

# Stem cell antigen-1 is necessary for cell-cycle withdrawal and myoblast differentiation in C2C12 cells

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Accepted 21 September 2004

Journal of Cell Science 117, 6185-6195 Published by The Company of Biologists 2004

doi:10.1242/jcs.01548

## Summary

Extracellular signaling pathways regulating myoblast differentiation and cell-cycle withdrawal are not completely understood. Stem cell antigen-1 (Sca-1/Ly-6A/E) is a glycosylphosphatidylinositol-anchored membrane protein known for its role in T-cell activation, and recently described as a marker for regeneration-competent myoblasts. We previously determined that expression of Sca-1/Ly-6A is transiently upregulated during myocyte cell-cycle withdrawal; however, a specific function for Sca-1 in myogenesis has not been described. Here, we show that Sca-1 expression on the surface of a subpopulation of differentiating C2C12 myoblasts is maximal at the time of cell-cycle withdrawal, and that blocking Sca-1 with monoclonal antibodies or downregulating Sca-1 expression by antisense both promotes proliferation and inhibits myotube formation.

Downregulating Sca-1 expression derepresses Fyn at the time of myoblast cell-cycle withdrawal, and dominant-negative and constitutively active Fyn mutants rescue and recapitulate the Sca-1 antisense phenotype, respectively. This suggests a Fyn-mediated mechanism for Sca-1 action. Thus, we demonstrate an unprecedented role for Sca-1 in early myogenesis in C2C12 cells, and propose a novel pathway from the myoblast cell surface to intracellular signaling networks controlling proliferation versus differentiation in mammalian muscle. These findings suggest that, beyond its role as a marker for muscle progenitors, Sca-1 may be an important therapeutic target for promoting muscle regeneration.

Key words: Sca-1, Myoblast fusion, Cell-cycle withdrawal, Ly-6, GPI-anchored proteins, Muscle regeneration

## Introduction

Myogenesis, the process of muscle cell determination, differentiation and fusion into multinucleated syncytia, is essential for normal muscle development and tissue regeneration following injury. Much is known about the transcriptional regulation of myogenesis. The myogenic regulatory factors MyoD, Myf-5, myogenin and MRF4 act to initiate and maintain the differentiated state (Puri and Sartorelli, 2000). Additionally, they inhibit cell-cycle progression by upregulating expression of the retinoblastoma protein and members of the Cip1/Kip1 family of cyclin-dependent kinase inhibitors, and downregulating the expression of the G1 effectors cyclin D1 and cyclin-dependent kinase 4 (Walsh and Perlman, 1997).

Although signaling events during myocyte differentiation have been studied in detail, cell-surface molecules that transmit signals from the extracellular milieu to these intracellular pathways are less well understood. Recent genetic studies in *Drosophila* have provided the first insights into mechanisms governing myoblast fusion. The Ig-like proteins Dumbfounded and Sticks and Stones support cellular adherence, whereas the membrane- and cytoskeletal-associated proteins Myoblast City and Rolling Stone facilitate formation of the fusion complex, which also requires cytoplasmic signaling proteins Rolling Pebbles and Antisocial (Taylor, 2002).

Other cell-surface molecules have been linked to muscle differentiation in vertebrates. Antibody blockade of ligand binding to  $\beta 1$  integrins in chick embryos results in a predominance of early myogenic precursors (Menko and Boettiger, 1987), and whereas  $\beta 1$  integrin-null myoblasts have been shown to be capable of fusion (Hirsch et al., 1998), mice lacking  $\beta 1$  integrin specifically in muscle have poorly developed muscle fibers (Schwander et al., 2003). By contrast, murine embryonic stem cells deficient in  $\alpha 4$  integrin can be differentiated into myoblasts that fuse to form myotubes (Yang et al., 1996). Antibody interference of N-cadherin during early chick embryogenesis disrupts formation of early myogenic precursors (George-Weinstein et al., 1997), and antibodies against N-cadherin inhibit cellular adherence in cultured chick myoblasts (Knudsen et al., 1990); however, N-cadherin-null embryonic stem cells can differentiate into myoblasts with normal fusion properties (Charlton et al., 1997).

During a screen for regulators of myoblast differentiation, we determined that expression of stem cell antigen-1 (Sca-1/Ly-6A/E), a stem cell marker, was transiently upregulated during myoblast cell-cycle withdrawal (Shen et al., 2003). Sca-1 is a member of the Ly-6 multigene family encoding several highly homologous, glycosylphosphatidylinositol (GPI)-anchored membrane proteins that are expressed on hematopoietic and lymphoid cells. The best characterized

family member is Sca-1/Ly-6A, an antigen commonly used for purification of murine pluripotent hematopoietic cells (Patterson et al., 2000). Although the ligand for Sca-1 is not known, studies have implicated Sca-1 and related family members in cell-cell adhesion among thymocytes (Bamezai and Rock, 1995; Classon and Boyd, 1998). Interestingly, ligands for other superfamily members contain epidermal growth factor (EGF)-like repeats, and a ligand for Ly-6D demonstrates significant homology to the Notch family responsible for somatic patterning in *Drosophila* (Aposolopoulos et al., 2000). More recently, Sca-1 has been reported as a marker for muscle-derived stem cells (Asakura et al., 2002; Jankowski et al., 2001), and Sca-1-positive myogenic precursor cells appear to be recruited from the circulation to sites of myocardial injury (Oh et al., 2003). However, a specific function for the Sca-1 antigen in muscle development has not been described.

We now report a previously unrecognized role for Sca-1 in myoblast differentiation and proliferation during myogenesis in C2C12 cells. Inhibition of Sca-1 expression using antisense or interference with Sca-1 activity using blocking antibodies prevent myoblast differentiation, causing sustained myoblast proliferation and producing a defect in myotube formation. Inhibition of Sca-1 expression also causes the derepression of Fyn kinase, and Fyn mutants recapitulate the phenotype of Sca-1 antisense-expressing cells. These results suggest both a mechanism for Sca-1 action and a novel pathway from the extracellular environment to myoblast differentiation.

## Materials and Methods

### Cell culture

Actively growing C2C12, Sol8 and L6 skeletal myoblasts (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle's medium with 10%, 20% or 10% fetal bovine serum (FBS), respectively. QM7 quail skeletal myoblasts, maintained in M199 medium with 10% tryptose phosphate and 10% FBS, were the generous gift of C. Ordahl (UCSF). Differentiation was induced by culture of confluent monolayers in Dulbecco's Modified Eagle's medium with 1% FBS for C2C12 and Sol8 cells, 2% horse serum for L6 cells, or M199 medium with 10% tryptase phosphate and 0.5% FBS for QM7 cells. For phosphatidylinositol-specific phospholipase C (PIPLC) treatment, 5 U/ml of PIPLC (Sigma) was added into the culture medium when cells reached confluence, and with daily media changes. For antibody treatments, a total of 5 µg/ml monoclonal anti-Sca-1 (i.e. 5 µg/ml of either clone E13-161.7 or D7 alone, or 2.5 µg/ml of each clone in combination) or anti-CD34 were added to medium when cells reached confluence, and with medium changes. Monoclonal rat anti-Sca-1 antibodies, clones E13-161.7 and D7, were obtained from BD Pharmingen (553333; 557403). Monoclonal rat anti-CD34 antibody was obtained from Santa Cruz Biotechnology (sc-18917). All three rat monoclonal antibodies were isotype IgG2a.

### Primary myoblast isolation

Primary skeletal myoblasts were isolated according to standard procedures (Blanco-Bose et al., 2001). Skeletal muscle harvested from the hindlimbs of 10-20 C57BL/6 neonatal (3-day-old) mice was minced in cold, sterile phosphate-buffered saline (PBS), digested with 1.5 U/ml collagenase D, 2.4 U/ml dispase II, 2.5 mM CaCl<sub>2</sub> at 37°C, filtered through 40 µm nylon mesh, centrifuged, resuspended in 80% Ham's F-10 nutrient mixture, 20% FBS, 2.5 ng/ml basic fibroblast growth factor (bFGF), and plated on laminin-coated dishes. After 24 hours, cells were harvested with trypsin-EDTA, incubated with

monoclonal rat anti- $\alpha$ 7 integrin antibody (clone CY8; gift of R. Kramer, UCSF) (Yao et al., 1996), selected at least 5 times with MACS goat anti-rat IgG MicroBeads on an autoMACS magnetic cell sorter (Miltenyi Biotec), and re-plated in 40% Ham's F-10 nutrient mixture, 40% Dulbecco's Modified Eagle's media, 20% FBS, 2.5 ng/ml bFGF (proliferation medium) on collagen-coated dishes. Purity was analyzed by flow cytometric detection of  $\alpha$ 7 integrin, using monoclonal rat anti- $\alpha$ 7 integrin antibody and phycoerythrin (PE)-conjugated donkey anti-rat F(ab')<sub>2</sub> fragment (Jackson ImmunoResearch). Sorted cells were grown in proliferation medium for 5 days, and then transferred to differentiation medium (80% Dulbecco's Modified Eagle's media, 2% horse serum) to stimulate myotube formation.

### Flow cytometric analysis

C2C12 cells and primary skeletal myoblasts were detached from culture with 0.25% trypsin, 2 mM EDTA at indicated times. Cells were washed with PBS, 10% FBS and fixed with 0.4% paraformaldehyde for 30 minutes at room temperature. After fixation, cells were washed with PBS, 2% calf serum (wash buffer) and then incubated in 100 µl wash buffer, 1% goat serum, 0.1% Triton X-100 containing primary antibodies at 4°C overnight. Cells were then washed with wash buffer and incubated in 100 µl wash buffer, 2 µg/ml DNAase-free RNase A (Sigma) containing secondary antibodies at 37°C for 1 hour. Prior to flow cytometric analysis, cells were washed with wash buffer, filtered through 40 µm nylon mesh to exclude cell clumps and large myotubes, and incubated with 5 µg/ml propidium iodide (PI) at room temperature for 30 minutes. Primary/secondary antibody combinations used included monoclonal rat anti-Sca-1 (clone E13-161.7; BD Pharmingen) with PE-conjugated donkey anti-rat F(ab')<sub>2</sub> fragment (Jackson ImmunoResearch), and monoclonal mouse anti-desmin (DAKO), anti-myosin (MF20; University of Iowa Developmental Studies Hybridoma Bank), or anti-MyoD (BD Pharmingen) with FITC-conjugated goat anti-mouse IgG (Sigma).

A Becton Dickinson FACSCalibur with 488 nm argon-ion laser was used to acquire PI, FITC and PE signals with 630/22 mm, 530/30 mm and 575/20 mm bandpass filters, respectively. Forward and side angle light scatter plots (as indicators of cell size and granularity), and PI, FITC and PE signals (to detect DNA content, desmin, myosin or MyoD, and Sca-1, respectively), were collected for at least 10,000 cells. PI area versus width plots were used to verify that our gating strategy included cell populations ranging from mononuclear cells with 2N DNA content to small, tetraploid myotubes containing 8N DNA content (Sharpless et al., 1975) that expressed MyoD and myosin. Data were analyzed using CellQuest software (Becton Dickinson).

### Generation of stable antisense Sca-1 transfectants of C2C12 cells

A 330 bp fragment encoding nucleotides -9 to +321 of the Sca-1 coding sequence (Stanford et al., 1992), and flanked by *Eco*RI sites, was cloned by PCR from total mouse cDNA, inserted into the *Eco*RI site of vector pcDNA3.1A (Invitrogen), and screened for antisense orientation. The newly generated pLy6AS or empty pcDNA3.1A were transfected into C2C12 cells with Lipofectamine Plus reagent (Invitrogen) as instructed by the manufacturer. After 10 days of incubation in medium containing 800 µg/ml G418 (Life Technologies), resistant clones numbered 1-8 were isolated, expanded and screened for attenuation of Sca-1 expression by immunostaining and immunoblot, as described below. Stable cell lines were maintained in culture medium with 500 µg/ml G418. Expression levels of Sca-1 in clones 1-8 were measured by flow cytometry as described below. Experimental results were verified in multiple clones to ensure that observations were not the result of clonal variation inherent in C2C12 myoblast lines. Typical results for clone 6, which

suppressed Sca-1 expression by 75% at two days in differentiation medium, are shown.

#### Immunostaining and immunoblot analysis

To detect Sca-1, Myf5, MyoD and myogenin in situ, wild-type and stably transfected C2C12 cells were plated on cover slips, grown as described for the indicated lengths of time, and stained with 2.5 µg/ml monoclonal anti-Sca-1 antibody (clone E13-161.7; Pharmingen) and 4 µg/ml FITC-conjugated goat anti-rat IgG (Pharmingen), or 10 µg/ml rabbit polyclonal anti-Myf5, anti-MyoD or anti-myogenin antibody (Santa Cruz Biotechnology) and 2 µg/ml FITC-conjugated goat anti-rabbit IgG (Molecular Probes), as previously described (Liu et al., 2003).

Immunoblot analysis was performed using previously described methods (Hlaing et al., 2002). Briefly, cells were lysed by sonication in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1% SDS, 10% glycerol), boiled for 5 minutes, and centrifuged at 16,000 *g* for 2 minutes. Protein concentrations in supernatants were measured by BCA assay (Pierce), and equal amounts of protein (100–200 µg) were separated by SDS-polyacrylamide gel electrophoresis. Immunoblots were analyzed with 5 µg/ml monoclonal antibody against Sca-1 (Pharmingen) or β-actin (Sigma), and 1 µg/ml horseradish peroxidase (HRP)-conjugated goat anti-mouse (Bio-Rad) IgG.

#### BrdU incorporation and myoblast fusion assays

Cells were plated on cover slips, cultured under conditions described above, and labeled with 5-bromo-2-deoxyuridine (BrdU) for 60 minutes using Labeling and Detection Kit I (Roche) according to the manufacturer's instructions. Cells were stained with 1:10 dilution of monoclonal anti-BrdU (Roche), followed by 4 µg/ml Alexa Fluor594-conjugated goat anti-mouse IgG in incubation buffer provided with the Labeling and Detection Kit I (Roche). Cells were co-stained with antibodies against Myf5, MyoD or myogenin as described above. Prior to mounting cover slips on slides, cells were incubated with 1 µg/ml DAPI (Sigma) for 5 minutes at room temperature. Immunofluorescence signals were acquired with a Nikon Microphot-FX fluorescence microscope and Spot imaging software. At least 1000 nuclei and 20 myotubes were counted from several random fields. Cell proliferation was calculated as percent BrdU-positive nuclei per field. Fusion index was calculated as number of nuclei in myocytes containing ≥2 nuclei/total number of nuclei. All experiments were performed at least three times with independent cultures, and quantification values are reported as the mean ± standard error of the mean (s.e.m.).

#### Fyn and Src kinase assays

Cells were cultured under conditions described above, lysed in lysis buffer [25 mM Tris, pH 7.2, 150 mM NaCl, 100 µM Na<sub>3</sub>VO<sub>4</sub>, 1% NP-40, 10 mM NaF, 1:1000 Protease inhibitor cocktail (Sigma)] for 30 minutes on ice, sheared by needle passage, then centrifuged at 11,750 *g* for 6 minutes at 4°C. Total supernatant protein was determined by BCA assay (Pierce). One mg total protein was incubated with 2 µg monoclonal anti-Fyn or anti-Src antibody (Santa Cruz Biotechnology) for 2 hours at 4°C, and 50 µl protein-A sepharose (Sigma) for an additional 30 minutes. Immune complexes were washed with ice-cold lysis buffer, then re-suspended in 40 µl ice-cold kinase reaction buffer (100 mM Tris, pH 7.2, 125 mM MgCl<sub>2</sub>, 25 mM MnCl<sub>2</sub>, 2 mM EGTA, 25 µM Na<sub>3</sub>VO<sub>4</sub>, 2 mM DTT) with 20 µM ATP and 4 µM [ $\gamma$ -<sup>32</sup>P]ATP (6000 mCi/mmol). Reactions were incubated with 150 µM substrate peptide [(K19) cdc2 (6-20)-NH<sub>2</sub>, KVEKIGEGTYGVVKK; Upstate] for 10 minutes at 30°C with vigorous agitation. Reactions were stopped on ice and proteins precipitated with 20 µl 40% trichloroacetic acid. Precipitates were

collected on phosphocellulose squares (Upstate), washed with 0.75% phosphoric acid then acetone, and dried prior to measuring incorporated radioactivity by Cerenkov counting. Counts were normalized against immunoprecipitated Fyn or Src protein determined by immunoblot with monoclonal anti-Fyn or anti-Src antibody and densitometry (Adobe Photoshop and NIH/Object-Image), and expressed as fold-increase over background (immunoprecipitation performed without primary anti-Fyn or anti-Src antibody). Experiments were performed at least three times and quantification values are reported as the mean ± s.e.m.

#### Fyn mutant assays

A 1615 bp fragment of pCMV-FynDN (Ko et al., 2002), containing the full-length, dominant-negative K299M mutant of human Fyn, was amplified by PCR using primers 5'-TTATGCGGCCGCATGGGCTGTGTGCAATGTAAGG-3' and 5'-GCATGCATGCTTACAGGTTTTCACCAGGTTGG-3' and cloned into *NotI/SphI* sites in pIRES-hrGFP-1a (Stratagene). pCMV-FynDN was the kind gift of M. Resh (Sloan-Kettering Cancer Center). The 1.6 kb *BamHI* fragment of Fyn-Y531F (Maulon et al., 2001), containing the full-length, constitutively active Y531F mutant of mouse Fyn, was cloned into the *BamHI* site in pIRES-hrGFP-1a (Stratagene). Fyn-Y531F was generously provided by P. Auberger (INSERM). Sequences for both plasmids expressing Fyn mutants and green fluorescent protein (GFP) were confirmed. Expression of mutants and GFP was determined by immunoblot of whole cell lysates from transfected C2C12 cells with anti-Fyn antibody (Santa Cruz Biotechnology), and immunofluorescence detection of GFP in live cells.

Cells were plated on cover slips and cultured under conditions described above. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions 24 hours prior to switching cultures to differentiation conditions. Prior to mounting cover slips on slides, cells were incubated with 1 µg/ml DAPI (Sigma) for 5 minutes at room temperature. Data were acquired with a Nikon Microphot-FX fluorescence microscope and Spot imaging software. Each set of transfections with mutant Fyn constructs was performed in triplicate, with data representing the mean of 10 medium-power (20×) fields (>2000 nuclei counted per experiment). The effect of Fyn mutants on cell fusion was measured as the ratio of GFP-positive mononuclear myoblasts:GFP-positive myotubes (cells with two or more nuclei). Experiments were performed at least three times and quantification values are reported as the mean ± s.e.m.

#### Statistical analysis

To determine significance between two groups, comparisons between means were made using the Student's *t*-test. Multiple group comparisons were performed by one-way ANOVA with Student-Newman-Keuls' post hoc test. Trendlines were generated by bivariate regression analysis using the Pearson coefficient. SPSS version 11 for Macintosh (SPSS) was used for all tests, with a 0.05 level of confidence accepted for statistical significance.

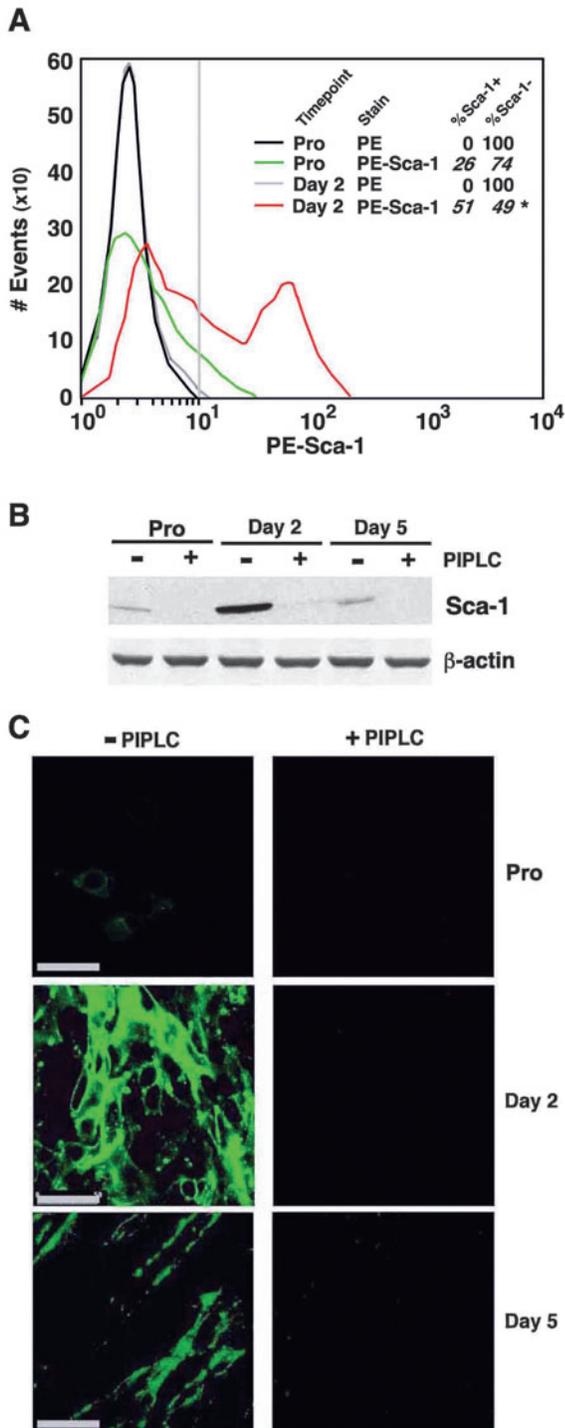
## Results and Discussion

### Sca-1 is expressed on a subpopulation of C2C12 myoblasts during differentiation

We identified Sca-1, a GPI-anchored cell-surface protein, during a screen for regulators of myogenesis (Shen et al., 2003). We determined that Sca-1 expression was significantly increased coincident with the time point at which cells expressed the first markers of both cell-cycle withdrawal and commitment to myocyte differentiation (Shen et al., 2003). By contrast, the Sca-1 transcript was downregulated in

proliferating myoblasts and differentiated myotubes relative to this intermediate time point (Shen et al., 2003).

To determine further the expression of Sca-1 on the surface of C2C12 myoblasts on a per cell basis, we performed flow cytometry on proliferating C2C12 cells, and cells grown in differentiation medium for two days, which is the time point of maximal Sca-1 expression (Fig. 1A). We observed that whereas the majority (74%) of proliferating myoblasts were Sca-1-negative, more than half of the cells (51%) were positive for Sca-1 surface expression at the time of cell-cycle withdrawal during myogenesis ( $P=0.008$ ). Although



immunoblot analysis suggested that Sca-1 expression declines overall by five days in differentiation medium (Shen et al., 2003), the heterogeneity of cell size in differentiated cultures precluded us from analyzing these cultures by flow cytometry. However, these data suggest that subpopulations of C2C12 cells (Sca-1-positive and Sca-1-negative) exist.

#### GPI-anchored proteins are necessary for myoblast cell-cycle withdrawal and myotube formation

To investigate the role of GPI-anchored cell-surface proteins in myoblast differentiation, we used the GPI-specific phospholipase, phosphatidylinositol-specific phospholipase C (PIPLC) (Griffith and Ryan, 1999), to strip GPI-anchored proteins from the surface of C2C12 myoblasts (Fig. 1B,C). Treatment with PIPLC had a profound effect on myotube formation (Fig. 2A), resulting in primarily mononuclear myoblasts even when grown under differentiation conditions. Specifically, removal of GPI-anchored proteins from the surface of C2C12 myoblasts resulted in a decrease in myoblast fusion index ( $0.08 \pm 0.07$  versus  $0.65 \pm 0.12$ ;  $P=0.004$ ) and an increased level of BrdU incorporation ( $37 \pm 2\%$  versus  $3 \pm 1\%$  BrdU-positive nuclei;  $P=0.003$ ) (Fig. 2B,C) under conditions that normally would produce terminally differentiated myotubes (Shen et al., 2003). This suggested that a GPI-anchored protein, such as Sca-1, might regulate cell-cycle withdrawal and myotube formation during myocyte differentiation.

To test whether GPI-anchored proteins play a similar role in myogenesis in other myoblasts and across species, we performed similar experiments in Sol8 murine myoblasts (Fig. 2D-F), L6 rat myoblasts (Fig. 2G-I) and QM7 avian myoblasts (Fig. 2J-L). Consistent with our findings in C2C12 cells, we observed that PIPLC decreased myotube formation and increased BrdU incorporation in Sol8 (fusion index  $0.13 \pm 0.03$  versus  $0.66 \pm 0.07$ ,  $P=0.004$ ;  $18 \pm 2\%$  versus  $1 \pm 1\%$  BrdU-positive nuclei,  $P=0.01$ ), L6 (fusion index  $0.05 \pm 0.03$  versus  $0.75 \pm 0.08$ ,  $P=0.024$ ;  $20 \pm 2\%$  versus  $2 \pm 1\%$  BrdU-positive nuclei,  $P=0.009$ ) and QM7 (fusion index  $0.06 \pm 0.03$  versus  $0.83 \pm 0.10$ ,  $P=0.031$ ;  $48 \pm 5\%$  versus  $1 \pm 0.5\%$  BrdU-positive nuclei,  $P=0.005$ ) cells. These results in different skeletal muscle types and species suggest a general role for GPI-anchored proteins in myogenesis.

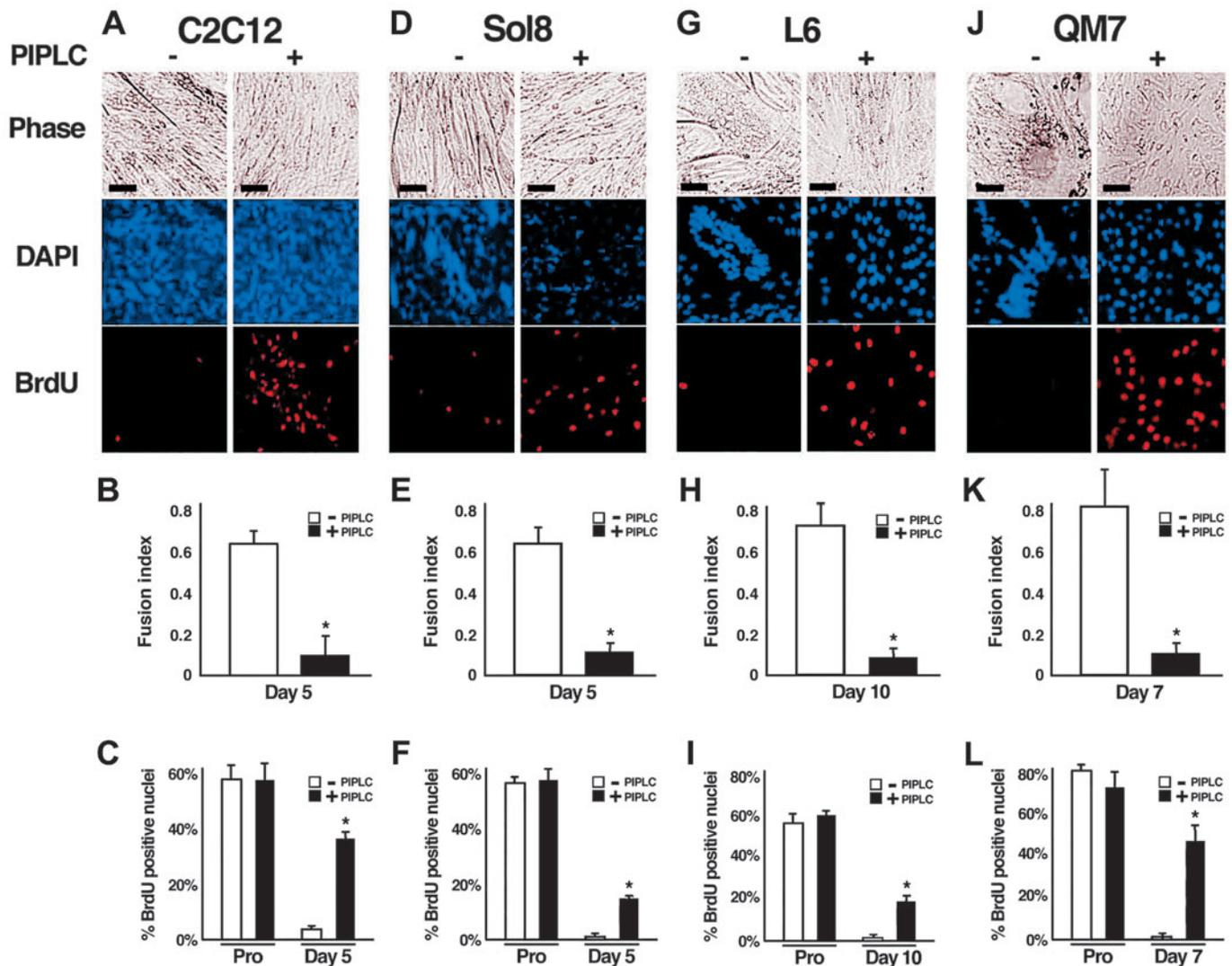
**Fig. 1.** Sca-1 is expressed on a subpopulation of C2C12 cells and is removed by PIPLC. (A) Flow cytometry of proliferating C2C12 cells (Pro; green) and cells grown in differentiation media for two days (Day 2; red) demonstrated that the percentage of cells with surface expression of Sca-1 increases during differentiation, but that Sca-1-positive and -negative populations persist. Gates (vertical gray bar) were established by nonspecific PE-conjugated antibody binding in each experiment (black and gray lines). A representative profile is shown with mean values for percent Sca-1-positive and -negative cells given ( $n=3$ ; \*significant difference,  $P<0.05$ ). (B) Immunoblot analysis of Sca-1 with specific monoclonal antibody E13-161.7 in proliferating C2C12 cells (Pro) and cells grown in differentiation media for 2 and 5 days demonstrated removal of Sca-1 protein from whole cell lysates with PIPLC (5 U/ml).  $\beta$ -actin was used as a control. Representative data are shown ( $n=4$ ). (C) Immunostaining of Sca-1 with monoclonal antibody E13-161.7 demonstrated stripping of Sca-1 (green) from the cell surface with PIPLC (5 U/ml). Bar, 50  $\mu$ m.

### Sca-1 inhibition results in defective myogenesis and sustained myoblast proliferation

T cells overexpressing Sca-1 homotypically aggregate when cultured *in vitro*, suggesting that Sca-1 is capable of mediating cell-cell adhesion (Bamezai and Rock, 1995). Anti-Sca-1 antibodies have been used to inhibit aggregation of thymocytes expressing Sca-1 on their surfaces (Bamezai and Rock, 1995; English et al., 2000), suggesting that specific antibodies block Sca-1 binding to its unidentified ligand. Whereas crosslinking of some anti-Sca-1 monoclonal antibodies have been shown to stimulate T-cell activation, binding of others, such as D7 and E13-derived antibodies, has been demonstrated to be non-activating (Bamezai and Rock, 1995), consistent with their role in inhibiting aggregation. To examine directly the role of Sca-

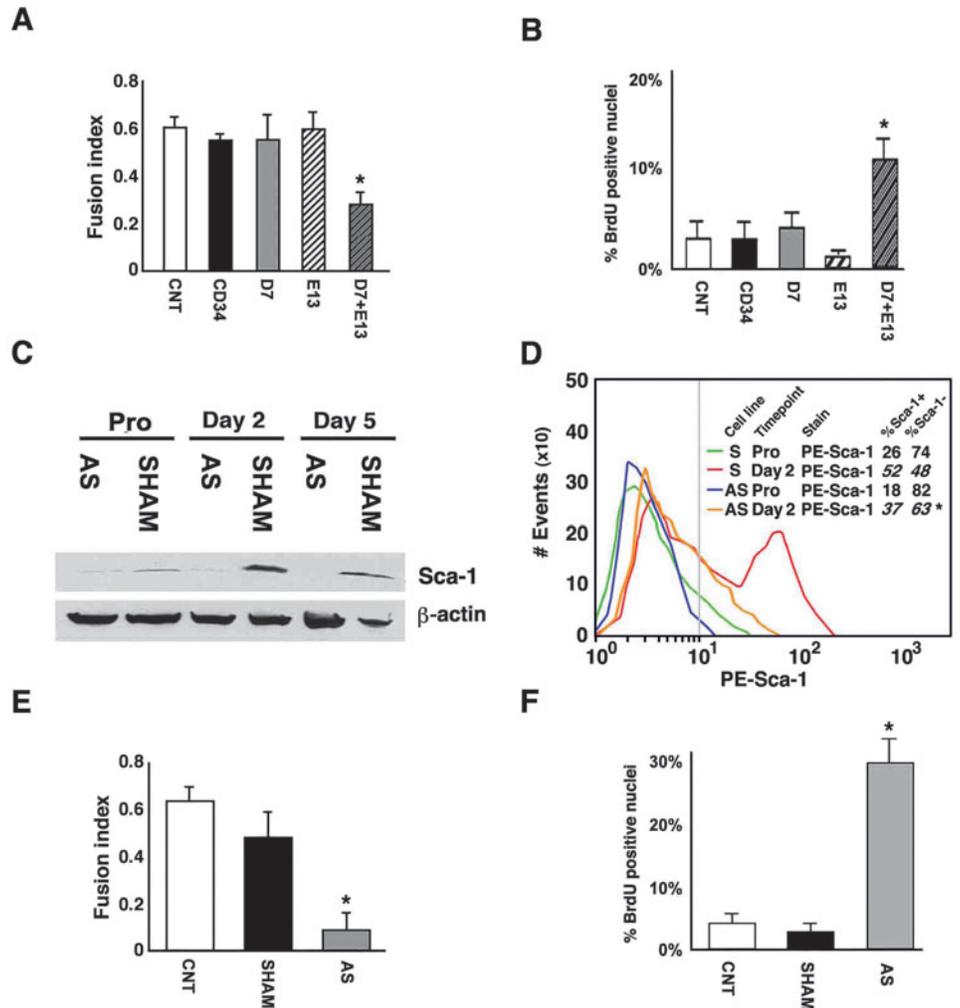
1 in myoblast cell-cycle withdrawal and differentiation, we cultured C2C12 cells in the presence of anti-Sca-1 antibodies that have been shown to block Sca-1 interactions *in vitro*.

Clone E13-161.7 originally was raised against BALB/c mouse 'pre-T' cells (Aihara et al., 1986), whereas clone D7 was raised against the interleukin 2 (IL-2)-dependent mouse T-cell line CTL-L (Ortega et al., 1986). Although both antibodies react with Sca-1, they recognize distinct epitopes as D7 is unable to block binding of E13-161.7 (Bamezai and Rock, 1995). In our experiments, antibody treatment did not alter Sca-1 expression, as determined by immunoblot (data not shown). Whereas treatment with either antibody alone produced no significant difference in myoblast fusion or DNA synthesis, compared with untreated cells or cells similarly



**Fig. 2.** PIPLC inhibits cell-cycle withdrawal and myotube formation in rodent and avian myoblasts. C2C12 (A-C), Sol8 (D-F), L6 (G-I), and QM7 (J-L) myoblasts were grown under differentiation conditions in the presence and absence of PIPLC (5 U/ml) until extensive myotube formation was observed in untreated cells: 5 days for C2C12 and Sol8 cells, 7 days for QM7 cells, and 10 days for L6 cells. All cultures were then labeled with BrdU. (A,D,G,J) Detection of all nuclei with DAPI and nuclei of cycling cells by immunostaining for BrdU demonstrated an increase in cycling cells and a decrease in multinucleated myotubes (as determined by phase microscopy) in the presence of PIPLC. Representative data are shown ( $n=5$ ). Bar, 50  $\mu\text{m}$ . (B,E,H,K) Fusion index [number of nuclei in fused cells ( $\geq 2$  nuclei)/total nuclei] was decreased in cells treated with PIPLC, compared with untreated cells. Data shown are mean  $\pm$  s.e.m. ( $n=4$ ; \*significant difference,  $P < 0.05$ ). (C,F,I,L) Percent BrdU-positive cells was increased in cells treated with PIPLC, compared with untreated cells. Data shown are mean  $\pm$  s.e.m. ( $n=5$ ; \*significant difference,  $P < 0.05$ ).

**Fig. 3.** Sca-1 is essential for myoblast cell-cycle withdrawal and myotube formation during differentiation. (A) Fusion index [number of nuclei in fused cells ( $\geq 2$  nuclei)/total nuclei] was decreased in cells grown for 5 days in differentiation media in the presence of E13-161.7 and D7 antibodies, compared with untreated cells (CNT) or cells treated with either antibody alone or antibody against CD34, a primitive stem cell marker also found on C2C12 myoblasts. Data shown are mean $\pm$ s.e.m. ( $n=3$ ; \*significant difference,  $P<0.05$ ). (B) Percent BrdU-positive cells was increased in cells grown for 5 days in differentiation media in the presence of E13-161.7 and D7 antibodies, compared with untreated cells (CNT) or cells treated with either antibody alone or anti-CD34 antibody. Data shown are mean $\pm$ s.e.m. ( $n=3$ ; \*significant difference,  $P<0.05$ ). (C) Immunoblot analysis demonstrated downregulation of Sca-1 expression in C2C12 cells stably transfected with Sca-1 antisense (AS), compared with cells transfected with empty vector (SHAM).  $\beta$ -actin was used as a control. Typical results from clone 6 are shown. (D) Flow cytometry demonstrated a decrease in the subpopulation of Sca-1-expressing C2C12 cells in cultures grown for 2 days in differentiation medium (Day 2) and stably transfected with Sca-1 antisense (AS; orange), compared with cells transfected with empty vector (S; red) (\*significant difference,  $P=0.035$ ). There was a small, but not statistically significant, decrease in the percent Sca-1-expressing cells in proliferating cultures (Pro) stably transfected with Sca-1 antisense (AS; blue), compared with cells transfected with empty vector (S; green) ( $P=0.4$ ). Gates (vertical gray bar) were established by nonspecific PE-conjugated antibody binding in each experiment. A representative profile is shown with mean values for percent Sca-1-positive and -negative cells given ( $n=3$ ). (E) Fusion index [number of nuclei in fused cells ( $\geq 2$  nuclei)/total nuclei] was decreased in cells expressing Sca-1 antisense (AS) and grown for 5 days in differentiation media, compared with cells expressing empty vector (SHAM). Data shown are mean $\pm$ s.e.m. ( $n=4$ ; \*significant difference,  $P<0.05$ ) (F) Percent BrdU-positive cells was increased in cells expressing Sca-1 antisense (AS) and grown for 5 days in differentiation media, compared with cells expressing empty vector (SHAM). Data shown are mean $\pm$ s.e.m. ( $n=4$ ; \*significant difference,  $P<0.05$ ).



treated with anti-CD34, which is a primitive stem cell marker also found on C2C12 myoblasts (Beauchamp et al., 2000), the two anti-Sca-1 antibodies in combination blocked myotube formation and sustained myoblast proliferation compared with untreated control cells, similar to the effects seen with PIPLC treatment (fusion index  $0.30\pm 0.06$  versus  $0.60\pm 0.05$ ,  $P<0.001$ ;  $11\pm 3\%$  versus  $3\pm 1\%$  BrdU-positive nuclei,  $P<0.001$ ) (Fig. 3A,B). These effects on myoblast differentiation and DNA synthesis could be seen as far out as at 10 days of antibody treatment, with complete reversal of these effects upon removal of antibody (data not shown).

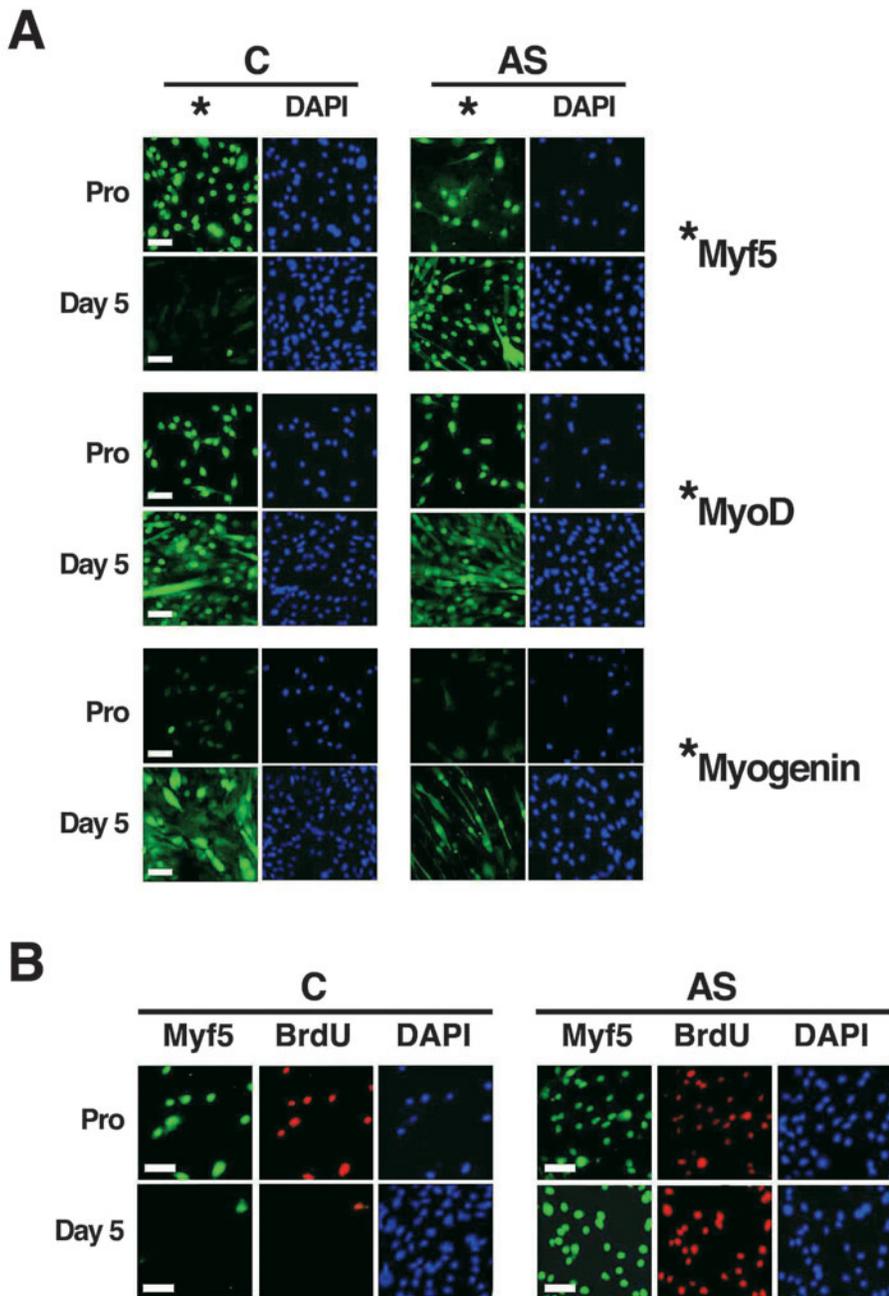
Previously, it has been shown that D7 antibody alone can inhibit thymocyte aggregation by 80-90% (Bamezai and Rock, 1995); however, D7 was unable to block myotube formation in our studies in the absence of E13-161.7 antibody. This suggests that the mechanism for Sca-1-mediated myoblast differentiation is different from its mechanism for aggregation

in thymocytes, perhaps involving other pro-differentiation events that are myoblast specific.

To determine whether Sca-1 expression is necessary for myoblast cell-cycle withdrawal and differentiation, we made stable cell lines expressing sense and antisense versions of full-length Sca-1. Sca-1-overexpressing cells behaved no differently than sham-transfected or untransfected cells (data not shown), and were not included in subsequent experiments. This suggested that Sca-1 does not confer a gain of function in cells already expressing Sca-1. Antisense-expressing cells showed a significant downregulation of Sca-1 expression, compared with a cell line stably transfected with empty vector (Fig. 3C,D). When grown in differentiation media, Sca-1 antisense-expressing cells displayed a fusion defect with short, paucinuclear myotubes (fusion index  $0.09\pm 0.07$  versus  $0.65\pm 0.05$ ;  $P<0.001$ ) (Fig. 3E), and retained a higher proliferation index ( $29\pm 4\%$  versus  $3\pm 1\%$  BrdU-positive nuclei;  $P<0.001$ ) (Fig. 3F), compared with

untransfected or sham-transfected cells. These data demonstrated that Sca-1 is necessary for proper cell-cycle withdrawal and myotube formation during C2C12 differentiation.

Because others have shown that myoblasts that fail to differentiate undergo apoptosis, we also analyzed Sca-1 antisense-expressing cells for cell death and p21 expression. Compared with control C2C12 cells, which showed appropriate induction of p21 expression and protection against apoptosis during differentiation, Sca-1 antisense-expressing cells, which continue to proliferate in differentiation medium, failed to induce p21 expression and demonstrated an increase in apoptosis (C.L.E., J.E.L. and H.S.B., unpublished). Although these data suggest that Sca-1 might influence p21 expression, and consequently protect against apoptosis, how Sca-1 couples to p21 expression remains to be determined.

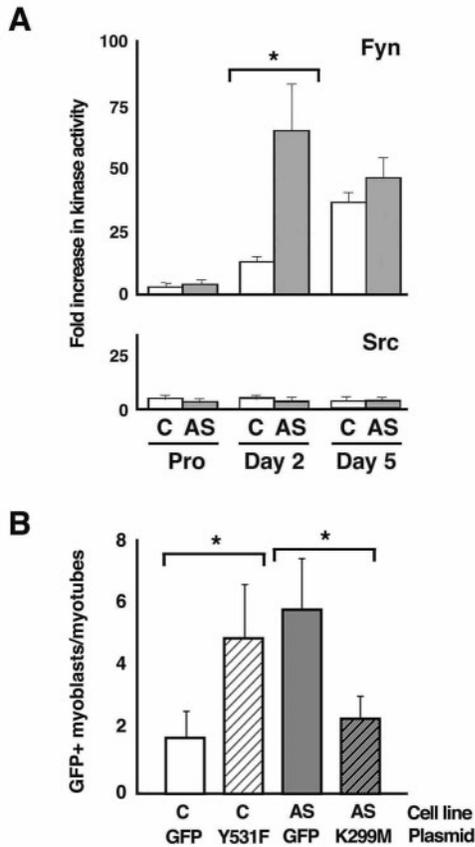


**Fig. 4.** Sca-1 downregulation blocks myogenesis. Untransfected, control C2C12 cells (C) and cells expressing Sca-1 antisense (AS) were grown under proliferation conditions and in differentiation medium for 5 days (as described for Fig. 3). They were then metabolically labeled with BrdU and stained with DAPI to identify nuclei, with antibody against BrdU (red) to detect DNA synthesis, and with antibodies against Myf5 (green), MyoD (green) and myogenin (green) as markers of myoblast differentiation. Bar, 50  $\mu$ m. (A) Whereas proliferating (Pro) control and antisense cells expressed Myf5 and MyoD, and myogenin was expressed under differentiation conditions in both cell lines, expression of the early myogenic marker Myf5 persisted in Sca-1 antisense cells under differentiation conditions. Quantitative analysis provided in text. (B) Wild-type cells were negative for Myf5 and BrdU after 5 days in differentiation medium, whereas Sca-1 antisense cells grown in differentiation medium for 5 days were positive for both Myf5 and BrdU. Quantitative analysis provided in text.

#### Sca-1 inhibition arrests myoblast differentiation

Since experiments with blocking antibodies and antisense directed against Sca-1 suggested that Sca-1 regulates myoblast cell-cycle withdrawal and myotube formation, we wanted to determine whether blocking Sca-1 affected the expression of skeletal muscle differentiation markers. We grew C2C12 cells expressing Sca-1 antisense in differentiation medium for five days and co-stained these cells with antibodies against Myf5, MyoD and myogenin, as markers of specific stages of C2C12 differentiation (Fig. 4) (Sabourin and Rudnicki, 2000; Shimokawa et al., 1998). Myf5 and MyoD were observed both in >90% of proliferating control and antisense-expressing cells ( $P>0.1$ ), whereas MyoD and myogenin were found in differentiated control (90% and 50%, respectively) and antisense (95% and 60%, respectively) cells, with no significant difference in the percent positive cells for either myogenic regulatory factor between control and antisense-expressing cells ( $P>0.1$ ) (Fig. 4A). However, expression of the earliest marker of myogenic commitment, Myf5 (Beauchamp et al., 2000), persisted in cells expressing Sca-1 antisense grown under differentiation conditions (78% $\pm$ 12% Myf5-positive nuclei in antisense-expressing cells at day 5 versus 4% $\pm$ 3% in control C2C12 cells;  $P=0.019$ ) (Fig. 4A). To determine whether the Myf5-positive, Sca-1 antisense-expressing cells grown under differentiating conditions comprised the pool of proliferating cells previously detected (Fig. 3F), we labeled cells with

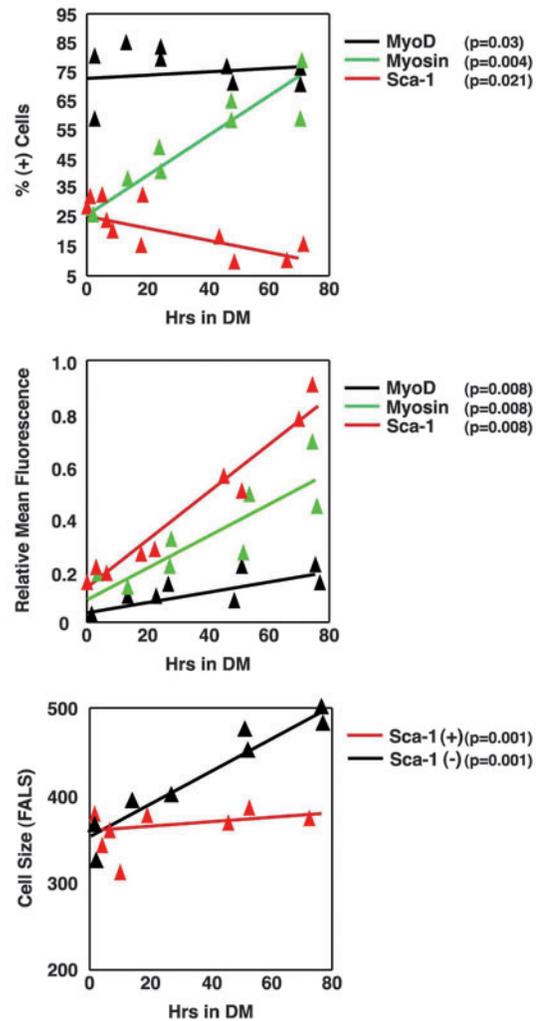
BrdU, and co-stained with antibodies against BrdU and Myf5. The percentages of BrdU-positive control and antisense-expressing cells in proliferating and differentiating cultures were the same as reported above (Fig. 3F). However, the majority of Sca-1 antisense-expressing, Myf5-positive cells were actively proliferating (Fig. 4B). These studies demonstrate that inhibiting Sca-1 expression results in a proliferating pool of mononuclear myoblasts that persist in an early stage of differentiation, despite culture under conditions that would result in myotube formation in wild-type cells.



**Fig. 5.** Fyn mediates Sca-1 function in myogenesis. (A) Fyn and Src kinase activities were measured in wild-type C2C12 cells (C; white bars) and cells expressing Sca-1 antisense (AS; gray bars). Cells grown in differentiation media for 2 and 5 days demonstrated an appropriate increase in Fyn activity in control and antisense-expressing cells at day 5 (top), low levels of Src activity at all time points (bottom), and an inappropriate increase in Fyn activity at day 2 in cells expressing Sca-1 antisense (top). Data shown are mean  $\pm$  s.e.m. ( $n=3$ ; \*significant difference,  $P<0.05$ ). Pro, proliferating cells. (B) Control (solid white or grey) or mutant-expressing plasmids (hatched bars), both co-expressing GFP, were transfected into Sca-1 antisense (AS) or wild-type C2C12 (C) cells. Cells were grown in differentiation media for 5 days. The ratio of GFP-expressing (i.e. transfected) mononuclear cells:myotubes ( $\geq 2$  nuclei) was lower in antisense cells (AS) transfected with dominant-negative Fyn-K299M compared with antisense cells transfected with control plasmid expressing GFP alone (GFP), and higher in wild-type (C) cells transfected with constitutively active Fyn-Y531F compared with GFP-transfected wild-type cells. Data shown are mean  $\pm$  s.e.m. ( $n=3$ ; \*significant difference,  $P<0.05$ ).

### Sca-1 is necessary for appropriate regulation of Fyn activity during myogenesis

Some GPI-anchored proteins signal through Src-family tyrosine kinases (Horejsi et al., 1998; Marmor and Julius, 2000). Specifically, Sca-1/Ly-6A/E has been shown to signal through Fyn during T-cell activation in vitro (Lee et al., 1994). However, whereas in vitro crosslinking of Sca-1 in T cells results in Fyn autophosphorylation (Lee et al., 1994), observations with Sca-1-null mice suggest that Sca-1 downmodulates lymphocyte responses (Stanford et al., 1997). Recently, Fyn activity has been implicated in the regulation of anti-apoptotic pathways in differentiated myotubes, but



**Fig. 6.** Sca-1 is expressed on a subpopulation of primary murine skeletal myoblasts. Flow cytometry of primary myoblasts isolated from the hindlimbs of C57BL/6 mice and cultured in differentiation medium (DM) for at least 3 days demonstrated that a subpopulation of Sca-1-positive cells and small myotubes, constituting 25% of selected myoblasts at the time of isolation, decreased in number with differentiation and expression of myosin (top). Of particular note, Sca-1 expression in this residual population of Sca-1 expressors increased on a per cell basis (middle), whereas the size of these cells, as a function of forward angle light scatter (FALS), remained constant (bottom). All data points are shown. Trendlines were generated by bivariate regression analysis using the Pearson coefficient with significance levels indicated.

specifically has been shown to be inactive during earlier stages of myogenesis (Laprise et al., 2002).

To determine whether Sca-1 function in myoblasts is mediated through Fyn, we assayed Fyn and Src activity in myoblasts stimulated to form myotubes. Both wild-type and Sca-1 antisense-expressing cells demonstrated constitutive Src activity, independent of differentiation stage or Sca-1 expression (Fig. 5A, bottom). Fyn activity was elevated in wild-type cells at day 5 in differentiation medium (Fig. 5A, top), consistent with the previously demonstrated role of Fyn in protecting differentiated myotubes against apoptosis (Laprise et al., 2002). By contrast, Fyn activity was elevated in Sca-1 antisense myoblasts at day 2 in differentiation medium, compared with wild-type cells ( $P=0.012$ ) (Fig. 5A, top). This time point coincides with the transient increase in Sca-1 expression observed during myoblast cell-cycle withdrawal and early differentiation (Shen et al., 2003). This finding suggested that inappropriate Fyn activity at an earlier stage of myogenesis might provide a mechanism for the sustained proliferation and arrest in myogenic differentiation seen in Sca-1 antisense cells.

We also tested whether anti-Sca-1 antibodies, capable of producing a similar though less-robust effect on myoblast differentiation, likewise affected Fyn activity. In contrast with the upregulation of Fyn activity in Sca-1 antisense-expressing cells at 2 days in differentiation medium, we observed no detectable difference in Fyn activity in cells treated with antibodies compared with untreated cells or cells treated with anti-CD34 antibody (data not shown). Since the alteration in Fyn activity with antisense expression was significant but modest (Fig. 5A), and antisense expression probably has a more consistent effect on Sca-1 than antibody blockade, the ability to detect an alteration in Fyn activity with antibody blockade might fall below our threshold for detection. We did observe that antisense expression had a more-significant effect on BrdU incorporation and myoblast fusion, compared with antibody treatment, as shown in Fig. 3 (fusion index  $0.30\pm 0.06$  with antibody treatment versus  $0.09\pm 0.07$  with antisense expression;  $11\pm 3\%$  BrdU-positive nuclei with antibody treatment versus  $29\pm 4\%$  with antisense expression), which is consistent with this explanation.

Why should there be such variability between antibody blockade and antisense expression? We showed that there is variability in Sca-1 expression, with Sca-1<sup>+</sup> and Sca-1<sup>-</sup> populations present at various times during differentiation (Fig. 1A). Such 'shifting' antigen concentrations might explain the incomplete effect of antibody treatment on BrdU incorporation and myoblast differentiation, and the failure to detect a measurable change in Fyn activity. To evaluate further the role of Fyn in Sca-1-mediated myogenesis, we co-expressed a dominant-negative mutant of Fyn and GFP (K299M) (Ko et al., 2002) in Sca-1 antisense-expressing cells and found that the ratio of transfected myoblasts:myotubes (as indicated by GFP expression) after five days in differentiation medium was lower in cells expressing dominant-negative Fyn compared with cells expressing GFP alone ( $2.3\pm 0.7$  versus  $5.7\pm 1.6$ ;  $P=0.006$ ) (Fig. 5B). Conversely, we co-expressed a constitutively active form of Fyn and GFP (Y531F) (Maulon et al., 2001) in wild-type C2C12 cells and found that the ratio of transfected myoblasts:myotubes at five days in differentiation medium was higher in cells expressing active Fyn compared with cells

expressing GFP alone ( $4.9\pm 1.7$  versus  $1.7\pm 0.8$ ;  $P=0.044$ ) (Fig. 5B). We also monitored BrdU incorporation in transfected cells, and observed that >90% of nuclei in mononuclear cells were BrdU positive (data not shown). These studies demonstrated that dominant-negative Fyn rescued the Sca-1 antisense phenotype, whereas active Fyn produced the Sca-1 antisense phenotype in wild-type C2C12 cells. It is important to note that whereas expression of Sca-1 antisense produced a tenfold change in myoblast fusion and BrdU incorporation, the effect of constitutively active Fyn was less pronounced. Although this may, in part, be explained by technical differences between transient transfection of mutant Fyn versus stable, constitutive expression of Sca-1 antisense, it also supports the possibility that Fyn alone may not be solely responsible for mediating Sca-1 effects. However, our observations suggest that the effects of Sca-1 in differentiating myoblasts are mediated at least in part by Fyn, that Sca-1 expression couples to the downregulation of Fyn activity during early myogenesis, and that inappropriate activation of Fyn is associated with sustained myoblast proliferation and a defect in myotube formation.

The role of Sca-1 in myoblast differentiation and cell-cycle withdrawal may be analogous to its role in T cells, where the absence of Sca-1 results in a more rapid and prolonged T-cell proliferative response (Stanford et al., 1997). Our findings also suggest that Fyn activity is temporally regulated during myoblast differentiation. Normally, Fyn activity is upregulated in differentiated myotubes, and is necessary for protecting post-mitotic muscle cells from apoptosis (Laprise et al., 2002). Downregulation of Sca-1 by antisense leads to premature activation of Fyn, which may be responsible for sustained proliferation. This suggests a role for Sca-1 in either suppressing Fyn activity or influencing the integration of Fyn with other signaling pathways that converge on the early myogenic program.

Although a role for Fyn in cellular proliferation and survival has been established in a variety of cell types (Resh, 1998), how Sca-1 couples to Fyn is not known. Plasma membrane microdomains couple signaling in space and time between GPI-anchored proteins on the extracellular surface and signaling proteins compartmentalized in the cytoplasmic leaflet of the plasma membrane, such as non-receptor protein tyrosine kinases and G proteins (Alonso and Millan, 2001; Matko and Szollosi, 2002). Transmembrane-spanning coreceptors also coalesce in these microdomains, and may be required for transmitting signals from GPI-anchored proteins (Werlen and Palmer, 2002). Whether Sca-1 signals directly through Fyn, or brings components of membrane microdomains together that ultimately regulate Fyn activation, remains to be demonstrated.

## Conclusions

Our data provide strong evidence that Sca-1 has a previously unrecognized role in myoblast proliferation and differentiation in C2C12 cells. Stripping Sca-1 from the extracellular surface, blocking Sca-1 with antibodies, or downregulating Sca-1 expression by antisense all produced a similar phenotype, that of sustained proliferation, interference with myotube formation and persistent expression of early markers of myogenic differentiation. Antisense-mediated downregulation of Sca-1 also derepressed Fyn kinase activity precisely during that time

when myoblasts withdraw from the cell cycle and begin to fuse, and Fyn mutants recapitulated and rescued the Sca-1 antisense phenotype.

Our findings suggest the question: is Sca-1 essential for normal muscle development in vivo? Sca-1-null mice have been reported as grossly normal, with T cells that demonstrate a more rapid and prolonged proliferative response (Stanford et al., 1997). Whether these mice have abnormal muscle phenotypes with respect to performance or regenerative capacity is not yet known. We have determined that, similar to our observations in C2C12 cells, Sca-1 is expressed on a subpopulation of primary murine skeletal myoblasts, and that this population decreases in number with myogenic differentiation and the expression of myosin (Fig. 6). Several recent studies have implicated Sca-1 as a marker of myogenic precursor cells that are recruited to a regeneration program following muscle injury (Jankowski et al., 2001; LaBarge and Blau, 2002; Oh et al., 2003; Poleskaya et al., 2003). Additional studies of muscle injury in Sca-1-null mice would be informative. Mice null for thymic shared antigen-1, another Ly-6 superfamily member, recently have been reported to exhibit growth delay at E14.5 and absorption by E16 (Zammit et al., 2002). This was preceded by the appearance of thinned ventricular myocardium with abnormal trabeculation, in the presence of otherwise normal atrioventricular and semilunar valves, at E14 (Zammit et al., 2002). These observations suggest a primary defect of myocardial development, and are consistent with an important role for Ly-6 family members in muscle development.

In conclusion, our studies describe a novel pathway from the extracellular environment to cell-cycle withdrawal and myogenic differentiation in C2C12 cells, and suggest that blocking Sca-1 should be explored as a strategy for muscle regeneration.

We thank M.-H. Disatnik (Stanford) and T. Rando (Stanford) for assistance with establishing primary myoblast culture in our laboratory; C. Ordahl (UCSF), A. Weiss (UCSF), A. DeFranco (UCSF), T. Rando (Stanford) and J. I. E. Hoffman (UCSF) for helpful discussion; and the anonymous referees for critical review of this manuscript. This work was supported by NIH grants HL62174 (H.S.B.), HL66727 (J.B.), and T32HL07544 (X.S.). C.L.E. is a March of Dimes Fellow of the Pediatric Scientist Development Program (NICHD K12-HD00850). H.S.B. is an Established Investigator of the American Heart Association.

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