

Long-term functional engraftment of mesenchymal progenitor cells in a mouse model of accelerated aging

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Methods

Animals

The University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) approved the use of mice described in this paper. Mutant mice had the *Wrn* and *Terc* alleles backcrossed onto the C57Bl/6 background (for > 11 generations). Fourth generation (G4) *Wrn*^{-/-} *Terc*^{-/-} mice were produced as previously described [1, 2]. Three-month old female enhanced green fluorescent protein-positive (GFP+) wild-type mice of the same C57Bl/6 strain for backcrossing the *Wrn* and *Terc* alleles were used as donors in BMT experiments. Three-month old male *Wrn*^{-/-} *Terc*^{-/-} mice used as recipients in BMT experiments were from G4 lineages. Animals were sacrificed at 42 weeks post-BMT or when they exhibited signs of significant suffering and impending demise, including severe lethargy and/or major (> 15%) weight loss. Based on our previous experience, animals exhibiting these features died within one week.

Bone marrow transplantation

Wrn^{-/-} *Terc*^{-/-} recipient mice received 1000 rads (10Gy), administered in two treatments from a ¹³⁷Cs source. For two weeks following irradiation animals were maintained on aqueous antibiotics by supplementing the water source with 2mg/ml neomycin sulfate. Whole BM aspirates from GFP+ animals were filtered through a 40µm strainer and then placed over a ficoll-paque cushion for isolation of buffy coat (mononuclear) cells. Approximately 5 x 10⁶ mononuclear cells were transplanted into recipients by tail vein injection in a total maximum volume of 0.2 ml, immediately after irradiation.

Isolation of mesenchymal progenitor cells (MPCs) and cell culture

Soft tissue was removed along the femoral shaft and metaphyses were removed. Bone marrow plugs were expelled by insertion of a 21 gauge needle into the marrow cavity and flushing the cavity with 10 ml of α -Minimal Essential Medium (α -MEM) with nucleosides (Invitrogen, Carlsbad, CA, USA). The bone marrow cells (marrow plugs) from femurs of the same animal were collected, pooled, and dispersed by repeat pipeting before centrifugation at 1000 x g for 10 minutes. The supernatant was removed and the pellet resuspended in α -MEM containing 10% fetal bovine serum (FBS, Invitrogen). Cells were plated at a density of 1×10^4 cells/cm² of tissue culture growth surface and maintained in α -MEM with nucleosides plus 10% FBS.

Osteoblast Differentiation

MPCs were seeded at 1×10^4 cell/cm² and refed three times weekly with α -MEM + 10% FBS + 50 μ g/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 10 mM β -glycerophosphate (Sigma-Aldrich, St. Louis MO, USA), and 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA). Medium containing differentiation factors was replaced every three days until mineralization was detected, usually within 2-4 weeks.

Adipocyte differentiation

Adipogenic differentiation was performed following a procedure previously described [3]. Briefly, MPCs were plated at a density of 1×10^4 cells/cm² in six well tissue culture dishes (Corning, NY, USA). The cultures were grown using basal medium (α -MEM;

Invitrogen, Carlsbad, CA, USA) supplemented with 10 % FBS, Invitrogen, Carlsbad, CA, USA). Once the cultures were confluent, cells were maintained in medium containing 0.5 mM IBMX (3-isobutyl-1-methylxanthine; Sigma-Aldrich, St. Louis, MO, USA) and 1 mM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) for 7 days. The medium was then replaced with basal medium containing 10 mg/mL insulin for 7 days, followed by maintenance in basal medium for 7 days with replenishment every 3 days.

Flow Cytometry

Approximately 0.5×10^6 GFP+ cells/sample were washed twice with FACS buffer (PBS + 2% FBS) and blocked with anti-mouse Fc receptor IgG (Miltenyl Biotec, Auburn, California, USA; 1:10 dilution.) for 10 minutes on ice. After two washes with PBS, cells were incubated with goat anti-mouse Sca1 Alexa® 647 (Invitrogen, Grand Island, New York, USA; 1:50 dilution) for 30 minutes and rat anti-mouse CD45 IgG (Invitrogen, Grand Island, New York, USA; 1:50 dilution) for 30 minutes. After 2 washes with FACS buffer, samples were incubated with goat anti-rat Alexa-fluor 555 (Invitrogen, Grand Island, New York, USA; 1:50 dilution). All manipulations were carried out at room temperature. The samples were analyzed using Becton Dickinson FACSCanto running DiVa software. A total of 10,000 events for each experimental sample were analyzed. Cell viability was assessed by negative staining using the Live/Dead aqua reagent (Life Technologies, Grand Island, NY). Offline analysis was performed by Flow-Jo analytical software (Treestar, Ashland, OR) and cells were gated to exclude dead cells, doublets, and higher order cell aggregates.

Immunofluorescence studies

Adherent cells grown on glass coverslips were washed twice with 1X phosphate-buffered saline (PBS) and fixed with 2 % paraformaldehyde containing Triton X-100 for 30 minutes. After rinsing with PBS, cells were placed in blocking solution containing 1% bovine serum albumin (BSA) in PBS for 30 minutes. Primary antibody against enhanced GFP (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:500 based on the manufacturer's recommendation and experience from previous use, and incubated with cells in a humidified container for 2 hours. Unbound primary antibody was washed out with three rinses of PBS before incubation of cells with fluorescently-tagged Alexa-fluor 647 (1:1000 dilution; Life Technologies, Grand Island, NY) secondary antibody. DAPI was added at a dilution of 1:500 for 1min. After rinsing cells three times with PBS, coverslips were mounted onto slides. All steps were performed at room temperature. Preimmune serum was used as a negative control at a dilution comparable to that of the primary antibody. All dilutions were made in blocking solution. Slides were stored at 4°C in the dark. Cells were visualized using a Nikon Eclipse 90i fluorescence microscope and Nikon Plan Fluor 10X0.30 and 20X0.45 objectives. Image capture was performed using NIS Elements Imaging Software 3.10 Sp2 and a Photometrics Coolsnap EZ camera.

For GFP staining in bone, sections were de-paraffinized, dehydrated and then re-hydrated, followed by permeabilization using Tween[®] 20 (0.2 % in distilled water) and blocking for 1 h with 10% normal goat serum (NGS). The samples were then incubated with rabbit anti-GFP antibody (1:1000 dilution; Abcam, Cambridge, MA) for 16 hours at

4°C. Following washes with PBS containing tween® 20, the samples were incubated with goat anti-rabbit Alexa® 647 antibody (1:1000 dilution; Life Technologies, Grand Island, NY) for 1 hour. Serial sections were similarly prepared before incubation with mouse anti-CD45 antibody (1:250 dilution; Life Technologies, Grand Island, NY) and Alexa 647 conjugated mouse anti-Sca-I antibody (1:500 dilution; Life Technologies, Grand Island, NY) for 16 hours at 4°C. For anti-CD45 antibody, the secondary antibody was goat anti-mouse Alexa 647, used according to the manufacturer's recommendations (Life Technologies, Grand Island, NY). Samples were stained with DAPI as above. Microscopy and image capture were performed as above.

Histology

Long bones were fixed in 4% paraformaldehyde for 3 days. Decalcification was performed using immunocal™ (Decal Chemical Corporation, Tallman, NY) for 3 days according to the manufacturer's instructions. The end point was monitored by analyzing calcium released in the solution every 24 hours using the ammonium oxalate method [4]. Decalcified sections were embedded with paraffin and 7 µm sections were cut and probed for the presence of GFP⁺ (donor) cells. Alternatively, isolated bone tissue was dehydrated in graded alcohols (70–100%), cleared in xylene and embedded in methyl methacrylate. Plastic tissue blocks were cut into 5µm sections using a Polycut-S motorized microtome (Reichert-Jung, Nossloch, Germany).

Alizarin red S staining was used to detect a mineralized matrix in vitro. Briefly, cells were fixed for 15 minutes with 3.7% formaldehyde at room temperature and then

washed with deionized water. After staining with alizarin red S at pH 4.3 for 10 minutes, cells were washed with deionized water and air-dried.

Oil red O staining was performed to detect adipocytes in vitro. Briefly, cells were fixed in 4% (w/v) paraformaldehyde solution for 20 min, washed twice with PBS, and treated with filtered 0.36% (w/v) Oil-Red O solution in 60% (v/v) isopropanol at 25°C for 15 min. After washing with 60% (v/v) isopropanol three times, the cells were washed three times with distilled water.

Briefly, for TRAP staining, sections were incubated with substrate solution (112mM sodium acetate, 77 mM L-(+) tartaric acid, 0.3% glacial acetic acid) at 37°C for 5 hrs. This solution was then replaced with substrate solution plus 11.6mM sodium nitrite and 2.6mM pararosaniline dye, and incubated at room temperature for an additional 2 hrs before rising and dehydration by standard procedures.

Goldner's Trichrome staining, and staining with hematoxylin and eosin, were performed by standard methods.

Cells were visualized on a Nikon Eclipse TS100 microscope using Nikon LWD 20x/0.40 Ph1 DL and 10x eye-piece (Nikon C-W10xB/22). Images were captured using a Nikon Digital Sight DS-Fi1 camera and Nikon NIS Elements software (F 3.0).

Bone Histomorphometry

Regions of interest (100 μm from the distal growth plate and 50 μm from the endosteal surface) on consecutive bone (femur) sections were visualized using a Nikon Eclipse 90i microscope. Image capture was performed using NIS Elements Imaging Software 3.10 Sp2 and a Nikon DS-Fi1 camera using x4 and x40 objectives. The Bioquant Osteo II digitizing system (R&M Biometrics, Nashville, TN) was used according to the manufacturer's instructions for image analysis. The number of osteoblasts/bone surface (N. Ob/BS) and number of osteoclasts/bone surface (N. Oc/BS) were quantified as recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research [5]. GFP⁺ cortical and trabecular osteocytes and GFP⁺ cortical and trabecular bone-lining osteoblasts were identified in femurs isolated from BMT recipients and expressed as the percentage among a total of 2000 cells scored.

MicroCT Analysis

High-resolution images of femurs were acquired by using a Scanco VivaCT 40 (Bruettisellen, Switzerland). The femurs were scanned with the source voltage 55 kV, a source current of 142 μA , and an isotropic voxel size of 10.5 μm . After scanning, 3D microstructural image data was reconstructed and structural indices were calculated using Scanco μCT V6.1 software.

The area for the trabecular analysis started 2940 μm proximal from the distal end of the femur and extended 1050 μm toward the proximal femoral head. The region of interest for trabecular microarchitectural variables was the bone within the endocortical margin.

An upper threshold of 1000 Hounsfield units and a lower threshold of 220 Hounsfield units was used to delineate each pixel as “bone” or “non-bone”. The tissue volume (TV), bone volume (BV), trabecular bone volume per total volume (BV/TV), mean trabecular thickness (Tb.Th), mean trabecular number (Tb.N), and mean trabecular separation (Tb.Sp) indices were computed.

The midshaft of the femur was determined as 50% of the entire length of the bone. Starting from the midshaft and extending 262.5 μ m distally and 262.5 μ m proximally, this area was defined as the cortical region of interest. An upper threshold of 1000 Hounsfield units and a lower threshold of 260 Hounsfield units was used to delineate each pixel as “bone” or “non-bone”. Total cross-sectional area (Tt. Ar), cortical bone area (Ct. Ar), cortical area fraction (Ct. Ar/Tt. Ar), and average cortical thickness (Ct. Th) indices were computed.

Statistics

The log-rank test was performed using R, version 2.15.0. (www.r-project.org). For comparison of bone parameters between the BMT and the non-BMT groups, two-sided, unpaired Student's *t*-tests were performed using Graphpad software (www.graphpad.com), with statistical significance set to $p < 0.05$. Error is expressed as standard error of the mean.

References

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