Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential

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Summary

We report here the isolation of a population of non-transformed pluripotent human cells from bone marrow after a unique expansion/selection procedure. This procedure was designed to provide conditions resembling the in vivo microenvironment that is home for the most-primitive stem cells. Marrow-adherent and -nonadherent cells were co-cultured on fibronectin, at low oxygen tension, for 14 days. Colonies of small adherent cells were isolated and further expanded on fibronectin at low density, low oxygen tension with 2% fetal bovine serum. They expressed high levels of CD29, CD63, CD81, CD122, CD164, hepatocyte growth factor receptor (cMet), bone morphogenetic protein receptor 1B (BMPR1B), and neurotrophic tyrosine kinase receptor 3 (NTRK3) and were negative for CD34, CD36, CD45, CD117 (cKit) and HLA-DR. The embryonic stem cell markers Oct-4 and Rex-1, and telomerase were expressed in all cultures examined. Cell-doubling time was 36 to 72 hours, and cells have been expanded in culture for more than 50 population doublings. This population of cells was consistently isolated from men and women of ages ranging from 3- to 72-years old. Colonies of cells expressed numerous markers found among embryonic stem cells as well as mesodermal-, endodermal- and ectodermal-derived lineages. They have been differentiated to bone-forming osteoblasts, cartilage-forming chondrocytes, fat-forming adipocytes and neural cells and to attachment-independent spherical clusters expressing genes associated with pancreatic islets. Based on their unique characteristics and properties, we refer to them as human marrow-isolated adult multilineage inducible cells, or MIAMI cells. MIAMI cells proliferate extensively without evidence of senescence or loss of differentiation potential and thus may represent an ideal candidate for cellular therapies of inherited or degenerative diseases.

Key Words: Stem cells, Aging, Bone marrow, Pluripotential, Reparative medicine, Neurogenesis

Introduction

We have isolated a unique subpopulation of human marrow stromal cells capable of differentiating in vitro into cell lineages derived from all three germ layers. Marrow stromal cells (MSCs) were first described by Friedenstein (Friedenstein et al., 1970) as fibroblast-like cells that reside in the bone marrow of vertebrate animals, including humans. MSCs are a heterogeneous population of uncommitted and lineage-committed cells with the potential to differentiate toward diverse somatic lineages. The plasticity of bone marrow stem cells has been demonstrated by recent studies showing that subpopulations of bone marrow cells appear to self-maintain throughout the organism's life, their progeny giving rise to a variety of tissues derived from other embryonic germ layers (Ferrari et al., 1998; Kopen et al., 1999; Asahara et al., 1999; Theise et al., 2000; Brazelton et al., 2000; Krause et al., 2001; Orlic et al., 2001; Mezey et al., 2003). Several investigators have reported the isolation of primitive human adult multipotent cells from different tissues, including bone marrow. Subpopulations of human MSCs (hMSCs) exhibiting features of primitive adult pluripotent or multipotent stem cells have been described (Young et al., 1998; Pittenger et al., 1999; Conget and Minguell, 1999; D'Ippolito et al., 1999; Colter et al., 2000; Colter et al., 2001; Reyes et al., 2001; Jiang et al., 2002; Hung et al., 2002; Gronthos et al., 2003). Cells with features of adult pluripotent stem cells have also been isolated from umbilical cord blood (Erices et al., 2000), peripheral blood (Huss, 2000; Zvaifler et al., 2000; Kuci et al., 2003), adipose tissue (Zuk et al., 2001), skeletal muscle and dermis (Young

et al., 1999; Young et al., 2001; Toma et al., 2001), as well as other tissues (Asakura and Rudnicki, 2002). Molecular and functional analyses demonstrate specific and clear differences among these pluripotent subpopulations of cells.

Although many of these primitive adult cell subpopulations have the potential to differentiate toward various cell lineages, it remains to be established if a particular population of adult human stem cells can maintain a broad and multilineage differentiation capacity, resembling the plasticity of embryonic stem cells, throughout the life of the individual.

We have combined specific culture conditions, including extracellular matrix substrata, oxygen tension, growth factors and vitamins, cell density and co-culture of cells to resemble the in vivo niche microenvironment in which the more primitive cells are expected to be found (Watt and Hogan, 2000). On the basis of a unique MSC molecular profile, we isolated a cell population that can be expanded for more than 50 population doublings. These cells, obtained from males and females 3- to 72-years old, have been maintained in vitro without detectable changes in their characteristic molecular profile. We show that this in vitro cell population can be differentiated into mesodermal lineage cells, as well as into cells with features of neuroectodermal- and endodermal-derived lineages. This cell population maintains a multi-germ layer differentiation potential in vitro. We have named these cells **m**arrow-**i**solated **a**dult **m**ultilineage **i**nducible cells, or MIAMI cells.

Materials and Methods

Cytokines

Human recombinant hepatocyte growth factor (HGF), basic-fibroblast growth factor (b-FGF), epidermal growth factor (EGF) and exendin-4 were from Sigma Chemical (St Louis, MO, USA); β-nerve growth factor (NGF), neurotrophin-3 (NT-3) and brain-derived neurotrophic factors (BDNF) were from Calbiochem (San Diego, CA, USA); and transforming growth factor-β3 (TGF-β3) and activin-A were from R&D (Minneapolis, MN, USA).

Antibodies

The following primary antibodies were used: for immunocytochemistry – neuron-specific enolase (NSE), neurofilament-M, nestin, neuronal nuclear protein (Neu-N), glial fibrillary acid protein (GFAP) and NGF receptor (TrkA) were from Chemicon (Temecula, CA, USA); neuron-specific class III β-tubulin (TuJ1) was from Covance (Princeton, NJ, USA); NT-3 receptor (NTRK-3) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); for immunoblotting – cMet from Santa Cruz Biotechnology; for flow cytometry – CD10, CD29, CD34, CD36, CD49e, CD45, CD54, CD56, CD63, CD81, CD103, CD117, BMPR-1B, CNFTR, Flt-1, Flk-1, NTRK3, and HLA-DR were from Santa Cruz Biotechnology and CD44, CD90, CD109, CD122, and CD164 were from BD-Pharmigen (San Diego, CA, USA).

Bone marrow and cells

Whole bone marrow was obtained from vertebral bodies (T1-L5) of cadaveric male and female donors who died of fatal traumatic injury (age range; 3- to 72-years old) following guidelines for informed consent set by the University of Miami School of Medicine Committee on the Use of Human Subjects in Research. Vertebral bodies were removed from normal healthy donors within 2 hours after the heart stopped beating. Whole bone marrow (adherent and nonadherent cells) was plated (without prior gradient centrifugation, immunoselection, or immunodepletion) at a constant density of 10^5 cells/cm^2 (D'Ippolito et al., 1999) on fibronectin-coated 15-cm dishes. Cell samples from 12 donors [#519 (3-year-old boy), #849 (7-year-old boy), #769 (10-year-old boy), #657 (3-year-old girl), #764 (11-year-old girl), 645 (14-year-old girl), #502 (40-year-old man), #869 (55-year-old man), #812 (72-year-old man), #619 (42-year-old woman), #507 (55-year-old woman), and #889 (59-year-old woman)] were selected for further analysis of their differentiation capacity. The human foreskin fibroblast cell line hTERT-BJ1 that stably expresses exogenous human telomerase reverse transcriptase (hTERT) was obtained from BD Bioscience Clontech (Palo Alto, CA, USA). The prostate cancer cell line PC-3, which expresses high levels of cMet, was obtained from Carlos Perez-Stable, at the University of Miami School of Medicine. The primary human pancreatic islet cells were obtained from Antonello Pileggi, at the University of Miami School of Medicine.

MIAMI cells and differentiation culture conditions

Selection/expansion of MIAMI Cells

Whole bone marrow cells (without gradient centrifugation, immunoselection, or immunodepletion) from cadaveric thoracolumbar (T1-L5) vertebral bodies were plated at a constant density of 10^5 cells/cm^2 in DMEM-low glucose medium, containing 5% fetal bovine serum (FBS) and 100 U/ml penicillin and 1000 U/ml streptomycin (pen-strep) (Gibco-BRL) in fibronectin-coated 10-cm dishes. Whole bone marrow cells, containing adherent and nonadherent cells, were maintained in a humidified incubator at 37°C undisturbed in an atmosphere of 3% O_2, 5% CO_2, 92% N_2; 7 days later half of the culture medium was replaced. Fourteen days after the initial plating, the nonadherent cells were removed. Under these conditions the highest number of colony-forming units (≥50 cells) per number of whole bone marrow cells plated could be obtained at 14 days after the initial plating. Single-cell-derived colonies were isolated by plating cells at a density of 0.1-0.2 cell/cm^2, marking the location of each individual adherent cell in the dish, microscopically monitoring the growth of each single-cell-derived colony each day, and isolating (using cloning rings) independent selected colonies (50-100 cells in size) in which the number of colonies was the same as the initial number of cells seeded per unit area. Single-cell-derived and pooled colonies of adherent cells were carefully rinsed in medium and removed by treatment with trypsin/EDTA. These cells were selected then plated ($1.3\text{-}1.4\times10^3$ cells/cm^2) and expanded at low density (≥30% confluency) as single-cell-derived or pooled colonies in fibronectin (10 ng/ml)-coated dishes using an expansion medium composed of: 98% DMEM-low glucose, 2% FBS and pen-strep at 3% O_2, 5%CO_2, 92% N_2. The selected and expanded population of cells, hereafter named MIAMI cells, was used for all studies.

Osteogenic differentiation

For osteogenic differentiation cells from single-cell-derived or pooled colonies were plated at 10,000 cells/cm^2 in 6-well plates (Costar, Cambridge, MA, USA) in the presence of α-MEM/10% FBS with pen-strep, 100 μM ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate (D'Ippolito et al., 1999; D'Ippolito et al., 2002). The next day, dexamethasone (Dex, Sigma, St Louis, MO, USA) dissolved in DMSO was added to a final concentration of 10 nM. DMSO at the final concentration of 0.001% was used as vehicle. The medium was changed twice a week.

Chondrogenic differentiation

For chondrogenic differentiation (Mackay et al., 1998), cells were trypsinized, washed in serum-containing medium, and resuspended in serum-free chondrogenic medium composed of DMEM high-glucose

(DMEM-HG), 100 nM Dex, 10 ng/ml TGF-β3, 50 μg/ml ascorbic acid 2-phosphate, 100 μg/ml sodium pyruvate, 40 μg/ml proline and ITS-plus (final concentrations: 6.25 μg/ml bovine insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenous acid, 5.33 μg/ml linoleic acid, and 1.25 mg/ml bovine serum albumin; Collaborative Biomedical Products, Bedford, MA, USA). Aliquots of 250,000 cells were suspended in 0.5 ml of chondrogenic medium and distributed between 15-ml conical polypropylene centrifuge tubes (Costar). The cells were centrifuged for 5 minutes at 600 ***g*** and left at the bottom of the tube. Tubes were incubated, with caps loosened, in a 100% humidified atmosphere of 95% air, 5% CO_2, at 37°C for up to 4 weeks. The medium was changed twice a week.

Adipogenic differentiation

For adipogenic differentiation (modified from DiGirolamo et al., 1999), cells were plated at 10,000 cells/cm^2 in 6-well plates in the presence of α-MEM, 10% FBS, 10% horse serum with 100 U/ml penicillin, 1 mg/ml streptomycin, 0.5 μM hydrocortisone, 0.5 mM isobutylmethylxanthine (Sigma), and 60 μM indomethacin (Sigma). Cells were incubated for 3 weeks in a 100% humidified atmosphere of 95% air, 5% CO_2, at 37°C. The medium was changed twice a week.

Neural differentiation

For neural induction cells were plated at low density on 6-well plates containing fibronectin (10 ng/ml-coated coverslips) in DMEM-HG, 20% FBS with 100 U/ml penicillin and 1 mg/ml streptomycin for 24 hours. Neural specification (step 1) was induced by exposing cells to DMEM-HG, 20% FBS, 10 ng/ml bFGF for 24 hours. At the end of the neural specification treatment cells were washed three times with PBS, and then neural commitment (step 2) was induced by exposing the cells to DMEM-HG, 1 mM β-mercaptoethanol (βME), 10 ng/ml NT-3 for 2 days. Finally, neural differentiation (step 3) was induced by first washing the cells three times with PBS and then exposing them to NT-3 (10 ng/ml), NGF (10 ng/ml) and BDNF (50 ng/ml) in DMEM-HG for 3 to 7 days.

Endodermal differentiation

Based on our data indicating that MIAMI cells express markers associated with endodermal precursors, we investigated the possibility of promoting the expression of genes associated with the phenotype of pancreatic islet cells. Cells were treated according to a sequential protocol of specification, commitment and differentiation with factors known to promote the expression of a β-like cell phenotype in other systems (Movassat et al., 2002; Hunziker and Stein, 2000; Zulewski et al., 2001; Lumelsky et al., 2001). Briefly, cells were plated at a density of 10,000 cells/cm^2 in the presence of DMEM-HG, 20% FBS, for 24 hours. On the next day, medium containing DMEM-HG, 20% FBS and 10 ng/ml b-FGF was added for 24 hours. Endodermal specification was induced by exposure to medium containing DMEM-HG, 1% DMSO, and 100 μM butylated hydroxyanisole (BHA), 10 nmol/l exendin-4, for 24 hours. Cells were washed three times, and endodermal commitment was induced by exposure to medium containing RPMI (Gibco), 11.1 mmol/l glucose, 10% FBS, 10 mmol/l Hepes, 1.0 mmol/l sodium pyruvate, 20 ng/ml b-FGF and 20 ng/ml EGF, 10 nmol/l exendin-4, for 4 days. Islet-like differentiation was induced by exposing the cells to medium composed of RPMI, 2.5 mmol/l glucose, 10 mmol/l Hepes, 10 mmol/l nicotinamide, 100 pmol/l HGF, 10 nmol/l exendin-4, and 2.0 nmol/l activin-A for 5-7 days.

RNA isolation and analysis

For RNA isolation, cells were grown in 60 mm dishes (Costar). At the indicated time points, the medium was removed, total RNA was extracted and reverse transcription and amplification were performed as previously described (Schiller et al., 2001a; Schiller et al., 2001b). Total RNA was extracted using Trizol reagent (Gibco-BRL) according to the manufacturer's instructions. RNA was quantified spectrophotometrically.

For reverse transcription-polymerase chain reaction (RT-PCR) analysis, total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA), and RNA was precipitated in a 2 mol/l LiCl (Sigma) solution. Five micrograms of high-molecular mass RNA was reverse transcribed using MuLV reverse transcriptase, 200 pmol random hexamer primer, and 50 pmol of oligo(dT). Aliquots (4%) of the total cDNA were amplified in each PCR in 20 μl of reaction mixture containing 10 pmol of 5′ and 3′ primers in a standard PCR buffer supplemented with 0.5 μCi [^{32}P]dCTP (10 μCi/μl; NEN, Boston, MA, USA). All PCR reagents were from Perkin-Elmer (Norwalk, CT, USA). Amplifications were performed in a GeneAmp 9600 thermal cycler (Perkin-Elmer) for 20-27 cycles (typically: 94°C/30 seconds; 55°C/45 seconds; 72°C/60 seconds) after an initial denaturation at 94°C of 2 minutes. PCR products in a 10 μl aliquot were size-separated by electrophoresis in precast 6% acrylamide/TBE gels (Bio-Rad, Hercules, CA, USA). The products of the amplification reactions were visualized by electronic autoradiography using an InstantImager analyzer (Packard Instrument Co., Meriden, CT, USA).

The following human-specific PCR primer sequences were used: *elongation factor 1-alpha* (accession no. L41490) hEF1α-F 5′-AGGTGATTATCCTGAACCATCC-3′ and hEF1α-R 5′-AAAGGTGGATAGTCTGAGAAGC-3′ (235-bp product); *telomerase reverse transcriptase* (accession no. NM_003219), hTERT-F 5′-AGCCAGTCTCACCTTCAACCGC-3′ and hTERT-R 5′-GGAGTAGCAGAGGGAGGCCG-3′ (272 bp product); *POU domain, class 5, transcription factor 1* (POU5F1/Oct-4) (accession no. NM_002701): hOct4-F 5′-CGACCATCTGCCGCTTTGAG-3′, hOct4-R 5′-CCCCCTGTCCCCCATTCCTA-3′ (577 bp product); *Rex-1* (accession no. AF450454): hRex1-F 5′-CAGATCCTAAACAGCTCGCAGAAT-3′, hRex1-R 5′-GCGTACGCAAATTAAAGTCCAGA-3′ (306 bp product); *osteocalcin* (accession no. NM_000711) hOC-F 5′-CATGAGAGCCCTCACA-3′ and hOC-R 5′-AGAGCGACACCCTAGAC-3′ (315 bp product); *bone sialoprotein* (accession no. J05213) hBSP-F 5′-TCAGCATTTTGGGAATGGCC-3′ and hBSP-R 5′-GAGGTTGTTGTCTTCGAGGT-3′ (667-bp product); *osteopontin* (accession no. X13694) hOP-F 5′-CCAAGTAAGTCCAAVGAAAG-3′ and hOP-R 5′-GGTGATGTCCTCGTCTGTA-3′ (348 bp product); *runt domain transcription factor Runx2* (accession no. L40992) hRunx2-F 5′-GTTTGTTCTCTGACCGCCTC-3′ and 5′-CCAGTTCTGAAGCACCTGA-3′ (318 bp product); *collagen type II-alpha 1* (accession no. NM_001844) hCOL2A1-F 5′-AACCAGATTGAGAGCATCCGC-3′ and hCOL2A1-R 5′-CCTTCAGGGCAGTGTACGTGA-3′ (517 bp product); *peroxisome proliferator-activated receptor gamma-2* (accession no. U79012), hPPAR-γ2-F 5′-ATTCTCCTATTGACCCAGAAAGCG-3′, hPPAR-γ2-R 5′-AGCTTTATCTCCACAGACACGACATT-3′ (419 bp product); *lipoprotein lipase* (accession no. X14390) hLPL-F 5′-GAGATTTCTCTGTATGGCACC-3′ and hLPL-R 5′-CTGCAAATGAGACACTTTCTC-3′ (276 bp product); *islet-1 transcription factor* (ISL-1) (accession no. BC017027) hISL-1-F 5′-CAACAAACAAAACGCAAAAC-3′ and hISL-1-R 5′-AAGTCAAACACAATCCCGA-3′ (542 bp product); *NK6 transcription factor related, locus 1* (accession no. NM_006168) hNkx6.1-F 5′-CTGGAGAAGACTTTCGAACAA-3′ and hNkx6.1-R 5′-AGAGGCTTATTGTAGTCGTCG-3′ (239 bp product); *beta-cell transcription factor Beta2/NeuroD* (accession no. NM_002500) hBeta2-F 5′-TCTTTCAAACACGAACCGT-3′ and hBeta2-R 5′-GCCTTTTGTAAACACGACAGT-3′ (295 bp product); *glucagon* (accession no. NM002054) hGLUC-F 5′-ATCTGGACTCCAGGCGTGCC-3′ and hGLUC-R 5′-AGCAATGAATTCCTTGGCAG-3′ (170 bp product); and *insulin* (accession no. NM_000207) hINS-F 5′-AGGCTTCTTCTACACA-3′ and hINS-R 5′-CAGGCTGCCTGCACCA-3′ (94 bp product).

Western blot analysis

Immunoblots were performed as previously described (D'Ippolito et al., 2002). Briefly, total cell extracts were prepared using NP-40 lysis buffer [50 mmol/l Tris, pH 8.0, 1.0% NP-40, 150 mmol/l NaCl, 2 mmol/l ethylene-glycol tetraacetic acid (EGTA), 2 mmol/l ethylene-diamine tetraacetic acid (EDTA), protease inhibitor tablet (Roche Molecular Biochemicals, Indianapolis, IN, USA), 50 mmol/l sodium fluoride, and 0.1 mmol/l sodium vanadate], and protein concentrations were determined using a protein assay (Bio-Rad Laboratories, Hercules, CA, USA). After separation of 10 μg protein by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred by electrophoresis to an Immobilon-P membrane (Millipore Corp., Bedford, MA, USA) and incubated in 5% nonfat dry milk, phosphate-buffered saline (PBS) and 0.25% Tween 20 for 1 hour. Antibodies were diluted in 5% nonfat dry milk, PBS and 0.25% Tween 20 and incubated overnight at 4°C. Membranes were washed in PBS and 0.25% Tween 20 (three times, 10 minutes each time) and incubated with horseradish peroxidase-conjugated secondary antibody (antirabbit; 1:2000 dilution; Santa Cruz Biotechnology) for 1 hour, washed in PBS and 0.25% Tween 20 and analyzed by exposure to X-ray film (X-Omat, Eastman Kodak Co., Rochester, NY, USA) using enhanced chemiluminescence plus (ECL Plus, Amersham Pharmacia Biotech, Arlington Heights, IL, USA). Membranes were subsequently washed for 1 hour in PBS and 0.25% Tween 20, and actin protein was measured using goat-polyclonal antibodies (1/1000 dilution; C-11, Santa Cruz Biotechnology) and anti-goat horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology).

Immunofluorescence

For staining of cytoskeletal proteins, cells were fixed with methanol at –20°C for 2 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. For other intracellular molecules, cells were fixed with 4% paraformaldehyde at 4°C for 10 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. For cell surface receptors, cells were fixed with 4% paraformaldehyde at 4°C for 10 minutes. Blocking and diluent solution consisted of phosphate-buffered saline (PBS), 1% BSA and 1% serum (Sigma) from the same species as the species in which the secondary antibody was raised. Fixed cells were blocked for 30 minutes, incubated sequentially for 30 minutes each with secondary specific antibodies, followed by incubation with fluorescein- or rhodamine-conjugated antimouse or anti-rabbit IgG antibody. PBS plus 0.3% BSA was use for the washes between each step. Specific immunostaining was demonstrated in control experiments in which cells were exposed to primary isotypic antibodies and then incubated with conjugated antibodies.

Flow cytometry analysis

Undifferentiated MIAMI cells grown from single-cell-derived colonies were plated in triplicate on 60 mm dishes in the presence of expansion medium. Five days later the cells were trypsinized, and 1×10^6 cells were placed into FACS tubes (BD Bioscience). Cells were rinsed twice with a cold buffer solution (DPBS, 0.5% BSA, 0.02% sodium azide, at pH 7.4) and then stained with the specific conjugated primary antibody. Cells were rinsed again twice with the cold buffer solution and fixed with 1% paraformaldehyde until analysis with FACScan (BD Bioscience).

Calcium accumulation assay

In vitro mineralization was evaluated on 6-well plates at the end of day 21, as previously described (Schiller et al., 2001b; D'Ippolito et al., 2002). Briefly, cells were rinsed three times with PBS at room temperature, fixed with ice-cold 70% ethanol for 1 hour at 4°C, rinsed three times with distilled water, and then stained with 40 mmol/l Alizarin Red sulfate (AR-S, Sigma), pH 4.2, for 10 minutes at room temperature using an orbital shaker (100 rpm). Nonspecifically bound stain was subsequently removed using five brief rinses with distilled water and one rinse for 15 minutes with PBS at room temperature. Extracellular matrix (ECM) mineral-bound stained nodules were photographed using an inverted light microscope. ECM mineral-bound AR-S was quantified by spectrophotometric evaluation at 562 nm (using an AR-S standard curve) after solubilizing the stain by a 15-minute incubation (orbital shaker) in 1 ml of 10% cetylpyridinium chloride in 10 mmol/l sodium phosphate, pH 7.0.

Cell staining

For Sudan-IV staining of cytoplasmic triglyceride lipid droplets, cells were fixed with 10% formalin at –20°C for 2 minutes and rinsed in water. Slides were stained in Sudan-IV (Sigma) for 10 minutes and rinsed in water (Schiller et al., 2001c).

Results

Isolation and expansion of MIAMI cells

Isolation of MIAMI-cell colonies was 100% more efficient when we used unfractionated cells that were not subjected to density gradient centrifugation or immunodepletion (Fig. 1A). Cell selection was based on the capacity of the cells to proliferate under specific conditions of low oxygen tension, ECM substratum, plating density and co-culture with nonadherent cells. Our unique isolation and culture conditions result in the selection/expansion of a population of cells with less cytoplasm (see Fig. 1B).

Single-cell-derived colonies (Fig. 1C-E) were molecularly defined by expression of the following combination of markers: CD29, CD49e, CD63, CD81, CD90, CD122, CD164, and CNTFR (Fig. 2A); they were also positive for c-Met (Fig. 2B), NTRK3 and BMP-receptor 1B (not shown). In addition, these cells were negative for CD36, ICAM-1 (CD54), N-CAM (CD56), CD 109 and HLA-DR (Fig. 2A). They were also negative for CD34, CD45, CD117 (cKit) and Class I-HLA (not shown). Oct-4, Rex-1 and *Telomerase* (hTERT) were expressed in all cultures examined (Fig. 2C,D). Expression of this marker combination, unique to our cells, is consistently found in all of our samples, regardless of donor age and gender. After culture expansion, undifferentiated MIAMI cells remained negative for CD34, CD45, HLA-DR, expressed low levels of Flt-l, and Flk-1/KDR and were positive for CD10, CD44 and CD103 in addition to the markers described above. The expanded MIAMI cells remained morphologically unchanged (Fig. 1G) and consistently expressed markers found in mesodermal, endodermal, and ectodermal lineages in samples taken from all ages (Table 1). After extensive expansion the cells were found to be positive for CD29, CD49e, CD63, CD81, CD122 and CD164.

Long-term expansion of MIAMI cells

We have established cultures of MIAMI cells capable of proliferating beyond 30 cell doublings and of differentiating to numerous cell types (see below) from all 12 donors, ages 3 to 72 years. MIAMI cells from at least three donors [#519 (3-year-old boy), #849 (7-year-old boy), and #869 (55-year-old man)] have been expanded more than 50 cell doublings, beyond the Hayflick limit for primary cells (Hayflick and

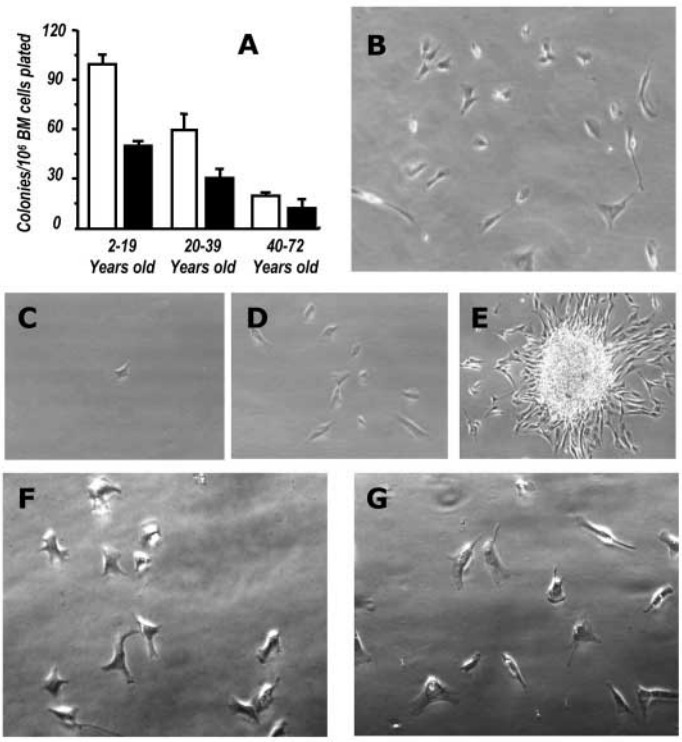

Fig. 1. MIAMI cells. (A) The number of colonies obtained after density gradient centrifugation (black bars) was significantly smaller than the number of colonies obtained without centrifugation (white bars) after plating the same number of bone marrow cells per dish using the conditions for expansion/selection of MIAMI cells. This result was observed in all cultures examined independent of age. The graph shows the mean±s.e.m. of more than 15 representative experiments. (B) MIAMI cells are enriched by a unique expansion/selection procedure to isolate a population of small (7-10 μm), highly proliferative cells with reduced cytoplasm. Essential in this procedure is a unique growth environment, with sequential culturing steps under defined oxygen tension, cell density, growth factors, serum concentration and substratum conditions. (C) MIAMI cells plated at low density (0.1-0.2 cells/cm^2) were allowed to proliferate (D) and when single-cell-derived colonies reached a size of 50-100 cells (E) the colonies where isolated using cloning rings, replated and expanded again at low density. The morphology of the cells is maintained after 5 cell doublings (F) and 52 cell doublings (G). (B,F,G) 20× magnification; (C-E) 10× magnification.

Moorhead, 1961). These results support the stem-like properties of the MIAMI cells. When MIAMI cells were cultured at low density (≤30% confluency) in expansion medium containing low serum (2%), cell doubling time increased to more than 60 hours. In cultures in which the low-serum medium was supplemented with 15% conditioned medium (from expanded MIAMI cell), cells could be expanded for more than 50 population doublings. As was seen for cells cultured for a low number of passages, cells expanded in excess of 50 population doublings remained small with reduced cytoplasm (Fig. 1G); no morphological changes could be detected. Expanded cells differentiated into osteoblastic and neural phenotypes (see below). A typical graph of the expansion potential profile of MIAMI cells is shown in Fig. 3. Several MIAMI cells isolated from donors of different ages [#869 (55-year-old man), #812 (72-year-old man) and #889 (59-year-old woman)] have responded identically to long-term expansion whether the cells were used immediately after isolation or after cryopreservation for periods of time ranging from 2-12 months. We observed that the MIAMI cells responded effectively to osteogenic and neural stimulation and

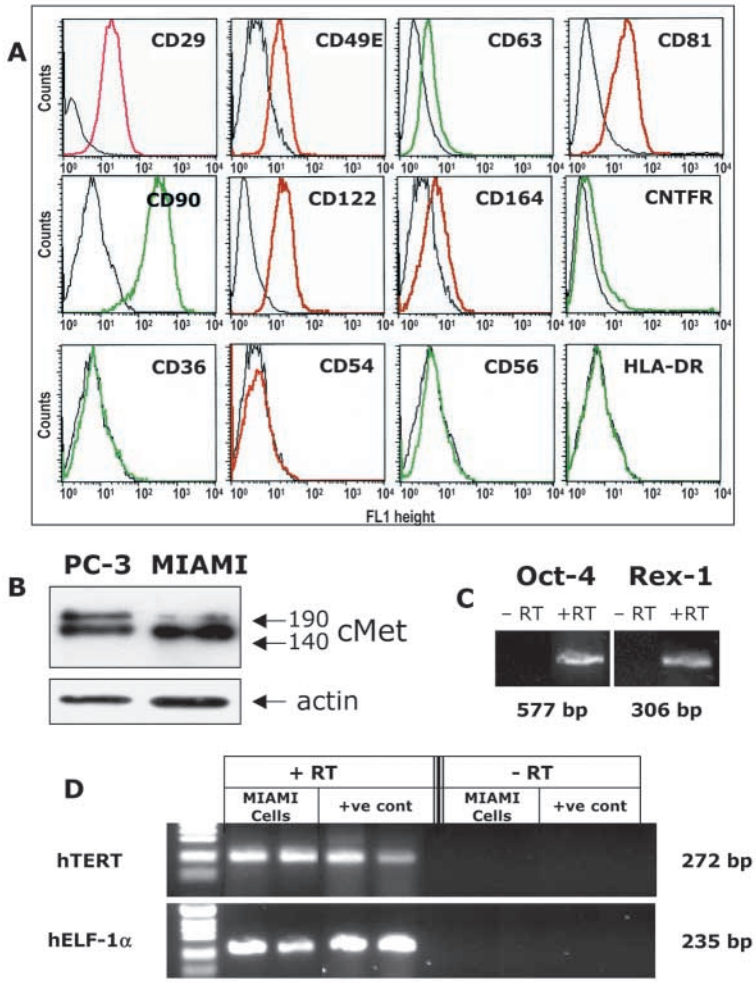

Fig. 2. Characterization of MIAMI cells. (A) MIAMI cells were harvested and labeled with antibodies against CD29, CD36, CD49E, CD54, CD56, CD63, CD81, CD90, CD122, CD164, CNTFR, and HLA-DR or control IgGs as indicated and analyzed by FACS. Plots show isotype control IgG-staining profiles (black lines) versus specific antibody staining profile (colored lines). (B) Ten micrograms of total protein was analyzed by western blot using an antibody that recognizes human cMet. Expression of the 190 kDa precursor and 140 kDa cleaved receptor was detected in MIAMI cells and in the PC-3 prostate cancer cell line (positive control). (C,D) RT-PCR analysis of expression of the stem cell markers *Oct-4* and *Rex-1* (C) in MIAMI cells and of *hTERT* mRNA in two separate MIAMI cell isolates (D, RNA isolated from the human foreskin fibroblast cell line hTERT-BJ1 was used as positive control; +ve cont). PCR products of specific sizes corresponding to each gene were obtained. RNA-specific amplification is demonstrated by the absence of a band when RT was excluded (– RT).

Table 1. Ectodermal, endodermal, and mesodermal gene products in the MIAMI cells

Ectoderm	Endoderm	Mesoderm
Neurotrophic tyrosine kinase, receptor, type 3	Hepatocyte growth factor (HGF)	Decorin
Ciliary neurotrophic factor receptor	cMet (HGF receptor)	Cadherin 11
BMP receptor, type IB	SL-1 (Islet-1) transcription factor	CD81/TAPA1
Neuron specific enolase	Beta-2 transcription factor	Runx2
POU4F1 transcription factor	Nkx6.1 transcription factor	Osteopontin
		CD63
		CD164

Cells were derived from males and females 3 to 72 years old.
Data presented in this table were obtained from RT-PCR, northern blot, immunocytochemistry, immunoblot and flow cytometry experiments.

differentiated to cells with phenotypes similar to those described above.

Multi-lineage potential of MIAMI cells in vitro

We have found that MIAMI cells isolated from a single young donor (#849, 7-year-old boy) can be expanded ex vivo and induced to differentiate to bone-forming osteoblasts, cartilage-forming chondrocytes, and fat-forming adipocytes as well as to neural cells (ectoderm-derived lineage) and pancreatic islet-like cells (endoderm-derived lineage).

Osteogenic differentiation

Cells with bone-forming capacity showed high alkaline phosphatase activity (not shown), produced and mineralized an extracellular matrix, and made bone nodules in vitro (see Fig. 4A). In addition, these cells expressed the osteoblast gene markers *Runx2*, *osteocalcin*, *collagen I α1* and *bone sialoprotein* (Fig. 4B).

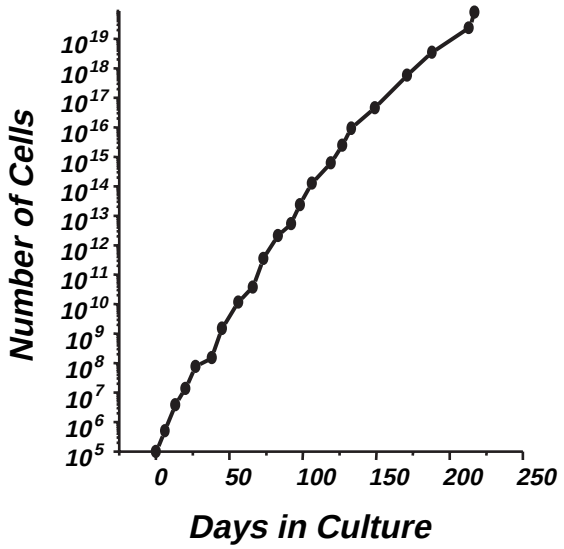

Fig. 3. Expansion capacity of long-term cultures of MIAMI cells. More than 50 cell doublings without detectable differentiation could be obtained in cultures grown in medium containing low serum (2%) supplemented with 15% conditioned medium (2% serum). Cells cultured for a few passages or cells expanded in excess of 50 population doublings remained small, with reduced cytoplasm, and differentiated into osteoblastic and neural phenotypes. Cells were counted at each passage with a hemacytometer.

Chondrogenic differentiation

For chondrogenic differentiation, cells were incubated in tubes containing the micromass cultures, with caps loosened, in a 100% humidified atmosphere of 95% air, 5% CO_2 at 37°C for various periods of time. Sedimented cells formed a spherical mass (1-2 mm^3) at the bottom of the tube within 24 hours. Medium was replaced three times a week. Virtually all of the cells showed cartilaginous tissue-forming capacity and produced a proteoglycan-rich soft collagen matrix. The cells expressed the chondrocyte gene markers collagen II (protein and mRNA) and *aggrecan* RNA (not shown).

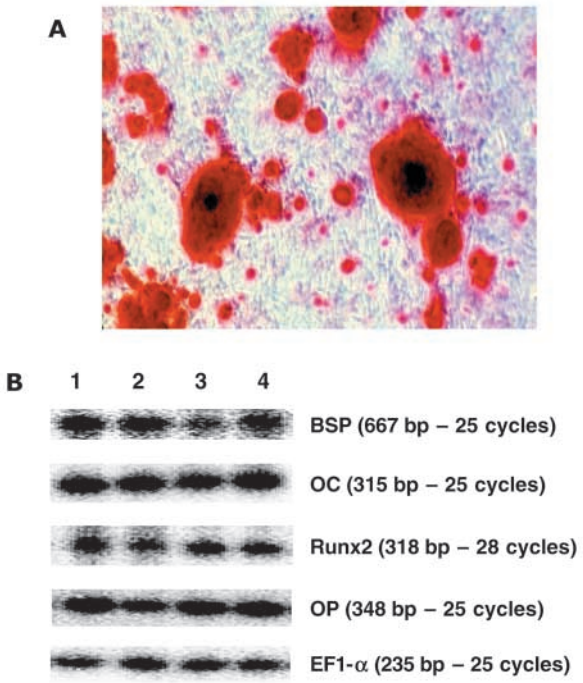

Fig. 4. Osteoblastic induction of MIAMI cells. (A) Alizarin Red-S staining of hydroxyapatite-associated calcium mineral deposited in the extracellular matrix by osteoblastic cells derived upon osteogenic induction (see Materials and Methods) of MIAMI cells. All plated cells induced to differentiate developed properties of functional osteoblasts. (B) RT-PCR analysis of transcripts present in osteoblast-induced MIAMI cells. MIAMI cells (lanes 1-4: different isolates) induced to differentiate to the osteoblastic lineage express the osteoblast phenotypic markers *bone sialoprotein* (BSP), *osteocalcin* (OC), *runt-homology domain transcription factor Runx2*, and *osteopontin* (OP). The size of the human-specific PCR products and the number of amplification cycles are in parentheses. BSP and OC transcripts were not detected in uninduced MIAMI cells (not shown).

Adipogenic differentiation

For adipogenic differentiation cells were plated in 6-well plates and incubated for 3 weeks in a 100% humidified atmosphere of 95% air, 5% CO_2 at 37°C. At the end of 3 weeks' incubation, cells were washed three times with PBS at room temperature, fixed with ice-cold 10% formalin buffer, and stained with fresh Sudan-IV solution. Plates were rinsed briefly with water. Nearly all the cells showed adipose tissue-forming capacity and accumulated large amounts of triglycerides in their cytoplasm (Fig. 5A). The cells expressed the adipocyte gene markers *lipoprotein lipase* and the pro-adipocytic transcription factor *peroxisome proliferators-activated receptor γ-2* (Fig. 5B).

Neural differentiation

For neural induction MIAMI cells (Fig. 6) were plated on 6-well plates. The next day a neuronal preinduction was performed for 24 hours. After 24 hours the cells were washed three times with PBS, and neuronal specification medium (DMEM-HG/bFGF) was added for 24 hours. Some of the cells acquired the bipolar spindle shape characteristic of neural cells within 2 hours (not shown). At this stage cells expressed nestin (Fig. 6, top left) and GFAP, but were negative for neuron-specific class III β-tubulin (TuJ1), neurofilament 160 (NF160) and neuronal nuclear protein (NeuN). After the neural specification treatment, cells were washed three times with PBS and neural commitment (step 2) was induced for 2 days. At this stage a morphologically homogeneous neural-like cell population was observed as a result of each neural commitment step (Fig. 6, middle). Expression of neuron-specific class III β-tubulin (TuJ1) and NGF receptor was detected in a fraction (40-50%) of these cells (Fig. 6, middle). We then induced neural differentiation (step 3) by exposing the cells to NT-3, NGF and BDNF for 3-7 days. At this stage, cells expressed NeuN and neurofilament-160 (Fig. 6, bottom), while expression of Nestin was not detected, consistent with a mature neural phenotype. Specific immunostaining was demonstrated in experiments in which no staining was detected when primary isotypic antibodies were used as negative controls (not shown) and fetal brain-derived human neuroepithelial progenitor cells (McCarthy et al., 2000) were used as positive controls (not shown). The morphology of the neural-induced MIAMI cells closely resembled that of mature neurons: they had a large number of neurites with significant branching (Fig. 6, bottom left). Moreover, the electrophysiological properties of the neural-like cells obtained after the final differentiation step were indistinguishable from properties of mature neurons (S.D., G.D'I., A. Valeyev, J. Hackman, M. McCarthy, P.M. and P.C.S., unpublished data). None of the mature neural markers was detected in the expanded MIAMI cells before their in vitro neural differentiation induction (not shown).

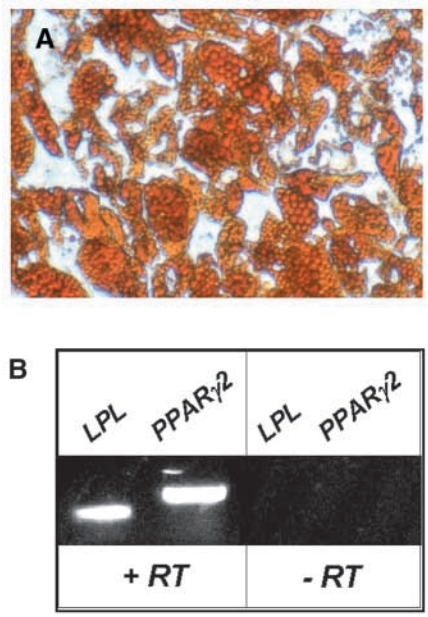

Fig. 5. Adipogenic induction of MIAMI cells. (A) Sudan-IV staining of triglyceride lipid droplets accumulated in the cytoplasm of adipocytic cells derived upon adipocytic induction (see Materials and Methods) of MIAMI cells. Functional differentiation was observed in all cells plated. (B) RT-PCR analysis of transcripts present in adipocyte-induced MIAMI cells. MIAMI cells induced to differentiate to the adipogenic lineage express the adipocytic phenotypic markers *lipoprotein lipase* (LPL) and pro-adipocytic transcription factor *peroxisome proliferator activated receptor γ-2* (PPAR-γ2), detected after 25 amplification cycles. These transcripts were not detected in uninduced MIAMI cells (not shown).

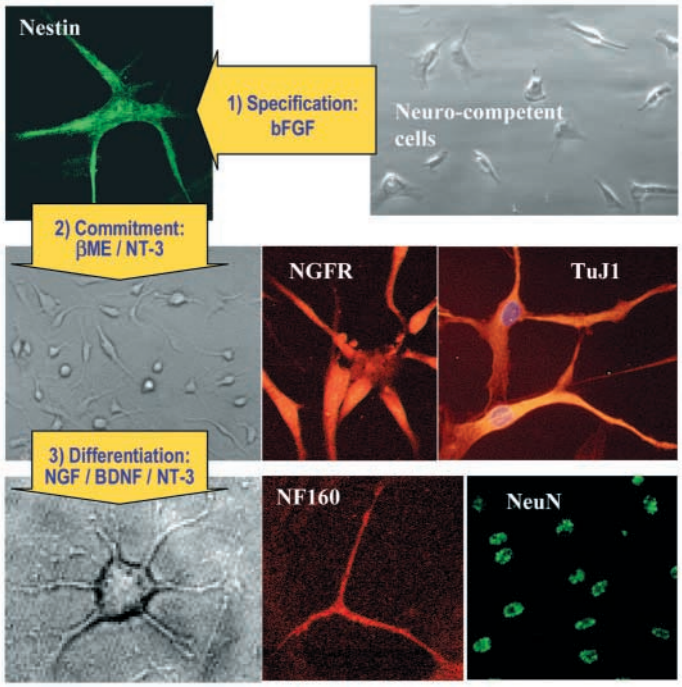

Fig. 6. Neural induction of MIAMI cells. Neural-competent cells require a sequential neuro-induction process of specification (step 1; bFGF), commitment (step 2; βME/NT-3), and differentiation (step 3; NT-3/NGF/BDNF) (see Materials and Methods). Each stage is characterized by the stage-specific expression of specific markers. Nestin (top left) is expressed first, followed by induction of β-III-tubulin (TuJ1) and NGF receptor (middle) in about 50% of the cells, and then expression of neurofilament-160 and NeuN (bottom) in nearly 40% of plated cells. Morphologically homogeneous cell populations are observed after each step.

Several MIAMI cells isolated from donors of various ages [#519 (3-year-old boy), #869 (55-year-old man), #812 (72-year-old man) and #889 (59-year-old woman)] responded in a similar fashion. Ten MIAMI cell colonies derived from single cells using cloning rings isolated from sample #519 (3-year-old boy) were expanded under the conditions described and were then tested for their ability to differentiate toward the ectodermal-derived neural lineage. These cells responded to differentiation in a manner similar to that described above. Characterization to other ectodermal-derived lineages is ongoing.

Differentiation of MIAMI cells to insulin-expressing structures similar to pancreatic islets

Our data showing that MIAMI cells express markers associated with an endodermal lineage and that precursors of pancreatic β cells and neural cells share common factors associated with their development and differentiation (Soria, 2001) led us to investigate the possibility of promoting the expression of genes associated with the phenotype of β cells in MIAMI cells. Cells were treated with factors known to promote the expression of a β-like cell phenotype in other systems (Movassat et al., 2002; Hunziker and Stein, 2000; Zulewski et al., 2001; Lumelsky et al., 2001). Culture conditions were developed (see above) in which cells proliferated, from attached cells, in an attachment-independent fashion, forming spherical clusters similar to embryonic stem cell-derived insulin-secreting structures (Fig. 7A,B). This process required the treatment of the cells with bFGF followed by exendin-4 [an agonist for glucagon-like peptide 1 receptor (GLP1R)]. Portions of the treated and untreated cells were used for total RNA isolation and the expression of transcripts encoding for insulin and glucagon (two hormones intimately associated with the phenotype of pancreatic islets) and for Beta2/NeuroD, Nkx6.1 and Isl1 (transcription factors involved in β-cell development). The RNAs were analyzed by RT-PCR using human-specific primers. Fig. 7C (bottom) shows the detection of specific bands corresponding to the β-cell-associated transcription factors *Beta2/NeuroD*, *Nkx6.1*, and *Isl1*, in both treated and control cells. The addition of forskolin to augment protein kinase A-mediated signaling did not increase the level of expression of these transcripts. However, transcripts for insulin and glucagon were observed only after sequential treatment with bFGF, followed by exendin-4 and then HGF/exendin-4/activin A (Fig. 7C, top). We used RNA from human pancreatic islet cells as a positive control.

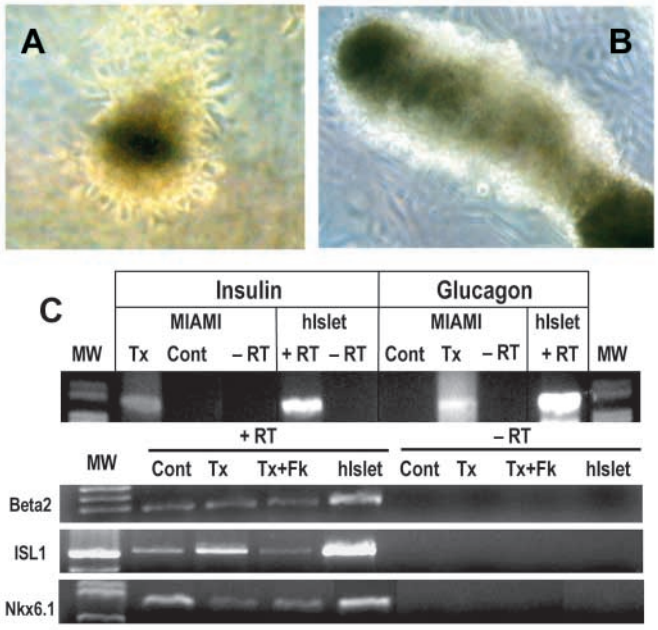

Fig. 7. MIAMI cells can be induced to develop features resembling those of pancreatic islets. MIAMI cells growing as adherent cultures can be induced to grow in an attachment-independent fashion, forming spherical or oblong clusters (A,B). Grown under endodermal-promoting conditions (see Materials and Methods), (C) MIAMI cells can be induced to express transcripts for insulin and glucagon, assayed by PCR of reverse transcribed (+RT) or control (–RT) RNA isolated from control (Cont) or treated (Tx) MIAMI cells or islet-positive (hIslet) controls. These islet-specific transcripts were not expressed under uninduced conditions (not shown). Control (Cont) and treated (Tx) MIAMI cells expressed *Beta2/NeuroD* (295 bp), *ISL1* (542 bp), and *Nkx6.1* (239 bp) transcripts (C). In all cases, bands were detected after 25 amplification cycles. MW, molecular size markers; Fk, forskolin.

Discussion

Based on molecular profile and differentiation potential, we have isolated a novel and distinctive population of multipotent human cells (not previously described) present in human bone marrow stromal cells. Our unique expansion/selection procedure involved specific culture conditions, including ECM substratum, oxygen tension, growth factors and vitamins, cell density and co-culture of cells, designed to resemble the in vivo niche microenvironment in which we expect to find the more primitive cells (Watt and Hogan, 2000). Via this process we isolated a population of bone marrow cells with broad differentiation potential, expressing markers found in embryonic stem cells as well as mesodermal-, endodermal-, and ectodermal-derived lineages. Although the expression of these markers in undifferentiated MIAMI cells suggests that they could be molecularly equipped to remain pluripotent or to progress to diverse lineages, this characteristic is not unique to MIAMI cells and is shared by other primitive adult progenitors (Jiang et al., 2002; Tremain et al., 2001; Colter et al., 2001). Using unfractionated whole bone marrow, we based our approach on our own preliminary findings that fractionation resulted in a significant decrease in the yield of primitive progenitor cells and on the premise that a stem cell niche microenvironment for the more primitive cells exists in vivo and will be better preserved in vitro under the conditions we have selected. The success of our approach may be attributed to preventing the loss of early progenitors caused by fractionation, or alternatively, the loss of the cells that support the survival and growth of the more primitive cells.

One of the most successful approaches to isolate primitive cells with broad differentiation potential from adult bone marrow has utilized cells obtained after fractionation of whole bone marrow (Reyes et al., 2001; Jiang et al., 2002). In those studies bone marrow mononuclear cells, obtained by Ficoll-Paque density gradient centrifugation, were depleted of CD45-positive and glycophorin-A-positive ($GlyA^+$) cells by means

of micromagnetic beads. The eluted cells were 99.5% $CD45^{-}GlyA^{-}$. These cells, termed multipotent adult progenitor cells (MAPCs), were cultured by plating 5 to 10×10^{3} $CD45^{-}GlyA^{-}$ cells in 1 ml expansion medium supplemented with EGF and PDGF-BB in wells coated with fibronectin (FN) (Reyes et al., 2001). MAPCs not only developed mesodermal-derived lineages (Reyes et al., 2001), they also developed ectodermal-derived lineages, such as immature neural-like cells and glial-like cells, in vivo (Zhao et al., 2002), as well as endodermal-derived lineages, such as hepatocyte-like cells, in vitro (Schwartz et al., 2002). Murine MAPCs have also been shown to engraft into various tissues in vivo (Jiang et al., 2002).

A critical aspect in our approach is that adherent cells are co-cultured with nonadherent cells for the initial 14 days in vitro. Nonadherent cells probably provide the cytokines needed for maintenance and proliferation of the more primitive stromal cells. The nonadherent cells are removed only after single-cell-derived colonies are established. We then continue the selection/expansion protocol with the adherent cells. A unique and essential aspect of our approach is that we perform this selection/expansion culture protocol under hypoxic conditions [3-5% (22.8-38 mmHg) oxygen], with the rationale of providing the conditions that resemble, as closely as possible, the in vivo niche of the most primitive stem cells (Chow et al., 2001; Cipolleschi et al., 1993; Lord, 1992). The standard approaches use air [21% (159 mmHg) oxygen] for expansion/selection of bone marrow progenitor cells (Friedenstein et al., 1970; Ferrari et al., 1998; Young et al., 1998; Conget and Minguell, 1999; Kopen et al., 1999; Pittenger et al., 1999; Asahara et al., 1999; Theise et al., 2000; Brazelton et al., 2000; Krause et al., 2001; Orlic et al., 2001; Mezey et al., 2003). Nevertheless even with our low oxygen conditions, the number of colonies that could be obtained decreased as a function of donor age, suggesting that in vivo the pool of these cells is depleted as we age. This is consistent with our previous observations (D'Ippolito et al., 1999). However, the differentiation capacity appears to be minimally affected by donor age.

The MIAMI cell population isolated in our approach is morphologically homogeneous and expresses a unique set of surface markers distinguishing MIAMI cells from those reported to be expressed in other primitive bone marrow stromal cell populations, such as RS-1 (Colter et al., 2000; Colter et al., 2001) and/or MAPCs (Reyes et al., 2001; Jiang et al., 2002). For example, CD10 (a neutral endopeptidase in B-cell progenitors and germinal center cells) was detected in MIAMI cells but not in MAPCs or RS-1 cells. MIAMI cells also express CD164, a sialomucin-like molecule found on early hematopoietic progenitors, as well as molecules involved in signaling of various types of progenitor and mature cells including CD122 (IL-2Rβ), CD81 (TAPA-1 involved in cell growth and signal transduction), bone morphogenetic receptor 1B and HGF receptor (cMet). Although the significance of the expression of these various markers related to stem cell properties awaits further clarification, MIAMI cells express markers found in embryonic stem cells (*Oct-4* and *Rex-1*) and appear to be equipped to respond to signals leading to differentiation toward diverse lineages. Like hMSCs, RS-1, or MAP cells, MIAMI cells can be differentiated to cells that express markers unique to bone-forming osteoblasts, cartilage-forming chondrocytes and fat-forming adipocytes and to cells with immature neural features. MIAMI cells, unlike RS-1 and/or MAP cells, have been induced to differentiate into attachment-independent spherical clusters, which express markers associated with endodermal derived-lineages and are found among cells in pancreatic islets. The data suggests that MIAMI, RS-1 and MAP cells are not identical but that they are potentially linked developmentally within the heterogeneous mesenchymal compartment. Since the expression of a particular immunophenotype depends more on the culture conditions than on the developmental status of a cell (Deschaseaux et al., 2003) it is hard to establish at this point how these different cells are positioned within a developmental hierarchy based on their molecular profile. However, based on their differentiation properties it would be reasonable to assume that MIAMI and MAP cells represent a more primitive developmental stage than RS-1 cells.

Mesodermal-derived osteoblastic cells and ectodermal-derived neural cells, with phenotypic and functional features of specialized mature cells, were obtained from MIAMI cells isolated from cloning-ring-derived colonies. Such colonies show extensive proliferative capacity and have been expanded in excess of 50 population doublings without detectable senescence or loss of differentiation potential. Differentiation to functionally mature phenotypes was achieved by the addition of cytokines, known to promote this process in vivo. The non-transformed nature of the MIAMI cells was demonstrated by the observation that no macroscopic or microscopic evidence of tumor formation could be found ten weeks after implanting 10^{6} MIAMI cells per animal in the tail vein of SCID/Beige immunodeficient mice (n=2, unpublished observation).

While cell fusion of somatic to ES cells has been suggested as an explanation for stem cell plasticity (Terada et al., 2002; Ying et al., 2002), our in vitro studies demonstrate that single cloning-ring-derived euploid MIAMI cells, never exposed to ES cells or other embryonic or mature somatic cells, differentiated into cells of at least two germ layers. Thus, the in vitro behavior of MIAMI cells cannot be attributed to cell fusion.

When MIAMI cells were cultured at high density (>10,000 cells/cm^{2}) and exposed to cytokines that induce their differentiation, the cells stopped proliferating and terminal differentiation was evident on both a molecular and functional basis. Long-term culture (>30 days) of differentiated cells led in most cases to apoptosis (not shown) with no evidence of transformation. Although it cannot be completely ruled out, the possibility that MIAMI cells are tissue-specific stem cells that undergo genetic reprogramming in culture is highly improbable, because freshly selected MIAMI cells cultured for a relatively short period of time homogeneously express markers found in embryonic stem cells as well as progenitors of all three germ layers. Although theoretically possible, genetic reprogramming in culture seems highly unlikely: the process would have to be very rapid, take place simultaneously in all the cells in culture, and be directed to a state of pluripotent differentiation potential in a very short period of time. Ongoing studies of the in vivo engraftment potential of MIAMI cells in immunodeficient rodents should further clarify this issue.

Although mesodermal-derived lineages could be obtained using primary marrow stromal stem cells isolated by standard

procedures for mesenchymal stem cell isolation (Pittenger et al., 1999), neural and endodermal differentiation could be obtained only with MIAMI cells. Treatment of marrow stromal stem cells or mesenchymal stem cells (isolated using standard published procedures) with the conditions used for the neural and endodermal differentiation of MIAMI cells did not cause endodermal or neural differentiation of hMSCs. Neural-like differentiation could be obtained using hMSCs, resulting in cells expressing some of the markers found in neurons. However, only when MIAMI cells were used were we able to derive neural cells with electrophysiological properties (resting membrane potential $\leqslant$–70 mV and ionic channel activity) indistinguishable from those of mature neurons (S.D., G.D'I., A. Valeyev, J. Hackman, M. McCarthy, P.M. and P.C.S., unpublished data). In a similar fashion, only MIAMI cells (but not hMSCs) were able to synthesize transcripts for insulin in response to exendin-4 following the protocol described.

MIAMI cells were isolated from vertebral bodies of cadaveric donors, confirming cadaveric vertebral bodies as an excellent source of material for the isolation of primitive bone marrow cells with extensive proliferative and differentiation potential. Preliminary studies indicate that MIAMI cells can be readily isolated from mobilized peripheral blood and iliac crest aspirates (data not shown). MIAMI cells hold strong promise in reparative medicine for the treatment of degenerative or inherited diseases and are free of the ethical concerns raised by the use of ES cells. Autologous ex vivo expanded MIAMI cells could be used for autologous implantation directed to repair damaged, aged or diseased tissues and organs. The ability to stably transduce MIAMI cells with specific genes using lentiviral vectors (S.D., B. Husta, G.D'I., G.A.H., P.M. and P.C.S., unpublished data) would also enable the genetic manipulation of autologous cells for the treatment of degenerative and congenital disorders. All of our results point to the capacity of MIAMI cells to be targeted to specific medical conditions and tailored to the needs of an individual person.

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References

Asahara, T., Takahashi, T., Masuda, H., Kalka, C., Chen, D., Iwaguro, H., Inai, Y., Silver, M. and Isner, J. M. (1999). VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J.* **18**, 3964-3972.

Asakura, A. and Rudnicki, M. A. (2002). Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. *Exp. Hematol.* **30**, 1339-1345.

Brazelton, T. R., Rossi, F. M., Keshet, G. I. and Blau, H. M. (2000). Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Science* **290**, 1775-1779.

Chow, D. C., Wenning, L. A., Miller, W. M. and Papoutsakis, E. T. (2001). Modeling pO(2) distributions in the bone marrow hematopoietic compartment. I. Krogh's model. *Biophys. J.* **81**, 675-684.

Cipolleschi, M. G., Dello-Sbarba, P. and Olivotto, M. (1993). The role of hypoxia in the maintenance of hematopoietic stem cells. *Blood* **82**, 2031-2037.

Colter, D. C., Class, R., DiGirolamo, C. M. and Prockop, D. J. (2000). Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc. Natl. Acad. Sci. USA* **97**, 3213-3218.

Colter, D. C., Sekiya, I. and Prockop, D. J. (2001). Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. *Proc. Natl. Acad. Sci. USA* **98**, 7841-7845.

Conget, P. A. and Minguell, J. J. (1999). Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J. Cell. Physiol.* **181**, 67-73.

Deschaseaux, F., Gindraux, F., Saadi, R., Obert, L., Chalmers, D. and Herve, P. (2003). Direct selection of human bone marrow mesenchymal stem cells using an anti-CD49a antibody reveals their $CD45^{med,low}$ phenotype. *Br. J. Haematol.* **122**, 506-517.

D'Ippolito, G., Schiller, P. C., Roos, B. A., Ricordi, C. and Howard, G. A. (1999). Age related changes in the osteogenic potential of human mesenchymal stem cells. *J. Bone Miner. Res.* **14**, 1115-1122.

D'Ippolito, G., Schiller, P. C., Perez-Stable, C., Balkan, W., Roos, B. A. and Howard, G. A. (2002). Cooperative anabolic actions of hepatocyte growth factor and 1,25-Dihydroxyvitamin D_3 in osteoblastic differentiation of human vertebral bone marrow stromal cells. *Bone* **31**, 269-275.

DiGirolamo, C. M., Stokes, D., Colter, D., Phinney, D. G., Class, R. and Prockop, D. J. (1999). Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br. J. Haematol.* **107**, 275-281.

Erices, A., Conget, P. and Minguell, J. J. (2000). Mesenchymal progenitor cells in human umbilical cord blood. *Br. J. Haematol.* **109**, 235-242.

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.

Friedenstein, A. J., Chailakhjan, R. K. and Lalykina, K. S. (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet.* **3**, 393-403.

Gronthos, S., Zannettino, A. C., Hay, S. J., Shi, S., Graves, S. E., Kortesidis, A. and Simmons, P. J. (2003). Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J. Cell Sci.* **116**, 1827-1835.

Hayflick, L. and Moorhead, P. S. (1961). The serial cultivation of human diploid cell strains. *Exp. Cell. Res.* **25**, 585-621.

Hung, S. C., Chen, N. J., Hsieh, S. L., Li, H., Ma, H. L. and Lo, W. H. (2002). Isolation and characterization of size-sieved stem cells from human bone marrow. *Stem Cells* **20**, 249-258.

Hunziker, E. and Stein, M. (2000). Nestin-expressing cells in the pancreatic islets of Langerhans. *Biochem. Biophys. Res. Commun.* **271**, 116-119.

Huss, R. (2000). Isolation of primary and immortalized $CD34^-$ hematopoietic and mesenchymal stem cells from various sources. *Stem Cells* **18**, 1-9.

Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M. et al. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**, 41-49.

Kopen, G. C., Prockop, D. J. and Phinney, D. G. (1999). Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc. Natl. Acad. Sci. USA* **96**, 10711-10716.

Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S. and Sharkis, S. J. (2001). Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105**, 369-377.

Kuci, S., Wessels, J. T., Buhring, H. J., Schilbach, K., Schumm, M., Seitz, G., Loffler, J., Bader, P., Schlegel, P. G., Niethammer, D. et al. (2003). Identification of a novel class of human adherent $CD34^-$ stem cells that give rise to SCID-repopulating cells. *Blood* **101**, 869-876.

Lord, B. I. (1992). The architecture of bone marrow cell populations. In *Concise Reviews in Clinical and Experimental Haematology* (ed. M. Murphy Jr), Dayton, OH, USA: AlphaMed Press.

Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R. and McKay, R. (2001). Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* **292**, 1389-1394.

Mackay, A. M., Beck, S. C., Murphy, J. M., Barry, F. P., Chichester, C. O. and Pittenger, M. F. (1998). Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue. Eng.* **4**, 415-428.

McCarthy, M., Auger, D. and Whittemore, S. R. (2000). Human cytomegalovirus causes productive infection and neuronal injury in differentiating fetal human central nervous system neuroepithelial precursor cells. *J. Hum. Virol.* **3**, 215-228.

Mezey, E., Key, S., Vogelsang, G., Szalayova, I., Lange, G. D. and Crain, B. (2003). Transplanted bone marrow generates new neurons in human brains. *Proc. Natl. Acad. Sci. USA* **100**, 1364-1369.

Movassat, J., Beattie, G. M., Lopez, A. D. and Hayek, A. (2002). Exendin 4 up-regulates expression of PDX 1 and hastens differentiation and maturation of human fetal pancreatic cells. *J. Clin. Endocrinol. Metab.* **87**, 4775-4781.

Orlic, D., Kajstura, J., Chimenti, S., Limana, F., Jakoniuk, I., Quaini, F., Nadal-Ginard, B., Bodine, D. M., Leri, A. and Anversa, P. (2001). Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc. Natl. Acad. Sci. USA* **98**, 10344-10349.

Pittenger, M. F., Mackai, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S. and Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147.

Reyes, M., Lund, T., Lenvik, T., Aguiar, D., Koodie, L. and Verfaillie, C. M., (2001). Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood* **98**, 2615-2625.

Schiller, P. C., D'Ippolito, G., Balkan, W., Roos, B. A. and Howard, G. A. (2001a). Gap-junctional communication mediates parathyroid hormone stimulation of mineralization in osteoblastic cultures. *Bone* 28, 38-44.

Schiller, P. C., D'Ippolito, G., Balkan, W., Roos, B. A. and Howard, G. A. (2001b). Gap-junctional communication is required for the maturation process of osteoblastic cells in culture. *Bone* **28**, 362-369.

Schiller, P. C., D'Ippolito, G., Brambilla, R., Roos, B. A. and Howard, G. A. (2001c). Inhibition of gap-junctional communication induces the trans-differentiation of osteoblasts to an adipocytic phenotype in vitro. *J. Biol. Chem.* **276**, 14133-14138.

Schwartz, R. E., Reyes, M., Koodie, L., Jiang, Y., Blackstad, M., Lund, T., Lenvik, T., Johnson, S., Hu, W. S. and Verfaillie, C. M. (2002). Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *J. Clin. Invest.* **109**, 1291-1302.

Soria, B. (2001). In-vitro differentiation of pancreatic beta-cells. *Differentiation* **68**, 205-219.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y., Meyer, E. M., Morel, L., Petersen, B. E. and Scott, E. W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* **416**, 542-545.

Theise, N. D., Badve, S., Saxena, R., Henegariu, O., Sell, S., Crawford, J. M. and Krause, D. S. (2000). Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. *Hepatology* **31**, 235-240.

Toma, J. G., Akhavan, M., Fernandes, K. J. L., Bernabé-Heider, F., Sadikos, A., Kaplan, D. R. and Miller, F. D. (2001). Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nature Cell Biol.* **3**, 778-784.

Tremain, N., Korkko, J., Ibberson, D., Kopen, G. C., DiGirolamo, C. and Phinney, D. G. (2001). MicroSAGE analysis of 2,353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells reveals mRNAs of multiple cell lineages. *Stem Cells* **19**, 408-418.

Watt, F. M. and Hogan, B. L. (2000). Out of Eden: stem cells and their niches. *Science* **287**, 1427-1430.

Ying, Q. L., Nichols, J., Evans, E. P. and Smith, A. G. (2002). Changing potency by spontaneous fusion. *Nature* **416**, 545-548.

Young, H. E., Steele, T. A., Bray, R. A., Detmer, K., Blake, L. W., Lucas, P. W. and Black, A. C. Jr (1999). Human pluripotent and progenitor cells display cell surface cluster differentiation markers CD10, CD13, CD56, and MHC class-I. *Proc. Soc. Exp. Biol. Med.* **221**, 63-71.

Young, H. E., Steele, T. A., Bray, R. A., Hudson, J., Floyd, J. A., Hawkins, K., Thomas, K., Austin, T., Edwards, C., Cuzzourt, J. et al. (2001). Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. *Anat. Rec.* **264**, 51-62.

Young, R. G., Butler, D. L., Weber, W., Caplan, A. I., Gordon, S. L. and Fink, D. J. (1998). Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. *J. Orthop. Res.* **16**, 406-413.

Zhao, L. R., Duan, W. M., Reyes, M., Keene, C. D., Verfaillie, C. M. and Low, W. C. (2002). Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Exp. Neurol.* **174**, 11-20.

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.

Zulewski, H., Abraham, E. J., Gerlach, M. J., Daniel, P. B., Moritz, W., Muller, B., Vallejo, M., Thomas, M. K. and Habener, J. F. (2001). Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes* **50**, 521-533.

Zvaifler, N. J., Marinova-Mutafchieva, L., Adams, G., Edwards, C. J., Moss, J. and Burger, J. A. and Maini, R. N. (2000). Mesenchymal precursor cells in the blood of normal individuals. *Arthritis Res.* **2**, 477-488.