

Mouse Adipose-Derived Stem Cells Undergo Multilineage Differentiation *in Vitro* but Primarily Osteogenic and Chondrogenic Differentiation *in Vivo*

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ABSTRACT

Human, rat, and mouse studies have demonstrated the existence of a population of adipose-derived adult stem (ADAS) cells that can undergo multilineage differentiation *in vitro*. However, it remains unclear whether these cells maintain their multilineage potential *in vivo*. The aim of this study was to examine the *in vitro* and *in vivo* characteristics and behavior of a potential population of murine ADAS (muADAS) cells isolated from the visceral fat of the abdominal cavity of C57BL/10J mice. We used flow cytometry to examine the cells' expression of CD29, CD31, CD45, CD34, CD44, CD144, CD146, Flk1, and Sca-1. The isolated cell population was CD45 negative, which precludes contamination by hematopoietic cells, but was partially positive for Sca-1 and CD34: 2 stem-cell markers. After induction in conditioned medium, the muADAS cells gained the ability to undergo adipogenic, osteogenic, chondrogenic, myogenic, and hematopoietic differentiation *in vitro*. The muADAS cells readily differentiated to form bone and cartilage *in vivo* for up to 24 weeks, but their ability to regenerate muscle or reconstitute bone marrow was found to be limited.

INTRODUCTION

RESEARCHERS TRADITIONALLY HAVE BELIEVED that stem cells in adult tissues can generate only the types of cells present within the tissues in which the stem cells reside; however, increasing numbers of papers have reported the detection of pluripotent stem and progenitor cells in various types of adult tissues, including bone marrow, muscle, brain, skin, and adipose tissue.¹⁻⁶ These cells, which purportedly can differentiate toward various lineages both *in vitro* and *in vivo*, could serve as the basis for a wide variety of tissue regeneration and repair applications. Unfortunately, the isolation of pluripotent stem cells from most of the tissue sources investigated has practical limitations. Often, the source tissue is not abundant, or cell harvesting relies on an invasive technique (e.g., bone marrow isolation) or is clinically impossible (e.g., brain tissue isolation). Ideal stem cell

sources would be easily accessible and available in unlimited amounts. Because adipose tissue fits these criteria, it is an attractive candidate as a stem cell source. Scientists recently have demonstrated that certain cells isolated from subcutaneous or visceral fat tissue can differentiate into a variety of cell types *in vitro*, including adipocytes, osteoblasts, chondrocytes, and myoblasts.⁶⁻⁸ These cells have been termed "adipose-derived adult stem (ADAS) cells" because they are self-renewing and can be induced to differentiate toward various mesodermal lineages.^{9,10}

Adipose tissue, which is abundant, expendable, and easy to obtain from the body, is derived from the mesoderm and contains 2 different cell populations: mature adipose cells and stroma vascular fraction (SVF) cells.¹¹ SVF cells isolated from rat adipose tissue have exhibited multipotent differentiation *in vitro*.^{10,12} The aim of our study was to determine whether visceral adipose tissue from mice contains

a population of adipose-derived mesodermal stem cells that can undergo multilineage differentiation *in vitro* and *in vivo*. The existence of such stem cells would validate the notion that these cells reside in adipose tissue and can be used to improve the healing of various types of tissues.

MATERIALS AND METHODS

Animals

The normal mice (C57BL/10J and BALB/cJ), *mdx* mice (C57BL/10ScSn DMD^{mdx}/J), SJL/J mice, and SCID mice used in this study were purchased from The Jackson Laboratory (Bar Harbor, ME). The Children's Hospital of Pittsburgh's Institutional Animal Care and Use Committee approved all animal protocols used for these experiments.

Cell harvest and culture

For isolation of murine adipose-derived adult stem (muADAS) cells, visceral adipose tissue was excised separately from six C57BL/10J mice and five BALB/cJ mice (4–8 weeks of age), finely minced, and digested using 0.075% collagenase type XI (Sigma, St. Louis, MO) for 45 min at 37°C. Enzyme activity was neutralized by treatment with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The cell suspension was centrifuged at 1200g for 10 min to separate the floating adipocytes from the SVF. The SVF was resuspended in ammonium chloride (160 mM), incubated at room temperature for 10 min to lyse contaminating red blood cells, and then centrifuged as described above. After filtration of the cell suspension through a 100- μ m nylon strainer to remove cellular debris, cell number and viability were determined using trypan blue exclusion. The SVF cells were plated in T75 flasks overnight in control medium (DMEM, 10% FBS, 1% penicillin/streptomycin) at 37°C in 5% carbon dioxide (CO₂). After incubation for 24 h, the flasks were washed extensively using phosphate buffered solution (PBS) to remove residual nonadherent cells. The adherent muADAS cells were expanded using serial passaging.

Characterization of muADAS cells

Flow cytometry was used to characterize muADAS cells obtained from C57BL/10J and BALB/cJ mice and grown for 4 passages and 10 passages under control conditions. Cultured cells were trypsinized, spun, and washed in cold PBS 1X (Mediatech, Herndon, VA) containing 2% FBS. The cells then were divided into aliquots and were spun to form a pellet. Cells were blocked using mouse serum (Sigma) diluted 1:10 in PBS and rat anti-mouse CD16/CD32 (BD PharMingen, San Diego, CA) for 10 min on ice. The primary antibodies (applied in optimal amounts) included a biotin-conjugated rat anti-mouse monoclonal antibody against CD34 and CD29 (surface proteins used to define mesenchymal stem cells and progenitor

cells) followed by streptavidin-APC, PE-conjugated mouse anti-Sca-1, and FITC-conjugated mouse anti-CD45 and anti-CD44 (all from BD PharMingen, San Diego, CA). To exclude dead cells, 7-aminoactinomycin D (Via-Probe, BD PharMingen) was added to each tube. Live cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and CellQuest software (Becton Dickinson). Analysis of surface protein expression was performed using appropriate gating on viable CD45-negative cells to eliminate contaminating hematopoietic cells. The isotype antibody control samples obtained for each individual cell population were used to set the dot-plot intercepts used for the analysis.

In vitro adipogenic differentiation assay

A previously described method was used to analyze adipogenesis.⁶ Trypsin/ethylenediaminetetraacetic acid was used to harvest the muADAS cells, which then were replated in 6-well plates (100,000 cells per well) in control medium to allow attachment. Twenty-four h later, the medium was replaced with new control medium or adipose medium (control medium plus insulin [10 μ M], dexamethasone [1 μ M], isobutyl-methylxanthine [0.5 mM], and indomethacin [200 μ M] [all from Sigma-Aldrich]). Cultures were maintained for 14 days, and medium was changed every 2 days. The cultures then were assessed using Oil Red O stain, which serves as an indicator of intracellular lipid accumulation. The cells were fixed for 10 min at room temperature in 10% neutral buffered formalin and were washed with distilled water. They then were incubated in Oil Red O reagent for 30 min and washed 3 times with distilled water. The cells were counterstained with hematoxylin for 1 min.

In vitro chondrogenic differentiation assay

Pellet culturing was performed as described previously.^{13–15} Cells ($n = 250,000$) were placed in a 15-mL conical polypropylene tube and were centrifuged at 600g for 5 min; they then were left on the bottom of the tube and cultured in control medium or defined medium: high-glucose DMEM supplemented with 1% ITS+Premix (BD Biosciences, Bedford, MA), L-ascorbic acid-2-phosphate (0.1 mM, Wako Pure Chemical Industries, Ltd. Osaka, Japan), dexamethasone (1×10^{-7} M, Sigma), proline (400 μ g/mL, Sigma), and bone morphogenetic protein 4 (BMP4) (500 ng/mL, R&D Systems). After the addition of 1 mL of chondrogenic medium to each tube, the pelleted cells were incubated at 37°C in 5% CO₂. The medium was changed every 2 to 3 days. Pellets were harvested at different time points (Days 7, 14, 21) and were embedded in paraffin as detailed in the standard protocol (available on the Cambrex Company website). Chondrogenesis was confirmed by use of the histologic stain Alcian blue at a low pH to stain the highly sulfated proteoglycans that are characteristic of cartilaginous matrix. After that, sections were counterstained with nuclear fast red, which specifically stains cell nuclei.

In vitro osteogenic differentiation assay

The *in vitro* osteogenic assay was performed as described previously.⁶ Osteogenic differentiation was induced by culturing muADAS cells in osteogenic medium (OM, control medium supplemented with dexamethasone [0.1 μ M], ascorbate-2-phosphate [50 μ M], and β -glycerophosphate [10 mM] [all from Sigma]). The medium was changed every 3 days. Osteogenesis was assessed using von Kossa staining and observation of alkaline phosphatase (ALP) activity 2 and 4 weeks after initial osteogenic induction, as described previously.¹⁶ The cells were incubated in OM for 2 weeks and rinsed with PBS. The commercially available Sigma AP kit 86-c (Sigma) then was used to detect ALP activity. The cells used for von Kossa staining were incubated in OM for 4 weeks and fixed at room temperature with 4% paraformaldehyde for 1 h. They were rinsed with distilled water and then were incubated for 30 min in 1% silver nitrate solution in the dark. The cells then were rinsed again with distilled water and exposed to ultraviolet light for 60 min. Secreted calcified extracellular matrix appeared as black nodules after von Kossa staining.

In vivo chondrogenic and osteogenic differentiation assays

muADAS cells were seeded at a density of 1.5×10^6 cells in 75-cm² flasks and were grown to 50% confluence. On the day of transduction, cells were washed twice with sterile PBS and were incubated for 16 h with a mixture of 2 mL of retro-*LacZ* viral suspension (1×10^6 – 5×10^6 cfu/mL), 18 mL of DMEM supplemented with 10% FBS, and polybrene (8 μ g/mL) in 5% CO₂ at 37°C. This same solution was replaced every 16 h for 32 h. The percentage of *LacZ*-positive transduced cells (muADAS-*LacZ* cells) was determined using X-galactosidase (gal) staining. The aforementioned technique also was used to transduce muADAS-*LacZ* cells with a retro-*BMP4* virus. Medium containing 10 mL of retro-*BMP4* viral suspension (5×10^5 cfu/mL), 10 mL of DMEM with 10% FBS, and polybrene (8 μ g/mL) was added to the flasks. muADAS-*LacZ* cells were cultured in this medium for 48 h, with medium changes performed every 16 h. After transduction, the muADAS-*LacZ*-*BMP4* cells were cultured in 6-well plates until the cultures reached confluence. The culture medium was collected after 48 h, centrifuged at 2000 rpm and 4°C for 5 min to remove cellular debris, and then used to perform a *BMP4* bioassay. A previously described *BMP4* assay was used to determine the level of functional *BMP4* secreted by the transduced cells.¹⁷ After detection of bioactive *BMP4* in the culture medium (100 ± 22 ng/million cells/24 h) of muADAS-*LacZ*-*BMP4* cells, 3×10^5 cells in a 100- μ L cell suspension were seeded on the surface of a 6 mm \times 6 mm piece of sterile collagen sponge (Gelfoam, Pharmacia & Upjohn Co., Ann Arbor, MI). After the Gelfoam absorbed all the cell suspension, 3 mL of DMEM supplemented with 10% FBS was added to

each well; the wells then were placed in a cell incubator overnight.

Animal surgery was performed on the following day. Samples were implanted into the skeletal muscle pouch of the gluteofemoral muscle of each SCID mouse. The mice were examined radiographically and then killed at different times (2, 4, 5, and 24 weeks) after cell implantation. The harvested tissue samples obtained 2, 4, and 5 weeks after cell implantation were treated with CRYO-GEL Embedding Medium (Cancer Diagnostics, Inc., Birmingham, MI), rapidly frozen, and stored at -80°C . Frozen sections were fixed in 1% glutaraldehyde for 1 min and then were washed 3 times in PBS. Some of the sections were stained in X-gal solution and counterstained with eosin. Other sections were stained in X-gal solution and immunostained with collagen type II (1:200 dilution, Santa Cruz Biotech, Santa Cruz, CA) and osteocalcin (1:200 dilution, Santa Cruz Biotech), as detailed in the manufacturer's protocol (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). Some of the 24-week samples were frozen and sectioned, whereas others were decalcified and embedded in paraffin. The frozen sections were stained in X-gal solution and counterstained with eosin. The paraffin-embedded sections were stained with Alcian blue and eosin. Some sections were immunostained for collagen type II or osteocalcin in addition to β -gal (1:200 dilution, Abcam, Inc., Cambridge, UK).

In vitro and in vivo myogenic differentiation assays

Myogenic differentiation was induced by culturing muADAS cells in myogenic medium (MM: control medium supplemented with dexamethasone [0.1 μ M, Sigma], hydrocortisone [50 μ M, Sigma], and 5% horse serum) for 4 weeks and was confirmed using desmin staining. The cells were rinsed twice with PBS, fixed for 1 min with methanol, and again washed several times with PBS. The cells were blocked with 5% horse serum at room temperature for 1 h and then were incubated with the primary antibody rabbit immunoglobulin G (IgG) anti-desmin (1:250 in PBS, Sigma) overnight at 4°C. After being rinsed thoroughly with PBS, cells were incubated with biotinylated anti-rabbit IgG antibody (1:250, Sigma) for 1 h at room temperature. The cells then were rinsed with PBS and incubated for 30 min at room temperature with streptavidin conjugated Cy3 fluorochrome (1:300, Sigma). Slides were viewed with a Nikon Diaphot 300 fluorescence microscope (Nikon, Garden City, NY).

In vivo myogenic differentiation was evaluated by injection of muADAS cells into the skeletal muscle of *mdx* mice, which model Duchenne muscular dystrophy.¹⁸ muADAS cells from passages 3 to 5 were grown to 60% confluence in control medium. To enable tracking of cells after injection into gastrocnemius muscles, muADAS cells were genetically engineered to express the *LacZ* reporter

gene (muADAS-*LacZ* cells), as described above for the *in vivo* chondrogenic and osteogenic assays. The cells were harvested, mixed with FluoSpheres (Molecular Probes, Eugene, OR) in 20 μ L of Hanks' balanced salt solution (HBSS, Invitrogen, Grand Island, NY), and injected (5×10^5 cells per injection) into a single site within the gastrocnemius muscles of 8-week-old *mdx* mice. Injected gastrocnemius muscles were harvested on Day 15 and were stained for *LacZ* and dystrophin. For *LacZ* staining, the sections were fixed in 1% glutaraldehyde, incubated overnight with X-gal substrate at 37°C, and counterstained with eosin. The sections were mounted in Gel/Mount (M01, Biomedica, Plovdiv, Bulgaria) and observed with a light microscope (Nikon Eclipse E800). For dystrophin staining, slides were fixed with acetone and blocked with 5% horse serum. The primary antibody was rabbit anti-dystrophin (1:1000, gift from Dr. T. Partridge). Sections then were washed in PBS and were incubated with biotinylated anti-rabbit IgG (1:300, Vector) followed by streptavidin-Cy3 (1:500, Sigma). The dystrophin-positive myofibers were visualized using fluorescence microscopy.

In vitro and *in vivo* hematopoietic differentiation assays

In vitro differentiation of the muADAS cells toward the hematopoietic lineage was induced by culturing the cells in methylcellulose as described previously.¹⁹ muADAS cells ($n = 1 \times 10^5$) in 1 mL of Methocult GF M3434 culture media (Stem Cell Technologies, Vancouver, Canada) were plated in a 35-mm petri dish and cultured in a humidified 5% CO₂ atmosphere maintained at 37°C. Colonies were counted 10 days after plating. Colonies from triplicate cultures were individually lifted from the methylcellulose culture and were centrifuged to glass slides with a Cytospin (Shandon Southern, Sewickley, PA). Cells then were stained using the HEMA 3 Stain Set (Biochemical Science, Inc., Swedesboro, NJ).

In vivo hematopoietic differentiation of the muADAS cells was determined by transplantation of muADAS cells into 8- to 10-week-old SJL/J mice that had been subjected to a single lethal dose of radiation (10 Gy) 1 day before cell transplantation. We used this alternative strain of mouse to distinguish the differentiated donor cells (CD45.2 positive) from the SJL/J host cells (CD45.2 negative), as described previously.¹⁹ Cells (1×10^5 , 5×10^5 , or 1×10^6 in 400 μ L of HBSS) were injected intraperitoneally or intravenously into the tail vein. Mice were given acidified water and autoclaved food.

RESULTS

Phenotypic characterization of the muADAS cells

The visceral adipose tissue from 1 mouse yielded approximately 7.0×10^6 nucleated cells. Within 4 passages

after the initial plating of the primary culture, muADAS cells appeared as a monolayer of large, flat cells. As the cells approached confluence, they acquired a spindle-shaped or fibroblast-like appearance. We used flow cytometry to evaluate the expression of CD45, Sca-1, CD34, CD44, and CD29 cell-surface antigens on the muADAS cells obtained from C57BL/10J and BALB/cJ mice. The muADAS cells from both mouse strains were negative for CD45, a hematopoietic cell surface marker (Fig. 1A). Approximately 31% and 10% of the muADAS cells were Sca-1 and CD34 positive, respectively (Fig. 1B). muADAS cells were negative for CD31, CD144, CD146, and flk-1 at the time of flow cytometry analysis (after 4 passages; data not shown). Because we observed similar results in cells obtained from both strains (Fig. 1C), we used only C57BL/10J mice for the remainder of the study. Flow cytometry analysis revealed that the muADAS cell marker profile changed during expansion. After passage 10, the cells showed increased Sca-1 expression (74.94%) and decreased CD34 expression (3.25%) (Fig. 1D).

muADAS cells undergo adipogenic differentiation in vitro

To determine whether muADAS cells undergo adipogenesis, we cultured muADAS cells in adipogenic medium. Oil Red O staining performed 2 weeks later revealed cytoplasmic lipid droplets, which are indicative of the adipogenic phenotype. These results indicate that the muADAS cells cultured in adipogenic medium underwent adipogenic differentiation (Fig. 2A), whereas muADAS cells maintained in control medium did not (Fig. 2B).

muADAS cells undergo chondrogenic differentiation in vitro

In the chondrogenic assay, the seeded muADAS cells initially formed a flat disk of cells that gradually expanded into a rounded pellet. After 21 days in culture, sections of pellet showed early cartilage formation, as indicated by Alcian blue staining (pH 1), which is specific for cartilaginous matrix (Fig. 2C). In contrast, cells cultured in control medium resulted in only light Alcian blue staining (Fig. 2D).

muADAS cells undergo osteogenic differentiation in vitro

muADAS cells cultured in osteogenic medium for 14 days expressed endogenous ALP (Fig. 2E). Moreover, mineralized nodular structures formed within 4 weeks, as confirmed by von Kossa staining (Fig. 2G). In contrast, muADAS cells cultured in control medium showed no evidence of ALP expression or formation of calcified extracellular matrix (Fig. 2F, H).

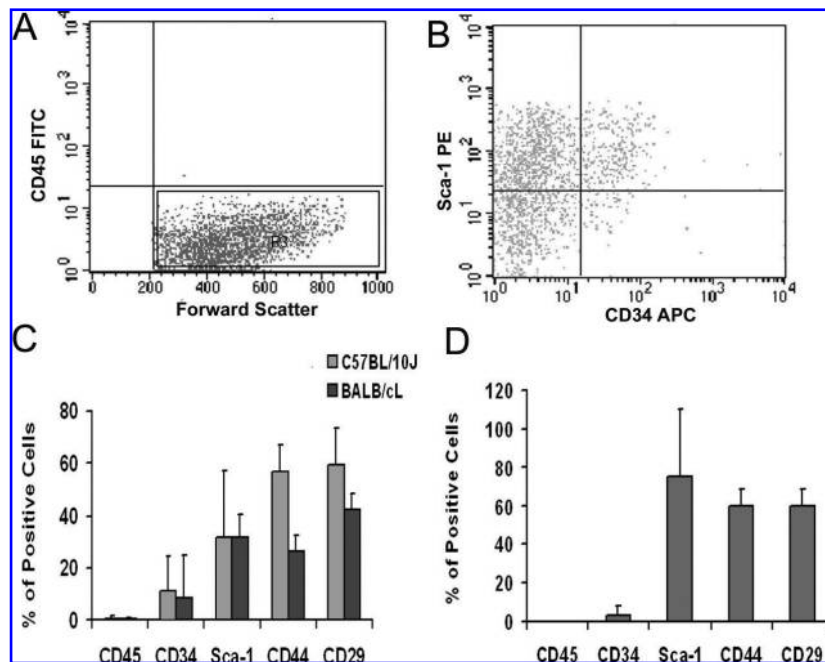


FIG. 1. (A) Flow cytometric analysis of CD45, (B) Sca-1, and CD34 expression on murine adipose-derived adult stem (muADAS) cells at passage 4. muADAS cells were CD45 negative, but 31% of them were Sca-1 positive and 10% were CD34 positive. (C) The marker profiles of muADAS cells from both C57BL/10J and BALB/cJ mice were similar. (D) CD34 expression was lower whereas Sca-1 expression was higher on C57BL/10J cells at passage 10.

muADAS cells undergo chondrogenic and osteogenic differentiation in vivo

We transduced the muADAS cells with a retrovirus containing the *LacZ* gene, a reporter gene that we used to track the fate of our donor cells *in vivo*. The *LacZ* gene transduction efficiency was 61%. X-gal staining enabled us to track the muADAS-*LacZ* cells on the basis of their β -gal expression (blue nuclei) (Fig. 3A). We then cotransduced these cells with a retrovirus containing the *BMP4* gene.¹⁷ The cotransduced muADAS cells secreted 100 ± 22 ng of *BMP4*/million cells/24 h. Twenty-eight days after implantation of 3×10^5 muADAS cells cotransduced with *LacZ* and *BMP4* into the host mice, bone formation was visible using radiographic examination (Fig. 3B). Histologic analysis showed that the muADAS cells differentiated toward chondrogenic and osteogenic lineages *in vivo* (Fig. 3 C–F). Radiographic analysis 24 weeks after cell implantation showed solid bone formation (Fig. 4A). Histologic results indicated that cortical-like bone tissue and cartilage formed in the area of ectopic ossification (Fig. 4B, C). Immunostaining of β -gal and collagen type II or osteocalcin supports the histologic findings that the implanted muADAS cells continued to survive and participate in the generation of ectopic bone and cartilage for up to 24 weeks (Fig. 4B–F).

muADAS cells' ability to undergo myogenic differentiation in vitro and in vivo

We examined the muADAS cells' myogenic differentiation *in vitro* by staining for desmin and *in vivo* by

staining for dystrophin and evaluating muscle regeneration after implanting muADAS cells into the skeletal muscle of *mdx* mice. muADAS cells cultured for 6 weeks in myogenic medium expressed desmin (Fig. 5A). To determine the *in vivo* myogenic capacity of muADAS cells, we injected 5×10^5 muADAS-*LacZ* cells into the gastrocnemius muscles of *mdx* mice. We killed the mice 15 days after injection and harvested the injected muscles to examine β -gal and dystrophin expression. Fifteen days after injection, we detected few *LacZ*-positive myofibers, which resulted from the fusion of donor-derived transduced muADAS cells with host myofibers (Fig. 5B, arrow). We also detected few dystrophin-positive myofibers in the injected muscle (data not shown). Our findings suggest that muADAS cells undergo limited myogenic differentiation *in vitro* and *in vivo*.

In vitro and in vivo hematopoietic differentiation by muADAS cells

To evaluate the hematopoietic differentiation of muADAS cells *in vitro*, we plated them in semisolid methylcellulose medium supplemented with cytokines known to promote myeloid differentiation. Of the 6 populations of muADAS cells examined, 1 formed typical hematopoietic colonies after 10 days in culture (Fig. 5C). HEMA 3 Stain Set staining also revealed granulomonocytic colonies (Fig. 5D).

We also injected 4 populations of muADAS cells intravenously (tail vein) or intraperitoneally into lethally

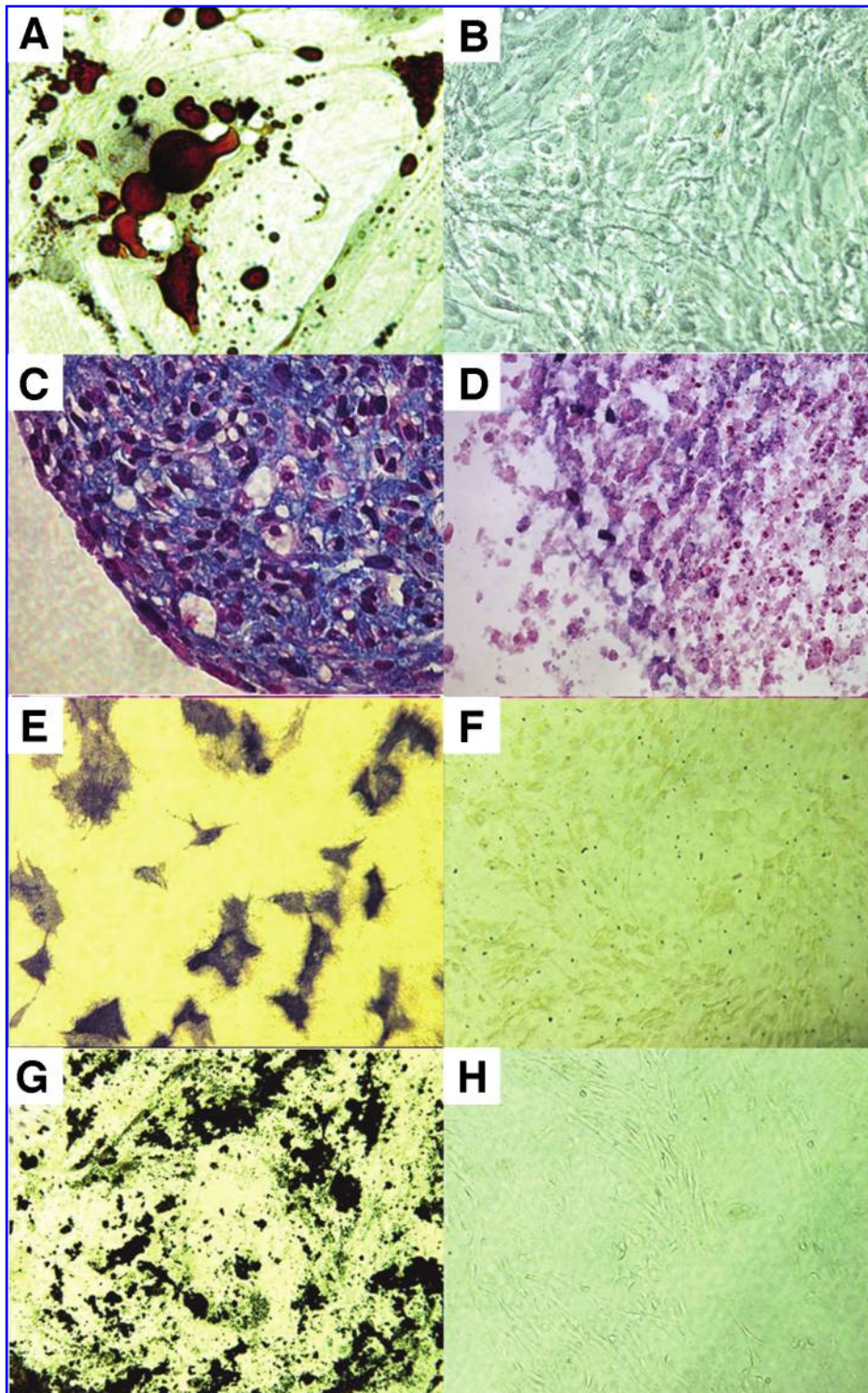


FIG. 2. Murine adipose-derived adult stem (muADAS) cells accumulated lipid-filled droplets when cultured for 2 weeks in adipogenic medium. **(A)** Oil Red O staining revealed lipid-filled intracellular vacuoles (magnification 40 \times). **(B)** muADAS cells maintained in control medium remained negative for Oil Red O staining (magnification 40 \times). **(C)** We observed positive Alcian blue staining, which indicates the presence of sulfated proteoglycans, in the cells cultured 3 weeks in chondrogenic medium (magnification 60 \times). **(D)** muADAS cells maintained in control medium were only slightly positive after Alcian blue staining (magnification 60 \times). **(E)** muADAS cells grown in osteogenic medium exhibited ALP activity (magnification 10 \times). **(F)** The cells cultured in control medium remained von Kossa negative (magnification 10 \times). **(G)** von Kossa staining revealed calcified extracellular matrix (black) in muADAS cells cultured for 4 weeks in osteogenic medium (magnification 10 \times). **(H)** The cells maintained in control medium remained von Kossa negative (magnification 10 \times). (Color images available online at www.liebertpub.com/ten.)

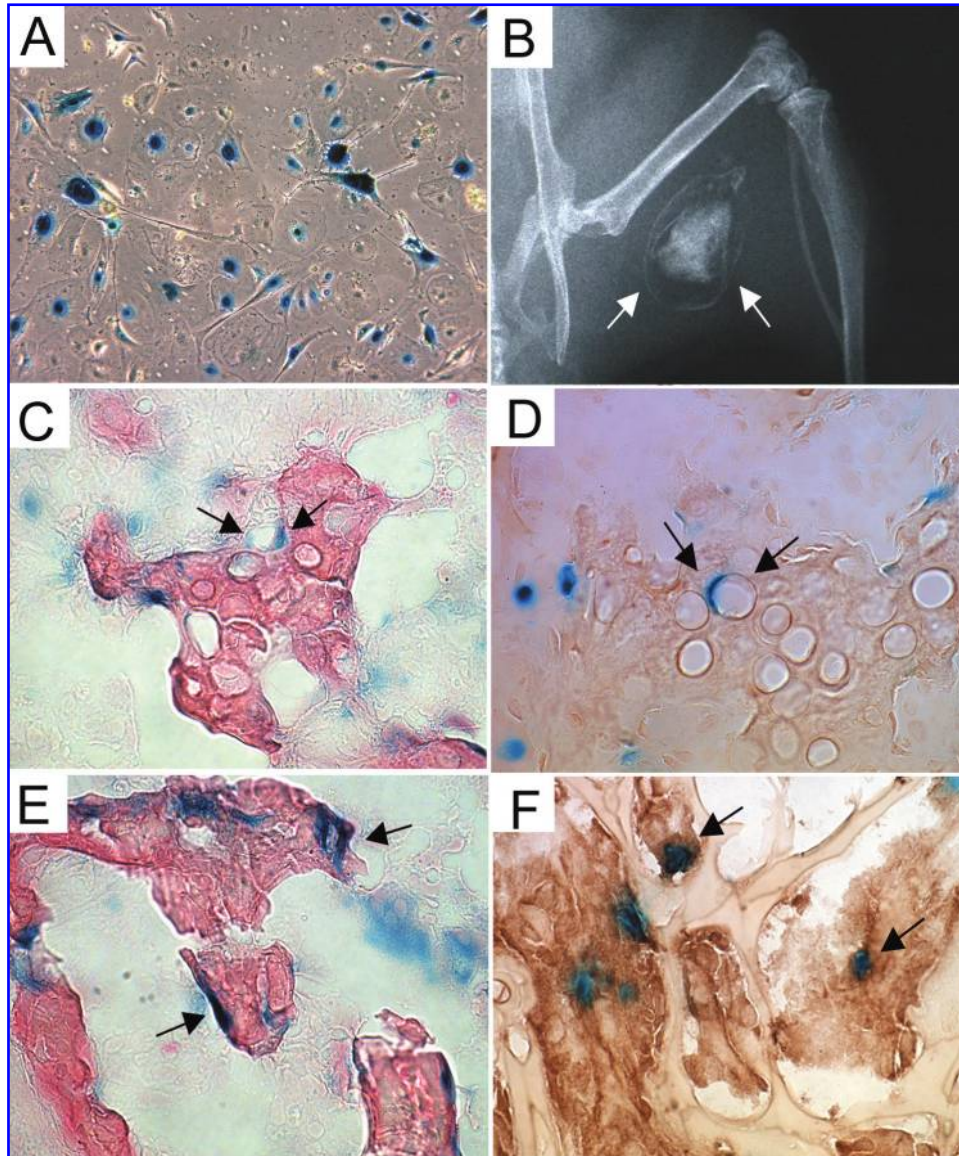


FIG. 3. (A) Murine adipose-derived adult stem (muADAS) cells were transduced with a retrovirus containing the *LacZ* gene and were identifiable by their *LacZ*-expressing nuclei after β -galactosidase staining (magnification 20 \times). (B) Radiographic examination showed intramuscular bone formation in SCID mice 28 days after implantation of muADAS cells cotransduced to express *LacZ* and *BMP4*. (C) *In vivo* chondrogenesis by muADAS cells was determined by colocalization of round chondrocytes with blue nuclei (arrow) after staining for *LacZ*/eosin (magnification 60 \times) and (D) by positive immunostaining for *LacZ*/collagen type II (brown) (magnification 60 \times). (E) *In vivo* osteogenesis by muADAS cells was confirmed by colocalization of blue cells in the newly formed bone tissue (arrow, magnification 60 \times) (F) with osteocalcin (brown) (arrow, magnification 60 \times).

irradiated SJL/J recipient mice. The intravenous injection of 1×10^6 muADAS cells per mouse ($n = 4$ mice) induced immediate mouse death, a result that parallels findings reported by other researchers.¹¹ After intraperitoneal transplantation of muADAS cells into 4 lethally irradiated SJL/J mice, the treated mice and control mice died within 3 weeks of transplantation. We also investigated the effect of intravenous injection (into the tail vein) of fewer cells (1×10^5 and 5×10^5) into lethally irradiated mice ($n = 8$ mice, 4 per quantity of cells). The mice did not immediately die, which indicates that injection of the decreased quantities of cells

did not cause fat boluses in these mice. However, the treated mice and control mice died within 3 weeks of transplantation. This finding demonstrates that the muADAS cells failed to reconstitute the bone marrow and hence failed to provide radiation protection to treated mice.

DISCUSSION

The results of this study demonstrate that a population of cells isolated from the visceral fat of mice can undergo

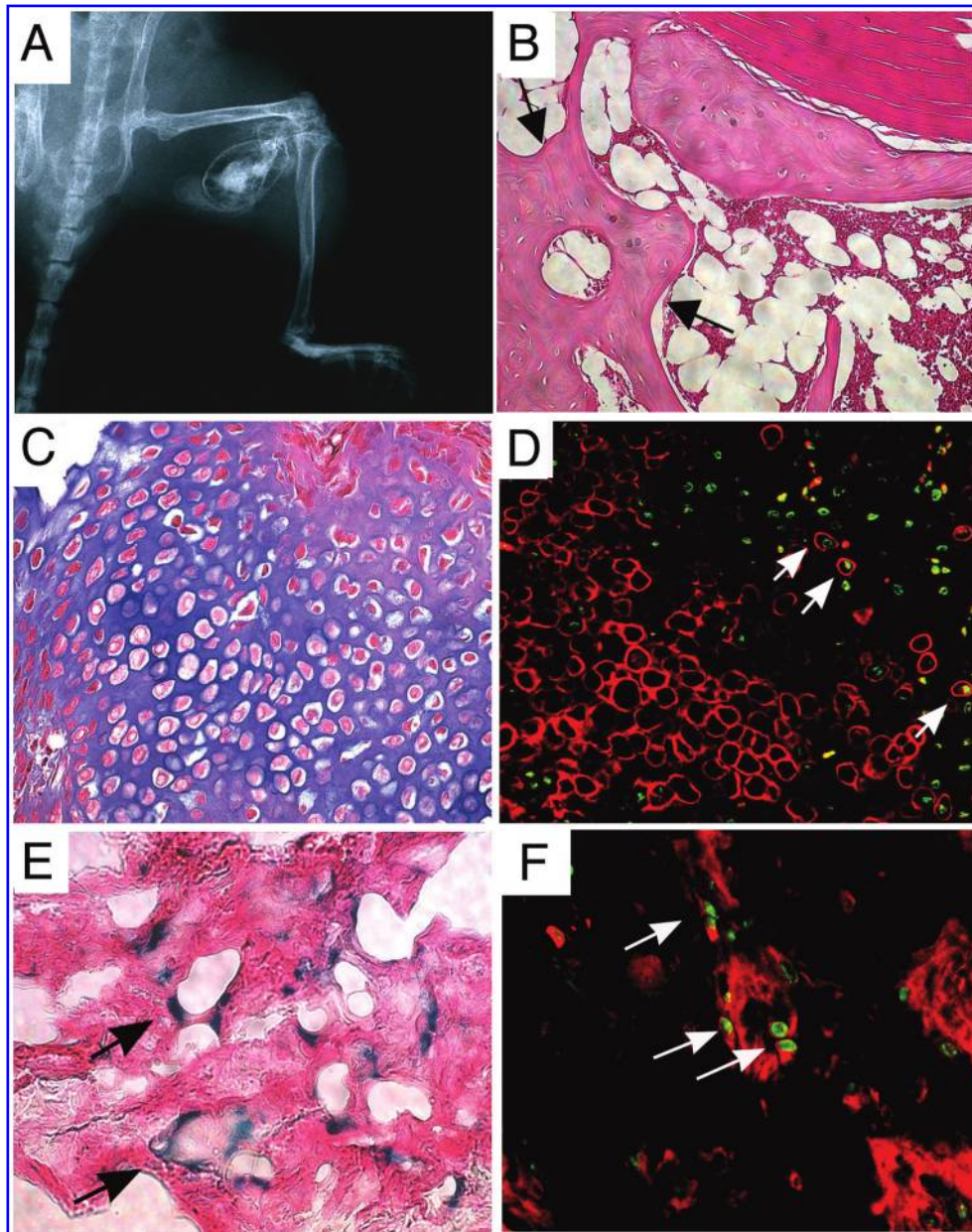


FIG. 4. Ectopic bone formation 24 weeks after implantation of muADAS-LacZ-BMP cells into muscle pockets of SCID mice. (A) Radiographic examination. (B) The histology of ectopic bone tissue (arrows, 20 \times). (C) *In vivo* ectopic bone tissue included some areas of cartilage, as shown by Alcian blue staining (magnification 40 \times). (D) Chondrogenesis of murine adipose-derived adult stem (muADAS) cells was shown by positive immunostaining for colocalization of collagen type II (red)/ β -galactosidase (green) (arrow, magnification 40 \times). *In vivo* osteogenesis by muADAS cells was confirmed (E) by LacZ/eosin (magnification 60 \times) and (F) by positive immunostaining for colocalization of osteocalcin (red)/ β -gal (green) (arrow, magnification 60 \times).

multilineage differentiation *in vitro* and *in vivo*. This muADAS cell population contains fibroblast-like cells that are easy to expand *in vitro* without specific serum supplementation. The muADAS cells used in our study differentiated toward the adipogenic, osteogenic, chondrogenic, and myogenic lineages when cultured in the presence of established lineage-specific differentiation factors. The ability of muADAS cells to undergo multilineage differentiation suggests that a stem cell population exists within

mouse adipose tissue. The observed multilineage differentiation may also be attributed to the presence of multiple populations of lineage-committed progenitor cells within adipose tissue (e.g., pre-osteoblasts, pre-myoblasts, or pre-adipocytes), multipotent cells from other sources (e.g., blood vessels or pericytes), or a combination of these populations. muADAS cell populations obtained from excised adipose tissue are known to contain pre-adipocytes that differentiate into mature adipocytes. The adipogenic differentiation

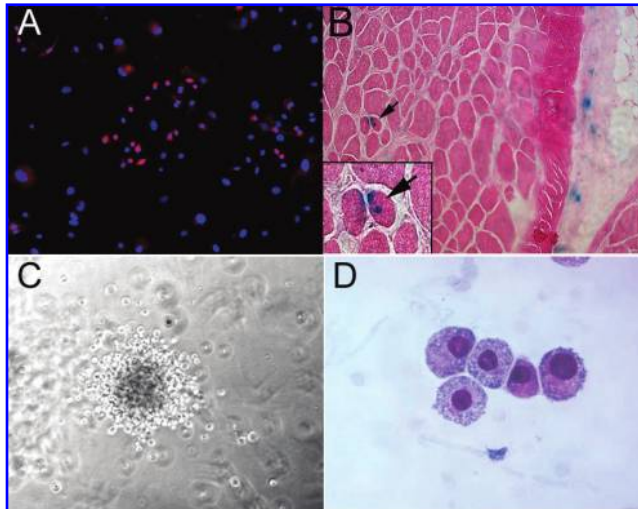


FIG. 5. (A) Murine adipose-derived adult stem (muADAS) cells cultured for 6 weeks in myogenic medium expressed desmin (red, magnification 20 \times). Hoechst staining (blue) revealed the cell nuclei. (B) Few muADAS cells participated in muscle regeneration, as determined by the number of *LacZ*-positive myofibers in the *mdx* mouse muscle 15 days after injection (magnification 20 \times , bottom left-hand corner insets, 60 \times). (C) Large colonies composed of small cells were found in methylcellulose cultures of muADAS cells (magnification, 5 \times). (D) After picking, cytopinning, and staining the colonies with Wright-Giemsa stain, we observed granulomonocytic cells (magnification 100 \times).

exhibited by the muADAS cells also could be due to the commitment of existing pre-adipocytes rather than to the differentiation of multipotent cells. However, such an explanation is unconvincing in the case of humans, because as few as 0.02% of the SVF cells in adipose tissue excised from humans are pre-adipocytes that can undergo adipogenic differentiation.⁶ Therefore, the large-scale adipogenesis observed in our study more likely resulted from the differentiation of muADAS cells themselves.

Our observations of muADAS cells under control conditions also revealed changes in their expression of CD34 and Sca-1, both of which are progenitor cell surface markers. Our analysis of muADAS cells at 10 passages indicated that 74.94% expressed Sca-1 and 3.25% expressed CD34. In comparison, the expression of Sca-1 and CD34 by muADAS cells at 4 passages was 31% and 10%, respectively. We are investigating whether these changes in the marker expression profile reflect the different proliferation rates of stem cells and differentiated cells. Because we isolated the cells from visceral fat, the involvement of myogenic precursor cells or satellite cells in the observed myogenic differentiation is unlikely. Furthermore, the freshly isolated muADAS cells did not express the myogenic marker desmin. Desmin was only expressed when muADAS cells were cultured in myogenic medium. This finding suggests that the myogenic differentiation observed in this study was not due to the activity of myogenic pre-

cursors or proliferating myoblasts within the muADAS cell population.

The circulatory system, which contains potentially multipotent cells such as pericytes and marrow-derived mesenchymal stem cells, is another possible source of stem cell contamination of the muADAS cell population. Disruption of the blood supply during isolation could have resulted in the release of hematopoietic progenitors, which are known to give rise to hematopoietic lineages *in vivo* and *in vitro*;¹¹ however, flow cytometry revealed no CD45-positive cells in the muADAS cell population. In addition, prior research revealed no hematopoietic cells in muADAS cell cultures at 2 to 3 passages.^{9,20} Although our *in vitro* data suggest that the muADAS cells might have at least limited hematopoietic potential, muADAS cells transplanted intravenously or intraperitoneally failed to prevent the death of lethally irradiated SJL/J mice. The immediate death of the mice after intravenous injection of muADAS cells could be due to the formation of a fat bolus in critical organs such as the brain or heart. The inability of intraperitoneally and intravenously injected muADAS cells to reconstitute the hosts' bone marrow suggests that the *in vivo* hematopoietic potential of these cells is, at best, limited. Because of these findings, definitive confirmation of the existence of multipotent stem cells within adipose tissue will require the isolation and characterization of multiple clones.

Researchers have demonstrated that bone marrow-derived mesenchymal stem cells can differentiate toward osteogenic and chondrogenic lineages *in vivo*, and preliminary data suggest that these cells can facilitate the repair of bone and cartilage defects.^{21–24} However, harvesting bone marrow is an invasive procedure, and the marrow contains only a limited number of progenitor cells. Many studies of ADAS cells have focused on the cells' ability to differentiate toward various lineages *in vitro* or their potential to repair bone and cartilage *in vivo*; few studies have investigated the long-term fate of donor ADAS cells influenced by *BMP4 in vivo*. The results presented here demonstrate that ADAS cells transduced with a retrovirus containing *LacZ* and *BMP4* genes and implanted into the skeletal muscle of mice differentiated toward the chondrogenic and osteogenic lineages and continued to survive within ectopic bone tissue 6 months after implantation. Moreover, radiographic analysis revealed that the ectopic bone formed *in vivo* maintained its size for 5 months (from 4 to 24 weeks after implantation, Figs. 3B, 4A). Histologic analysis also revealed a greater amount of dense bone and cartilage in the samples obtained 24 weeks after implantation than in those harvested 4 weeks after implantation. These observations indicate that the ectopic cartilage and bone tissue formed by the transduced muADAS cells was stable and persistent, which suggests that ADAS cells could serve as a good cell source for bone and cartilage regeneration.

In conclusion, these observations validate the notion that muADAS cells could serve as a cell source for gene

therapy. The limited ability of those cells to undergo myogenic and hematopoietic differentiation restricted their utility for muscle regeneration and reconstitution of bone marrow. On the other hand, their high capacity for bone and cartilage repair and their long-term survival under the influence of *BMP4 in vivo* suggest that they might be a useful cell source for osteogenic and chondrogenic applications. The origin of these multipotent cells isolated from adipose tissue remains unclear and will be the focus of future research.

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