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SPARC is up-regulated during skeletal muscle regeneration and inhibits myoblast differentiation

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Summary. Skeletal muscle repair is mediated primarily by the muscle stem cell, the satellite cell. Several factors, including extracellular matrix, are known to regulate satellite cell function and regeneration. One factor, the matricellular Secreted Protein Acidic and Rich in Cysteine (SPARC) is highly up-regulated during skeletal muscle disease, but its function remains elusive.

In the present study, we demonstrate a prominent yet transient increase in *SPARC* mRNA and protein content during skeletal muscle regeneration that correlates with the expression profile of specific muscle factors like *MyoD, Myf5, Myf6, Myogenin, NCAM, CD34*, and *M-Cadherin*, all known to be implicated in satellite cell activation/proliferation following muscle damage. This up regulation was detected in more cell types. Ectopic expression of SPARC in the muscle progenitor cell line C2C12 was performed to mimic the high levels of SPARC seen in muscle disease. SPARC overexpression almost completely abolished myogenic differentiation in these cultures as determined by substantially reduced levels of myogenic factors (P*ax7, Myf5, Myod, Mef2α, Myogenin,* and *Myostatin*) and a lack of multinucleated myotubes. These results demonstrate that there is a delicate temporal regulation of SPARC to which more sources in the micro environment contribute, and that disturbances in this, such as extensive up regulation, may have an adverse effect on muscle regeneration.

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Introduction

Following injury, adult skeletal muscle has the capacity to regenerate (Charge and Rudnicki, 2004) primarily by activation, proliferation and fusion of resident skeletal muscle stem cells, the satellite cells (Seale and Rudnicki, 2000). Besides satellite cells, a surrounding myogenic extracellular matrix (ECM) milieu is obligatory for reconstitution of normal skeletal muscle architecture (Sorokin et al., 2000; Lewis et al., 2001). SPARC (Secreted Protein Acidic and Rich in Cysteine) is a multifunctional calcium-binding glycoprotein associated with the ECM (Lane and Sage, 1994). During embryonic development SPARC is expressed in many tissues, including the somites (Holland et al., 1987; Sage et al., 1989a), while limited to tissues undergoing remodeling and repair in adults (Reed et al., 1993; Blazejewski et al., 1997; Lussier et al., 2001; Delany et al., 2003). SPARC has anti-adhesive properties, possibly facilitating cell shape plasticity and cell migration (Sage et al., 1989b), and it also appears to be a direct modulator of several mitogenic factors (Motamed et al., 2003). The literature reports that SPARC is expressed by malignant melanoma tumor cells and is secreted by surrounding fibroblasts and endothelial cells (Fukunaga-Kalabis and Herlyn, 2007). The subcellullar localization of SPARC has previously

been reported to be in cell nuclei associated with the nuclear matrix (Gooden et al., 1999) suggesting a direct effect on gene expression. Yan et al. (2005) observed SPARC both in the cytoplasm and in culture medium (Yan et al., 2005; Fukunaga-Kalabis and Herlyn, 2007). Furthermore, recently it has been suggested that SPARC functions as a matricellular chaperone, which shuttles between the cell and the ECM (Chlenski et al., 2011).

In vivo, SPARC appears to be essential for musculoskeletal development in invertebrates, since abolition of the SPARC homologue from Xenopus laevis embryos (Purcell et al., 1993) and over expression of the SPARC homologue in *Caenorhabditis elegans* (Schwarzbauer and Spencer, 1993) both result in developmental muscle and motility defects. Nonetheless, SPARC-null mice appear to develop normally without muscle defects (Gilmour et al., 1998) suggesting either compensatory mechanisms in mouse muscle development or species-specific differences in SPARC function. In humans, we have recently reported SPARC to be expressed in satellite cells and muscle fibers in developing and diseased dystrophic skeletal muscle (Jorgensen et al., 2009) in line with a few other studies (Chen et al., 2000; Haslett et al., 2002; Noguchi et al., 2003) showing SPARC present in skeletal muscle as well as during regeneration of porcine skeletal muscle (Ferre et al., 2007). Only a few functional studies exist on SPARC in muscle. Accordingly, one study has shown that SPARC may promote myoblast differentiation (Motamed et al., 2003), and another suggests that SPARC up-regulation is indispensable for C2C12 myoblast differentiation (Cho et al., 2000).

Thus, although several studies link high levels of SPARC to skeletal muscle disease and regeneration, its myogenic role remains largely unknown. We therefore set forth to examine the temporal expression of SPARC during *in vitro* and *in vivo* muscle remodeling, as well as to mimic the scenario of muscle disease with increased levels of SPARC by determining the effect of SPARC overexpression in skeletal muscle myoblasts.

Materials and methods

Animal experiments

Skeletal muscle regeneration in m. gastrocnemius was performed as previously described (Jorgensen et al., 2007; Andersen et al., 2009). In brief, hindlimb muscles of anesthetized mice were carefully exposed and two small sutures placed in the outer fascia. A stab wound was introduced between the two stitches, starting from the outer fascia to the widest part of m. gastrocnemius until reaching the calf bone, after which the skin incision was sutured. The m. gastrocnemius was dissected out from mice on days 0-14 following muscle damage. For immunohistochemistry, the tissues were snap-frozen or fixed for 24 hours in 4% phosphate buffered formalin. For qRT-PCR, the central part of m. gastrocnemius, which included the lesion, was excised. For each time point 2-6 muscles were pooled and kept in RNA Lysis buffer (Applied Biosystems, ABI, Foster City, CA) until further analysis. All animal experiments were performed in accordance with Danish Legislation on animal welfare and approved by the Danish Council for Supervision with Experimental Animals.

Plasmids and stable transfection

Stable transfection of SPARC was made in the C2C12 myoblast cell line (ATCC, CRL-1772™, LGC Promochem, Boras, Sweden) using the Flp-In™ System (Invitrogen, Taastrup, Denmark). Briefly, full-length mouse *SPARC* was stably inserted into the genome of C2C12 cells under control by the constitutive CMV promoter (human cytomegalovirus immediate-early enhancer/promoter). A FLP-In™ C2C12 host cell line was established according to manufacturer's recommendation. In brief, the host cell was established by inserting the plasmid pFRT™/lacZeo into the genome of C2C12 cells according to the manufacturer's instructions using Lipofectamine2000™ Reagent (Invitrogen). The plasmid was linearized using Sca1 (Invitrogen) prior to transfection. Obtained clones were screened for number of integration sites by southern blotting using a probe directed against the LacZ gene in the inserted fragment(s). The transcriptional activity of the integrated sites was tested by ß-galactosidase assay and the myogenic commitment of the cells was tested in a differentiation assay to ensure no loss of function due to the genomic integration. The host cell line is, as described, clonal in origin. Full length murine SPARC was obtained from adult mouse testis tissue (Holland et al., 1987; Sage et al., 1989a) using PCR primers covering the 3' and 5' end of the mRNA. The fwd primer included a HindIII restriction site, a ribosomal Binding Site and a start codon: 5' CCC AAG CTT ATC ATG AGG GCC TGG AT 3', whereas the rev primer contained an Xho1 restriction site and a stop codon: 5' CCC CTC GAG TTA GAT CAC CAG ATC CT 3'. The cloning PCR was performed at 59°C annealing temperature using the error-correcting, proof-reading Phusion™ DNA polymerase (Finnzymes, Medinova). The PCR reaction was visualized on a 2% agarose gel with EtBr staining; full length SPARC cDNA was extracted from the gel, purified and digested with HindIII and XhoI (Invitrogen, Taastrup, Denmark) at 37°C for 2 hours. The vector pcDNA™/FRT was digested under the same conditions and SPARC was inserted into the plasmid using T4 DNA Ligase (Invitrogen, Taastrup, Denmark). The ligated plasmid containing *SPARC* was transformed into E. coli for sequencing, determination of orientation of the inserted fragment and amplification followed by plasmid purification.

The obtained SPARC-pcDNA™/FRT was co-transfected into the C2C12 host cell line with pOG44, expressing the FRT Integrase (Invitrogen, Taastrup, Denmark), using Lipofectamine2000™ Reagent

according to the manufacturer's instructions. Selection was performed using 200μg/ml Hygromycin and resulting clones were screened for *SPARC* expression using qPCR. ß-Galactosidase assay was performed to test the transcriptional activity of the integrated sites as co-transfection of SPARC-pcDNA5™/FRT and pOG44 into the obtained Flp-In host cell line disrupts the LacZ locus due to Flp Integrase mediated homologous recombination between the FRT target sites of the pFRT™/lacZeo and the SPARC-pcDNA5™/FRT vectors. Following successful recombination the modified cells no longer express ß-Galactosidase. In brief, the staining confirms the insertion of pFRT™/lacZeo into C2C12 cells and the generation of the Flp-In host cell line carrying the LacZ gene on the genome. The positive reaction for ß-Galactosidase also ensures that the inserted locus is transcriptionally active.

Cell differentiation assay

C2C12^{SPARC} and control cells were plated in monolayer at a density of 2000 cells/cm^2 and cultured as previously described in Andersen 2009 (Andersen et al., 2009). Cells were harvested after 1, 3, 5 and 8 days in culture for qPCR or immunofluorescence.

RNA isolation and real-time PCR analysis

Total RNA was isolated as previously described (Andersen et al., 2009) using the ABI PRISM™ 6100 Nucleic Acid PrepStation system, and RNA was reverse transcribed to single-stranded cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed on an ABI Prism 7900HT Sequence Detection System using a custom designed Applied Biosystems TaqMan® Array (Andersen et al., 2009) or the TaqMan® Gene Expression Assays for *SPARC* (secreted acidic cysteine rich glycoprotein, Mm00486332_m1), *Pgk1* (phosphoglycerate kinase 1, Mm00435617_m1), *Tfrc* (transferrin receptor, Mm00441941_m1), and *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase, Mm99999915_g1). Robust and reliable qRT-PCR data was obtained using the following primer/probe sets from Applied Biosystems' TaqMan® Arrays: *Myf5* (myogenic factor 5, Myf5-Mm00435125_m1), *Fgf2* (fibroblast growth factor 2, Fgf2-Mm00433287_m1), *Myod1* (myogenic differentiation 1, Myod1-Mm00440387_m1), *Pax-7* (paired box gene 7, Pax7-Mm00834079_m1), *Mef2B* (myocyte enhancer factor 2B, Mef2B-Mm00488969_m1), *Myogenin* (Myog-Mm00446194_m1), Myostatin (growth differentiation factor 8, Gdf8-Mm00440328_m1), *C-met* (met proto-oncogene, Met-Mm00434924_m1), *Fgfr1* (fibroblast growth factor receptor 1, Fgfr1-Mm00438923_m1), *Cdkn1b* (cyclin-dependent kinase inhibitor 1B, Cdkn1b-Mm00438168_m1), *Id1* (inhibitor of DNA binding 1, Id1-Mm00775963_g1) and normalizing against the geometric mean of multiple stably expressed endogenous control genes, *Beta-actin* (actin, beta, Actb-Mm00607939_s1), *Gusb* (glucuronidase, beta, Gusb-Mm00446953_m1), *Hprt1* (hypoxanthine guanine phosphoribosyl transferase 1, Hprt1-Mm00446968_m1), and *Tbp* (TATA box binding protein, Tbp-Mm00446973_m1) (Applied Biosystems, Foster City, CA, USA) using the qBase platform (Vandesompele et al., 2002; Hellemans et al., 2007; Andersen et al., 2008).

Immunofluorescence and immunohistochemistry

Immunofluorescence and immunohistochemistry were performed as previously described (Andersen et al., 2008, 2009) using the primary antibodies; mouse-α-myogenin clone F5D, (DAKO, Glostrup, Denmark) at 1:50,mouse-α-fast myosin clone MY32 1:8000 (Sigma, Sant Louis, Missouri, USA), mouse-α-myoD 1:20 (Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK), mouse-α-Pax7 (Developmental Studies Hybridoma Bank, Iowa, USA) at 1:50, rat-α-SPARC 1:1000 (MAB942, clone 124413, R&D systems), rabbit-α-FGFR1 1:50 (GeneTex, San Antonio, USA) and mouse-α-desmin (DAKO, Glostrup, Denmark) at 1:25. For immunohistochemistry, antigen retrieval was performed by heating in TEG (10 mM Tris, 0.5 mM EGTA, pH 9.0) and antigen-antibody complex visualized by Envision+ (DAKO, Glostrup, Denmark) in combination with PowerVision (ImmunoVision Technologies, Co).

Western Blot

Total protein was extracted from the harvested pellets and samples were added 6x loading buffer (DTT, 0.5 M Tris Base (pH 6.8), SDS, glycerol, Bromphenol blue), heated to 95°C for 5 min and then loaded on a 12% Bis-Tris gel. The gel was run using running-buffer (Tris-base, Glycine, SDS, and H_2O) for 2 h at 130 V constant and transferred to a PVDF membrane (0.45 um pore size) using electro-blotting for 30 min. at 0.1 mAmp constant. The membrane was blocked for 1 h at room temperature with 5% skimmed milk/TBS-T and incubated with primary antibody overnight at 4°C, shaking rat-anti-SPARC 1:1000 (R&D systems) or ß-Actin (cat. no. 4967S, Cell signaling Technology (1:1000)). The following day blots were washed using TBS-T and incubated with goat-anti-rat-HRP or goat-anti-rabbit-HRP (Lifetechnologies) 1:5000 for 1 h at room temperature and developed using Enhanced Chemiluminescence.

Statistical analysis

For all experiments 2-4 independent experiments were performed each comprising 3 technical replicates. Statistical analysis was performed using a Two-Way factorial ANOVA analysis with Bonferroni post-testing.

Results

SPARC expression coincides with myogenic transcription factors during skeletal muscle regeneration

We have previously found that mouse skeletal muscle regeneration following a knife-cut lesion is characterized by different phases, including inflammation/degeneration, myoblasts activation/proliferation as well as a stage of differentiation (Andersen et al., 2009). At present, relative quantitative qRT-PCR revealed that *SPARC* mRNA levels started to increase at day 2 post injury, peaking ~4-fold in level at day 5, and declined thereafter reaching control levels at day 8-14 (Fig. 1A). The expression profile of *SPARC* thus more or less mirrored our recent results on myogenic regulatory factors (Andersen et al., 2009), such as *MyoD, Myf5, Myf6, Myogenin, NCAM, CD34,* and *M-Cadherin*, which are known to be implicated in satellite cell activation and proliferation (Molkentin and Olson, 1996a,b; Seale and Rudnicki, 2000).

Immunohistochemistry confirmed the pattern of SPARC expression at the protein level during regeneration (Fig. 1B). In uninjured muscle, SPARC protein was observed in a few cells located in the endomysium, whereas muscle lesion induced SPARC protein to be evident from day 3-14 in both regenerating

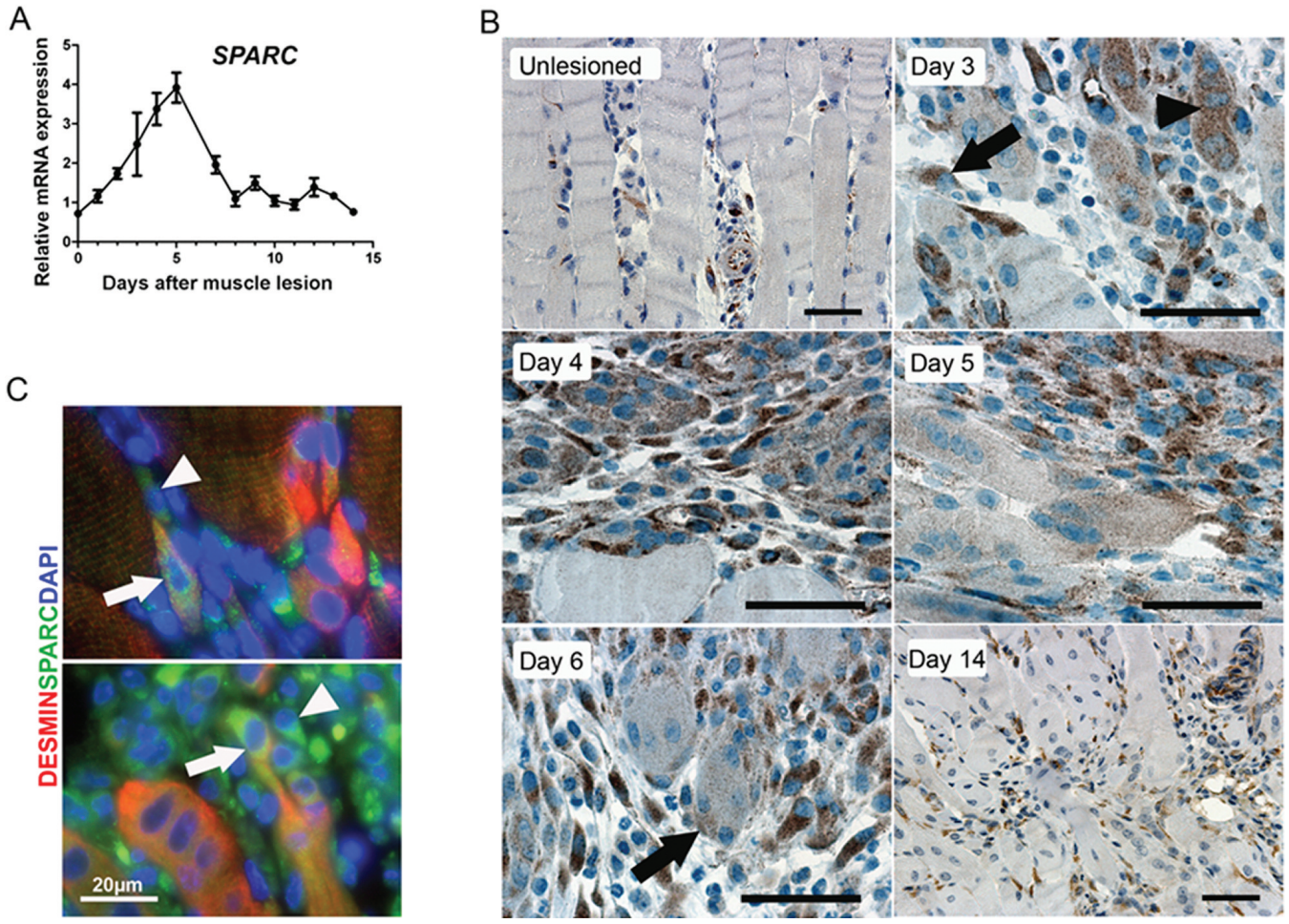

Fig. 1. SPARC mRNA and protein expression is regulated during muscle regeneration. **A.** Foldexpression (qPCR) of *SPARC* mRNA to uninjured muscle (calibrator set to 1). Error bars represent 1 SD. During early regeneration myogenic progenitor cells are activated and start to proliferate. This regenerative phase is characterized by a prominent remodelling of the ECM early in the repair processes. A previous study (Goetsch et al., 2003) showed increased ECM/cell adhesion-associated gene expression in mouse muscles on day 5 post cardiotoxin-induced injury. This is in accordance with our findings of *SPARC* mRNA expression. The timing of increased *SPARC* expression corresponds to the peak for reorganization of the ECM and differentiation of the myogenic progenitor cells (Charge and Rudnicki, 2004). **B.** Immunohistochemistry showing the development in SPARC protein expression from day 0 to day 14 during skeletal muscle regeneration. SPARC is expressed by mononuclear cells (black arrows) and myotubes (black arrow-head) with most prominent expression at day 3-14 confirming the mRNA expression pattern in the regenerative phase and suggesting a role for SPARC during skeletal muscle repair. **C.** Immunofluorescense showing SPARC with (white arrows) and without (white arrow-heads) co-localisation with desmin. Scale bar: B, 50 μm; C, 20 μm

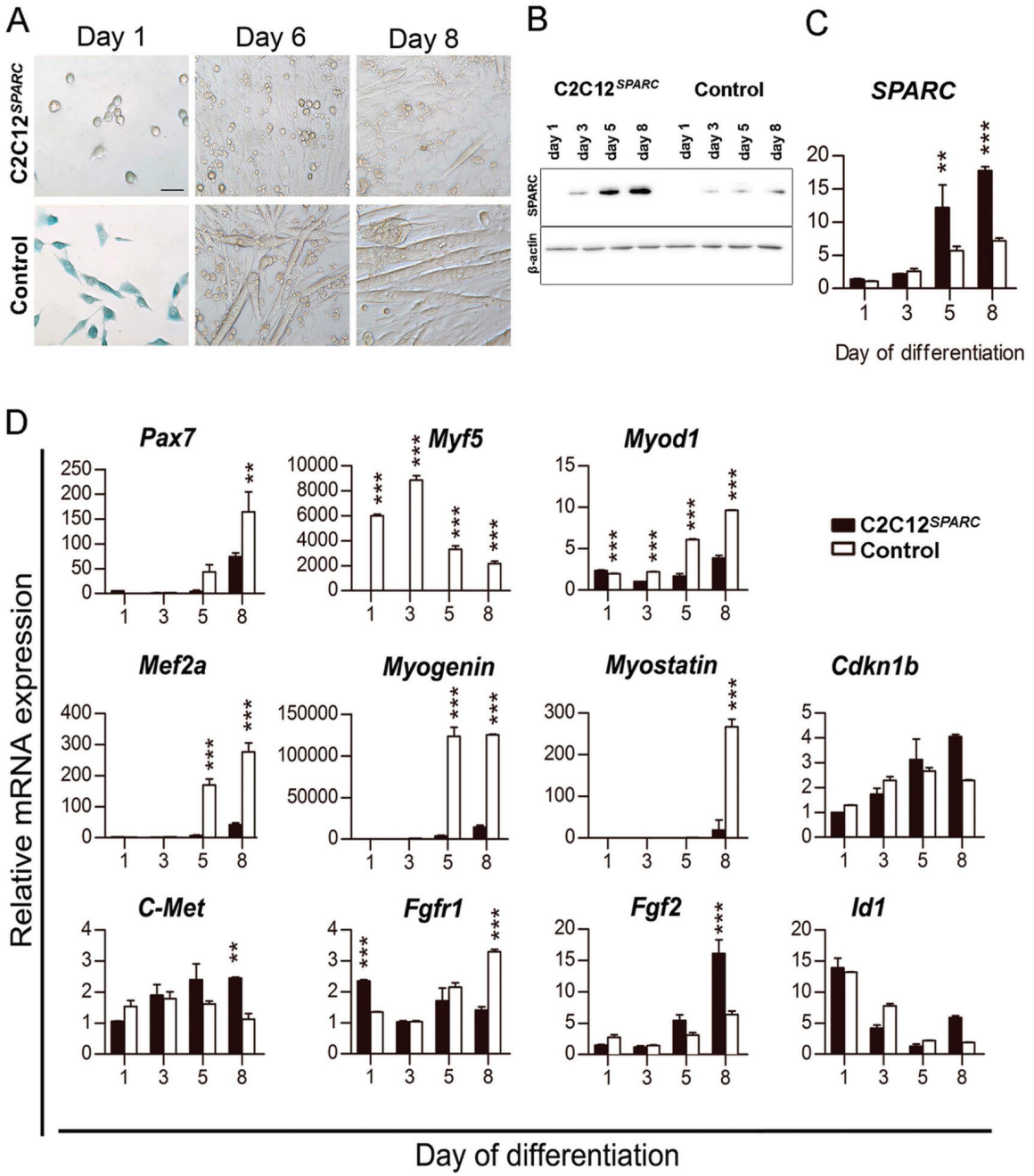

Fig. 2. Ectopic expression of SPARC alters the phenotype of C2C12 cells. **A.** Phase-contrast microscopy of C2C12^{SPARC} and control cells in culture in the proliferative state and during differentiation. ß-galactosidase assay of SPARC-transfected clone in the proliferative state showing loss of activity compared to the control cells. This was a measure for successful incorporation of the SPARC expressing construct, since correct incorporation disrupted the lacZ locus inserted in the C2C12^{SPARC} cells. The C2C12^{SPARC} cells appear smaller and more rounded in shape than the control cells. During differentiation, the C2C12SPARC cells maintain a high proliferative potential and do not form myotubes to the same extent as the controls as observed at day 6 and 8 of in vitro differentiation. C2C12^{SPARC} cells are shown in the upper panel and control cells in the lower panel. **B.** Representative Western blot of SPARC during differentiation of C2C12^{SPARC} and control cells showing up regulation of SPARC in C2C12^{SPARC} compared to controls. SPARC expression at day 1 was too low to be detected in both C2C12^{SPARC} and control cells when loading an amount of 10 μg. **C.** The mRNA expression of *SPARC* increased throughout the differentiation study in both C2C12^{SPARC} and control cells with a significantly higher level overall in the C2C12^{SPARC} cells (p=0.0001). **, p<0.01; ***, p<0.001. **D.** The expression of the myogenic genes (*Pax7 Myf5, MyoD, Mef2*α*, Myogenin,* and *Myostatin*) were significantly down-regulated in the C2C12^{SPARC} cells compared to the control cells. *C-Met* however, was significantly up-regulated in the C2C12^{SPARC} cells compared to a more stabilized level in the control cells. The relative level of *Fgf2* was significantly up-regulated at day 5-8 in the C2C12^{SPARC} cells and this could result in the observed suppression or stabilization of its receptor *Fgfr1*. B-C. Filled dark bars represent C2C12^{SPARC} and white bars represent control cells. The data-sets are normalized to four reference genes; *Actb, Gusb, Hprt1,* and *Tbp*, and the lowest expression level set to one. The four reference genes had an average expression stability M=0.4097 and coefficient of variance CV=17.45% (Hellemans et al., 2007). The graphs show means of the two data-sets and error bars represent 1 SD. Statistical analysis was performed by ANOVA with Bonferroni posttest. **, p<0.01; ***, p<0.001. Scale bar: 50 μm.

myofibers as well as mononuclear cells and vessel walls within the lesion area (Fig. 1B). Additionally, we observed numerous mononuclear SPARC positive cells in the muscle fascia and connective tissue (data not shown), which is in agreement with its known implication in fibrosis (Frizell et al., 1995; Kos et al., 2009; Kos and Wilding, 2010) as well. Immunofluorescence double staining verified that SPARC was expressed in desmin-positive muscle cells, but also in some non-myogenic, desmin-negative cells (Fig. 1C).

This temporary expression profile of SPARC may suggest this molecule to be implicated in the proliferation and differentiation commitment of myogenic cells during muscle regeneration.

Ectopic expression of SPARC compromises differentiation of myoblasts

We next aimed to mimic the increased SPARC levels seen in muscle disease (Jorgensen et al., 2009), and extend the published reports on beneficial effects on myoblast differentiation (Cho et al., 2000; Motamed et al., 2003) by over expressing SPARC in C2C12 myoblasts. C2C12 cells, having inserted full-length mouse SPARC into the genome (C2C12^{SPARC}), were morphologically smaller and more round in shape as compared to control cells (Fig. 2A). SPARC is expressed in C2C12 myoblasts (Cho et al., 2000) and we found protein (Fig. 2B) and mRNA (Fig. 2C, p=0.001) levels increased upon differentiation like in human myoblasts. A 2-3 fold increased *SPARC* mRNA level was detected in differentiating C2C12^{SPARC} cells as compared to control cells (Fig. 2C), which together with the up regulation of *SPARC* protein during differentiation of the SPARC-transfected cells (Fig. 2B) confirmed our SPARC overexpression setup. Furthermore, there seemed to be a delayed differentiation of C2C12^{SPARC} myoblasts into myotubes (Fig. 2A) as compared to control cells. To further characterize this inhibition of myogenic differentiation, we isolated RNA from C2C12^{SPARC} and control cells at day 1, 3, 5, 8 of differentiation, and performed relative qRT-PCR of genes known to be specific for the myogenic cell lineage (Fig. 2D). Significantly, lower mRNA levels of the early

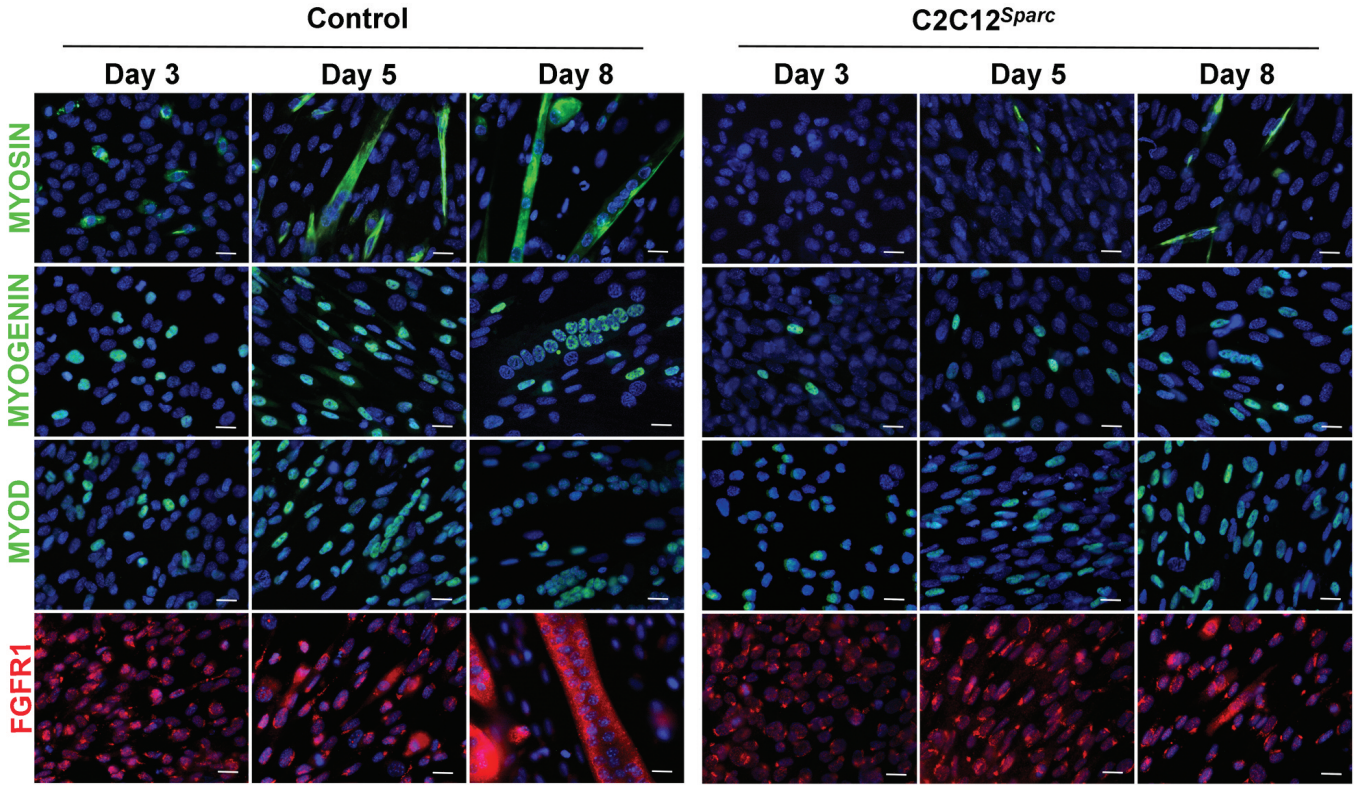

Fig. 3. Downregulation of expression of myogenic proteins during differentiation of C2C12^{SPARC} cells. C2C12^{SPARC} and control cells were harvested during a differentiation assay and the myogenic potential assessed. As previously noticed there is a decreased formation of myotubes in the C2C12^{SPARC} cell cultures and the myotubes that form are not as multi-nucleated as observed for the control cells. The expression of FAST MYOSIN appears late around day 5-8 in the C2C12^{SPARC} cells compared to the early appearance at day 3 in the control cells. A considerably lower level of expression of the two myogenic proteins MYOGENIN and MYOD is also observed, reflecting either a delayed myogenic response or an alternative mesenchymal fate. There is a consistent expression of the fibroblast growth factor receptor 1 (FGFR1) from day 1 (data not shown) and throughout the differentiation assay in both C2C12^{SPARC} and control cells. Scale bars: 25 μm.

satellite/myoblast markers *Pax7* (p=0.0036), *Myf5* (p<0.0001), *Myod* (p<0.0001), and *Mef2a* (p<0.0001) were found in C2C12^{SPARC} versus control cells, as were the level of the later differentiation markers *Myogenin* (p<0.0001) and *Myostatin* (p<0.0001). Of note, the level of *Myf5, Myogenin*, and *Myostatin* were almost completely abolished in C2C12^{SPARC} cells, whereas no significant difference before day 8 was observed in the level of *C-met* (p=0.0088 at day 8) (Fig. 2D), a growth factor receptor gene known to be expressed in myogenic as well as many other types of cells (Vigna et al., 1994; Cornelison and Wold, 1997; Ma et al., 2003). To exclude a possible toxicity effect in the C2C12^{SPARC} we measured the transcript levels of cell cycle parameter *cdkn1b* and the DNA-binding protein *Id1* and detected no difference between the C2C12^{SPARC} and control cells (Fig. 2D). To verify that myogenic factors were down-regulated in C2C12^{SPARC} cells, we next performed immunofluorescence of C2C12^{SPARC} and control cells at day 1, 3, 5, 8 of differentiation (Fig. 3). Accordingly, the late myogenic marker MYOSIN was found to be expressed in mononuclear cells already at day 3 of differentiation, as well as in numerous prominent multinucleated myotubes at day 5-8 in control cells, whereas only a small number of small MYOSIN-positive myotubes with a few nuclei were observed in C2C12^{SPARC} cells at day 5 and 8 of differentiation (Fig. 3). Likewise, we found a clear delay and apparent reduction in the appearance of MYOGENIN- and MYOD-positive nuclei in the C2C12^{SPARC} cells, confirming that increased level of SPARC inhibits myogenic differentiation.

SPARC has previously been shown to be implicated in FGFR1 mediated FGF2 signaling (Motamed et al., 2003). FGF2 is a mitogenic factor that is known be a strong inhibitor of myogenic differentiation (Dollenmeier et al., 1981). We therefore examined the mRNA level of Fgfr1 and Fgf2 in C2C12^{SPARC} as well as control cells during myogenic differentiation. Two-three fold higher levels of Fgf2 were found in C2C12^{SPARC} as compared with control cells at day 8 of differentiation, whereas C2C12^{SPARC} exhibited 2-3 fold less Fgfr1 mRNA (Fig. 2D). Immunofluorescence for FGFR1 verified its high expression in differentiated control myofibers at the protein level (Fig. 3). These findings indicate that FGF modulation might be a factor in the SPARC induced changed myogenic capacity of the C2C12^{SPARC} cells.

Discussion

In our previous study on human, myopathic muscle (Jorgensen et al., 2009), we found that expression of SPARC reflected the degree of muscle injury and severity of muscle disease. In the present mouse study we show that SPARC expression is induced when the muscle is damaged using a knife stab wounding method. Moreover, SPARC exhibits a temporal pattern of expression that peaks around day 5, indicating a possible relation to proliferation and transition to differentiation. In accordance with this a previous study showed that addition of soluble SPARC to cultures of the myogenic mouse MM14 cell line promoted muscle differentiation (Motamed et al., 2003). Moreover, treatment of muscle cultures with anti-SPARC antibodies has been suggested to inhibit muscle differentiation from C2C12 myoblasts (Cho et al., 2000).

The temporal pattern indicates that SPARC production undergoes regulation. Since many sources of SPARC exist in the microenvironment, a complex, dynamic mechanism could be active during skeletal muscle regeneration. Specifically, SPARC could have a non cell-autonomous effect on immune cells, but also on other cell types in the regenerative area, since SPARC is known to bind to the stabilin-1 scavenger receptor on both macrophages and endothelial cells (Workman and Sage, 2011; David et al., 2012). Furthermore, SPARC has been shown to exhibit a dual role in relation to regeneration of induced pulmonary damage, where the effect of SPARC on inflammation and fibrosis was dependent on the cell type expressing SPARC (Sangaletti et al., 2011).

Lack of SPARC apparently does not affect formation of muscle, as SPARC null mice do form muscle (Gilmour et al., 1998). We therefore focused our studies on over expression of SPARC and the direct effect on isolated myoblasts. Doubling the gene expression in C2C12 myoblasts resulted both in delay and reduction of myotube formation and a severe inhibition of myogenic gene activity. This indicates that SPARC production in the different compartments of a muscle lesion must be balanced so that SPARC is kept below a certain level. It is possible that the exact level and timing of SPARC is obligatory for its ability to retain normal muscle function.

Changes in SPARC expression seen during muscle disease may therefore have a large impact on muscle regeneration. Local aggregations of lesions might result in high SPARC levels inhibiting differentiation. The loss of myogenic gene expression is also of interest in connection with a study (Shefer et al., 2004) reporting that skeletal myoblasts can spontaneously enter an alternative mesenchymal pathway, resulting in adipocytes and fibroblasts with reduced numbers of muscle cells as a consequence. SPARC has recently been suggested to mediate the disassembly and degradation of ECM networks by functioning as a matricellular chaperone (Chlenski et al., 2011). Furthermore, SPARC has both been implicated in adipogenesis as well as fibrosis, possibly through ECM regulation (Lane and Sage, 1994; Blazejewski et al., 1997; Motamed, 1999; Alford and Hankenson, 2006; Nie and Sage, 2009ab; Song et al., 2010). We therefore speculate whether abnormal, high levels of SPARC in muscle disease may reduce the regenerative capacity of satellite cells and direct them into these alternative differentiation pathways, leading to incomplete muscle repair, and to increased fibrosis and formation of adipose tissue seen in several muscle diseases (Manzur et al., 2008; Russell,

2010). However, this needs further investigation.

The mechanism by which SPARC functions in skeletal muscle remains to be elucidated.

There is an overall agreement that SPARC is necessary for correct extracellular matrix formation, which is supported by the observation that SPARC-null mice have slack skin and smaller collagen fibrils (Bradshaw et al., 2003). Our study showed that SPARC overexpressing cells were more round than control cells, which correlates with previous studies (Porter et al., 1995; Mothe and Brown, 2001), and may be explained by SPARC facilitating changes in muscle cell shape and diminishing the number of focal adhesions as suggested by others (Murphy-Ullrich et al., 1995; Porter et al., 1995). A previous study (Motamed et al., 2003) has suggested that SPARC promotes skeletal myoblast differentiation through interference with the autophosphorylation of FGFR-1. Our results additionally indicate that over expression of SPARC down regulates *Fgfr-1* gene expression, and induces a massive mRNA up regulation of the FGFR-1 ligand fibroblast growth factor 2 (*Fgf2*). FGF2 is known to stimulate myoblast proliferation and repress differentiation at the same time (Motamed et al., 2003). Whether a high level of SPARC directly interferes with the expression of both *Fgfr1* and *Fgf2*, disturbing the balance in this system, and thereby inhibiting myogenic differentiation, or alternatively that *Fgf2* expression increases as a compensatory mechanism due to low *Fgfr1* signaling, remains to be determined.

Our study is the first to demonstrate the inhibitory effect of high levels of SPARC directly on muscle precursors and their differentiation into myofibres, and this, together with our demonstration of a distinct temporal and topographical expression pattern, indicates that SPARC expression level during muscle repair is subject to close regulation.

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