MUSCULOSKELETAL PATHOLOGY

SPARC Interacts with Actin in Skeletal Muscle *in Vitro* and *in Vivo*

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The cytoskeleton is an integral part of skeletal muscle structure, and reorganization of the cytoskeleton occurs during various modes of remodeling. We previously found that the extracellular matrix protein secreted protein acidic and rich in cysteine (SPARC) is up-regulated and expressed intracellularly in developing muscle, during regeneration and in myopathies, which together suggests that SPARC might serve a specific role within muscle cells. Using co-immunoprecipitation combined with mass spectrometry and verified by staining for direct protein-protein interaction, we find that SPARC binds to actin. This interaction is present in regenerating myofibers of patients with Duchenne muscular dystrophy, polymyositis, and compartment syndrome. Analysis of the α -, β -, and γ -actin isoforms in SPARC knockout myoblasts reveals a changed expression pattern with dominance of γ -actin. In SPARC knockout mice, we performed an injury study to investigate whether lack of SPARC would compromise the ability to repair muscle. We report that these mice develop normal skeletal muscle with retained ability to regenerate. However, when we subject muscle from SPARC-deficient mice to an *in vitro* fatigue stimulation protocol, we find a defective force recovery. Therefore, SPARC appears to be an important modulator of the actin cytoskeleton, implicating maintenance of muscular function. This direct interaction with actin suggests a new role of SPARC during tissue remodeling. (*Am J Pathol* 2017, 187: 457–474; <http://dx.doi.org/10.1016/j.ajpath.2016.10.013>)

The myopathies constitute a group of degenerative disorders that affect the skeletal muscle system, thereby causing varying degrees of weakness and impairment, depending on the underlying molecular mechanism and severity state.¹ We previously found that the multifunctional Ca²⁺-binding protein secreted protein acidic and rich in cysteine (SPARC) is expressed by regenerating muscle fibers and myoblasts in patients with different types of myopathies, such as Duchenne muscular dystrophy, inclusion body myositis, and congenital muscular dystrophy.² Moreover, expression level of SPARC correlated with damage severity of the affected muscles.² In normal adult muscle, SPARC is not detectable except for a very few cells located in the interstitium.² The finding of SPARC within damaged muscle fibers and myoblasts is intriguing because SPARC has generally been described in many tissues as an extracellular located protein involved in regulation of matrix reorganization, cell adhesion, and proliferation.³

Substitution of muscle tissue for fat and connective tissue is prominent, especially in severe dystrophies, where continued cycles of degeneration and regeneration exhaust the satellite cell pool.⁴ SPARC has been associated with a number of fibrotic conditions, including scleroderma,⁵ lung,⁶ liver,⁷ and adipose tissue⁸ fibrosis. Hence, SPARC expression in myopathies could relate to formation of fibrosis,⁹ possibly through extracellular matrix reorganization,¹⁰ because SPARC is known to bind thrombospondin, fibrillar collagens I, III, V, and basement membrane collagen type IV.^{11,12}

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During mouse development SPARC is detected in the somites,¹³ and in human fetal muscle, we detect a prominent expression of SPARC in early myotubes with expression decreasing as the muscles mature.² SPARC null mice have severe cataracts¹⁴ and osteopenia,¹⁵ but they do not appear to have an overt muscle phenotype,¹⁴ even though it was suggested that SPARC null mice have a decreased muscle mass.¹⁶

The potential effect on muscle mass by lack of SPARC was further implied by a study that found that siRNA knock down of SPARC *in vivo* causes myofiber atrophy, and it was suggested that reduced SPARC in aging muscle might correlate with sarcopenia.¹⁷ Further substantiating a role for SPARC in skeletal muscle function is the observation that eradication of SPARC in *Xenopus laevis* resulted in developmental defects in many tissues after hatching, including the myotome, which appears disordered,¹⁸ as well as a study that found that in *Caenorhabditis elegans* the SPARC homologue is expressed by body wall and sex muscle cells, and overexpression results in deformed embryos, affected mobility, and even paralysis.¹⁹

In cell culture, we detected SPARC during proliferation with a prominent up-regulation during differentiation in both mouse and human myoblasts.^{2,20} In line with the effects of overexpression of SPARC in *C. elegans*, we have observed that overexpression of SPARC in C2C12 cells inhibits muscle cell differentiation.²⁰ Contrary, another study found that the addition of SPARC protein to muscle cells facilitated differentiation,²¹ suggesting diverse effects, depending on SPARC being present intracellularly or extracellularly and supporting that SPARC could have a direct cellular function.

An actual shuttling effect of SPARC has recently been found, where SPARC appears to bring extracellular matrix proteins into fibroblasts by acting as a chaperone and facilitating extracellular matrix disassembly,²² thereby serving a specific role within the cells. The ability of SPARC to function as a chaperone is also implied by SPARC's ability to mediate a cell survival effect, which has been observed in mouse lens epithelial cells²³ and human cancers, such as melanoma and leukemia.^{24,25} Furthermore, SPARC has also been described as a heat shock protein (HSP),²⁶ and its expression has been associated with other stress-induced proteins, such as HSP47 and HSP70.^{27,28} Thus, a general role of SPARC could be of a physiologic nature with the specific function to protect cells or tissues during a temporarily stressful or unfit state. We investigated the specific function of SPARC in skeletal muscle. We found that SPARC interacts directly with actin and that the absence of SPARC results in a changed expression pattern of the different actin isoforms *in vitro* and force generation deficit after muscle fatigue *in vitro*. Our results therefore indicate a new mechanistic intracellular role for SPARC, where SPARC might serve to modulate cytoskeletal structure and function in skeletal muscle.

Materials and Methods

Mice and Animal Experiments

C57BL/10ScSn-DMD^{mdx}/J (*mdx*) mice, wild-type control C57BL/10 (wt), and B6;129S-SPARC^{tm1Hwe}/J mice were obtained from The Jackson Laboratory. B6;129S-SPARC^{tm1Hwe}/J mice were bred as heterozygotes (SPARC^{+/-}) to obtain wild-type littermate controls (SPARC^{+/+}) and SPARC knockout mice (SPARC^{-/-}); genotyping was performed according to The Jackson Laboratory protocol (Bar Harbor, ME).

Tibialis anterior (TA) were harvested from 7- to 10-week-old and 24- to 30-week-old *mdx* and wt mice for histologic examination (*n* = 4 to 5 in each group).

Extensor digitorum longus (EDL) and soleus muscle were excised from SPARC^{-/-} and SPARC^{+/+} adult mice after weighing for *in vitro* force measurements (*n* = 6 to 11) and myofiber phenotype and diameter measurements (*n* = 3 to 4).

Muscle regeneration was studied after an injection with 50 μ L of cardiotoxin (10 μ mol/L; Sigma-Aldrich, St Louis, MO) in the right TA muscle of adult SPARC^{+/+} and SPARC^{-/-} mice. The left TA muscle served as contralateral control. Mice were sacrificed with cervical dislocation after 3 and 7 days (*n* = 5 in each group) and TAs harvested.

An exercise study (short-term eccentric running) and force grip measurements before and after running were performed in adult SPARC^{+/+} and SPARC^{-/-} mice (*n* = 3 in each group). The mice were placed in an electric treadmill (IITC Life Science 800 Series Treadmill; IITC Inc. Life Science, Woodland Hills, CA) adjusted to a 15° angle to allow downhill running and thus subject the mice to eccentric (lengthening) strain. The top speed was set at 18 m per minute with an initial speed of 1 m per minute and a ramp speed of 60 seconds. The duration of the run was 25 minutes. Before the actual short-term running experiments, the mice were familiarized with the treadmill a few days before so they knew what to expect and what to do when placed in the treadmill.

Force grip measurements were collected before the short-term exercise regimen and after running (1 minute, 10 minutes, 30 minutes). The mice were held by the tail and moved over a T-bar mounted on a force measuring device (IITC Life Science Grip Strength Meter), and the grip force was recorded when the mice were holding the bar with both front paws and pulled before letting go of the bar. At least 5 measurements were performed, and the maximum force recorded was used.

All animal experiments were performed in accordance with Danish legislation on animal welfare and approved by the Danish Council for Supervision with Experimental Animals (license 2012-15-2934-00739). Mice were housed under standard conditions, kept in a 12-hour light/dark cycle, had access to food and water *ad libitum*, and were provided with enrichment for improved care. Mice with any type of distress or illness were immediately euthanized by cervical dislocation.

In Vitro Force Measurements

Immediately after excision, the muscles were placed in Krebs-Ringer bicarbonate solution that contained 122 mM sodium chloride, 25 mM sodium bicarbonate, 2.8 mM potassium chloride, 1.2 mM monopotassium phosphate, 1.2 mM magnesium sulfate, 1.3 mM calcium chloride, and 5 mM D-glucose. A small loop of surgical silk was tied to each tendon of the muscle, and the muscles were mounted vertically between a fixed hook and an isometric force transducer (K30 type 351; Hugo Sachs Elektronik, March-Hugstetten, Germany) in a temperature-controlled chamber at 30°C (Schuler Organbad, Hugo Sachs Elektronik) and gassed with a mixture of 95% oxygen and 5% carbon dioxide (pH 7.4) throughout the experiments. Muscles were gently adjusted to the length eliciting maximum force with a single-twitch stimulus, equilibrated for 30 minutes before stimulation with a test stimulus (60 Hz, 0.2-millisecond duration for 1.5 seconds). To test the muscle force-frequency relationship, a series of stimulations at 10, 20, 30, 40, 60, 80, 100, 150, 200, and 250 Hz for 1.5 seconds were elicited, with 1-minute rest in between. After a 5-minute rest, the muscles were subjected to a 3-minute fatigue protocol in which contractions were evoked by 100 Hz on a duty cycle of 400 milliseconds every 5 seconds (EDL) or 60 Hz on a duty cycle of 500 milliseconds every 3 seconds (soleus). To test muscle contractile properties after the fatigue protocol, test stimuli were elicited after 1-, 10-, and 30-minute recovery.

SPARC Knockdown Cells

In the C2C12 myoblast cell line (CRL-1772, LGC Promochem, ATCC, Manassas, VA), SPARC protein was knocked down using the BLOCK-iT expression system (Life Technologies, Carlsbad, CA) according to the manufacturers' instruction. Predesigned DNA oligos (Sparc BLOCK-IT miR RNAi select, Life Technologies) were used. In brief, the single-stranded DNA oligos were annealed and ligated with the linearized pcDNA6.6-GW/EmGFP-miR vector followed by transformation into competent TOP10 *Escherichia coli*. Resulting clones were sequenced and plasmids were purified and transfected into C2C12 cells using Lipofectamine 2000 Reagent (Life Technologies) according to the manufacturers' instruction. Clones were screened for expression of Emerald Green Fluorescent Protein, and a stable cell line was established by selective growth using Blasticidine (5 µg/mL).

C2C12 Culture

C2C12 and C2C12 SPARC knockdown cells (C2C12^{SPARC KD}) were cultured at 37°C with 5% carbon dioxide in a humidified chamber. For proliferation, cells were grown in Dulbecco's modified Eagle's medium with high glucose (Life Technologies) supplemented with 10% fetal bovine serum and

antibiotics (50 U/mL of penicillin and 50 µg/mL of streptomycin); in addition, C2C12^{SPARC KD} cells were continuously grown with 5 µg/mL of Blasticidine in the medium.

To induce myofiber formation (differentiation), cells were cultured to approximately 90% confluence in proliferation media then changed to Dulbecco's modified Eagle's medium with high glucose supplemented with 2% fetal bovine serum and antibiotics.

Human Samples

Biopsy specimens for isolation of primary myoblasts were obtained from voluntary participants after they gave written informed consent. The Regional Scientific Ethical Committee for Southern Denmark (S-20070079) approved the use of the biopsy specimens. Biopsy specimens from dystrophic patients were obtained from the archive at the Department of Clinical Pathology, Odense University Hospital, Odense, Denmark. The use of human archival tissue for protein expression analyses during this study was approved by The Regional Scientific Ethical Committee for Southern Denmark (20070075).

Primary Human Myoblast Culture

The primary human myoblast culture was established from a muscle biopsy specimen taken from the vastus lateralis as described previously.²⁹ The isolated cells were grown (maximum of 7 passages) in extracellular matrix (Sigma-Aldrich, Copenhagen, Denmark)-coated T75 flasks (Nunc, VWR, Radnor, PA) in growth medium that consists of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics.

Real-Time Quantitative PCR

Total RNA was extracted from C2C12 and C2C12^{SPARC KD} cells using TRIzol (Life Technologies), quantified using the NanoDrop Spectrophotometer ND-1000 and 500 ng reverse transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a total volume of 100 µL. TaqMan Gene Expression Assays (Applied Biosystems) were used to analyze *Sparc* and the reference genes *Hprt1* and *Tfrc1*. The real-time quantitative PCR reactions were run in triplicates on duplicate samples using the ABI PRISM 7900 HT Real Time PCR System and analyzed using SDS software version 2.1 (Applied Biosystems) and qBase software.³⁰

Tissue Preparation

Formalin-fixed, paraffin-embedded mouse (TA from wt and *mdx* mice) and human muscle tissue as well as mouse tissue (TA from the regeneration study, EDL and soleus muscle) fixated in 4% normal buffered formaldehyde (TA only), embedded in Tissuetek, and frozen in nitrogen cooled

isopentane was cut in 4- to 5- μ m sections and stained with hematoxylin and eosin for morphologic analysis and Sirius red for analysis of fibrosis. For immunohistochemical or Duolink analysis, heat-induced antigen retrieval was performed on paraffin-embedded sections by boiling the sections in Tris-EGTA buffer (pH 9.0) for the first 15 minutes at 900 W then for 9 minutes at 440 W. The sections were allowed to cool in the buffer before the staining protocol was continued. All sections, paraffin and cryosections, were blocked for endogenous peroxidase and biotin activity.

Immunohistochemistry

Human tissue sections were incubated with mouse—anti-human SPARC (ncl-o-nectin; Novocastra, Wetzlar, Germany) 1:50 with rabbit—anti-human Hsp27 (Spa-803; Stressgen, Victoria, British Columbia, Canada) 1:2000 and detected using Alexa fluor 555 goat—anti-rabbit IgG (Life Technologies) 1:200 and Alexa fluor 488 goat—anti-mouse IgG 1:200 (Life Technologies).

Mouse tissue sections from paraffin-embedded tissue were incubated with rat—anti-mouse SPARC (R&D Systems, Minneapolis, MN) 1:500, mouse—anti-rat myogenin (F5D, Dako Cytomation, Carpinteria, CA) 1:200, or rat—anti-mouse CD45 [30-F11 (Ly 5); BD Pharmingen, San Jose, CA] 1:50 and detected using biotinylated rabbit—anti-rat IgG (Dako Cytomation) 1:200 with streptavidin—horseradish peroxidase (HRP) (Dako Cytomation) or the ARK kit (K3954, Dako Cytomation) and DAB⁺ as chromogen (Dako Cytomation). Nuclei were counterstained using Mayer's hemalum with 4.5% chloralhydrate.

Frozen mouse tissue sections (TA regeneration study) was fixated in 4% normal buffered formaldehyde and incubated with rat—anti-mouse CD45 [30-F11 (Ly 5); BD Pharmingen] 1:100, mouse—anti-human desmin (Dako Cytomation, M0760) 1:50, or mouse—anti-rat myogenin (F5D, Dako Cytomation) 1:200. The CD45 reaction was detected using rabbit—anti-rat antibody (Dako Cytomation) diluted 1:200 followed by ready-to-use anti-rabbit HRP-labeled polymer (EnVision+ System, Dako Cytomation), and the desmin and myogenin reactions were detected using the ARK kit. All reactions were counterstained as above.

For double fluorescence staining, mouse sections were incubated with rat—anti-mouse SPARC (R&D Systems) 1:500 or 1:100.000 (for detection with tyramide-signal amplification) with rabbit—anti-human S100A4 (Abcam, Cambridge, United Kingdom) 1:200, rabbit—anti-chicken neural cell adhesion molecule (NCAM) (AB5032; Chemicon International, Billerica, MA) 1:1000, rat—anti-mouse F4/80 (macrophage) (AbD Serotec, Hercules, CA) 1:2000 overnight, rat—anti-mouse Ly-6G (BD Pharmingen) 1:1000 overnight, or rabbit—anti-rat Hsp27 (Cell Signaling Technology, Danvers, MA) 1:50. Secondary antibodies used were Alexa fluor 555 goat—anti-rabbit IgG (Life Technologies) 1:200, Alexa fluor 488 or 555 goat—anti-rat (Life Technologies) 1:200, and Alexa fluor 488 goat—anti-mouse IgG (Life

Technologies) 1:200 were used. For analysis of SPARC with Ly6G or F4/80, antibody reactions were performed sequentially, including a denaturing step where sections were boiled in TEG buffer between reactions to avoid cross-reactions of the two secondary antibody steps. For double staining with Ly-6G, the SPARC antibody reaction was detected using Tyramide-Alexa 488 1:50 (Life Technologies), and for analysis of SPARC with F4/80, the F4/80 antibody reaction was detected with Tyramide-Alexa 488 1:50.

All fluorescence staining was mounted using Vectashield with DAPI (Vector Laboratories Inc, Burlingame, CA).

For fiber-type determination, frozen mouse sections, either fixated before freezing (TA contralateral and day 7 after injury) or fresh frozen (EDL and soleus), were brought to room temperature and then placed in 95°C Tris-EGTA buffer, pH 9.0, for 15 minutes, followed by blocking for endogenous biotin and peroxidase. Sections were incubated with 2% bovine serum albumin (BSA) or Tris-buffered saline (TBS) for 10 minutes followed by either myosin heavy chain (MHC) fast (MY32, Sigma-Aldrich) 1:1000 or MHC slow (MEDCLA67-1, Accurate Chemicals, Westbury, NY) 1:25 for 30 minutes. The reactions were detected using the ARK kit (K3954, Dako Cytomation) according to standard protocol with DAB⁺ as chromogen. Nuclei were counterstained as above.

Image Acquisition and Data Preparation

Epifluorescent images were obtained with a Leica DM LB2 microscope and the digital camera Leica DFC 300FX (Leica, Wetzlar, Germany). Images were acquired with the Leica Application Suite software version 3 at identical settings to allow a direct comparison and analyzed as TIFF files using Adobe Photoshop CS5 software version 12 (Adobe, San Jose, CA). Images were used either directly with changes in size and resolution (from 300 to 600 dpi) only or equally improved in all channels by using the image tab followed by “adjustments,” and then using automatic tone or color, light or contrast, or shadow or highlight changes. Scans of Western blot films were imported in 1000-dpi resolution, changed to gray scale, and improved using light or contrast changes. The Leica epifluorescent objectives [HC/HCX (Harmonic Compound System, X indicates compatibility with past optics) plan APO (Apochromats)] used in the study had the following numerical apertures: 20 $\times$ /0.70, 63 $\times$ /1.40 to 0.60 oil and 100 $\times$ /1.40 to 0.70 oil. The filter cubes used for visualizing fluorescent protein staining had the following specifications: L5; excitation filter (EF) BP480/40, dichromatic mirror (DM) 505, suppression filter (SF) BP 527/30, N2.1 (equivalent to Texas Red); EF BP 562/40, DM LP 593, SF BP 624/40, A4 (equivalent to DAPI); EF BP 360/40, DM 400, SF BP 470/40 and Y3; EF BP 545/40, DM 565, SF BP 610/75.

Electron microscopic images were obtained using the JEM 1400 Plus transmission electron microscope (JEOL,

Freising, Germany) and the bottom-mounted digital camera Quemesa (Olympus, Southend-on-Sea, United Kingdom). Images were acquired using iTEM software Olympus 2010 version 5.2 (Build 3554) and analyzed as JPEG files using Adobe Photoshop CS5 software.

Analysis of Necrosis

The presence of necrosis after regeneration was analyzed by incubating frozen mouse muscle sections with a 1:200 solution of donkey anti-mouse IgG (Alexa Fluor 555, Life Technologies) and mounting the sections with DAPI (Vectashield, Vector Laboratories Inc). Analysis was performed using the program VIS version 4.5.6.440 (Visio-pharm, Hoersholm, Denmark) and a fluorescence microscope (Leica DM6000B) coupled to a computer and a camera (Olympus DP72). For this analysis, a 20 $\times$ objective and the filter cubes Y3 and DAPI were used (see specifications above). Measurements of the entire cross-sectional area and the necrotic area were performed automatically. The microscope slides were placed in the microscope (8 at the same time), then fluorescence filters (Y3, DAPI) were chosen and focus and exposure time fine-tuned and saved. Then the microscope was briefly changed to bright field and a small magnification, after which a super image was obtained for every tissue section on the 8 microscope slides. This allowed for marking the entire tissue section on the computer screen using the super images. The microscope was changed back to the fluorescence filters and the chosen settings, and at $\times 20$ magnification images were obtained to cover each entire tissue section and compiled automatically into one image showing both fluorophores at the same time. With the use of the VIS software, the whole region was measured by manually drawing around the tissue section, and by the same method, the area of interest (here necrosis) was marked in a similar way, allowing for quantification of necrosis per area.

Morphometrics

The number of cells expressing SPARC, myogenin, and CD45 protein in *mdx* and wt mice and the number of cells expressing CD45 or myogenin protein in regenerating muscle was estimated on sections ($n = 4$ to 5 mice) by counting cells using a microscope (Olympus BX50) equipped with a camera (Olympus U-PMTVC) connected to a motorized cross board and a computer. The CAST software version 2.1.6.0 (Olympus Danmark A/S, Ballerup, Denmark) was used to systematically select fields to include the entire muscle section. A cross-sectional area was measured by drawing around the area of interest (entire section or regenerating area) by means of the computer screen and the CAST software. CAST was then able to calculate the size of the area, and this measurement was used for normalization of counts.

For analysis of fiber-type composition (fast or slow) and fiber diameter, CAST was used to count and measure a

minimum of 100 individual fibers for each fiber type on each section (TA, EDL, and soleus muscles). The magnification used was $\times 10$, and the entire section was included as counting area. Diameters were measured by manually drawing transverse lines across all fibers.

The objectives used had the following numerical apertures: Uplan/APO 10 $\times$ /0.40 and Uplan/APO 20 $\times$ /0.70.

Immunocytochemistry

C2C12 and C2C12^{SPARC KD} cells grown on glass coverslips were fixated in 4% normal buffered formaldehyde, permeabilized in 0.5% Triton-X, and blocked with 2% BSA/TBS. The samples were incubated with the following primary antibodies: rat—anti-mouse SPARC (R&D Systems) 1:500, rabbit—anti-actin (all isoforms) (1844-1, Epitomics, Burlingame, CA) 1:250, mouse—anti-rabbit α -actin (sc-58671, Santa Cruz Biotechnology, Santa Cruz, CA) 1:100, or mouse—anti-bovine γ -actin (sc-65638, Santa Cruz Biotechnology) 1:100 for 1 hour. For staining of β -actin, cells were fixated in 100% methanol for 5 minutes, permeabilized in 0.5% Triton-X, and blocked with 2% BSA/TBS. Cells were incubated with rabbit—anti-human β -actin (ab8227, Abcam) overnight at 4°C. After incubation with primary antibody, samples were washed in Tris-buffered saline with Tween 20 before incubation with the secondary antibodies goat—anti-mouse—Alexa 488 or Alexa 555 1:200, goat—anti-rat—Alexa 555 1:200 or goat—anti-rabbit—Alexa 555 1:200 (Life Technologies) for 1 hour, after which they were mounted with SlowFade Diamond Antifade Mountant with DAPI (Life Technologies).

For staining of human myoblasts, cells were treated as above and incubated with mouse—anti-human SPARC (ncl-o-nectin; Novocastra) 1:50 and rabbit—anti-human Hsp27 (Spa-803; Stressgen) 1:300 followed by Alexa fluor 555 goat—anti-rabbit IgG (Life Technologies) 1:200 and Alexa fluor 488 goat—anti-mouse IgG 1:200 (Life Technologies) and mounted with SlowFade Diamond Antifade Mountant with DAPI (Life Technologies).

Duolink Interaction Studies

Specific protein-protein interactions between SPARC and Hsp27 or SPARC and actin were analyzed using Duolink *in situ* reagents with proximity ligation assay probes (Olink Bioscience, Uppsala, Sweden) on human primary myoblasts and human muscle tissue. Human myoblasts were fixated in 4% formaldehyde and permeabilized with 0.5% Triton X followed by incubation with Duolink blocking solution in a preheated humidified chamber at 37°C for 30 minutes. Primary antibody solution containing mouse—anti-human SPARC (ncl-o-nectin; Novocastra) 1:50/rabbit—anti-human Hsp27 (SPA-803; Stressgen) 1:2000 or mouse—anti-human SPARC (ncl-o-nectin; Novocastra) 1:50/rabbit—anti-human actin (1844-1; Epitomics) 1:250 was added for 1 hour at room temperature.

Tissue sections were treated as described, and then the sections were incubated with Duolink blocking solution followed by primary antibody solution containing mouse—anti-human SPARC (ncl-o-nectin; Novocastra) 1:25/rabbit—anti-human actin (1844-1; Epitomics) 1:20 as above. Then, cells and sections were incubated with proximity ligation assay probes PLUS and MINUS diluted 1:5 for 1 hour at 37°C, followed by incubation with ligation-ligase solution (Duolink) for 30 minutes at 37°C. After ligation, the samples were incubated with an amplification polymerase solution for 100 minutes at 37°C.

Myoblasts was counterstained for 1 hour at room temperature with Vimentin Dylight 488 (Epitomics) diluted 1:100. Tissue sections were counterstained for NCAM by blocking with 2% BSA/TBS and then incubating with mouse—anti-human NCAM (ms-1149-p; Thermo Fisher Scientific, Waltham, MA) 1:25 for 45 minutes at room temperature followed by Alexa flour 488 goat—anti-mouse IgG (Life Technologies) 1:200 for 45 minutes at room temperature. All samples were mounted with Mounting medium (Duolink) containing DAPI before microscopic analysis.

Co-Immunoprecipitation and Mass Spectrometry

Magnetic beads (Dynabeads, Life Technologies) were coupled with 5 µg/mg of SPARC antibody (rat—anti-mouse SPARC IgG2B, R&D Systems) on a roller overnight at room temperature. Beads incubated with vehicle only were used as control. Protein was extracted from differentiated C2C12 and C2C12^{SPARC KD} cell pellets by incubating the cell pellets in a 1:9 ratio with extraction buffer (110 mmol/L potassium acetate, 0.5% Triton X-100, 2 nmol/L magnesium chloride, 1 mmol/L dithiothreitol, 100 mmol/L sodium chloride, 1 µL/mL of protease inhibitor, pH 7.4), followed by incubation on ice for 15 minutes and centrifugation at 2600 × *g* for 5 minutes at 4°C. The supernatants were used immediately for immunoprecipitation. Then 1.5-mg antibody and control coupled beads were incubated with cell lysate and placed on a roller for 30 minutes at 4°C. Beads were magnetically removed and washed, and then the immunoprecipitation products were eluted and run on a 4% to 12% Bis-Tris SDS-PAGE gel for protein separation under reducing conditions. The gel was silver stained according to the standard protocol, and relevant protein bands (present in C2C12 but absent from C2C12^{SPARC KD}) were excised from one-dimensional -gel and subsequently in-gel digested. Briefly described, the gel pieces were washed twice in 50 mmol/L ammonium bicarbonate and 50% ethanol and then dehydrated in ethanol before reduction with 10 mmol/L dithiothreitol and alkylation with 50 mmol/L iodoacetamide in 50 mmol/L ammonium bicarbonate. The alkylated gel pieces were washed and dehydrated as above and digested with trypsin overnight at 37°C followed by

acidification with 1% trifluoroacetic acid. The tryptic peptides were desalted using the reverse phase microtip purification technique and subsequently eluted onto Bruker UltraFlex polished steel target. Peptide mass fingerprints (PMFs) and MALDI-TOF/TOF spectra were obtained on an UltraFlex matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF/TOF) instrument (Bruker Daltonics, Billerica, MA). All PMFs were three points externally calibrated using known masses of tryptic peptides and annotated by the flex analysis software version 3.4 (Bruker Daltonics). The data were searched against an in-house NCBI database (<ftp://ftp.ncbi.nlm.nih.gov/refseq/release/complete>, release 53, last accessed June 28, 2012) using an in-house MASCOT server (Matrix Science, Columbia, SC). Peptide tolerance was set to 80 ppm for PMFs and 0.9 Da for MALDI-TOF/TOF spectra. Cysteine carbamidomylation was set as fixed modification. Methionine oxidation was set as variable modification. One missing cleavage was allowed per peptide. For positive protein identification, the score of the result of $(-10 \times \log(p))$ had to be over the significance threshold level ($P = 0.05$).

Western Blot

Cell samples were washed twice in phosphate-buffered saline. Total protein was extracted by resuspending cell pellets in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) containing 1× Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and 1× Halt phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) followed by incubation on ice for 30 minutes. The lysate was centrifuged (15 minutes, 12,000 × *g*, 4°C) and the supernatant was stored at −80°C until use. Protein concentration was determined using Pierce BCA protein assay kit (VWR).

For analysis, approximately 10 µg of protein was added to the loading buffer (Thermo Fisher Scientific) and sample reducing agent (Thermo Fisher Scientific), heated to 95°C for 5 minutes, and loaded onto 4% to 12% Bis-Tris gels (Thermo Fisher Scientific). The gel was run using 1× MES buffer (Thermo Fisher Scientific) for 1 hour at 120 V constant and electroblotted onto 0.45-µm polyvinylidene difluoride membranes (Millipore, Billerica, MA). Transfer and equal loading were validated using a Ponceau S staining of the membranes after transfer. The membranes were blocked with either 5% skimmed milk and Tris-buffered saline with Tween 20 or 5% BSA and Tris-buffered saline with Tween 20 for 1 hour at room temperature and incubated overnight at 4°C with the following primary antibodies: rat—anti-mouse SPARC (1:1000, R&D Systems), rabbit—anti-human β-actin (1:1000, ab8227, Abcam), mouse—anti-rabbit α-actin (1:200, sc-58671, Santa Cruz Biotechnology), mouse—anti-bovine γ-actin (1:2000, sc-65638, Santa Cruz Biotechnology), rabbit—anti-human vinculin (1:1000, 4650, Cell Signaling Technology), rabbit—anti-human RhoA (1:1000, no. 2117,

Cell Signaling Technology), rabbit-anti-human glyceraldehyde-3-phosphate dehydrogenase (1:1000, sc-25778, Santa Cruz Biotechnology). The following day blots were washed in Tris-buffered saline with Tween 20 (3×)

and incubated for 1 hour at room temperature with goat-anti-rabbit-HRP (1:1000, p0448, Dako Cytomation), goat-anti-mouse-HRP (1:1000, p0447, Dako Cytomation), or goat-anti-rat-HRP (1:5000, A10549, Life

Figure 1 Secreted protein acidic and rich in cysteine (SPARC) expression correlates with active regeneration. Morphometric analysis of cells expressing SPARC (A), CD45 (B), and myogenin (C) in 7- to 10-week-old (young) and 24- to 30-week-old (older adults) dystrophic (*mdx*) and wild-type (wt) mice. The number of cells expressing SPARC decreased significantly with age in both wt (7 to 10 weeks: 59.5 ± 12 versus 24 to 30 weeks: 10.3 ± 1.57 , $P < 0.01$, unpaired *t*-test) and *mdx* (7 to 10 weeks: 144 ± 25 versus 24 to 30 weeks: 41 ± 7.1 , $P < 0.01$, unpaired *t*-test) mice. Myogenin-positive cells were mostly absent from wt mice regardless of age (7 to 10 weeks: 0.29 ± 0.29 versus 24 to 30 weeks: 0.33 ± 0.22 , $P > 0.05$, unpaired *t*-test), whereas the number of myogenin-positive cells was reduced in 24- to 30-week-old *mdx* mice compared with 7- to 10-week-old *mdx* mice (7 to 10 weeks: 10.9 ± 2.1 versus 24 to 30 weeks: 4.7 ± 1.5 , $P < 0.05$, unpaired *t*-test). Few CD45-positive cells were observed in both 7- to 10-week-old and 24- to 30-week-old wt mice; still the number was reduced with age (wt: 7 to 10 weeks: 53.7 ± 9.3 versus 24 to 30 weeks: 7.2 ± 2.9 , $P < 0.01$), whereas in the *mdx* mice, the number of leukocytes remained high, both in the 7- to 10-week-old group and the 24- to 30-week-old group (7 to 10 weeks: 82.7 ± 12.5 versus 24 to 30 weeks: 115.4 ± 30.3 , $P > 0.05$, unpaired *t*-test). Correlation analysis between SPARC and CD45 (D) as well as SPARC and myogenin (E) revealed a direct correlation between SPARC and myogenin ($P < 0.01$, Pearson correlation) but there was no correlation between SPARC and CD45 ($P > 0.05$, Pearson correlation). **F:** Representative images of SPARC, neural cell adhesion molecule (NCAM) and Sirius red staining in young (7- to 10-week-old) wt and *mdx* mice. The figure depicts the SPARC staining used for quantification in A, and NCAM reveals active regeneration in 7 to 10-week-old *mdx* mice. Sirius red is a fibrosis marker revealing beginning thickening of connective tissue in the young *mdx* mice. **G:** Representative images of SPARC, NCAM and Sirius red staining in older adult (24- to 30-week-old) wt and *mdx* mice. The figure depicts the SPARC staining used for quantification in A, and NCAM reveals that active regeneration is barely present in the 24- to 30-week-old *mdx* mice. Sirius red reveals increased thickening of connective tissue in the 24- to 30-week-old *mdx* mice. Scale bar = 50 μ m (F and G).

Figure 2 Secreted protein acidic and rich in cysteine (SPARC) is predominantly expressed by myoblasts and regenerating myofibers. **A:** SPARC protein expression was analyzed in various cell types during regeneration of 7- to 10-week-old dystrophic (*mdx*) mice using double immunostainings for SPARC combined with cell markers for neutrophils (Ly6G), monocytes/macrophages (F4/80), fibroblasts (S100A4), myogenic cells [neural cell adhesion molecule (NCAM)] and stress [heat shock protein 27 (Hsp27)]. SPARC predominantly co-localized with NCAM (white arrow points toward myofiber with expression of both SPARC and NCAM) and Hsp27 in regenerating myofibers (white arrows point toward co-localization in regenerating myofibers and single cells in the regenerating area). Co-expression between SPARC and Hsp27 was evaluated further and detected in human primary myoblasts (B) and Duchenne muscular dystrophy patient muscle (C). White arrow points towards a regenerating myofiber expressing both SPARC and Hsp27. D: The co-localization between SPARC and Hsp27 in human myoblasts was analyzed for the possibility of a direct protein-protein interaction using Duolink. Few direct interactions sites were detected (white arrow). E: In comparison, no positive signal is observed in a negative control with omission of Hsp27 antibody. Scale bars = 20 μ m.

Technologies), washed in Tris-buffered saline with Tween 20 (3 $\times$) and developed using Novex ECL HRP Chemiluminescent Substrate kit (Life Technologies) and standard X-ray film. Glyceraldehyde-3-phosphate dehydrogenase was used as loading control.

Transmission Electron Microscopy

Muscle tissue (EDL and soleus) from *SPARC*^{+/+} and *SPARC*^{-/-} mice were fixated in 2% glutaraldehyde/phosphate-buffered saline and stained with 1% osmiumtetroxide/phosphate-buffered saline, dehydrated, and

embedded in epon (epoxyresin). Ultrathin sections (60 nm) were cut on a Leica Ultracut Ultramicrotome and placed on a grid, contrasted using 3% uranylacetate/water and lead citrate (Leica Ultrastain 2) and analyzed.

Statistical Analysis

The unpaired *t*-test, multiple *t*-test with statistical significance determined using the Holm-Sidak method, 2-way analysis of variance, and Pearson correlation analysis with *P* < 0.05 as the significance level were performed using GraphPad Prism software version 6 (GraphPad Software Inc, San Diego, CA).

Results

SPARC Expression Is Predominantly Associated with Myoblasts and Immature Myofibers

We know that SPARC is highly expressed by actively regenerating skeletal muscle tissue in both Pax7-positive cells and regenerating fibers.² However, we do not know whether this expression relates to the regeneration process only or if SPARC is also present in, for example, fibroblasts and is implicated in fibrosis. To investigate this further, we analyzed SPARC protein expression in TA from dystrophic (*mdx*) and control (wt) mice at a young age (7 to 10 weeks) and an older age (24 to 30 weeks). We observed that SPARC expression was prominent in young dystrophic mice, which have active, ongoing regeneration (NCAM) (Figure 1F) with the number of positive cells decreasing as the disease progressed in older dystrophic mice (7 to 10 weeks versus 24 to 30 weeks *mdx*: $P < 0.01$) (Figure 1A), where the regeneration process is substituted for fibrosis (NCAM and Sirius red) (Figure 1G). Moreover, SPARC expression was reduced with age in wt mice as well (7 to 10 weeks versus 24 to 30 weeks wt: $P < 0.01$) (Figure 1A), suggesting that SPARC generally decreased with age, as has previously been published,¹⁷ and this decrease was not countered by the more severe dystrophic state of the adult *mdx* mice³¹ as observed by increased

fibrosis (Sirius red) (Figure 1, F and G), pronounced, continued inflammation (CD45) (Figure 1B), and decreased regeneration (Myogenin and NCAM) (Figure 1, C and G). The highest number of SPARC-positive cells was detected in young dystrophic mice, and generally SPARC expression correlated with the marker for actively differentiating myoblasts, myogenin ($**P < 0.01$) (Figure 1E), and not the common leukocyte marker, CD45 ($P > 0.05$) (Figure 1D), suggesting a direct relation between SPARC expression and muscle tissue repair. This finding was further substantiated by double immunostaining for cell-specific markers (fibroblasts: S100A4, activated satellite cells, myoblasts, and regenerating myofibers: NCAM, neutrophils: Ly6G, and macrophages: F4/80) and SPARC, which revealed that intracellular SPARC expression during regeneration is associated directly with the muscle cells and immature, newly formed myofibers and not other components of the regeneration process, for example, fibrosis and inflammation (Figure 2A).

SPARC Protein Is Co-Expressed with the Small Hsp27 in Myoblasts and Regenerating Fibers

Next we investigated if the presence of SPARC in muscle cells and regenerating myofibers correlated with cellular stress. We

Figure 3 Identification of actin as a binding partner to secreted protein acidic and rich in cysteine (SPARC). **A:** To investigate the functional role of SPARC *in vitro*, SPARC was knocked down in C2C12 cells (C2C12^{SPARC KD}) using shRNA. Using real-time quantitative PCR, we confirmed that Sparc expression was reduced on the mRNA level. **B:** Reduced expression of SPARC protein after knock down was observed using immunocytochemistry. We wanted to detect whether possible intracellular binding partner(s) to SPARC exist, and we therefore performed mass spectrometry analysis of proteins co-immunoprecipitating with SPARC. **C:** Silver staining of the gel used in identification of the interaction between SPARC and actin. The band identified as actin is enclosed by the black box. The gel visualizes the band spectra present in whole cell lysates from C2C12 and C2C12^{SPARC KD} cells, a negative control (N.C.) from immunoprecipitation with naked (vehicle-treated) beads from C2C12 control cells and with SPARC antibody-coupled beads from C2C12 and C2C12^{SPARC KD} cells. Cell samples used for immunoprecipitation were from differentiating cells. **D:** Immunocytochemical staining for localization and presence of SPARC (green) and actin (red) in C2C12 and C2C12^{SPARC KD} proliferating cells. Expression of both proteins is clearly reduced in C2C12^{SPARC KD}. Data are expressed as means $\pm$ SEM (A). Scale bars: 50 μ m (B); 20 μ m (D). co-ip, co-immunoprecipitation.

Figure 4 The interaction between actin and secreted protein acidic and rich in cysteine (SPARC) is present during proliferation and differentiation *in vitro* and in regenerating myofibers of dystrophic/myopathic patients *in vivo*. The interaction between actin and SPARC was analyzed in human muscle cells *in vitro* and *in vivo* in patients with muscle disorders all causing muscle damage using the Duolink proximity ligation assay. Red dots are equivalent to specific Duolink interactions, and each dot represents one interaction between actin and SPARC. Green indicates the vimentin (cells) or neural cell adhesion molecule (NCAM) (tissue) counterstaining, and blue is DAPI expression in nuclei. SPARC and actin is expressed and interacts in proliferating primary human satellite cells (**A**) and in myotubes derived from differentiating primary human satellite cells (**B**). The interactions are distributed throughout the cells both during proliferation and differentiation. **C** and **D**: Two different patients with Duchenne muscular dystrophy have a prominent number of interactions in regenerating myofibers (**white arrows**). **E**: A patient with compartment syndrome in the leg has the same interactions in regenerating myofibers (**white arrows**) (**F**) as does a patient with polymyositis (**white arrow**, this patient's biopsy specimen has not been counterstained with NCAM). **G**: A control staining for the interaction with omission of the actin antibody. **H**: A control staining for the interaction with omission of the SPARC antibody. **I**: A control staining for the interaction with omission of both primary antibodies. Scale bars = 20 μ m (**A–I**).

detected co-expression of SPARC with the small heat shock molecule Hsp27 in human myoblasts (Figure 2B) and in regenerating myofibers of both *mdx* mouse muscle (Figure 2A) and Duchenne muscular dystrophy patient muscle (Figure 2C). Using Duolink, we analyzed whether the co-localization of SPARC and Hsp27 could be associated with a direct interaction between the two proteins. We only observed few sites of close proximity in human myoblasts, indicating no direct interaction between SPARC and Hsp27 (Figure 2, D and E).

Identification of Actin as a Binding Partner to SPARC

Next, we wanted to identify whether specific intracellular SPARC interaction partners exist because of the consistent presence of this extracellular matrix protein within muscle cells and regenerating myofibers. To identify these, we performed SPARC pull-down experiments from C2C12 control and C2C12^{SPARC KD} differentiated cell extracts using beads coupled with SPARC antibody. Knock-down efficiency of SPARC was confirmed at the mRNA (Figure 3A) and protein (Figure 3B) levels.

All proteins were visualized by silver staining, and bands reduced in or absent from C2C12^{SPARC KD} cells were subjected to mass spectrometry for protein identification. As shown in Figure 3C and identified using mass spectrometry, actin (boxed) is enriched in a SPARC-dependent manner and hence constitutes a potential new SPARC-binding partner.

Actin expression was analyzed in C2C12 and C2C12^{SPARC KD} cells using a pan-actin antibody, and we observed that SPARC was important for general expression of actin and thus also the structure of the cytoskeleton, which was severely compromised in proliferating C2C12^{SPARC KD} cells (Figure 3D).

Our initial findings of SPARC were based on human tissue; thus, we wanted to investigate whether this interaction was also present in human tissue. We verified the interaction between SPARC and actin in human myoblasts during proliferation and differentiation using the Duolink proximity ligation assay (Figure 4, A and B), suggesting that our previous observation of SPARC in these cells² could be correlated to control of cytoskeletal arrangement during regeneration.

The Interaction between SPARC and Actin Is Directly Correlated with Active Regeneration in Patients with a Muscle Degenerative Condition

To investigate whether the interaction between SPARC and actin observed in human myoblasts translated into a physiologic setting, we analyzed muscle biopsy specimens from patients with various muscle degenerative disorders, all with a regenerative component. Again using Duolink, we found that the direct interaction between SPARC and actin was present in all types of muscle-damaging disorders analyzed,

Figure 5 Absence of secreted protein acidic and rich in cysteine (SPARC) affects localization of the three actin isoforms (α , β , and γ) and the expression of α -actin. The observation that SPARC interacts with actin prompted us to investigate in more detail which actin isoforms might be affected by the absence of SPARC. **A:** C2C12 control and C2C12^{SPARC KD} cells were analyzed using immunocytochemistry for the three isoforms (α , β , and γ) during proliferation and differentiation. All three isoforms changed in use observed as changing localization patterns between C2C12 control and C2C12^{SPARC KD} cells during proliferation and differentiation. **B:** Western blot was used to investigate expression level of the 3 actin isoforms (α , β , and γ) and expression of SPARC and proteins involved in actin filament formation (RhoA) and focal adhesions (vinculin). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. The experiment was run in duplicate and representative images are shown. Western blot confirms the effect of SPARC absence on α -actin, which is only expressed in C2C12 control cells during differentiation. Expression of both β - and γ -actin appears to remain constant between cell lines and during proliferation and differentiation. Vinculin is not affected by absence of SPARC, whereas RhoA is reduced in C2C12^{SPARC KD} cells during differentiation. Scale bar = 20 μ m (**A**).

which included Duchenne muscular dystrophy (Figure 4, C and D), compartment syndrome (Figure 4E), and polymyositis (Figure 4F). Interestingly, the interaction was only present in actively regenerating muscle structures observed

using a counterstain for NCAM (Figure 4, C–E). We did not detect the interaction in necrotic areas or in undamaged muscle fibers within the biopsy specimens, suggesting that SPARC function is limited to fibers undergoing remodeling.

Moreover, control experiments with omission of the primary actin antibody (Figure 4G), the primary SPARC antibody (Figure 4H), or both (Figure 4I) confirmed the specificity of the Duolink reaction.

Absence of SPARC Modifies the Expression Pattern of α -, β -, and γ -Actin Isoforms during Proliferation and Differentiation of Myoblasts

Next, we wanted to investigate the expression and localization pattern of the α -, β -, and γ -actin isoforms in the presence or absence of SPARC. For this purpose, we used the C2C12 and C2C12^{SPARC KD} cells. We observed that α -actin was only present in very mature differentiating myotubes in the C2C12 control cells (Figure 5A), and we could not detect α -actin in the C2C12^{SPARC KD} cells. This was confirmed by Western blotting of total α -actin protein level (Figure 5B). Both cell lines expressed β -actin (Figure 5, A and B); however, the distribution pattern was different between C2C12 and C2C12^{SPARC KD}. In C2C12 cells, β -actin was expressed in a dot-like pattern close to the nuclei during proliferation, and the pattern became more filamentous and elongated and followed the shape of the cells during differentiation. In the C2C12^{SPARC KD} cells, β -actin was present in a cloud-like pattern located more to the periphery of the cells during proliferation, and during differentiation β -actin was more diffusely localized. However, as revealed by Western blotting (Figure 5B), β -actin was expressed equally between the cell lines during both proliferation and differentiation, suggesting that the difference lies in localization. Both cell lines also expressed γ -actin (Figure 5, A and B), and again there was a marked difference in localization pattern

(Figure 5A) because Western blotting revealed equal amounts of protein present between the cell lines (Figure 5B). In the C2C12 cells, γ -actin was expressed in the entire cell, clearly marking the cell filaments during proliferation, whereas expression changed markedly during differentiation. In the C2C12^{SPARC KD} cells, γ -actin was also present in the entire cell during proliferation; however, the expression pattern was not filamentous. This changed during differentiation, where γ -actin was now expressed in a pattern where cell filaments and contact points were clearly marked. This was in direct contrast to the C2C12 control cells. The analysis of the different isoforms reveals a clear difference in localization pattern dependent on the presence of SPARC. Moreover, this analysis also reveals that the C2C12^{SPARC KD} cells are incapable of forming myotubes during differentiation, and the effect on the isoform expression pattern suggests that in the absence of SPARC, the cells switch to dominantly use the γ -actin isoform for filament formation because α -actin is not expressed at all. In addition to investigating expression levels of the actin isoforms using Western blotting, we further analyzed expression of RhoA, a key regulatory protein for actin stress filament formation,³² and vinculin, a focal adhesion protein that interacts with F-actin.³³ Our results indicate that vinculin expression is not affected by absence of SPARC or regulated between proliferation and differentiation in either C2C12 or C2C12^{SPARC KD} cells (Figure 5B). However, expression of RhoA is markedly reduced in C2C12^{SPARC KD} during differentiation compared with C2C12 control cells. Thus, absence of SPARC *in vitro* appears to affect actin filament formation during differentiation both on the key regulatory protein RhoA and actin isoform use.

Figure 6 Skeletal muscle regeneration is not affected by absence of secreted protein acidic and rich in cysteine (SPARC). To investigate whether the absence of SPARC influenced the muscle's ability to regenerate after severe trauma, regeneration was induced in SPARC^{-/-} and SPARC^{+/+} mice using a cardiotoxin injection. **A:** Muscle regeneration was assessed 3 and 7 days after injury using hematoxylin and eosin (H&E) staining. There was no apparent difference in ability to regenerate based on histologic analysis. The absence of SPARC-positive cells was verified using an immunofluorescence staining against SPARC protein 3 days after injury (**bottom left white arrows** point toward SPARC-positive cells, and **bottom right white arrow** points toward unspecific staining of a necrotic myofiber). **B:** Representative images depicting an entire muscle section from day 3 and day 7 after a cardiotoxin injection. The images show how the entire muscle cross-sectional area and the area of necrosis have been marked for automatic quantification using the VIS software. **C:** Quantification of the necrotic area following muscle damage. There was no difference in the size of the necrotic area between SPARC^{-/-} and SPARC^{+/+} mice (day 3: SPARC^{-/-}: 29.21 ± 7.46 versus SPARC^{+/+}: 38.21 ± 9.09, $P > 0.05$, day 7: SPARC^{-/-}: 7.24 ± 2.89 versus SPARC^{+/+}: 7.59 ± 2.48, $P > 0.05$, SPARC^{-/-} versus SPARC^{+/+}, unpaired t -test). **D:** The mice were analyzed for ability to elicit an inflammatory response by staining for the common leukocyte marker CD45 followed by quantification. The quantification was performed on the entire section because inflammatory cells are present throughout the muscle tissue due to recruitment and activation from uninjured sites of the tissue as well. There was no difference in presence of inflammatory cells between SPARC^{-/-} and SPARC^{+/+} mice (day 3: SPARC^{-/-}: 1052.75 ± 216.3 versus SPARC^{+/+}: 1272.75 ± 297.7, $P > 0.05$, day 7: SPARC^{-/-}: 176.59 ± 11.32 versus SPARC^{+/+}: 114.60 ± 55.57, $P > 0.05$, SPARC^{-/-} versus SPARC^{+/+}, unpaired t -test). **E:** The mice were analyzed for the presence of myogenin-positive nuclei following regeneration to assess the myoblasts' ability to enter differentiation during regeneration. This quantification was performed in the regenerative area only to ascertain that any differences in size of the lesions would not affect the result. There was no difference in the number of myogenin-positive nuclei in the regenerative area between SPARC^{-/-} and SPARC^{+/+} mice (day 3: SPARC^{-/-}: 71.33 ± 8.99 versus SPARC^{+/+}: 68.29 ± 11.99, $P > 0.05$, day 7: SPARC^{-/-}: 84.23 ± 9.71 versus SPARC^{+/+}: 60.83 ± 3.83, $P > 0.05$, SPARC^{-/-} versus SPARC^{+/+}, unpaired t -test). **F:** The contralateral, uninjured tibialis anterior (TA) muscles were analyzed for fiber phenotypes, and it was observed that both SPARC^{-/-} and SPARC^{+/+} mice predominantly expressed type 2 myosin heavy chain (MHC) fast-positive fibers. **G:** The TA muscles 7 days after cardiotoxin injection was further examined for fiber phenotypes to investigate whether any fiber type switching had occurred during regeneration in SPARC^{-/-} versus SPARC^{+/+} mice. No fiber-type switching was observed, and all regenerative fibers expressed MHC fast. **H:** The contralateral, uninjured control TA muscles were further analyzed to determine fiber diameters. There was no difference in myofiber size between SPARC^{-/-} and SPARC^{+/+} mice ($P > 0.05$, 2-way analysis of variance). **I:** In support of the morphologic and morphometrical analyses of regeneration, desmin staining was performed to visualize the presence of actively regenerating myoblasts and the formation of new myofibers in a similar manner between SPARC^{-/-} and SPARC^{+/+} mice 3 days after cardiotoxin injection. Data are expressed as means ± SEM (C–E, H). Scale bars = 50 μ m (A, F, G, I); 500 μ m (B).

SPARC^{-/-} Mice Are Fully Capable of Undergoing Regeneration *in Vivo*

The effect on isoform expression *in vitro* prompted us to investigate whether lack of SPARC directly inhibits skeletal muscle regeneration *in vivo*. For this purpose, we used SPARC^{-/-} mice and SPARC^{+/+} littermate controls. These mice had a cardiotoxin injection in the right TA muscle and were analyzed for the ability to regenerate after 3 and 7 days. We observed no histologic difference (hematoxylin and eosin staining) in regenerative ability between SPARC^{-/-} and SPARC^{+/+} mice (Figure 6A). We analyzed the ability to regenerate further by investigating the amount of necrosis (Figure 6, B and C), the elicited inflammatory response (Figure 6D), and the number of myogenin-positive nuclei (Figure 6D) as a marker for actively differentiating myoblasts. We found that none of these parameters were affected by the absence of SPARC. We also performed a desmin staining to further evaluate the regenerative ability (Figure 6I), and we observed that already 3 days after injury there was presence of desmin-positive cells in both SPARC^{-/-} and SPARC^{+/+} mice, supporting that absence of SPARC does not affect regeneration.

Fiber-type analysis revealed that there was no difference in fiber-type composition in uninjured, contralateral muscles between SPARC^{+/+} and SPARC^{-/-} mice. TA muscle from both presented predominantly with MHC fast fibers (Figure 6F), and there was no difference in fiber diameter (Figure 6H). Moreover, fiber-type analysis of the regenerating muscles revealed that after injury and during regeneration no isotype shift occurred; thus, the regenerating fibers also predominantly expressed MHC fast (Figure 6G).

The SPARC^{-/-} phenotype was verified (in addition to genotyping PCR, not shown) by the absence of SPARC-positive cells in the regenerating area (day 3) in SPARC^{-/-} mice compared with SPARC^{+/+} mice (Figure 6A). This finding suggested that even though muscle cells lacking SPARC *in vitro* had a disturbed actin cytoskeleton, this did not translate to an *in vivo* setting, indicating that there might be compensatory mechanisms present *in vivo*.

Lack of SPARC in Skeletal Muscle Causes Inability to Recover following Fatigue *in Vitro*

To further investigate the physiologic role of SPARC within skeletal muscle, we analyzed muscle function using *in vitro* force measurements on EDL and soleus muscles from SPARC^{-/-} and SPARC^{+/+} mice. There were no differences in force-frequency relationship between SPARC^{-/-} and SPARC^{+/+} mice for either EDL ($F_{50} = 65$ Hz for SPARC^{+/+}, $F_{50} = 59$ Hz for SPARC^{-/-}) or soleus ($F_{50} = 43$ Hz for SPARC^{+/+}, $F_{50} = 46$ Hz for SPARC^{-/-}) (Figure 7A). Furthermore, there was no difference in the maximum tetanic force between SPARC^{-/-} and SPARC^{+/+} mice for either EDL or soleus. Thus, the force-generating ability in resting, uninjured muscles appeared to be independent of SPARC, which also correlates with the observation that SPARC is not detectable in normal, healthy, uninjured muscle.² However, after the 3-minute fatigue stimulation protocol, SPARC^{-/-} mice had a significant impairment of force recovery for both EDL (Figure 7B) and soleus (Figure 7C), with SPARC^{+/+} mice fully recovering within 30 minutes. To investigate this force recovery defect, we analyzed EDL and soleus muscles from SPARC^{-/-} and SPARC^{+/+} mice using electron microscopy (Figure 7D). We did not observe any difference in sarcomere structure and found no abnormalities in SPARC^{-/-} mice in undamaged muscle, which correlated well with the normal ability to generate force. Next, we wanted to investigate whether this force deficit after fatigue translated into an *in vivo* setting. We therefore subjected SPARC^{+/+} and SPARC^{-/-} mice to eccentric exercise on a treadmill and measured force grip strength before exercise and at 1, 10, and 30 minutes after exercise. We observed that the short-term exercise protocol did not fatigue the mice in a manner that caused any decrease in the ability to elicit force, and there was no difference between SPARC^{+/+} and SPARC^{-/-} mice in their ability to generate force *in vivo* either before or after short-term exercise (Figure 7E). To further analyze the background for the *in vitro* force decrease results, we investigated the muscle phenotypes in SPARC^{+/+} and SPARC^{-/-} using MHC fast and slow staining and

Figure 7 Recovery of force *in vitro* is compromised in secreted protein acidic and rich in cysteine (SPARC) knockout mice (SPARC^{-/-}) mice after fatigue. To test whether absence of SPARC had an influence on force transmission in skeletal muscle, we analyzed extensor digitorum longus (EDL) and soleus muscle using *in vitro* force measurements. **A:** Force-frequency analysis of EDL and soleus muscles revealed no difference between SPARC^{-/-} and SPARC^{+/+} mice. The ability to generate force after a fatigue protocol was analyzed in EDL (**B**) and soleus (**C**) muscles from SPARC^{-/-} and SPARC^{+/+} mice. There was a significant difference in the ability to recover force generation in SPARC^{-/-} in EDL and soleus. **D:** Electron microscopy of representative EDL muscle from SPARC^{-/-} and SPARC^{+/+} mice showing normal sarcomeric units in both. **E:** On the basis of the results *in vitro*, we tested if a short-term eccentric exercise protocol-induced fatigue earlier in SPARC^{-/-} mice and whether there was a similar decrease in force grip of the front paws combined with inability to recover. We observed that the short-term exercise protocol did not induce measurable fatigue and therefore also no deficit in recovery between SPARC^{-/-} and SPARC^{+/+} mice. To further investigate the EDL and soleus muscle phenotypes, we performed analyses of fiber types in EDL (**F**) and soleus (**G**) muscles. We observed no difference in fiber types between SPARC^{-/-} and SPARC^{+/+} mice in EDL ($P > 0.05$, 2-way analysis of variance, Sidak's multiple comparison) or soleus ($P > 0.05$, 2-way analysis of variance, Sidak's multiple comparison). We further analyzed the muscles by measuring diameters of all EDL fibers (**H**), myosin heavy chain (MHC) fast-positive fibers in soleus (**I**), and MHC slow-positive fibers in soleus (**J**). We did not detect any differences in fiber diameters between SPARC^{-/-} and SPARC^{+/+} mice ($P > 0.05$, 2-way analysis of variance for all analyses) (**H–J**). In support of the quantifications in **F** and **G**, we show representative images of the MHC fast and MHC slow staining of EDL and soleus muscles in SPARC^{+/+} mice (**K**) and SPARC^{-/-} mice (**L**). * $P < 0.05$ at 1 minute versus SPARC^{+/+}; ** $P < 0.01$ at 30 minutes versus SPARC^{+/+} (multiple *t*-tests); *** $P < 0.001$ at 10 minutes versus SPARC^{+/+}; **** $P < 0.0001$ at 30 minutes versus SPARC^{+/+} (multiple *t*-tests). Scale bars = 500 nm (left panel); 1000 nm (right panel) (**D**); 50 μ m (**K** and **L**).

measurements of fiber diameters of both EDL and soleus muscle. There was no difference in fiber-type composition (Figure 7, F, G, K, and L) or diameter (Figure 7, H–J) of either EDL or soleus between *SPARC*^{+/+} and *SPARC*^{-/-}; thus, the decrease in force after the *in vitro* protocol is not based on differences in fiber types or diameters and must relate to other intrinsic effects dependent on SPARC within the muscle tissue, which most likely are compensated *in vivo*.

Discussion

SPARC is an evolutionarily conserved protein among all phyla³⁴ and belongs to a group of extracellular matrix proteins that are regulatory but do not contribute significantly to extracellular matrix structure.³⁵ The fact that SPARC is evolutionarily conserved and expressed by multiple species suggests that this protein has important regulatory role(s). Our previous finding of SPARC expression during muscle development and in patients with myopathy² is consistent with the expression pattern of matricellular proteins, which are generally present during embryogenesis, not expressed in adult tissues but reexpressed in damaged or remodeling tissues, as well as in response to inflammation or cancers.³⁵ In line with this, SPARC is expressed in a regulated manner during regeneration in mice as well.²⁰ In more primitive species SPARC function in skeletal muscle has been associated with reduced motility and developmental muscle defects,^{18,19} whereas in mice, absence of SPARC has previously not been correlated with a muscle phenotype.¹⁴

However, the exact role of SPARC within mammalian skeletal muscle tissue remains unknown because the presence of SPARC in single cells and in developing or regenerating myofibers does not correlate with previously described general effects of SPARC, such as inhibition of proliferation³⁶ and collagen deposition in connective tissue.³⁷

In the current study, we therefore investigated the role of SPARC in skeletal muscle tissue. We made several interesting observations: i) SPARC interacts directly with actin in myoblasts, and lack of SPARC results in a changed localization pattern of the different actin isoforms *in vitro*; ii) SPARC interacts directly with actin in the regenerating myofibers in patients with muscular dystrophies and myopathies; iii) SPARC absence in mice results in defects in force recovery after a fatigue stimulation protocol *in vitro*; and iv) SPARC is not necessary for the regeneration process after muscle damage with toxin that completely destroys the myofibers. Because SPARC is not expressed by normal, adult healthy muscle,² our results indicate that the function of SPARC in skeletal muscle is temporary, occurring only during remodeling or regeneration of the skeletal muscle with a possible physiologic role to recover the ability to generate force after damage. The indication that the function of SPARC is temporary suggests that SPARC needs to be synthesized according to need and maybe even be transported from the extracellular to the intracellular

compartment. This agrees with the observed shuttling mechanism of SPARC in fibroblasts.²²

SPARC protein structure has been extensively studied, and it is well known that SPARC contains an extracellular Ca²⁺ domain containing 2 EF-hand motifs,³⁸ a follistatin-like domain and a kazal-like domain.³⁹ We have analyzed this protein for presence of conserved actin-binding domain(s) using the Conserved Domain Database, National Center for Biotechnology Information,⁴⁰ and the only other conserved domains were a collagen binding site and a putative K⁺-binding site. Thus, SPARC is not a classic actin-binding protein, suggesting that a more general mechanism is involved in the specific interaction between actin and SPARC. The role of SPARC as a molecular chaperone has been suggested previously, where it is proposed that SPARC is a scavenger chaperone mediating disassembly of the extracellular matrix networks.²² SPARC has chaperone-like activity in a thermal aggregation assay. Here, SPARC remained stable at 50°C, where it was capable of protecting alcohol dehydrogenase from temperature-induced aggregation.²⁶ In line with this, our results could suggest a protective chaperone-like activity of SPARC in remodeling skeletal muscle.

Interestingly, the interaction between SPARC and actin is present in such diverse muscular disorders as Duchenne muscular dystrophy, a genetic disorder with mutated dystrophin,⁴¹ polymyositis, an autoimmune inflammatory disorder that affects the skeletal muscles⁴² and compartment syndrome, an illness occurring in response to compromised blood supply to the muscle.⁴³ This finding suggests that the interaction between these proteins represents a common mechanism related to muscle damage and not a specific type of injury.

During muscle cell differentiation, major changes in cytoskeletal architecture occur, involving all actin isoforms, and during formation of myotubes there is a shift in expression and localization pattern from β - and γ -actin to α -actin.^{44,45} In agreement with this, we observed in the C2C12 control cells that β - and γ -actins are predominantly expressed during proliferation but with very different localization patterns. Where γ -actin clearly outlines the cytoskeletal structure, β -actin is present in a more dot-like pattern. During myotube formation, α -actin is now highly expressed in mature myotubes, whereas β - and γ -actin are still present but change the localization pattern during differentiation. These morphologic actin changes are not observed in the C2C12^{SPARC KD} cells. β -actin is present in a cloud-like pattern and is barely observable during differentiation, and α -actin is not detectable at all. The fact that we do not see α -actin could be a result of the C2C12^{SPARC KD} cells inability to differentiate and form myotubes. However, it might be the lack of interaction between SPARC and actin that results in the inability of the C2C12^{SPARC KD} cells to differentiate and hence the absence of α -actin expression in C2C12^{SPARC KD} cells. Interestingly, the C2C12^{SPARC KD} cells appear to compensate for the lack

of SPARC by expressing and using γ -actin during differentiation. In the proliferative state, γ -actin is present throughout the cells, but the localization pattern suggests that these cells have a compromised cytoskeletal structure. However, during differentiation, this changes, and γ -actin reveals a cytoskeletal network both inside cells and between cells, suggesting that during differentiation these cells compensate through the use of γ -actin to form filaments and attach to each other instead of α -actin. Interestingly, though, the amount of γ -actin remains the same during proliferation and differentiation, suggesting that it is most likely use and localization that are changed. The fact that C2C12^{SPARC KD} uses γ -actin in this manner is exciting because γ -actin is generally considered one of the nonmuscle actins, yet γ -actin is a component of the z disk.⁴⁶

Furthermore, we detect the direct interaction between actin and SPARC during both proliferation and differentiation of primary derived human muscle cells. This observation, together with the shift in isoform localization and use in response to lack of SPARC, points toward SPARC as a protector or stabilizer of the cytoskeleton during myogenesis.

The obvious effect of lack of SPARC on differentiation and localization of the actin isoforms is interesting, considering that we previously reported an inhibition of differentiation in C2C12 cells when SPARC was overexpressed in these cells.²⁰ This finding suggests that expression of SPARC during differentiation needs to be well regulated possibly to aid actin during myogenesis. It has recently been found that SPARC has a specific effect on collagen deposition in the basement membrane. Under normal circumstances, SPARC appears to facilitate collagen trafficking from sites of production to sites not expressing collagen; however, when SPARC is overexpressed, the effect is disrupted, and collagen deposition into the basement membrane is reduced, thus facilitating invasive processes instead.⁴⁷ Therefore, regardless of cellular context, it appears that SPARC needs to be tightly controlled to avoid detrimental effects.

On the basis of the inability of C2C12^{SPARC KD} cells to differentiate, SPARC^{-/-} mice would be expected to have a regenerative defect; however, this is not the case. We also did not observe any cytoskeletal defects with electron microscopy in skeletal muscle from SPARC^{-/-} mice, which have normal sarcomeric units. These mice are also capable of generating normal force; thus, the effect of SPARC on force appears not to be related to normal function and general force generation. This is further implied by our results from the short-term, eccentric exercise study, which indicates that SPARC^{-/-} do not fatigue faster *in vivo*; thus, redundant systems could be in play.

Because our short-term exercise protocol was not capable of inducing fatigue that was measurable using force grip analysis in either SPARC^{+/+} or SPARC^{-/-}, this issue needs to be addressed further, possibly through long-term exercise studies combined with strength analyses over time. On the basis of the *in vivo* results from the regeneration study and

the exercise and force grip study, it appears that the isolated effects of SPARC observed *in vitro*, such as the changes in the cytoskeleton, inhibition of differentiation, and force decrease after fatigue, are not transferable to an *in vivo* setting, pinpointing the fact that compensatory mechanisms must be present *in vivo*.

In summary, this study provides new insight into the mechanistic function of SPARC in skeletal muscle and highlights how a multidomain protein can exert very different functions, depending on the compartmental presence and the tissue or disease context in which it is expressed.

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