PlGF–MMP-9–expressing cells restore microcirculation and efficacy of cell therapy in aged dystrophic muscle

Cesare Gargioli1,2, Marcello Coletta1, Fabrizio De Grandis1,2, Stefano M Cannata2 & Giulio Cossu1,3,4

Sclerosis and reduced microvessel density characterize advanced stages of muscular dystrophy and hamper cell or gene delivery, precluding treatment of most individuals with Duchenne muscular dystrophy. Modified tendon fibroblasts expressing an angiogenic factor (placenta growth factor, PlGF) and a metalloproteinase (matrix metalloproteinase-9, MMP-9) are able to restore a vascular network and reduce collagen deposition, allowing efficient cell therapy in aged dystrophic mice. These data open the possibility of extending new therapies to currently untreatable individuals.

Among experimental therapies for muscular dystrophy1,2, the transplantation of mesangioblasts, vessel-associated progenitors3, has reduced disease severity in α-sarcoglycan–null dystrophic mice and golden retriever dystrophic dogs4,5. For both gene and cell therapies, efficient delivery to skeletal muscle represents a major challenge, as this muscle type is the most abundant tissue in the body and is composed of multinucleated cells that do not divide. Sclerosis and fat infiltration that occur in the advanced stages of the disease6 further reduce the efficacy of gene or cell delivery. Therefore, even if current trials are successful, they are unlikely to elicit a significant benefit when extended to people at more advanced stages of disease, currently the vast majority of affected individuals. Here we show that tendon fibroblasts engineered to express a metalloproteinase and an angiogenic factor can restore microcirculation and reduce connective tissue deposition after intramuscular delivery to aged dystrophic muscle, resulting in a substantial improvement of subsequent cell therapy that reaches the same efficacy as observed with young dystrophic muscle.

Aged (12-month-old) dystrophic (α-sarcoglycan–null) mice showed more severe degeneration in their skeletal muscles than their young (2-month-old) counterparts, with enhanced variability in fiber diameter, massive accumulation of collagen and adipose tissue and frequent necrotic fibers (Supplementary Fig. 1a–d online), consistent with data previously reported in other muscular dystrophies7–10. Likewise11,12, leukocyte infiltration, as revealed by immunostaining with antibodies to CD11b, was also increased (Supplementary Fig. 1e,f).

We used cells as vectors for molecules that may reduce fibrosis and stimulate angiogenesis. We transduced tendon fibroblasts, characterized by FACS analysis and immunocytochemistry (Supplementary Fig. 2a–d online), with lentiviral vectors expressing MMP-9 (ref. 13) or PlGF (ref. 14) and the blasticidin resistance gene. We chose tendon fibroblasts because they reside in avascular tissue and can survive under very hypoxic conditions15; moreover, they may be easily available during the surgical correction of tendon retractions that occur during the progression of muscular dystrophy16. Transduced tendon fibroblasts were selected in 2 μg ml–1 blasticidin and then tested by RT-PCR for expression of the vector-encoded genes (Supplementary Fig. 2e). Wild-type tendon fibroblast cells (TFb-WT cells), tendon fibroblasts expressing MMP-9 (TFb–MMP-9 cells) and tendon fibroblasts expressing PlGF (TFb–PlGF cells) proliferated at similar rates (Supplementary Fig. 2f). We injected 5 × 10^5 TFb-WT cells and 5 × 10^5 TFb–MMP-9 cells, previously labeled with octadecyl (C18) indocarbocyanine (DiI), into the right and left tibialis anterior muscles, respectively, of six 12-month-old, α-sarcoglycan–null mice. Six hours after the injection, the muscles were collected, frozen, cryosectioned and stained with antibodies to laminin (to reveal basal lamina of muscle fibers and blood vessels). Control TFb-WT cells were mainly distributed around the injection area, whereas TFb–MMP-9 cells migrated more than 1 mm away from the site of injection (Fig. 1a–c). Because the needle had been inserted along the longitudinal axis of the muscle, diffusion could not be dependent upon the needle track in transverse sections. We also tested (by quantitative RT-PCR for lentiviral sequences) whether injected tendon fibroblasts could reach neighboring muscles or distant filter organs such as the liver, lung or kidney (possibly consequent to passage into the circulatory system). TFb–MMP-9 cells were detected in the injected tibialis anterior (at a level corresponding to 20% of total injected cells) but also, albeit in much smaller amounts, in the neighboring gastrocnemius and quadriceps (Supplementary Fig. 3e online). This dispersed requirement MMP-9, as TFb–PlGF cells were essentially confined to the injected tibialis anterior (data not shown). Cells could not be detected in other organs (Supplementary Fig. 3g).

One month after injection, less than 2% of the initially detected cells were still present in the injected tibialis anterior (Supplementary Fig. 3f), indicating that tendon fibroblasts do not survive for a long period of time. Nevertheless, one week after the injection, they produced a large amount of MMP-9 that was readily detected.
by zymography in extracts of muscles injected with TFb–MMP-9 cells but not with tendon fibroblasts or TFb-PIGF cells (Supplementary Fig. 3a). Although not a quantitative method, the zymography revealed a strong signal in the adjacent muscles as well (gastrocnemius and quadriceps), suggesting that the enzyme becomes more widely distributed than the cells.

We then investigated whether PlGF-expressing tendon fibroblasts could promote angiogenesis alone or in combination with TFb–MMP-9 cells. As a preliminary test of activity, we grew human umbilical vein endothelial cells (HUVECs) in the presence of medium conditioned by either TFb–WT or TFb–PIGF cells. HUVECs responded to TFb–PIGF-conditioned medium, but not to TFb–WT-conditioned medium, by creating an intricate network of vessel-like structures (Supplementary Fig. 4 online), as has been reported to occur in response to angiogenic factors17.

Therefore, we injected 12-month-old mice (n = 8 per experimental group) twice (with an interval of 15 d) in the tibialis anterior with 5 × 10^5 TFb–WT, TFb–MMP-9 or TFb–PIGF cells or with a combination of 2.5 × 10^5 TFb–MMP-9 cells and 2.5 × 10^5 TFb–PIGF cells. Fifteen days after the second injection, the mice were killed and the tibialis anterior, gastrocnemius and quadriceps were collected and either cryosectioned or used to prepare muscle protein extracts. Injection of TFb–WT cells did not reduce collagen accumulation (as revealed by Azan–Mallory staining), whereas injection of TFb–MMP-9 cells did (Fig. 1d,e). We quantified this result by western blot analysis with an antibody to collagen I (Fig. 1g,i) and by spectrophotometric hydroxyproline quantification (Fig. 1f). Only muscles injected with TFb–MMP-9 cells alone or together with TFb–PIGF cells showed reduced collagen accumulation at a level comparable to, though still higher than, that in untreated young dystrophic muscle (Fig. 1f–j and Supplementary Fig. 3b,c). Leukocyte infiltration (quantified by staining of both tissue sections (Supplementary Fig. 1e,f) and protein extracts (Fig. 1j and Supplementary Fig. 3b–d) with an antibody to CD11b) was reduced only by the combined transplantation of TFb–MMP-9 and TFb–PIGF cells. The number of leukocytes per cross-sectional area (CSA) counted in three different nonserial sections of the tibialis anterior muscle of three mice for each experimental group was 2 ± 1 in aged WT mice (n = 5), 45 ± 7 in aged α-sarcoglycan–null untreated mice (n = 5) and 10 ± 3 in aged α-sarcoglycan–null mice treated with TFb–MMP-9 and TFb–PIGF cells (n = 5; Supplementary Fig. 3b–d). Lipid accumulation, as revealed by Sudan Black staining, appeared to be reduced in all sections (five non serial sections for each of three separate muscles of three mice per experimental group) injected with modified tendon fibroblasts (particularly
Figure 2  Intramuscular injection of modified TFb results in increased blood vessel density. (a) A representative western blot analysis of total protein extracts of the TA muscle of aged mice (one mouse per experimental group; total number of mice per group was five). Lanes: Aged WT mice (1); aged α-sarcoglycan–null mice left untreated (2); aged α-sarcoglycan–null mice transplanted with TFb-WT cells (3); aged α-sarcoglycan–null mice transplanted with TFb–MMP-9 cells (4); aged α-sarcoglycan–null mice transplanted with TFb–PlGF cells (5); aged α-sarcoglycan–null mice transplanted with TFb–MMP-9 and TFb–PlGF cells (6); aged α-sarcoglycan–null mice left untreated (7). The filter was hybridized with antibodies to vascular endothelial cadherin (VE-cad) and actin as an internal control. (b) Capillary/muscle fiber ratio was calculated by scoring PECAM-positive capillaries. Each bar represents the mean ± s.d. of positive capillaries counted in eight randomly selected fields of three nonserial sections from each sample compared to the number of muscle fibers in the same section (n = 5 for each group). The values for aged WT and aged dystrophic mice transplanted with both TFb–MMP-9 and TFb–PlGF are statistically different (P < 0.05) from those of all other dystrophic samples. (c,d) Whole-mount staining of TA muscles from an aged α-sarcoglycan–null mouse injected with TFb–MMP-9 in the TA muscle of the left leg (c) and with TFb–MMP-9 and TFb–PlGF in the TA muscle of the right leg (d) and injected with China ink immediately before killing, revealing increased vascularization after MMP-9 and PlGF treatment. (e–g) Double immunofluorescence staining with antibodies to laminin (green) and PECAM (red). Shown are aged WT (e) and aged dystrophic (f,g) TA muscle transplanted with TFb–MMP-9 and TFb–PlGF (g). Numerous PECAM-positive capillaries are indicated in e and g by arrows, whereas large vessels are indicated in f by arrowheads. Insets show details of laminin and PECAM staining. Scale bars: 2.5 mm (c,d); 50 μm (e–g), 20 μm (insets in f,g).

by both TFb–MMP-9 and TFb–PlGF cells together) but not with TFb–WT cells (Fig. 1h).

Two weeks after the second injection, we injected three transplanted α-sarcoglycan–null mice (per each group) with black ink in the heart and immediately killed them; we collected tibialis anterior muscles and fixed them to observe vessel distribution in the whole-mount preparation. Five different mice (for each experimental group), similarly injected in tibialis anterior muscles, were collected, frozen, cryosectioned and stained with antibodies to platelet/endothelial cell adhesion molecule (PECAM) and laminin. Only injection of both TFb–MMP-9 and TFb–PlGF cells induced a notable enhancement of the vascular network (Fig. 2), as shown by western blot analysis with antibody to vascular endothelial cadherin (Fig. 2a), whole-mount staining after black ink injection (Fig. 2c,d) and immunocytochemistry with antibody to PECAM (Fig. 2e–g). No single population of cells stimulated vessel formation (data not shown). Immunofluorescence analysis of the tibialis anterior with antibody to PECAM showed an even distribution of the capillary network in only aged WT mice (Fig. 2e) and aged α-sarcoglycan–null mice injected with both TFb–MMP-9 and TFb–PlGF cells (Fig. 2g). The extent of vascular network distribution was calculated by scoring the number of PECAM-positive capillaries per muscle fiber in mice treated with both TFb–MMP-9 and TFb–PlGF cells (Fig. 2b) and was found to be similar to that observed in aged WT mice, highlighting the effect of the combination of MMP-9 and PlGF on dystrophic muscle angiogenesis.

To test whether prior intramuscular transplantation could ameliorate subsequent intra-arterial cell delivery, we injected D16 mesoangioblasts3 expressing nuclear β-galactosidase as a reporter (D16-nLacZ cells) into the femoral artery of either control or treated (one month earlier with TFb–MMP-9 and TFb–PlGF cells) 13-month-old α-sarcoglycan–null mice (n = 3 per experimental group). Mesoangioblasts can cross the vessel endothelium and colonize surrounding tissues3. Six hours after intra-arterial injection, we collected the tibialis anterior, gastrocnemius and quadriceps muscles for RNA extraction or cryosectioning. Histochemistry for β-galactosidase expression revealed widespread distribution of D16-nLacZ cells in aged dystrophic muscles previously treated with TFb–MMP-9 and TFb–PlGF cells (Supplementary Fig. 5a,b online) but not with control TFb–WT cells; β-galactosidase–positive cells were detected mainly around blood vessels but were also adjacent to muscle fibers (Supplementary Fig. 5c). By quantitative real-time RT-PCR for LacZ expression, we confirmed that mesoangioblasts specifically colonized the muscles previously subjected to intramuscular injection of TFb–MMP-9 and TFb–PlGF cells (Supplementary Fig. 5d). To our surprise, in three separate experiments, the highest accumulation of D16-nLacZ cells occurred in the gastrocnemius muscle that had not been previously injected with TFb–MMP-9 and TFb–PlGF cells (Supplementary Fig. 5d). This may be the result of the spread of the TFb–MMP-9 and TFb–PlGF cells or of the secreted PlGF and MMP-9 proteins from the neighboring tibialis anterior. The high blood demand of the gastrocnemius muscle, which is crucial for hind limb motility, may facilitate such spreading.

In a similar experiment, 13-month-old mice, also pretreated with TFb–MMP-9 and TFb–PlGF cells as described above (n = 3 for each experimental group), were killed one month after the intra-arterial injection of D16 mesoangioblasts. Muscles were collected and analyzed for α-sarcoglycan expression by immunofluorescence (Fig. 3a–c). Aged WT mice uniformly expressed α-sarcoglycan on the sarcolemma (Fig. 3a). In contrast, untreated α-sarcoglycan–null mice did not express α-sarcoglycan (Fig. 3b). However, the tibialis anterior muscle of mice pretreated with TFb–MMP-9 and TFb–PlGF, showed substantial, though incomplete, α-sarcoglycan expression (Fig. 3c). α-sarcoglycan protein accumulation was confirmed by western blot
analysis (Fig. 3d), which showed expression of α-sarcoglycan protein in the aged dystrophic muscles pretreated with TFb–MMP-9 and TFb–PIGF in amounts comparable to those detected in young dystrophic muscles directly injected with donor mesangioblasts in the femoral artery (approximately 30% of WT muscle). The level of α-sarcoglycan mRNA expression was quantified by real-time RT-PCR in extracts from similarly treated muscles (Fig. 3e). Only the α-sarcoglycan–null mice previously treated with intramuscular injection of both TFb–MMP-9 and TFb–PIGF cells and then with intrafemoral injection of D16-nLacZ cells expressed α-sarcoglycan at a level comparable with that observed in young dystrophic muscle treated only by intrafemoral injection (approximately 35% of WT muscle) (Fig. 3e). The highest amount of α-sarcoglycan mRNA was detected in gastrocnemius muscle (Fig. 3e), as seen previously (Supplementary Fig. 5d). Restored α-sarcoglycan expression corresponded to a general amelioration of the histopathology (Fig. 3f). The total number of fibers in tibialis anterior, gastrocnemius and quadriceps muscles from aged α-sarcoglycan–null mice transplanted with either TFb-WT (n = 3), TFb-PIGF (n = 3), TFb-MMP-9 (n = 3) or both TFb-PIGF and TFb–MMP-9 (n = 3) cells was counted in transverse cryosections stained with H&E. Four sections from the portion of the muscle with the largest CSA were chosen for analysis. The total number of fibers per CSA in muscle transplanted with control tendon fibroblasts was found to be 830 ± 188 fibers in tibialis anterior, 1,400 ± 305 fibers in gastrocnemius and 1,090 ± 234 fibers in quadriceps (Fig. 3h), approximately 30% of the number of fibers found in these muscles in WT mice15. Muscles transplanted with fibroblasts expressing either PIGF or MMP-9 alone showed similar numbers of fibers as those transplanted with control tendon fibroblasts (Fig. 3h). In contrast, muscles transplanted with tendon fibroblasts expressing both proteins had double the number of fibers (Fig. 3h), reaching approximately 60–70% of the numbers in WT untreated mice.

Figure 3 Mesoangioblast intra-arterial transplantation in aged dystrophic muscle. (a–c) α-SG immunofluorescence analysis in aged WT (a) or aged α-SG–null TA muscle left untreated (b) or previously injected intramuscularly with both TFb–MMP-9 and TFb–PIGF cells and then intra-arterially injected with WT D16-nLacZ mesangioblasts (c). Insets: high magnification images also showing DAPI-positive blue nuclei, centrally located in c. (d) Western blot analysis on total protein extracts from TA muscles of mice treated with intra-arterial injection of D16-nLacZ mesangioblasts (lanes 2–7) or left untreated (lane 1). Lanes: aged WT TA (untreated) (1); aged α-SG–null TA, no pretreatment (2); aged α-SG–null TA pretreated with TFb–WT cells (3); aged α-SG–null TA pretreated with TFb–MMP-9 cells (4); aged α-SG–null TA pretreated with TFb–PIGF cells (5); aged α-SG–null TA pretreated with TFb–MMP-9 and TFb–PIGF cells (6); young α-SG–null TA, no pretreatment (7). The filter was hybridized with antibodies to α-SG and laminin as an internal control. (e) Real-time RT-PCR for α-SG expression in the TA, quadriceps (Quad) and gastrocnemius (Gast) of mice treated as described in d. Values from young dystrophic muscle or aged dystrophic muscle pretreated with TFb–MMP-9 and TFb–PIGF cells were significantly different (P < 0.01) from those of all other samples. Rel-ampl, relative amplification. (f,g) H&E staining of transverse sections of TA muscle from aged α-SG–null mice left untreated (f) or treated with TFb–MMP-9 and TFb–PIGF cells and then with D16-nLacZ mesangioblasts (g). (h) Histogram showing the total number of fibers per CSA scored on H&E-stained cryosections. (i) Range of CSA values of muscle fibers from TA muscles from aged α-SG–null mice left untreated or treated with TFb–MMP-9 and TFb–PIGF cells, as determined by counting 800 fibers in three different mice per group. Scale bars: 100 μm (a,f); 30 μm (insets a–c).
Moreover, muscles transplanted with both types of transduced tendon fibroblasts had a larger percentage of small, presumably regenerating, fibers and a lower percentage of very large, degenerating fibers than the corresponding muscle from aged, untreated dystrophic mice (Fig. 3i). Consistent with this result, there was a fourfold increase in the number of centrally nucleated (regenerating) fibers in the tibialis anterior treated with both types of transduced tendon fibroblasts compared to untreated muscles (data not shown). Histology confirmed that treatment with the transduced tendon fibroblasts ameliorated pathology (Fig. 3f–g) and decreased leukocyte infiltration (Supplementary Fig. 5c.f.). Injection of tendon fibroblasts or injection of tendon fibroblasts followed by injection of mesoangioblasts did not result in lymphocyte infiltration (as assessed by staining with antibodies to CD4 and CD8) at higher levels than in untransplanted muscle (data not shown), in agreement with previous results.

These data show that treatment of aged dystrophic muscle with metalloproteinase and angiogenic factors, delivered by intramuscularly injected tendon fibroblasts, makes this muscle amenable to cell therapy with intra-arterially injected mesoangioblasts. This treatment results in decreased collagen and fat accumulation, decreased leukocyte infiltration and increased fiber numbers. As satellite cell–derived regenerating fibers incorporate exogenous mesoangioblasts and thus express z-sarcoglycan derived from these injected cells, the increase in fiber number may depend upon better regeneration of surviving satellite cells in an environment enriched in blood vessels. Based on our findings that the migration of fibroblasts expressing MMP-9 is extensive, and that secreted factors are widely distributed, the number of intramuscular injections needed in a large muscle might not be unreasonably high. This would make it possible in the future to treat patients with dystrophic muscular dystrophy at an advanced stage of the disease that otherwise would exclude them from new cell therapies.

METHODS

Cells and culture conditions. We isolated tendon fibroblasts from patellar tendons from 6–8-week-old C57BL/6J mice. We cut the tendon into small pieces and used 3 mg ml\(^{-1}\) collagenase type I (Worthington) and 4 mg ml \(^{-1}\) dispase (Roche) in PBS for 1 h at 37 °C to digest the tendon\(^{19}\). We cultured cells in α-MEM (Gibco) supplemented with heat-inactivated 20% FCS, 100 international units ml\(^{-1}\) penicillin and 100 mg ml \(^{-1}\) streptomycin. We grew the cells under low oxygen conditions (90% N\(_2\), 5% CO\(_2\) and 5% O\(_2\); Rivoira analyzed gas mixture). We transduced tendon fibroblasts in vitro with third-generation lentiviral vectors from Invitrogen (ViraPower Lentiviral Expression System) encoding the MMP-9 and PGF proteins (from Open Biosystems clones) and then used the cells for intramuscular injection. Both control and modified tendon fibroblasts were labeled with 2 μM Dil according to the protocol supplied by the manufacturer (Molecular Probes). We cultured mesoangioblasts expressing nuclear LacZ (D16-nLacZ) and used them for systemic injection as described\(^{3}\).

Cell injection into z-sarcoglycan–null mice. We anesthetized mice with an intramuscular injection of physiologic saline (10 ml kg\(^{-1}\)) containing ketamine (5 mg ml\(^{-1}\)) and xylazine (1 mg ml\(^{-1}\)) and then injected approximately 5 × 10\(^5\) tendon fibroblasts into the tibialis anterior muscle\(^{20}\) via a 0.20-mm diameter needle inserted along the cranio-caudal axis of the muscle. We repeated the intramuscular injection after 15 d. We performed intra-arterial injection of mesoangioblasts as previously described\(^{2}\). Briefly, we made a limited incision on the medial side of the leg and gently separated the femoral artery from surrounding muscle and connective tissue. We inserted the needle of an insulin syringe into the femoral artery and delivered the cells slowly (over approximately 30–40 s) in a volume of 30–40 μl. There was no visible damage to the vessel wall during or after the operation. Unless otherwise stated, we killed the mice 1 month after the intra-arterial injection for molecular and morphological analyses.

Ink injection. Fifteen days after the second of the two intramuscular injections, we anesthetized treated mice as described above. Using a 1-ml syringe, we withdrew 0.5 ml of mouse blood from the heart ventricle and injected 1 ml of China ink (Pelikan) to label the blood vessels. We allowed the heart to beat for 1 min (until the eyes, mouth and legs became black) and then killed the mouse. We quickly dissected the muscles and fixed them in 4% paraformaldehyde overnight at 4 °C. The fixed muscles, showing black vessels, were treated with a solution of 0.5% ROH for 1 d and in the following days were treated with the same solution with an increasing glycerol concentration (every other day in increments of 30%, 60% and 100%) to visualize blood vessels.

Immunofluorescence. We carried out immunofluorescence according to standard procedures\(^{21,22}\); further details are available in the Supplementary Methods.

Immunoblotting. We carried out immunoblotting using standard procedures; further details are available in the Supplementary Methods.

Real-time reverse transcription PCR. We carried out RT-PCR according to standard procedures\(^{23}\); further details are available in the Supplementary Methods.

Zymography. We determined MMP-9 activity by gelatin zymography as previously described\(^{24}\) with a few modifications. Briefly, we separated the samples on a 10% polyacrylamide gel with 0.05% gelatin type A from porcine skin (Sigma). After electrophoresis, we washed the gels for two 30-min periods with 50 mM Tris-HCl, pH 7.2, containing 10 mM CaCl\(_2\), 0.02% Na\(_3\) and 2.5% Triton X-100 (Sigma). Then, we incubated the gels for 18 h at 37 °C in the same buffer except with 1% Triton X-100. We stained the gels with 0.1% Coomassie Blue R-250 in 30% ethanol and 10% acetic acid and destained them in 30% ethanol and 10% acetic acid. MMP-9 activity was detected as clear bands on the blue background. Enzymes were identified by comigration with recombinant mouse MMP-9 (Sigma).

Angiogenesis assay. We coated 24-well plates with growth factor–reduced matrigel (BD Biosciences) and incubated them for 30 min at 37 °C. We plated HUVECs (a gift from E. Dejana) at 2 × 10\(^5\) cells per well and cultured them in RPMI (Gibco) supplemented with heat-inactivated 2% horse serum in the presence of TFG-\(\alpha\)- or TFG-PlGF–conditioned medium at a 1:4 dilution for 24 h at 37 °C. We then photographed the wells for morphological analysis of capillary-like structure formation, as previously described\(^{27}\).

Morphometry. We carried out morphometrical analyses to evaluate the distribution of fiber CSA on 800 fibers per muscle, on H&E-stained sections using Image J.63 software (Scion) as previously described\(^{25}\). We counted the total number of fibers in two different nonadjacent transverse sections from the largest muscle portion for three mice per experimental group. The number of muscle fibers scored in the quadriceps refer only to the vastus medialis.

Determination of hydroxyproline content. We carried out hydroxyproline analysis according to a standard method modified in ref. 26. Briefly, we dried tissue samples from tibialis anterior (30 to 50 mg) on paper, weighed and homogenized them in liquid nitrogen and then placed them in 6 N HCl in a glass Pyrex tube. The tube was placed at 100 °C overnight to hydrolyze the tissue. We dried the solution in NaOH at 50 °C under vacuum and then re-suspended in 5 mM HCl. An aliquot of the sample was combined with milliQ water (Millipore) H\(_2\)O in a glass tube. To this we added chloramine T solution (Sigma) in hydroxyproline assay buffer (0.17 M citric acid, 0.8% acetic acid, 0.6 M sodium acetate, 0.57 M sodium hydroxide and 20% propan-1-ol pH 6). After adding the chloramine T solution, we added Erlich’s reagent (Sigma). We then incubated the samples at 55 °C for 25 min. Once the samples had cooled to 25 °C, we read the absorbance at 558 nm. We ran a standard curve with trans-4-hydroxy-l-proline (Sigma). We normalized readings to original tissue weight.

Statistical analyses. We expressed values as means ± s.d. We assessed statistical significance of the differences between means by one-way analysis of variance followed by the Student-Newman-Keuls test to determine which
groups were statistically significantly different from the others. When only two groups had to be compared, we used the unpaired Student’s t-test. We considered a probability of less than 5% (P < 0.05) to be statistically significant.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We would like to thank E. Dejana (University of Milan) for the gift of HUVECs, BV13 antibody to vascular endothelial cadherin and for helpful discussion; S. Bernardini for advice on histology; and S. Iacovelli for technical help. This work was supported by grants from Duchenne Parent Project Onlus, BMW, Association Française contre les Myopathies, the Italian Ministry of Health and of Research (grant RBINO63EWP) and the European Community (MyoAmp).

AUTHOR CONTRIBUTIONS

C.G. prepared vectors, transduced cells and carried out most of the experimental work; S.M.C. helped with data analysis and interpretation; E.D.G. did the histology; M.C. isolated the cells and did the work on mice; G.C. coordinated the project and wrote the manuscript.

Published online at http://www.nature.com/naturemedicine/
Reprints and permissions information is available online at http://hpg.nature.com/reprintsandpermissions/