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# Safety and Efficacy of AAV-Mediated Calpain 3 Gene Transfer in a Mouse Model of Limb-Girdle Muscular Dystrophy Type 2A

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**Calpainopathy (limb-girdle muscular dystrophy type 2A, LGMD2A) is a recessive muscular disorder caused by deficiency in the calcium-dependent cysteine protease calpain 3. To date, no treatment exists for this disease. We evaluated the potential of recombinant adeno-associated virus (rAAV) vectors for gene therapy in a murine model for LGMD2A. To drive the expression of calpain 3, we used rAAV2/1 pseudotyped vectors and muscle-specific promoters to avoid calpain 3 cell toxicity. We report efficient and stable transgene expression in muscle with restoration of the proteolytic activity and without evident toxicity. In addition, calpain 3 was correctly targeted to the sarcomere. Moreover, its presence resulted in improvement of the histological features and in therapeutic efficacy at the physiological levels, including correction of atrophy and full rescue of the contractile force deficits. Our results establish the feasibility of AAV-mediated calpain 3 gene transfer as a therapeutic approach.**

**Key Words: limb girdle muscular dystrophy type 2A, gene transfer, calpain 3, AAV, muscle**

## INTRODUCTION

Calpainopathy (LGMD2A; OMIM 253600) is one of the recessive limb-girdle muscular dystrophies (LGMD2), a group of genetically distinct disorders affecting predominantly the proximal limb muscles [1]. It results from mutations in calpain 3, a calcium-dependent cysteine protease whose function in the muscle is not fully elucidated [2,3]. LGMD2A was first discovered in a genetic isolate from Réunion Island and later found to have a worldwide distribution with more than 150 loss-of-function mutations identified to date [4,5] (Leiden Muscular Dystrophy Pages, http://www.dmd.nl/). It represents between 30 and 90% of the LGMD2 cases, depending on the geographic area, with an estimated prevalence from 10 to 70 per million. The mean age of onset is 13 years of age. The disease progresses with gradual atrophy and weakness of the muscles of the lower and upper girdles, especially those from the posterior compartment, leading to the loss of walking ability after 10 to 20 years [6]. Muscle impairment is associated with an elevation of serum creatine kinase and dystrophic features on muscle biopsies such as area of degeneration/regeneration, variation in fiber size, inflammatory infiltrates, and fiber splitting. There is no facial or cardiac involvement and respiratory impairment is minimal. A murine model of calpainopathy was constructed by homologous recombination showing that calpain 3 deficiency in mice parallels the muscle histopathological signs; their level is variable depending on the muscle [7]. These mice present no obvious defect of locomotor activity but a 20% decrease in force when tested on isolated muscles [8].

Calpain 3 is a member of the calcium-dependent cysteine protease family of calpains [3,9]. Calpain 3 is expressed mainly in skeletal muscle where it has a sarcomeric localization through binding to several regions of titin, a giant elastic protein spanning half a sarcomere [10]. It has also been observed in the nuclear compartment in human biopsies [11]. We and others have shown that calpain 3 has a specific mechanism of activation consisting of the removal of an internal peptide by autolysis to free the catalytic site [12,13]. An elevation of myonuclear apoptosis associated with perturbation of the NF-κB pathway and a down-regulation of components of the ubiquitin–proteasome system were observed in human LGMD2A biopsies and in calpain 3-deficient murine muscles, respectively [7,11,14]. In addi-



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tion, we observed that the proteolytic activity of calpain 3 is directed against proteins of the sarcomere and costamere such as talin, vinexin, ezrin, filamin-C, and titin [12], suggesting that it may be involved in muscle remodeling [15].

At present, no treatment exists for LGMD2A. Considering the loss-of-function nature of this disorder, a sensible therapeutic strategy would be via calpain 3 gene transfer in skeletal muscle. The recent improvement of muscle gene transfer brought by use of new adeno-associated virus (AAV) serotypes and development of systemic delivery methods strengthens the rationale of this approach [16–23]. In this study, we have designed, constructed, and evaluated recombinant (r) AAV2/1-mediated calpain 3 transfer to complement calpain 3 deficiency in a murine model. We report efficient transgene expression in muscle tissue at the RNA and protein levels with restoration of the proteolytic activity and without evident toxicity. In addition, the calpain 3 protein product seems to be correctly targeted to the sarcomere. More importantly, calpain 3 transfer resulted in improvement of the histological features and in therapeutic efficacy at physiological levels, including correction of atrophy and full rescue of the contractile force deficits.

## RESULTS

### Design of AAV Vectors Encoding Calpain 3

We constructed and produced two rAAV2/1 vectors carrying the human calpain 3 cDNA under the control of either the ubiquitous CMV (AAV1-CMV-CAPN3) or a synthetic muscle-specific promoter, C5-12 (AAV1-C5-12-CAPN3). Determination of titers showed that viral preparations corresponding to the CMV promoter gave low titers in both viral genomes (vg) and infectious genomes (ig), with means of $4.4\times10^{7}$ ig/ml and $2.7\times10^{10}$ vg/ml ($n$ = 8), whereas titers of the viral preparations corresponding to the C5-12 promoter were in the normal range: $7.9\times10^{9}$ ig/ml and $1.0\times10^{12}$ vg/ml ($n$ = 5). We hypothesized that expression of calpain 3 could be toxic to the cells used for vector preparation due to a disruption of the cytoskeleton [12]. We subjected protein extracts of AAV1-CMV-CAPN3- or AAV1-CMV-GFP-transduced cells to Western blotting and probed with an antibody against talin, a cytoskeletal protein substrate of calpain 3. A 190-kDa cleaved fragment of talin was detected only in the lane corresponding to AAV1-CMV-CAPN3, suggesting that the cytoskeleton may be disrupted in these cells as the consequence of calpain 3 expression, thus preventing the use of a ubiquitous promoter (Fig. 1).

We then compared the expression efficacy of three muscle-specific promoters, CK6, desmin, and C5-12, using murine embryonic secreted alkaline phosphatase (mSeAP) as a reporter gene after single intramuscular administration of an equal amount of infectious AAV particles ($2\times10^{6}$ ig per injection) in the tibialis anterior (TA) muscle of wild-type mice. The day of injection and every week afterward for 2 months, we took blood samples from mice and analyzed them for mSeAP secretion. At the endpoint of the experiments, we sampled, cryosectioned, and stained TA muscles for mSeAP activity. With all three vectors, we obtained high levels of gene expression (Fig. 2A) and of fiber transduction (Fig. 2B). Fibers were evenly stained with the C5-12 and desmin rAAV2/1 vectors, but we observed a high variability of expression between fibers with the CK6 rAAV2/1 vector. Slow-fiber typing of serial sections indicated a lower expression in slow fibers with this promoter (Fig. 2C). Considering this result, we excluded the CK6 promoter and chose to construct AAV calpain 3 vectors with one of the two remaining promoters (AAV1-C512-CAPN3fsr) to be used in subsequent experiments. To be able to distinguish between the transgenic and the endogenous calpain 3 messengers, we introduced several silent mutations (indicated by the suffix fsr in the name of the vector) in the human calpain 3 coding sequence that would correspond to primers and probe for a specific quantitative PCR assay (Fig. 3A).

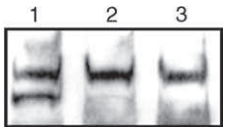


**FIG. 1.** Western blot analysis of HEK293 cell extracts transfected with AAV-CMV-C3 (lane 1), AAV-C512-C3 (lane 2), and AAV-CMV-GFP (lane 3). At the end of the viral preparation procedure, proteins were extracted from cells and subjected to Western blot against talin, a calpain 3 substrate. A 190-kDa cleaved fragment was observed only in the lane corresponding to the AAV-CMV-C3-producing cells (lane 1).

### AAV1-C512-CAPN3fsr-Mediated Calpain 3 Expression in Muscle by Intramuscular Administration

We injected $4\times10^{10}$ AAV1-C512-CAPN3fsr vg (corresponding to $13.2\times10^{7}$ ig) into the TA muscle of 7-month-old normal and calpain 3-deficient mice. Muscles were sampled 35 days after injection and processed for expression analysis at the RNA level using specific TaqMan quantitative assays (see Materials and Methods). We obtained $4.8\times10^{6}$ copies of transgene-specific transcripts per microgram of muscle RNA in calpain 3-deficient mice and $7.7\times10^{6}$ in normal mice (Fig. 3B). The observed difference between calpain 3-deficient and normal muscles has been found repeatedly in all our experiments with all transgene. We determined the level of the endogenous calpain 3 in normal muscles to be in the range of $1\times10^{6}$ copies/μg of RNA, showing that an expression level 8-fold higher can be achieved after AAV injection of calpain 3. This high expression was maintained for up to 90 days without any signs of decrease (data not shown).

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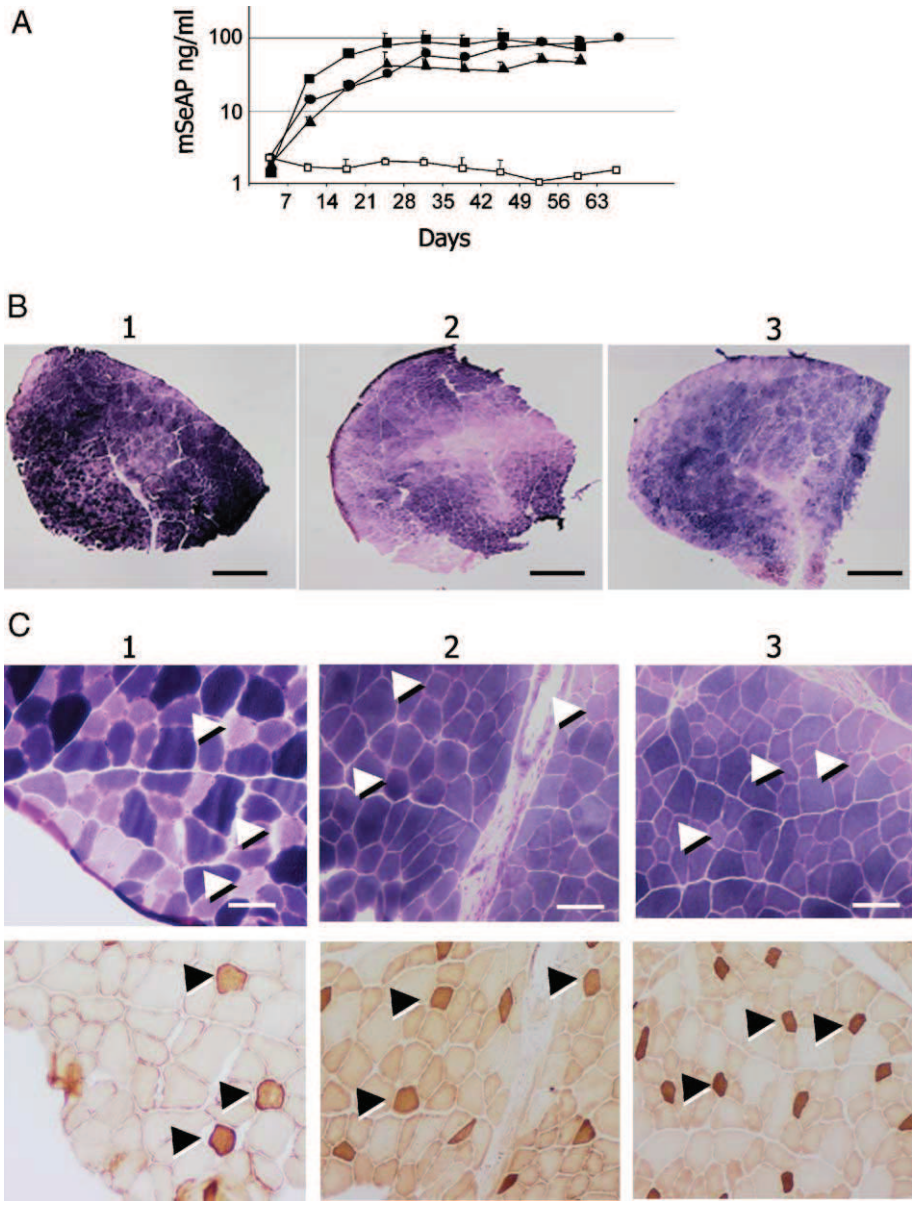


**FIG. 2.** Comparison of expression driven by three muscle-specific promoters after a single intramuscular injection. (A) Kinetics of mSeAP blood levels after intramuscular injection of AAV1-CK6-mSeAP (black square), AAV1-des-mSeAP (black triangle), AAV1-C5-12-mSeAP (black circle), or PBS (white square) ($n$ = 5 mice for each promoter tested). Mice received $2 \times 10^6$ ig of each viral preparation in the TA muscle. Plasma alkaline phosphatase activity was monitored every week for 2 months. Blood from animals injected with PBS was used as control. (B) Cryosections stained for alkaline phosphatase activity at the endpoint of the experiment described in A (scale bars, 1 mm). (1) AAV1-CK6-mSeAP, (2) AAV1-des-mSeAP, and (3) AAV1-C5-12-mSeAP. (C) Top: Higher magnifications of sections stained in B. Bottom: Serial sections immunostained with an antibody against the skeletal slow myosin. Arrowheads indicate slow fibers. (1) AAV1-CK6-mSeAP, (2) AAV1-des-mSeAP, and (3) AAV1-C5-12-mSeAP (scale bars, 100 μm).

We also evaluated gene transfer efficiency by Western blot. Analysis of AAV1-C512-CAPN3fsr-injected TA muscles showed a level of calpain 3 protein expression that exceeded the natural level of calpain 3 (Fig. 3C). The presence of autolysed forms originating from the transgenic human calpain 3 indicated that the proteins produced from AAV represent potentially active molecules. To assess the distribution of transgenic calpain 3 within the injected muscle, we obtained and immunostained cross and longitudinal sections from wild-type and vector-treated and untreated dystrophic mice (Fig. 4). The AAV-treated muscles showed widespread positive staining with a sarcomeric pattern, indicating correct localization of the newly expressed protein (Figs. 4C, 4F, and 4G).

**Restoration of a Proteolytic Activity After Calpain 3 Transfer in Mature Muscle**

We next examined whether the calpain 3 transferred by rAAV2/1 vector was functional in terms of proteolytic activity. We assayed the activity *in vitro* on a reporter protein in the form of V5-tagged human calpain 3 that was rendered inactive by introduction of the C129S mutation but that is still a substrate for active calpain 3 molecules [12]. We mixed extracts from pcDNA3-CAPN3C129S-transfected cells with muscle extracts from wild-type, untreated, and treated calpain 3-deficient muscles. We then performed Western blot using a V5-tag-specific antibody and demonstrated that the reporter molecules were processed with wild-type or

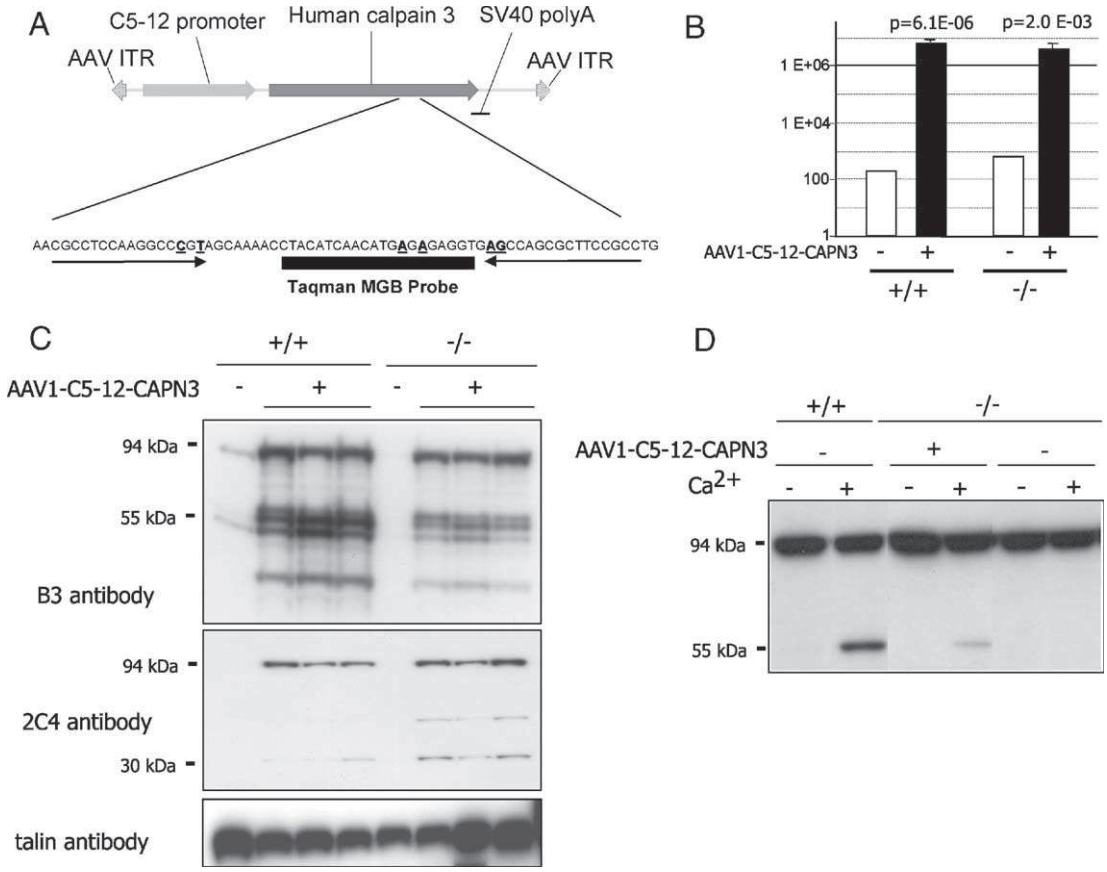


**FIG. 3.** Detection of calpain 3 mRNA, protein, and activity after intramuscular delivery of a calpain 3 AAV vector in normal and calpain 3-deficient muscles. (A) Diagram of the AAV1-C512-CAPN3fsr construct. Silent mutations (in underlined bold along the sequence) were introduced into exon 13 at positions corresponding to the 3′ end of both primers and to the middle of the TaqMan MGB probe that were used in real-time RT-PCR to detect specifically the transgene. Sequence of the mutated calpain 3 (CAPN3fsr) is indicated with the positions of the primers and probe. (B) Evaluation of copy number of calpain 3 expressed from the rAAV2/1 vector by real-time RT-PCR. Five normal and calpain 3-deficient mice 7 months of age received $4\times10^{10}$ AAV1-C512-hCAPN3fsr physical particles (corresponding to $13.2\times10^{7}$ ig) by intramuscular injection into the TA muscle. The endogenous level of calpain 3 in normal muscle is indicated by a solid black bar and corresponds to $1\times10^{6}$ copies/μg of RNA. Differences compared to uninjected animals were statistically significant ($P < 0.01$) for both groups of animals. (C) Western blot detection of calpain 3 in normal and calpain 3-deficient muscles treated and not treated with calpain 3 rAAV2/1 vector. The polyclonal B3 antibody recognizes both the murine and the human proteins and the monoclonal antibody 2C4 recognizes only the human form. Top: Detection of both endogenous and transferred calpain 3. Middle: Detection of transferred calpain 3 using the 2C4 antibody. Bottom: Normalization with an anti-talin antibody. (D) Detection of calpain 3 proteolytic activity in normal and calpain 3-deficient muscles treated and not treated by calpain 3 rAAV2/1 vector. Activity in muscle extracts was assessed by *in vitro* proteolysis of a reporter protein consisting of V5-tagged inactive calpain 3. The reaction was run for 1 h at 37°C in the presence or absence of calcium. Western blot using an anti-V5 antibody was processed to visualize the extent of cleavage. The blot corresponds to a representative of a total of eight independent experiments. The corresponding treated muscles expressed $1.9\times10^{8}$ copies of transgene per microgram of RNA.

calpain 3-transduced deficient muscles but not with untreated deficient muscle (Fig. 3D). This result showed that it is possible to detect specifically the calpain 3 proteolytic activity and that this activity is restored in deficient muscle after calpain 3 transfer by rAAV2/1 vector.

### Overexpression of Calpain 3 in Mature Muscle Does Not Disrupt the Cytoskeleton

Because calpain 3 is a protease and induces disruption of the cytoskeleton when overexpressed in nonmuscle cells, we investigated whether calpain 3 transfer using rAAV2/1 vector could lead to cytoskeleton disorganization. We injected the AAV1-C512-CAPN3fsr vector ($1.3\times10^{10}$ vg) into the TA muscle of 6-month-old normal mice. One month after injection and 1 week before sacrifice, we electrotransferred a plasmid encoding α-actin fused to GFP to serve as a reporter of sarcomere organization. We observed muscles under a confocal microscope on living mice after resection of the skin without detecting any difference between untransduced and transduced muscles (Fig. 5). We then collected muscles and processed them for calpain 3 Western blotting to confirm calpain 3 expression (Fig. 5,

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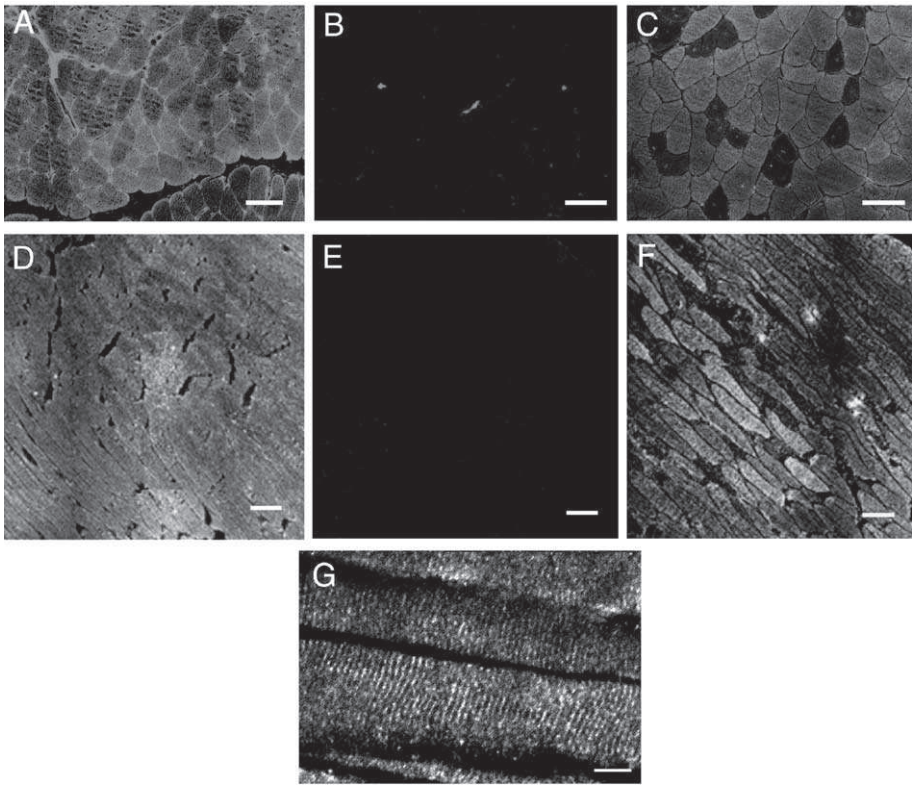


**FIG. 4.** Immunodetection of calpain 3 in normal and calpain 3-deficient muscles treated and not treated with calpain 3 rAAV2/1 vector using a polyclonal antibody directed against the calpain 3 protein (D1; Calbiochem). (A, B, and C) Transverse sections. (D, E, F, and G) Longitudinal sections. (A, D) Normal muscles. (B, E) Calpain 3-deficient muscles. (C, F) Calpain 3-deficient muscle injected with AAV1-C512-hCAPN3fsr. (G) A higher magnification shows a sarcomeric pattern. Scale bars: 80 μm for A, B, and C; 50 μm for D, E, and F; and 6 μm for G.

insets). Those results rendered us confident that overexpression of calpain 3 in muscle does not induce apparent sarcomere disorganization. In addition to these analyses, we performed immunostaining for titin, a partner of calpain 3, without noticing any alterations in calpain 3-deficient muscles before and after AAV1-C512-CAPN3fsr transfer (see Supplementary Fig. 2).

### Histological Analysis

We carried out histological studies on sections from the different mice by hematoxylin and eosin (H&E) staining and inflammatory response monitoring (CD11b marker staining) 1 month after injection. Whereas calpain 3-deficient muscles (Fig. 6A) presented dystrophic features like variation in fiber cross-sectional area, centrally located nuclei, and fibrosis compared to wild type (Fig. 6B), AAV1-C512-CAPN3fsr-injected muscle phenotype was almost like the wild type (Fig. 6C). We still observed a few centrally located nuclei, possibly due to insufficient delay between the injection and the time when analyses were performed. CD11b-positive cells were present in clusters or dispersed throughout the sections in calpain 3-deficient mice (Fig. 6D), while we observed no staining in wild-type mice (Fig. 6E). In muscle from mice injected with AAV1-C512-CAPN3fsr, few scattered CD11b-positive cells were still observed but clusters were never noticed (Fig. 6F). These results demonstrated that AAV1-C512-CAPN3fsr vector injection improved the dystrophic phenotype in calpain 3-deficient muscles.

### Increased Muscle Mass and Force After Calpain 3 Intra-arterial Administration

Previous characterization of the mechanical properties of calpain 3-deficient mice revealed a 20% muscle-specific force deficit in the soleus muscle evidenced between 7 and 12 months of age without any difference in susceptibility to stretch-induced damage [8]. To test if calpain 3 rAAV2/1 transfer might reverse this decrease of zforce, we injected $1.15 \times 10^{12}$ viral genomes of AAV1-C512-CAPN3fsr through the femoral artery of the right limb of 7- to 8-month-old calpain 3-deficient males. Eight weeks later, we anesthetized the mice and excised the extensor digitorum longus (EDL) and soleus of both limbs for measurement of calpain 3 expression, muscle weight, and isometric contractile properties (Fig. 7). The mean copy number of calpain 3 transgene messenger measured by TaqMan quantitative RT-PCR was $2.6 \times 10^6$ and $5.7 \times 10^5$ per microgram of RNA in EDL and soleus, respectively. The EDL muscle from the uninjected limb weighed 9.17 ± 0.72 mg, while EDL from the treated limb weighed 9.46 ± 1.80 mg ($P$ = 0.60) (Fig. 7A). The soleus muscle from the uninjected limb weighed 4.75 ± 0.50 mg, while soleus from the

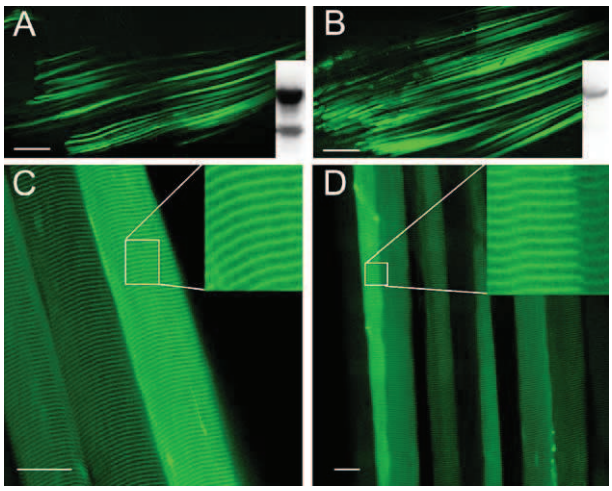


**FIG. 5.** Absence of cytoskeleton disorganization induced by an overexpression of calpain 3. Normal TA muscles were injected with (A and C) $1.4 \times 10^{10}$ vg of AAV1-C512-hCAPN3fsr or (B and D) PBS. One month later, a reporter plasmid carrying α-actin fused to GFP was electrotransferred into the same muscles. The day of sacrifice, mice were anesthetized and skin was carefully dissected to expose the transduced muscle. Efficiency of electrotransfer was evaluated under the microscope and revealed widespread plasmid transduction with about a third of the fibers estimated to be transduced by the plasmid actin–GFP (A and B). A control Western blot was subsequently performed to assess the amount of calpain 3 expression (insets of A and B). (C and D) Sarcomere organization at 60× original magnification (insets correspond to a 3× zoom showing 1 representative fiber of 50 that were examined). No difference in the actin cytoskeleton was observed between (C) calpain 3- and (D) PBS-injected muscles. Scale bars: A and B, 1 mm; C and D, 50 μm.

treated limb weighed 5.88 ± 1.60 mg ($P$ = 0.037) (Fig. 7B). This result indicates that calpain 3 injection has a positive influence on weight, at least on soleus muscles. Maximum absolute tetanic force was 208 ± 31 mN in EDL from untreated limb versus 234 ± 69 mN ($P$ = 0.31) from treated limb of the same mouse. Maximum absolute tetanic force was 61 ± 12 mN in soleus from untreated limb versus 100 ± 36 mN from treated limb of the same mouse ($P$ = 0.006). When the force was normalized for the muscle cross-sectional area, the specific isometric tetanic force was 125 ± 26 mN/mm$^2$ versus 135 ± 26 mN/mm$^2$ ($P$ = 0.40) in untreated and treated EDL, respectively (Fig. 7C), and 88 ± 23 mN/mm$^2$ versus 113 ± 27 mN/mm$^2$ in untreated and treated soleus, respectively ($P$ = 0.01) (Fig. 7D). Thus, the increase in force after calpain 3 administration is 12.5% (8% when normalized) and 64% (28% when normalized) in EDL and soleus muscles, respectively. To represent the extent of correction, the relative specific force of treated and untreated calpain 3-deficient muscles compared to wild-type muscles are displayed along arrows with values obtained for wild-type muscles set to 100% (Figs. 7C and 7D). The increase in force led to full recovery of the force of the soleus fibers.

## DISCUSSION

In this paper, we have presented evidence that injection of rAAV2/1 vectors expressing the human calpain 3 cDNA results in therapeutic effects in a dystrophic model of LGMD2A, suggesting that AAV gene transfer may be a strategy of choice to treat this disease. Among the muscular dystrophies, LGMD2A represents a favorable case for gene therapy. Its restricted pattern of involvement suggests that it would necessitate only reaching proximal limb muscles to improve considerably the clinical state of the patients. In fact, transduction of whole groups of muscle in human no longer seems an

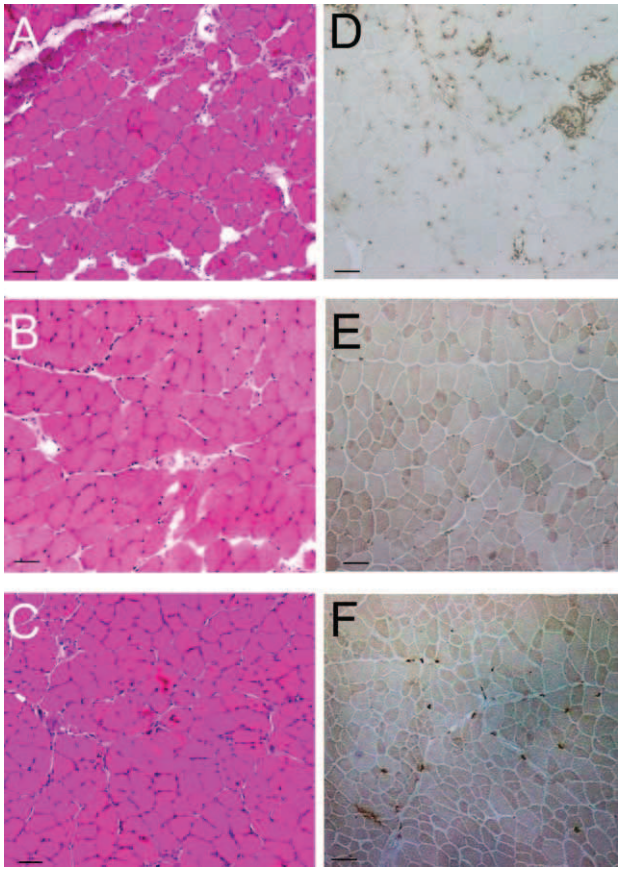


**FIG. 6.** Histological analyses of muscles. (A, B, and C) Frozen sections of TA muscles stained with H&E. (D, E, and F) The same muscle stained with CD11b antibodies. (A, D) Calpain 3-deficient muscle. (B, E) Muscle from wild-type mouse. (C, F) Calpain 3-deficient muscle injected with AAV1-C512-hCAPN3fsr. Dystrophic changes were evident in calpain 3-deficient muscle with variation in fiber cross-sectional area, centrally located nuclei, and fibrosis (A). Macrophage infiltrations are also observed (D). AAV1-C512-CAPN3fsr-injected muscle histology was almost like the wild type with residual centrally located nuclei (C). Few scattered macrophage cells were still observed but clusters were never noticed (F). These observations demonstrated an improved histological phenotype and a decreased in inflammatory response in AAV treated muscle (scale bars, 50 μm).

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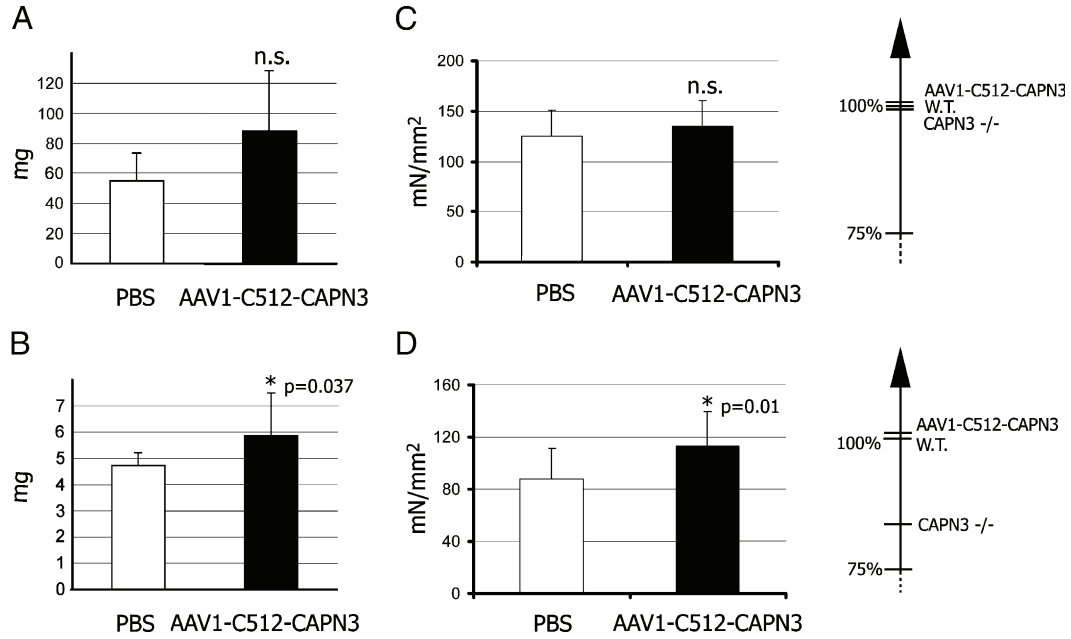


**FIG. 7.** Evaluation of muscle mass and contractile force after intra-arterial AAV calpain 3 treatment. Fifteen calpain 3-deficient male mice 7 to 8 months of age received $1.15 \times 10^{12}$ viral genomes by intra-arterial delivery in the right limb. Weight and force were assessed 8 weeks after injection. Mean copy numbers per microgram of RNA were determined to be $2.6 \times 10^{6}$ for EDL and $5.5 \times 10^{5}$ for soleus. (A and B) Histograms presenting the mean weight (±SD) of the EDL (A) and soleus (B) muscles from the uninjected and injected limb of the mice ($n$ = 15). (C and D) Histogram presenting the mean contractile specific force (±SD) of the EDL (C) and soleus (D) from the uninjected and injected limb of the mice ($n$ = 15). Differences with a $P$ value <0.05 are indicated with asterisks and the actual value is displayed. Inversely, n.s. indicates nonsignificant difference. Arrows represent the relative specific force of treated and untreated calpain 3-deficient muscles compared to wild-type muscles for EDL (C) and soleus muscles (D). Values obtained for wild-type muscles have been set to 100%.

unattainable challenge, considering the recent development of efficient techniques for systemic delivery [16,17,22–25].

Due to the proteolytic nature of calpain 3, a demonstration of the safety and efficacy of calpain 3 gene transfer presented specific particularities. In muscle gene transfer, it was previously shown that use of a muscle-specific promoter prevents expression in antigen-presenting cells and subsequent immune response directed toward the transgene product [26]. In fact, it should be noted that we did not detect any antibodies directed against calpain 3 in AAV1-C512-CAPN3fsr-treated mice (see Supplementary Fig. 1). Muscle-specific promoters may also help avoid toxicity thanks to the lower efficiency of these promoters compared to ubiquitous ones. In our case, another reason to avoid the use of a ubiquitous promoter was calpain 3 toxicity in cells used for vector preparation. This toxicity raised some concern about potential harmful effects of calpain 3 transfer in muscle. However, we observed a high transgene expression with the C5-12 promoter, without any evidence of toxicity and with persistence of expression over a 3-month period. This promoter obviously lacks the regulation of calpain 3 transcription but the absence of toxicity suggests that it is not necessary to use the native calpain 3 promoter. In fact, in addition to transcriptional regulation, calpain 3 has many other levels of regulation to ensure a specificity of timing and location of its proteolytic activity, even when overexpressed [12,13,27]. The transferred calpain 3 seems correctly localized, as a sarcomeric pattern can be observed, suggesting a correct binding to titin. This is an important point as it has been proposed that titin has a role in the inhibition of the proteolytic activity of calpain 3 [28]. Therefore, correct binding may imply correct regulation, which is particularly crucial to avoid any unwanted proteolysis. In addition, the level of proteolytic activity that we detected was only a fraction of that of a normal muscle even in the presence of high expression. This observation implies a high buffering capacity of the transfected fibers for the uptake of a high level of calpain 3. The fact that a transgenic mouse model overexpressing calpain 3 does not present any obvious muscle phenotype is another positive argument concerning the safety of calpain 3 gene transfer [29].

To assess the efficiency of transfer in future human clinical trials, we cannot rely on protein detection because some calpain 3 mutations have been shown to result in normal expression of inactive calpain 3 [30]. To bypass this hindrance, we developed two different assays aimed at the specific detection of the transgene messen-

ger and at the detection of the proteolytic activity of calpain 3. These assays will enable us to determine precisely, using dose escalation studies, the minimal level of expression and proteolytic activity needed to achieve a correction of atrophy and reversal of force deficit. It will then be possible to extrapolate the therapeutic efficiency after quantification in human samples.

The histology of calpain 3-deficient mouse muscles is characterized by moderate and highly variable pathological signs including central nuclei, mononucleated cell infiltrates, and variation in fiber cross-sectional area. Interestingly, the treatment used partially alleviated the phenotype as demonstrated by the absence of clusters of mononucleated inflammatory cells and a decrease in fiber size heterogeneity. LGMD2A is a purely atrophic phenotype with no pseudo-hypertrophy as in the case of Duchenne muscular dystrophy or sarcoglycanopathies. Calpain 3 transfer in mice induced an increase in soleus muscle mass, suggesting that gene transfer in humans would be beneficial with respect to the atrophy parameter. However, the ultimate proof of pathological reversion is a restoration of the force of the muscle, as it was achieved in the soleus. This increase in force is found when we consider the raw force that corresponds to the global force of the muscle and the normalized force that reflects the specific force of the fibers. It is interesting to note that both effects (atrophy and force) were observed in soleus muscle, one of the most affected muscle in the calpain 3-deficient animal model, whereas calpain 3 expression has minimal effects in EDL, a relatively spared muscle.

In conclusion, our work represents the first example of phenotypic correction of a LGMD2 caused by mutations in a protein with enzymatic properties and establishes the feasibility of AAV-mediated calpain 3 gene transfer as a therapeutic approach for this disease.

## Materials and Methods

***Vector construction and production.*** Four AAV plasmids, pAAV-CMV-MCS, pAAV-CK6-MCS, pAAV-C5-12-MCS, and pAAV-desmin-MCS, were obtained from Dr. Jeng-Shin Lee (HGTI, Harvard). The last three were constructed by replacing the CMV promoter of pAAV-CMV-MCS, an AAV-based pSMD2-derived vector, with muscle-specific promoters [31]. The CK6 and C512 promoters have been previously described [32,33]. The desmin promoter consists of a fragment derived from the pD-1738cat plasmid, which contains the region −974 to +75 bp from the transcription initiation site [34].

The human calpain 3 cDNA previously cloned in a pBluescript plasmid [2] was subcloned by PCR with specific oligonucleotides containing restriction enzyme sites in two of the plasmids described above to obtain the plasmids pAAV-CMV-CAPN3 and pAAV-C512-CAPN3. The plasmids pAAV-CK6-muSeAP, pAAV-C5-12-muSeAP, and pAAV-desmin-muSeAP were constructed by subcloning a *Hin*dIII–*Xho*I fragment containing the muSeAP coding sequence (obtained from the pVT20 plasmid, a gift from Vincent Thuiller [35]) into the corresponding vectors. The pAAV-CMV-eGFP (pSMD2-eGFP) was previously described [36].

Silent mutations were introduced into the human calpain 3 coding sequence using Quick Change site-directed mutagenesis kits (Stratagene). A first mutagenesis was performed with the primers TU1.f, 5′-AAAACCTACATCAACATGAGAGAGGTGTCCCA-3′, and TU1.r, 5′-GAAGCGCTGGGACACCTCTCTCATGTTGATGT-3′, and modifies the codon encoding the R540 residue from CGG to AGA. These mutations are situated in the middle of the MGB probe that was used to detect the transferred calpain 3 (see RT-PCR). This was followed by a second mutagenesis using the Quick Change multi-site-directed mutagenesis kit (Stratagene) with the primers TUmutpa.f, 5′-AACGCCTC-CAAGGCCCGTAGCAAAACCTACATC-3′, and Tumutpm.f, 5′-AACATGA-GAGAGGTGAGCCAGCGCTTCCGC-3′ to introduce mutations at the 3′ sites of the primers. TUmutpa.f is aimed at modifying the codon encoding the R532 residue from AGA to CGT and Tumutpm.f the codon encoding S543 from TCC to AGC. The mutated calpain 3 insert was subcloned by PCR in the plasmid pAAV-C512-MCS to obtain the plasmid pAAV-C512-CAPN3fsr.

To obtain the plasmid pcDNA3-hCAPN3-C129S, the cysteine at position 129 of the human calpain 3 coding sequence was converted to serine using the Quick Change site-directed mutagenesis kit (Stratagene) and the following oligonucleotides: hCAPN3-mutag-C129S.f, 5′-TG-TCAAGGAGAGCTAGGGGACTCCTGGTTTCTCGCAGCCATT-3′, and hCAPN3-mutag-C129S.r, 5′-AATGGCTGCGAGAAACCAGGAGTCCCC-TAGCTCTCCTTGACA-3′. The mutated calpain 3 was then subcloned by PCR in pcDNA3.1D/V5-His-TOPO. The plasmid pGFP-actin was purchased from Clontech.

Plasmid DNA was prepared using the Maxiprep kit or, for *in vivo* injection, the EndoFree Megaprep kit from Qiagen. All constructs were subjected to automated sequencing to verify the integrity of the constructs.

Adenovirus-free AAV2/1 viral preparations were generated by packaging AAV2-ITR recombinant genomes in AAV1 capsids using a three-plasmid transfection protocol as described [37]. Viral genomes were quantified by dot blot against a standard plasmid serial dilution and infectious titer was measured by a modified replication center assay [38].

***In vivo vector delivery into muscle tissue.*** The construction and characterization of the calpain 3-deficient model have been previously described [7,8]. Control mice from the 129SV or the C57BL/6 strain were purchased from Charles River Laboratories (Les Oncins, France). All mice were handled according to the European guidelines for the humane care and use of experimental animals.

For intramuscular injection, 129SV or calpain-deficient mice were injected with 25 μl of rAAV2/1 viral preparation or with 50 μg of plasmid into the left tibialis anterior muscle. In the case of plasmid injection, transcutaneous electric pulses were applied on the hind limb according to previously described conditions [12]. The detailed procedure for intra-arterial injection has been previously described [23].

Force measurements after transfer were done according to our previous study [8].

***RT-PCR analysis.*** Quantitative RT-PCR analyses were done as previously described [37]. The primer pairs and TaqMan MGB probe used for the mutated calpain 3 were as follows: MGBCAPN3.f, 5′-CGCCTCCAAGGC-CCGT-3′; MGBCAPN3.r, 5′-AGCCAGCGCTTCCGC-3′; MGBTUCAPN3.s, 5′-CTACATCAACATGAGAGAGGT-3′. The primer pairs and TaqMan probe used for the normal calpain 3 were as follows: M811CAPN3.f, 5′-AAACAATCAGCTGGTTTTCAC-3′; M954CAPN3.r, 5′-GGAGGGGTGACA-GAGTTTTTTG-3′; and M884CAPN3.p, 5′-TGCCAAGCTCCATGGCT-3′. The ubiquitous acidic ribosomal phosphoprotein (PO) was used to normalize the data across samples [37].

To bypass the variability in the efficiency of the cDNA preparation and the PCR and to be able to compare different experiments, we prepared a RNA stock by an *in vitro* transcription reaction using the Megascript T7 kit (Ambion) and a plasmid carrying the mutated calpain 3 (CAPN3fsr). Serial dilutions ($1\times10^7$ to $1\times10^2$) of this control RNA were used in each experiment and processed along with the experimental samples. Since the precise size of this RNA is known (1795 bases), calculation of the copy

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number can be performed according to the following formula: (weight in g × Avogadro's number)/(1795 × 330).

***Determination of muSeAP secretion.*** Detection of alkaline phosphatase activity was carried out with the Phospha-Light kit (Tropix Perkin–Elmer) following the manufacturer's instructions except for the modifications as previously described [37].

***In vitro detection of calpain 3 proteolytic activity.*** Fifty micrograms of muscle protein extract (about 1/200 of the whole muscle) was mixed with 600 ng of protein extract from NIH3T3 cells transfected with V5-tagged calpain 3 C129S as described in the supplementary methods and processed for 2 h at 37°C in reaction buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton) in a final volume of 100 μl. The reaction was performed with and without calcium at 5 mM final. The reaction was subjected to Western blot using an anti-V5-tag antibody at a dilution of 1/5000 (Invitrogen).

***Western blot, immunofluorescence, and histological analyses.*** Western blots were performed according to the protocol described in the supplementary methods. Cryosections (8 or 10 μm thickness) were prepared from frozen muscles. Transverse sections were processed for H&E histological staining. For detection of alkaline phosphatase activity, cryosections were treated as presented in [37]. For immunohistochemical detection of calpain 3, unfixed transverse or longitudinal sections were blocked with PBS 1× +FCS 20% for 1 h and then incubated with 1/300 dilutions of calpain 3 primary antibodies for 2 h at room temperature (D1; Calbiochem). After being washed with PBS 1×, sections were incubated with a goat anti-rabbit secondary antibody conjugated with Alexa 633 dye diluted 1/1000 (Molecular Probe) for 1 h at room temperature. For immunohistochemical detection of CD11b, acetone-fixed transverse sections were blocked with PBS 1× + rabbit serum 10% for 30 min and then incubated with 1/20 dilutions of rat anti-mouse monoclonal CD11b antibodies (BD Pharmingen) for 1 h at room temperature. After being washed with PBS 1×, sections were incubated with a rabbit anti-rat HRP-conjugated secondary antibody diluted 1/200 for 1 h at room temperature and revealed with the DAB–peroxidase kit from DAKO. Slow-fiber staining was performed using the monoclonal antibody anti-skeletal slow myosin (dilution 1/1000; Sigma; M-8421) according to the protocol of the ARK peroxidase kit (DAKO). Sections were mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA) and visualized on a Leica confocal microscope.

***Statistical analysis.*** Differences between treated and untreated muscles were determined using the Student paired *t* test. Statistical significance was defined as $P < 0.05$.

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## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymthe.2005.09.017.

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