Myogenic potential of adipose-tissue-derived cells

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Summary

Adipose-tissue-derived mesenchymal stem cells can be directed towards a myogenic phenotype in vitro by the addition of specific inductive media. However, the ability of these or other adipose-tissue-associated cells to respond to 'natural' myogenic cues such as a myogenic environment has never been investigated in detail. Here, we provide evidence that a restricted subpopulation of freshly harvested adipose-tissue-derived cells possesses an intrinsic myogenic potential and can spontaneously differentiate into skeletal muscle. Conversion of adipose-tissue-derived cells to a myogenic phenotype is enhanced by co-culture with primary myoblasts in the absence of cell contact and is maximal when the two cell types are co-cultured in the same plate. Conversely, in vitro expanded adipose-tissue-derived mesenchymal stem cells require direct contact with muscle cells to generate skeletal myotubes. Finally, we show that uncultured adipose-tissue-associated cells have a high regenerative capacity in vivo since they can be incorporated into muscle fibers following ischemia and can restore significantly dystrophin expression in mdx mice.

Key words: Adipose tissue, Myogenic differentiation, Cell transplantation, Muscular dystrophy

Introduction

The ability of adult skeletal muscle to generate new fibers resides in a reserve population of mononucleated precursors termed satellite cells, which lie underneath the basal lamina. In healthy adult muscles, most satellite cells are in a quiescent, non-proliferative state. In response to environmental cues such as injury, satellite cells become activated and proliferate. After division, the satellite cell progeny, termed myoblasts, undergo terminal differentiation and ultimately fuse to each other to form new myotubes or become incorporated into pre-existing muscle fibers.

Cell therapy approaches for degenerative muscle diseases aim at rescuing muscle damage by delivery of cells that can differentiate into skeletal muscle. Satellite cells represent one choice because of their intrinsic myogenic potential. However, in several myopathies, including Duchenne muscular dystrophy, continuous muscle degeneration-regeneration cycles lead to a depletion of the satellite cells pool. As a consequence, satellite cells are recovered in low number from dystrophic muscle biopsies. Moreover, they are poorly expandable in vitro and rapidly undergo senescence (Cossu and Mavilio, 2000).

Alternative sources are represented by other pluripotent stem cells that can be isolated from skeletal muscle as well as from several other tissues. Recent reports have suggested that adult skeletal muscle progenitor cells, distinct from satellite cells, may function in some models of muscle injury and repair. For example muscle-resident side population (SP) cells, defined by their ability to exclude the Hoechst dye, have been shown to contribute to myofibers when injected intramuscularly (McKinney-Freeman et al., 2002) or intravenously (Bachrach et al., 2004). Similarly, bone-marrow-derived cells (Ferrari et al., 1998; Fukada et al., 2002; Gussoni et al., 1999; LaBarge and Blau, 2002), and even single hematopoietic stem cells (Camargo et al., 2003; Corbel et al., 2003; Sherwood et al., 2004b) also have been reported to contribute to myofibers when injected directly into injured muscle or intravenously into irradiated injured or dystrophic animals. Analogous results have been obtained with stromal cells isolated from synovial membrane (De Bari et al., 2003), and with cord-blood-derived or adult circulating cells expressing the hematopoietic/endothelial markers CD34 and AC133 (Pesce et al., 2003; Torrente et al., 2004). However, in all cases, the frequency of stem cell incorporation into skeletal muscle is too low to result in an important amelioration of the dystrophic phenotype. A more significant skeletal muscle recovery has been obtained by intra-arterial delivery of embryonic-vessel-derived stem cells termed mesoangioblasts (Sampaolesi et al., 2003). Yet the existence of mesoangioblasts in adult tissues has not been reported.

Adipose tissue (AT) has recently been identified as an alternative, uniquely abundant and accessible source of pluripotent cells. In adult mammals, AT is an association of adipocytes that are held together in a framework of collagen fibers. In addition to mature adipocytes, AT contains the stromal vascular fraction (SVF), which is composed of a heterogeneous cell population including adipose precursors cells of varying degrees of differentiation, vascular cells and a supportive stroma. Similarly to bone-marrow-derived stromal cells, in the presence of lineage-specific inductive media, AT-derived stromal cells can differentiate in vitro into adipogenic, chondrogenic, osteogenic and myogenic cells (Zuk et al., 2001) as well as other non-mesenchymal lineages such as neurons and hepatocytes (Safford et al., 2002; Seo et al., 2005). Also,

the AT-SVF has been shown to include progenitors of endothelial cells (Miranville et al., 2004; Planat-Benard et al., 2004). Recently, short-term cultures of AT-SVF cells have been shown to incorporate into regenerating muscle fibers with low efficiency after transplant into damaged muscles (Bacou et al., 2004). However, the full myogenic potential of freshly isolated AT-SVF cells and their ability to respond to myogenic cues producing terminally differentiated myotubes is largely unknown.

In the present work, we have characterized the myogenic potential of AT-SVF cells both in vitro and in vivo. We provide evidence that subpopulations of AT-SVF cells are able to differentiate spontaneously into skeletal muscle and may be further induced to adopt a myogenic phenotype by co-culture with primary myoblasts. Moreover, we show that AT-SVF cells possess a high regenerative capacity in vivo since they are able to participate in the formation of new muscle fibers following ischemia and to restore significantly dystrophin expression in mdx mice.

Results

AT-derived SVF cells co-cultured with primary myoblasts incorporate into differentiating skeletal myotubes

Non-myogenic cells of different origin, including muscle-associated and bone-marrow-derived cell populations, have been shown to be able to form skeletal muscle fibers when co-cultured with myoblasts. To test whether a similar population of cells competent for the myogenic lineage was also present in the stromal vascular fraction (SVF) of AT, freshly harvested, uncultured SVF cells from inguinal AT depots from GFP mice were co-cultured with equal amounts of wild-type (GFP negative) primary myoblasts. Muscle-derived non-myogenic fibroblastic cells, obtained from pre-platings of primary myoblasts preparations (Materials and Methods), were used instead of primary myoblasts in control experiments. Cells were first maintained in growth medium (GM) for 2 days and then switched to differentiation medium (DM). After 2-3 days in DM, in culture plates containing primary myoblasts, but not in control plates where co-culture was performed with non-myogenic cells, we started to observe the formation of GFP-positive contracting myotubes whose number increased with time. Myogenic differentiation of AT-SVF cells was confirmed by immunofluorescence experiments with an antibody against Troponin-T (TnT, Fig. 1). After 7 days in DM, the number of myotubes expressing both GFP and TnT corresponded to approximately 0.2% (0.25±0.08 s.e.m., n=3 experiments) of input cells. However, this frequency of myogenic conversion could be an underestimate of the actual efficiency since GFP is expressed in only 50% (as determined by FACS analysis) of AT cells derived from GFP mice and plating efficiency is quite low, ranging between 10 and 20% of total seeded cells. Analogous results were obtained when the same experiment was performed with AT-SVF derived from visceral (gonadal or omental) fat indicating that AT-SVF cells obtained from different locations are equally able to contribute to myotube formation in co-culture assays (data not shown).

Contact-independent myogenic conversion of AT-SVF cells

Double GFP/TnT-positive cells mostly consisted of multinucleated myotubes containing both GFP-negative and GFP-positive nuclei, probably originating by fusion of AT-derived cells with differentiating myotubes. However, the presence of GFP/TnT-positive mononucleated cells was also detected (Fig. 1). This indicates that at least some AT-SVF cells can be induced to differentiate into skeletal muscle via a fusion-independent mechanism and that they can subsequently divide and/or fuse to one another to generate terminally differentiated myotubes. To confirm this hypothesis, we performed co-culture experiments on transwell plates, where inguinal AT-SVF cells were separated from primary myoblasts by a 0.4 μm porous filter which does not allow cell and/or nuclear passage. As in the previous experiment, muscle-derived non-myogenic fibroblastic cells were used in place of primary myoblasts in control plates. As shown in Fig. 2A,B, although with a lower efficiency compared with direct co-culture (tenfold lower), we observed the formation of many TnT-expressing myotubes when AT-SVF cells seeded on transwell filters were cultured in the presence of differentiating primary myoblasts. After 7 days in DM, the number of TnT-positive cells corresponded approximately to 0.02% of input cells (0.02±0.01 s.e.m., n=3 experiments). In many cases, TnT-positive cells appeared as clusters of multiple myotubes, which suggested a certain degree of cell proliferation before the formation of differentiated multinucleated cells (Fig. 2A,B).

RT-PCR analysis of cells cultured on transwell filters for 1 week in the presence of primary myoblasts, revealed the expression of early (MyoD, Myogenin) and late (alpha-skeletal actin) skeletal-muscle-specific markers (Fig. 2C, lane 2). Freshly harvested, uncultured SFV cells were instead always negative for all the skeletal markers tested (Fig. 2C, lane 1).

Together these data indicate that soluble factors secreted by differentiating myogenic cells are sufficient to promote the expression of muscle-specific proteins by a subpopulation of AT-SVF cells. Moreover, the presence of such myogenesis-competent cells does not seem to be restricted to subcutaneous fat, since we obtained comparable results when visceral (gonadal) instead of inguinal fat was used (data not shown).

Sporadic spontaneous myogenic differentiation of AT-SVF cells

Control plates from both direct and transwell-mediated co-culture experiments, in which AT-SVF cells were cultured in the presence of muscle-derived fibroblastic non-myogenic cells, were mostly negative for TnT. However, to our surprise, in some cases (5 plates out of 30 and 6 plates out of 20 for direct and transwell co-culture, respectively), TnT staining revealed the presence of a few AT-SVF-cell-derived myotubes. Overall, the number of TnT-positive cells corresponds approximately to 0.001% of total plated cells. Since we are able to exclude any possible contamination of AT samples with skeletal muscle tissue, this result seems to suggest that a restricted population of AT-AVF cells is endowed with an autonomous myogenic potential and can, in our culture conditions, spontaneously differentiate into skeletal muscle. To verify this hypothesis, and to exclude any possible residual inductive activity by the fibroblastic cells, we cultured AT-SVF cells from either inguinal or gonadal fat pads alone, in the absence of any other cell type. Culture conditions were the same as those used for co-culture assays, consisting of 2 days culture in GM and an additional 7 days in DM. A typical group of TnT-positive myotubes that we obtained in this assay is

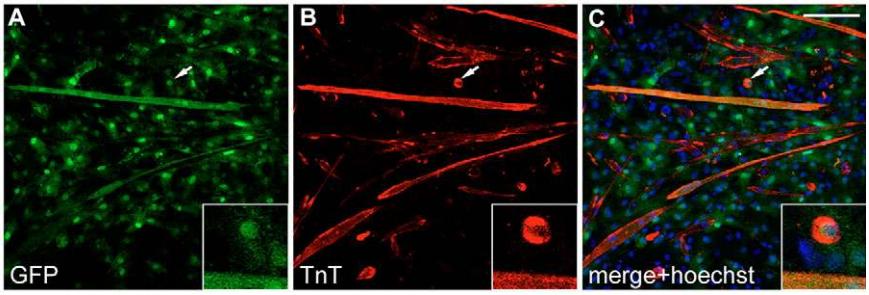

Fig. 1. Direct co-culture of AT-SVF cells with primary myoblasts. Fresh inguinal AT-SVF cells from GFP mice were co-cultured with wild-type primary myoblasts for three days in GM and then switched to DM. Myogenic differentiation was revealed after one week by staining with an anti Troponin-T antibody (TnT). Several GFP cells (green) that have been incorporated into multinucleated myotubes expressing TnT (red) are visible. The arrow indicates a double GFP/TnT-positive mononucleated cell, shown in the inset at larger magnification, suggesting that AT-derived cells can differentiate into skeletal muscle in the absence of cell fusion with skeletal myotubes. (A) Fluorescence image of GFP positive cells, (B) fluorescence image of TnT-positive cells, (C) overlay of A and B; Hoechst staining was used to visualize nuclei (blue). Magnification, 20×. Bar, 100 μm.

shown in Fig. 2D,E. As in the case of co-culture with non-myogenic cells, the percentage of TnT-positive cells obtained in these experiments corresponds approximately to 0.001% of total plated cells and is comparable in inguinal and gonadal AT-SVF cells.

Myogenic potential of in vitro expanded AT-derived mesenchymal stem cells

Together our results show that AT-SVF cells possess an intrinsic myogenic potential and that, albeit at very low rate, they can spontaneously differentiate into skeletal muscle. AT-SVF conversion to a myogenic phenotype is enhanced by a myogenic environment even in the absence of cell-cell contact and is maximal when the two cell types are co-cultured in the same plate. This property makes such cells good candidates for use in clinical applications for cell therapy of degenerative muscle diseases. However, an easier use for clinical purposes would require the expansion of stem cells in culture and an increase in their number. It is well known that a population very similar to bone-marrow-derived mesenchymal stem cells (BM-MSCs) can be obtained by the adherent population of AT-SVF cells of both murine and human origin (Safford et al., 2002; Zuk et al., 2001).

To test whether AT-SVF cells maintained their full myogenic potential after sequential culturing and expansion in vitro, AT-SFV cells obtained from GFP mice were plated on uncoated tissue culture dishes and maintained in culture for several passages under conditions that are generally used to amplify populations of mesenchymal stem cells. After 4 or 5 passages, cells were quite homogenous in shape. Cells between passage 8 and 12 were used for further experiments and referred to as adipose-tissue-derived mesenchymal stem cells (AT-MSCs).

When co-cultured with primary myoblasts for 1 week in DM, approximately 1% of input AT-MSCs gave rise to GFP-positive myotubes, as confirmed by TnT staining (Fig. 3A-C). However, in contrast to what observed with freshly harvested AT-SVF cells, we were never able to detect the presence of double GFP/TnT-positive mononuclear cells. Accordingly, in co-culture experiments performed with transwell filters, AT-MSCs never gave rise to TnT-positive myotubes (Fig. 3D-F). Finally, we never detected TnT-positive myotubes in the absence of co-culture with myogenic cells, indicating that the AT-MSC population does not contain cells that can spontaneously differentiate into skeletal muscle cells.

Characterization of AT-SVF myogenic cells

Sca1 and CD34 are two surface antigens that, in addition to being generally expressed in hematopoietic stem cells, have been very often associated with cells with in vitro and/or in vivo myogenic potential. These myogenic cells include 'canonical' sublaminal satellite cells (which express CD34 but not Sca1) (Asakura et al., 2002; Beauchamp et al., 2000; Sherwood et al., 2004b), other muscle-derived cells (which express Sca1 or both Sca1 and CD34) (Polesskaya et al., 2003;

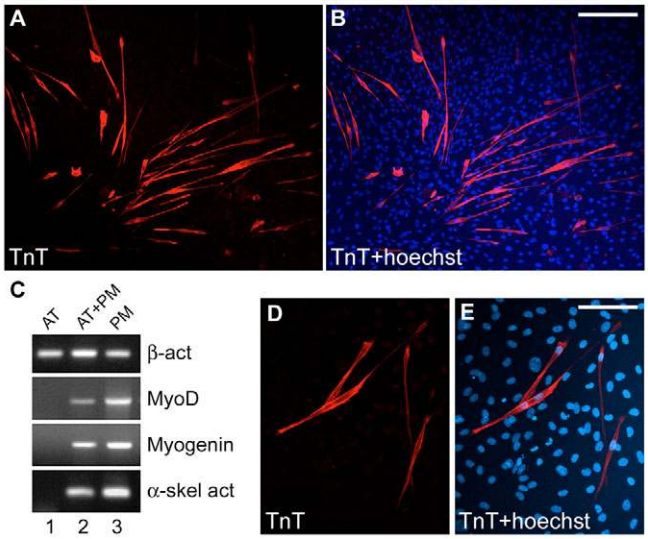

Fig. 2. Cell-autonomous myogenic differentiation of AT-SVF cells. Fresh inguinal AT-SVF cells were plated on fibronectin-coated transwell filters floating over a layer of primary myoblasts (A-C) or simply on a fibronectin-coated tissue-culture dish (D,E). Cultures were maintained for three days in proliferation medium and then switched to differentiation medium. Myogenic differentiation was revealed after 1 week by staining with an anti TnT antibody (red). (A,B) The image shows one of the several clusters of skeletal myotubes found on a transwell filter in a typical experiment. (C) RT-PCR analysis for the indicated skeletal muscle markers of inguinal AT-SFV cells. AT, fresh, uncultured AT-SVF cells; AT+PM, AT-SVF cells cultured on transwell filters in the presence of primary myoblasts. Differentiating primary myoblasts (PM) were used as positive controls. (D,E) Spontaneous myogenic differentiation of AT-SVF cells. (A,D) Fluorescence image of TnT positive cells; (B,E) merge with Hoechst to visualize nuclei (blue). (A,B) Magnification, 10×. Bar, 200 μm. (D,E) Magnification, 20×. Bar, 100 μm.

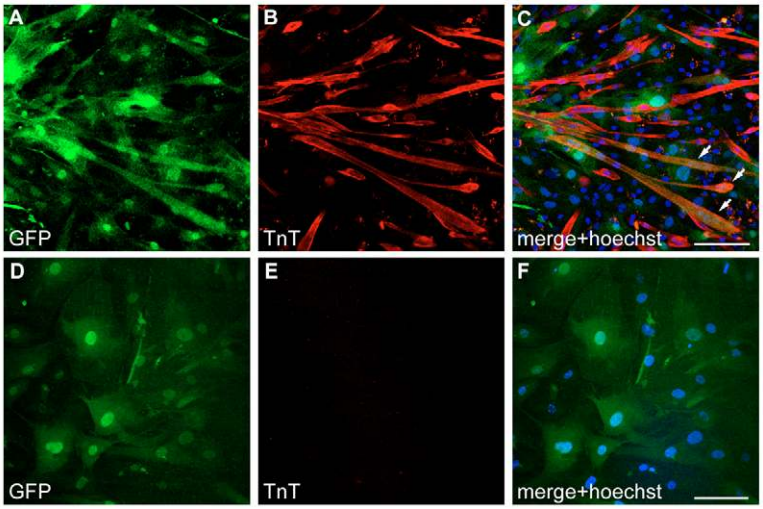

Fig. 3. Myogenic conversion of AT-MSCs induced by primary myoblasts requires cell contact with myogenic cells. GFP-positive AT-MSCs were either seeded in the same dish together with primary myoblasts (top panels) or plated on 0.4 μm porous transwell filters floating on a layer of primary myoblasts (bottom panels). Myogenic differentiation was revealed by TnT staining (red) after 1 week in DM. Hoechst was used to visualize nuclei (blue). Double GFP/TnT-positive cells indicating myogenic differentiation of AT-MSCs are observed only when contact between the two cell types is allowed (A-C). (A,D) Fluorescence image of GFP-positive cells; (B,E) fluorescence image of TnT-positive cells; (C,F) overlay of GFP, TnT and Hoechst staining. Magnification, 20×. Bars, 100 μm.

Qu-Petersen et al., 2002; Torrente et al., 2004), as well as cells from different sources such as bone marrow (Gussoni et al., 1999), blood (Torrente et al., 2004) and embryonic vasculature (Minasi et al., 2002). FACS analysis on freshly harvested, uncultured AT-SVF cells from inguinal fat depots revealed that approximately 8% (8±1.15 s.e.m., *n*=5) of the AT-SVF cells express Sca1 but not CD34, 3% (3.33±0.67 s.e.m., *n*=5) express CD34 but not Sca-1 and 12% (12.67±1.76 s.e.m., *n*=5) express both Sca1 and CD34 (data not shown).

To test whether the myogenic potential of AT-derived cells correlates with the expression of CD34 and/or Sca1, $Sca1^+$ and $CD34^+$ cells were independently purified from the inguinal AT-SVF by magnetic sorting and cultured either alone, or in the presence of primary myoblasts (either as direct or transwell-mediated co-culture). For direct co-culture experiments, AT-SVF cells were obtained from GFP transgenic mice. Purity of sorted cell fractions was checked by FACS and reached values between 80 and 85% in each experiment. The antigen-depleted fraction of each sorting was used as a control.

Probably due to the fact that many cells express both Sca-1 and CD34, we obtained comparable results with each of the two sorted populations. In the case of a direct co-culture, the number of GFP-positive myotubes formed by the Sca1 or CD34-enriched cell population did not differ from the one obtained with the unsorted cell population (approximately 0.2% of seeded cells). This result indicates that, in those conditions where fusion with myotubes is allowed, the Sca-1 and/or CD34-negative cells are equally able to take part in skeletal myotube formation compared with Sca-1 and/or CD34-positive cells. When co-culture experiments were performed on transwell plates, we obtained several TnT positive myotubes from both Sca-1 and CD34-sorted cells. However, quite surprisingly, their number was always lower (approximately 50%, as an average from three different experiments for each antigen) than that obtained with the antigen-depleted fraction or with unsorted cells. Since we did not observe any relevant difference in the plating efficiency of sorted and unsorted cells, this result seems to suggest not only that the ability to respond to myogenic cues is not restricted to cells expressing either Sca-1 or CD34, but also that only a fraction of cells expressing one or both of these two antigens may indeed be susceptible to contact-independent myogenic induction by differentiating myogenic cell. Finally, quite unexpectedly, we could not detect spontaneously differentiating skeletal muscle fibers from Sca1 or CD34-enriched cell populations. Conversely, a few TnT-positive myotubes were always found in control plates, suggesting that the ability to spontaneously form skeletal myotubes in vitro resides in a small subset of SVF cells, which, at least before culturing, are negative for both Sca1 and CD34.

Mesenchymal or fibroblast-like-cells are known to quickly attach to uncoated tissue culture plates. Myogenic progenitors, such as satellite cells or muscle-derived stem cells, by contrast, are slowly adherent cells and require a matrix coating to efficiently adhere to culture dishes. In order to test whether it could be possible to enrich AT-SFV cell preparation for spontaneously differentiating myogenic precursors by mean of differential adhesion properties, we performed pre-plating experiments where AT-SVF were first seeded on uncoated dishes and after a short time (1-2 hours) the unattached cells (supernatant) were transferred on a fibronectin-coated second dish. Both dishes were then left in GM for 3 days and then switched to DM. TnT staining revealed that, although roughly 70-80% of the total adherent cells attached to the uncoated pre-plating dish, spontaneously differentiating cells were always found on the fibronectin-coated secondary dishes.

Supernatant cells depleted from fast adherent, non-myogenic cells, were then plated at low densities in order to allow isolated clone formation from single cells and kept in GM for several days. With this method, we were able to distinguish at least ten cell types based on their different morphology, and among these, one type (Fig. 4A) which was able to generate contractile, myotubes after switching into DM (Fig. 4B) as confirmed by TnT staining (not shown). Such myogenic clones consist of both flat, spindle-shaped and more rounded cells that tend to stay separate one from the other. Proliferating myogenic clones express Pax7, as revealed both by immunostaining (Fig. 4C,D) and RT-PCR (Fig. 4E, lane 2) as well as other markers that are also expressed by muscle satellite cells such as Pax3, Myf5, the VEGF receptor Flk-1 and the HGF receptor c-met, as shown by RT-PCR. Flk-1 and c-met, but not Pax3, Pax7 and Myf5 were also detectable in uncultured AT-SVF cells (Fig. 4E). Although we were able to obtain a certain degree of proliferation, attempts to expand such myogenic clones, at least in these culture conditions, have been unsuccessful given that even if maintained in GM, these cells invariably stop proliferating and terminally differentiate as soon as transferred to a new plate.

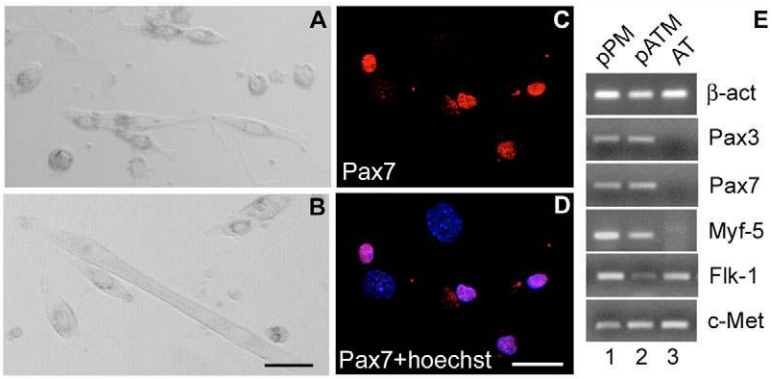

Fig. 4. Spontaneously differentiating AT-SVF myogenic cells express satellite-cell-specific markers. (A) Transmission light image of a typical AT-derived group of proliferating myogenic cells, 5 days after plating in GM. (B) The same group of cells shown in A after 24 hours in DM, when myotubes start to form (magnification, 20×; Bar, 50 μm). (C,D) Immunofluorescence staining with an anti-Pax7 antibody of proliferating myogenic cells. To highlight the specificity of the Pax7 staining, we chose an image field where myogenic cells groups were close to morphologically different, non-myogenic cells. Small nuclei of myogenic cells express Pax7 while bigger nuclei of adjacent, non-myogenic cells, are negative for Pax7. (C) Fluorescence image of Pax7-positive cells (red). (D) merge with Hoechst (blue) to visualize nuclei. Magnification, 40×. Bar, 25 μm. (E) RT-PCR analysis for the indicated markers of isolated myogenic clones. pPM, proliferating, skeletal-muscle-derived, primary myoblasts; pATM, proliferating AT-derived myogenic clones; AT, fresh, uncultured AT-SVF cells.

AT-SVF cells participate in skeletal muscle regeneration in vivo

To establish their in vivo myogenic potential, AT-SVF cells were assayed for the capacity to differentiate through the myogenic lineage and contribute to muscle regeneration in a mouse model of hind-limb ischemia. Since the uncultured AT-SVF cells seem to include the population with the highest myogenic potential in vitro, we performed in vivo experiments with freshly harvested AT-SVF cells. Inguinal AT-SVF cells were isolated from 6-week-old GFP-positive mice, and injected into the adductor muscle of GFP-negative syngenic mice immediately after femoral artery removal. Engrafted GFP-expressing cells were visualized 1 week after injection. Representative images from control PBS injected and AT-SVF injected hind limbs are shown in Fig. 5A and 5B, respectively. A wide region of GFP-positive fibers covering up to 20% of the total area (38.33±8.82 GFP-positive fibers per mm^2 of section area calculated as an average from n=8 experiments) is present in adductor muscle sections from treated hind limbs while no GFP staining is observed in control sections from PBS-injected limbs. These results demonstrate that uncultured AT-SVF cells can be efficiently incorporated into skeletal muscle fibers in vivo and effectively contribute to skeletal muscle regeneration.

AT-SVF restore dystrophin expression in mdx mice

To further assess the therapeutic value of AT-SVF cells in correcting muscle disorders, we wanted to investigate whether administration of AT-SVF cells may induce the formation of dystrophin-expressing muscle fibers in dystrophin-deficient mdx mice. Inguinal AT-SVF cells were isolated from 6-week-old wt C57BL/10SnJ mice (syngenic to mdx) and delivered intramuscularly into 2-month-old mdx mice. Three weeks after injection, less than 0.1% dystrophin positive fibers, corresponding to spontaneously revertant fibers, were observed in sections from age-matched PBS-injected mice. Conversely, dystrophin was detected in up to 10% of the myofibers analysed on sections from AT-SVF transplanted muscles (11.7±2.94 dystrophin positive fibers per mm^2 of section area, calculated as an average from n=6 experiments), as revealed by immunofluorescence with an antibody against the C-terminal portion of mouse dystrophin (Fig. 6). Dystrophin-positive fibers were organized in clusters, suggesting clonal proliferation of donor cells. Nuclei were located both centrally and at the periphery of myofibers, indicating the existence of regeneration and maturation processes. These data show that transplantation of AT-SVF cells may restore dystrophin expression in mdx mice and therefore may represent a promising tool for the cellular therapy of muscle diseases.

Discussion

During vertebrate embryogenesis, mesodermal progenitors give rise to distinct mesenchymal lineages, including skeletal myocytes, osteocytes, chondrocytes and adipocytes. The commitment and subsequent differentiation of a mesenchymal stem cell toward a particular lineage is regulated by the coordinated action of extracellular signals, some of which, such as IGF-1, are shared by adipocytes and myocytes and can promote the production of one or the other cell type, depending on the state of activation of Rho GTPase (Sordella et al., 2003). In vitro data suggest that not only uncommitted mesenchymal progenitors, but also more committed myogenic precursors distinct from the mesenchymal compartment, such as satellite cells and primary myoblasts, display a high degree of plasticity and can differentiate into adipocytes (Asakura et al., 2001; Csete et al., 2001). Moreover, recent studies have indicated that single cells from within the satellite cell compartment exhibit mutually exclusive abilities to generate either myogenic or fibroblastic and adipogenic colonies in clonal in vitro assays (Shefer et al., 2004; Sherwood et al., 2004a).

Several in vivo observations have also suggested the existence of adipogenesis-competent cells within adult skeletal muscles. For example, expansion of AT within skeletal muscles occurs in response to denervation (Dulor et al., 1998) and in some muscle diseases including muscular dystrophy (Cossu and Sampaolesi, 2004). Similarly, a replacement of muscle mass by AT is observed in double MyoD:Myf5 mutant mice (Rudnicki et al., 1993). However, much less is known about the ability of uncommitted or more committed adipocyte precursors to differentiate into skeletal muscle.

Conversion of AT-MSCs to a myogenic phenotype has been obtained in vitro by the addiction of 'artificial' inductive media (Zuk et al., 2001), but the ability of AT-MSCs or other AT-associated cells to respond to 'natural' myogenic cues such as a myogenic environment has never been investigated. In the present work we show that subpopulations of freshly harvested

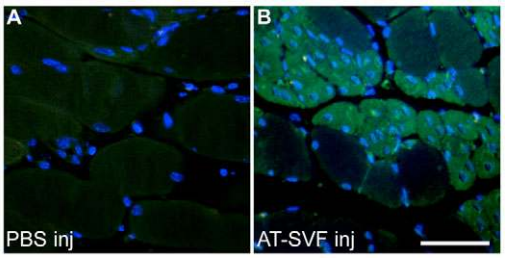

Fig. 5. AT-SVF cells participate in skeletal muscle regeneration. AT-SVF cells from GFP mice were injected into the adductor muscle of GFP-negative syngenic mice were ischemia was induced by femoral artery removal. Engrafted GFP-expressing cells in the injected muscle were visualized by an anti-GFP antibody (green) 7 days after injection. A wide region of GFP positive fibers are present in muscle sections from treated hind limbs (B) while no GFP staining is observed in control sections from PBS-injected limbs (A). Nuclei are visualized by Hoechst (blue). Magnification, 40×. Bar, 50 μm.

GFP-labeled AT-SVF cells derived from inguinal or visceral fat pads can differentiate into skeletal muscle cells when cultured in the presence of differentiating primary myoblasts. As demonstrated by experiments performed on transwell filters, some AT-SVF cells can be induced to become myocytes by a fusion-independent, cell-autonomous mechanism. Moreover the spontaneous formation of rare skeletal muscle cells was sporadically observed in the absence of inducing myogenic cells. The relative number of GFP-positive cells incorporated into skeletal myotubes during direct co-culture with primary myoblasts is higher (tenfold) compared with the percentage of myotubes we obtained performing co-cultures on transwell plates. We think that this can only in part be explained by the distance between the responder and inducer cell compartments on transwell plates. The SVF consists of a heterogeneous population with the majority of cells being mesenchymal. We show that in vitro expanded mesenchymal stem cells derived from AT can be incorporated into contractile myotubes when directly co-cultured with primary myoblasts, similarly to and even more efficiently than uncultured AT-SVF cells. However, they are not able to differentiate into myotubes when cell contact is not allowed. Recently Schulze et al. convincingly demonstrated both in vitro and in vivo that BM-MSCs are unable to acquire a fully differentiated skeletal muscle phenotype in the absence of cell fusion to predetermined muscle cells (Schulze et al., 2005). We therefore suggest that AT-SVF contains at least three different cell populations that can be directed towards a myogenic lineage. A main one, composed of mesenchymal cells, requires direct contact with myogenic cells to acquire a skeletal muscle phenotype. Cells belonging to this population probably become incorporated into muscle fibers only via cell fusion with differentiating myotubes. A less represented, different cell population, can instead be induced to the myogenic phenotype by soluble factors secreted by muscle cells. In addition, we identified a very rare population of myogenesis-committed cells that spontaneously differentiate into skeletal muscle. These last two cell populations could be lost during sequential passages in culture conditions that favor mesenchymal-like cell expansion.

To our knowledge, most (if not all) cell populations that have been reported to have an in vitro and/or in vivo myogenic potential express either CD34 or Sca-1 or both. However, we were not able to isolate prospectively the rare spontaneously differentiating myogenic precursors from AT-SVF by means of selection for CD34 or Sca-1 expression. By seeding AT-SVF cells at low density after partial depletion of non-myogenic cells via differential adhesion, we have been able to isolate small clones of spontaneously differentiating myogenic cells that express markers characteristic of muscle satellite cells. We are currently setting up expansion conditions for these clones by testing different substrates (i.e. collagen or laminin versus fibronectin) as well as different growth factors known to stimulate proliferation and inhibiting myogenic differentiation of muscle precursors. Preliminary experiments show that, similarly to muscle satellite cells, AT-derived myogenic cells extensively proliferate in the presence of bFGF although its presence is still not sufficient to totally inhibit myogenic differentiation on any of the different substrate tested.

Altogether our in vitro data suggest that, in the presence of a myogenic environment, AT-SVF cells can efficiently convert to a myogenic phenotype and generate functional skeletal myotubes. To test whether AT-SVF cells would be equally able to contribute to skeletal muscle in vivo, uncultured inguinal AT-SVF cells from GFP mice were injected in a wild-type mouse where skeletal muscle damage was induced by ischemia. After one week, we observed that GFP positive fibers represented up to 20% of the total area of sections from treated hind limbs, thus demonstrating that AT-SVF cells can significantly participate in skeletal muscle regeneration in vivo.

To further assess the therapeutic value of AT-SVF cells in correcting muscle disorders, we transplanted uncultured AT-SVF cells into untreated syngenic dystrophin-deficient mdx mice. Our results show that administration of AT-SVF cells efficiently induce the formation of dystrophin-expressing muscle fibers in mdx mice. We exclude that dystrophin-expressing cells derive from spontaneous revertants, since in

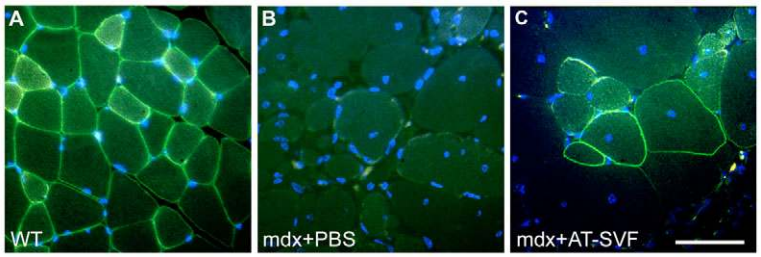

Fig. 6. Transplantation of wild-type AT-SVF cells rescues dystrophin expression in mdx mice. Immunostaining of adductor muscles transverse sections with an antibody against the C-terminal portion of dystrophin. Fresh AT-SVF cells were injected into the left adductor muscle of mdx mice. PBS injected age-matched mdx mice were used as controls. Staining for dystrophin (green) was performed 21 days after injection. Nuclei are visualized by Hoechst (blue). Dystrophin expression is totally absent in PBS-injected muscles while clusters of dystrophin-positive fibers are with both centrally and peripherally located nuclei in mice injected with AT-SVF cells. (A) Wild-type uninjected; (B) mdx injected with PBS; (C) mdx injected with AT-SVF cells. Magnification, 40×. Bar, 50 μm.

control PBS-injected limbs we never detected more than 0.1% of dystrophin-positive fibers. Also, the relatively high number of dystrophin-positive cells (up to 10% in sections overlapping the injected region) argues against this possibility. These results strongly suggest that AT-derived cells are promising candidates for the cellular therapy of degenerative muscle diseases.

While this work was in preparation, Rodriguez at al. reported that injection into immunocompetent mdx mice of a multipotent population of human AT-derived cells which had been extensively amplified in vitro (referred to as hMADS), resulted in a substantial expression of human dystrophin (Rodriguez et al., 2005). However, whether in vitro expanded hMADS can contribute to muscle fiber formation only by fusion to existing myotubes or by a cell-autonomous mechanism is still to be determined. Our results confirm Rodriguez et al. findings and strongly support the idea that AT-derived cells can be an important tool for muscle cell therapy. However our in vitro data seem to suggest that those cells that can respond to myogenic cues and generate skeletal muscle cells by a fusion-independent mechanism are not included in the mesenchymal cell population. We are currently investigating whether the presence of such cells is required to regenerate the muscle progenitor cell pool after transplantation of AT-SVF cells in vivo.

Although for some time the therapeutic potential of satellite cells did not seem very promising, mostly due to their low viability after transplant, very recent data seem to show that, provided that the right isolation procedure is used, the regenerative ability of satellite cells is maximal compared with other myogenesis competent cells (Collins et al., 2005; Montarras et al., 2005). This is probably explained by the fact that cells already committed to the myogenic phenotype and therefore able to autonomously differentiate into skeletal muscle in vitro, possess the highest regenerating potential in vivo. Indeed, clonally expanded, human bone-marrow-derived stromal cells that have been exposed to a mix of various growth factors and constitutively express an activated Notch have been shown to possess an intrinsic myogenic activity in vitro and to potently regenerate myofibers in immunocompromised dystrophic mice (Dezawa et al., 2005). Significantly the myogenic contribution observed in that study was much more robust than those typical of BM-derived hematopoietic stem cells or untreated stromal cells. Given the similarity to BM-MSCs, it is tempting to speculate that a similar treatment would be effective in the myogenic induction of AT-MSCs.

Materials and Methods

Cells isolation and culture

All experimental procedures complied with the Guidelines of the Italian National Institutes of Health and were approved by the Institutional Animal Care and Use Committee.

To obtain AT-SVF cells, subcutaneous (inguinal) or visceral (gonadal or omental) fat pads were removed from 6-week-old wild-type Swiss CD1 or ubiquitous GFP-expressing mice (Okabe et al., 1997) and carefully screened under a dissection microscope to exclude the presence of contaminating (non-adipose) tissue fragments. Samples were then digested at 37°C in PBS containing 2% BSA and 2 mg/ml collagenase A (Roche) for 45 minutes. SVF crude preparation was filtered through a 40 μm cell strainer (Falcon) and pelleted at low speed (500 ***g***) to remove floating mature adipocytes. SVF cells were then washed with PBS, counted, and resuspended in culture medium.

To derive and expand the mesenchymal cell fraction (AT-MSCs), SVF obtained from inguinal AT cells from GFP transgenic mice, were plated at a density of 2.5×10^4 cells/cm^2 on uncoated tissue culture dishes in α-MEM (Sigma) supplemented with 20% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin (all from Gibco). After an overnight incubation, non-adherent cells were discarded and medium replaced. Cells were passaged when they reached 80% confluence. Cells from passage 8 to 12 were used in all experiments.

For primary myoblast preparations, hind-limb muscles from 4-week-old Swiss CD1 mice were removed, minced by scissors and pre-digested with collagenase type A (2 mg/ml) and then digested with collagenase/dispase (1 mg/ml, both from Roche) for 1 hour a 37°C. A single cell suspension was obtained after filtering the samples through 40 μm cell strainer. Isolated cells were pre-plated overnight on uncoated tissue culture dishes in Ham-F10 (Gibco) supplemented with 20% FBS 2 mM L-glutamine, 1% penicillin-streptomycin. Cells from the non-adherent fraction, which is enriched in primary myoblasts were then collected, counted and used as 'inducer' cells in co-culture experiments. Cells from the adherent fraction, which are mostly fibroblastic were kept in culture till they reached confluence and then passaged. Second and third passage cells were used as control non-myogenic cells.

For direct co-cultures, mixed equal amounts of GFP-negative primary myoblasts and GFP-positive AT-SVF cells were plated at a density of 5×10^4 cells/cm^2 on fibronectin-coated culture slides (BD Falcon) in growth medium [GM:DMEM high glucose (Gibco), 20% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin]. For indirect co-cultures, six-well Transwell-clear dishes (0.4 μm porous filter, Costar) were used. Primary myoblasts collected from the pre-plating step (or control non-myogenic cells) were seeded in Ham-F10, 20% FBS, 5 ng/ml bFGF at a density of 2×10^4 cells/cm^2 on the collagen-coated bottom compartment of transwell dishes. After two days, medium was changed to GM and AT-SVF cells (2×10^4 cells/cm^2) were seeded on fibronectin-coated top compartments (porous filters). All cells were washed 24 hours after plating, kept in GM for an additional 2 days and then switched to differentiation medium (DM:DMEM high glucose, 5% horse serum, 2 mM L-glutamine, 1% penicillin-streptomycin). After 4-10 days cells were lysed and processed for RT-PCR or fixed and processed for immunofluorescence.

For AT-SVF myogenic clone isolation, inguinal AT-SVF cells were seeded on uncoated culture dishes in GM and after 1-2 hours transferred on fibronectin-coated plates. After at least 1 week in GM, well isolated clones were picked up with the aid of cloning cylinders and processed for RT-PCR.

FACS analysis and cell purification

PE- or FITC-conjugated anti-mouse CD34 (RAM34) and anti-mouse Sca-1 (Ly-6A/E) from BD-Pharmingen were used for cell labeling. FACS analysis was performed on a Coulter Epics XL cytometer (Becton Dickinson). Magnetic sorting was performed with the mini-MACS kit (Miltenyi) according to manufacturer's instructions and antigen expression of cell populations after sorting was tested by FACS.

Immunofluorescence

Cells were washed with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After incubation overnight at 4°C with primary antibody (1:100, Troponin-T, Santa Cruz; Pax7, R&D), cells were washed and incubated with a Texas-Red-conjugated anti-mouse IgG antibody (1:100, Vector) for 1 hour. Antibody dilutions and washes were all in PBS with 2% BSA and 0.1% Triton X-100. Nuclei were visualized by staining with Hoechst. Cells were observed under an Olympus confocal microscope and images were acquired and stored with an image analyzer Fluoview software.

RT-PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was reverse-transcribed with M-MLV Reverse Transcriptase (Promega) using random examers. PCR primers were as follows (5′-3′): MyoD: Fw CGCTCCAACTGCTCTGATGGCA, Rev TGCTGCTGCAGTCGATCTCTCA; Myogenin: Fw GAGCGCGATCTCCGCTACAGAGG, Rev TCTGGCTTGTGGCAGCCCAGG; Alpha-skeletal actin: Fw AGCACGATTGTCGATTGTCG, Rev ACCACCATGTACCCTGGTAT; Myf5: Fw TGAATGTAACAGCCCTGTCTGGTC, Rev CGTGATAGATAAGTCTGGAGCTGG; Pax3: Fw AGGAGGCGGATCTAGAAAGGAG, Rev TGTGGAATAGACGTGGGCTGGTA; Pax7: Fw GACAAAGGGAACCGTCTGGATGA, Rev TGTACTGTGCTGCCTCCATCTTG; Flk-1: Fw GTGATCAGGGGTCCTGAAAT, Rev GCAAACATAGTCGCCTTGGT; c-Met: Fw GAATGTCGTCCTACACGGCC, Rev CAGGGGCATTTCCATGTAGG. PCR conditions for amplification consisted in an initial denaturation step at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and a final extension step at 72°C for 7 minutes.

Surgical procedures for in vivo experiments

All mice were anesthetized with 2.5% Avertin (100% Avertin: 10 ***g*** 2,2,2-tribromoethyl alcohol, 10 ml tert-amyl alcohol, Sigma). Animals were housed at constant room temperature (24±1°C) and humidity (60±3%).

Hind limb ischemia was induced by femoral artery dissection as described (Germani et al., 2003). AT-SVF cells (5×10^5) from 6-week-old GFP$^+$ mice (Okabe et al., 1997) were delivered by 5 injections (1×10^5/injection in 10 μl of PBS) in the left adductor muscle of GFP-negative syngenic mice immediately after femoral

artery removal. Control animals were injected with PBS only. Ischemic limbs were processed for immunoistochemistry 1 week after injection.

For transplantation into dystrophic mice, 5×10^5 AT-SVF cells from 6-week-old C57BL/10SnJ (wt) mice were delivered by 5 injections (1×10^5/injection in 10 μl of PBS) into the left adductor muscle of 2-month-old C57BL/10SnJ-*mdx* mice (The Jackson Laboratory). Age-matched mdx control animals were injected with PBS only. Injected limbs were processed for immunohistochemistry 3 weeks after injection.

Immunohistochemistry

Anesthetized mice were perfused with phosphate buffer (0.2 mol/l, pH 7.4) containing 5000 U/ml heparin (Roche) followed by 10% buffered formalin for 10 minutes via the abdominal aorta at 100 mm Hg. Adductor muscles were then removed, fixed in formalin for 48 hours and embedded in paraffin (Bio-plast). Sections from each sample were cut at a thickness of 3 μm.

To visualize donor-derived fibers, sections were deparaffinized, microwave treated, and incubated overnight at 4°C with rabbit polyclonal antibody against GFP (Abcam Ab290, 10 μg/ml) or against the carboxy-terminal portion of dystrophin (Abcam, Ab15277, 2 μg/ml) for ischemic or mdx mice, respectively. Sections were subsequently incubated at room temperature for 1 hour with FITC-conjugated anti-rabbit antibody (1:40 dilution, Dako), stained with Hoechst to identify nuclei and mounted in Vectashield (Vector Laboratories). Images were acquired under a ZEISS Axiovert fluorescence microscope with an image analyser KS300 software.

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