb muscles, the EDL and the PA, which were only mildly and more severely affected, respectively, and compared muscle size and number of muscle fibres of DKO with *mdx* and wild-type mice in comparable sections (disappearance of the popliteus muscle and appearance of the soleus muscle). At P10, the sizes of the two muscles were indistinguishable between the genotypes (Fig. 4A) and also the number of muscle fibres did not significantly differ in DKO mice (Fig. 4C). At P20, the EDL muscle of DKO mice was similar in size to wild-type and *mdx* controls. In contrast, their PA muscle was dramatically smaller at this age (Fig. 4A). We also observed that despite substantial myopathic

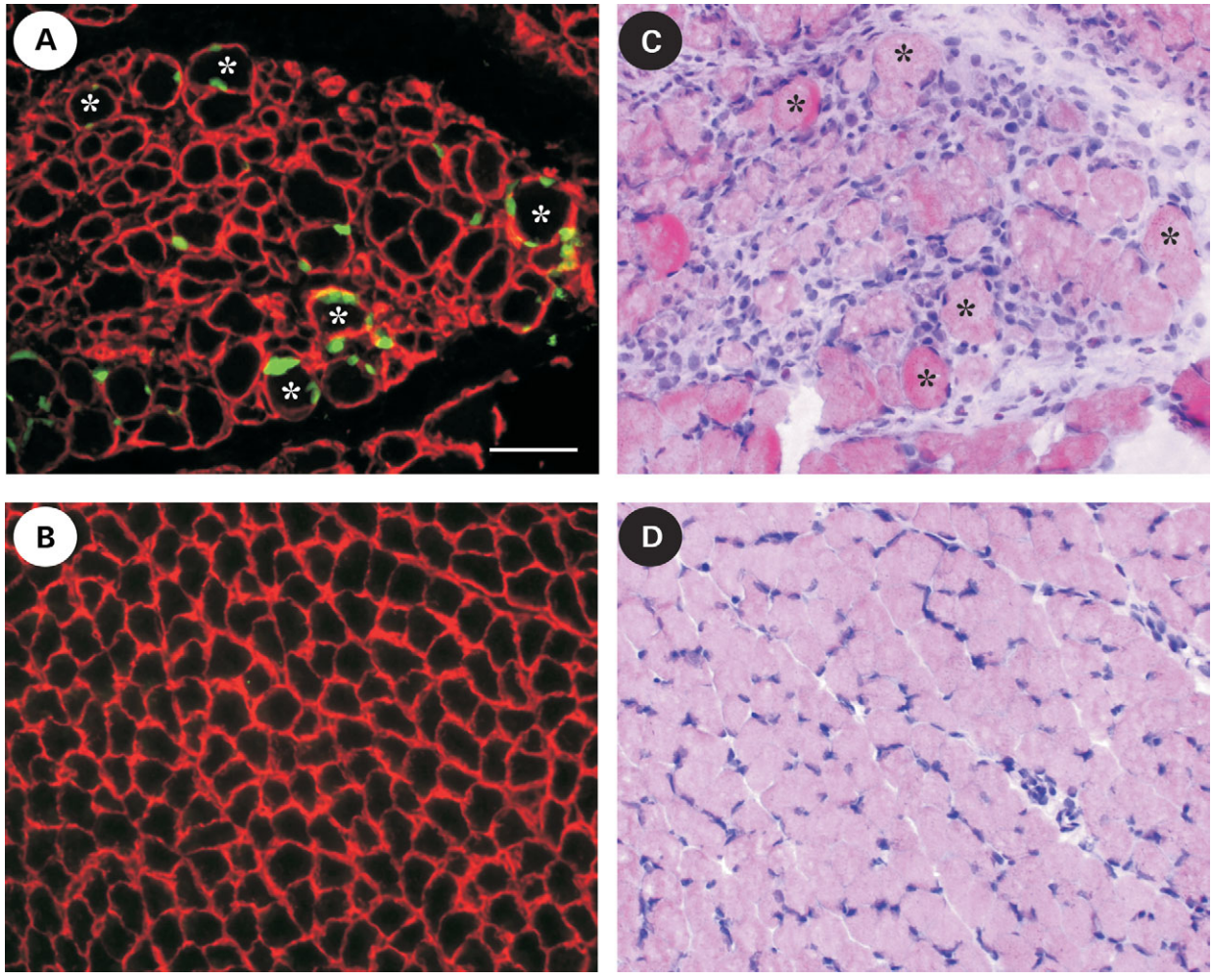

Figure 5. Detection of apoptotic nuclei. Transverse sections of GC muscle of 15-day-old DKO (**A** and **C**) and *mdx* (**B** and **D**) mice were double stained for apoptotic nuclei by TUNEL-labelling (green) and the laminin α2 chain (red) (A and B). Adjacent sections are H&E stained (C and D). Corresponding areas in DKO mice are marked by asterisk. Bar: 50 μm.

changes, there were only few fibres with centrally located nuclei in DKO mice and only around 10% were identified in DKO PA muscles at P20 (Fig. 4B). Counting of muscle fibres demonstrated similar numbers in EDL muscles, whereas a 50% loss of fibres in DKO PA muscle was noted (Fig. 4C). Together, these findings demonstrate that lack of integrin α7 and dystrophin does not interfere with muscle development but results in muscle wasting due to fibre loss.

Apoptosis has been described in muscles from both *mdx* mice (27) and DMD patients (28). To assess the possible role of apoptosis in the loss of DKO muscle fibres, we used TUNEL labelling. Focal staining of some muscle fibres was only detectable in lower hindlimb muscles of DKO mice at P15 (Fig. 5). At P20, the number of TUNEL positive nuclei did not increase and labelled nuclei were also detected in *mdx* controls (data not shown). Staining for activated caspase-3 only occasionally detected positive muscle cells in DKO mice, suggesting that mainly necrotic cells were labelled by TUNEL and further indicates that apoptosis is not essentially contributing to muscle fibre loss in DKO mice.

To evaluate muscle regeneration in DKO mice, we examined the expression of developmental myosin heavy chain (MHCd), a marker for newly regenerated fibres (Fig. 6). At P10, few MHCd-positive small calibre fibres were detected near to sites of degeneration in DKO mice and more evident at P15. Costaining by TUNEL labelling further demonstrated that newly regenerated fibres did not undergo apoptosis. At P20, expression of this myosin isoform became diffuse, but the number of MHCd-positive fibres did not exceed that observed at P15. In view of the low percentages of fibres with centrally located nuclei, these results suggest that loss of muscle fibres in the double mutant was likely due to inadequate regeneration following muscle degeneration.

Death of DKO mice results from cardiac and/or respiratory failure

DKO mice die prematurely 24–27 days after birth. We have, therefore, examined the respiratory and cardiac system, whose failure is the predominant cause of death in DMD patients.

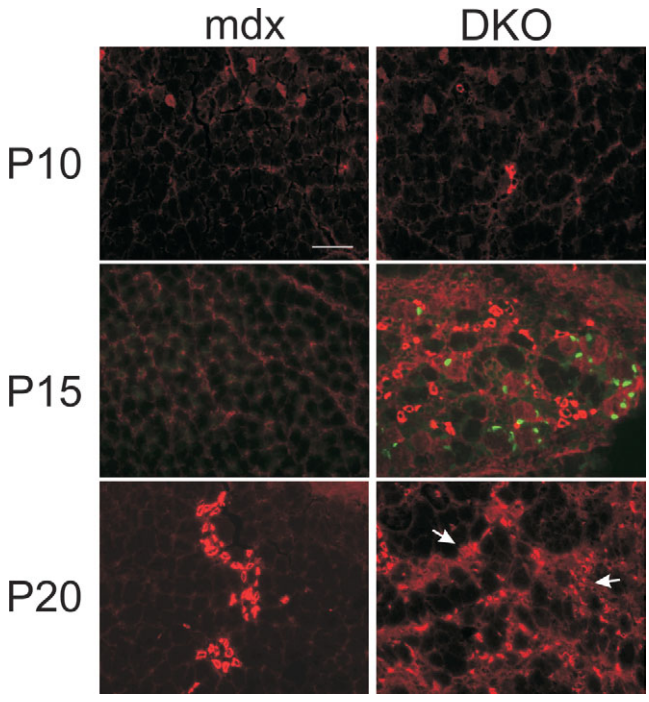

Figure 6. Regeneration in DKO skeletal muscles. Sections from the lower hindlimb of DKO and *mdx* mice were stained for MHCd, highlighting newly regenerated muscle fibres. The first MHCd-positive fibres (red) are detected at P10 and become widespread at P15 in DKO mice. Newly regenerated fibres do not contain TUNEL-positive nuclei (green). Some MHCd-positive areas are found at P20 in *mdx* mice, whereas MHCd staining in DKO mice is becoming less pronounced and more diffuse. Bar: 50 μm.

From P10 onwards, a severe muscular dystrophy was seen in the diaphragm of DKO mice and the pathology in the DKO diaphragms was temporally and spatially more evident when compared with *mdx* controls, at all the ages examined (Fig. 7A). Similarly, the intercostal muscles of DKO mice were more severely affected at P20 than that of *mdx* controls (Fig. 7B). Massive calcification was detected by van Kossa staining in the diaphragm of DKO mice as early as P15, whereas calcification was only occasionally seen in P20 *mdx* diaphragm (in one of three mice examined) (Fig. 7C). This finding implies that absence of α7 integrin may aggravate the abnormal calcium homeostasis in dystrophin-deficient muscles as demonstrated by previous studies (29).

The cardiac muscle of DKO mice appeared generally normal in histological analysis at P20–24, although some eosinophilic myocytes were identified in all hearts analyzed ($n = 9$) (Fig. 8). Similarly, echocardiography did not reveal any significant changes in ventricular diameter, wall thickness or ejection fraction in DKO mice when compared with controls (data not shown). Further analysis by high-resolution electron microscopy, however, demonstrated substantial changes only in DKO mice. Large necrotic areas in both ventricular walls became obvious and disarray of cardiomyocytes was observed ($n = 4$). In addition, accumulation and size variation of mitochondria were only present in the DKO myocardium (Fig. 8). Post-mortem examination showed that DKO lungs were imbued with blood, although no obvious abnormality has been observed in lungs of P20 DKO mice (data not shown). However, we also observed that the size of the atria was increased post-mortem.

These data, along with a more acidic pH measured in the blood and a higher breathing frequency seen in double mutants, indicate congestive heart failure as a likely cause of death (30). In addition, respiratory failure due to the severe myopathy seen in the diaphragm could exacerbate this condition and further contribute to premature death.

DISCUSSION

In this paper, we provide novel data showing that mice lacking both integrin α7 and dystrophin develop a much more severe progressive muscular dystrophy than mice lacking either of the two individually. Thus, mild myopathic phenotypes seen in *mdx* mice result at least in part from the beneficial effect of the presence of α7β1 integrin. Likewise, the presence of the DGC supports muscle integrity in integrin α7-deficient mice. This mutually beneficial effect reinforces the significance of the mechanical link between the basement membrane and the sarcolemma provided by both complexes. It is well established that α7β1 integrin is essential for MTJ stability and the DGC for the integrity of the sarcolemmal membrane of muscle fibres. However, in addition to severe MTJ pathology, a pervasive extrajunctional dystrophic phenotype seen in DKO muscles, together with the sarcolemmal expression of α7 integrin in WT and *mdx* controls, supports a role for α7β1 integrin in maintaining the integrity of muscle fibres.

The loss of a mechanical link between α7β1 integrin and laminin(s) presumably predisposes dystrophin-deficient muscles to additional shear stress, which then leads to exacerbated muscle degeneration. This would provide an explanation for the overall severe muscular dystrophy in integrin α7/dystrophin double-mutant mice. Moreover, because we could not establish a correlation between muscle pathology and muscle fibre type composition in DKO mice (data not shown), differential myopathic severities in the lower hindlimbs of DKO mice are thus likely to reflect the consequences of exposing individual muscles to different mechanical stress. It is unclear, at present, why the EDL is relatively spared from muscle degeneration. However, the EDL muscle is the last lower hindlimb muscle affected in *mdx* mice and is able to tolerate increased mechanical stress in response to repeated tetanic stimulation comparable to wild-type (31). Therefore, it is reasonable to assume that the ability of the extensor to adapt to dorsiflexion stress remains largely intact in the absence of both integrin α7 and dystrophin.

Unexpectedly, immunofluorescence analysis indicates that the absence of integrin α7, the secondary loss of α-DG or both do not affect the expression of the laminin α2 chain in the basement membrane, thereby excluding the possibility of mutual regulation of receptors and the laminin α2 chain. Considering a marginal role of the laminin α4 chain in binding to α-dystroglycan (32), persistent presence of this laminin chain in the basement membrane in *mdx* muscles and at a higher level in DKO muscles may be regarded as either a sign of delayed muscle maturation or a feedback mechanism to promote regeneration upon shear stress injury.

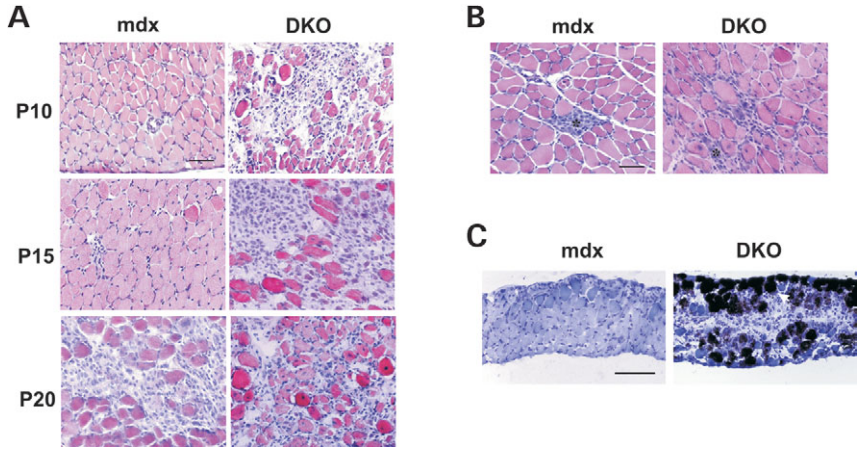

Figure 7. Respiratory muscles in integrin α7/dystrophin-deficient mice. (**A**) H&E-stained diaphragms of *mdx* and DKO mice. DKO mice show already at 10 days of age massive muscle fibre degeneration, whereas mild signs of pathology in the *mdx* diaphragm are only visible at P15 and are extensive by P20. (**B**) H&E-stained intercostal muscles of DKO mice show signs of infiltration (asterisk), fibre size variation and central nuclei at P20, whereas in *mdx* mice, the intercostal muscles are only mildly affected, with some areas of infiltration (asterisk). (**C**) Van Kossa staining shows calcification of muscle fibres in the diaphragm of DKO but not of *mdx* mice at P15. Bar: 50 μm.

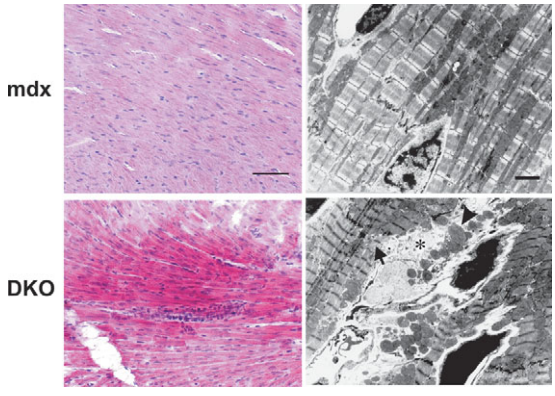

Figure 8. Integrin α7/dystrophin-deficient mice develop a cardiomyopathy. *Left*: H&E stained sections of P20 *mdx* and DKO mice. Eosinophilic cardiomyocytes are seen in DKO but not in mdx mice. *Right*: Ultrastructural analysis of P20 ventricular myocardium shows necrotic areas (asterisk), cardiomyocyte disarray (arrow) and variable size of mitochondria (arrowhead). Bars: 50 μm (left); 1.6 μm (right).

Apparently, normal histology in muscles lacking α7 integrin and dystrophin before P10 suggests that the presence of either protein is not essential for embryonic and post-natal myogenesis, although the possibility of other compensatory mechanisms cannot be excluded at the moment. In contrast, a severe progressive muscular dystrophy from P10 onwards in DKO mice indicates that the presence of α7 integrin is vital for the protection of muscles against early necrosis in the absence of dystrophin. Evidence has accumulated that the presence of integrin α7 is indispensable for the maintenance of muscles following the identification of α7 integrin-null disease in mice and humans (14,15). A recent study demonstrated that the absence of α7 integrin leads to aggravated muscle pathology and early lethality within 4 weeks in γ-sarcoglycan-deficient mice (33), suggesting a compensatory role for α7 integrin upon disruption of the sarcolgycan complex. Further evidence of a protective role of α7 integrin in muscle with a disrupted DGC comes from mice lacking integrin α7 and dystroglycan specifically in skeletal muscle (Kanagawa M. and Campbell K.P., personal communication).

In the absence of dystrophin, the structurally related protein utrophin is upregulated and partially compensates functionally. Indeed, *mdx* mice that also lack utrophin display a deteriorated muscular dystrophy and only survive up to 36 weeks (21,34,35). It has been suggested that utrophin/dystrophin double-mutant mice closely resemble DMD, but the muscle wasting phenotype we observed in *mdx* mice lacking α7 integrin in terms of permanent fibre loss, interstitial fibrosis and dramatically reduced life span is by far more severe. Therefore, integrin α7/mdx double-mutant mice could be regarded as a novel model for DMD, resembling the progression and pathology in human patients, although it differs genetically and biochemically. Moreover, despite the fact that utrophin is upregulated in α7 integrin-deficient *mdx* muscles and partially retains components of the DGC at the cell membrane, utrophin appears to be insufficient to wholly restore the integrity of dystrophin-deficient muscles in the absence of α7 integrin. Mechanisms underlying the differential progression of dystrophin-null disease between mice and humans may be more complex than expected. Interestingly, however, the results obtained in this study raise the possibility that a fine-tuned variable expression in functionally related proteins such as dystrophin and integrin α7 may account for the difference in clinical severity of the disease between humans and mice.

It has been suggested that progressive fibre loss in DMD patients is due to inadequate regeneration (36,37). In contrast, the high regeneration capacity of *mdx* muscles, probably based on an expansion of muscle satellite cell populations, is also believed to account for the milder murine dystrophin-null disease (17,38–40). However, inactivating myogenic regulatory factors, such as MyoD or MNF in *mdx* mice, resulted in

markedly impaired muscle regeneration, increased myopathic severity and reduced life span (41,42), further supporting the previous finding that the DGC has a direct role in satellite cell activation (43). In the present study, we have identified impaired regenerative capacity of *mdx* muscles in the absence of α7 integrin, although muscle development proceeds normally. Our finding, for the first time, unveils a novel function of α7 integrin in muscle regeneration and suggests a role for α7 integrin in muscle satellite cell biology, in line with reports relating α7β1 to injury-related regeneration of rat skeletal muscles (44,45).

In the present study, we have excluded the possibility that apoptosis is the primary cause for impaired muscle regeneration in DKO mice by demonstrating that apoptosis and muscle regeneration are independent cellular events. Instead, impaired capacity of DKO muscles to regenerate likely results from impaired muscle satellite cell function. Despite the fact that lack of integrin α7 and dystrophin does not apparently affect muscle development, the possibility exists that their absence has an effect on the expression of myogenic markers in satellite cells, thereby indirectly perturbing muscle regeneration. Alternatively, as proliferating wild-type myoblasts express high levels of α7 integrin *in vitro* (46), it is tempting to speculate that the absence of α7 integrin may limit cell-cycle potential of dystrophin-deficient myoblasts and this may in turn result in inadequate muscle regeneration. Additionally, it has been demonstrated that overexpression of the disintegrin and metalloproteinase ADAM12 in *mdx* muscles upregulates α7 integrin (47). As ADAM12 has been shown to be involved in myoblast fusion *in vitro*, the absence of integrin α7 could negatively regulate expression of ADAM12 and thereby indirectly compromises fusion of myoblasts with segmentally damaged muscle fibres.

Impaired muscle regeneration may be the reason for early mortality of DKO mice. First, severe muscle wasting could cause respiratory pump collapse, resulting in hypoventilation and finally respiratory failure. However, the presence of necrotic areas and cardiomyocyte disarray, together with lung congestion but otherwise normal pulmonary structure, suggests cardiac failure as the predominant cause of death and suggests a crucial function for α7β1 integrin and dystrophin in maintaining architecture and function of the heart.

Together, integrin α7/dystrophin double-mutant mice represent a unique strain that resembles human DMD in skeletal and cardiac muscle pathology and progression of the disease. These animals will be useful in further studies to establish the role of integrin α7 and dystrophin in satellite cell activation and for testing the efficacy of new therapeutics.

MATERIALS AND METHODS

Breeding schemes

Mice lacking the α7 integrin gene have been previously described (14). 129Sv integrin α7 null mice were crossed with C57BL/10 *mdx* mice and backcrossed onto a 129Sv background. DKO mice were then generated by crossing female and male *mdx* mice being heterozygous for integrin α7 deficiency. Genotyping was performed as previously described (14,48). All experiments were performed in accordance with the Animals (Scientific Procedures) Act, UK, 1986.

Histological analysis

Mice were sacrificed and the lower hindlimb muscles, diaphragms, oesophaguses, lungs and hearts were excised and either frozen in liquid nitrogen-cooled isopentane or paraffin-embedded according to standard protocols. Transverse sections (10 μm) were cut and collected onto TESPA-coated glass slides. For general morphology, the sections were stained with haematoxylin and eosin (H&E). For the visualization of calcification and fibrosis, sections were stained using the von Kossa and Van Gieson procedures, respectively.

Immunofluorescence analysis

Cryosections (10 μm) were air dried and used either with or without fixation. For fixation, sections were incubated for 10 min in 1% paraformaldehyde in PBS at room temperature, followed by 8 min incubation in methanol at −20°C. After blocking with 5% normal goat serum (NGS) in PBS containing 0.1% Tween-20 (PBS-T) for 1 h at 37°C, sections were incubated with appropriate primary antibodies in 2% NGS/PBS-T. Primary antibodies employed were rabbit polyclonal antibodies, peptide 31 (1:50) for dystrophin (49), peptide 44 (1:20) for α-sarcoglycan (50) (kind gifts of Dr Kevin Campbell), U31+ (1:400) for integrin α7B (51), 1100+ (1:500) for laminin α4 chain (32), 1124+ (1:2000–10 000) for laminin α2 chain (52) (kind gifts of Drs Rupert Timpl and Takako Sasaki), a rat monoclonal antibody (5 μg/ml; Pharmingen) for the integrin α5 subunit, Urd40 (1:500) for utrophin (53) (kind gift of Dr Kay Davies), a sheep polyclonal antibody (1:100 dilution) for α-dystroglycan (54) (kind gift of Dr Stephen Kröger) and a mouse monoclonal antibody for MHCd (1:40; Novocastra). After incubation with respective Cy2- or Cy3-conjugated secondary antibodies (Dianova), sections were analyzed with a motorized Axioplan fluorescence microscope (Zeiss).

Detection of apoptosis

To visualize the outline of muscle fibres, paraffin sections of hindlimb muscles were stained with a polyclonal rabbit antiserum against the laminin α2 chain (1:2000) or the mouse monoclonal antibody against MHCdev (1:40 dilution). Apoptotic nuclei were then detected using the TUNEL procedure according to manufacturer's (Roche) protocol. Muscle nuclei were counterstained with 4′,6′-diamidino-2-phenylindole.

Electron microscopy

Whole mouse hearts were fixed in 100 mM cacodylate buffer (pH 7.0) containing 2% (w/v) glutaraldehyde, 4% (w/v) formaldehyde and 0.5% $CaCl_2$. Ventricular specimens were fixed in fresh fixative for 16 h at 4°C and post-fixed with 1% osmium tetroxide in 100 mM cacodylate buffer (pH 7.4) containing 0.5 mM $CaCl_2$ for 2 h at 4°C. The specimens were stained with 1% (v/v) uranyl acetate for 16 h at 4°C,

dehydrated in acetone and propylene oxide and finally embedded in Spurr's resin. Ultrathin sections of tissue blocks were cut with a Reichert ultramicrotome and collected on formvar coated copper grids, stained for 10 min with uranyl acetate, 5 min with lead citrate and examined with a Philip 400 electron microscope.

Statistics

To quantify muscle fibre numbers at comparable anatomical locations in P10 and P20 mice, whole EDL and PA were photographed and muscle fibres in two sections of 100 μm apart were then counted, the average of which was taken as the number of muscle fibres. To evaluate muscle regeneration, the percentages of fibres with centrally located nuclei were calculated. For comparison of muscle fibre numbers and percentages of fibres with central nuclei between WT ($n = 3$ at P10; $n = 4$ at P20), *mdx* ($n = 3$ at P10; $n = 5$ at P20) and DKO mice ($n = 3$ at P10; $n = 4$ at P20), ordinary one-way analysis of variance followed by Bonferroni post-test was used to compare any two groups. All values are presented as the mean $\pm$ standard deviation. For comparison of body weight between two time-matched groups, a paired Student's test with two-tail *P*-value was used.

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Conflict of Interest statement. The authors declare that they have no conflict of interest.

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