Supplementary Materials

Bone marrow skeletal stem/progenitor cell defects in patients with dyskeratosis congenita and telomere biology disorders

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Supplementary Methods

Colony forming efficiency (CFE) assays

To ascertain the approximate number of skeletal stem cells (SSCs) in bone marrow from normal donors and patients with bone marrow failure, bone fragments from surgical waste (normal donors) and when available, bone fragments from the Jamshidi needle used for aspiration (normal donors and patients) were processed as previously described. The bone fragments in growth medium (α-MEM, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 20% lot-selected fetal bovine, non-heat inactivated serum, 10^{-8} M dexamethasone and 10^{-4} M ascorbic acid-2-phosphate) were gently scraped with a surgical blade and washed extensively, but gently, with the same medium to remove marrow. The cell suspensions were then passed through cell strainers (70 µm, Falcon) to yield single cell suspensions. Nucleated cells were counted via a hemocytometer, and T25 flasks were in plated in triplicate with 1x10^4, 1x10^5 and 1x10^6 nucleated cells. The next day, the cultures were washed with nutrient medium to remove red blood cells and non-adherent nucleated cells, and re-fed with growth medium. After
incubation at 37 C in a humidified atmosphere of 5% CO₂ for 10-14 days without medium change, cultures were washed with Hank’s Balanced Salt Solution (HBSS), then fixed with 100% methanol and stained with an aqueous solution of saturated methyl violet. Using a dissecting microscope, colonies with greater than 50 cells were counted, and the CFE was determined per 1x10⁵ nucleated cells plated.

Secondary CFE was also studied by plating 500 cells of passaged BMSCs/100 cm³ dishes in growth medium in order to compare the survival of CFU-Fs (an approximation of SSC numbers) with time in culture. The number of cells plated in secondary CFE assays is reduced compared with the number cell plated for primary CFE assays due to the fact that hematopoietic cells are no longer present in the passaged population. However, it should be noted that the number of colonies formed increased due to the presence of transiently amplifying cells that are not present in the freshly isolated cell suspension. Yet, due to lack of specific markers, the secondary CFE is the only assay available. Secondary CFE was also evaluated after transfection with a negative control siRNA or with TERC-siRNA as described in the methods section.

Establishment of non-clonal bone marrow stromal cell (BMSC) cultures

Single cell suspensions of bone marrow from normal donors (bone fragments from surgical waste and aspirates) and aspirates from patients were prepared as previously described. For bone fragments, suspensions were prepared as described above. For aspirates ~2.0 cc were immediately placed into a tube and heparin was added to a final concentration of 100 U/ml). The tube was mixed well to avoid clotting, and the contents
were subsequently combined with nutrient medium (serum-free α-MEM, 20 mls). The suspensions obtained from bone fragments and aspirates were centrifuged, and the resulting cell pellets were resuspended in fresh growth medium and plated at 5x10^6-5x10^7 nucleated cells per 75 cm^2 flask. After one day of incubation, the medium was replaced, and changed three times per week until the cultures become ~70% confluent (between 12 and 14 days). Cells were passaged by washing extensively with HBSS followed by two treatments with 0.05% Trypsin/0.53 mM EDTA for 10-15 minutes at room temperature. Trypsin was inhibited by the addition of fetal bovine serum (final concentration of 3%) as each fraction was collected. After combination of fractions, cell aggregates were broken up by pipetting, collected by centrifugation, and resuspended in fresh growth medium.

For osteogenic differentiation, 10 mM β-glycerophosphate was added to the growth medium (which already contained 10^{-8} M dexamethasone and 10^{-4} M ascorbic acid-2-phosphate). Cells were used between P2 and P4.

**In vitro colorimetric assays**

**Oil red O staining** – In order to verify adipogenic differentiation, cultures were fixed with neutral buffered formaldehyde for 1 hour, followed by 30 minutes with 60% isopropanol, and then stained with Oil Red O. The Oil Red O stock solution was prepared by dissolving 0.5 g of Oil Red O in 100 mls of isopropanol. The working solution was prepared fresh by diluting 30 mls of stock with 20 mls of distilled water.

**Senescence associated (SA)-β-galactosidase staining** – SA-β-galactosidase staining
was detected using a senescence detection kit (Biovision Research Products, Mountain View, CA) per the manufacturer's protocol. Briefly, the cells were plated in chamber slides (3-4 chambers per slide). At confluency, cultures were fixed and incubated in the reconstituted staining solution at 37 C overnight. The chambers were then dissociated from the slide and the slides were observed under the microscope to capture a representative photo for each chamber. The senescent cells, with blue-green staining, were then counted and the average cell number of positive cells was reported.

**qRT-PCR**

Total RNA was extracted from cells using the RNeasy MiniKit (Qiagen, Valencia CA) following the manufacturer’s instructions. RNA (1.0 µg) was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad, #170-8891). Quantitative RT-PCR (qPCR) was performed using a CFX-96 Real Time System paired with a C1000 Thermal Cycler (Bio-Rad, Hercules CA). qPCR reactions were set up using iQ SYBR Green Supermix (BioRad #170-8882) according to the kit instructions. Primers were designed using Beacon Designer 6 software (Premier Biosoft International, Paolo Alto CA). qPCR results, expressed as critical threshold (C\text{T}) values, were normalized to the levels of GAPDH, generating ΔCT values; levels of relative expression were calculated as $2^{-\Delta CT}$.

**In vivo transplantation assay**
For *in vivo* transplantation, 40 mg of heat-sterilized hydroxyapatite/tricalcium particles (HA/TCP, 65%/35%), 0.5-1.0 mm in size (Zimmer, Warsaw, IN), were incubated with BMSCs (2x10^6 cells/1 ml of growth medium) at 37 C for 70-100 minutes with slow rotation (25 rpm) to allow cells to attach. The slurry was briefly centrifuged at 135 x g, and the supernatant was carefully removed. The cell/particle constructs were transplanted subcutaneously into immunocompromised mice (NIH-Lystbg Foxn1nu Btkxid, Charles River Laboratories). Following euthanasia at 8 wks, transplants were harvested and fixed for standard histological evaluation. All procedures were performed under NIDCR ACUC approved animal study protocols.

**Cytokine secretion analysis**

N-BMSCs, siNC-BMSCs, and siTERC-BMSCs were incubated for 72 hours, and for the last 16 hours they were incubated with serum-free medium. Conditioned medium (1 ml) was incubated with a RayBio® Human Cytokine Antibody Array 6 (60), according to the manufacturer’s instructions. After exposure of the membranes to Kodak X-Omat film, quantitation was performed by densitometry and use of the RayBio Analysis Tool (n=4 independent experiments for each group). Briefly, values were selected that best represent the background (Negative Control Spots and/or Blank Spots). Signal intensity of each sample was normalized with the intensity of positive control spots on the membrane. The intensity of Positive Control signals (coated with biotinylated IgGs) were used to normalize signal responses for comparison of results across multiple arrays, much like housekeeping genes and proteins are used to normalize results of PCR gels and
Western Blots, respectively. To normalize array data, one array is defined as "Reference Array" to which the other arrays are normalized to.

REFERENCES


Supplementary Table 1. Genes under-represented and over-represented by 2-fold in siTERT-BMSCs compared with siNC-BMSCs and N-BMSCs

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**BMSC DEFECT IN DYSKERATOSIS CONGENITA**
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Supplementary Figure Legends

Supplementary Figure 1. Colony forming efficiency (CFE) of bone marrow from a patient with untreated AAA and subsequent growth of AAA-BMSCs. (A) Bone marrow from a 40 year old female diagnosed with AAA (but untreated) was analyzed for CFE, which was found to be equivalent to that of normal controls (n=16, mean age=49.81 yrs, range 18-80 yrs). (B) AAA-BMSCs proliferated normally, and were morphologically similar to N-BMSCs, and did not take on a senescent morphology, form fibrotic areas or spontaneously differentiate into adipocytes as noted for TBD-BMSCs (see Figure 2).

Supplementary Figure 2. Differential gene expression patterns associated with the WNT signaling pathway exhibited by negative control siNC-BMSCs (NC), untreated N-BMSCs (N), and siTERC-BMSCs (T).

Supplementary Figure 3. Genes associated with lipoprotein biosynthetic pathways were changed significantly upon TERC knock-down (siNC-BMSCs - NC, untreated N-BMSCs - N, and siTERC-BMSCs - T).

Supplementary Figure 4. Expression pattern of genes associated with membrane lipid metabolic processes were significantly changed upon TERC knock-down (siNC-BMSCs - NC, untreated N-BMSCs - N, and siTERC-BMSCs - T).
**Supplementary Figure 5.** Cytokine array map showing the cytokines and location on the blot, and representative results generated using 1 ml serum-free medium conditioned for 16 hours by N-BMSCS, siNC-BMSCs and siTERT-BMSCs.
Supplementary Figure 1

A. Primary Colony Forming Efficiency

- Number of colonies per $10^5$ BMNCs
- Comparison between Normal and AAA groups

B. Images of BMSCs at P4

- N BMSCs
- AAA BMSCs
Supplementary Figure 2

WNT Pathway

N – N-BMSCs
NC – siNC-BMSCs
T – siTERC-BMSCs
GO Group: Lipoprotein biosynthetic pathway

N – N-BMSCs
NC – siNC-BMSCs
T – siTERC-BMSCs
Supplementary Figure 4

Genes Associated with Membrane Lipid Metabolic Processes

N – N-BMSCs
NC – siNC-BMSCs
T – siTERC-BMSCs
**RayBio™ Human Cytokine Antibody Array 6 (60) (TGFβ detects only the active form)**

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