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# Phenotypic Correction of $\alpha$ -Sarcoglycan Deficiency by Intra-arterial Injection of a Muscle-specific Serotype 1 rAAV Vector

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$\alpha$ -Sarcoglycanopathy (limb-girdle muscular dystrophy type 2D, LGMD2D) is a recessive muscular disorder caused by deficiency in  $\alpha$ -sarcoglycan, a transmembrane protein part of the dystrophin-associated complex. To date, no treatment exists for this disease. We constructed recombinant pseudotype-1 adeno-associated virus (rAAV) vectors expressing the human  $\alpha$ -sarcoglycan cDNA from a ubiquitous or a muscle-specific promoter. Evidence of specific immune response leading to disappearance of the vector was observed with the ubiquitous promoter. In contrast, efficient and sustained transgene expression with correct sarcolemmal localization and without evident toxicity was obtained with the muscle-specific promoter after intra-arterial injection into the limbs of an LGMD2D murine model. Transgene expression resulted in restoration of the sarcoglycan complex, histological improvement, membrane stabilization, and correction of pseudohypertrophy. More importantly,  $\alpha$ -sarcoglycan transfer produced full rescue of the contractile force deficits and stretch sensibility and led to an increase of the global activity of the animals when both posterior limbs are injected. Our results establish the feasibility for AAV-mediated  $\alpha$ -sarcoglycan gene transfer as a therapeutic approach.

## INTRODUCTION

$\alpha$ -Sarcoglycanopathy (LGMD2D, OMIM608099) is one of the recessive limb-girdle muscular dystrophies (LGMD2s), a group of genetically distinct disorders affecting predominantly the proximal limb muscles.<sup>1</sup> The clinical pattern presents as symmetric involvement of trunk and limb muscles predominating in pelvic girdles, frequent calf hypertrophy, occasional cardiomyopathy, and absence of mental retardation.<sup>2,3</sup> Age of onset and severity of LGMD2D is variable, ranging from severe forms (with onset in the first decade and rapid evolution) to

milder forms (with later onset and slower progression). Muscle impairment is associated with a substantial elevation of serum creatine kinase levels and dystrophic features on muscle biopsies such as area of degeneration/regeneration, variation in fiber size, and fibrosis.

LGMD2D results from mutations in  $\alpha$ -sarcoglycan (SGCA), a 50 kDa type I transmembrane protein mainly expressed in skeletal muscle and to a lesser extent in heart tissue.<sup>4,5</sup> It consists of a highly hydrophobic N-terminal region that acts as a signal sequence, an extracellular N-terminal domain, a single membrane-spanning domain, and a short intracellular domain.<sup>6</sup>  $\alpha$ -Sarcoglycan together with  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycans, dystroglycans, and syntrophins form the dystrophin-glycoprotein complex, which plays a crucial role in maintaining the linkage between the intracellular cytoskeleton and the extracellular matrix.<sup>7</sup> This link confers stability to the sarcolemma and protects muscle fibers from contraction-induced damage. It has also been suggested that sarcoglycans participate in signaling pathways as potential receptors and through binding with other proteins such as filamin-C or integrins.<sup>8,9</sup>

Two murine models for LGMD2Ds have been established.<sup>10,11</sup> Mice present a clinical phenotype very similar to the human condition, with a severe pattern of regeneration/degeneration cycles with numerous centronucleated fibers, wide regions of mononucleated-cell infiltrate, fiber splitting, and fatty infiltrations. In addition to this altered histological phenotype, mice exhibit a muscle-specific force decrease.<sup>10-12</sup> In an attempt to define a therapeutic strategy for this myopathy, gene transfer experiments have been previously performed in mice by intramuscular administration of viral vectors expressing a normal copy of  $\alpha$ -sarcoglycan.<sup>10,13,14</sup> In the first two studies, an adenovirus carrying the human  $\alpha$ -sarcoglycan under the control of the ubiquitous Rous sarcoma virus promoter was injected into the muscles of neonates, leading to genetic, biochemical, and histological restoration. In the third  $\alpha$ -sarcoglycan gene transfer study, a recombinant pseudotype-2 adeno-associated virus (rAAV2) carrying the human  $\alpha$ -sarcoglycan cDNA under the control of the CMV promoter was injected into the

muscles of 2- and 6-week-old animals.<sup>14</sup> An initial biochemical rescue of the muscle was obtained, but rapid loss of the transduced fibers occurred 6 weeks post-injection. Subsequent analysis in immunodeficient mice showed fiber cytotoxicity of  $\alpha$ -sarcoglycan overexpression.

In our study, we combined the use of rAAV2/1 vector and a muscle-specific promoter to administrate the  $\alpha$ -sarcoglycan cDNA by intra-arterial injection into the hind limbs of the *Sgca*-null model. We report stable and efficient transgene expression in muscle tissue, resulting in correction at the biochemical, histological, and functional levels without evident toxicity. Our results demonstrate feasibility for clinical application of AAV-mediated  $\alpha$ -sarcoglycan gene transfer in human patients.

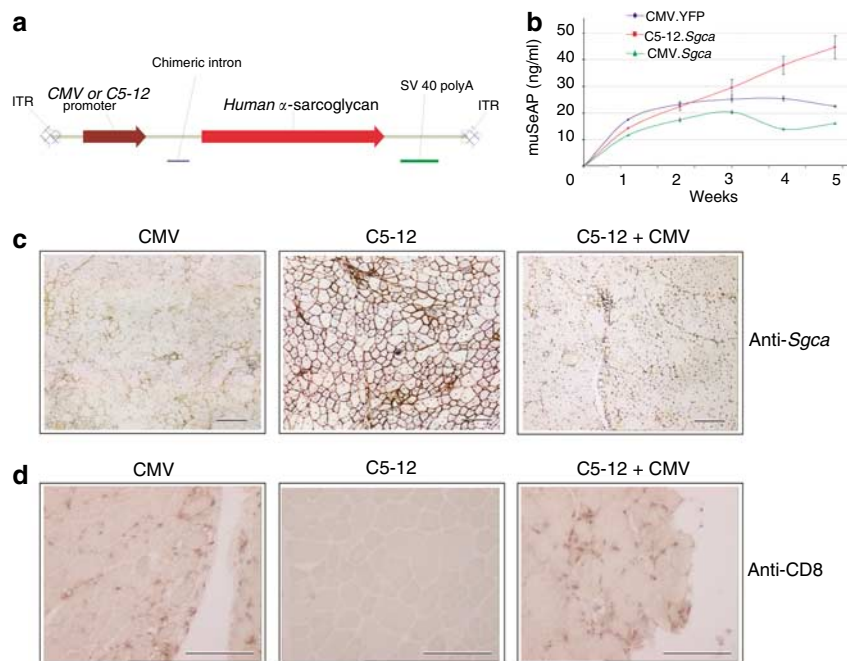
## RESULTS

### Comparison of $\alpha$ -sarcoglycan expression driven by ubiquitous versus muscle-specific promoters after AAV-mediated gene transfer

We constructed two rAAV2/1 vectors carrying the human  $\alpha$ -sarcoglycan cDNA under the control of either a ubiquitous (CMV, rAAV2/1.CMV.SGCA) or a synthetic muscle-specific (C5-12, rAAV2/1.C5-12.SGCA) promoter (Figure 1a). We previously demonstrated that it is possible to monitor cell survival and therefore the extent of muscle repair by monitoring murine secreted alkaline phosphatase (muSeAP) secretion in blood after administration of an muSeAP reporter vector.<sup>15</sup> Therefore, to compare the efficiency of both SGCA vectors, we co-injected

$2 \times 10^{10}$  viral genomes (vg) of each SGCA vector together with  $1 \times 10^{10}$  vg of an rAAV expressing muSeAP (rAAV2/1.C5-12 muSeAP) into the left quadriceps muscle of 5-week-old  $\alpha$ -sarcoglycan-deficient male mice (*Sgca*-null). A control condition without therapeutic vector consisted of injection of rAAV2/1.C5-12.muSeAP with an rAAV expressing the yellow fluorescent protein (rAAV2/1.CMV.YFP). We quantified muSeAP in blood from the mice every 5 days for more than 1 month after the injection (Figure 1b). With all three conditions, a similar rise in level of expression was observed up to day 12. After that time point, the level of secreted muSeAP stopped increasing for rAAV2/1.CMV.SGCA and rAAV2/1.CMV.YFP, while it continued to increase for rAAV2/1.C5-12.SGCA (Figure 1b). The similar absence of positive outcome with rAAV2/1.CMV.YFP and rAAV2/1.CMV.SGCA indicated that rAAV2/1.CMV.SGCA had no therapeutic effect. Immunostaining of the corresponding muscle slices with an  $\alpha$ -sarcoglycan polyclonal antibody showed an almost complete absence of staining for rAAV2/1.CMV.SGCA-injected muscles, while widespread staining was obtained for rAAV2/1.C5-12.SGCA-injected muscles (Figure 1c, left and middle panels). These results indicated that only rAAV2/1.C5-12.SGCA improved survival of the fibers.

Further experiments implied that the result obtained with the CMV promoter is related to an immune response. First, CD8<sup>+</sup> T lymphocytes were detected in muscle sections injected with rAAV2/1.CMV.SGCA (Figure 1d). Second, when the rAAV2/1.C5-12.SGCA vector was injected in one leg with a concomitant injection of rAAV2/1.CMV.SGCA in the contralateral muscle,



**Figure 1 Analysis of promoter.** AAV-mediated  $\alpha$ -sarcoglycan expression driven by a ubiquitous versus muscle-specific promoters. **(a)** Diagram of virus genomes used in this study. The human or mouse  $\alpha$ -sarcoglycan cDNAs were expressed under the control of the CMV or the C5-12 promoter. **(b)** Kinetics of muSeAP secretion in mouse blood after co-injection of rAAV2/1.C5-12.muSeAP vector ( $1 \times 10^{10}$  vg) with rAAV2/1.CMV.YFP, rAAV2/1.CMV.SGCA, or rAAV2/1.C5-12.SGCA ( $2 \times 10^{10}$  vg) into the quadriceps muscle of *Sgca*-null mice. Plasma concentration of muSeAP was quantified every week for 5 weeks. **(c)** Immunodetection of  $\alpha$ -sarcoglycan using a polyclonal antibody (AC-ahSarco57) in deficient muscles treated by either rAAV2/1.CMV.SGCA (left panel) or rAAV2/1.C5-12.SGCA (middle and right panels). The right panel corresponds to a mouse that was co-injected in the contralateral limb with rAAV2/1.CMV.SGCA (Bar = 100  $\mu$ m). **(d)** Immunodetection of CD8<sup>+</sup> T lymphocytes on muscle sections. A higher number of CD8<sup>+</sup> T lymphocytes was detected in muscles injected with rAAV2/1.CMV.SGCA compared with muscles injected with rAAV2/1.C5-12.SGCA alone (Bar = 100  $\mu$ m).

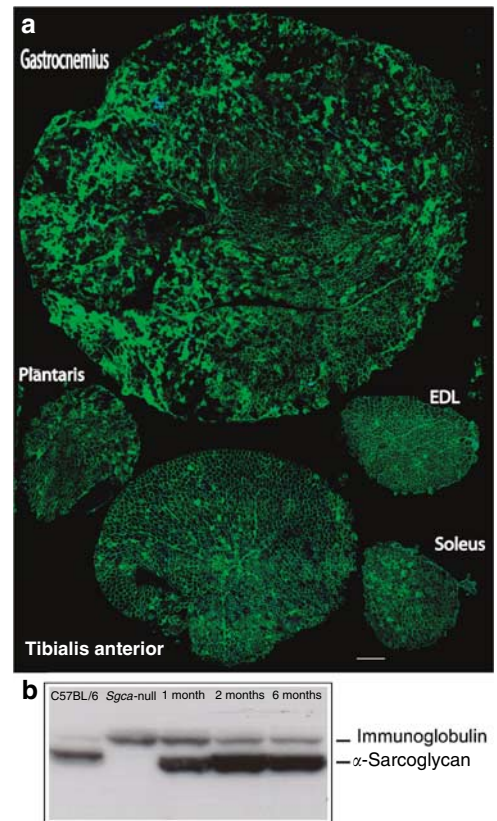
disappearance of  $\alpha$ -sarcoglycan-positive fibers was observed in both limbs (**Figure 1c**, right panel). Considering these results, we chose the C5-12 promoter to carry out the subsequent experiments.

### Restoration of the sarcoglycan complex after intra-arterial injection of the AAV.C5-12.SGCA vector

We injected  $3.8 \times 10^{12}$  vg of rAAV2/1.C5-12.SGCA into the femoral artery of the right limb of 5-week-old *Sgca*-null male mice. Previous work has shown that most muscles of the leg are effectively transduced after such intra-arterial injection.<sup>16</sup> The gastrocnemius, extensorum digitorum longus (EDL), soleus, plantaris, and tibialis anterior (TA) muscles of both limbs were sampled 30 days after injection and processed for evaluation of gene transfer efficiency. To assess the distribution of  $\alpha$ -sarcoglycan within the corresponding muscles, cross sections from injected and non-injected muscles were immunostained using a polyclonal antibody against  $\alpha$ -sarcoglycan. All the sampled muscles from the rAAV-treated legs showed extensive positive sarcolemmal staining (corresponding to nearly 100% of the fibers), indicating that the transgene protein was expressed and correctly localized (**Figure 2a**). We also performed Western blot analysis of *Sgca*-null TA muscles at 1, 2, and 6 months after injection. We observed sustained expression of  $\alpha$ -sarcoglycan that exceeded the level observed in normal muscle (**Figure 2b**). A positive effect on the level of immunoglobulin present in the muscle was also noticed (**Figure 2b**). Further transfer experiments indicated that protein expression is maintained for up to 1 year after injection but that the injection has to be performed on young animals, as only weak expression was obtained when 4-month-old animals were used (data not shown). It should be noted that a similar observation was reported for  $\gamma$ -sarcoglycan gene transfer by an AAV vector.<sup>17</sup> As is often the case in humans, the deficiency in  $\alpha$ -sarcoglycan in mice is associated with secondary loss of other components of the dystrophin-glycoprotein complex. After injection, the presence of  $\alpha$ -sarcoglycan at the membrane allowed the reconstitution of the complex as shown by the positive immunostaining for the other sarcoglycans and dystrophin (**Figure 3**).

### Restoration of the sarcoglycan complex improved the histology, decreased the membrane permeability, and reversed the pathologic hypertrophy

Histological analyses of the injected muscles indicated the benefit of  $\alpha$ -sarcoglycan transfer as there is less interstitial tissue and fewer degeneration/regeneration areas on muscle sections of injected animals (**Figure 4a**). This decrease was correlated with an important reduction of the inflammatory response as indicated by the disappearance of CD11b cellular infiltrations (**Figure 4b, c**). The improvement of the phenotype was also evidenced by a decrease in cells positive for Evans blue, a dye indicative of cell necrosis (**Figure 4d, e**). It should be noted that the improvement of tissue histology was not associated with a significant decrease in the percentage of fibers with central nuclei and that a number of fibers with more than one central nucleus could be observed, although this particularity decreased later on (data not shown).



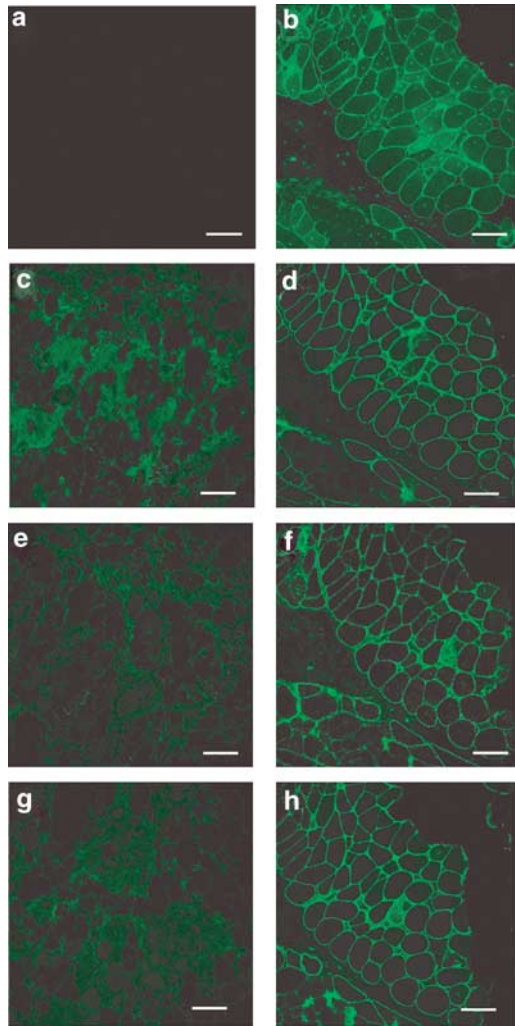
**Figure 2**  $\alpha$ -Sarcoglycan expression after intra-arterial injection. **(a)** Widespread expression of  $\alpha$ -sarcoglycan in limb muscles. Gastrocnemius, plantaris, TA, EDL, and soleus from *Sgca*-null mice injected with rAAV2/1.C5-12.SGCA vector ( $3.8 \times 10^{12}$  vg) were immuno-labeled with the AC-ahSarco57  $\alpha$ -sarcoglycan antibody (Bar = 250  $\mu$ m). **(b)** Western blot analysis of  $\alpha$ -sarcoglycan expression in *Sgca*-null TA muscle 1, 2, and 6 months (mo) after injection with rAAV2/1.C5-12.SGCA. C57BL/6 and *Sgca*-null muscle tissues were used as positive and negative controls, respectively. It is noteworthy that the immunoglobulins present in these highly inflammatory muscles were detected as a nonspecific band at 50 kDa by the anti-Ig mouse secondary antibodies. The decrease of Ig over time among the different injected samples underlined the positive effect of  $\alpha$ -sarcoglycan expression on the level of inflammation of the muscle.

Next, we characterized the effect of the injection on the pseudo-hypertrophic manifestation of the disease. When we compared the muscle weights from treated and untreated *Sgca*-null mice, we observed a significant decrease of 10–30% for every sampled muscle of the injected limb (**Table 1**). This effect is in fact readily visible in **Figure 4a** when comparing the muscle cross sections. Correction of hypertrophy was examined at the fiber-scale level by morphometric analysis of the muscles, demonstrating an important decrease in the number of small fibers, indicative of a reduction of the regeneration process (**Figure 5a**). This observation was confirmed by a reduction in the number of developmental myosin heavy chain (MHCd)-positive cells (**Figure 5b**).

### Increased force after $\alpha$ -sarcoglycan intra-arterial gene transfer

To test whether rAAV2/1.C5-12.SGCA transfer might be beneficial at the functional level, we performed another set of

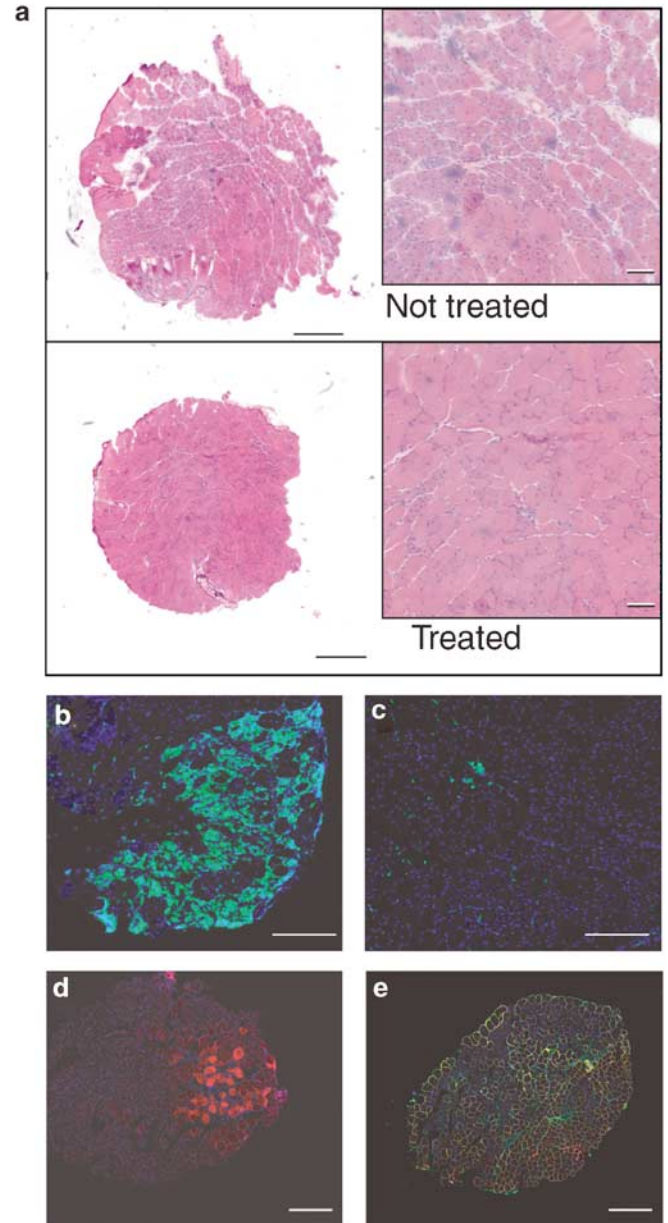




**Figure 3** Restoration of the dystrophin-glycoprotein complex after intra-arterial injection. **(a, c, e, g)** Muscle sections from untreated *Sgca*-null mice. **(b, d, f, h)** Muscle sections from treated *Sgca*-null mice. The immunostaining was performed using the following antibodies: **a, b**  $\alpha$ -sarcoglycan; **c, d**  $\beta$ -sarcoglycan; **e, f**  $\gamma$ -sarcoglycan; **g, h** dystrophin (Bar = 50  $\mu$ m).

injections ( $3.8 \times 10^{12}$  vg of rAAV2/1.C5-12.SGCA) in the femoral artery of the right limb of eight 5-week-old male *Sgca*-null mice. Eight weeks later, mice were anesthetized and the EDL and soleus of both limbs were surgically excised for *in vitro* measurement of force. The contractile properties of *Sgca*-null muscles revealed a specific isometric force normalized for the muscle cross-section area of 31 and 19% in the EDL and soleus, respectively ( $P = 0.04$  and  $0.03$ ; **Table 2** and **Figure 6a**). The resistance to mechanical stress was also corrected in injected EDL as shown by the force drops after five eccentric contractions that decreased from  $-45\%$  to  $-15\%$  after vector injection (**Figure 6b**).

Nevertheless, these improvements were not associated with a better performance in animal mobility that had been assessed by the “escape test” before the killing of the animals, probably because muscles from only one lower limb do not participate appreciably in whole body force. Therefore, we administered rAAV2/1.C5-12.SGCA by intra-arterial injection simultaneously

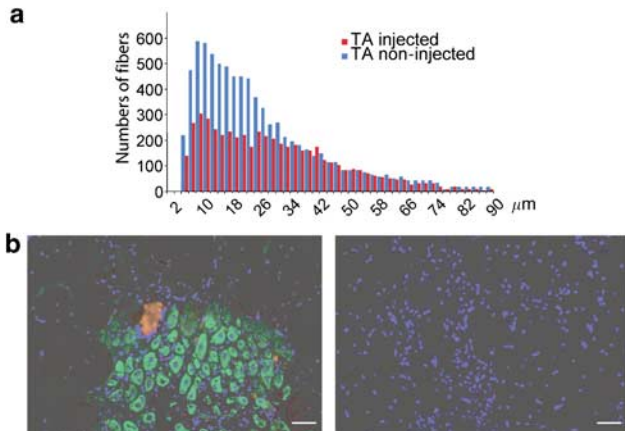


**Figure 4** Correction of histological aspect and decrease of membrane permeability. **(a)** Histological analyses of muscle. Muscle histology (hematoxylin and eosin staining) after rAAV2/1.C5-12.SGCA vector injection ( $3.8 \times 10^{12}$  vg) was much improved with fewer mononucleated cells and more homogeneous fibers within the cross-section area. However, residual centrally located nuclei were observed (see inset for a higher magnification) (Bars = 120  $\mu$ m for the whole section and 30  $\mu$ m for the inset). **(b, c)** Muscles from the same animals were stained for CD11b, a macrophage-specific marker. Macrophage infiltrations are clearly observed in non-treated animals. Inversely, a few scattered macrophage cells were still observed, although clusters were never noticed, in treated animals in transduced areas. **(d, e)** Membrane permeability was assessed with Evans blue staining (in red) with a concomitant labeling of  $\alpha$ -sarcoglycan (in green). A decrease in necrotic fibers is observed in treated animals compared with controls. For rAAV2/1.C5-12.SGCA-injected muscle, all Evans blue-positive fibers were devoid of  $\alpha$ -sarcoglycan staining and inversely all  $\alpha$ -sarcoglycan-positive fibers were negative for Evans blue.

**Table 1** Weight of the muscles sampled from the hind limbs of untreated and treated *Sgca*-null mice

Weight (mg)	EDL	Soleus	TA	Quadriceps	Gastrocnemius	Plantaris
<i>Sgca</i> -null	14.79 ± 3.93	10.59 ± 3	96.2 ± 19.27	305.76 ± 74.68	166.48 ± 23.08	22.61 ± 5.34
<i>Sgca</i> -null treated	10.11 ± 3.53	9.26 ± 2.47	68.34 ± 25.51	271.59 ± 70	143.41 ± 30.9	18.74 ± 6.04
<i>P</i> -value paired	0.0002	0.02	0.002	0.04	0.02	0.04
C57BL/6	12.40 ± 1.2	8.4 ± 0.8	56.5 ± 4.4	222.1 ± 12	144.5 ± 8.5	17.2 ± 2.1

EDL, extensorum digitorum longus; TA, tibialis anterior. Weights are indicated in mg ± SD. The *P*-value of the Student's test between treated and non-treated limb are also indicated.



**Figure 5** Reversion of pseudohypertrophy. **(a)** Evaluation of fiber size 8 weeks after intra-arterial rAAV2/1.C5-12.SGCA injection. The histogram represents the fiber minimum diameter. Injection of rAAV2/1.C5-12.SGCA induced a large decrease in the number of fibers with sizes less than 30 μm compared with non-injected animals. **(b)** The developmental stages of these muscles were assessed by MHCd staining. A large decrease of MHCd-positive fibers was noticed in treated (right panel) when compared with non-treated mice (left panel) (Bar = 50 μm).

**Table 2** Isolated muscle kinetic properties of EDL and soleus in *Sgca*-null mice treated with the AAV vector in the right limb (*Sgca*-null treated) or with PBS (*Sgca*-null) in the left limb

	Isometric contraction			
	Tension (mN/mm <sup>2</sup> )			
	EDL	N	Soleus	N
<i>Sgca</i> -null	72.6 ± 21.1	6	92.2 ± 31.1	6
<i>Sgca</i> -null treated	110.6 ± 40.2* ( <i>P</i> =0.04)	6	102.3 ± 23.9* ( <i>P</i> =0.03)	6

AAV, adeno-associated virus; EDL, extensorum digitorum longus; PBS, phosphate-buffered saline. Parameters are presented as mean ± SD. Tension is the normalized tetanic force (*n*=number of tested muscles) (\**P*<0.05).

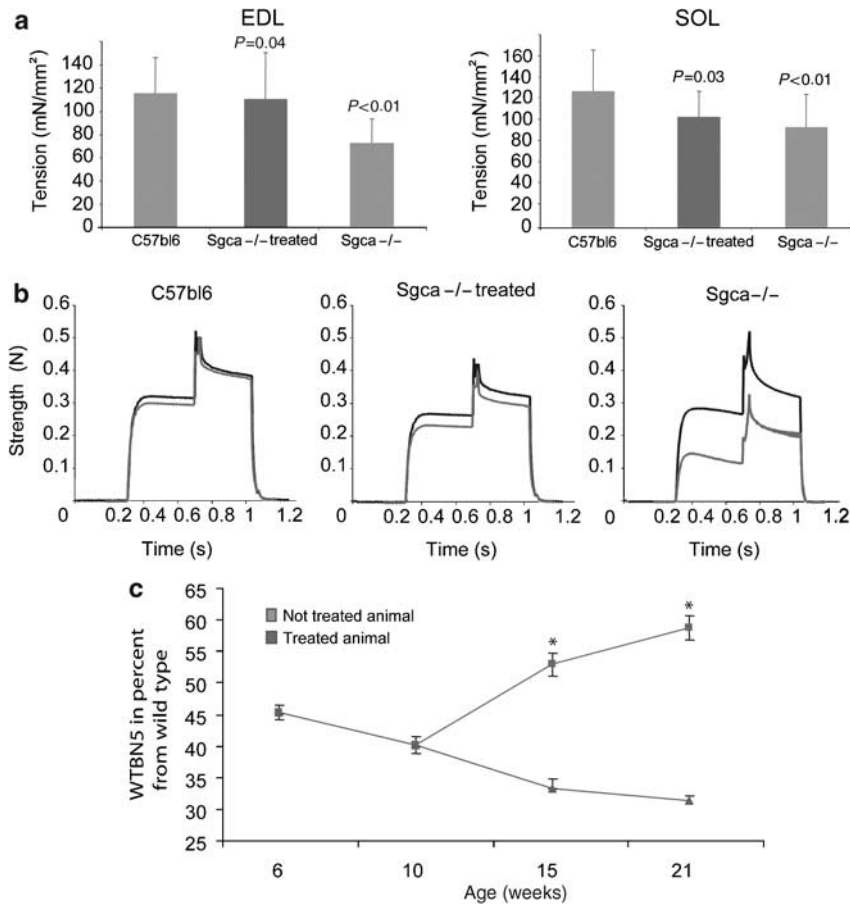
in the right and left posterior limbs of 5-week-old male *Sgca*-null mice ( $1.9 \times 10^{12}$  vg in each limb). A control group consisted of phosphate-buffered saline (PBS)-injected animals. Escape test analyses performed 6, 10, 15, and 21 weeks after the injection showed that the slope of the global force decreased in non-treated animals, whereas the slope for treated animals initially followed the same evolution and then increased (*P* = 0.04 at 15 weeks and *P* = 0.01 at 21 weeks; **Figure 6c**). This result illustrates a positive effect for  $\alpha$ -sarcoglycan gene transfer in disease evolution.

## DISCUSSION

In this report, we present evidence that intra-arterial injection of an rAAV2/1 vector expressing the human  $\alpha$ -sarcoglycan cDNA via a muscle-specific promoter can achieve efficient therapeutic effects in a dystrophic model of LGMD2D, suggesting that AAV-mediated transfer may be a strategy of choice to treat this disease. In addition, our data suggest that the difficulty to obtain  $\alpha$ -sarcoglycan expression in previous gene transfer experiments may be related to an immune response against the transgene.

$\alpha$ -Sarcoglycan transfer has been previously reported in an LGMD2D model by means of adenovirus or AAV-derived vectors.<sup>10,13,14</sup> In the two studies using adenovirus, it was necessary to use neonatal animals to take advantage of the immaturity of the immune system and therefore to circumvent the strong immune response elicited by this type of vector. This element, together with the fact that adenovirus has low transduction efficiency in adult skeletal muscles owing to the lack of appropriate receptors at the myofiber surface,<sup>18</sup> precludes the use of this type of vector for treatment of LGMD2D. In contrast, rAAV, which has been more widely used in the previous gene transfer studies in LGMD2 animal models including this one,<sup>14,17,19-24</sup> is non-pathogenic, has low immunogenicity, and has been shown to confer long-term gene expression in muscles of various species. In particular, transgene expression was shown to last at least 2 years in the dog<sup>25</sup> and 6 years in non-human primates.<sup>26</sup> Moreover, encouraging results have been obtained in Phase I clinical trials using rAAV vectors for cystic fibrosis<sup>27</sup> and hemophilia B.<sup>28,29</sup> Therefore, rAAV appears as one of the most promising vectors for gene therapy of muscular dystrophies, including Duchenne muscular dystrophy.<sup>30</sup>

In a third study, an rAAV2 expressing  $\alpha$ -sarcoglycan via the CMV promoter was not able to produce sustained transduction of the muscle for more than 6 weeks after injection.<sup>14</sup> Data obtained in immunodeficient mice led to the suggestion that  $\alpha$ -sarcoglycan cytotoxicity causes the loss of the vector through the death of the transduced fibers.<sup>14</sup> Similarly, we observed that  $\alpha$ -sarcoglycan expression is progressively lost when driven with the ubiquitous CMV promoter while it is not when the C5-12 promoter was used. The C5-12 promoter was reported to be stronger than the CMV promoter in muscles,<sup>31</sup> an observation that we confirmed independently (**Supplementary Figure S1**). High activity of C5-12 was also shown by the presence of intracytoplasmic accumulation of the transgene product in some muscles (e.g. gastrocnemius; **Figure 2**). However, this expression was not associated with any evidence of important toxicity as indicated by persistence of expression over a 6-month period. In



**Figure 6** Rescue of contractile force and stretch sensibility. Evaluation of contractile force of the EDL and soleus of C57BL/6 wild-type ( $n=36$  muscles) and *Sgca*-null male mice (non-injected muscles = 29 and injected muscles = 6). **(a)** The histogram presents the mean contractile normalized specific force ( $\pm$ SD) from the injected limb of injected mice compared with non-injected mice. Force was assessed 8 weeks after injection. The  $P$ -values are indicated and show significance between, on the one hand, *Sgca*-null and C57BL/6 mice ( $P<0.01$ ) and, on the other hand, *Sgca*-null treated and untreated mice ( $P=0.04$  for EDL,  $P=0.03$  for soleus). **(b)** In resistance to stretch experiments, the force drops of EDL muscles from C57BL/6, *Sgca*-null treated and *Sgca*-null mice are, respectively,  $3 \pm 3.3$ ,  $13 \pm 4$ , and  $30 \pm 5.7\%$ . Graphs show data obtained from representative mice. This result indicated that treatment restored a normal resistance (force drop inferior to 20%). **(c)** Global activity of treated and non-treated animals evaluated by the escape test 6, 10, 15, and 21 weeks after injection. WBTNS (whole body tension normalized 5) is the average of the five maximal developed forces after a pinching of the tail. The curves represent the WBTNS of treated and non-treated in percent from wild-type mice ( $*P<0.05$ ).

addition, detailed examination of fibers overexpressing  $\alpha$ -sarcoglycan indicated that this did not preclude correct formation of the dystrophin-glycoprotein complex. A more probable scenario is that the numerous antigen-presenting cells present in those dystrophic muscles could direct a strong immune response against the transgene product when the CMV promoter was used. In fact, it was previously shown that immature dendritic cells can capture rAAV vectors.<sup>32</sup> The use of the CMV promoter would allow expression of  $\alpha$ -sarcoglycan in those cells, which in turn would elicit an immune response against the transgene product. Assumption of an immune response is supported by the observation of disappearance of AAV-C5-12 transduced fibers when the contralateral muscle was injected with an AAV-CMV vector as well as by the presence of CD8<sup>+</sup> T lymphocytes in AAV-CMV-injected muscles. It seems that in the case of  $\alpha$ -sarcoglycan transfer, the immune response could cause more damage than toxicity, although we cannot excluded the possibility of the deleterious effect of overexpres-

sion toxicity over time. It would be critical to ensure restriction of expression in muscle cells in any gene therapy protocol. This would be even more crucial for systemic injection, which would be the method of choice if one wished to treat more than a local region of a single muscle.

Our data clearly show that  $\alpha$ -sarcoglycan expression directed by a muscle-specific promoter can stably correct the clinical phenotype of *Sgca*-null mice after intra-arterial delivery of an rAAV2/1 vector. Correction of the phenotype occurred at expression, biochemical, histological, and functional levels. In particular,  $\alpha$ -sarcoglycan expression reversed the pseudo-hypertrophy in *Sgca*-null muscles, which was mainly due to an extensive inflammatory infiltrate and extensive regeneration as seen by a large number of MHCd-positive myotubes. Fiber branching may also contribute to hypertrophy and may explain why, when the degeneration/regeneration process was halted, we observed fibers with more than one centrally located nucleus in transverse sections. Such multinucleation tends to regress with

time, but the fibers retained central nuclei for at least 4 months. It should be noted that we injected muscles that had already undergone cycles of degeneration/regeneration, which was different from all the previous studies of  $\alpha$ -sarcoglycan transfer in which only animals at the pre-necrotic stage were injected. In fact, persistence of centronucleation is a normal phenomenon following regeneration. For example, it was reported that the nuclei of many fibers remained in a central position even 6 months after a notexin injection.<sup>33</sup> As far as we know, no molecular explanation exists and it remains to be seen whether such events could be eventually reversed. Nevertheless, our observation indicates that it is possible to treat muscles already engaged in the dystrophic process.

Our study has demonstrated an improvement of the force of the muscles and a decreased sensibility to stretch in an  $\alpha$ -sarcoglycan-deficient model after gene transfer. Importantly, the increase in tension indicated a boost in the mean force of individual fibers that can be consecutive to membrane stabilization. Moreover, the transduction obtained with a dose of about  $2 \times 10^{12}$  vg in each hind limb led to transduction of approximately 50% of fibers and was sufficient to increase the global force of the animals as visualized by the escape test. Nevertheless, detailed dose-effect studies would be needed to determine the minimal level of fibers to be transduced to prevent clinical manifestations.

In conclusion, the results obtained herein with rAAV2/1-mediated transfer of the  $\alpha$ -sarcoglycan gene using an intravascular route represent a good prospect for gene therapy of LGMD2D. However, some critical points remain to be solved before application of such a strategy in humans. First, it will be important to assess the possibility of an immune response against the newly synthesized transmembrane  $\alpha$ -sarcoglycan because of the level of inflammation at the time of injection. Second, it may be necessary to define the therapeutic window because significantly dystrophic muscle appears to be refractory to gene transfer, as seen by the absence of expression in older animals. Concerning this particular point, several hypotheses can be given: (i) the presence of fibrosis in the muscle might constitute a barrier for viral delivery, (ii) the high level of inflammation might clear the viral vector before its entry into the fibers, or (iii)  $\alpha$ -sarcoglycan deficiency might induce a loss of AAV receptors at the plasma membrane. Lastly, efficient systemic or loco-regional delivery methods in humans will be needed in order to treat sufficient muscle to improve the clinical condition.

## MATERIALS AND METHODS

**Vector construction and production.** A plasmid carrying the coding sequence of human  $\alpha$ -sarcoglycan was obtained from Dr Jeng-Shin Lee (HGTI, Harvard). It was used to construct the pAAV.CMV.hSGCA and pAAV.C5-12.hSGCA plasmids that consist of an AAV-based pSMD2-derived vector<sup>34</sup> where the human  $\alpha$ -sarcoglycan is placed under the control of a CMV or C5-12 promoter.<sup>22</sup> The plasmid pAAV.C5-12.muSeAP was constructed by subcloning a *HindIII-XhoI* fragment containing the muSeAP coding sequence (obtained from the pVT20 plasmid, a gift from Vincent Thuiller<sup>35</sup>) in place of  $\alpha$ -sarcoglycan in pAAV.C5-12.hSGCA. A control rAAV vector carrying the YFP under the control of the CMV promoter was also constructed. Plasmids were

prepared using the Nucleobond PC2000EF kit from Macherey-Nagel (Germany). All constructs were subjected to automated sequencing to verify their integrity. Adenovirus-free AAV2/1 viral preparations were generated by packaging AAV2-inverted terminal repeat recombinant genomes in AAV1 capsids using a three-plasmid transfection protocol.<sup>15</sup> After DNA extraction by successive treatments with DNase I and proteinase K, viral genomes were quantified by a TaqMan real-time PCR assay using primers and probes corresponding to the inverted terminal repeat region and to the albumin gene (Alb) for normalization of the data across samples.<sup>36</sup> The primer pairs and TaqMan MGB probes used for inverted terminal repeat amplification were: 1AAV65/Fwd: 5'-CTCCATCACTAGGGGTTCCCTTGTA-3'; 64AAV65/rev: 5'-TGGCTACGTAGATAAGTAGCATGGC-3'; and AAV65MGB/taq: 5'-GTTAATGATTAACCC-3'. The primer pairs and TaqMan MGB probes used for Alb amplification were: ALB.Fwd: 5'-GCTGTCATCTCTGTGGGCTGT-3'; ALB.Rev: 5'-ACTCATGGGAGCTGCTGGTTC-3'; and AlbVic/taq: 5'-CCTGTCATGCCACACAAATCTCTCC-3'.

**In vivo experiments.** A murine knockout for  $\alpha$ -sarcoglycan was obtained from K Campbell (University of Iowa, USA<sup>10</sup>). Control mice from 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, animals were anesthetized by intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg) and the left TA was injected with 20  $\mu$ l of viral preparation. The intra-arterial injection procedure has been previously described.<sup>16</sup> Briefly, mice under isoflurane anaesthesia underwent femoral artery and vein isolation of the right hind-limb for single injection or both limbs for double injection. After clamping the femoral vein and two collaterals, a catheter was introduced in the femoral artery and the rAAV preparation was injected in a volume of 1 ml/20 g of body weight at a rate of 100  $\mu$ l/s. In case of double injection, a lapse-time of 20 h separated the two injections. Blood samples for muSeAP-injected animals were obtained every week by retro-orbital puncture of anesthetized animals and detection of alkaline phosphatase activity was carried out as previously described.<sup>15</sup> One day before the endpoint of the experiment, the mice were injected intraperitoneally with Evans blue dye (0.5 mg/g of body weight). The following day, mice were killed and the hind-limb muscles were removed and quickly frozen in liquid nitrogen-cooled isopentane.

**Immunofluorescence, histology, and morphometrical analyses.** Cryosections (8  $\mu$ m thickness) were prepared from frozen muscles. Transverse sections were processed for hematoxylin and eosin histological staining.

For colorimetric immunodetection of  $\alpha$ -sarcoglycan, unfixed transverse cryosections were rehydrated with PBS for 5 min and then incubated with H<sub>2</sub>O<sub>2</sub> to inhibit endogenous peroxidases 20 min at room temperature (RT). After washing with PBS, sections were blocked with PBS/10% goat serum for 30 min and then incubated with 1/1,000 dilution of a rabbit polyclonal primary antibody directed against amino acids 366-379 of the human  $\alpha$ -sarcoglycan sequence (AC-ahSarco57) 1-2 h at RT. After washing with PBS, sections were incubated with secondary antibody conjugated with horseradish peroxidase (HRP) diluted 1/200 for 1 h at RT. Sections were washed three times with PBS and then incubated with diluted diaminobenzidine (DAKO, Trappes, France) for 2-5 min. Then, sections were successively treated with ethanol (5 min), twice in xylene (5 min), mounted with Eukkit (Labonord, France), and visualized on a Nikon microscope.

For fluorescent immunohistochemical detection of sarcoglycans, unfixed transverse cryosections were blocked with PBS/20% fetal calf serum for 1 h and then incubated with a 1/1,000 dilution of primary antibodies for 2 h at RT. The sarcoglycan, dystrophin, and MHCd monoclonal antibodies used in this study were purchased from



Novocastra (Antony, France, NCL-a-, NCL-b-, NCL-g-SARC, NCL-Dys2, NCL-MHCd) and the rat anti-mouse CD11b monoclonal antibody from BD Pharmingen (Le pont de Claix, France, 550282). After washing with PBS 1 ×, sections were incubated with a goat anti-mouse secondary antibody conjugated with Alexa488 dye diluted 1/1,000 (A-11070, Molecular Probes, Cergy Pontoise, France) for 1 h at RT. Sections were mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL) after 4',6 diamidino-2-phenylindole staining and visualized on a Leica confocal fluorescence microscope.

Evans blue positive fibers were revealed by fluorescence excitation at 633 nm on a Leica confocal fluorescent microscope. The final images represent an assembly of several images taken using a motorized stage at an original × 40 magnification.

For determination of the number and minimal diameter of fibers, immunological staining with a polyclonal antibody against laminin (P-4417, Progen, Heidelberg, Germany), used to delimit each fiber, was performed as described above for colorimetric immunodetection of α-sarcoglycan. Digital images of a slice corresponding to the muscle midsection were acquired with a 4 × objective, a CCD camera (Sony) and a motorized stage. Images were then analyzed with the help of Ellix software (Microvision, Evry, France). For determination of percentage of fibers with central nuclei, 10 randomly selected fields of a section stained with hematoxylin and eosin were acquired with a 20 × objective lens. The total number of fibers and those with a central nucleus were determined with the help of Saisam software (Microvision, Evry).

**Western blot analysis.** Frozen sections corresponding to approximately 1-mm-thick muscle were solubilized in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM ethyleneglycol tetraacetate, 1% Triton containing the Complete Mini Protease Inhibitor Cocktail and 2 μM E64. After centrifugation at 8,000 g for 5 min at +4°C, the supernatants were quantified using the bicinchoninic acid Protein Assay (Pierce, Rockford, IL) and then mixed with 1 mM dithiothreitol and 1 × Lithium dodecyl sulfate NuPAGE Buffer (Invitrogen, Cergy Pontoise, France) before they were denaturated at 70°C for 10 min. Ten microgram of protein was then processed for Western blotting using a monoclonal antibody against α-sarcoglycan (Novocastra, NCL-a-SARC, dilution 1:100) and revealed using the anti-mouse immunoglobulin secondary antibodies (Amersham, Buckinghamshire, UK) according to standard procedure.

**In vitro and in vivo force measurement.** Animals were anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg). The EDL and soleus muscles were surgically excised and maintained in Krebs buffer. Measurement of isometric contractile properties and resistance to eccentric contractions of EDL have been described previously.<sup>37</sup>

Global activity of mice was evaluated by the escape test.<sup>38</sup> Mice were placed on a platform facing the entrance of a tube that was 30 cm long. A cuff was wrapped around the tail and connected to a fixed force transducer. In response to gentle pinching of the tail, mice tried to escape within the tube. This was prevented by attaching the tail to the force transducer and a short peak of force was recorded. Maximal peak and the average of the five highest peaks normalized to animal body weight are reported.

**Statistical analysis.** Data for each group are represented as the means plus SD. Differences between treated and untreated muscles were determined using the Student's paired *t*-test. All statistical tests were considered non-significant when the error was above 5%.

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