Stem Cell Research (2010) 4, 77–91

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REGULAR ARTICLE

An adult tissue-specific stem cell in its niche: A gene profiling analysis of *in vivo* quiescent and activated muscle satellite cells

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Received 9 October 2009; received in revised form 21 October 2009; accepted 21 October 2009

Abstract The satellite cell of skeletal muscle provides a paradigm for quiescent and activated tissue stem cell states. We have carried out transcriptome analyses on satellite cells purified by flow cytometry from *Pax3*$^{GFP/+}$ mice. We compared samples from adult skeletal muscles where satellite cells are mainly quiescent, with samples from growing muscles or regenerating (*mdx*) muscles, where they are activated. Analysis of regulation that is shared by both activated states avoids other effects due to immature or pathological conditions. This *in vivo* profile differs from that of previously analyzed satellite cells activated after cell culture. It reveals how the satellite cell protects itself from damage and maintains quiescence, while being primed for activation on receipt of the appropriate signal. This is illustrated by manipulation of the corepressor Dach1, and by the demonstration that quiescent satellite cells are better protected from oxidative stress than those from *mdx* or 1-week-old muscles. The quiescent versus *in vivo* activated comparison also gives new insights into how the satellite cell controls its niche on the muscle fiber through cell adhesion and matrix remodeling. The latter also potentiates growth factor activity through proteoglycan modification. Dismantling the extracellular matrix is important for satellite cell activation when the expression of proteinases is up-regulated, whereas transcripts for their inhibitors are high in quiescent cells. In keeping with this, we demonstrate that metalloproteinase function is required for efficient regeneration *in vivo*.

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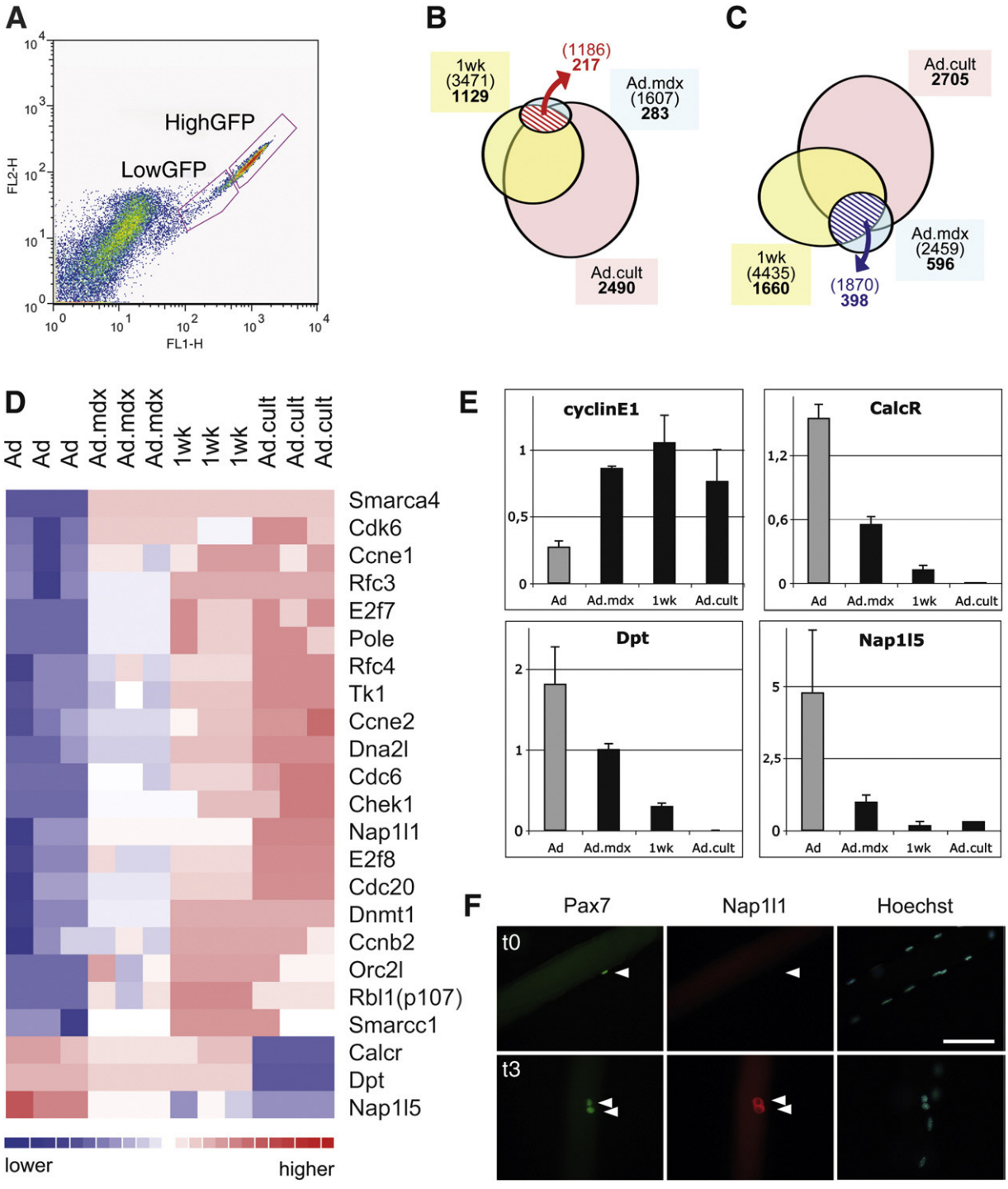
doi:10.1016/j.scr.2009.10.003

Introduction

Stem cells in many adult tissues are in a quiescent state. Their contribution to tissue repair depends on activation, which leads to proliferation and subsequent differentiation or return to quiescence. Skeletal muscle provides a model system for studying this pattern of cell behavior. This tissue regenerates after injury, and the muscle satellite cell is key to this process (Collins et al., 2005; Montarras et al., 2005). These quiescent cells lie under the basal lamina of the muscle fiber until activated in response to injury, when they leave this niche and proliferate before differentiating to form new muscle fibers or reverting to the quiescent satellite cell state on the newly formed fibers (Montarras and Buckingham, 2008). The satellite cell is characterized by the expression of *Pax7* (Seale et al., 2000) and also *Pax3* in many muscles (Relaix et al., 2006).

With the advent of transcriptome analyses, it has become possible to perform gene profiling on satellite cells and hence to further investigate their regulation. Previous transcriptome analyses on skeletal muscle were carried out either with whole muscle tissue in the course of regeneration (Zhao and Hoffman, 2004; Porter et al., 2004; van Lunteren and Leahy, 2007) or on cultured muscle



cells (Seale et al., 2004; McKinnell et al., 2008; Kumar et al., 2009). The former includes several cell types in addition to differentiating muscle fibers, while with the latter the question of quiescence cannot be addressed since culture leads to immediate activation.

Strategies have now been developed which permit characterization of satellite cells from adult muscle. They rely on the direct isolation of cells by flow cytometry on the basis of the expression of surface markers or targeted reporter expression (Montarras et al., 2005; Fukada et al., 2007; Day et al., 2007; Cerletti et al., 2008; Bosnakovski et al., 2008; Tanaka et al., 2009). Fukada et al. (Fukada et al., 2007) isolated quiescent satellite cells *in vivo* and compared their transcriptome to that of activated satellite cells under *in vitro* culture conditions. We now take this analysis an important step further by comparing the transcriptomes of quiescent with activated states *in vivo*. Our approach depends on the purification of satellite cells by flow cytometry from *Pax3*$^{GFP/+}$ mice (Montarras et al., 2005). Such cells from adult skeletal muscle, which are mainly quiescent, were compared to satellite cells, from 1-week postnatal muscle and from adult dystrophic muscle of *mdx* mice (Ikemoto et al., 2007), that are undergoing activation. Pax3GFP-positive cells from adult muscle were also cultured, providing a population of activated satellite cells, but with potential modifications due to *in vitro* culture conditions. Our analysis provides novel insight into the nature of the quiescent state and the response of the satellite cell to activation *in vivo*.

Results and discussion

Experimental strategy

We selected diaphragm, pectoralis, and abdominal muscles to purify satellite cells from *Pax3*$^{GFP/+}$ mice by flow cytometry (Montarras et al., 2005). In order to compare quiescent versus activated satellite cell states, we isolated these cells from adult (6-week-old) mice (Ad), in which most satellite cells are quiescent, confirmed by Ki67 labeling for which 97% of the GFP-positive satellite cells were negative (Supplementary Fig. 1). These adult cells were compared to cells isolated from *Pax3*$^{GFP/+}$:*mdx*/*mdx* (Ad.mdx) mice at the same stage, where the lack of dystrophin has engendered major skeletal muscle degeneration/regeneration, with activation of satellite cells (30% Ki67 positive, Supplementary Fig. 1), (Ikemoto et al., 2007). The *mdx* mouse represents a pathological situation and we therefore also used postnatal (1-week-old) muscles of *Pax3*$^{GFP/+}$ mice (1wk), where many satellite cells are activated (80% Ki67-positive cells, Supplementary Fig. 1), to ensure muscle growth (Relaix et al., 2006). The same FACS criteria of GFP fluorescence, size, and granularity, excluding other cell types and muscle fibers, were applied in all cases. The GFP reporter is relatively stable and continues to be detectable at a low level in differentiating cells when *Pax3* is down-regulated. We took advantage of this to separate the satellite cells into high and low GFP-positive fractions (Fig. 1A). Antibody staining of cells isolated by cytospin after flow cytometry revealed expression of some differentiated muscle markers in the low GFP fraction (results not shown). In the comparative analysis reported here, we have concentrated on the transcriptome of the high GFP fraction. Also included in this analysis is the transcriptome of cultured satellite cells, for comparison with previously published work. These *ex vivo* activated cells correspond to the progeny of satellite cells from the high GFP fraction of normal adult muscles, after 3 days in culture under conditions that promote their proliferation.

Analyses of the microarray data are presented in Supplementary Tables 1 to 6 and are summarized in Figs. 1B and C. In each comparison a minimum of 1.5-fold change was retained. We used both Benjamini-Hochberg (BH) and Bonferroni adjustments (see Materials and Methods), except for the comparison with *ex vivo* activated cells (Supplementary Table 3) where only the latter was applied because of the much greater number of differences. The highly stringent Bonferroni adjustment reduces the number of genes identified in the comparisons, but with a risk of eliminating transcripts of interest. When the two *in vivo* states are compared, this results in 217 genes which can be regarded as potential markers of quiescent satellite cells in adult muscles (Fig. 1B, Supplementary Table 4) and 398 which are up-regulated on *in vivo* activation (Fig. 1C, Supplementary Table 5). We then employed the more commonly used BH

Figure 1 Scheme of the experimental strategy and validation of the *in vivo* models: genes associated with proliferation are down-regulated in quiescent satellite cells. (A) Flow cytometry diagram showing high and low GFP fluorescent fractions of satellite cells from adult skeletal muscle. (B) Comparison of the transcriptome of *Pax3*$^{GFP/+}$ cells from adult muscle (Ad), in which most satellite cells are quiescent, with those from regenerating *Pax3*$^{GFP/+}$:*mdx*/*mdx* (Ad.mdx, blue), growing 1-week-old muscle (1wk, yellow), or adult *Pax3*$^{GFP/+}$ cells after 3 days in culture (Ad.cult, pink). The number of genes with significant differential expression was identified using two algorithms for multiple comparisons: Benjamini-Hochberg (BH), results within parentheses, and the more stringent Bonferroni algorithm, results shown in bold. The numbers of genes which are up-regulated in Ad satellite cells compared to Ad.mdx and 1wk activated states are shown hatched in red. (C) A similar comparison showing the numbers of genes up-regulated in activated Ad.mdx and 1wk compared to Ad satellite cells (hatched in blue). (D) An intensity map, showing differences in transcript levels in satellite cells from Ad, Ad.mdx, 1wk, and Ad.cult experimental groups. Triplicate samples are shown for each group. Blue color indicates low and red indicates high relative transcript levels. Genes shown were selected from the list presented in Supplementary Tables 4 and 5 and include those involved in cell cycle progression and cell division (*cyclins*/*Ccn*, *Cdc*, *Cdk*), DNA replication (*Rfc*, *Orc*, *Pole*, *Dna2l*), or transcriptional activity (*E2f*, *Rbl1*/*p107*). (E) Transcript levels measured by qPCR for cyclin E1, calcitonin receptor (CalcR), dermatopontin (Dpt), and Nap1l5. Samples are as in D. (F) Immunofluorescence on a single fiber preparation at time zero (t0) and after 3 days in culture (t3) showing satellite cells labeled with a Pax7 or a Nap1l1 antibody. Hoechst staining of Pax7-positive satellite cell nuclei (arrow heads) and myonuclei is shown. Bar = 100 μm.

adjustment to analyze the microarray data, in order to include more genes of potential interest in our comparisons. With the BH adjustment, these numbers are respectively 1186 (Fig. 1B, Supplementary Table 4) and 1870 (Fig. 1C, Supplementary Table 5). This common fraction obviates variations due to the pathological state of adult *mdx* mice, or to physiological conditions associated with growth of 1-week postnatal mice, as well as the many changes which are introduced as a result of culture. In subsequent analyses, we focused our attention on genes showing changes in both Ad-Ad.mdx and Ad-1wk comparisons after the BH adjustment and at least in one of the two after Bonferroni adjustment.

Genes associated with proliferation

When quiescent cells are activated in response to a requirement for muscle growth or repair, they proliferate. Examination of transcripts of genes associated with proliferation shows that they are up-regulated in cells from postnatal or regenerating muscle, as well as in culture, thus

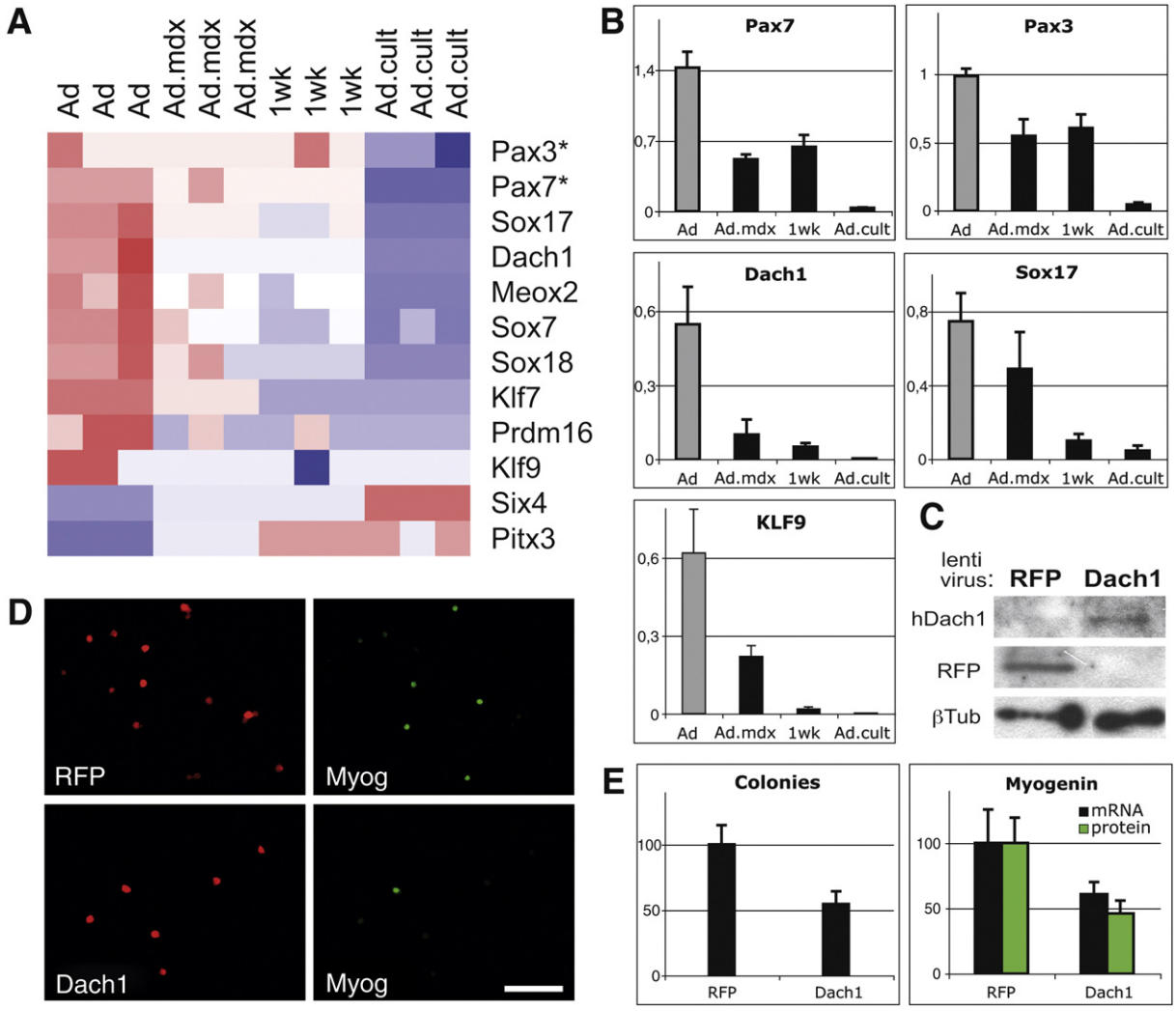


Figure 2 Changes in the expression of genes encoding transcription factors associated with Pax-positive progenitor cell behavior: involvement of Dach1 in the inhibition of satellite cell proliferation and differentiation. (A) An intensity map, showing differences in transcript levels, presented as in Fig. 1D. * did not show differences in the *in vivo* Bonferroni comparisons. (B) Transcript levels measured by qPCR for Pax7, Pax3, Dach1, Sox17, and Klf9. Samples are as in Fig. 1E. (C–E) Transduction of freshly isolated satellite cells from adult *Pax3*$^{GFP/+}$ muscles by a lentiviral vector expressing human (h) Dach1 or control RFP. (C) Western blot analysis showing overexpression of h Dach1 and RFP proteins. β-Tubulin is shown as a control for protein loading. (D) Immunofluorescence staining of satellite cells using anti-RFP or anti-hDach1 antibodies (red) or anti-myogenin (Myog) antibodies (green) after 4 days of culture. Bar = 100 μm. (E) Proliferation (left panel), after 3 days of culture, is expressed as the percentage of colonies formed by Dach1-transduced cells compared to RFP-transduced cells. Differentiation (right panel), measured by the level of myogenin mRNA determined by qPCR (black bars) and the number of myogenin expressing cells (green bars) detected by immunofluorescence, is similarly expressed as a percentage. Two independent experiments with triplicate samples were carried out for each measurement. Error bars represent standard errors.

validating the *in vivo* models and demonstrating that normal adult satellite cells are mainly quiescent. This is the case for the Gene Ontology classes referring to cell cycle progression. Supplementary Table 6 shows, as an example, the list of genes found in the DNA replication class. A selection of genes involved in cell cycle progression is shown in the intensity map (Fig. 1D) with *Cyclin E1* (*Ccne1*) shown as an example of qPCR analysis (Fig. 1E). Transcripts of the calcitonin receptor (*Calcr*), which was identified as a quiescent satellite cell marker based on *ex vivo* comparison (Fukada et al., 2007), are also lower in activated cells *in vivo*, but the comparison shows a much more striking (160-fold) down-regulation in cultured cells (Fig. 1E). *Dermatopontin* (*Dpt*) also referred to as *"Early quiescence 1"* (Supplementary Reference 1, SR1) provides another example of a gene that is more highly expressed in adult quiescent satellite cells (Fig. 1E) and strikingly down-regulated in culture.

Transcripts for major chromatin remodeling complexes associated with cell proliferation/transcription are up-regulated in Ad.mdx and 1wk satellite cells. This is illustrated by Smarca4/Brg1 and Smarcc1, components of the SWI/SNF complex (Fig. 1D), also directly implicated in the promotion of myogenesis (Ohkawa et al., 2007). An interesting example is provided by members of the Nap family of nucleosome assembly factors (SR2). Nap1l1 accumulates after satellite cell activation in culture (Fig. 1F), as expected for the role of this protein in chromatin assembly after cell division. However, Nap1l5 transcripts are high in quiescent satellite cells (Fig. 1E), suggesting a different role.

Transcription factors associated with Pax-positive myogenic progenitor cells

In Fig. 2A, the expression of genes encoding transcription factors potentially involved in maintenance of the muscle progenitor cell state, as illustrated by the presence of Pax3/7, is compared. *Pax7*, and to a lesser extent *Pax3*, transcripts are relatively higher in Ad satellite cells (Figs. 2A and B). Six homeodomain proteins, Six1 and Six4, are important upstream regulators of myogenesis intervening with Pax3 and Pax7 in the embryo (Buckingham and Relaix, 2007). *Six1* transcripts and those for the Six coactivators *Eya1, 3,* and *4,* are present, but show little differences, except in cultured cells (Supplementary Tables 1–3). *Six4*, also transcribed in Ad cells, shows up-regulation in Ad.mdx and 1wk samples (Fig. 2A). Transcripts for Dach1 are notably up-regulated in quiescent adult satellite cells (Fig. 2B). Dach1 is potentially involved in repressing Six activity (Li et al., 2003), thus avoiding myogenic activation by Six in quiescent satellite cells. Dach1 also inhibits cyclin D1 and cell cycle progression (Wu et al., 2006), which may reflect a role in maintenance of quiescence. In order to address these issues, freshly isolated adult satellite cells were transduced with a lentiviral vector carrying human (h) Dach1 cDNA and their proliferation and differentiation potential was assayed in culture (Figs. 2C–E). We observed a 46% reduction in the capacity of the cells to proliferate as measured by the number of colonies formed in culture (Fig. 2E). An effect on differentiation was detected at the onset of terminal differentiation, marked by the expression of myogenin. A 2.2-fold reduction in myogenin expression was found at both the transcript and the protein level in Dach1-transduced cells, compared to control cells transduced with a lentiviral vector encoding RFP (Figs. 2D and E). Taken together, these results argue in favor of a role of Dach1 in the control of proliferation and differentiation in muscle satellite cells.

Meox2, expressed in myogenic progenitor cells in the embryo and implicated in limb myogenesis (Mankoo et al., 1999), is transcribed at a high level in Ad satellite cells and, like *Dach1*, is down-regulated in Ad.mdx and 1wk samples and notably in cultured satellite cells. *Pitx3*, which encodes another homeobox transcription factor active in later fetal myogenesis, is expressed at a lower level in adult quiescent satellite cells compared to activated cells, in keeping with its role in myogenesis (L'Honore et al., 2007).

Transcripts of a number of *Sox* genes are present in satellite cells (Supplementary Tables 1–3). *Sox7, Sox17*, and *Sox18* transcripts are higher in quiescent adult satellite cells (Figs. 2A and B). The Sox family of transcription factors acts with Pax factors in the specification of a number of tissues (Buckingham and Relaix, 2007). This might suggest that these members of the Sox family are involved in the maintenance of myogenic identity and/or quiescence of adult satellite cells. Our analysis also revealed increased levels of transcripts encoding Prdm16 (Fig. 2A), which is a cotranscriptional activator of the nuclear receptor Pparγ, a master regulator of adipocyte differentiation. *Pparγ* is also transcribed at higher levels in Ad cells (Supplementary Tables 1, 2, and 4). Prdm16 has been implicated in switching myogenic precursor cells to the brown fat lineage (Kajimura et al., 2009) and the presence of these transcripts probably reflects some degree of intrinsic plasticity of satellite cells, which can undergo adipogenesis under certain conditions (Asakura et al., 2001; Shefer et al., 2004).

This may also be suggested by the high levels of Klf7 and Klf9 transcripts in these cells (Figs. 2A and B). Klf4, another member of this family of Kruppel-like transcription factors, is associated with the acquisition of pluripotency (Takahashi and Yamanaka, 2006). Klf9, like Klf4, also inhibits proliferation (Good and Tangye, 2007), thus pointing to a potential role in quiescence.

Signaling pathways

Canonical Wnt and Notch signaling pahways have been implicated in satellite cell activation (Brack et al., 2008). Consistent with this, we see higher levels of transcripts for Wisp1 (Wnt induced signaling pathway protein 1) (Figs. 3A and B) and Hes6, a readout of Notch signaling (Figs. 3C and D) in Ad.mdx and 1wk samples. In contrast, transcripts for *Axud1*, also referred to as *Csrpn1*, are higher in Ad cells (Figs. 3A and B). This gene is up-regulated by Axin 1, an inhibitor of Wnt signaling. Noncanonical Ca^{2+}/PKC-dependent Wnt signaling, on the other hand, may be involved in the maintenance of quiescence (Otto et al., 2008), as indicated by transcript levels of PKCη (*Prkch*) (Fig. 3A). Small changes (detected after the BH adjustment) were observed in transcripts for Frizzled7 and Prickle3, components of the noncanonical Wnt planar cell polarity pathway (Supplementary Tables 1 and 2). Interestingly *Lnx1*, which encodes a Numb interacting scaffold protein, is highly expressed in

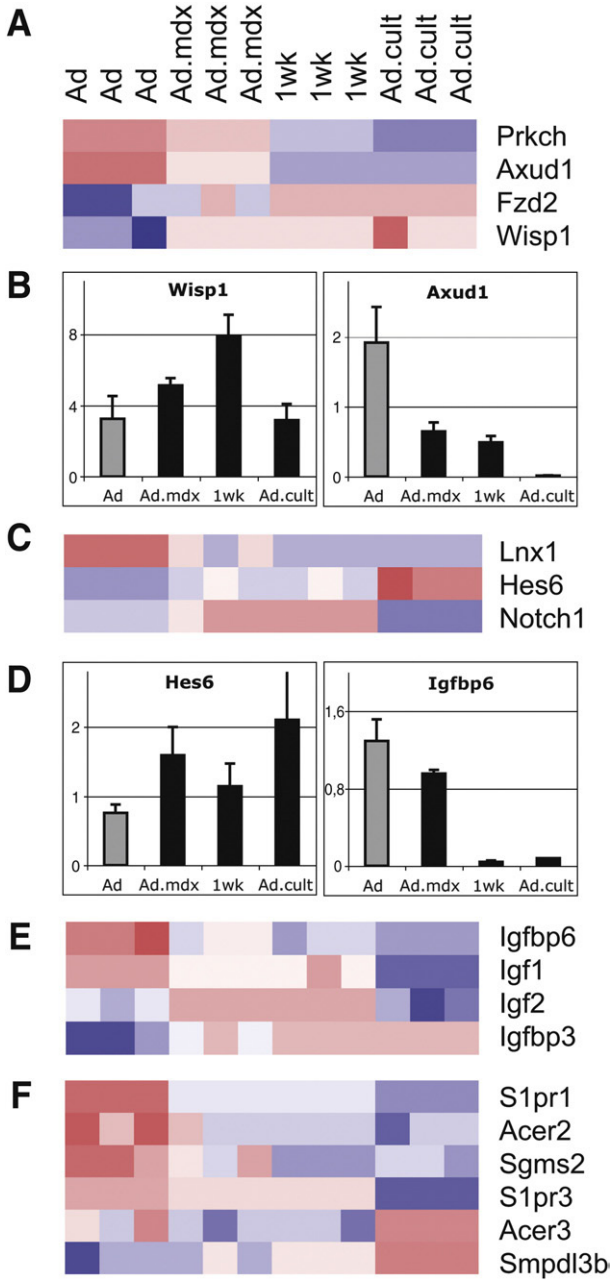


Figure 3 Transcriptome differences in signaling pathways during *in vivo* activation. (A, C, E, F) Intensity maps, showing examples of differences in the level of transcripts for components of Wnt (A), Notch (D), Igf (E), and sphingolipid (F) signaling pathways, presented as in Fig. 1D. (B, D) Levels of transcripts measured by qPCR, for genes implicated in Wnt (B, *Wisp1*, Wnt1 inducible signaling pathway protein 1, and *Axud1*, Axin-1 up-regulated gene 1), Notch and Igf (D, *Hes6*, Hairy and enhancer of split 6 and *Igfbp6*, Igf binding protein 6) signaling.

quiescent satellite cells. Transcripts for Numb, which antagonizes Notch activity, are higher in Ad cells (BH adjustment only). Lnx1 also interacts with cell adhesion molecules involved in apical tight junction formation (SR3) and may therefore play a role in asymmetric cell division of satellite cells on the muscle fiber, observed at the onset of activation (Kuang et al., 2007).

Changes in the expression of the Igf (Figs. 3D and E) and Pdgf (Supplementary Figs. 2A and B) signaling pathways reflect their involvement in satellite cell activation. This is illustrated by the up-regulation of the gene for Igf binding protein, Igfbp3, that transports Igf and potentiates its signaling (SR4), whereas transcripts for Igfbp6, that sequesters Igfs (SR4) and thus prevents activation, are high in Ad cells (Figs. 3D and E).

Tgfβ/Bmp signaling tends to antagonize myogenesis (Kollias and McDermott, 2008) and transcripts for components of this pathway are highly expressed in Ad cells (Supplementary Figs. 2C and D), in keeping with a role in preventing myogenic progression in quiescent satellite cells.

Signaling through sphingolipids is also a feature of Ad satellite cells (Fig. 3F), which express sphingomyelin in their plasma membrane (Nagata et al., 2006). Transcripts for sphingomyelin synthetase (*Sgms2*), that converts ceramide to sphingomyelin, are high in quiescent cells, whereas the gene encoding sphingomyelin phospodiesterases (*Smpdl3b*) is down-regulated. Accumulation of ceramide triggers apoptosis and is also avoided by high level expression of genes for ceramidases (*Acer2* and *3*) that convert it to sphingosine (SR5). Transcripts for sphingosine receptors (*Sr1pr1* and *3*) (SR5) are high in quiescent satellite cells. Signaling through these receptors is likely to promote survival and activation when spingosine-1-phosphate becomes available (Nagata et al., 2006).

Resistance to xenobiotics and oxidative stress

Quiescent satellite cells have developed strategies that confer resistance to xenobiotics, genotoxics, and oxidative stress. This is illustrated in Fig. 4 (see also Supplementary Tables 4 and 5). They express high levels of genes such as *Abcb1a*, *Abca5*, and *Abcc9* encoding efflux channels of the multidrug resistance family that pump toxic substances out of the cell. Transcripts for proteins involved in the solubilization of toxins by hydroxylation, such as many cytochrome mono-oxygenases of the p450 family (examples, *Cyp4b1*, *Cyp26b1*) or the Flavin mono-oxygenase (*Fmo2*), are also high. This is also the case for other modifying enzymes that increase solubilization of xenobiotics, such as UDP-glucuronosyl transferase (*Ugt1*), glutathione transferase (*Gstm1*), or sulfotransferase (*Sult1a1*) (SR6). Transcriptional activation of further detoxification enzymes depends on the gene encoding the Aryl hydrocarbon receptor (*Ahr*) expressed at a higher level in quiescent, compared with activated, satellite cells. Interestingly, in screening for Pax3/7 target genes in the C2 muscle cell line, *Ahr* and *Abcb1a* were identified (Kumar et al., 2009; McKinnell et al., 2008). The Ahr receptor translocates to the nucleus on binding toxic molecules such as dioxin derivatives or polycyclic aromatic hydrocarbons, where it interacts with transcription factors of the Aryl hydrocarbon nuclear translocator (*Arnt*) family (SR7). *Arnt2* transcripts are higher in activated satellite cells, indicating that this form is more important for cell activation. The Arnt2 factor also interacts with hypoxia inducible factor, which is expressed after muscle injury and during growth, when the vasculature is damaged or still developing and tissue oxygenation is compromised. In this context, transcripts for angiotensin II

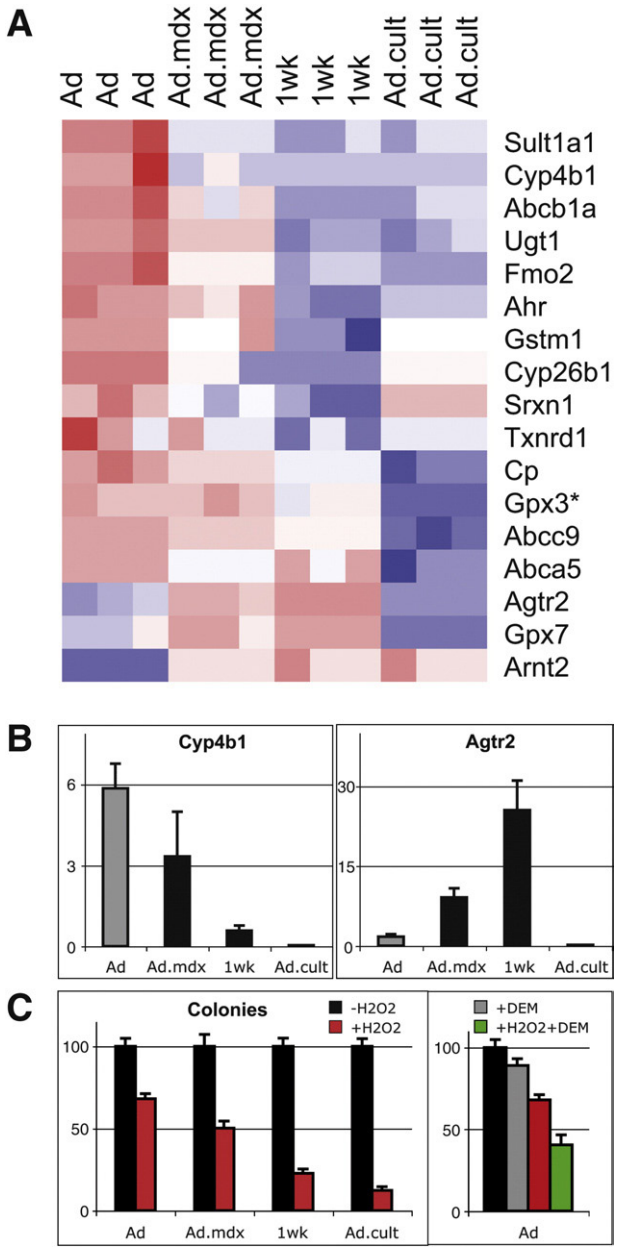


Figure 4 Quiescent satellite cells up-regulate genes for detoxification, protection from xenobiotics, and resistance to oxidative stress. (A) An intensity map, showing differences in transcript levels, presented as in Fig. 1D. * Levels were not detected as different with the BH adjustment in Ad versus Ad.mdx cells. (B) Transcript levels, measured by qPCR, for a member of the cytochrome P450 family (*Cyp4b1*) and for the angiotensin type 2 receptor (*Agtr2*). Samples are indicated as in Fig. 1E. (C) Left panel, the antioxidant capacity of each satellite cell sample is expressed as the percentage of colonies formed after 3 days in culture in cells after treatment with 50 μM H_2O_2 (red bars) compared to untreated cells (black bars). Right panel, treatment with the reduced glutathione depleting agent, diethylmaleate (DEM), increases the sensitivity of adult quiescent cells to H_2O_2 (green bar), compared to controls: nontreated cells (black), cells treated with DEM alone (gray), and H_2O_2 alone (red). Results are expressed as in the left panel. Data presented are from a minimum of three independent experiments, performed in triplicate. The error bars represent standard errors.

type 2 receptor (*Agtr2*) are markedly up-regulated in *in vivo* activated cells (Figs. 4A and B) where it may protect them from DNA damage and senescence, as shown for vascular smooth muscle cells (Min et al., 2008).

Genes for enzymes implicated in the response to oxidative stress (SR8) are also highly expressed in quiescent cells, as exemplified by *Srxn (Sulfiredoxin)* (Jonsson and Lowther, 2007) which controls reactive oxygen species-mediated cytotoxicity, glutathione peroxidase 3 (*Gpx3*), a secreted form, which scavenges oxygen radicals as part of the reactive oxygen species-redox system, or the intracellular thioredoxin reductase 1 (*Txnrd1*). Transcripts for ceruloplasmin (*Cp*), which chelates metals such as copper or iron required for redox reactions, are also high in quiescent satellite cells. Administration of hydrogen peroxide to satellite cells shows that adult quiescent cells are better protected from oxidative stress, as measured by the number of cells that form colonies (Fig. 4C). The role of Gstm1, Gpx3, and Srxn is demonstrated by increased sensitivity of these cells on addition of the reduced glutathione depleting agent, diethylmaleate (DEM) (Plummer et al., 1981) (Fig. 4C). This is in keeping with observations on *Gpx1* mutants (Lee et al., 2006).

Cell adhesion and the extracellular matrix

Genes for cell–cell adhesion molecules (Figs. 5A and B), such as claudins (*Cldn1,5*), intercellular adhesion molecule 2 (*Icam2*), epithelial V-like antigen 1 (*Eva1/Mpzl2*), and endothelial specific adhesion molecule 1 (*Esam1*), are notably expressed in Ad satellite cells (Figs. 5A and B). Esam1 and claudin 5 were thought to be confined to blood vessels where they mark tight junctions between endothelial cells (SR9). Their expression, together with CD38 that is the receptor for Pecam1, on satellite cells may be important in promoting contiguity with blood vessels (Christov et al., 2007); see also (Abou-Khalil et al., 2009). *Esam1* has also recently emerged as a marker of adult hematopoietic stem cells (SR10). Integrins on the cell surface promote adhesion and signaling through interaction with fibronectin, collagens, and laminins (SR11, SR12). Integrin β1 (*Itgb1*) transcripts are high in quiescent cells, as are those for syndecan 4 (*Sdc4*), a satellite cell marker which promotes integrin-mediated cell adhesion and is required for a rapid satellite cell response to activation (Cornelison et al., 2004). In contrast, genes for the laminins, such as *Lamb1* and the integrin β3 binding protein (*Itgb3 bp*) that promotes Integrin reactions with substrates and integrin signaling (*Integrin* β3 is also transcribed in satellite cells), are up-regulated in Ad.mdx and 1wk cells. The *Collagen* genes, *Col1α1* and *Col1α2*, are also more highly expressed in these cells, as is the gene for *Syndecan 1* (*Sdc1*) which enhances integrin α2β1 interactions with collagens, leading to up-regulation of *matrix metalloproteinase* genes (SR13, Fig. 6).

The genes for N-cadherin (*Cdh2*) and its intracellular interactor, α-catenin (*Ctnna2*) are up-regulated in Ad.mdx and 1wk satellite cells. N-cadherin in the embryo marks cells that will enter myogenesis (Cinnamon et al., 2006) and is also associated with their migration (Brand-Saberi et al., 1996) (Fig. 5A). Transcripts for the surface glycoprotein CD24 that prevents proliferation of progenitor cells in self-renewing

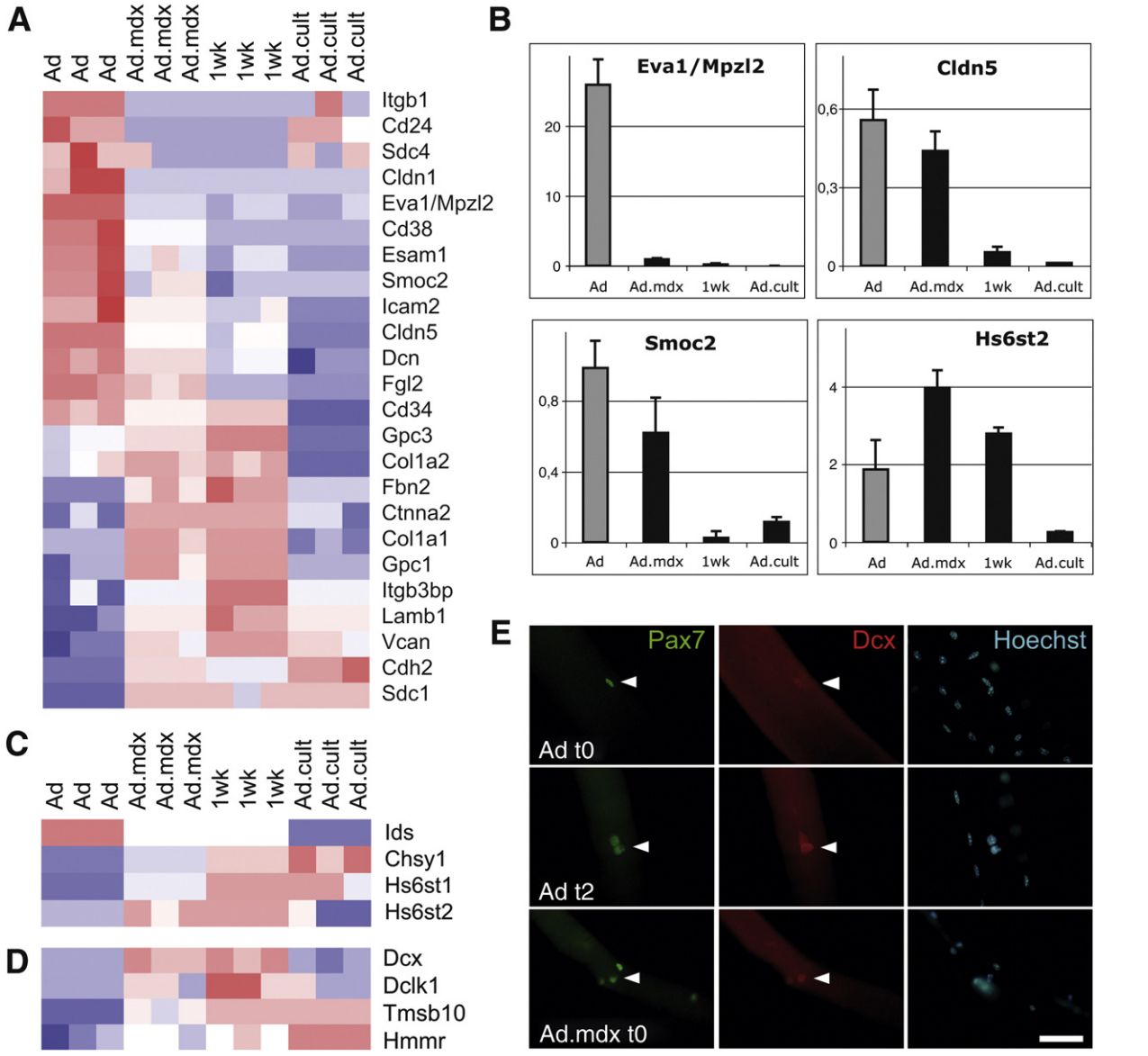


Figure 5 Genes implicated in cell adhesion, extracellular matrix, and cell mobility are modulated during satellite cell activation. (A, C, and D) Intensity maps, showing differences in transcript levels of genes for adhesion molecules and extracellular matrix components (A), proteoglycan modifying enzymes (C), and proteins implicated in cell mobility (D). Samples are as in Fig. 1D. (B) Transcript levels, measured by qPCR, for cell adhesion (*Eva1*, *Cldn5*), matrix component (*Smoc2*), and heparan sulfotransferase (*Hs6st2*) genes. (E) Immunofluorescence staining with Pax7 and doublecortin (Dcx) antibodies of satellite cells, marked by Pax7 (arrowheads), on single fibers from adult EDL muscle immediately after isolation (Ad t0), and after 2 days in culture (t2). A fiber from an adult *mdx* EDL muscle at t0 (Ad.mdx t0) shows that satellite cells already express detectable levels of Dcx. Hoechst staining marks nuclei. Bar = 50 μm.

tissues (SR14) are higher in Ad cells (Fig. 5A), providing another potentially interesting surface marker.

Many genes encoding extracellular matrix proteins (Fig. 5A) are highly expressed by Ad satellite cells. This is exemplified by *Fgl2*, encoding fibrinogen-like 2, involved in cell adhesion (SR15) and not previously associated with muscle, or *Smoc2* (Figs. 5A and B), encoding a calcium binding protein which regulates cell/matrix interactions (SR16). As previously noted, transcripts for the small secreted proteoglycan, Decorin (*Dcn*), that potentiates TGFβ signaling (Cabello-Verrugio and Brandan, 2007) are also very high in Ad cells (Supplementary Fig. 2D). Genes for extracellular matrix proteins that bind growth factors modulating their availability (SR17), such as Versican (*Vcan*), Glypicans 1 and 3 (*Gpc1,3*), or Fibrillin 2 (*Fbn2*), are more highly expressed in Ad.mdx and 1wk cells.

Expression of genes encoding enzymes involved in proteoglycan sulfation also undergoes major changes (Figs. 5B and C). Ad.mdx and 1wk satellite cells express high levels of heparan sulfate 6-*O* sulfotransferase (*Hs6st1* and 2) transcripts (Figs. 5A and B). Hs6st transfers sulfate groups to heparan, allowing it to bind signaling molecules, promoting their activity and presentation to cellular receptors. Genes encoding components of the Fgf signaling pathway, such as Fgf receptors and Sproutys, implicated in

myogenesis (Buckingham and Relaix, 2007; Lagha et al., 2008), did not show significant changes in expression. We conclude that in the postnatal *in vivo* context, Fgf signaling is mainly regulated by heparan sulfation orchestrated by the satellite cell. Indeed, sulfatase deficiency negatively affects muscle regeneration (Langsdorf et al., 2007). The up-regulation of the chondroitin sulfate synthase 1 gene (*Chsy1*) in Ad.mdx and 1wk cells is also indicative of matrix remodeling (Izumikawa et al., 2008). On the other hand, transcripts for iduronate-2 sulfatase (*Ids*), an enzyme responsible for the degradation of glycosaminoglycan (Moro et al., 2010), are high in Ad satellite cells (Fig. 5C).

On activation, as the niche environment breaks down, satellite cells move from the fiber. Transcripts for factors associated with motility are up-regulated. Examples are shown in Fig. 5D. *Doublecortin (Dcx)* is 14- to 16-fold up-regulated in Ad.mdx and 1wk cells, an effect, like for others in Fig. 5A, not reproduced in culture. Doublecortin is a microtubule stabilizing protein, indispensable for cortical migration of neurons (SR18). Its unexpected presence on activated satellite cells is confirmed by immunodetection (Fig. 5E). Transcripts for a protein very similar to doublecortin, Dclk1, also show up-regulation in activated satellite cells, where it may also be involved in migration, as well as mitosis (SR18). Up-regulation of the gene for the hyaluronan mediated motility receptor (*Hmmr*), required for migration of fibroblasts in injured skin (SR19) and for thymosin β10 (*Tmsb10*), that regulates actin dynamics critical for motility (SR20), is also high in Ad.mdx and 1wk cells and provides another example of the potentiation of satellite cell migration.

Proteinases and inhibitors involved in the regulation of the niche

Strikingly, a number of genes encoding inhibitors of proteinases, such as *Tfpi2* or *Serpin1*, that prevent extracellular matrix remodeling, are up-regulated in Ad satellite cells, whereas transcripts for proteinases are up-regulated in Ad.mdx and 1wk cells (Figs. 6A and C). Proteinases of the matrix metalloproteinase (MMP) and Adam families and their inhibitors illustrate this aspect. Transcripts for the former (ex. *Adam12* and *19*, *Adamts2* and *7*, *Mmp11*) are low in quiescent cells and increase on activation. This is illustrated for Adam19 at the protein and transcript level (Figs 6A, B and C). Exceptions are *Mmp2* and *Adamts1*; however, the former encodes an enzyme that requires proteolytic cleavage to be active (SR21) and the latter an enzyme involved in the release of Tgfβ (SR22), implicated in quiescence. Many genes for the inhibitors of matrix degrading enzymes (*Timp2*, *3*, *4*) or proteins involved in their association with the extracellular matrix (*Efemp1*) are up-regulated in Ad cells, also shown by qPCR for *Timp4* and *Tfpi2* (Fig. 6C). The requirement for MMPs in satellite cell activation and muscle regeneration is substantiated by the result of systemic delivery of the MMP inhibitor, AM409 (Defamie et al., 2008), in *mdx* mice (Figs. 6D–G). We found a 3.5-fold decrease in the percentage of regenerating fibers in the diaphragm of AM409 treated *mdx* mice, together with a 2.5-fold decrease in the percentage of activated MyoD-positive cells (Fig. 6F). This suggests that MMPs are involved in the activation of satellite cells required for regeneration. We also found that muscle regeneration was delayed in cardiotoxin-injured tibialis anterior muscle of AM409 treated wild-type mice (Supplementary Fig. 3). MMPs, implicated in regeneration (Oh et al., 2004; Li et al., 2009), are also activated in other cell types at the site of injury, such as macrophages. In order to show that the effect on regeneration also reflects a direct role of MMPs in satellite cell activation, we treated cultured single fibers from normal adult muscle for 2 days with 10 μM AM409. This led to a marked reduction in the expansion of MyoD-positive cells along the fibers, measured as the number of clustered MyoD-positive cells that have progressed beyond the first cell division. The number of clusters with more than two cells was reduced from 59 to 34% on treatment with AM409. A significant reduction in the average number of MyoD-positive cells per fiber was also observed in AM409-treated cultures (Fig. 6G, left panel). These results suggest a cell autonomous requirement for MMP activity in satellite cell expansion.

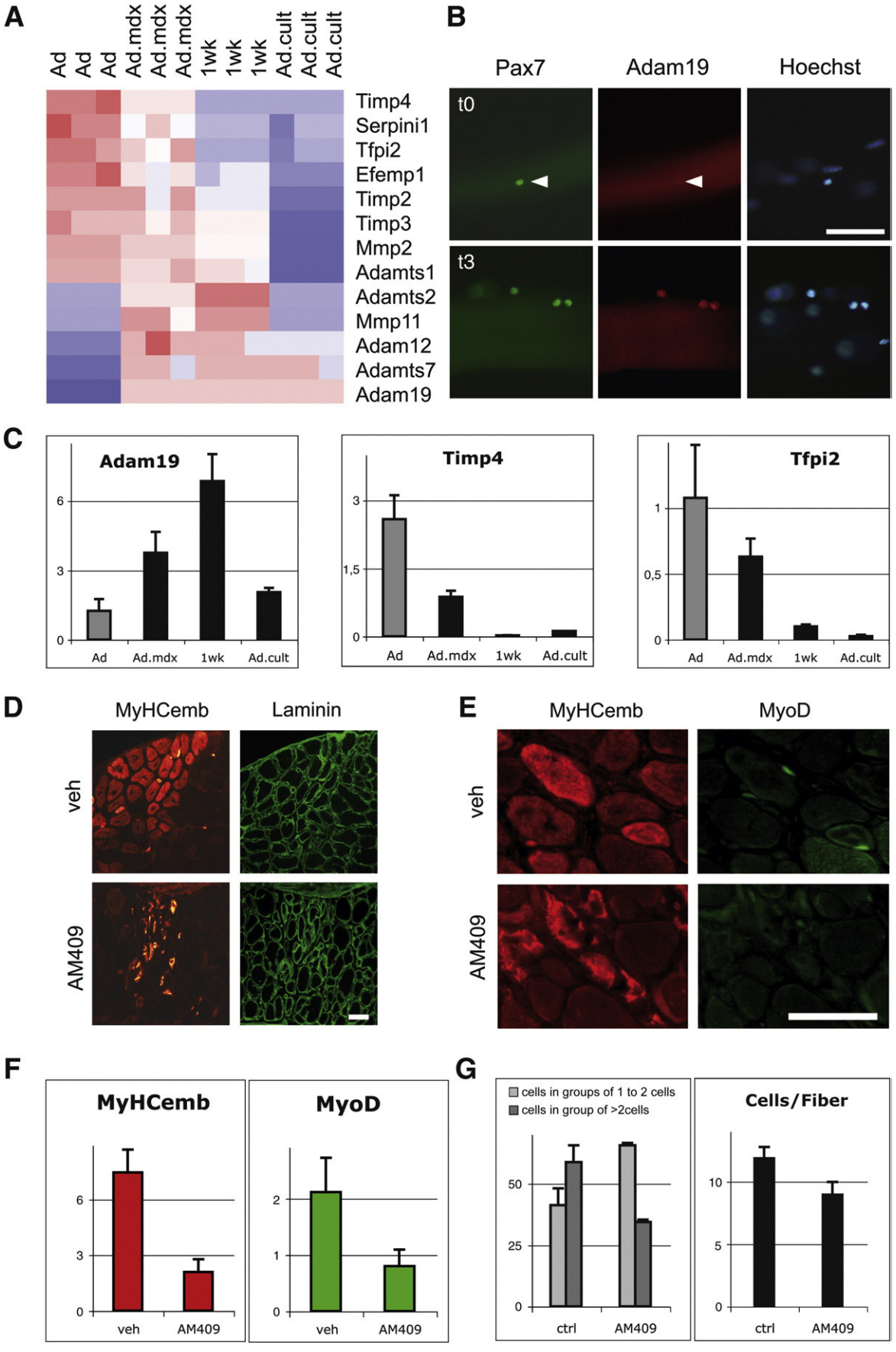
Conclusions

We report here the first transcriptome analysis of quiescent versus *in vivo* activated satellite cells. As shown by Ki67 labeling and expression of genes associated with proliferation, most adult satellite cells are quiescent. Satellite cells from *mdx* and growing muscle, which show different degrees of activation, are subject to particular environmental influences; however, the comparison of transcripts that show changes between adult and both adult *mdx* and 1week satellite cells selects those involved in quiescence versus activation. Proliferating cultured satellite cells display accentuated differences in the expression of many genes, as already noted (Fukada et al., 2007), but this comparison can be misleading in terms of the member of a gene family that changes (e.g., *Notch3* instead of *Notch1*, or *Igfbp7* instead of *Igfbp6*) or of genes that show no or different changes in expression on *in vivo* versus *in vitro* activation (e.g., *Dcx*, involved in satellite cell mobilization, or *Gcp3* implicated in extracellular matrix modifications that affect signaling).

Our analysis reveals expression of genes for novel cell surface markers (e.g., *CD24*) and regulatory factors (e.g., *Prdm16*) in quiescent satellite cells. Accumulation of transcripts for family members of factors associated with pluripotency suggests that satellite cells, like neural stem cells (Kim et al., 2009), may be prone to reprogramming. High levels of transcripts for Sox17, and the closely related Sox18, in quiescent cells, may also be important for protection from apoptosis, as shown for hematopoietic stem cells (Kim et al., 2007). This is a function that Pax7 performs before and at birth (Relaix et al., 2006). However, Pax7 is not essential later (Lepper et al., 2009), when Sox17/18 may ensure survival, together with other protective mechanisms, such as that of sphingolipid signaling.

Protection of stem cells from aggressive agents is critical, particularly in a tissue like muscle where there is little turnover and satellite cells remain quiescent for long periods. The transcriptome analysis shows that adult satellite cells are well armed in this respect, expressing a battery of genes that confer resistance to toxic molecules.

Adult satellite cells are primed to respond rapidly to activation signals, but are held back by expression of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Serpini1
Tfpi2
Efemp1
Timp2
Timp3
Mmp2
Adamts1
Adamts2
Mmp11
Adam12
Adamts7
Adam19
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Pax7
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veh
AM409
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cells in groups of 1 to 2 cells
cells in group of >2cells
50
25
0
ctrl
AM409
Cells/Fiber
10
5
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ctrl
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inhibitors. This is illustrated by high levels of transcripts for the insulin binding protein, Igfbp6, or by Dach1 that can act as a co-repressor of Six transcription factors.

Another characteristic feature of stem cells *in vivo* is that they are sequestered in a particular environment or "niche." Our comparison shows down-regulation of genes encoding adhesion molecules on activation and changes in transcripts for extracellular matrix components that will also affect the response to growth factors, such as Fgfs. Notably, proteinase activity, required for breakdown of the niche and efficient regeneration, is inhibited in quiescent satellite cells.

Our analysis underlines the importance of *in vivo* experiments for understanding the muscle stem cell. We provide new insight into how these cells orchestrate their own maintenance and protection as quiescent satellite cells within the niche where they are poised for activation and also how they respond in order to leave the niche to become activated, proliferating myogenic cells. The skeletal muscle paradigm has its special features—a single quiescent stem cell within its niche, for example. However the control of quiescence versus activation is a central issue in stem cell biology. Characterization of the muscle stem cell in this respect reveals underlying general principles.

Materials and methods

Transgenic mice

The *Pax3*$^{GFP/+}$mouse line (Montarras et al., 2005) and *Pax3*$^{GFP/+}$*:mdx/mdx* mice obtained by crossing *mdx/mdx* (Bulfield et al., 1984) with *Pax3*$^{GFP/+}$mice were used to prepare satellite cells. All animal experiments were carried out according to the regulations of the French Ministry of Agriculture and Fisheries practiced by the Institut Pasteur animal facility.

Flow cytometry, satellite cell isolation, and cell culture

Satellite cells were isolated from a pool of diaphragm, pectoralis, and abdominal muscles of *Pax3*$^{GFP/+}$ mice, by flow cytometry as previously described (Montarras et al., 2005), on the basis of size and granularity and GFP fluorescence. The window of GFP positivity was subdivided into the same high and low GFP fractions for all samples (Fig. 1A). Muscle satellite cells were isolated from three experimental groups: (1) adult 6-week-old *Pax3*$^{GFP/+}$(Ad), (2) adult 6-week-old *Pax3*$^{GFP/+}$*:mdx/mdx* (Ad.mdx), and (3) postnatal 1-week-old *Pax3*$^{GFP/+}$ (1wk) mice. Cells were directly collected in RLT buffer (Qiagen) for RNA extraction or plated on gelatin-coated dishes in growth medium (Relaix et al., 2006) for 3 days before RNA extraction, to obtain the fourth experimental group of *ex vivo* activated satellite cells (Ad.cult).

For each experimental group three independent samples were prepared for analysis. For each sample, cells were isolated from at least six mice (males and females in equal proportion).

The percentage of GFP-positive cells in each sample was 100%, as expected since this was the basis on which cells were purified by flow cytometry. The GFP-positive cells that we analyzed corresponded to 10–15% of the small, nongranular population.

As a control of viability and purity, cells from each sample were plated at low density. All the colonies formed after 3 to 5 days were myogenic on the basis of MyoD, myogenin, or troponin T expression (data not shown), indicating that we had isolated pure samples of muscle progenitor cells.

RNA collection and microarray analysis

Total RNA was extracted and purified after DNase treatment (Promega) using the RNAeasy Micro kit (Qiagen). RNA and cRNA quality was monitored on Agilent RNA Pico LabChips (Agilent Technology). cRNAs obtained from 100 ng of RNA were amplified by using the GeneChip Expression Two-Cycle 3′ amplification system (Affymetrix). Fragmented biotin-labeled cRNA samples were hybridized on Mouse 430_2.0 GeneChip Genome arrays, according to the manufacturer's protocol. Three independent isolates were analyzed for each experimental group. The generation of cell intensity files and the quality control of hybridizations were performed with GeneChip Operating Software (Affymetrix).

Figure 6 Expression analysis of genes encoding extracellular proteinases and their inhibitors: matrix metalloproteinases (MMP) are required for efficient satellite cell activation and muscle regeneration. (A) An intensity map, showing differences in the transcript level of genes for proteinases (Adam and MMP families) and inhibitors (Timps, Tfpi2, Serpin), presented as in Fig. 1D. (B) Immunofluorescence of a single fiber preparation showing Adam19 protein in activated Pax7-positive satellite cells (arrowhead) after 3 days in culture (t3), while it is absent in quiescent cells (t0). Hoechst staining marks nuclei. Bar = 100 μm. (C) Transcript levels, measured by qPCR, for the proteinase Adam19, the MMP inhibitor Timp4, and the proteinase inhibitor Tfpi2. (D and E) *In vivo* treatment of *mdx* mice with the AM409 MMP inhibitor (see Materials and Methods for details) affects regeneration as shown by the altered morphology of regenerating fibers in diaphragm muscle after treatment with the inhibitor for 14 days (AM409), compared to control untreated muscle (veh), labeled with antibodies to embryonic myosin heavy chain (MyHCemb, red) that marks newly regenerating fibers and to laminin (green) that marks the fiber outline (D). (E) Sections stained with MyHCemb (red) and MyoD (green) antibodies from diaphragm muscles as in D. Bar in D and E = 50 μm. (F) Quantification of the number of regenerating fibers after AM409 treatment, expressed as the percentage of MyHCemb-positive fibers in the total number of fibers in the *mdx* diaphragm muscle (red bars). MyoD quantification is expressed as the number of positive satellite cells per 100 fibers in treated (AM409) and control (veh) sections, encompassing the entire width of the diaphragm muscle (green bars). (G) AM409 treatment impairs satellite cell activation on single fiber preparations. Satellite cell activation was assayed as the percentage of cells in groups of 1–2 cells or of more than 2 cells along the fibers (left panel), as well as the number of satellite cells per fiber found in AM409-treated compared to nontreated preparations, after 2 days in culture. Three sets of single fiber preparations, 35 fibers, and a total of 300 satellite cells were scored for each sample.

Microarray data processing

Differentially expressed transcripts between samples were analyzed using SPlus ArrayAnalyser software (Insightfull Corporation) and processed by the Robust Multichip Analysis (RMA) algorithm in order to correct the background, to adjust the intensity distribution over the arrays, and to convert probe intensity summarization into a unique probe set signal. These normalized data sets are expressed on a logarithmic scale and presented as intensity map diagrams, using DNA-Chip Analyzer (dChip) software (available at http://www.hsph.harvard.edu/~cli/complab/dchip/ (Li and Wong, 2003)) for genes selected from Supplementary Tables 4 and 5, according to our stringency criteria (see below). Rows in intensity maps represent genes and columns represent samples (analyzed in triplicate). Two-way clustering was performed using a correlation-based metric and the unweighted pair group method with the arithmetic mean for gene color assignment. Redundant probe sets were omitted in the analysis.

Differential expression between samples was calculated using the local pool error test for pairwise sample comparisons. To determine statistical significance, the Benjamini-Hochberg (BH) algorithm for multiple testing was implemented to adjust the *P* value. We also used the more stringent Bonferroni algorithm for *P* value adjustment, indicated in bold and with a symbol in Supplementary Tables 1, 2, 4, and 5. As expected, fewer genes were selected by this method. The threshold *P* value was set at 0.05 for both BH and Bonferroni adjustments, which assigns a 95% confidence interval to the microarray analysis. For the Ad-Ad.cult comparison, only genes significant after the Bonferroni adjustment were retained, since the number of genes after the BH method was very large. Only transcripts showing at least 1.5-fold difference between samples were retained. Genes that satisfy the BH stringency criteria and that are in common in the two *in vivo* comparisons (Ad-Ad.mdx and Ad-1wk, Supplementary Tables 1 and 2) are presented in Supplementary Tables 4 and 5. Gene function enrichment using dChip software was performed on genes form Supplementary Tables 4 and 5 to obtain Gene Ontology classification of genes associated with DNA replication presented in Supplementary Table 6.

Accession numbers

The complete microarray data set, including the RMA data used to produce intensity maps, have been uploaded onto the GEO (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo) website under Accession Number GSE15155.

Single fiber preparation and culture

Single fibers were prepared from EDL muscles as described (Collins et al., 2005) and immediately fixed or transferred into growth medium (see cell culture section).

Table 1 Primer sets used in the qPCR experiments. Table columns from left to right correspond to: gene symbol, transcript accession number, forward and reverse primer sequence from 5' to 3'

Gene Symbol	Acc Num	Forward primer	Reverse primer
Actb	NM_007393	CAAACATCCCCCAAAGTTCTAC	TGAGGGACTTCCTGTAACCACT
Adam19	NM_009616	GCAGAATGAAACGGGAAGAT	CATCCTGGTCATGTCGATTC
Agtr2	NM_007429	TTCAGCCTGCATTTTAAGGA	TGCAGCAACTCCAAATTCTT
Axud1	NM_153287	TCCGCCGCCGTTTAAAG	ATCGATGGCATCCGCTGT
Calcr	NM_007588	AGTGAAGTCTGCGTTCCTGA	GCGCTCTAATGGCACTTACC
Cldn5	NM_013805	TTAAGGCACGGGTAGCACTCA	GCCCAGCTCGTACTTCTGTGA
CyclinE1	NM_007633	GTGGCTCCGACCTTTCAGTC	CACAGTCTTGTCAATCTTGGCA
CypA	NM_008907	AGCATGTGGTCTTTGGGAAGGTG	CTTCTTGCTGGTCTTGCCATTCC
Cyp4b1	NM_007823	CTCACACCTGGCTTCCATTA	CCCACGTCACAGAAGATGTC
Dach1	NM_007826	GATTCTGAAAACGGGGACAT	TGACAGATGCTGGAGGTAGG
Dcn	NM_007833	CCGTTATGGAGAATGGCAGT	GCGGAGATGTTGTTGTTGTG
Dpt	NM_019759	AATCAGTGCTGGATCGTGAG	GTCTTCCCCATAGTGGCTTG
Eva1/Mpzl2	NM_007962	TCACAGCCCTTTGTCCTACA	AGTTAGCGCATCTCCCACAG
GAPDH	XM_001476037	CACCATCTTCCAGGAGCGAG	CCTTCTCCATGGTGGTGAAGAC
Hes6	NM_019479	CGGATCAACGAGAGTCTTCA	TCACGGTCAGCTCCAGAAC
Hs6st2	NM_001077202	purchased from Applied Biosystem TaqMan® Gene Expression Assays	
Igfbp6	NM_008344	TGTGCCAAACTGTGACCTCAG	CCCTGCGAGGAACGACACT
Klf9	NM_010638	TGGCTGTGGGAAAGTCTATG	AGCGCGAGAACTTTTTAAGG
Nap1l5	NM_021432	GCCCAATTCCGTGAAATGC	TTCTCCAGAGCCTGGAATTCC
Pax3	NM_008781	TCCATCCGACCTGGTGCCAT	TTCTCCACGTCAGGCGTTG
Pax7	NM_011039	AGGCCTTCGAGAGGACCCAC	CTGAACCAGACCTGGACGCG
Pdgfrb	NM_008809	AGCCAGAAGTAGCGAGAAGC	GGCAGTATTCCGTGATGATG
Smoc2	NM_022315	CACCAAATGGAAGACCCATCA	ATCATCTGCTTTCCCTGCTCC
Sox17	NM_022454	CAGCAGAACCCAGATCTGCA	GTCAACGCCTTCCAAGACT
Tfpi2	NM_009364	purchased from SuperArray Bioscience Corporation	
Tgfbr3	NM_011578	AGGTGGTCAAAAACCTCGTC	GCCAATACTGTCAGGAGCAA
Timp4	NM_080639	TGCAGAGGGAGAGCCTGAA	GGTACATGGCACTGCATAGCA
Wisp1	NM_018865	AGCAGTGGGTGTGTGATGAT	GTTCTCATACCGTTGCTCCA

Immunofluorescence analysis

Immunodetection was as described on single fibers (Collins et al., 2005), on cultured cells, and on sections (Relaix et al., 2006). Antibodies and procedures are in Supplementary Information.

Quantitative real-time PCR

Total RNA was isolated as described for the microarray analysis experiments from freshly prepared satellite cells. The list of primers used for quantitative real-time PCR (qPCR) is shown in Table 1; procedures are in Supplementary Information.

Lentiviral vector production and satellite cell transduction

The human Dach1 full-length cDNA was cloned into a lentiviral vector using the Gateway system (Invitrogen) and the strategy designed by Charneau and colleagues (Arhel et al., 2007). A lentiviral vector coding for the red fluorescent protein, RFP, was used to monitor cell transduction. This type of vector permits efficient transduction of freshly isolated satellite cells. For detailed procedures see Supplementary Information.

Western blot analysis

Protein extracts were obtained from lentivirus transduced satellite cell cultures, separated on SDS-PAGE gels, and transferred to nitrocellulose membranes as described (Crist et al., 2009) and membranes were probed with antibodies (see Supplementary Information).

In vivo muscle regeneration and matrix metalloproteinase (MMP) inhibitor treatment

Cardiotoxin injury of tibialis anterior (TA) muscles was as described (Cooper et al., 2001). *In vivo* MMP inhibitor treatment of injured and *mdx* mice was as in (Defamie et al., 2008). See Supplementary Information.

Antioxidant capacity of satellite cells

Treatment of freshly isolated satellite cells in culture with 50 μm H_2O_2 provoked oxidative stress, estimated as a percentage of colonies formed compared to untreated cells. Prior treatment with 50 μm diethylmaleate (DEM) was used to deplete reduced glutathione; see also Supplementary Information.

Acknowledgments

M.B.'s laboratory is supported by the Pasteur Institute, the CNRS, and by grants from the AFM and the E.U. through the MYORES network of excellence (6th F.R.P.) and the EuroSyStem and Optistem projects (7th F.R.P.). G.P. was the recipient of an FRM postdoctoral fellowship and later, was supported by EuroSyStem. The funding sources had no role in the design or execution of this work. We are grateful to the Pasteur Institute staff of the DNA microarray platform and Imagopole and also to Pierre Charneau, Yves Jacob, and Philippe Souque for help with lentivirus production. We also thank Catherine Bodin for technical assistance with the preparation of sections.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2009.10.003.

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