Human muscle satellite cells show age-related differential expression of S100B protein and RAGE

Sara Beccafico · Francesca Riuzzi ·
Cristina Puglielli · Rosa Mancinelli ·
Stefania Fulle · Guglielmo Sorci · Rosario Donato

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Abstract During aging, skeletal muscles show reduced mass and functional capacity largely due to loss of the regenerative ability of satellite cells (SCs), the quiescent stem cells located beneath the basal lamina surrounding each myofiber. While both the external environment and intrinsic properties of SCs appear to contribute to the age-related SC deficiency, the latter ones have been poorly investigated especially in humans. In the present work, we analyzed several parameters of SCs derived from biopsies of vastus lateralis muscle from healthy non-trained young (28.7 ± 5.9 years; $n=10$) and aged (77.3 ± 6.4 years; $n=11$) people. Compared with young SCs, aged SCs showed impaired differentiation when cultured in differentiation medium, and exhibited the following: (1) reduced proliferation; (2) higher expression levels of S100B, a negative regulator of myoblast differentiation; (3) undetectable levels in growth medium of full-length RAGE (receptor for advanced glycation end products), a multiligand receptor of the immunoglobulin superfamily, the engagement of which enhances

myoblast differentiation; and (4) lower expression levels of the transcription factors, MyoD and Pax7. Also, either overexpression of full-length RAGE or knockdown of S100B in aged SCs resulted in enhanced differentiation, while overexpression of either a non-transducing mutant of RAGE (RAGE Δ cyto) or S100B in young SCs resulted in reduced differentiation compared with controls. Moreover, while aged SCs maintained the ability to respond to mitogenic factors (e.g., bFGF and S100B), they were no longer able to secrete these factors, unlike young SCs. These data support a role for intrinsic factors, besides the extracellular environment in the defective SC function in aged skeletal muscles.

Keywords Muscle satellite cells · Aging · Proliferation · Differentiation · S100B · RAGE

Introduction

Elderly people typically experience a progressive loss of muscular mass and strength (an age-specific muscle atrophy known as sarcopenia), reduction in muscle regenerative abilities, and susceptibility to muscular pathologies (Lahoute et al. 2008; Musarò and Rosenthal 1999; Shefer et al. 2006). These hallmarks of aging are believed to be largely due to age-related changes in the biology of muscle satellite cells (SCs), the principal stem cell component of skeletal muscles (Buckingham 2006; Chargé and

S. Beccafico · F. Riuzzi · G. Sorci · R. Donato (✉)
Department of Experimental Medicine and Biochemical
Sciences, IIM, University of Perugia,
Via del Giochetto,
06122 Perugia, Italy
e-mail: donato@unipg.it

C. Puglielli · R. Mancinelli · S. Fulle
Department of Neuroscience and Imaging, CeSI, IIM,
University G d'Annunzio,
Chieti-Pescara, Italy

Rudnicki 2004; Zammit et al. 2006). SCs are responsible for the postnatal growth of skeletal muscle tissue, the maintenance of muscular mass in the adulthood, and tissue regeneration in case of muscle damage. SCs are located beneath the basal lamina surrounding each myofiber and are normally quiescent; in case of damage, they become rapidly activated, emigrate, proliferate, and fuse with the damaged myofibers to repair them and/or with each other to form new myofibers. However, a fraction of mitotically arrested SCs do not differentiate in fusion-competent myocytes and return to a quiescent status thus reconstituting the pool of quiescent SCs.

SC differentiation and self-renewal are governed by a number of extracellular factors [i.e., insulin and insulin-like growth factors, basic fibroblast growth factor (bFGF), hepatocyte growth factor, myostatin, transforming growth factor- β , follistatin, Notch signaling, members of the Wnt family, leukemia inhibitory factor, and several cytokines] that are released by the damaged myofibers and infiltrating macrophages and act via cell surface receptors to drive the muscle regeneration process; these extracellular signals trigger biochemical responses in SCs ultimately leading to a timely regulation of transcription factors of which some are muscle-specific (i.e., Myf5, MyoD, myogenin, and MRF4) while others are not (i.e., NF- κ B, Pax3 and Pax7, β -catenin, serum response factor; Chargé and Rudnicki 2004).

There is debate as to whether an age-related reduction in the number of SCs occurs, some works reporting a decrease in the SC number with aging (Brack et al. 2005; Carlson et al. 2009; Kadi et al. 2004; Renault et al. 2002a, b; Sajko et al. 2004; Verdijk et al. 2007) and some others reporting no age-related changes (Brooks et al. 2009; Dreyer et al. 2006; Petrella et al. 2006; Roth et al. 2000). By contrast, there is consensus regarding an age-related decline in the performance of SCs as a primary cause of sarcopenia. In this regard, both extrinsic factors of the extracellular environment (the so-called SC “niche”) and intrinsic properties of the SCs have been proposed to contribute to sarcopenia (Brack and Rando 2007; Conboy and Rando 2005; Gopinath and Rando 2008). While several works have documented relevant changes in the SC niche during aging (Brack and Rando 2007 and Refs. therein), relatively little is known about changes in the intrinsic cellular properties of the SCs, especially in humans. In the

present study, we analyzed human muscle SCs isolated from healthy non-trained young and aged subjects in terms of proliferation and differentiation and for the expression of the Ca²⁺-binding protein of the EF-hand type, S100B (Donato et al. 2009), and RAGE (receptor for advanced glycation end products) (Bierhaus et al. 2005; Schmidt et al. 2001), two factors recently implicated in the regulation of the biology of myoblast proliferation and differentiation.

S100B exerts a dual role in myogenic differentiation, acting as an extracellular signal that stimulates myoblast proliferation and inhibits myogenic differentiation (Riuzzi et al. 2006b; Sorci et al. 2003), and as an intracellular factor that represses MyoD (and hence, myogenin) expression in an NF- κ B-dependent manner (Tubaro et al. 2010). Also, we showed that RAGE, activated by its ligand, high mobility group box 1 (HMGB1), reduces myoblast proliferation and promotes myogenic differentiation (Riuzzi et al. 2006a, 2007; Sorci et al. 2004). RAGE is expressed in developing myofibers and proliferating and differentiating myoblasts but not in mature myofibers or quiescent SCs (Sorci et al. 2004). Of note, primary myoblasts from *Rage*^{-/-} mice show enhanced proliferation and reduced differentiation in accordance with the promyogenic role of this receptor, and regeneration of damaged muscles is delayed in *Rage*^{-/-} mice, compared with wild-type controls (manuscript in preparation). Thus, intracellular S100B and RAGE are likely to represent two myoblast intrinsic factors, among others, involved in the regulation of myogenic differentiation.

In the present study, we analyzed human muscle SCs isolated from young and aged subjects for expression of S100B and RAGE and the roles of these factors in proliferation and differentiation. Aged SCs showed lower and higher levels of RAGE and S100B, respectively, proliferated and differentiated less efficiently than young SCs, and released small, if any amounts of S100B and bFGF. However, either transient transfection with RAGE or knocking down S100B switched aged SCs to a young phenotype and conversely functional inactivation of RAGE or transient transfection with S100B switched young SCs to an aged phenotype. Lastly, treatment of aged SCs with S100B, bFGF, or young SC-conditioned medium enhanced aged SC proliferation and their subsequent differentiation when switched to a differentiation medium (DM).

Materials and methods

Muscle samples

Vastus lateralis muscle biopsies were obtained from young (28.7 ± 5.9 years; $n=10$) and aged (77.3 ± 6.4 years; $n=11$) healthy untrained patients (Table 1). Some subjects (both young and old) underwent orthopedic surgery within 1–2 days by accidental femur fracture, during which a biopsy was performed immediately before surgery. They were healthy subjects with no other pathologies, who hold regular physical activity, but were not trained. Other subjects (both young and old) participated in a voluntary training protocol approved by the Ethics Committee for Biomedical Research, University of Chieti (PROT 1884 COET), and complied with the Declaration of Helsinki (amended in 2000); these subjects underwent voluntarily biopsy before the training. All individuals

Table 1 List of human subjects undergoing muscle tissue biopsy

Subjects	Age (years)	Gender	Type of recruitment ^a
Young			
1	20	M	Volunteer
2	22	M	Volunteer
3	25	M	Volunteer
4	25	M	Volunteer
5	28	M	Femur fracture
6	29	M	Femur fracture
7	30	M	Femur fracture
8	35	M	Femur fracture
9	36	M	Femur fracture
10	37	M	Volunteer
Aged			
1	70	M	Volunteer
2	71	M	Femur fracture
3	73	F	Femur fracture
4	73	M	Volunteer
5	74	M	Volunteer
6	75	F	Volunteer
7	76	F	Volunteer
8	81	M	Femur fracture
9	81	F	Femur fracture
10	87	F	Femur fracture
11	89	M	Femur fracture

^a See [Materials and methods](#)

provided written informed consent before participating in the study. Elderly subjects involved in the present study had a diagnosis of sarcopenia according to the criteria of the Centers for Disease Control and Prevention (CDCP). The inclusion criteria were as follows: normal ECG and blood pressure; and absence of metabolic, cardiovascular, or chronic bone/joint diseases. Exclusion criteria were the presence of metabolic and/or cardiovascular diseases, evidence of hereditary or acquired muscular disease, or diagnosis of respiratory or psychiatric disorders. No subject was under treatment with testosterone, other pharmacological interventions known to influence muscle mass, or nonsteroidal anti-inflammatory drugs. Biopsies were obtained as described (Engel 1994), and samples were immediately treated to obtain explants placed in culture as described (Decary et al. 1996). The first mononucleated cells migrated out of the explants within 7 to 13 days from the beginning of the culture, independent of the donor age.

Cell culture conditions and transfections

Isolated SCs were grown in HAM's F-10 (Invitrogen) supplemented with 20% fetal bovine serum (FBS; Hyclone), 20 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamycin [growth medium (GM)] in a H₂O-saturated 5% CO₂ atmosphere at 37°C. To induce differentiation, cultures were switched to high-glucose Dulbecco's Modified Eagle Medium (HG-DMEM; Invitrogen) supplemented with 2% horse serum (HS; Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin, 50 µg/ml gentamycin, 10 µg/ml insulin, and 100 µg/ml apo-transferrin (DM). For C2C12 myoblasts, GM was HG-DMEM supplemented with 20% FBS (Euroclone), 20 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. DM was obtained by substitution of FBS with 2% HS. In some experiments, SCs were cultivated in the absence or presence of the proteasome inhibitor, MG132 (20 µM, Calbiochem), for 6 h and then lysed for Western blotting. Where required, the p38 MAPK inhibitor, SB203580 (Calbiochem), was added to cultures of 10 µM.

Cells were transiently transfected in GM with expression vectors for the following proteins: S100B (pcDNA3/S100B), RAGE (pcDNA3/RAGE), RAGEΔcyto (pcDNA3/RAGEΔcyto), or empty vec-

tor (pcDNA3) in GM using jetPEI™ (Polyplus Transfections) as recommended by the manufacturer. After 48 h, the cells were switched to DM for 6 days, washed, lysed, and processed for Western blotting, reverse transcription PCR (RT-PCR), or immunocytochemistry (Figs. 3 and 6).

Effects of conditioned media from young and aged SC cultures

Young (Y) and aged (A) SCs were cultivated in GM for a total of 7 days during which time culture media were aspirated every other day and added to parallel cultures so as to obtain four combinations: young SCs treated with conditioned medium from young SCs (YY); young SCs treated with conditioned medium from aged SCs (YA); aged SCs treated with conditioned medium from aged SCs (AA); and aged SCs treated with conditioned medium from young SCs (AY). After three such treatments, the cultures were either analyzed as described in the legend to Fig. 8c or switched to DM and left undisturbed for 7 days before analyses as described in the legend to Fig. 8d, e.

Cell proliferation assay

Cell proliferation was measured by bromodeoxyuridine (BrdU) incorporation assay. BrdU was added to cultures 2 hours before fixation with cold methanol at -20°C and processing by immunofluorescence using a monoclonal anti-BrdU antibody (1:50, Santa Cruz Biotechnology). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1:20,000, Fluka). BrdU⁺ and total cells were counted and the percentage of BrdU⁺ cells determined.

Expression and purification of S100B

Recombinant bovine S100B was expressed and purified as reported (Donato 1988; Huttunen et al. 2000).

Western blotting

SCs were cultivated as detailed in the legend of pertinent figures and lysed. Cell lysates were subjected to Western blotting as described (Sorci et al. 2003). The following antibodies were used: mono-

clonal anti-myogenin (1:1,000; Santa Cruz Biotechnology), monoclonal anti-myosin heavy chain (MyHC; 1:500, Novocastra), polyclonal anti-S100B (1:1,000, Epitomics), monoclonal anti-MyoD (1:1,000; Santa Cruz Biotechnology), polyclonal anti-phosphorylated (Ser 536) NF- κ B p65 (1:1,000; Cell Signaling Technology), polyclonal anti-NF- κ B p65 (1:1,000; Santa Cruz Biotechnology), polyclonal anti-cyclin D1 (1:1,000; Cell Signaling Technology), polyclonal anti-RAGE (N-16; 1:1,000, Santa Cruz Biotechnology), polyclonal anti-RAGE (C-20; 1:1,000, Santa Cruz Biotechnology), polyclonal anti-phosphorylated (Thr180/Tyr182) p38 (1:1,000; Cell Signaling Technology), polyclonal anti-p38 (1:2,000; Cell Signaling Technology), polyclonal anti-phosphorylated (Ser 473) Akt (1:1,000, Cell Signaling Technology), polyclonal anti-Akt antibody (1:1,000; Cell Signaling Technology), polyclonal anti-phosphorylated (Thr202/Tyr204) ERK1/2 (1:1,000; Cell Signaling Technology), polyclonal anti-ERK1/2 (1:10,000; Sigma), anti-phosphorylated (Thr183/Tyr185) SAPK/JNK (1:1,000; Cell Signaling Technology), monoclonal anti-Pax7 (1:500; R&D Systems), and polyclonal anti-bFGF (1:1,000; Santa Cruz Biotechnology).

Reverse transcription PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions and reversed by MMLV reverse transcriptase (Invitrogen). cDNAs were tested in PCR by using the following primers: human S100B mRNA (5' ATGTCTGAGCTGGAGAAAGG3' and 5' CTCATGTTCAAAGAACTCGTG3'); bovine S100B (5'CATGTCTGAACTCGAGAAAGC3' and 5' GCTTATTCATGTTCTGAAGAACTC3'); full-length RAGE (5'TGTCGGGATCCAGGATGAGG3' and 5' ACTACTCTCGCCTGCCTCAG3'); C-truncated RAGE (5'GCTGTGTCAGCATCAGCATCAT3' and 5' CCCTGACTTTATCAAACCCC3') (Lieuw-a-Fa et al. 2006); N-truncated RAGE (5'AGTGCCTTTCAAGGTCCTC3' and 5'GGAAAGGGAATGAGGGCTAAC3') (Lieuw-a-Fa et al. 2006); and, glyceraldehyde-3-phosphate dehydrogenase (5'AGGTGAAGGTCGGAGTCAAC3' and 5'AGGATGATGTTCTGGAGAG3') used as a housekeeping gene. After a 10-min incubation at 95°C , 35–40 cycles were performed as follows: denaturation at 95°C

Fig. 1 Aged SCs show impaired proliferation and differentiation. **a** Young and aged human SCs on glass coverslips were fixed and subjected to immunofluorescence staining using an anti-c-Met antibody. Nuclei were counterstained with DAPI. **b** Young and aged human SCs were cultivated in either GM or DM for the indicated time and subjected to BrdU incorporation assay to measure proliferation. **c** Young and aged human SCs were cultivated in DM for 6 days and viewed by phase-contrast microscopy. **d** Young and aged human SCs were cultivated in either GM or DM. At intervals, the cells were lysed, and cell lysates were subjected to Western blotting using anti-myogenin or anti-MyHC antibody. Asterisk indicates significantly different from internal control (**b**)

for 30 s, annealing at 56°C for 45–60 s, and extension at 72°C for 45–60 s. The amplification products were resolved on 1.2–3.0% agarose gel and visualized by ethidium bromide staining.

Immunofluorescence and immunocytochemistry

Immunofluorescence was performed as described (Sorci et al. 2004) using the following antibodies: polyclonal anti-S100B (1:30, SWant), polyclonal anti-RAGE (N-16; 1:20, Santa Cruz Biotechnology), and polyclonal anti-c-Met (1:20, Santa Cruz Biotechnology). For detection of Pax7 and MyoD and for double myogenin/S100B immunofluorescence, SCs were fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), washed three times with PBS and incubated with 0.4% Triton X-100, 1% BSA, and 10% horse serum in PBS for 1 hour at room temperature. Then, the cells were incubated overnight at 4°C with a monoclonal anti-Pax7 (1:20, R&D Systems), monoclonal anti-MyoD (1:10, Santa Cruz Biotechnology), or monoclonal anti-myogenin (1:20, Santa Cruz Biotechnology) antibody in combination with the polyclonal anti-S100B (1:30, SWant) antibody. After washes with 0.1% Tween-20 in PBS, the cells were incubated with Alexa fluor 488 donkey anti-mouse antibody (1:200, Invitrogen) for 1 hour at room temperature. Nuclei were counterstained with DAPI. After mounting, the cells were viewed on a DM Rb epifluorescence microscope (Leica, Wetzlar, Germany) equipped with a digital camera. MyHC was detected by immunocytochemistry as described (Sorci et al. 2003).

Release of S100B and bFGF

To determine the basal release of S100B and bFGF, nearly confluent SCs grown in GM were transferred

to serum-free HAM's F-10 medium for 24 h. Individual culture medium was clarified by centrifugation, added of 1/100 volume of 2% sodium deoxycholate and subjected to precipitation with 1/10 volume of

100% trichloroacetic acid. Pellets were resuspended in sodium dodecyl sulfate buffer and titrated with 1 N NaOH to obtain the normal blue color of the sample buffer, boiled for 5 min, and subjected to Western blotting analysis. Purified S100B or bFGF was used as a marker.

Membranes/cytosol separation

Human SCs and C2C12 myoblasts cultivated for 24 hours in GM or DM were lysed in 20 mM Tris–HCl (pH 7.4) in the presence of a mixture of protease inhibitors. Cells were sonicated and centrifuged at $100,000\times g$ for 1 hour at 4°C. The resultant pellets (membrane fraction) were resuspended in sodium dodecyl sulfate buffer. The cytosolic fractions were concentrated as described for S100B and bFGF release.

RNA interference

The siRNA specific sequences to target human S100B in SCs were from Dharmacon and were used in parallel with non-targeted Negative Universal Control Stealth (Invitrogen). Transfections of siRNA (10 nM) were performed by using the INTERFERin™ Transfection reagent (Polyplus Transfection) for 72 hours following the manufacturer's instructions. Expression of S100B transcripts in transfected cells was evaluated by RT-PCR and Western blotting.

Results

As in humans, age-related changes in skeletal muscle become evident after the age of 70 years (Kadi et al. 2004); we decided to compare SCs from aged, over-seventy people (77.3 ± 6.4 years; $n=11$) to SCs from young, under-forty people (28.7 ± 5.9 years; $n=10$).

Aged SCs show impaired proliferation and differentiation

SCs from both young and aged individuals were characterized for expression of c-Met. Greater than 95% of cells were myogenic cells by this criterion (Fig. 1a). Compared with young SCs, aged SCs showed reduced proliferation in both, GM, and DM (Fig. 1b), as well as impaired differentiation, i.e., reduced myotube formation and reduced expression

Fig. 2 Young and aged SCs express different levels of S100B. ►

a Young and aged human SCs were cultivated in either GM or DM and analyzed for S100B mRNA (*left panel*) or protein (*right panel*) expression levels. **b** Same as in (**a**) except that the cells were analyzed by immunofluorescence using anti-S100B antibody. Nuclei were counterstained with DAPI. **c** Same as in (**b**) except that young SCs were used, cultivation in DM was prolonged to 6 or 8 days, and the cultures were subjected to double immunofluorescence using anti-S100B antibody (*red signal*) and anti-myogenin antibody (*green signal*). Shown are merged (S100B/myogenin/DAPI) images only (*left side of the panel*). Notice that at 6–8 days in DM S100B expression is restricted to myotubes as is expression of myogenin (*light-blue signal*). Parallel 6-d samples in DM received the p38 MAPK inhibitor, SB203580 (*SB*), during the 6-day cultivation time in DM and subjected to double S100B/myogenin immunofluorescence (*top right image*). Notice the absence of myogenin expression and myotube formation and the presence of S100B in mononucleated cells in SB203580-treated cultures. Still other samples were transferred to GM for 2 days after a 6-day cultivation in DM before double S100B/myogenin immunofluorescence (*bottom right panel*). Notice that non-fused cells are now S100B-positive. **d** Same as in (**c**) except that cultures were subjected to RT-PCR (*top panel*) or Western blotting (*bottom panel*) for detection of S100B mRNA and protein, respectively. **e** Aged SCs were treated as described in (**d**) and lysed for detection of S100B by Western blotting. Notice that treatment with SB203580 does not prevent S100B downregulation in DM and that switching the cells to GM for 2 days after a 6-day cultivation in DM (GM*) results in a partial rescue of S100B expression

levels of the early myogenic differentiation marker, myogenin, and the late differentiation marker, MyHC, and reduced myotube formation when cultured in DM (Fig. 1c, d). No significant differences were detected between satellite cells from volunteers and those from subjects with femur fracture in either young or aged samples in terms of proliferation rate (Fig. 1b) and myogenic potential (Fig. 1c). These results pointed to a different sensitivity of young and aged SCs to the same microenvironment in vitro and, hence, to different intrinsic properties.

Young and aged SCs express different levels of S100B

Next, we examined SCs for expression levels of S100B, a Ca^{2+} -binding protein of the EF-hand type shown to interfere with myoblast differentiation via activation of NF- κ B transcriptional activity (Tubaro et al. 2010). Young and aged SCs exhibited relatively low and high levels of S100B (mRNA and protein) in GM, respectively (Fig. 2a). Upon switching to DM, a tendency of S100B (mRNA and protein) abundance

to decrease was noted in young SCs (Fig. 2a) and this decrease was restricted to non-fused SCs (Fig. 2b, c), in accordance with observations made using myoblast cell lines (Sorci et al. 1999). Thus, either differentiation cues or the reduced mitogen content of DM determined a decrease in S100B expression levels.

Indeed, inhibition of SC differentiation obtained by treating young SCs in DM with SB203580, a specific inhibitor of the promyogenic p38 MAPK (Guasconi and Puri 2009; Lluís et al. 2006), resulted in reduced S100B downregulation (Fig. 2d), supporting the possibility that downregulation of S100B in non-

Fig. 3 Transfection of young SCs with S100B expression vector results in defective myogenic differentiation. **a** Young SCs transiently transfected with bovine S100B expression vector show expression of bovine S100B mRNA as opposed to cells transfected with empty vector (*top panel*) and enhanced expression levels of S100B protein compared with cells transfected with empty vector (*bottom panel*). **b** Young SCs

were transiently transfected with bovine S100B expression vector or empty vector and cultivated in DM. Cultures were either viewed by phase-contrast microscopy (*top panel*) or subjected to MyHC immunocytochemistry (*bottom panel*). **c** Same as in (**b**) except that young SCs were cultivated in GM or DM and subjected to Western blotting using anti-myogenin, anti-MyoD, or anti-MyHC antibody

fused young SCs in DM was consequent to differentiation cues in part and might be required for myoblast differentiation. However, switching young SCs back to GM after an interval of 6 days in DM resulted in restoration of S100B expression levels in non-fused myoblasts in part (Fig. 2c, d). This suggested that the reduction of serum mitogens occurring upon switching SCs from GM to DM was another important factor determining S100B down-regulation in non-fused SCs.

Aged SCs also showed a decreased S100B (mRNA and protein) abundance upon transfer to DM although less rapidly and less markedly than young SCs (Fig. 2a, b). Also, aged SCs after 3 days in DM

showed association of residual S100B with filamentous structures running parallel to the long axis of the cells as opposed to the more diffuse (and abundant) cytoplasmic localization of the protein in GM (Fig. 2b). Association of S100B with microtubules and type III intermediate filaments has been described (Sorci et al. 1998, 1999, 2000). Thus, the decrease in S100B expression levels in aged SCs in DM appeared to correlate more with the decrease in serum mitogens than with differentiation cues as these cells differentiated poorly (Fig. 1b–d). Indeed, switching aged SCs back to GM after an interval of 6 days in DM resulted in a significant extent of restoration of S100B expression levels (Fig. 2c, e). Also, the substantial

Fig. 4 Knockdown of S100B in young and aged SCs results in enhanced myogenic differentiation. **a** Aged SCs were treated with S100B siRNA or control siRNA and analyzed for S100B mRNA (*top panel*) or protein (*bottom panel*) content. **b** Same as in (**a**) except that aged SCs were cultivated in DM for 7 days and either viewed by phase-contrast microscopy (*top panel*) or subjected to MyHC immunocytochemistry (*bottom panel*). **c** Same as in (**b**) except that cultures were subjected to Western blotting using anti-MyoD, anti-myogenin, or anti-MyHC

antibody. **d** Same as in (**b**) except that young SCs were used and cultures were either viewed by phase-contrast microscopy (*left panel*) or subjected to Western blotting using anti-MyoD, anti-myogenin, or anti-MyHC antibody (*right panel*). **e** Conditions were as in (**a**) except that aged cells were cultivated in GM or DM and subjected to Western blotting using anti-phosphorylated NF- κ B (p65), anti-NF- κ B (p65), or anti-cyclin D1 antibody

lack of restoration of S100B expression levels in aged SCs treated with SB203580 (Fig. 2e; as opposed to the relatively high expression levels of S100B in SB203580-treated young SCs) supported the conclusion that the decrease in serum mitogens rather than differentiation cues were a major cause of reduction of S100B levels in these cells in DM.

To examine whether S100B levels affected SC differentiation, we transfected young SCs with bovine S100B expression vector or knocked down S100B in aged SCs by RNA interference (RNAi). Transient

transfection of young SCs with S100B expression vector resulted in reduced differentiation compared with controls, i.e., acquisition of an aged phenotype (Fig. 3a–c). Specifically, S100B-transfected young SCs (Fig. 3a) exhibited reduced myotube formation and MyHC immunostaining (Fig. 3b) as well as reduced MyoD, myogenin, and MyHC levels (Fig. 3c). Conversely, knocking down S100B in aged SCs by RNAi (Fig. 4a) resulted in enhanced differentiation, i.e., acquisition of a young phenotype, as evidenced by an increase in the number of myotubes

Fig. 5 Young and aged SCs show differential expression of RAGE. **a** Young and aged SCs were cultivated in GM or DM and either subjected to Western blotting using anti-RAGE antibody (left panel) or analyzed for expression of full-length (fl) RAGE, C-terminal-truncated RAGE, or N-terminal-truncated RAGE (right panel). **b** Same as in (a) except that SC cultures in GM or DM were analyzed by immunofluorescence using anti-RAGE antibody. Nuclei were counterstained with DAPI. **c** C2C12 myoblasts and young and aged SCs cultivated in GM or DM were fractionated to obtain a cytosolic

(Cyt) and a membrane (M) fraction. Individual fractions were subjected to Western blotting using an anti-RAGE antibody directed against RAGE N-terminal domain. **d** Aged SCs were cultivated in GM or DM and subjected to Western blotting using an anti-RAGE antibody directed against RAGE C-terminal domain. **e** Aged SCs were cultivated in GM and treated for 6 hours with the proteasome inhibitor, MG132, or vehicle before Western blotting using an anti-RAGE antibody directed against RAGE N-terminal domain

Fig. 6 Effects of transfection of aged and young SCs with full-length RAGE or RAGE Δ cyto. **a** Aged SCs were transiently transfected with full-length RAGE or empty vector in GM, transferred to DM for 7 days and either viewed by phase-contrast microscopy (*top left panel*), subjected to immunocytochemistry using anti-MyHC antibody (*bottom left panel*) or analyzed by Western blotting using anti-MyHC antibody (*right panel*). **b** Young SCs were transiently transfected with RAGE Δ cyto or empty vector in GM, transferred to DM for 7 days and either viewed by phase-contrast microscopy (*left panel*) or analyzed by Western blotting using anti-MyHC antibody (*right panel*)

and enhanced MyHC immunostaining (Fig. 4b) as well as increased MyoD, myogenin, and MyHC levels compared with controls (Fig. 4c). Of note, knockdown of S100B in young SCs resulted in enhanced myotube formation and expression levels of MyoD, myogenin, and MyHC upon switching to DM (Fig. 4d), in agreement with data obtained using myoblast cell lines (Tubaro et al. 2010). Moreover, knockdown of S100B in aged SCs resulted in reduced expression of the proliferation factor, cyclin D1, and reduced phosphorylation (activation) levels of NF- κ B (p65), in both GM and DM (Fig. 4e), which raises the possibility that S100B supports cyclin D1 expression via activation of NF- κ B (p65). However, the relatively high expression levels of S100B alone appeared to be insufficient to ensure an optimal proliferation of aged SCs (see Fig. 1b). Incidentally, in aged SCs, NF- κ B (p65) activation was higher in DM than in GM (Fig. 4e), raising the possibility that the stress consequent to the reduction of serum mitogens occurring upon switching the cells from GM to DM resulted in a high NF- κ B (p65) activity (see Discussion). Moreover, such a high NF- κ B (p65) activity

might contribute to the defective differentiation of aged SCs (Bakkar and Guttridge 2010). Yet, reducing S100B expression levels in aged SCs in DM resulted in a dramatic decrease in NF- κ B (p65) activity (Fig. 4e), suggesting that the relatively high S100B levels under these conditions contributed significantly to the elevated activation of NF- κ B (p65).

Young and aged SCs show differential expression of RAGE

Whereas young SCs showed expression of full-length RAGE (~50 kDa) in GM and, to a larger extent, DM in accordance with previous findings (Sorci et al. 2004), a truncated form (~32.5 kDa) and a minor fraction of full-length RAGE were detected in aged SCs in GM (Fig. 5a). However, aged SCs showed RAGE mRNA of normal size in GM and DM with comparable levels of C-terminal truncated RAGE and undetectable levels of N-terminal truncated RAGE (Fig. 5a). Upon switching aged SCs from GM to DM the amount of truncated RAGE rapidly decreased and full-length RAGE was expressed. By immunofluorescence, RAGE was

Fig. 7 Aged SCs exhibit defective activation of ERK1/2, p38 MAPK and Akt and low expression levels of the transcription factors, MyoD and Pax7. **a** Young and aged SCs were cultivated in GM or DM and subjected to Western blotting using anti-phosphorylated p38 MAPK, total p38 MAPK, phosphorylated Akt, total Akt, phosphorylated ERK1/2, total

ERK1/2, or anti-phosphorylated JNK antibody. **b** Same as in **(a)** except that anti-Pax7 or anti-MyoD antibody was used. **c** Same as in **(b)** except that SCs were analyzed by immunofluorescence. Nuclei were counterstained with DAPI. Arrows point to Pax7-positive or MyoD-positive nuclei

detected in the forms of punctae throughout individual cells in young SCs in GM and exclusively in myotubes from young SCs in DM, being almost completely absent in non-fused myoblasts (Fig. 5b). By contrast, RAGE was occasionally detected in aged SCs in GM and found as punctae throughout the cytoplasm in DM (Fig. 5b). Also, whereas in young SCs (as well as in the C2C12 myoblast cell line) RAGE was detected almost exclusively in the membrane fraction with trace amounts present in the cytosolic fraction in both GM and DM, in aged SCs, truncated RAGE was mainly recovered in the membrane fraction in GM, and full-length RAGE was detected in the cytosolic fraction to a large extent and in the membrane fraction to a lesser extent in DM (Fig. 5c). When cell lysates from aged SCs were subjected to Western blotting using an antibody directed to the RAGE C-terminal domain, full-length RAGE was detected in GM with no traces of the 32.5-kDa truncated form (Fig. 5d). The full-length RAGE so detected likely corresponded to the small amount of full-length RAGE found in these cells in GM. Lastly, treatment of aged SCs in GM with the proteasome inhibitor, MG132, resulted in the detection of full-length RAGE in whole cell lysates (Fig. 5e), suggesting that truncated RAGE in these cells resulted from proteosomal degradation.

Yet, transfection of aged SCs with full-length RAGE expression vector (Fig. 6a) resulted in enhanced differentiation (i.e., acquisition of a young phenotype) as investigated by phase-contrast microscopy, MyHC immunostaining, and Western blotting with anti-MyHC antibodies (Fig. 6a). These latter results suggested that aged SCs might lack optimum amounts and/or appropriate subcellular (i.e., membrane) localization of full-length RAGE, thus explaining their defective differentiation potential in part. In the converse experiment, transient transfection of young SCs with an expression vector carrying a RAGE mutant lacking the cytoplasmic and transducing RAGE domain (RAGE Δ cyto; Huttunen et al. 1999) resulted in defective differentiation (Fig. 6b) in accordance with previous work on myoblast cell lines (Riuzzi et al. 2006a; Sorci et al. 2004).

Aged SCs exhibit defective activation of ERK1/2, p38 MAPK and Akt, and low expression levels of the transcription factors, MyoD and Pax7

An analysis of the promyogenic p38 MAPK, the pro-survival and promyogenic Akt, the pro-survival and

mitogenic ERK1/2, and the anti-myogenic JNK, revealed that (Fig. 7a): (1) p38 MAPK and Akt phosphorylation (activation) levels increased upon transfer of young SCs from GM to DM in accordance with the high differentiation potential of these cells, while low or no phosphorylation of these kinases could be documented in aged SCs; (2) the phosphorylation pattern of ERK1/2 in GM and DM in young SCs were identical to those of myoblast cell lines (i.e., a decrease at early differentiation stages followed by an increase at late differentiation stages; Wu et al. 2000), while, in aged SCs, a marked decrease in ERK1/2 phosphorylation levels was detected upon transfer from GM to DM; and (3) JNK activation was detected in both young and aged SCs at relatively late time points in DM only. Thus, aged SCs showed a low differentiation potential in consequence of defective activation of p38 MAPK and Akt (whose activity is required for efficient myoblast differentiation; Serra et al. 2007) and exhibited a low proliferation potential in GM in spite of comparable ERK1/2 activation to young SCs.

Besides expressing relatively low amounts of myogenin in DM, thus explaining their reduced ability to differentiate, to express MyHC and to form myotubes, aged SCs also showed a reduced ability to express the transcription factors Pax7 and MyoD in GM (Fig. 7b). As Pax7 and MyoD characterize proliferating myoblasts and SCs and are required for efficient myoblast proliferation and subsequent differentiation (Cornelison et al. 2000; Kitzmann and Fernandez 2001; Kuang and Rudnicki 2008; Zammit 2008), these results might explain the reduced ability of aged SCs to proliferate even in GM (see Fig. 1b). Thus, again, aged SCs exhibited altered intrinsic properties compared with young SCs in the same microenvironment in vitro. However, upon transfer to DM, aged SCs did express comparable amounts of MyoD to young SCs (Fig. 7b), pointing to an attempt to differentiate although unsuccessfully (see Fig. 1).

Aged SCs maintain the ability to respond to but lose the ability to release mitogenic factors

The results illustrated thus far indicated that reduced proliferation (and hence, a reduced expansion) might be one major functional defect of aged human SCs. As the proliferation rate is strongly affected by mitogenic factors in the medium, some of which are

being secreted by myoblasts themselves, we decided to verify if aged SCs maintain the ability to respond to mitogens. To address this point, bFGF or S100B, two factors reported to stimulate myoblast proliferation via activation of ERK1/2 signaling (Riuzzi et al. 2006b; Yoshida et al. 1996), were added to young and aged SCs cultured in medium containing 2% FBS, and the cells were then tested for BrdU incorporation (Fig. 8a). Both SC types increased their proliferation rate in response to either factor, with aged SCs showing a larger increase in the percentage of proliferating cells with respect to basal level than the young counterpart (~85% and ~121% vs. ~54% and ~41% increment in the presence of 10 nM bFGF or S100B, respectively; Fig. 8a). Interestingly, in the presence of 10 nM S100B the percentage of proliferating (BrdU⁺), aged SCs was very similar to that of young SCs, indicating a substantially unchanged responsiveness to mitogens by human SCs during aging. These results suggested that the difference in the proliferation rate observed in aged vs. young SCs (Fig. 1b) could be due to a defective secretion and hence, a defective autocrine effect of mitogenic factors including bFGF and S100B. Indeed, aged SCs were found to secrete extremely low amounts of bFGF and S100B, as opposed to the remarkably high amounts of either factor secreted by young SCs (Fig. 8b).

To further support the possibility that young and aged SCs might differentially condition their own behavior via an autocrine mechanism, we performed experiments in which both type of cells were cultured either with young or with aged SC-conditioned GM with renewal of conditioned media every other day. After 7 days of culture in these conditions, ERK1/2 phosphorylation levels were lower in young SCs treated with aged SC-conditioned medium (YA) and enhanced in aged SCs treated with young SC-conditioned medium (AY) while p38 MAPK phosphorylation levels were unchanged in young SCs treated with aged SC-conditioned medium (YA) and lower in aged SCs treated with young SC-conditioned medium (AY), compared with their respective controls (Fig. 8c). After the 7-day conditioning period, the SC cultures were switched to fresh, unconditioned DM for a further 7 days (with no renewal of culture media) to test SC myogenic potential. At this time point, young SCs previously treated with aged SC-conditioned medium (YA) showed unaltered ability to

Fig. 8 Aged SCs maintain the ability to respond to but lose the ability to release mitogenic factors. **a** Young and aged SCs were cultivated in GM for 1 day in the presence of increasing concentrations of bFGF or S100B and subjected to BrdU incorporation assay. **b** Conditioned medium from young and aged SCs cultures (1 day in serum-free medium) were analyzed for bFGF and S100B content by Western blotting. **c** Young (Y) and aged (A) SCs in GM were treated for 7 days with conditioned medium from GM-cultured young (Y) or aged (A) SCs with renewal of conditioned media every other day and analyzed for phosphorylation levels of ERK1/2 or p38 MAPK by Western blotting. **d**, **e** Same as in (c) except that after treatment with conditioned media the cultures were switched to DM, left undisturbed for an additional 7 days and either viewed by phase-contrast microscopy (**d**) or subjected to Western blotting using anti-myogenin antibody (**e**). **f** Young and aged SCs were cultivated for 1 day in GM in the absence or presence of 10 nM S100B, switched to DM for 7 days with no additions and either subjected to immunocytochemistry using anti-MyHC antibody or subjected to Western blotting using anti-MyHC antibody

form myotubes compared with the young SCs previously treated with young SC-conditioned medium (YY; Fig. 8d). By contrast, aged SCs previously treated with young SC-conditioned medium (AY) showed a partially rescued ability to differentiate and to form myotubes, compared with aged SCs previously treated with aged SC-conditioned medium (AA; Fig. 8d, f). Thus, the observed myogenic potential in each group appeared to be mainly linked to the activation status of ERK1/2 at the end of the conditioning time and was in line with the observation that young SCs secrete larger amounts of mitogenic factors including bFGF and S100B than do aged SCs; by expanding the SC population, mitogenic factors might contribute to myoblast fusion. This conclusion was supported by the finding that adding the mitogenic S100B to aged SCs in GM followed by cultivation in DM without additions resulted in a significant stimulation of MyHC expression and myotube formation (Fig. 8f) similar to what was described using bFGF (Shefer et al. 2006).

Discussion

It is widely accepted that defective proliferation and/or differentiation potential of SCs represent a major cause of sarcopenia in otherwise healthy aged people (Brack and Rando 2007; Conboy and Rando 2005; Gopinath and Rando 2008). However, the molecular mechanisms underpinning these age-related changes

in SC function are not completely understood. Herein, we have studied some general properties of human SCs isolated from young (<40 years) and aged (>70 years) healthy subjects and correlated these properties with S100B protein and RAGE, an intracellular/extracellular factor and a membrane receptor, respectively, recently proposed to play a role in the

regulation of myoblast proliferation and differentiation. We have shown that isolated human muscle SCs exhibit age-related differential expression of S100B and RAGE and that these changes correlate with the reduced proliferation and differentiation potential exhibited by aged SCs compared with young SCs. Specifically, aged SCs show higher levels of S100B,

an intracellular protein shown to inhibit myoblast differentiation via NF- κ B-dependent repression of MyoD (and myogenin) expression (Tubaro et al. 2010), and lower levels of membrane-bound RAGE, a receptor promoting myoblast differentiation (Riuzzi et al. 2006a; Sorci et al. 2004), compared with young SCs. Thus, the altered levels of S100B and RAGE correlated positively with the defective differentiation of aged SCs, a notion reinforced by the observation that knockdown of S100B in or transient transfection with RAGE of aged SCs resulted in restoration of the differentiation potential in part. Conversely, transient transfection of young SCs with either S100B or a dominant negative RAGE mutant resulted in the acquisition of an aged phenotype. We also noted a remarkably reduced ability of aged SCs to secrete bFGF and S100B, which are both potent stimulators of myoblast proliferation (Riuzzi et al. 2006b; Yoshida et al. 1996), and we found that treatment of aged SCs with either bFGF or S100B or with conditioned medium from young SCs resulted in a nearly complete restoration of the proliferation potential along with an enhanced differentiation when aged SCs were shifted to DM. Thus, it is tempting to speculate that the reduced ability of aged SCs to release bFGF and/or S100B is a one major cause of defective proliferation (i.e., expansion), which is essential for the attainment of the critical cell density necessary for subsequent myotube formation. On the other hand, the reduced ability of aged SCs to express the promyogenic RAGE on the plasma membrane might be another major cause of defective differentiation. Thus, the defective myogenic potential of aged SCs appears to be dependent on a combination of insufficient secretion of mitogens such as bFGF and S100B and defective expression of the promyogenic RAGE at the appropriate cellular site.

We also found that aged SCs do not express MyoD in GM while expressing normal levels of MyoD in DM, and express lower levels of Pax7 compared with young SCs. Moreover, aged SCs are defective in the activation of ERK1/2, p38 MAPK, and Akt, i.e., kinases whose optimal and timely activity is required for myoblast proliferation/survival and differentiation (Chargé and Rudnicki 2004; Guasconi and Puri 2009; Lluís et al. 2006; Serra et al. 2007; Wu et al. 2000). By contrast, JNK activation levels were similar in young and aged SCs, thus establishing an important difference between SCs (either young or aged)

isolated from healthy subjects and those from subjects affected by chronic inflammatory diseases or cancer, in which case, high JNK activation levels represent a major feature (Grounds et al. 2008; Perdiguerro et al. 2007).

Our findings suggest that aged SCs show altered expression levels of S100B, RAGE, and Pax7 and a reduced ability to secrete bFGF and S100B among other factors, which appear to be responsible for the defective proliferation and differentiation. Indeed, the proliferation and differentiation defects can be rescued by cultivation of aged SCs in young SC-conditioned media which contain bFGF and S100B among others. Thus, intrinsic properties appear to negatively condition the myogenic potential of aged SC. However, our results cannot exclude that the altered expression levels of S100B, RAGE, and Pax7 are consequent to changes in the aged SC microenvironment. For example, while the reduced ability of aged SCs to release S100B might be consequent to the defective expression levels and/or subcellular localization of RAGE [in that S100B secretion has been proposed to be dependent on RAGE (Perrone et al. 2008)], one wonders what factors and/or conditions are responsible for the higher levels of S100B detected in aged SCs compared with young SCs. However, aged SCs are defective in the secretion of the mitogenic bFGF as well, suggesting that aged SCs exhibit a generally compromised ability to secrete factors capable of supporting SC expansion in an autocrine manner. By the same reasoning, one wonders what factors and/or conditions are responsible for the altered expression levels and/or subcellular localization of RAGE and the altered expression levels of Pax7 in aged SCs. The relatively high activation state of NF- κ B, a known anti-myogenic factor (Bakkar and Guttridge 2010), correlate positively with the higher expression levels of S100B in aged SCs compared with young SCs, which is, in turn, consistent with S100B's ability to activate NF- κ B in myoblasts (Tubaro et al. 2010). However, while knockdown of S100B expression in aged SCs results in reduced activation of NF- κ B and acquisition of a young phenotype (Fig. 4; also see Tubaro et al. 2010), the question as to what mechanism is responsible for the higher S100B levels in aged SCs compared with young SCs still remains unresolved. Incidentally, enhanced expression levels of S100B are also found in the aging brain (Akhisaroglu et al. 2003;

Katoh-Semba and Kato 1994) again with no data about the mechanism underlying this event. The *s100b* promoter contains recognition sites for NF- κ B raising the possibility that the relatively high NF- κ B activity in aged SCs might result in upregulation of S100B which, in turn, would activate NF- κ B in a feed-forward loop. On the other hand, the altered redox status of aged SCs (Beccafico et al. 2007; Fulle et al. 2005; Renault et al. 2002a, b) could be one cause of high NF- κ B activity. We have shown that either treatment of myoblasts with the NF- κ B inhibitor, sulfasalazine, or transfection of myoblasts with the NF- κ B inhibitor, I κ B α -SR, results in enhanced myogenic differentiation (Tubaro et al. 2010).

In conclusion, we have identified some major intrinsic differences that contribute to explain the different proliferation and differentiation behaviors between young and aged SCs, namely a lower and higher expression level of Pax7 and the anti-myogenic S100B, respectively, an altered subcellular localization of the promyogenic RAGE, and a reduced ability to secrete mitogenic factors, including S100B, in aged SCs compared with young SCs. The extent to which the changes in these intrinsic factors reflect changes in the SC microenvironment or alterations of the cellular machinery regulating the expression levels of those factors remains to be established.

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