

# Human multipotent adipose-derived stem cells restore dystrophin expression of Duchenne skeletal-muscle cells *in vitro*

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**Background information.** DMD (Duchenne muscular dystrophy) is a devastating X-linked disorder characterized by progressive muscle degeneration and weakness. The use of cell therapy for the repair of defective muscle is being pursued as a possible treatment for DMD. Mesenchymal stem cells have the potential to differentiate and display a myogenic phenotype *in vitro*. Since liposuctioned human fat is available in large quantities, it may be an ideal source of stem cells for therapeutic applications. ASCs (adipose-derived stem cells) are able to restore dystrophin expression in the muscles of *mdx* (X-linked muscular dystrophy) mice. However, the outcome when these cells interact with human dystrophic muscle is still unknown.

**Results.** We show here that ASCs participate in myotube formation when cultured together with differentiating human DMD myoblasts, resulting in the restoration of dystrophin expression. Similarly, dystrophin was induced when ASCs were co-cultivated with DMD myotubes. Experiments with GFP (green fluorescent protein)-positive ASCs and DAPI (4',6-diamidino-2-phenylindole)-stained DMD myoblasts indicated that ASCs participate in human myogenesis through cellular fusion.

**Conclusions.** These results show that ASCs have the potential to interact with dystrophic muscle cells, restoring dystrophin expression of DMD cells *in vitro*. The possibility of using adipose tissue as a source of stem cell therapies for muscular diseases is extremely exciting.

## Introduction

Muscular dystrophies are a clinically and genetically heterogeneous group of disorders characterized by progressive degeneration and loss of skeletal muscle (reviewed by Zatz et al., 2003). Adult skeletal muscle has the potential to regenerate new muscle

fibres by activating a population of mononucleated precursors, which otherwise remain in a quiescent and non-proliferative state (Schultz and McCormick, 1994). However, the continuous and gradual muscle degeneration in progressive muscular dystrophies leads to a depletion of satellite cells and, consequently, the capacity to restore the skeletal muscle is lost (Laguens, 1963; Heslop et al., 2000).

DMD (Duchenne muscular dystrophy), an X-linked lethal disorder that affects 1 in 3–4000 male births, is the most prevalent form of muscular dystrophy (Leturcq and Kaplan, 2005). DMD is caused by genetic mutations in the dystrophin gene at Xp21, resulting in the absence of this protein in muscle. Dystrophin is a component of the dystrophin-associated glycoprotein complex and

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**Key words:** adipose, Duchenne muscular dystrophy (DMD), DMD therapy, *in vitro* study, skeletal-muscle cell, stem cell.

**Abbreviations used:** ASC, adipose-derived stem cell; DAPI, 4',6-diamidino-2-phenylindole; DMD, Duchenne muscular dystrophy; DMEM-HG, Dulbecco's modified Eagle's medium with high glucose; FBS, fetal bovine serum; FM, fusion medium; GFP, green fluorescent protein; GM, growth medium; hASC, human ASC; HLA-ABC, human leucocyte antigens, MHC class I; HLA-DR, human leucocyte antigens, MHC class II; hMSC, human mesenchymal stem cells; HS, horse serum; *mdx*, X-linked muscular dystrophy; PLA, processed lipoaspirate; RT, reverse transcription; SH3 domain, Src homology 3 domain; SVF, stromal-vascular fraction.

links the muscle fibre cytoskeleton to the extracellular matrix (Burghes et al., 1987).

A promising approach to the treatment of DMD is to restore dystrophin expression by repairing the defective muscle through cell therapy. Previous studies have suggested that haematopoietic stem cells can contribute to skeletal-muscle regeneration (Gussoni et al., 1999; McKinney-Freeman et al., 2002; Camargo et al., 2003; Corbel et al., 2003; Bachrach et al., 2004). In normal and *mdx* (X-linked muscular dystrophy) mice, BM (bone marrow)-derived stem cells were shown to participate in skeletal-muscle repair after induced damage (Ferrari et al., 1998; Fukada et al., 2002; LaBarge and Blau, 2002). However, the clinical usefulness of haematopoietic cell transplantation in muscular dystrophies, including DMD, has been a subject of great controversy (Lakshminpathy and Verfaillie, 2005; Sampaolesi et al., 2006).

An abundant and accessible source of stem cells is adipose tissue. Several groups have demonstrated that mesenchymal cells within the SVF (stromal-vascular fraction) of subcutaneous adipose tissue [PLA (processed lipoaspirate) cells] are capable of differentiation along multiple lineages, including myocytes, in the presence of lineage-specific inductive medium (Halvorsen et al., 2001a, 2001b; Zuk et al., 2001, 2002; Erickson et al., 2002; Mizuno et al., 2002; Safford et al., 2002, 2004; Justesen et al., 2004; Miranville et al., 2004; Planat-Benard et al., 2004; Rehman et al., 2004; Brzoska et al., 2005; Seo et al., 2005; Rodriguez et al., 2006).

Recently, the ability of hASCs [human ASCs (adipose-derived stem cells)] to differentiate into muscle was demonstrated *in vitro* (Lee and Kemp, 2006; Rodriguez et al., 2006). *In vivo* studies showed that implantation of ASCs in *mdx* mice restored dystrophin expression in the dystrophic mouse cells (Rodriguez et al., 2005).

In the present study, we explore the concept of using ASCs to restore dystrophin expression, but in the context of human DMD muscle cells. We also demonstrate that the participation of ASCs in human myogenesis occurs through cellular fusion.

## Results

### Characterization of ASCs

ASCs from six unrelated donors were characterized by flow cytometry for the expression of 12 cell sur-

face proteins [HLA-DR (human leucocyte antigens, MHC class II), HLA-ABC (human leucocyte antigens, MHC class I), CD13, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105 and CD117]. Cell viability was above 96% by Guava ViaCount reagent (Guava Technologies).

At passage 4, hASCs did not express either endothelial markers (CD31-PECAM1) or haematopoietic markers (CD34, CD45 and CD117-c-kit). Most of the ASCs expressed high levels of CD13, CD44, adhesion markers (CD29-integrin  $\beta$ 1 and CD90-Thy-1) and mesenchymal stem cell marker CD73 [SH3 domain (Src homology 3 domain)]. Expression of some markers, such as CD105 (SH2), was variable among the donors. ASCs were negative for HLA-class II (HLA-DR), but positive for HLA-class I (HLA-ABC) (Figure 1).

The plasticity of ASCs was assessed 3 weeks after lineage induction. Myogenic, adipogenic, chondrogenic and osteogenic differentiations were demonstrated by the expressions of myogenic markers (myosin and desmin), lipid vacuoles, mucopolysaccharide-rich extracellular matrix and calcium deposits respectively (Figure 2). These results confirmed the mesenchymal nature of the isolated cells as well as their multipotent potential.

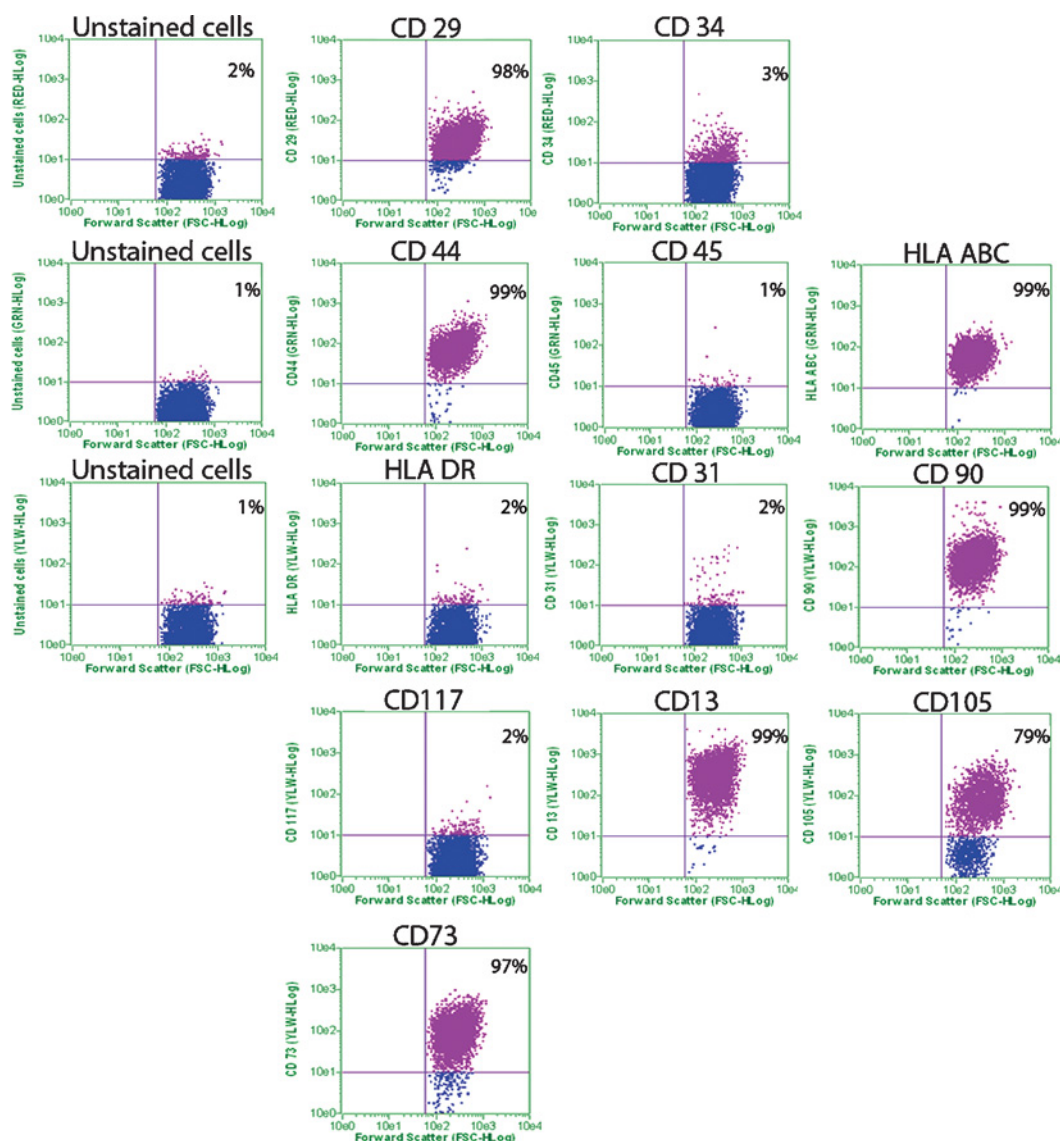
### Spontaneous myogenic differentiation of ASCs

The undifferentiated control ASC cultures were maintained in GM (growth medium) to preserve their multipotent capacity (Zuk et al., 2001). Surprisingly, we observed spontaneous fusion between ASCs and the formation of myotube-like cells (Figures 3B and 3E) in the control culture plates from two of the donors (fresh or at passages 1 and 2). However, differentiation was not observed in cultures maintained in a low cell density (Figure 3A).

Immunofluorescence confirmed the expression of myosin in the differentiated ASC culture (Figure 3D). RT (reverse transcription)-PCR revealed the expression of MyoD, telethonin and dystrophin in first passage/high density culture but not at first passage/low density nor in passage 4/high density cultures (Figure 3F). Western blot confirmed the presence of dystrophin in the differentiated cells (Figure 3G). Since uncultured ASCs do not show expression of these markers (Figures 3F and 3G), these results suggest that ASCs when submitted to culture

**Figure 1 | Immunophenotyping of ASCs at passage 4**

Values represent the mean percentage of positively stained cells as analysed by flow cytometry. Graphs of forward scatter against fluorescence intensity of the indicated antigen are shown.



conditions in earlier passages and in high density show autonomous myogenic potential and can spontaneously differentiate into skeletal muscle. On the other hand, since we observed such a spontaneous differentiation only in the initial passages and not in later stages, even at a high cell density (Figure 3C), we decided to use ASCs at passages 4 or later in the co-culture experiments.

#### ASCs co-cultured with primary DMD myoblasts differentiate into dystrophin-positive myotubes

To test whether the ASCs were able to interact with DMD myoblasts and acquire a myogenic phenotype, we co-cultured equal amounts of ASCs expressing GFP (green fluorescent protein) along with primary, GFP-negative DMD myoblasts previously stained with DAPI (4',6-diamidino-2-phenylindole;

**Figure 2 | Differentiation potential of ASCs at passage 4**

(A) The adipogenic differentiation was detected by the formation of intracytoplasmic lipid droplets stained with Oil Red O. Scale bar, 200  $\mu\text{m}$ . (A') Undifferentiated ASCs stained with Oil Red O. Scale bar, 200  $\mu\text{m}$ . (B) Osteogenic differentiation was demonstrated by calcium deposition, which was shown by von Kossa stain. Scale bar, 200  $\mu\text{m}$ . (B') Undifferentiated ASCs stained with von Kossa. Scale bar, 200  $\mu\text{m}$ . (C) Chondrogenic differentiation in monolayer culture was demonstrated by staining with Toluidine Blue. Scale bar, 200  $\mu\text{m}$ . (C') Undifferentiated ASCs stained with Toluidine Blue. Scale bar, 200  $\mu\text{m}$ . (E, F) Myogenic differentiation was assessed by immunofluorescence. Induced cells were labelled with anti-human myosin monoclonal antibody (E) or with anti-human; desmin monoclonal antibody (F). (E') Undifferentiated ASCs labelled with anti-human myosin monoclonal antibody. (F') Undifferentiated ASCs labelled with anti-human desmin monoclonal antibody. Scale bar, 50  $\mu\text{m}$ .

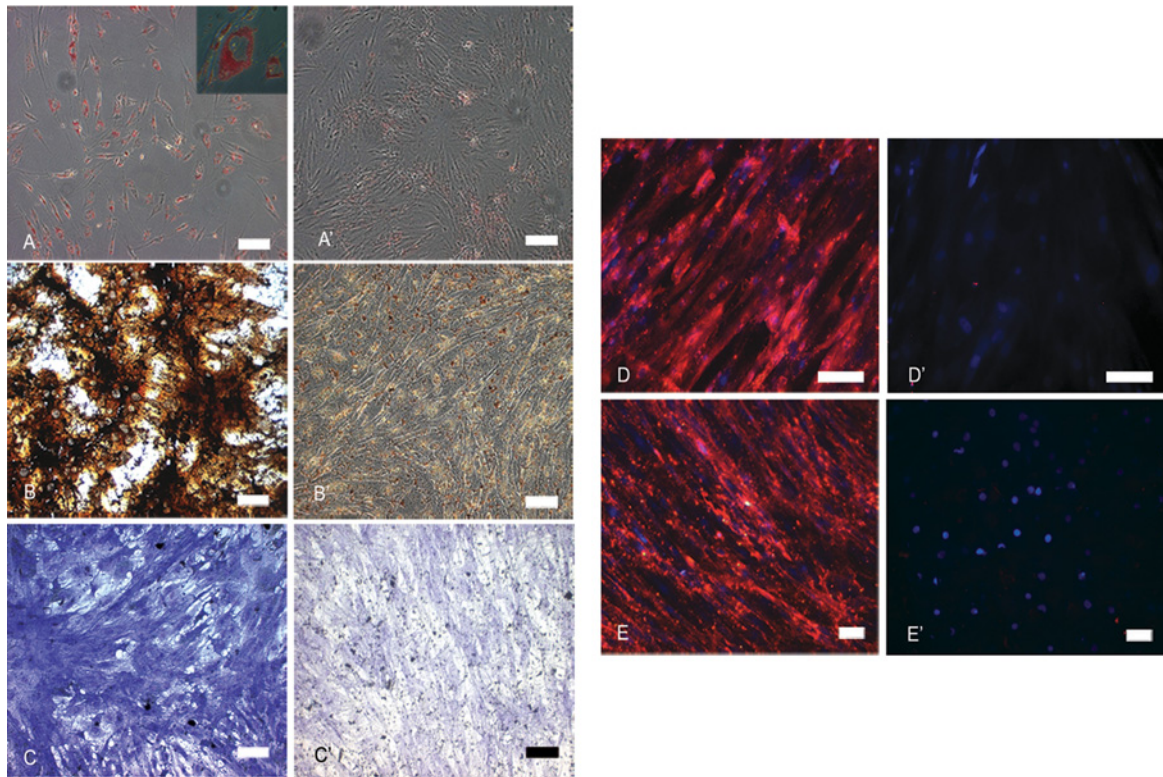


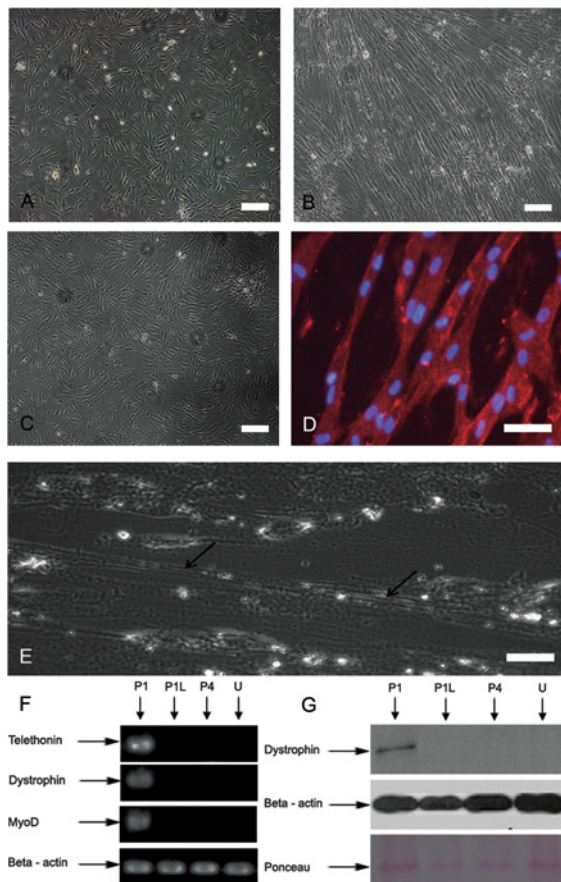
Figure 4A). The ASCs (passage 4) were stably transfected with GFP in order to distinguish them from DMD myoblasts in the co-cultures. After establishment of the mixed cultures, the cells were exposed to FM (fusion medium) that induces myoblasts to coalesce and form multinucleated structures. After 10 days, multinucleated myotubes were observed with areas of GFP-positive syncytia in the resulting cultures (Figure 4B). Controls containing exclusively GFP-positive ASCs subjected to the same experimental conditions did not contain any multinucleated structures, whereas syncytium formation in cultures of DMD myoblasts occurred to the same extent as in the mixed cultures.

To evaluate whether ASCs contributed to the pool of human myotubes by differentiation, fusion, or both, the co-culture was kept under myogenic differentiation conditions and visualized through direct fluorescence microscopy analysis (Figure 4C). We concluded that ASCs participate in the generation of human myotubes through cellular fusion, because all GFP-positive syncytia presented at least one DAPI-stained nucleus.

To confirm that the fluorescently labelled multinucleated structures were expressing the heretofore absent dystrophin, an immunofluorescence assay was performed for this protein after 45 days (Figure 4D). The specificity of this assay was corroborated by the

**Figure 3 | Spontaneous differentiation**

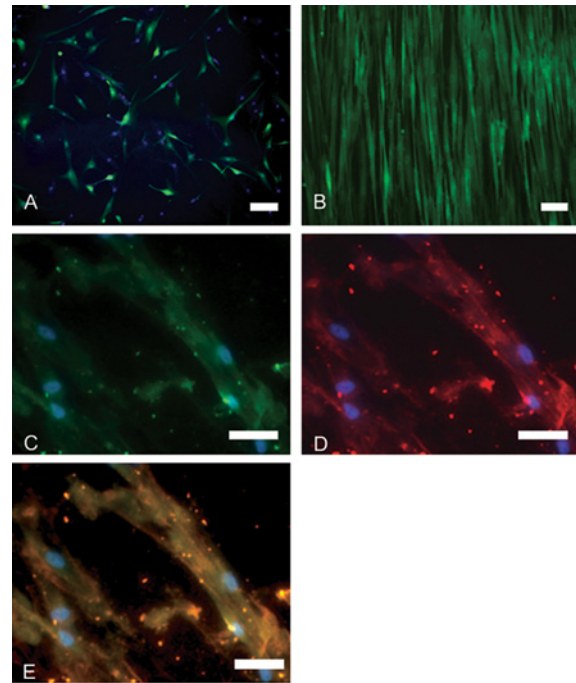
(A) Typical morphology of ASCs at passage 1/low-density culture. Scale bar, 200  $\mu\text{m}$ . (B) ASCs at passage 1/high-density culture show the characteristic pattern of muscle fibres. Scale bar, 200  $\mu\text{m}$ . (C) Spontaneous differentiation was not observed in passage 4/high-density culture. Scale bar, 200  $\mu\text{m}$ . (D) Expression of myosin in passage 1/high-density culture. Scale bar, 50  $\mu\text{m}$ . (E) Phase-contrast microscopy image showing that at passage 1/high-density culture, ASCs fuse, forming myotube-like structures (arrows). Scale bar, 200  $\mu\text{m}$ . (F) Gene expression of skeletal-muscle markers at passage 1/high-density (P1), passage 1/low-density (P1L), passage 4 (P4) and uncultured ASCs (U) determined by RT-PCR using  $\beta$ -actin as a control gene. (G) Dystrophin expression confirmed by Western-blot analysis.



absence of staining ASCs and DMD myotubes. No revertant fibres that spontaneously expressed dystrophin were found in the DMD myotube cultures. Merging the DAPI, GFP and Dys1-specific signals revealed that the GFP-positive myotubes, containing DAPI-stained nuclei, were expressing dystrophin

**Figure 4 | ASCs co-cultured with primary DMD myoblasts**

(A) DMD myoblasts stained with DAPI and GFP-positive ASCs on the first day in culture. Scale bar, 200  $\mu\text{m}$ . (B) GFP-positive fibres observed after 10 days in culture. Scale bar, 50  $\mu\text{m}$ . (C) GFP-positive myotubes. Scale bar, 50  $\mu\text{m}$ . (D) Expression of dystrophin in myotubes containing blue DMD nuclei by immunofluorescence. Scale bar, 50  $\mu\text{m}$ . (E) Merged image confirming the fusion between the two cell types. Scale bar, 50  $\mu\text{m}$ .



(Figure 4E). These results demonstrated that ASCs can contribute to the generation of human myotubes in the presence of differentiating myoblasts.

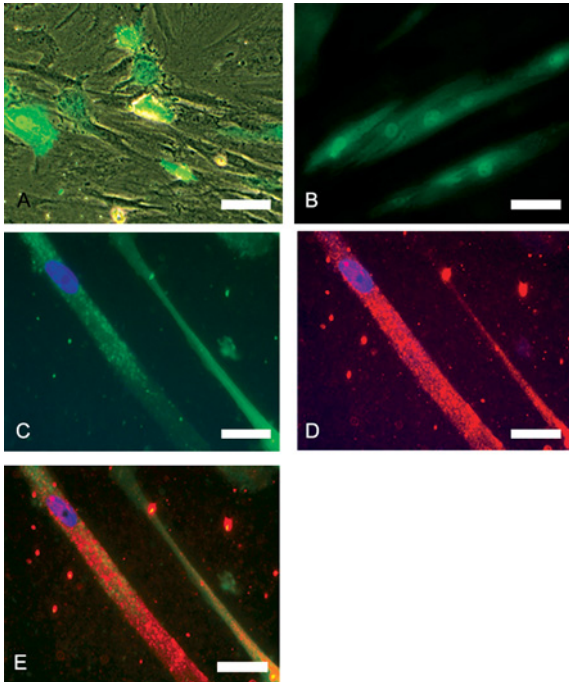
**ASCs plated on DMD myotubes restore dystrophin expression**

In order to investigate the behaviour of ASCs in the presence of DMD myotubes, we differentiated DAPI-stained DMD myoblasts into myotubes and added undifferentiated GFP-positive ASCs to the myotube cultures. The co-cultures were maintained only with GM to exclude the possibility of myogenic induction by the medium.

After 1 h of co-culture, the ASCs adhered to the DMD myotubes (Figure 5A). GFP-positive myotubes were found after 10 days (Figure 5B). Immunofluorescence showed GFP-positive myotubes containing DAPI-stained nuclei and expressed dystrophin on

**Figure 5 | ASCs plated on DMD myotubes**

(A) GFP-positive ASCs adhered to the DMD myotubes after 1 h. Scale bar, 50  $\mu$ m. (B) GFP-positive fibres observed within 10 days in culture. Scale bar, 50  $\mu$ m. (C) GFP-positive myotubes. Scale bar, 50  $\mu$ m. (D) Expression of dystrophin in myotubes containing blue DMD nuclei by immunofluorescence. Scale bar, 50  $\mu$ m. (E). Merged image confirming fusion between the two cell types. Scale bar, 50  $\mu$ m.



day 45 (Figure 5C), which was not limited to the proximity of the normal nuclei (Figure 5D). This observation demonstrates that ASCs are able to fuse to DMD myotubes and restore dystrophin expression.

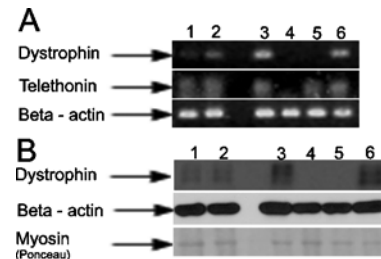
**Dystrophin expression through RT-PCR and Western-blot analyses**

Dystrophin expression was evaluated by RT-PCR. In order to assess whether the expression of dystrophin was proportional to the amount of ASCs present in the co-culture, we plated equal proportions of DMD myoblasts and ASCs. In this co-culture assay, we observed a greater dystrophin expression than that found when the ratio of ASCs to plated myoblasts was 1:3 (Figure 6A).

In addition, the co-culture of ASCs with normal myoblasts showed a dystrophin expression similar to that found in normal myotubes (Figure 6A). This indicates that ASCs, when differentiated into muscle

**Figure 6 | Gene expression in the co-culture experiments**

Samples shown are the following: (1) co-culture of DMD myoblasts/GFP-positive ASCs at a ratio of 1:1. (2) Co-culture of DMD myotubes/GFP-positive ASCs at a ratio of 1:3. (3) Co-culture of normal myoblasts/GFP-positive ASCs at a ratio of 1:1. (4) GFP-positive ASCs. (5) DMD myotubes. (6) Normal myotubes. (A) Dystrophin and telethonin gene expressions determined by RT-PCR by using  $\beta$ -actin as a control gene. (B) Dystrophin expression confirmed by Western-blot analysis.



cells, can express dystrophin at the same level of differentiated myoblasts.

To determine whether ASCs express skeletal-muscle proteins, we analysed the expression of telethonin, which is expressed exclusively in skeletal-muscle fibres (Moreira et al., 2000). We observed that the telethonin expression in the co-cultures was comparable with that found in normal controls or DMD myoblasts cultures. This result indicates that these cells can differentiate into skeletal-muscle cells (Figure 6A).

Finally, we analysed the dystrophin protein levels in the two different co-cultivation assays. As seen in Figure 6(B), the level of dystrophin protein was quite similar as assessed by Western-blot analysis between the co-cultivation assays and as compared with normal myotubes. This indicates that the vast majority of co-cultivated cells, including ASCs, were expressing this protein.

**Discussion**

In response to muscle injury, muscle satellite cells are activated to become myogenic precursor cells. These cells divide and fuse to repair the damaged muscle. However, the mature muscle satellite cells represent only 1–5% of the total muscle cells and their potential for self-renewal decreases with age (Schultz and Lipton, 1982). In DMD patients, the intense degeneration that occurs in muscle fibres exhaust the ability

of satellite cells to proliferate and replace damaged fibres (Heslop et al., 2000).

The possibility of stem cell-mediated myogenesis is very exciting. It has been previously suggested that hMSC (human mesenchymal stem cells) from bone marrow and human circulating AC133<sup>+</sup> stem cells, when co-cultured with mouse skeletal myoblasts, form myotubes by fusion and commit functionally in the myogenic environment (Torrente et al., 2004; Schulze et al., 2005; Goncalves et al., 2006). However, obtaining cells from bone marrow is a painful procedure and yields a low number of hMSC.

Zuk et al. (2001) have shown that human stem cells derived from adipose tissue were able to differentiate along the myogenic lineage when exposed to inductive media. The cells expressed muscle-specific proteins and fused to form multinucleated myotubes when plated under promyogenic conditions (Mizuno et al., 2002). More recently, the potential of human PLA to differentiate into smooth-muscle cells in addition to a trilineage differentiation capacity was reported (Rodriguez et al., 2006).

It was also shown that mouse adipose tissue stem cells were able to differentiate into skeletal muscle and restore dystrophin expression in *mdx* mice (Di Rocco et al., 2006). On the other hand, ASCs when injected into *mdx* mice revealed dystrophin expression just at the site of injection (Rodriguez et al., 2005). The authors suggest two mechanisms to account for the contribution of ASCs to muscle regeneration: *de novo* generation of muscle-specific cells from ASCs or modification in gene expression after direct fusion of ASCs with host cells. The relative contribution of transdifferentiation compared with nuclear fusion in the long-term engraftment of ASCs into the host muscle was not investigated by these authors.

However, to the best of our knowledge, the interaction between human adipose stem cells and human skeletal-muscle cells *in vitro* has not yet been reported. In the present study, we first co-cultured human ASCs with human DMD myoblasts or DMD myotubes and assessed whether dystrophin expression was restored *in vitro*. We observed that ASCs were able to fuse into myoblasts as well as myotubes and express dystrophin in both situations. Whether ASCs directly fuse to DMD muscle cells or first acquire muscle progenitor phenotype remains to be investigated.

We found that human ASCs show spontaneous skeletal-muscle differentiation in early passages when

cultured in high cell density without any induction, as observed for mouse ASCs (Di Rocco et al., 2006). Therefore we decided to use ASCs in passage 4 or later for co-culture experiments. Apparently, sequential passages favour hMSC expansion since we did not find any spontaneous differentiation after passage 4.

Assessment of dystrophin and telethonin expression in co-cultures of DMD myoblast/ASCs as compared with normal myoblast/ASCs suggests that most of the ASCs, under the co-culture conditions used in the present study, were involved in myotube formation.

Previous studies reported that ASCs are composed of heterogeneous cell populations including blood-derived cells, endothelial, pericytes and other progenitors (Yoshimura et al., 2006). According to Sampaoli et al. (2006), transplantation of mesoangioblasts, a class of vessel-associated stem cells, into dystrophic dogs can achieve an extensive reconstitution of the muscle with up to 70% of total fibres expressing dystrophin. ASCs present similarities to mesoangioblasts. Both types of cells show fibroblast-like morphology, proliferate efficiently and display a similar surface protein profile. However, while mesoangioblasts were isolated from the outgrowth of small, vessel-containing, tissue fragments from muscle biopsies, which are not easily obtainable, human liposuctioned fat is available in large quantities. If the *in vivo* potential of ASCs is similar to that found with mesoangioblasts, it might be an ideal source of stem cells for therapeutic applications.

Our *in vitro* results reinforce the observation that ASCs are able to restore dystrophin expression in *mdx* mice (Rodriguez et al., 2005) and support the idea that ASCs could be an important source of muscle cell therapy. In short, we demonstrated here that ASCs, when co-cultured with primary DMD myoblasts, fuse and generate dystrophin and skeletal myotubes. We also show that ASCs plated on DMD myotubes fuse into muscle cells and re-establish dystrophin expression. This suggests that cellular fusion of ASCs with muscle cells takes place during the process of syncytium formation.

The *in vitro* expression of dystrophin supports the hypothesis that ASC cells have the potential to be used for DMD therapy. Adipose tissue is abundant and liposuction procedures are relatively safe. Therefore the possibility of using adipose tissue as a source of stem cell therapies not only for DMD but also

for different muscular diseases is extremely exciting. However, further *in vivo* studies, which are currently under way, will be essential for identifying the factors determining their definitive myogenic differentiation and 'homing' as well as potential clinical effects in animal models before any therapeutic trial in DMD patients.

## Materials and methods

All experiments were approved by the research ethics committee of the Biosciences Institute, University of São Paulo. All the present studies were performed in the Human Genome Research Center, at the Biosciences Institute, University of São Paulo.

### Myoblasts cell culture and differentiation

The human DMD skeletal-muscle cells were obtained from primary cultures of muscle biopsies taken for diagnostic purposes, following informed consent. Normal skeletal muscle was obtained from healthy DMD fathers. The biopsies were processed according to protocol 11.4 described in Freshney (2000).

The myoblasts were cultured in DMEM-HG (Dulbecco's modified Eagle's medium with high glucose; Gibco) supplemented with 20% (v/v) FBS (fetal bovine serum; Gibco), 100 units/ml of penicillin and 100 g/ml of streptomycin (Gibco). Cells were maintained at 37°C and 5% CO<sub>2</sub>. To differentiate human myoblasts into myotubes, the cells were rinsed twice in PBS and cultured in FM [DMEM-HG containing 3% (v/v) HS (horse serum; Gibco)] for 1 week.

### ASC isolation and expansion

Human adipose tissue was obtained from elective liposuction procedures. Cells were isolated using methods previously described (Gimble and Guilak, 2003). Briefly, the unprocessed lipoaspirate was washed extensively with equal volumes of PBS containing antibiotics (100 units/ml of penicillin and 100 g/ml of streptomycin) and then dissociated with 0.075% collagenase (Sigma). Enzyme activity was neutralized with DMEM-HG containing 10% FBS. The infranatant was centrifuged at 1200 *g* for 5 min to pellet the cells. The cells from the pellet SVF were filtered to remove debris and seeded on to tissue culture plates (Nunc) at 1000–3500 cells/cm<sup>2</sup> in DMEM-HG containing 10% FBS. Cultures were washed with PBS 24–48 h after plating to remove unattached cells and fed with fresh medium.

The cultures were maintained at 37°C with 5% CO<sub>2</sub>, in GM (DMEM-HG containing 10% FBS). When they achieved approx. 70% confluence, the cells were trypsinized (0.025%; Invitrogen) and plated at a density of 5000/cm<sup>2</sup>. Cultures were passaged repeatedly after achieving a density of 70–80% until passage 4. The remaining cells were cryopreserved in cryopreservation medium (10% DMSO, 10% DMEM-HG and 80% FBS), frozen at –80°C in a cryo 1°C freezing container (Nalgene) and stored in liquid nitrogen the next day.

### Multilineage differentiation

Cells were analysed for their capacity to differentiate towards adipogenic, osteogenic, chondrogenic and myogenic lineages as described by Zuk et al. (2001).

### Adipogenic differentiation

Subconfluent cells were cultured in GM supplemented with 1 µM dexamethasone (Sigma), 500 µM IBMX (isobutylmethylxanthine; Sigma), 60 µM indomethacin (Sigma) and 5 µg/ml insulin (Sigma). Adipogenic differentiation was confirmed on day 21 by intracellular accumulation of lipid-rich vacuoles stainable with Oil Red O (Sigma). For the Oil Red O stain, cells were fixed with 4% (w/v) paraformaldehyde for 30 min, washed and stained with a working solution of 0.16% Oil Red O for 20 min.

### Chondrogenic differentiation

Subconfluent cells were cultured in chondrogenic differentiation medium consisting of DMEM with low glucose supplemented with 100 nM dexamethasone, 50 µM ascorbic acid-2 phosphate (Sigma), 1 mM sodium pyruvate (Gibco), 10 ng/ml TGF-β1 (transforming growth factor-β1; R&D Systems) and 1% ITS-Premix (Becton Dickinson). Medium was changed every 3–4 days, and cells were fixed on day 21 with 4% paraformaldehyde. Chondrogenesis was demonstrated by staining with Toluidine Blue.

### Osteogenic differentiation

To promote osteogenic differentiation, subconfluent cells were treated with GM supplemented with 50 µM ascorbate-2 phosphate, 10 mM β-glycerophosphate (Sigma) and 0.1 µM dexamethasone for 21 days. Osteogenesis was demonstrated by the accumulation of mineralized calcium phosphate, which was shown by von Kossa stain. Briefly, cells were stained with 1% silver nitrate (Sigma) for 45 min under UV light, followed by 3% sodium thiosulfate (Sigma) for 5 min, and then counterstained with van Gieson.

### Myogenic differentiation

For myogenic differentiation, ASC cells passage 4 were cultured in GM supplemented with 0.1 µM dexamethasone (Sigma), 50 µM cortisol (Sigma) and 5% HS (Gibco) for 45 days.

### Flow cytometry

Early passages from six different donors (aged 19–40 years) were evaluated for cell surface protein expression using flow cytometry. The flow cytometry was performed on a Guava EasyCyte system (Guava Technologies) using a blue laser (488 nm). Cells were pelleted, resuspended in PBS at a concentration of 1 × 10<sup>5</sup> cells/ml and stained with saturating concentration of antibodies. Cells were incubated in the dark for 45 min at room temperature (20°C). After incubation, cells were washed three times with PBS and resuspended in 0.25 ml of cold PBS. Cell viability was assessed with Guava ViaCount reagent (Guava Technologies).

ASCs were incubated with the following primary antibodies: HLA-DR-PE, HLA-ABC-FITC, CD13-PE, CD29-PECy5, CD31-PE, CD34-PerCP, CD44-FITC, CD45-FITC, CD73, CD90-PE, CD105 and CD117-PE (Becton Dickinson) for 45 min. Unconjugated markers were reacted with anti-mouse PE secondary antibody (Guava Technologies) for 15 min.

Flow cytometer gates were set using unstained cells. Cells were gated by forward scatter to eliminate debris. To eliminate the possible autofluorescence of ASCs, we removed the contribution of unstained cells in the measurement channel. A minimum of 10 000 events was counted for each analysis.



### ASCs transduction with lentivirus vector

Supernatant containing the FUGW lentiviral vector (Lois et al., 2002) was produced as described previously (Strauss et al., 2006) and concentrated by ultracentrifugation. Undifferentiated ASCs at passage 2 were incubated at 37°C, in a 6-well plate (Nunc), using a minimal volume of GM in the presence of vector particles [20 pfu (plaque-forming units) per cell] and 8 µg/ml Polybrene (Sigma). After 4 h, 2 ml of GM was added and the medium was changed on the next day. At 72 h post-transduction, transgene expression was examined by flow cytometry. Approx. 80% of the cells were GFP-positive, and GFP expression did not decline during culture passages. To evaluate whether GFP interfered with the multipotent capacity of ASCs, both GFP-positive and -negative cells at successive passages were analysed by flow cytometry and multilineage differentiation.

### Co-cultures

Two different types of co-culture were tested. In the first, we mixed equal amounts of GFP-negative myoblasts stained with DAPI and GFP-positive ASCs plated at a density of 5000 cell/cm<sup>2</sup> on culture dishes in GM. After 90% confluence was reached, the cultures were washed with PBS and fed with FM to promote myotube differentiation. Fresh medium was added every 2–3 days for 6 weeks.

In the second type of co-culture, DMD myotubes stained with DAPI were cultured and the GFP-positive ASCs were added to the myotube cultures at a ratio of 3:1 of plated myoblasts and ASCs. The co-cultures were maintained in GM, and fresh medium was added every 2–3 days for 6 weeks.

For co-culture experiments, we used DMD muscle cells from patients with known deletions, which allowed the use of primers containing the mutation and quantify the mRNA of the dystrophin gene by RT-PCR.

Co-culture control experiments were: GFP-positive ASCs maintained undifferentiated; normal control myoblasts co-cultured with GFP-positive ASCs; normal control myoblasts; and DMD myoblasts. The myoblast controls were exposed to FM as the co-culture experiments.

The visualization of ASC cell differentiation and their identification in the myoblasts co-cultures were performed with GFP transduction since it has been shown that GFP did not influence the multilineage potential of human ASCs (Lin et al., 2006) although it had been reported that GFP might impair actin-myosin interaction in muscle cells (Agbulut et al., 2006).

### RNA isolation and RT-PCR

Total RNA was harvested from cultured cells using TRIzol<sup>®</sup> (Invitrogen) following the manufacturer's instructions. The RNA was treated with DNase (Invitrogen). A total of 5 µg of total RNA was reverse-transcribed with a SuperScript<sup>™</sup> III First-strand Synthesis System (Invitrogen). All amplifications were performed in an MJ Research PTC-200 thermocycler (MJ Research) for 24 cycles after the initial 2 min denaturation at 94°C. The PCR primers are listed in Table 1. The PCR products were analysed by electrophoresis of 5 µl aliquots in 2% agarose gel, and the amplicons were visualized by ethidium bromide staining.

### Immunofluorescence

Cells grown in a one-chamber slide (Nalgene; Nunc) were fixed in 4% paraformaldehyde in PBS for 20 min at 4°C and per-

**Table 1 | Oligonucleotide primer sequences and expected PCR product sizes**

Gene	Oligonucleotide primers	Product size (bp)
Dystrophin		196
Sense	5'-ttgctggtctcctgctggtcagtg-3'	
Antisense	5'-caaagccctcactcaaacatcaagc-3'	
MyoD		430
Sense	5'-cgatataccaggtgctctgaggg-3'	
Antisense	5'-gggtgggttacaggtacacctgc-3'	
Telethonin		150
Sense	5'-ggctgctccctgcatga-3'	
Antisense	5'-ctgtagggcagctggta-3'	
β-Actin		242
Sense	5'-gtggggcgccccaggcaccac-3'	
Antisense	5'-ccccctgacccccaggccaa-3'	

meabilized in 0.05% Triton X-100 in PBS for 5 min. Non-specific binding was blocked with 10% FBS in PBS for 1 h at room temperature. Cells were incubated with primary antibody overnight at 4°C and with secondary antibody for 1 h at room temperature. The following primary antibodies were used: anti-desmin (1:100; Sigma), anti-myosin, skeletal (1:100; Sigma), NCL-Dys1 (1:20; Novocastra), NCL-Dys2 (1:20; Novocastra) combined with a rabbit anti-mouse IgG secondary antibody, Cy3-conjugated (1:100; Chemicon). The fluorescence signal was examined in Axiovert 200 (Carl Zeiss) and in an ApoTome imaging system (Carl Zeiss).

### Western-blot analysis

Cells were harvested from culture samples and proteins were extracted by treatment with a buffer containing 10 mM Tris/HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 60 mM octyl glucoside. Samples were centrifuged at 13 000 g for 10 min to remove insoluble debris. Soluble proteins were resolved by SDS/PAGE (6 or 13% gel) and transferred on to nitrocellulose membranes (Hybond; Amersham). All membranes were stained with Ponceau (Sigma) to confirm equal loading and transfer of protein. Blots were blocked for 1 h in TBST (Tris-buffered saline+Tween) containing 5% (w/v) non-fat dried skimmed milk powder and reacted overnight with the following primary antibodies against dystrophin: NCL-Dys1 (1:100; Novocastra), NCL-Dys2 (1:25; Novocastra) and anti-β-actin (1:10000; Sigma) kindly provided by Dr Patrícia Gama (Instituto Ciências Biomédicas, São Paulo, SP, Brazil). Blots were incubated for 1 h at room temperature with secondary antibodies and immunoreactive bands were detected with an ECL<sup>®</sup> chemiluminescence detection system (GE Healthcare).

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## References

- Agbulut, O., Coirault, C., Niederlander, N., Huet, A., Vicart, P., Hagege, A., Puceat, M. and Menasche, P. (2006) GFP expression in muscle cells impairs actin–myosin interactions: implications for cell therapy. *Nat. Methods* **3**, 331
- Bachrach, E., Li, S., Perez, A.L., Schienda, J., Liadaki, K., Volinski, J., Flint, A., Chamberlain, J. and Kunkel, L.M. (2004) Systemic delivery of human microdystrophin to regenerating mouse dystrophic muscle by muscle progenitor cells. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 3581–3586
- Bzroska, M., Geiger, H., Gauer, S. and Baer, P. (2005) Epithelial differentiation of human adipose tissue-derived adult stem cells. *Biochem. Biophys. Res. Commun.* **330**, 142–150
- Burghes, A.H., Logan, C., Hu, X., Belfall, B., Worton, R.G. and Ray, P.N. (1987) A cDNA clone from the Duchenne/Becker muscular dystrophy gene. *Nature* **328**, 434–437
- Camargo, F.D., Green, R., Capetanaki, Y., Jackson, K.A. and Goodell, M.A. (2003) Single hematopoietic stem cells generate skeletal muscle through myeloid intermediates. *Nat. Med.* **9**, 1520–1527
- Corbel, S.Y., Lee, A., Yi, L., Duenas, J., Brazelton, T.R., Blau, H.M. and Rossi, F.M. (2003) Contribution of hematopoietic stem cells to skeletal muscle. *Nat. Med.* **9**, 1528–1532
- Di Rocco, G., Iachininoto, M.G., Tritarelli, A., Straino, S., Zacheo, A., Germani, A., Crea, F. and Capogrossi, M.C. (2006) Myogenic potential of adipose-tissue-derived cells. *J. Cell Sci.* **119**, 2945–2952
- Erickson, G.R., Gimble, J.M., Franklin, D.M., Rice, H.E., Awad, H. and Guilak, F. (2002) Chondrogenic potential of adipose tissue-derived stromal cells *in vitro* and *in vivo*. *Biochem. Biophys. Res. Commun.* **290**, 763–769
- Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G. and Mavilio, F. (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* **279**, 1528–1530
- Freshney, R.I. (2000) *Culture of Animal Cells: A Manual of Basic Technique*, pp. 158, Wiley-Liss, Wilmington, DE, U.S.A.
- Fukada, S., Miyagoe-Suzuki, Y., Tsukihara, H., Yuasa, K., Higuchi, S., Ono, S., Tsujikawa, K., Takeda, S. and Yamamoto, H. (2002) Muscle regeneration by reconstitution with bone marrow or fetal liver cells from green fluorescent protein-gene transgenic mice. *J. Cell Sci.* **115**, 1285–1293
- Gimble, J. and Guilak, F. (2003) Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytherapy* **5**, 362–369
- Goncalves, M.A., de Vries, A.A., Holkers, M., van de Watering, M.J., van der Velde, I., van Nierop, G.P., Valerio, D. and Knaan-Shanzer, S. (2006) Human mesenchymal stem cells ectopically expressing full-length dystrophin can complement Duchenne muscular dystrophy myotubes by cell fusion. *Hum. Mol. Genet.* **15**, 213–221
- Gussoni, E., Soneoka, Y., Strickland, C.D., Buzney, E.A., Khan, M.K., Flint, A.F., Kunkel, L.M. and Mulligan, R.C. (1999) Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* **401**, 390–394
- Halvorsen, Y.D., Bond, A., Sen, A., Franklin, D.M., Lea-Currie, Y.R., Sujkowski, D., Ellis, P.N., Wilkison, W.O. and Gimble, J.M. (2001a) Thiazolidinediones and glucocorticoids synergistically induce differentiation of human adipose tissue stromal cells: biochemical, cellular, and molecular analysis. *Metabolism* **50**, 407–413
- Halvorsen, Y.D., Bond, A., Sen, A., Franklin, D.M., Lea-Currie, Y.R., Sujkowski, D., Ellis, P.N., Wilkison, W.O. and Gimble, J.M. (2001b) Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. *Tissue Eng.* **7**, 729–741
- Heslop, L., Morgan, J.E. and Partridge, T.A. (2000) Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. *J. Cell Sci.* **113**, 2299–2308
- Justesen, J., Pedersen, S.B., Stenderup, K. and Kassem, M. (2004) Subcutaneous adipocytes can differentiate into bone-forming cells *in vitro* and *in vivo*. *Tissue Eng.* **10**, 381–391
- LaBarge, M.A. and Blau, H.M. (2002) Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. *Cell* **111**, 589–601
- Laguens, R. (1963) Satellite cells of skeletal muscle fibers in human progressive muscular dystrophy. *Virchows Arch. Pathol. Anat. Physiol. Klin. Med.* **336**, 564–569
- Lakshminpathy, U. and Verfaillie, C. (2005) Stem cell plasticity. *Blood Rev.* **19**, 29–38
- Lee, J.H. and Kemp, D.M. (2006) Adipose-derived stem cells display myogenic potential and perturbed function in hypoxic conditions. *Biochem. Biophys. Res. Commun.* **341**, 882–888
- Leturcq, F. and Kaplan, J.C. (2005) Molecular bases of dystrophinopathies. *J. Soc. Biol.* **199**, 5–11
- Lin, Y., Liu, L., Li, Z., Qiao, J., Wu, L., Tang, W., Zheng, X., Chen, X., Yan, Z. and Tian, W. (2006) Pluripotency potential of adipose-derived stem cells marked with exogenous green fluorescent protein. *Mol. Cell. Biochem.* **291**, 1–10
- Lois, C., Hong, E.J., Pease, S., Brown, E.J. and Baltimore, D. (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295**, 868–872
- McKinney-Freeman, S.L., Jackson, K.A., Camargo, F.D., Ferrari, G., Mavilio, F. and Goodell, M.A. (2002) Muscle-derived hematopoietic stem cells are hematopoietic in origin. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 1341–1346
- Miranville, A., Heeschen, C., Sengenès, C., Curat, C.A., Busse, R. and Bouloumie, A. (2004) Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. *Circulation* **110**, 349–355
- Mizuno, H., Zuk, P.A., Zhu, M., Lorenz, H.P., Benhaim, P. and Hedrick, M.H. (2002) Myogenic differentiation by human processed liposiphate cells. *Plast. Reconstr. Surg.* **109**, 199–209
- Moreira, E.S., Wiltshire, T.J., Faulkner, G., Nilforoushan, A., Vainzof, M., Suzuki, O.T., Valle, G., Reeves, R., Zatz, M., Passos-Bueno, M.R. and Jenne, D.E. (2000) Limb-girdle muscular dystrophy type 2G is caused by mutations in the gene encoding the sarcomeric protein telethonin. *Nat. Genet.* **24**, 163–166
- Planat-Benard, V., Silvestre, J.S., Cousin, B., Andre, M., Nibbelink, M., Tamarat, R., Clergue, M., Manneville, C., Saillan-Barreau, C., Duriez, M. et al. (2004) Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. *Circulation* **109**, 656–663
- Rehman, J., Traktuev, D., Li, J., Merfeld-Claus, S., Temm-Grove, C.J., Bovenkerk, J.E., Pell, C.L., Johnstone, B.H., Considine, R.V. and March, K.L. (2004) Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* **109**, 1292–1298

- Rodriguez, A.M., Pisani, D., Dechesne, C.A., Turc-Carel, C., Kurzenne, J.Y., Wdziekonski, B., Villageois, A., Bagnis, C., Breittmayer, J.P., Groux, H. et al. (2005) Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. *J. Exp. Med.* **201**, 1397–1405
- Rodriguez, L.V., Alfonso, Z., Zhang, R., Leung, J., Wu, B. and Ignarro, L.J. (2006) Clonogenic multipotent stem cells in human adipose tissue differentiate into functional smooth muscle cells. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 12167–12172
- Safford, K.M., Hicok, K.C., Safford, S.D., Halvorsen, Y.D., Wilkison, W.O., Gimble, J.M. and Rice, H.E. (2002) Neurogenic differentiation of murine and human adipose-derived stromal cells. *Biochem. Biophys. Res. Commun.* **294**, 371–379
- Safford, K.M., Safford, S.D., Gimble, J.M., Shetty, A.K. and Rice, H.E. (2004) Characterization of neuronal/gliial differentiation of murine adipose-derived adult stromal cells. *Exp. Neurol.* **187**, 319–328
- Sampaolesi, M., Blot, S., D'Antona, G., Granger, N., Tonlorenzi, R., Innocenzi, A., Mogno, P., Thibaud, J., Galvez, B.G., Barthélémy, I. et al. (2006) Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. *Nature* **444**, 574–579
- Seo, M.J., Suh, S.Y., Bae, Y.C. and Jung, J.S. (2005) Differentiation of human adipose stromal cells into hepatic lineage *in vitro* and *in vivo*. *Biochem. Biophys. Res. Commun.* **328**, 258–264
- Schultz, E. and Lipton, B.H. (1982) Skeletal muscle satellite cells: changes in proliferation potential as a function of age. *Mech. Ageing Dev.* **20**, 377–383
- Schultz, E. and McCormick, K.M. (1994) Skeletal muscle satellite cells. *Rev. Physiol. Biochem. Pharmacol.* **123**, 213–257
- Schulze, M., Belema-Bedada, F., Technau, A. and Braun, T. (2005) Mesenchymal stem cells are recruited to striated muscle by NFAT/IL-4-mediated cell fusion. *Genes Dev.* **19**, 1787–1798
- Strauss, B.E., Patricio, J.R., de Carvalho, A.C. and Bajgelman, M.C. (2006) A lentiviral vector with expression controlled by E2F-1: a potential tool for the study and treatment of proliferative diseases. *Biochem. Biophys. Res. Commun.* **348**, 1411–1418
- Torrente, Y., Belicchi, M., Sampaolesi, M., Pisati, F., Meregalli, M., D'Antona, G., Tonlorenzi, R., Porretti, L., Gavina, M., Mamchaoui, K. et al. (2004) Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. *J. Clin. Invest.* **114**, 182–195
- Yoshimura, K., Shigeura, T., Matsumoto, D., Sato, T., Takaki, Y., Aiba-Kojima, E., Sato, K., Inoue, K., Nagase, T., Koshima, I. and Gonda, K. (2006) Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. *J. Cell. Physiol.* **208**, 64–76
- Zatz, M., de Paula, F., Starling, A. and Vainzof, M. (2003) The 10 autosomal recessive limb-girdle muscular dystrophies. *Neuromuscul. Disord.* **13**, 532–544
- Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P. and Hedrick, M.H. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.* **7**, 211–228
- Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P. and Hedrick, M.H. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* **13**, 4279–4295

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