Musculoskeletal Pathology

Transgenic Overexpression of ADAM12 Suppresses Muscle Regeneration and Aggravates Dystrophy in Aged *mdx* Mice

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Muscular dystrophies are characterized by insufficient restoration and gradual replacement of the skeletal muscle by fat and connective tissue. ADAM12 has previously been shown to alleviate the pathology of young dystrophin-deficient *mdx* mice, a model for Duchenne muscular dystrophy. The observed effect of ADAM12 was suggested to be mediated via a membrane-stabilizing up-regulation of utrophin, $\alpha 7$ B integrin, and dystroglycans. Ectopic ADAM12 expression in normal mouse skeletal muscle also improved regeneration after freeze injury, presumably by the same mechanism. Hence, it was suggested that ADAM12 could be a candidate for nonreplacement gene therapy of Duchenne muscular dystrophy. We therefore evaluated the long-term effect of ADAM12 overexpression in muscle. Surprisingly, we observed loss of skeletal muscle and accelerated fibrosis and adipogenesis in 1-year-old *mdx* mice transgenically overexpressing ADAM12 (ADAM12⁺/*mdx* mice), even though their utrophin levels were mildly elevated compared with age-matched controls. Thus, membrane stabilization was not sufficient to provide protection during prolonged disease. Consequently, we reinvestigated skeletal muscle regeneration in ADAM12 transgenic mice (ADAM12⁺) after a knife cut lesion and observed that the regeneration process was significantly impaired. ADAM12 seemed to inhibit the satellite cell response and delay myoblast differentiation. These results discourage long-term therapeutic use of ADAM12. They also point to impaired regeneration as a possible factor in development of muscular dystrophy. (*Am J Pathol* 2007, 171:1599–1607; DOI: 10.2353/ajpath.2007.070435)

Skeletal muscle is capable of regeneration after injury, such as in trauma or disease. This process is dependent on tissue-specific skeletal muscle stem cells, the satellite cells.^{1–3} These cells are normally mitotically quiescent cells situated close to the muscle fibers between the sarcolemma and basal lamina.⁴ On injury they are activated and give rise to the myogenic precursor cells, which multiply, differentiate, and fuse with damaged fibers or generate new fibers.^{4–6} Several factors are involved in control of the regenerative processes, and the protein ADAM12, belonging to the ADAM (a disintegrin and metalloprotease) family, has been implicated in myogenesis and skeletal muscle repair.^{7–10}

ADAM12 is expressed in skeletal muscle at the embryonic stage and in the early postnatal period, where it is present in the developing myofibers.^{11–14} It is absent from normal adult skeletal muscle^{11,12} but re-expressed in the fibers during skeletal muscle regeneration.¹⁵ ADAM12 protein was proposed to participate directly in the cell to cell fusion process to generate multinucleated cells, both during embryonic growth and regeneration.^{14–16}

Studies on ADAM12-null mice suggested a regulatory role for ADAM12 in development of both skeletal muscle and adipose tissue because viable mutants showed reductions in interscapular muscles and brown adipose tissue; however, the ADAM12 gene was proven dispensable for myoblast fusion.⁹ Ectopic expression of ADAM12 in an ADAM12 transgenic (TG) mouse model¹⁰ resulted in a faster and more efficient regeneration after freeze injuries.¹⁰ Overexpression of ADAM12 also induced adipogenesis in these TG mice,¹⁷ in accordance with the ADAM12-null mouse study.⁹

ADAM12 was shown to alleviate the pathology of the *mdx* mouse when overexpressed in this model for Duchenne muscular dystrophy.^{10,12} Analyses indicated that

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ADAM12 stabilized the sarcolemma through an up-regulation of utrophin thereby protecting the *mdx* fibers and thus modulating the effect of lack of dystrophin.¹² In accordance with this, the effect of ADAM12 on muscle damage seemed to be specific for the *mdx* phenotype, because overexpression of ADAM12 in the laminin $\alpha 2$ -deficient (*dy/dy*) muscular dystrophic mice had no effect on the damaged muscle.¹⁸ Based on the ability of ADAM12 to boost utrophin gene levels, ADAM12 was suggested as a candidate for therapy of Duchenne muscular dystrophy.⁷

With the aim of further characterizing the therapeutic potential of ADAM12 in substitution or nonreplacement gene therapy in dystrophinopathies, we decided to evaluate the long-term effect of ectopic ADAM12 expression. Analysis of skeletal muscle samples obtained from 1-year-old ADAM12⁺/*mdx* mice, however, revealed an aggravating effect of the transgene on the *mdx* muscle pathology, and investigations indicated a reduction of muscle stem cell mobilization response in these old mice. This prompted us to investigate skeletal muscle regeneration after major acute injury in ADAM12⁺ TG mice, with focus on satellite cell mobilization. The results point to ADAM12 as a negative regulator of the satellite cell response during regeneration promoting adipogenic and fibrogenic substitution of muscle tissue.

Experimental Procedures

Animal Models

ADAM12⁺ TG mice and age-matched normal littermate controls (LC), 10 to 12 weeks of age, were used for the knife injury experiment. The generation of TG mice expressing human ADAM12 under the control of the muscle creatine kinase promoter has been described previously.¹² ADAM12⁺/dystrophin⁻ animals were obtained by mating homozygous female *mdx* mice [C57BL/10ScSn-Dmd(*mdx*); Jackson Laboratories, Bar Harbor, ME] with male ADAM12⁺ mice.¹² One-year-old individuals were used for analysis along with 1-year-old homozygous *mdx* mice and normal controls.

Animal Experiments

All animal experiments were performed in accordance with Danish legislation on animal welfare and approved by the Danish Council for Supervision with Experimental Animals. ADAM12⁺ TG ($n = 23$) and LC mice ($n = 24$) (10 to 12 weeks of age) were anesthetized using Hypnorm/Dormicum (fentanyl:fluanisone/midazolam), and experimental regeneration of skeletal muscle was induced by a stab incision in both calf muscles (musculus triceps surae). The mice were sacrificed by cervical dislocation 0.25, 1, 2, 3, 4, 5, 7, 10, and 14 days after injury. The injured muscles were removed and processed for either histological analysis or RNA purification. Uninjured muscle tissue was obtained from three ADAM12⁺ mice and three control mice. For histological analysis, muscles were fixed in formalin and embedded in paraffin and for

RNA analysis kept in RNAlater (Invitrogen, Tåstrup, Denmark) until further processing. From the 1-year-old mice (ADAM12⁺/*mdx*, *mdx*, and control) musculus quadriceps and musculus tibialis muscles were formalin-fixed and embedded in paraffin whereas musculus gastrocnemius and musculus tibialis muscles were snap frozen for cryosectioning.

RT-PCR and Quantitation

Total RNA from muscle samples kept in RNAlater was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. One μ g of pooled total RNA was used to synthesize cDNA using the Superscript-III first-strand synthesis system (Invitrogen). The RNA samples from each genotype were pooled (10 μ g of each sample) for each time point after surgery.

Two μ l of cDNA were used as template for each PCR experiment, and primers were added at a concentration of 5 pmol/20 μ l each. Primer sequences used: Pax7 sense: 5'-GCTACCACTACAGCCAGTATG-3', Pax7 antisense: 5'-GTCCTAAGCATGGGTAGATG-3', MyoD sense: 5'-GCCCCGCGCTCCAAGTCTCTGAT-3', MyoD antisense: 5'-CCTACGGTGGTGGCCCTCTGC-3', Myogenin sense: 5'-GGGCCCCCTGGAAGAAAAG-3', Myogenin antisense: 5'-AGGAGGCGCTGTGGGAGT-3', NCAM sense: 5'-ATTGTCTGCTCCTCGGTCCATT-3', NCAM antisense: 5'-GAGGGTAGAGGAGTCGTGTCATCA-3', GAPDH sense: 5'-AACGACCCCTTCATTGAC-3', GAPDH antisense: 5'-TCCACGACATACTCAGCAC-3', 18s rRNA sense: 5'-GTAACCCGTTGAACCCATT-3' and 18s rRNA antisense: 5'-CCATCCAATCGGTAGTGACG-3'. All PCR reactions were run as multiplex reactions with GAPDH. 18s rRNA was run as an additional control gene (not shown). Reaction conditions: initial denaturation 94°C for 5 minutes; denaturation: 94°C for 30 seconds; annealing: specific temperature for 30 seconds; extension: 72°C for 30 seconds; and final extension: 72°C for 7 minutes; Pax7: 56°C, 35 cycles; MyoD: touchdown 67 to 57°C, 35 cycles; Myogenin: 60°C, 35 cycles; NCAM: 57°C, 31 cycles; and 18s rRNA: 55°C, 30 cycles. Both a nontemplate (water) control and a reverse transcriptase control (-RNA) were run as negative controls for all primer pairs. Uninjured muscle was included in the analysis as normal background control. PCR products were visualized on a 2% agarose gel stained with ethidium bromide. DNA sequence analysis confirmed the specificity of the PCR reaction products. Each data point was quantified using the GelDoc 1000 and the software program Quantity One (Bio-Rad, Herlev, Denmark) and normalized to the abundance of GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) expressed as log² ratio followed by calculation of fold expression compared with normal uninjured muscle.¹⁹

Histological Analysis and Immunohistochemical Stainings

Formalin-fixed and paraffin-embedded muscle tissue was cut in 4- μ m sections in a longitudinal direction, to ensure inclusion of the zones of interest in the injured area, and stained with hematoxylin and eosin (H&E) for morphological analysis. A Sirius red staining was performed to detect

fibrosis. For immunohistochemistry (IHC), antigen retrieval was done by microwave oven treatment, and sections were subsequently blocked to eliminate endogenous peroxidase and endogenous biotin. The following primary antibodies were used: monoclonal mouse- α -myogenin at 1:200 (F5D; DAKO, Glostrup, Denmark); monoclonal mouse- α -pax7 at 1:10 (Developmental Studies Hybridoma Bank, Iowa City, IA); polyclonal rabbit- α -chicken NCAM (neural cell adhesion molecule) at 1:2000 (AB5032; Chemicon International, Inc., Temecula, CA); rat- α -mouse ki67 at 1:50 (Tec3; DAKO), monoclonal mouse- α -dystrophin at 1:10 (MAB 1692; Chemicon, AH Diagnostics, Arhus, Denmark), polyclonal rat- α -mouse CD45 at 1:50 [30-F11 (Ly 5); BD Pharmingen, Brondby, Denmark], polyclonal rabbit- α -human myostatin 1:200 (Chemicon, AH Diagnostics), monoclonal mouse- α -human transforming growth factor (TGF)- β 1 at 1:10 (NCL-TGFB; NovoCastra, Newcastle, UK), monoclonal mouse- α -human follistatin at 1:10 [85918 (MAB669); R&D Systems, TriChem ApS, Skanderborg, Denmark).

For detection of mouse monoclonal antibodies, the ARK kit (DAKO) was used. The labeled streptavidin-biotin-system with goat- α -rat (E468 at 1:200; DAKO) and the Envision⁺ horseradish peroxidase system (K4003, DAKO) were used as detection systems for polyclonal antibodies. DAB⁺ was used as chromogen. Nuclei were counterstained using Mayer's hemalum with 4.5% chloral hydrate. Controls with exclusion of the primary antibodies were included in all experiments.

Immunofluorescence Stainings

Crysections of musculus tibialis ($n = 6$) and musculus gastrocnemius ($n = 6$) from ADAM12⁺/*mdx* and *mdx* mice were stained with monoclonal utrophin at 1:50 (NCL-DRP2; NovoCastra) and monoclonal β 1D integrin at 1:25 [2B1 (MAB1900); Chemicon, AH Diagnostics] using an ARK-immunofluorescence protocol using a fluorescein isothiocyanate-labeled Tyramid (TSA kit, NEL701; Perkin-Elmer, Waltham, MA) procedure. Before staining, sections were blocked to eliminate endogenous biotin and peroxidase. Nuclei were detected with 4,6-diamidino-2-phenylindole mounting medium (Vectashield; Vector Laboratories, Burlingame, CA).

Morphometrics

The number of cells expressing a particular protein during the course of regeneration was estimated on sections by counting the number of positive nuclei (pax7, ki67, and myogenin) or nuclei surrounded by positive cytoplasm in mononuclear cells (NCAM). Morphometrics was performed using a Leica microscope equipped with a camera connected to a computer and a motorized cross board. By means of the CAST software (2000; Olympus Denmark A/S) systematic random fields for counting were selected. Fields with positive events (positive nuclei or stained cytoplasm) were accepted as lesion area and included. The number of nuclei in cells with positive events were compared with the total number of nuclei in the counted fields to give an estimate of the number of

cells expressing the various proteins at the time points investigated.

Statistics

P values were calculated for the comparison of TG animals with LC animals using two-way factorial analysis of variance analysis with an α -value of 0.05. *P* values <0.05 were considered statistically significant.

Results

Long-Term Transgenic Expression of ADAM12 in *mdx* Muscles Has a Negative Impact on Muscle Regeneration

Having seen the beneficial effect of short-term ADAM12 expression on the *mdx* phenotype,¹² the next step in evaluating the therapeutic potential of ADAM12 was to study its long-term effects. For this purpose musculus quadriceps and musculus tibialis from 1-year-old ADAM12⁺/*mdx* mice, *mdx* mice, and normal mice was analyzed by IHC (Table 1). A Sirius staining performed on muscle in the 1-year-old ADAM12⁺/*mdx* mice to detect fibrosis (Figure 1, A–C) demonstrated that the ADAM12/*mdx* mice had more extensive fibrosis and more adipocytes, thus having a more dystrophic phenotype compared with the *mdx* mice. A recent study indicated a relationship between myostatin, fibrosis, and TGF- β 1 in skeletal muscle.²⁰ Therefore we decided to evaluate the expression of myostatin, a known inhibitor of myogenesis,²¹ TGF- β 1, and follistatin, a myostatin inhibitor,²⁰ because these factors could participate as co-regulators in the fibrosis reaction observed. TGF- β 1 (data not shown) and follistatin (data not shown) were both expressed in the connective tissue of ADAM12⁺/*mdx* and *mdx* mice. However, we did not observe any difference in expression of either TGF- β 1 or follistatin in ADAM12⁺/*mdx* mice compared with *mdx* mice. Thus, expression of these two factors seems to have no apparent correlation to the ADAM12⁺/*mdx* phenotype observed in this study.

Table 1. Results from Immunohistochemical Stainings of Young (6 Weeks) and Old (1 Year) *mdx* and ADAM12⁺/*mdx* Mice Analyzed by Light Microscopy According to Number of Stained Cells/Nuclei

	mdx, 6 weeks ($n = 2$)	mdx, 1 year ($n = 6$)	ADAM12 ⁺ / mdx 6 weeks ($n = 3$)	ADAM12 ⁺ / mdx, 1 year ($n = 6$)
ki67	+	++	+	+
CD45	+++	++	+	+++
pax7	++	+	+	(+)
myogenin	+	(+)/+	+++	+
NCAM	++	++	+++	+
myostatin	++	+	++	+++
Sirius	+	+	++	+++

Expression of the markers was analyzed according to a semi-quantitative scale where zero protein expression was scored as –, weak expression as (+), modest expression as +, marked expression as ++, and pronounced expression as +++.

Figure 1. Sirius staining of 1-year-old normal (A), *mdx* (B), and ADAM12⁺/*mdx* (C) mice showing considerable fibrosis and adipose infiltration in the ADAM12⁺/*mdx* mice compared with both normal and *mdx* mice. Original magnifications, $\times 100$.

However, in contrast to *mdx* mice, most of the myofibers in the ADAM12⁺/*mdx* mice expressed myostatin.

Muscle in ADAM12⁺/*mdx* mice also showed more inflammation, seen as an increase in CD45⁺ cells. The number of pax7⁺ nuclei was the same as in control mice, indicating that the degeneration was not followed by satellite cell activation and proliferation in ADAM12⁺/*mdx* mice at this age. A few myogenin⁺ nuclei could, however, be detected suggesting a slight regenerative activity. Compared with the *mdx* mice, ADAM12⁺/*mdx* mice had lower numbers of NCAM⁺ mononuclear cells and ki67⁺ proliferating cells, indicating a more active regeneration in the *mdx* mice than in ADAM12⁺/*mdx* mice.

These results implied that long-term expression of ADAM12 in *mdx* dystrophic mice compromised muscle restoration seen as reduced stem cell function, increased inflammation, fibrosis, and adipogenesis. The mechanisms underlying this could be exhaustion of the stem cell population; however, the expression patterns seen, in particular the myostatin expression, could suggest that the ADAM12⁺/*mdx* mice had an overall suppressed regeneration.

Membrane stabilization, rather than an increase in regeneration capacity, was proposed to be the mechanism behind early alleviation of the dystrophic phenotype in the ADAM12⁺/*mdx* mice.¹⁰ We therefore analyzed the expression of utrophin (Figure 2) and β 1D integrin (data not shown) in the membranes and found that expression of utrophin seemed mildly elevated in the aged ADAM12⁺/*mdx* mice compared with age-matched controls. Expression of β 1D integrin reflected the fibrosis reaction observed in the Sirius stain (data not shown). Thus a down-regulation of these compounds could not account for the late dystrophic phenotype.

To re-evaluate the regenerative capacity, satellite cell activation and subsequent proliferation were analyzed in young ADAM12⁺/*mdx* and *mdx* mice with focus on the same markers used for the old mice (Table 1). In brief, we found few pax7⁺ but many myogenin⁺ nuclei in 6-week-old ADAM12⁺/*mdx* compared with age-matched *mdx* mice. Identical staining patterns were observed for NCAM and myostatin in fibers from both genotypes. Although the presence of many CD45⁺ nuclei indicated pronounced inflammation in *mdx* mice, a Sirius staining revealed more fibrosis in the young ADAM12⁺/*mdx* mice than in the *mdx* mice. These results altogether suggested an altered regeneration in the young ADAM12⁺/*mdx* mice, too.

The Regeneration Process Is Impaired in ADAM12 TG Mice after Severe Injury

To address the question about stem cell function in an ADAM12-enriched milieu, we studied the regenerative response after a stab lesion in 10- to 12-week-old ADAM12⁺ mice. Regeneration after freeze injury has previously been reported for ADAM12⁺ mice¹⁰ but not with specific focus on myogenic cell parameters. The knife cut injury resulted in a total loss of architecture of the injured muscle, but on day 5, the injured area in the control mice had regained most of its original structure (Figure 3A), and on day 10 (Figure 3B) the regeneration was almost complete in these animals. The only indication of a previous injury was the centrally located nuclei in the repaired fiber segments (Figure 3B). By contrast, no obvious regeneration had occurred in the ADAM12⁺ mice on day 5 (Figure 3C), and on day 10 their muscle structure was still incomplete, although some regenerative activity could be observed (Figure 3D). These findings strongly indicated an impaired regenerative capacity in the ADAM12⁺ mice compared with their LCs. To analyze this effect further, immunohistochemical stainings and RT-PCR were performed.

Lower numbers of ki67⁺ proliferating cells seemed to be present in ADAM12 mice at days 4 to 5 compared with control mice (Figure 4); however, the difference between the two groups was not statistically distinguishable (*P* value, 0.104). Compared with uninjured control muscle, the fold-expression of pax7, myoD, and myogenin mRNA differed significantly between ADAM12⁺ and LC mice (Figure 5, A–C; *P* < 0.01 for all three markers). In ADAM12⁺ mice, the mRNA expression of pax7 (Figure 5A) varied little during the regeneration period. By contrast, the LCs displayed an up-regulation of pax7 initiating a few hours after surgery, peaking at days 3 to 5 and declining slowly during the later phases of regeneration (Figure 5A). The same expression pattern was observed for myoD (Figure 5B). The difference in pax7 expression was also visualized immunohistochemically (Figure 6A, *P* < 0.01). These data indicated that the recruitment of activated satellite cells/proliferating myogenic cells was depressed in ADAM12⁺ mice compared with the controls.

Analysis of myogenin at the mRNA level (Figure 5C) and by IHC (Figure 6B) revealed that fold expression of myogenin mRNA when compared with uninjured control muscle was significantly different between ADAM12⁺

Figure 2. Utrophin staining of 1-year-old ADAM12⁺/mdx (**A**) and mdx (**B**) mice. Utrophin expression remains mildly elevated in the old ADAM12⁺/mdx mice compared with the age-matched controls. Negative controls for ADAM12⁺/mdx (**C**) and mdx (**D**). Original magnifications, $\times 200$.

and LC mice (Figure 5C, $P < 0.01$). The analysis of cells expressing myogenin protein in their nuclei showed a similar expression pattern (Figure 6B, $P < 0.05$). Thus, fewer differentiating myogenic cells were present during regeneration in the ADAM12⁺ mice, which supported the result from the pax7 and myoD analyses.

The expression pattern of NCAM followed that of pax7, myoD, and myogenin on both mRNA and protein level during the regeneration process in LC mice (Figures 5D and 6C). In the ADAM12⁺ mice (Figure 5D) we observed a constitutive expression of NCAM mRNA, and the difference in NCAM mRNA expression patterns between LC and TG mice were supported statistically ($P < 0.0001$) (Figure 5D). The number of NCAM⁺ cells during regeneration recorded by IHC appeared reduced in the ADAM12⁺ mice compared with the controls even though no significant difference could be observed when the entire observation period was included ($P = 0.0265$).

Myotubes were visualized by NCAM IHC (Figure 7, A and B). On day 5, the LC mice showed regenerating areas filled with NCAM⁺ myotubes (Figure 7A). Such fibers were scarcely present in ADAM12⁺ mice; instead,

we observed mononuclear, nonfusing NCAM⁺ cells (Figure 7B). The NCAM patterns thus indicated a difference in the temporal development of regeneration in ADAM12⁺ mice compared with controls, with a delayed fusion and myotube formation in ADAM12⁺ mice.

Disorganized Muscle Repair and Fibrosis after Severe Injury in the ADAM12⁺ Mice

The analyses of ki67, pax7, myoD, myogenin, and NCAM expression indicated that satellite cell activation and subsequent differentiation was repressed during skeletal muscle regeneration in the ADAM12⁺ mice. The morphological investigations (Figure 3) also supported these findings, suggesting that the normal regeneration was substituted by connective tissue formation. To confirm this, a Sirius red staining was performed (Figure 8).

Formation of connective tissue was found in the transgenic muscles (Figure 8, A–C) but could not be observed in the control mice (Figure 8, D–F). The ADAM12⁺ mice displayed massive connective tissue reaction within the

Figure 3. H&E stainings of ADAM12⁺ TG mice (**B** and **D**) and their LCs (**A** and **C**). Original magnifications, $\times 200$.

regenerating area on day 5 (Figure 8B), whereas the control mice formed new myotubes and only normal amounts of connective tissue at the same time point (Figure 8E). The connective tissue component was still very prominent day 10 after injury in the ADAM12⁺ mice (Figure 8C), whereas the LCs completely regenerated the destroyed muscle tissue leaving no excess connective tissue at day 10 (Figure 8F). Differences in the late stages

Figure 4. Morphometrical analysis of ki-67 protein expression pattern during regeneration of ADAM12⁺ and normal LCs. **Black squares:** ADAM12⁺ mice ± 1 SE; **open squares:** LC mice ± 1 SE ($P = 0.1$) ($n = 2$ to 3).

of regeneration could also be seen from the dystrophin IHC staining. The control mice had at day 10 regained their muscle architecture completely (Figure 8F, inset), whereas the ADAM12⁺ mice displayed a compromised regeneration and less structured skeletal muscle compartment at this time point (Figure 8C, inset).

Figure 5. Semiquantitative analysis of mRNA expression levels of pax7 (**A**), myoD (**B**), myogenin (**C**), and NCAM (**D**) during regeneration of ADAM12⁺ and normal LC mice ($P < 0.01$ for all markers). **Black bars:** ADAM12⁺ mice; **white bars:** LC mice ($n = 2$ to 3).

Figure 6. Morphometrical analysis of pax7 (A), myogenin (B), and NCAM (C) protein expression patterns during regeneration of ADAM12⁺ and normal LC mice. **Black squares:** ADAM12⁺ mice $\pm$ 1 SE; **open squares:** LC mice $\pm$ 1 SE (Pax7, $P < 0.001$; myogenin, $P < 0.05$; NCAM, $P = 0.27$) ($n = 2$ to 3).

Discussion

It is well recognized that ADAM12 is involved in muscle formation as well as regeneration.^{9,12,13,15} However, con-

cerning the specific role of ADAM12 in the skeletal muscle there are diverging indications. *In vivo* studies have shown ADAM12 able to recruit myogenic cells²² and to support regeneration,¹⁰ whereas *in vitro* studies²³ indicate an inhibitory effect. Our *in vivo* results are in accordance with the reported²³ inhibitory *in vitro* effects of ADAM12, because both satellite cell recruitment and terminal maturation appeared suppressed. This was seen as reduced up-regulation of the responses of the stem cell-associated myogenic factors, myoD, pax7, and myogenin, to injury and as an increased expression of myostatin. Both the acute stab injuries performed in ADAM12⁺ mice and the long-term experiments with ADAM12⁺/mdx mice indicated this inhibitory effect of ADAM12 on the satellite cells. But when satellite cell recruitment was specifically addressed in the young ADAM12⁺/mdx mice, indications of suppression could be seen even at this early age.

The mechanism of ADAM12 alleviation in the young mdx mice has been linked to the up-regulation of utrophin,¹⁰ a functional homologue of dystrophin, known to reduce the myopathic effect resulting from lack of dystrophin in mdx muscle.²⁴ Although there continues to be mildly elevated utrophin expression in aged ADAM12⁺/mdx mice compared with their age-matched mdx controls, it seems from our studies that the ADAM12 induced inhibition of the regeneration throughout time overrules the positive effect of utrophin up-regulation. This may be attributable to the mild degree of up-regulation achieving only subtherapeutic levels. When a negative effect is not seen initially, this could be attributable to a higher regeneration capacity in young animals in combination with the retardation of the degeneration provided by utrophin up-regulation.

Previously a positive effect of ADAM12 on regeneration has been reported.¹⁰ Interestingly, it has been found that different types of muscle injury may result in different regenerative responses.²⁵ The study in which ADAM12 was reported to support regeneration¹⁰ used smaller freeze injuries, which leaves the basement lamina as a

Figure 7. NCAM staining of the regenerating area at day 5 after injury reveals differing morphologies in ADAM12⁺ mice (A) and normal LCs (B). Original magnifications, $\times 400$.

Figure 8. Sirius staining of the regeneration process after massive, acute injury in ADAM12⁺ (A–C) and LC (D–F) mice. **Insets** in C and F show dystrophin expression in the membranes at day 10 after injury to illustrate the end-point of regeneration. Original magnifications, $\times 200$.

scaffold, whereas the lesions in our present study completely disrupted the basal lamina. This difference could to some extent explain the observed difference because the conserved basal lamina scaffolds in the smaller freeze injuries could create a more myogenic milieu and thus assist the myogenic cells²⁶ in muscle repair. Moreover, ADAM12 is a membrane-spanning protein, which in itself could provide a structural membrane support when the basement membrane is intact.

The skeletal muscle of mice transgenically overexpressing ADAM12 demonstrates a slightly myopathic pattern observed as centrally placed nuclei, scattered necrosis, and accumulation of adipose tissue in the muscles.¹⁰ In our study, we found a constitutive expression of NCAM mRNA, further supporting the myopathic phenotype of the ADAM12⁺ transgenic muscles. If ADAM12 has an inhibitory effect on regeneration, it could explain this observed myopathic phenotype. The negative effect would accumulate throughout time and become accelerated when combined with the *mdx* phenotype, resulting in an overt dystrophic phenotype. Thus, the ADAM12⁺/*mdx* mouse seems to develop its more severe myopathy because of inhibition of the recruitment of progenitor cells and fusion/maturation of myogenic cells into fibers.

Lack of, or mutations in proteins necessary for maintenance of the mature muscle fiber integrity are common defects observed in different kinds of myopathies.²⁷ The present observations suggest inhibition of stem cell activation as a novel mechanism that could lead to myopathy/dystrophy. It has previously been observed that transgenic expression of ADAM12 in skeletal muscle results in the occurrence of adipocytes within the muscles.¹⁷ In our study we observed fibrosis and adipose accumulation in an environment where the satellite cells are not lost but rather

inhibited, thus indicating that adipogenesis is not linked to loss of myogenic stem cells/precursors.

ADAM12 has been presented as a possible candidate in nonreplacement gene therapy of muscular dystrophies providing structural support alleviating the *mdx* phenotype.⁷ However, as observed in the present study, a structural support is not enough if the satellite cell response is constantly depressed not allowing an effective regeneration to occur. In long-term disease, this will eventually result in adipogenesis and fibrogenesis. The use of ADAM12 as booster gene thus needs to be revised. Perhaps, ADAM12 could be useful in short-term boosting to yield a short-term inhibition of satellite cell recruitment combined with a structural support both directly and through utrophin up-regulation to protect the stem cell population against exhaustion. However, based on the present results, long-term treatment is not recommended.

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