

Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse

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Here, we report the isolation of a human multipotent adipose-derived stem (hMADS) cell population from adipose tissue of young donors. hMADS cells display normal karyotype; have active telomerase; proliferate >200 population doublings; and differentiate into adipocytes, osteoblasts, and myoblasts. Flow cytometry analysis indicates that hMADS cells are CD44⁺, CD49b⁺, CD105⁺, CD90⁺, CD13⁺, Stro-1⁺, CD34⁺, CD15⁺, CD117⁺, Flk-1⁺, gly-A⁺, CD133⁺, HLA-DR⁺, and HLA-I^{low}. Transplantation of hMADS cells into the mdx mouse, an animal model of Duchenne muscular dystrophy, results in substantial expression of human dystrophin in the injected tibialis anterior and the adjacent gastrocnemius muscle. Long-term engraftment of hMADS cells takes place in nonimmunocompromised animals. Based on the small amounts of an easily available tissue source, their strong capacity for expansion *ex vivo*, their multipotent differentiation, and their immune-privileged behavior, our results suggest that hMADS cells will be an important tool for muscle cell-mediated therapy.

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Abbreviations: aFABP, adipocyte fatty acid-binding protein; CA, fast-adherent; CS, slow-adherent; DEX, dexamethasone; EGFP, enhanced GFP; hFGF, human fibroblast growth factor; hMADS, human multipotent adipose-derived stem; hMAPCs, human multipotent adult progenitor cells; LPA, lipoaspirate; PD, population doubling; PE, phycoerythrin; SA, senescence-associated; SV, stromal-vascular; SVF, SV fraction; WAT, white adipose tissue.

Recently, multipotent adult stem cell populations have been obtained from various tissues of human and rodent origin (1). Adult stem cells can differentiate *in vitro* to cells of the three germ layers and *in vivo* in tissue-specific cells (2–5). For postnatal tissue-specific stem cells to be used for the clinical treatment of degenerated or inherited diseases, many criteria must be fulfilled. These cells should be isolated from a large reservoir of an easily available source from human, and should ideally exhibit the following: (a) long-term expansion *in vitro* accompanied by normal karyotype; (b) multilineage potential of a single cell *in vitro*, and (c) capacity for long-term engraftment and tissue regeneration after transplantation into recipients. Human mesodermal progenitor cells isolated from bone marrow (human multipotent adult progenitor cells [hMAPCs]) can be cul-

ture expanded >70 population doublings (PDs; reference 6). Given the medical implications, identification of hMAPCs has evoked significant excitement. However, isolation of bone marrow is frequently painful and yields low doses of mesenchymal stem cells. White adipose tissue (WAT) represents a major source of expendable tissue. Stromal-vascular cells from lipoaspirate (LPA) of human WAT have been shown to contain multipotent stem cells, but their ability to be maintained in culture with a normal karyotype and to differentiate *in vivo* remains unknown (7). We report herein the isolation, from adipose tissue of young donors, of a nonimmunogenic human multipotent adipose-derived stem (hMADS) cell population that is able to undergo >200 PDs and to differentiate into cells of the adipogenic, osteogenic, and myogenic lineages as well as the

characterization of derived clones. After transplantation into muscles of the nonimmunocompromised mdx mouse, an animal model of Duchenne muscular dystrophy, a long-term engraftment occurs and a high proportion of the myofibers expresses human dystrophin.

RESULTS

Isolation of a hMADS cell population

To isolate hMADS cells, first we modified the previous published protocol used to isolate adipocyte precursors from the stromal-vascular (SV) fraction (SVF) (see Materials and methods). Next, we used the crude SVF of WAT from young donors (1 mo-old to 7 yr-old) to avoid potential aging effects on stem cell properties (8, 9). Two cell populations were isolated based on adhesion properties on uncoated culture dishes. Fast-adherent (CA) cells and slow-adherent (CS) cells were collected 12 and 72 h after plating, respectively. At early passages, CA and CS cells showed similar properties. Both exhibited a fibroblast-like morphology and had a doubling time of 36 h. At this stage, CA and CS cells were able to differentiate with a similar efficiency into adipocytes and osteoblasts (Fig. 1 A). After 60–80 PDs, marked changes were observed between the two populations. CS cells ceased to proliferate, lost their differentiation properties (unpublished data), and exhibited senescence-

associated (SA) β -galactosidase activity. In contrast, slow growth (doubling time ~ 72 h) and flatness of CA cells occurred whereas SA β -galactosidase activity remained undetectable (Fig. 1 B). CA cells expressed significant levels of telomerase activity (23% of the activity of control HK 293T cells), whereas CS cells showed no activity. To promote the proliferation of CA and CS cells, various mitogens were tested. Unlike hMAPCs (6), CA cells did not expand in response to epithelial growth factor and/or platelet-derived growth factor but responded to human fibroblast growth factor (hFGF)-2. A strong proliferative response of CA cells was obtained upon addition of this growth factor, in contrast with the weak response of CS cells (Fig. 1, C and D). CA cells were culture expanded >200 PDs by passaging them every 4–5 d. CA cells frozen and thawed after 3 and 18 mo retained all the characteristics of the original population. CA cells were named hMADS cells.

Using this technique, we were able to expand hMADS cells from small pieces of adipose tissue (from 200 to 2,100 mg) of different anatomical sites of young donors of either sex. As shown in Table I, of the six samples used in the experiment, we succeeded in expanding hMADS cells beyond 150 PDs from four donors (hMADS-1, hMADS-2, hMADS-3, and hMADS-6 cells). hMADS-4 and hMADS-5 cells were not studied beyond 40 and 20 PDs, respectively.

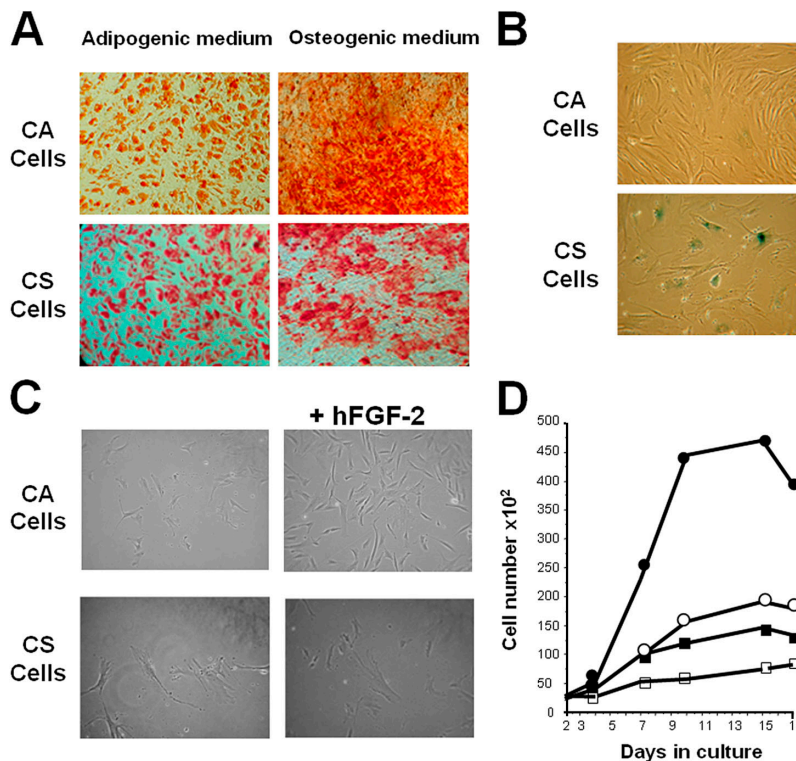


Figure 1. Isolation of cell populations and establishment of hMADS-2 cells. (A) In vitro differentiation of CA and CS cells at early passages (10 PDs). Cultures were stained after 15 d with Oil-red O for adipocytes and with Alizarin red for osteoblasts. (B) Cell morphology and

SA β -galactosidase activity of CA and CS cells after 60 PDs. (C) Cell morphology and (D) proliferative response of CA (○, ●) and CS (□, ■) cells in the absence (○, □) or the presence of 5 ng/ml hFGF-2 (●, ■).

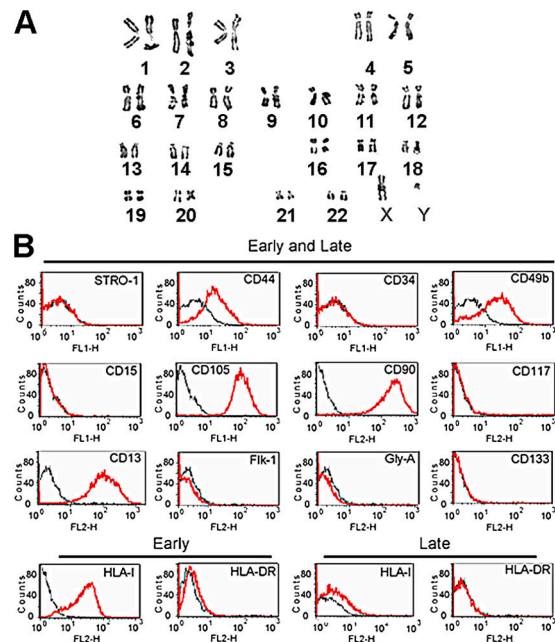


Figure 2. Characteristics of culture-expanded hMADS cells. (A) A representative result of cytogenetic analysis of hMADS-2 cells ($n = 3$). (B) hMADS-2 cells cultured for 40 (early) or 160 (late) PDs ($n = 4$) were labeled with FITC-conjugated antibodies against STRO-1, CD44, CD34, CD49b, CD15, CD105, and with PE-conjugated antibodies against CD90, CD117, CD13, Flk-1, Gly-A, CD133, class I-HLA, and HLA-DR or immunoglobulin isotype control antibodies ($n = 4$). Black line, control immunoglobulins; red line, specific antibodies.

The karyotype of hMADS cells has been investigated (Fig. 2 A). Except for hMADS-4 cells that had an abnormal karyotype at 40 PDs, other hMADS cells did not show any karyotype abnormalities. No karyotype abnormalities were detected for hMADS-2 cells at 70, 80, and 150 PDs; for hMADS-1 and hMADS-3 cells at 90 PDs; and for hMADS-6 cells at 110 PDs. Of note, during isolation, both a quiescent state and a reversible response to hFGF-2 were also observed for hMADS-1 and hMADS-3 cells.

The phenotype of hMADS-2 cells at early (before 40 PDs) and late passages (between 80 and 160 PDs) was analyzed for cell surface markers (Fig. 2 B). In both cases, they were Flk-1 (VEGF-R2), glycophorin A, CD34, CD15 (SSEA-I), CD117 (c-Kit), CD133, STRO-1 negative, and CD13, CD49b, CD44, CD90 (Thy-1), and CD105 positive. The overlap of CD44 and CD49b curves with the negative control curve was due to a low expression of these two antigens at the cell surface. After amplification of the signals, which do not alter autofluorescence, the positive and negative histograms were clearly distinct (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20042224/DC1>). To show directly that hMADS-2 cells coexpressed the latter markers, double staining experiments were performed. The results demonstrate coexpression of CD105, CD44, CD49b, and CD90 (Fig. S1). Importantly, hMADS-2 cells were both

Table I. Isolation of hMADS cells from different donors

Donor age	Sex	Anatomical area	Sample weight	SV fraction	PDs (CA cells)	Cell population
			mg	cell no. $\times 10^5$		
2 yr, 7 mo	F	umbilical	300	4	>160	hMADS-1
5 yr	M	pubic	400	5	>200	hMADS-2
4 mo	M	prepubic	210	4	>160	hMADS-3
7 yr	F	inguinal	2,100	20	40	hMADS-4
1 mo	F	inguinal	200	3.5	20	hMADS-5
1 yr, 6 mo	M	scrotum	200	3.5	>150	hMADS-6

class I HLA positive and class II HLA negative at early passages, but class I HLA low and class II HLA negative at late passages; a similar phenotype was observed with hMADS-1 and hMADS-3 cells at early and late passages (unpublished data).

hMADS cell differentiation into adipocytes, osteoblasts, and skeletal myocytes

The ability of hMADS-2 cells at 100 PDs to differentiate was tested under conditions promoting different lineages (Fig. 3). When exposed to adipogenic medium, >90% of the cells differentiated into adipose cells that were stained with Oil red O for triglycerides (Fig. 3 A, left and middle) and expressed PPAR γ 2 and the adipocyte fatty acid-binding protein (aFABP) mRNAs specific of adipocytes (Fig. 3 A, right). Differentiated cells underwent lipolysis in response to β_1 - and β_2 -adrenoreceptor agonists and were able to secrete adiponectin and leptin, whereas glycerol-3-phosphate dehydrogenase activities increased from 20–30 mU/mg to 1,000–2,000 mU/mg (10). Osteogenesis occurred in the presence of osteogenic medium as seen by alkaline phosphatase and Alizarin red staining (Fig. 3 B, right and middle), and by detection of matrix-associated calcium (not depicted). In contrast with cells maintained under adipogenic conditions, these cells expressed reduced levels of PPAR γ 2 and aFABP mRNAs, but instead expressed high levels of osteocalcin mRNA (Fig. 3 B, right). When exposed to myogenic conditions, immunochemical staining showed that cells expressed myogenin, a transcription factor of myogenesis (Fig. 3 C, left). Expression of MyoD1 and of desmin was also detected (Fig. 3 C, right). Subsequently, flow cytometry analysis showed that >95% of the cells were fast-twitch myosin (Fig. 3 C, middle) and cell surface class I HLA positive (not depicted). However myotube formation was not observed. Similar to hMADS-2 cells, hMADS-1, -3, and -6 cells differentiated into adipose cells (10) and gave rise to osteoblasts and myocytes (unpublished data).

Next, we wished to determine whether individual hMADS cell clones could differentiate to different lineages. For that purpose, clones were isolated by two different methods; i.e., by limiting dilution from wild type and LacZ genetically marked hMADS cells and by automated cloning of enhanced GFP (EGFP)-positive cells sorted by FACS.

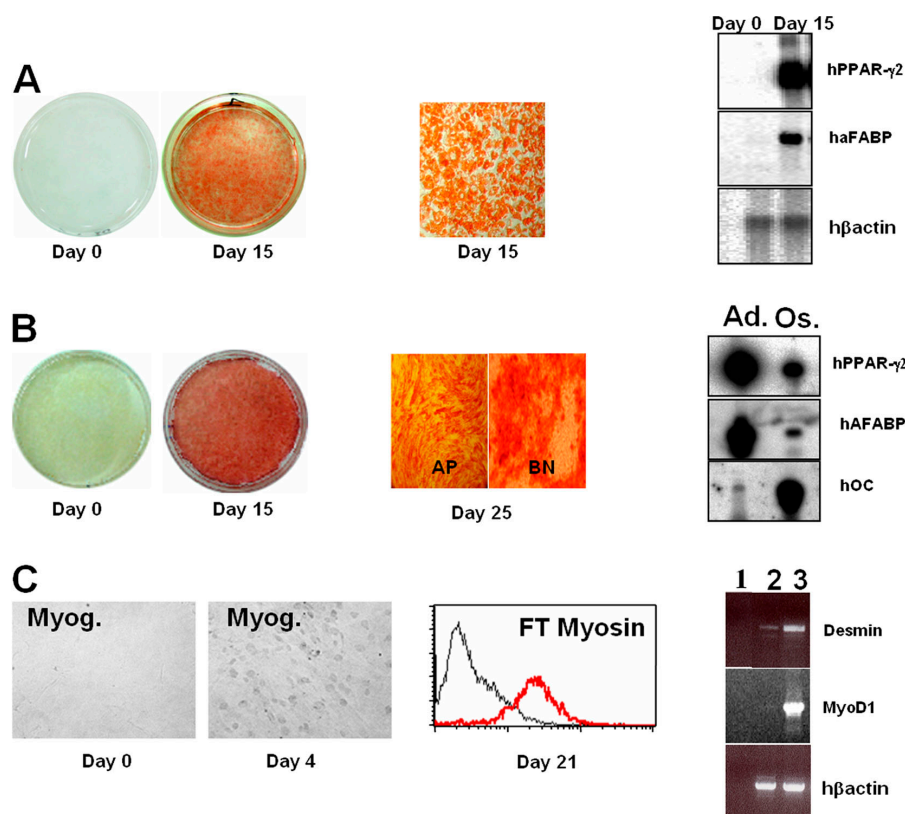


Figure 3. In vitro differentiation of hMADS cells (80–120 PDs) to adipocytes, osteoblasts, and myoblasts. (A) Adipogenic differentiation. Confluent hMADS-2 cells before (day 0) and 15 d after treatment with adipogenic medium were stained with Oil red O for lipid droplets. RNAs were prepared and analyzed by RT-PCR for expression of PPAR γ 2 and aFABP. (B) Osteogenic differentiation. Confluent hMADS-2 cells before (day 0) and 15 d after treatment with osteogenic medium were stained with Alizarin red for bone nodules. At day 25 after osteogenic induction, cells were stained for alkaline phosphatase (AP) or with Alizarin red for bone nodules (BN). RNAs were prepared and analyzed by RT-PCR for ex-

pression of PPAR γ 2 and aFABP (adipocyte-specific genes) and osteocalcin (OC) (osteoblast-specific gene) in adipogenic (Ad.) and osteogenic (Os.) conditions. (C) Myogenic differentiation. Confluent hMADS-2 cells before (day 0) and 4 d after treatment with myogenic medium were stained for myogenin (Myog.). Fast-twitch myosin of permeabilized cells was detected by FACS 21 d after treatment with myogenic medium. RNAs were prepared and analyzed by RT-PCR for expression of MyoD1 and desmin. (1) No RNA; (2) RNAs from undifferentiated hMADS cells; (3) RNAs from hMADS cells in myogenic medium for 4 d.

The hMADS-2 cell population, at 60 PDs, was first subjected to classical limiting dilution. Starting after 2 wk of culture, differentiation of 12 individual clones has been investigated. As shown in Table S1 (available at <http://www.jem.org/cgi/content/full/jem.20042224/DC1>), 10 clones were bipotent and 2 were able to undergo differentiation into adipocytes, osteoblasts, and myoblast-like cells. In a separate experiment, hMADS cells have been transduced with a lentiviral vector expressing nlsLacZ. After dilution cloning, three LacZ-expressing clones were able to undergo differentiation into adipocytes and osteoblasts. Southern blot analysis has been performed and indicated that only one retroviral insertion site was present and different for each clone (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20042224/DC1>). In a third series of experiments, hMADS cells were transduced with a lentivirus vector expressing EGFP. Then, EGFP-expressing cells were sorted by FACS and cloned in an automated fashion. Three clones

were expanded and maintained in adipogenic and osteogenic conditions. Data are summarized in Table S1. Altogether, these data are in favor of the presence of single multipotent cells in the hMADS cell population.

Cell engraftment and expression of human dystrophin in the mdx mouse

As hMADS cells differentiated in vitro into myocytes, we determined whether these cells were able in vivo to express human dystrophin in dystrophin-deficient mdx mice. The finding that undifferentiated hMADS cells expressed low levels of class I HLA and were class II HLA negative led us to address the issue of immune-privileged behavior using mdx mice treated or not with immunosuppressive drug (10 mg of cyclosporine A/kg, daily i.p. injection). hMADS-2 cells (1.5×10^5), obtained after 160 PDs, were transplanted in the left tibialis anterior muscle of 3-mo-old mdx mice. As expected, no dystrophin-positive fibers were observed in the

noninjected muscle other than the occasional revertant fibers that represent not >1% of the analyzed sections (Fig. 4 A, a). In contrast, 10 d after transplantation, dystrophin was detected in up to 50% of the myofibers analyzed on two consecutive sections. Dystrophin fibers were mainly organized in clusters and no difference in the histological aspect and number of dystrophin-positive myofibers was observed in transplanted muscles of immunosuppressed or immunocompetent mdx mice (Fig. 4 A, b, b', c, and c'). Similar results were obtained 10 d after transplantation of hMADS-1 and hMADS-3 cells in immunocompetent mdx mice (Table S2, available at <http://www.jem.org/cgi/content/full/jem.20042224/DC1>). Most of the dystrophin-positive fiber sections contained both human and mouse nuclei as revealed by DAPI and by fluorescent in situ hybridization analysis when using a probe specific to human centromeres (Fig. 4 A, b' and c'). Human nuclei were also observed outside myofibers (Fig. 4 A, b'). Nuclei were located both centrally and at the periphery of the myofibers, indicating the existence of regeneration and maturation processes. A higher proportion of human versus murine nuclei was observed in injected muscles (~70 vs. ~30% in dystrophin-positive myofibers). Analysis of serial sections showed that dystrophin expression was taking place all along the same myofiber. These results strongly suggesting that hMADS cells were not rejected after transplantation in tibialis anterior muscle, long-term engraftment had been investigated in the absence of cyclosporin A treatment. 50 d (Fig. 4 A, e and e') and 80 d (Fig. 4 A, g) after transplantation of hMADS cells, we observed a time-dependent pattern of dystrophin-positive myofibers. An increase in the percentage of peripheral nuclei (outside and within fibers) of human origin and a decrease in the percentage of central nuclei were observed from 10 to 50 d post-transplantation (from 73 to 85% and from 27 to 15%, respectively), providing evidence that injected cells participated in the terminal differentiation of myofibers. Interestingly, the presence of dystrophin-positive myofibers was observed in the adjacent gastrocnemius muscle at 50 d (Fig. 4 A, f and f') and 80 d (Fig. 4 A, h). As this was not observed 10 d after transplantation, this result indicates cell migration from the injection site to other dystrophic muscles. 6 mo later, dystrophin was detected in up to 90% of the myofibers of transplanted tibialis anterior muscles. Serial cross sections indicated a more homogeneous distribution of dystrophin than at prior times (Fig. 4 A, i compared with Fig. 4 A, b, c, e, and g). In contrast with tibialis anterior muscles of mdx mice of the same age, necrotic myofibers were not observed, suggesting a protective role of hMADS cells after long-term transplantation (unpublished data). As expected, human dystrophin appeared located under the sarcolemma, in contrast with mouse collagen III present between myofibers in the extracellular space (Fig. 4 B, c–e).

To determine whether hMADS cells induce a proliferative response of lymphocytes in vitro, splenocytes from mdx or from OF1 mice were cultured with irradiated hMADS

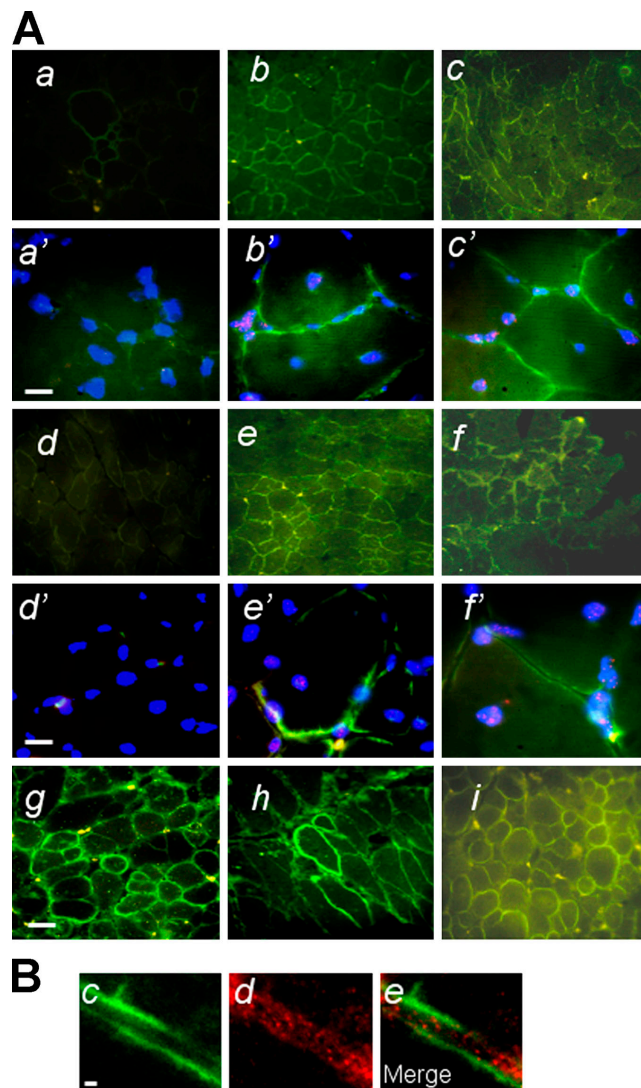


Figure 4. Human dystrophin and hMADS cells in skeletal muscle of mdx mice. (A) Expression of human dystrophin and detection of human nuclei 10 d (a–c), 50 d (d–f), 80 d (g and h), and 180 d (i) after transplantation of hMADS-2 cells. Human nuclei (nuclei counterstained with DAPI in blue and human centromeres as red hybridization signals) were found present within dystrophin-positive myofibers (green) and also at the periphery of myofibers using dystrophin antibody NCL-DYS2. (a, a', d, d') Right tibialis anterior muscle (control); (b, b') left tibialis anterior muscle of cyclosporin A-treated mdx mice; (c, c', e, e') left tibialis anterior muscle of immunocompetent mdx mice; (f, f'), left gastrocnemius muscle of immunocompetent mdx mice; g, Left tibialis anterior muscle; h, left gastrocnemius; i, left tibialis anterior muscle. Bar, 20 μ m (a–c, d–f, g–i) and 5 μ m (a'–c', d'–f'). (B) Dystrophin-positive myofibers and dystrophin subcellular localization were analyzed in the tibialis anterior muscle 10 d after transplantation using antibodies directed against (c, e) NH₂ terminus of human dystrophin and (d, e) mouse collagen III. Bars, 1 μ m.

cells. No proliferation of mdx or OF1 splenocytes cultured with hMADS cells was observed, in contrast with control experiments that show that allogenic BALB/c splenocytes or

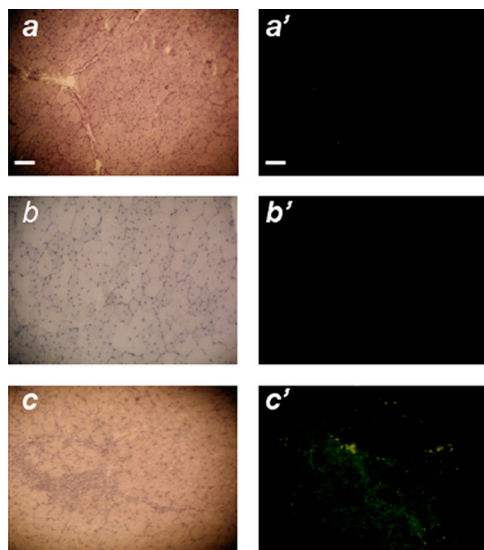


Figure 5. Absence of infiltration by mCD3-positive lymphocytes in muscle of immunocompetent mdx mice after transplantation with hMADS cells. Staining was performed with hematoxylin (a–c) or with antibodies against mouse CD3 (a', c'); left tibialis anterior muscle nontransplanted (a, a') or 10 d after transplantation of hMADS-2 cells (b, b') ($n = 8$) and CA cells at 40 PDs (c, c') ($n = 5$). Bar, 50 μm (a–c) and 20 μm (a'–c').

xenogenic human peripheral blood lymphocytes induced proliferation of mdx or OF1 splenocytes (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20042224/DC1>). Consistent with an absence of an immune recognition, transplantation of hMADS cells did not lead within 10 d to infiltration by mCD3-positive lymphocytes (Fig. 5, b and b') nor at 50 and 80 d after transplantation (not depicted). In contrast, transplantation of the same number of CA or CS cells at early passage (40 PDs), which expressed high levels of class I HLA (Fig. 2 B), did not restore dystrophin expression in muscle of mdx mice (not depicted) and elicited a strong immune response (Fig. 5, c and c'). Similar observations were made with SV cells at 10 PDs without prior separation of CA and CS cells.

DISCUSSION

In the present study, we isolated, independently of sex and adipose depot of young donors, a population of cells that could be extensively expanded *ex vivo* and that could undergo differentiation into various cell types. Our method took advantage of the extensive lifespan of stem cells as compared with other cell types. hMADS cells were generated from adipose tissue of young donors. Whether hMADS cells also can be generated from older persons has not been tested. hMADS cells shared many properties described for hMAPCs (extensive expansion capacity *in vitro*, clonal multilineage differentiation potential, telomerase activity with normal karyotype; references 5, 6), but differed with respect to (a) their maintenance in 10% serum-supplemented medium

with no loss of their differentiation potential after 200 PDs; (b) expression of cell surface markers revealed by immunophenotypic analysis; and (c) their response to various growth factors and extracellular matrix components. In addition, hMADS cells are easy to isolate and to expand. Frozen and thawed cells kept the same characteristics than the original population. The hMADS cell population contained single cells able to differentiate *in vitro* into mesodermal lineages. The hMADS cells were obtained after 160–200 PDs and differed clearly from SV cells (11), adipose tissue-derived stromal cells (12), and processed LPA cells (7), which were studied at either the first (7, 11) or the second to third passage (i.e., after a few PDs; reference 12). Although the population of LPA cells exhibited multipotency (7, 13), the coexistence at that stage of single lineage and multiple lineage-committed progenitor cells was not ruled out. Moreover, among clones exhibiting differentiation into mesodermal lineages, differentiation into myocytes was not reported. In contrast with LPA cells, hMADS cells expressed high levels of cell surface CD105 and did not express STRO-1. Furthermore, expression of class I and class II HLA and regenerative properties *in vivo* were not reported for LPA cells.

When injected into mdx mice, hMADS cells gave rise to higher levels of dystrophin-positive myofibers than previously reported for primary myoblasts or muscle stem cells isolated from wild-type mice (14), indicating substantial recruitment of hMADS cells to the dystrophic muscle. Interestingly, in wild-type mice suffering from partial muscle necrosis after bupivacain treatment, hMADS cells did not induce after 10 d a detectable expression of human dystrophin in the transplanted tibialis anterior muscle despite the presence of human nuclei in the regenerating zone (unpublished data). Thus, the apparent selective advantage of hMADS cells appears related to the inefficiency of mdx satellite cells to generate mature and stable myofibers. Remarkably, hMADS cells were able to migrate within 50 d from the tibialis anterior to the adjacent gastrocnemius muscle. This spatial-temporal regenerative behavior of the cells suggests a possible delivery of dystrophin to other skeletal muscles and the restoration of a normal phenotype including reduced necrosis. Based on this, hMADS cells could be considered as alternative cell source for muscle replacement therapy. Two mechanisms can be suggested to account for the contribution of hMADS cells to muscle regeneration: *de novo* generation of muscle-specific cells from hMADS cells or changes in gene expression after direct fusion of hMADS cells with host cells. Preliminary data suggest that the muscle phenotype of hMADS cells after transplantation may be acquired through both commitment into the myogenic lineage and direct cell fusion as nuclei of hMADS cells could fuse spontaneously with unidentified host cell nuclei at a high rate 7 d after transplantation. The relative contribution of transdifferentiation versus nuclear fusion in the long-term engraftment of hMADS cells into the muscle remains to be investigated.

The cells display an immunoprivileged behavior and were not rejected in nonimmunocompromised mdx mice up to 6 mo after transplantation. The absence of an immune recognition may be due in part to the low level of cell surface class I and the absence of class II HLA, similar to the lack of allogeneic rejection in mdx mice reported for (MHC) class I-negative muscle stem cells (15). Nevertheless, our observations obviously raise a serious immunological challenge as (a) class I HLA expression should be increased in vivo during the formation of dystrophin-positive myofibers as it is the case for hMADS cells in vitro under myogenic conditions and (b) human dystrophin is expressed at high levels in mdx mice despite the fact that it has been reported to elicit potent cytotoxic and humoral immune responses (16, 17). The immunoprivileged behavior of hMADS cells is reinforced by the lack of recognition by T lymphocytes, as shown by the absence of infiltration by mCD3 positive cells. The mechanisms by which hMADS cells escape immune surveillance remain unclear. When present outside the myofibers, these cells could facilitate the reeducation of the recipient immune system as suggested for rat embryonic stem-like cells (18). Alternatively, or in addition, hMADS cells could exhibit immunosuppressive properties, as recently described for mesenchymal multipotent stem cells from human and mouse bone marrow (19, 20). Such immunosuppression is likely to occur via the production of cytokines, such as IL-4, IL-10, and TGF β , responsible for the inhibition of alloreactive lymphocyte proliferation (21). Gene expression analysis indicate that hMADS cells do not express either IL-4 or IL-10 but express TGF β 1, TGF β 2, and TGF β 3 (unpublished data). Whether TGF β may control the immunosuppression induced by hMADS cells has still to be demonstrated.

Together, the properties of hMADS cells, combined with the small amount of an easily available tissue needed for their isolation make these cells a suitable tool for autologous and heterologous transplantation in humans. Autologous transplantation combined with gene therapy has often led to deleterious immune responses, in particular in Duchenne muscular dystrophy patients (22). In this context, the non-immunogenic and regenerative properties of hMADS cells hold great promise with respect to this pathology and other inherited diseases.

MATERIALS AND METHODS

Isolation of hMADS cells. For the isolation of hMADS cells from young donors, adipose tissue was obtained with the informed consent of the parents as surgical scraps from surgical specimen of various surgeries, as approved by the Centre Hospitalier Universitaire de Nice Review Board. We modified a previous published protocol used to isolate adipocyte precursors from adipose tissue (23). In brief, 200 mg/ml adipose tissue was dissociated for 5–10 min in DMEM containing antibiotics (100 U/ml of penicillin and 100 μ g/ml of streptomycin), 2 mg/ml collagenase, and 20 mg/ml bovine serum-albumin. The crude SVF was separated from the adipocyte fraction by low speed centrifugation (200 g, 10 min). The adipocyte fraction was discarded and cells from the pelleted SVF were seeded onto uncoated tissue culture plates (Greiner) at 1,000–3,500 cells/cm² in low glucose DMEM (Invitro-

gen) supplemented with 10% heat-inactivated fetal bovine serum (D. Dutcher) and antibiotics as described before. Fast-adherent cells, termed CA cells, were separated from slow-adherent cells, termed CS cells. CA and CS cells were expanded in the same culture medium as described before. After reaching 70% confluence, cells were dissociated (0.25% trypsin EDTA; Invitrogen) and replated at 1,000–3,000 cells/cm². Telomerase activity was determined by means of TeloTAGGG Telomerase PCR Elisa^{PLUS} kit from Roche Diagnostic, according to the manufacturer's recommendations. SA β -galactosidase activity was determined according to Dimri et al. (24).

Karyotyping. Dividing cells at various PDs were arrested in metaphase with colcemid overnight, hypotonically shocked with KCl, and fixed with methanol/acetic acid (3/1; vol/vol). Chromosomes were identified using RHG-banding technique. At least 30 mitoses were examined for each karyotype.

hMADS cell differentiation. For adipocytes, confluent cells were cultured in DMEM/Ham's F12 media supplemented with 10 μ g/ml transfer-rin, 100 μ M ascorbic acid 2-sodium, 0.85 μ M insulin, 20 nM sodium selenite, 0.2 nM triiodothyronine, 1 μ M dexamethasone (DEX), 100 μ M isobutyl-methylxanthine and 1 μ M rosiglitazone. 3 d later, the medium was changed (DEX and isobutyl-methylxanthine were omitted). Neutral lipid accumulation was assessed by Oil red O staining and glycerol-3-phosphate dehydrogenase activity assays were performed as previously described (25). For osteoblasts, confluent cells were cultured in 10% FCS supplemented with 0.1 μ M DEX, 10 mM β -glycerophosphate, and 50 μ g/ml ascorbic acid 2-phosphate. Alkaline phosphatase and Alizarin red staining were performed as previously described (26), and matrix-associated calcium was determined using a commercial detection kit (Sigma-Aldrich). For myocytes, confluent cells were maintained in Skeletal Muscle Cell Differentiation medium (PromoCell). Myogenin and fast-twitch myosin expression was determined at indicated times.

Immunocytochemistry analysis. Cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with PBS/0.1% Triton X-100. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min. Slides were incubated sequentially for 1 h with mouse monoclonal antimyogenin (1:100) and anti-mouse IgG antibodies and for 10 min with 3,3'-diaminobenzidine (Vector Laboratories). Antibodies against myogenin, fast-twitch myosin, control mouse, and goat IgGs were obtained from Santa Cruz Biotechnology, Inc.

Flow cytometric analysis. Cells were detached and treated sequentially with primary antibodies and, if necessary, immunofluorescent secondary antibodies were then fixed with 1% formaldehyde until analysis with a FACS-Calibur (Becton Dickinson). Antibodies against human CD105 and secondary anti-mouse antibody conjugated with FITC were obtained from R&D Systems and Caltag, respectively. Phycoerythrin (PE)-conjugated antibody against human CD133 was obtained from R&D Systems, and those against human CD13, CD90, CD117, class I HLA, HLA-DR, Flk1, glycophorin A, and isotype control were obtained from BD Biosciences. FITC-conjugated antibodies against human CD15, CD34, CD44, CD49b, and isotype control were obtained from BD Biosciences. Antibody against STRO-1 was obtained from R&D Systems and secondary anti-mouse IgM antibody conjugated with FITC was obtained from BD Biosciences. Amplification of positive signals was performed by adding FITC-labeled rabbit anti-mouse IgG (Caltag) after CD44 and CD49b staining. For detection of intracellular fast-twitch myosin, cells were previously fixed with 1% paraformaldehyde in phosphate-buffered saline and permeabilized with 10 μ g/ml of digitonin for 7 min at 20°C. Fast-twitch myosin was labeled with PE-conjugated specific antibodies and analyzed by FACS.

Reverse transcription PCR analysis. Total RNA was isolated using Tri agent (Euromedex), according to manufacturer's instructions, separated by gel electrophoresis, blotted on Hybond N⁺, and hybridized with specific

radiolabeled probes (ICN Biomedicals) using the ready-prime TM II Random Prime Labeling system (Amersham Biosciences). The primers used for PCR amplification and hybridization (internal primers) were: aFABP, forward, 5'-GCTTTGCCACCAGGAAAGTG-3', and reverse, 5'-ATG-ACGCATTCCACCACCAG-3', internal primer, 5'-TGCAGTGA-CTT-CGTCAAATT-3' for a 280-bp fragment; osteocalcin, forward, 5'-CGC-AGCCACCGAGACACCAT-3', and reverse, 5'-GGGCAAGGGCAA-GGGGAAGA-3', internal primer, 5'-GCTGCCCTCCTGCTTAA-3' for a 320-bp fragment; MyoD1, forward, 5'-AAGCGCCATCTCTTGAG-GTA-3', and reverse, 5'-GCGCCTTTATTTTGATCACC-3', for a fragment of 490 bp; desmin, forward 5'-CCTACTCTGCCCTCAACTTC-3', and reverse 5'-AGTATCCCAACACCCTGCTC-3' for a fragment of 519 bp; and β -actin, forward, 5'-CCGACAGGATGCAGAAGGAG-3', and reverse, 5'-GGCACGAAGGCTCATCATTC-3' for a fragment of 662 bp.

Transplantation of hMADS cells into mdx mice. C57BL/10ScSN-Dmd^{mdx/J} (X-linked muscular dystrophy) mice were obtained from Charles River Laboratories. 3-mo-old mdx mice were injected with 1.5×10^5 hMADS cells resuspended in 50 μ l of HBSS (Invitrogen) in the left tibialis anterior muscle. 50 μ l of HBSS were injected in the right tibialis anterior muscle used as control. Both muscles and adjacent gastrocnemius muscles were removed and snap frozen in isopentane precooled in liquid nitrogen. Cryostat serial sections of 12 μ m were prepared from frozen muscles. Animal experiments were performed in accordance with the recommendations of the French Accreditation of the Laboratory Animal Care and were approved by the local Centre National de la Recherche Scientifique ethics committee.

Analysis of hMADS cells engraftment and human dystrophin expression. The human origin of dystrophin was demonstrated by comparative immunodetection using antibodies against mouse and human dystrophin at the COOH terminus and against human dystrophin at the NH₂ terminus, which gave similar results. Antibodies against NH₂ terminus of human dystrophin (NCL-DYS3) or directed toward COOH terminus of human and mouse dystrophin (NCL-DYS2) were obtained from Novocastra. Before use, NCL-DYS2 and NCL-DYS3 were conjugated using, respectively, Zenon Alexa Fluor 488 mouse IgG1 and Zenon Alexa Fluor 488 mouse IgG2 labeling kit (Interchim). Antibody against mouse collagen type III and secondary goat anti-rabbit Alexa 568 antibody were obtained from Rockland and Interchim, respectively. FITC-conjugated antibody against mouse CD3 was obtained from BD Biosciences. For fluorescent in situ hybridization analysis and colocalization with dystrophin-positive myofibers, a digoxigenin-labeled all human centromere probe (α -satellite) was used. Detection was performed with rhodamine-labeled antidigoxigenin antibodies (Quantum Appligen). Samples were counterstained with DAPI and slides were examined using a Zeiss Axiophot fluorescent microscope.

For double staining, sections were incubated with collagen III antibody, followed by incubation with Alexa 568 secondary antibody, before proceeding to dystrophin detection as described before. For mapping regeneration or infiltration zones, 1 section out of 50 sections was stained with Harris' hematoxylin, rinsed with water, and dehydrated with ethanol (50, 75, and 100%) for 10 min. For single staining, frozen sections were first fixed with methanol/acetic acid (75/25 vol/vol) at -20°C for 15 min. All the following steps were performed at room temperature. Subsequently, after incubation for 1 h with mCD3 antibody labeled with FITC, sections were mounted with antifading solution to be visualized on an Olympus BH2 epifluorescence microscope.

Online supplemental material. Fig. S1 shows the coexpression of CD90, CD49b, CD44, and CD105 by FACS analysis. Table S1 and Fig. S2 include additional data on the cloning of hMADS cells. Fig. S3 shows the effect of hMADS cells on the proliferation of OF1 and mdx splenocytes. Table S2 includes additional data on the transplantation of hMADS cells in mdx mice. Materials and methods for the cloning of hMADS cells and the assay for proliferation of OF1 and mdx splenocytes are available, in addition

to all the other supplemental material, online at <http://www.jem.org/cgi/content/full/jem.20042224/DC1>.

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Dilution cloning	Automated cloning	Retroviral marking	Clone	Adipogenic Differentiation (Oil Red O stain)	Osteogenic Differentiation (Alizarin Red stain)	Myogenic Differentiation
			1 clone(CA1)	+	+	MyoD1-positive cells in vitro Dystrophin-positive cells in vivo
			1 clone	+	+	MyoD1-positive cells in vitro
			10 clones	+	+	ND
			3 LacZ-marked clones	+	+	ND
			3 GFP-marked clones sorted by FACS	+	+	ND

Table S1. Results of cell differentiation assays performed with expanded clones. Clones were isolated by dilution cloning or automated cloning from wild-type or transduced hMADS-2 cells. Criteria were as follows: adipogenic differentiation: microscopic detection of Oil red O-positive cells; osteogenic differentiation: microscopic detection of bone nodules after Alizarin red staining; and myogenic differentiation: detection by RT-PCR of expression of MyoD1 after in vitro differentiation and detection of dystrophin by immunohistochemistry after transplantation in mdx muscle.

Cloning of hMADS cells.

Cell cloning was performed by limiting dilution of wild-type cells, by limiting dilution of LacZ genetically marked cells, and by automated cell sorting of GFP-positive cells. For limiting dilution, cells were seeded at a nominal cell density of 0.5 cell per well into 24-well plates in DMEM supplemented with 10% FBS and 5 ng/ml⁻¹ hFGF-2. Cultures were examined daily by careful microscopic examination for the appearance of a single colony per well. Starting after 2 wk of culture, differentiation of several clones has been investigated. In a separate experiment, hMADS cells have been transduced with a lentiviral virus expressing nbLacZ. At a multiplicity of infection of 3, ~25% of the cells expressed the β-galactosidase (Fig. S2 A). After limiting dilution as indicated, 25% of the clones (3 clones out of 14 clones analyzed) were positive for β-galactosidase. More importantly, 100% of the cells of these three clones were β-gal positive (i.e., not

Cells injected	Number of mdx mice injected		Time of euthanasia (days)
	Dystrophin positive	Dystrophin negative	
hMADS-2	10	2	10
	2	0	50
	1	1	80
	1	0	180
hMADS-2-CA1 clone	1	0	10
hMADS-3	3	0	10
hMADS-1	1	0	10

Table S2. Results of transplantation of hMADS cells in the tibialis anterior muscle of immunocompetent mdx mice. The number of mice injected with hMADS cells is indicated. The result is considered as dystrophin positive when on two serial sections >5% of myofibers expressed human dystrophin. When detected, revertant myofibers represented <1% of the fibers on the section.

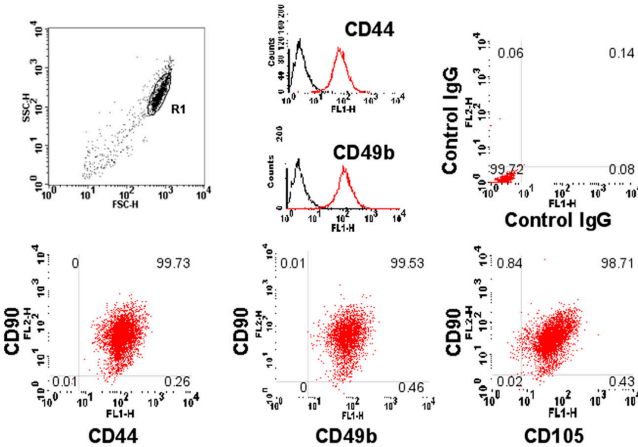


Figure S1. Co-expression of cell surface markers by hMADS. The R1 gate was established on forward versus side scatter parameters to exclude dead cells and debris from subsequent analysis. hMADS-2 cells cultured for 160 PDs and stained for CD44 and CD49b. The signals were amplified by adding FITC-labeled rabbit anti-mouse IgG after CD44 and CD49b staining. Double labeling were performed with CD90 and CD105 or CD90 and CD105, or CD90 and CD49b. The percentages of gated cells coexpressing the surface antigens are shown.

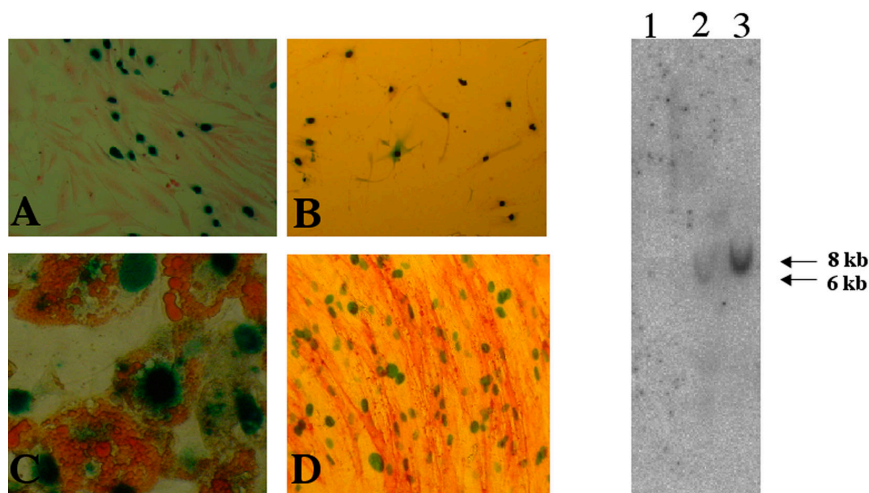


Figure S2. Single cell origin of hMADS clones. (A) hMADS cells were transduced with a lentiviral vector expressing LacZ. Cells were stained for β -galactosidase and counterstained with eosin; (B) one clone expressing β -galactosidase and isolated by limiting dilution is shown; (C) Differentiation of one β -galactosidase expressing clone is shown. Clonal cells were induced to undergo differentiation into adipocytes containing lipid droplets stained with Oil red O or (D) into osteoblasts expressing alkaline phosphatase. (E) DNA of two clones was extracted, digested with Not1 (cut only once in the retrovirus vector) and Southern blot was hybridized with a LacZ probe. A single retroviral insert at 6 kb and 8 kb for clone 2 and clone 3, respectively, is shown. 1, DNA of wild-type hMADS cells; 2, DNA of clone 2; 3, DNA of clone 3.

contaminated with wild-type cells representing 75% of the population before cloning). This observation indicated that, under our conditions, clones were likely formed from a single cell. LacZ-expressing clones were able to undergo differentiation into adipocytes and osteoblasts as shown in the Fig. S2. Southern blot analysis of LacZ marked clones has been performed and indicated that the integration of the marker was different for each clone. In a third series of experiments, hMADS cells have been transduced with a lentivirus expressing GFP. GFP-expressing cells were selected (FACSCalibur; Becton Dickinson) and a single cell was automatically deposited in each well of 96-well plates. From 192 wells, three clones were expanded and maintained in adipogenic and osteogenic conditions. Data are summarized in Table S1.

Proliferation of OF1 and mdx splenocytes.

OF1 and Mdx splenocytes were purified and stimulated at 10^6 cells/ml with an equivalent number of irradiated (500 rad) hMADS cells (at 120 PDs) or with human peripheral blood lymphocytes (hPBLs), or with mouse BALB/c splenocytes. On day 4, 1 mCi/ml tritiated thymidine was added and cells were harvested 24 h after.

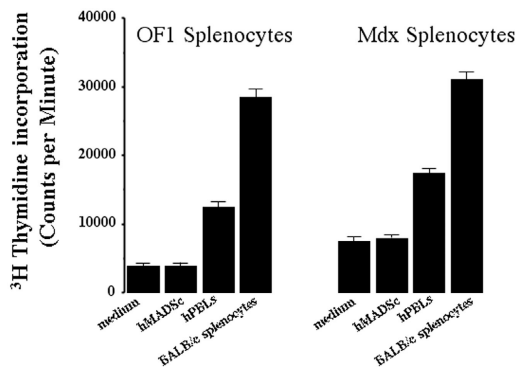


Figure S3. The effect of hMADS cells on the proliferation of OF1 and mdx splenocytes. OF1 and Mdx splenocytes were stimulated with an equivalent number of irradiated (500 rad) hMADS cells (at 120 PDs) or with human peripheral blood lymphocytes (hPBLs) or with mouse BALB/c splenocytes. Thymidine incorporation, determined 24 h after 1 $\mu\text{Ci/ml}$ tritiated thymidine, is expressed as counts per minute. Data are the mean of triplicate cultures.