**Otsuru et al. Supplemental Figure Legends**

**Supplemental Figure 1.** Characterization of MSCs and chondrocytes. (A) Expanded MSCs were characterized by flow cytometry with hematopoietic markers (CD45, TER119, CD3, B220, CD11b and Gr-1) and MSC markers (CD29, CD49e, CD90, CD105 and Sca-1) (MSC: solid line, isotype control: filled with grey). (B) MSCs were cultured in osteogenic medium, adipogenic medium and chondrogenic medium. Differentiation ability was confirmed by Alizarin Red staining (Left), Oil Red O staining (Middle) and Alcian Blue staining (Right), respectively. (C) The photomicrograph of cultured primary chondrocytes (Left). The production of sulfated proteoglycans was demonstrated by Alcian Blue staining after 10 days of culture (Right). (D) The expression of genes characteristic of chondrocytes was demonstrated by RT-PCR analysis in both freshly isolated chondrocytes (Lane 3) and in two preparations of chondrocytes after 10 days of culture (Lanes 4 and 5) simulating the conditions of the chondrocyte proliferation assay. Specific primers were used to detect the IIa and IIb splice forms of collagen type II (*Col2a1IIa, Col2a1IIb*), SRY-box 5 and 6 (*Sox5, Sox6*), collagen type IX (*Col9a1*), aggrecan (*Agc*) and β-actin (control for quality and quantity of RNA). Lane 1 is a no RNA control; Lane 2 is RNA from cultured MSCs (negative RNA control).

**Supplemental Figure 2.** Parameters of the chondrocyte proliferation assay. (A) Time course analysis assessing chondrocyte proliferation stimulated by serum taken from mice 3 to 21 days after MSC infusion. These data show that an interval of 7 days after MSC infusion is the optimal time point to detect the chondrocytes proliferation in our model system using our bioassay. (B) Analysis of the minimal concentration of test

murine serum (0.125% to 1% (v/v)) that can be added to the chondrocyte growth medium to generate a significant difference between the positive, second mediator-containing serum, and negative control serum. All data are expressed as the mean ± SEM.

**Supplemental Figure 3.** Fluorescence in situ hybridization analysis of culture-expanded osteoblasts. (A) Two X (red) chromosomes without any Y (green) chromosomes were evident in 0.8% of 1000 examined cells obtained from a male patient infused with female MNCs. Of the 500 male control cells counted, all demonstrated an XY pattern (not shown). (B) Flow cytometric analysis using side scatter properties and CD45 expression to distinguish white blood cells (WBC) from osteoblasts in tissue culture. The positive (upper left panel) and negative controls (upper right panel) demonstrate the WBC region and a limiting detection control (lower left panel) reveals a detection sensitivity of 0.05% WBC within an osteoblast culture. Flow cytometric analysis of the culture-expanded osteoblasts used for the fluorescence in situ hybridization study in (A) are displayed in the lower right panel.

**Supplemental Figure 4.** Patient and MNC Graft characteristics. Graphical representation of the responders (R) and nonresponders (NR) according to the patients' gender (male, M versus female, F), duration (months) since the original BMT[5,8], age (months) and blood chimerism (depicted as percent donor cells) at the time of MNC infusion, as well as the doses (cells/kg) of CD34 expressing cells and MSCs within the MNC graft.

**Supplemental Figure 5.** Donor derived osteopoiesis after bone marrow cell fusion with or without a marrow boost. (A, B) The percentage of GFP positive osteopoietic cell engraftment was evaluated at 8 weeks after bone marrow infusion without (A) or with (B) a marrow boost. All data are mean ± SEM.

## Supplemental Materials and Methods

### Flow cytometry of expanded MSC.

Expanded MSCs in culture were reacted with anti-CD45, anti-TER119, anti-CD3, anti-B220, anti-CD11b, anti-Gr-1, anti-CD49e, anti-Sca-1 (BD Biosciences, San Jose, CA), anti-CD29, anti-CD105 (eBioscience, San Diego, CA) or anti-CD90 (Abcam, Cambridge, MA). Flow cytometric analysis was performed with FACS Calibur (BD Biosciences).

### Differentiation of MSC

For osteogenic or adipogenic differentiation, expanded MSCs were plated into a 6 well culture dish at a density of 7,000 cells/$cm^2$ in α-MEM supplemented with 10 % FBS. When the cell expansion attained ~90% confluence, the culture media were changed to NH OsteoDiff Medium or NH AdipoDiff Medium (Miltenyi Biotec Inc, Auburn, CA) for 14 days. For chondrogenic differentiation, $1x10^6$ MSCs were pelleted in 15 ml tube by centrifuging at 300g for 5 min. Pelleted MSCs were cultured in NH ChondroDiff Medium (Miltenyi Biotec Inc) for 28 days. Calcium deposition from osteoblasts was stained with 1% alizarin red S (Sigma-Aldrich, St. Louis, MO) solution for 10 min. Adipocytes were

stained with 0.18% oil red O (Sigma-Aldrich) solution for 5 min. The chondrogenic pellet was embedded in paraffin after the fixation with formalin. The section was stained with 1% alcian blue (Sigma-Aldrich) solution (pH1.0) for 6 hours at room temperature.

**Expansion of Osteoblasts**

Osteoblasts were isolated and expanded by our standard laboratory protocol[5,8] as originally described by Robey and Termine (CalcifTissue Int. 1985;37:453-460). Briefly, bone sample from the patient (approximately 15mm x 5mm x 3mm) were dissected from soft tissue under a stereoscope, and progressively minced to a fine granular consistency. The resulting bone granules were digested with collagenase P (0.2 mg/mL; Roche Diagnostics, Mannheim, Germany) at 37°C for 3 hours, and plated into two 15 cm dishes. The cells were maintained in DMEM/F-12 (1:1, v/v) (Mediatech. Inc., Manassas, VA) at 37°C and passaged by splitting 1:3 when the cell cultures appeared approximately 80% confluent. The cells after passage 2 (24 days in culture) were used for fluorescence in situ hybridization and flow cytometric analysis.

**Fluorescence in situ hybridization (FISH)**

Fluorescence in situ hybridization for the X and Y chromosomes of culture expanded osteoblasts was performed with a commercially available kit (VYSIS, Downers Grove, IL) according to the manufacturer's instructions.

**Flow cytometric analysis of osteoblasts.**

Osteoblasts from bone biopsy were maintained in culture as previously described[5] and analyzed for hematopoietic contamination by flow cytometry.

**RNA isolation and reverse transcription-PCR.**

Total RNA was isolated using TRIzol® Reagent (Invitrogen, Carlsbad, CA) following the manufacture's protocol. Then, cDNA was generated from 2 µg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and then analyzed by PCR using specific primers described as follows: 5'-GTCCTCTGCGACGACATAATCTGTGAA-3' and 5'-AGGGGTCCCAGGTTCTCCATCTCTG-3' for the IIa splice form of mouse type 2 collagen (*Col2a1IIa*), 5'-TTCGGTGTCAGGGCCAGGATGT-3' and 5'-TCCCAGGTTCTCCATCTCTGCCAC-3' for the IIb splice form of mouse type 2 collagen (*Col2a1IIb*), 5'-ATGATTCCCGTGTTCCCTCCTGAC-3' and 5'-TGGTGGACTGTTTGTGCTCTTGTCTG-3' for mouse SRY-box 5 (*Sox5*), 5'-GCAGGACTGAGAAGGAAAGAACACGC-3' and 5'-CGCATTCATTGGTCGCTTGATGTG-3' for mouse SRY-box 6 (*Sox6*), 5'-CTTGTGGACAATCCTCAGGTTTCTGTTC-3' and 5'-TCGGTCACCATCAATGCCATCTATG-3' for mouse type 9 collagen (*Col9*a1), 5'-TCT GGA AATGAC AAC CCC AAG CAC A-3' and 5'-TGG CGG TAA CAGTGA CCC TGG AAC T-3' for mouse aggrecan (*Agc*), 5'-CATTGTGATGGACTCCGGAGACGG-3' and 5'-CATCTCCTGCTCGAAGTCTAGAGC-3' for mouse β-actin. The amplification was

performed with HotStarTaq (Qiagen, Valencia, CA) by incubating at 95°C for 3 min, 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 60 s followed by incubation at 72°C for 10 min. The electrophoresis was performed with 1.5% agarose gel in Tris/Borate/EDTA (TBE) buffer.

**Time course analysis of serum-inducing chondrocyte proliferation.**

Mice (n=4 per group) were infused with 1 x $10^6$ MSCs or PBS by tail vein injection and then blood was collected on days 3, 7, 10, 14 and 21 by retro-orbital bleed. Serum, prepared from the sampled blood, was used at a standard concentration of 1% (v/v) in the chondrocyte proliferation assay.

**Analysis of the serum concentraiotn in the chondrocyte proliferation assay.**

Mice (n=3 per group) were infused with 1 x $10^6$ MSCs or PBS by tail vein injection. Blood was collected on day 7 by retro-orbital bleed and test serum prepared. Freshly isolated murine chondrocytes (5,000 cells/well, 96 well plate) were maintained in culture for 6 days using medium supplemented with graded concentrations (0.125%, 0.25%, 0.5% and 1.0%) of the test serum. The proliferation was assessed as described in “Chondrocyte proliferation assay” in Materials and Methods.

| Patient[a] | Before (cm/month) | After |
|---|---|---|
| A | 0.00 | 0.36 |
| B | 0.00 | 0.54 |
| C | 0.12 | 0.36 |
| D | 0.14 | 0.42 |
| E | 0.23 | 0.60 |

Supplemental Table 1. Linear growth velocity before and after MSC therapy. Reanalyzed data from a previously published clinical trial[11]. Growth expressed as centimeters of growth per month for each patient over 6 months immediately prior to and the immediately after infusion of ex vivo expanded MSCs isolated from the original bone marrow donor.

[a]To ensure anonymity, the patients are identified by letters and do not correspond to patient numbers in Table I or previously published trials[5,8,11].

| Patient[a] | Boost (cm/month) | Mean[b] (cm/month) |
|---|---|---|
| 1 | 0.10 | 0.42 |
| 2 | 0.07 | 0.50 |
| 3 | 1.00 | 0.42 |
| 4 | 1.10 | 0.48 |
| 5 | 0.97 | 0.44 |

Supplemental Table 2. Linear growth velocity for each patient after NAMBC therapy compared with the population mean. [a]Patient number is for the current trial corresponding to Table 1. [b]The mean growth velocity listed for each patient corresponds to the mean of the unaffected general population according to gender as well as their age at the time of the cell therapy[24].

Figure S1.

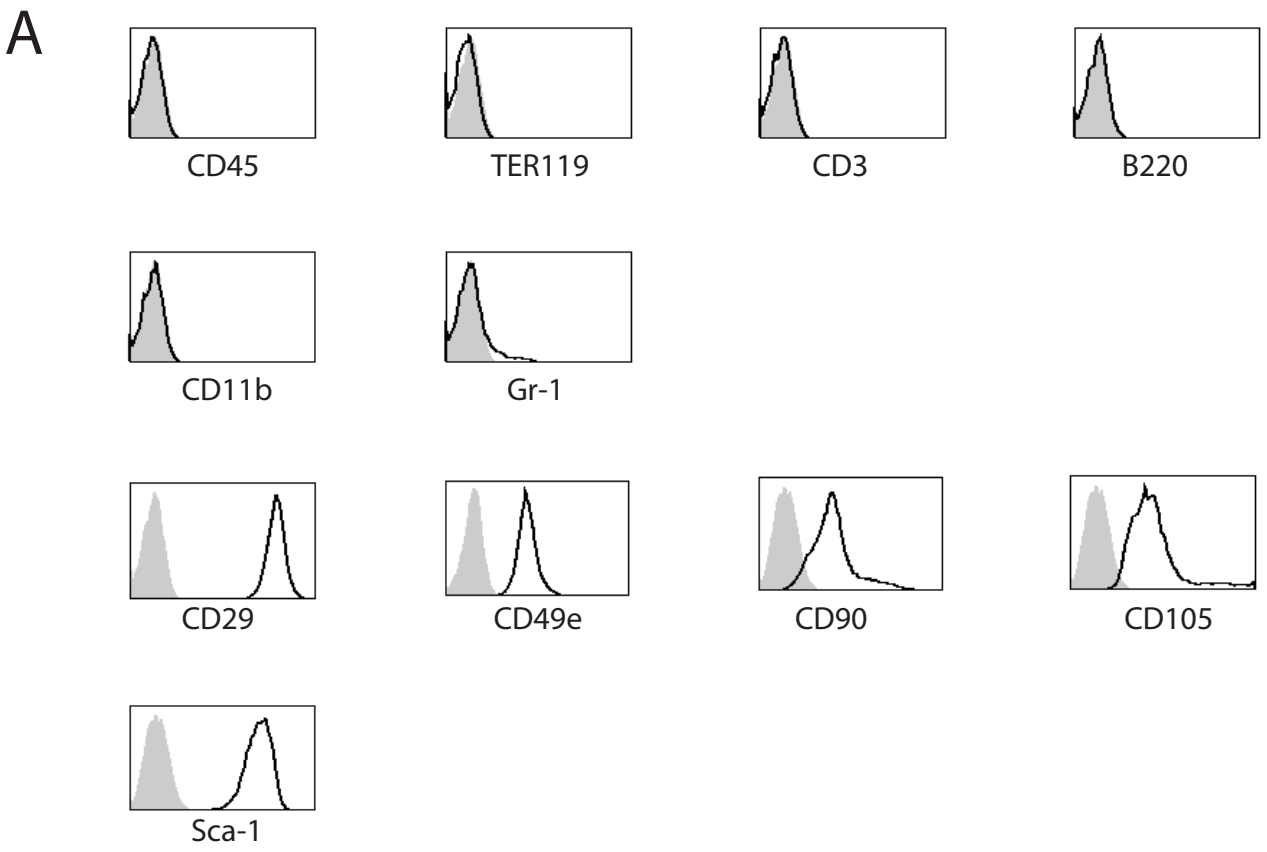
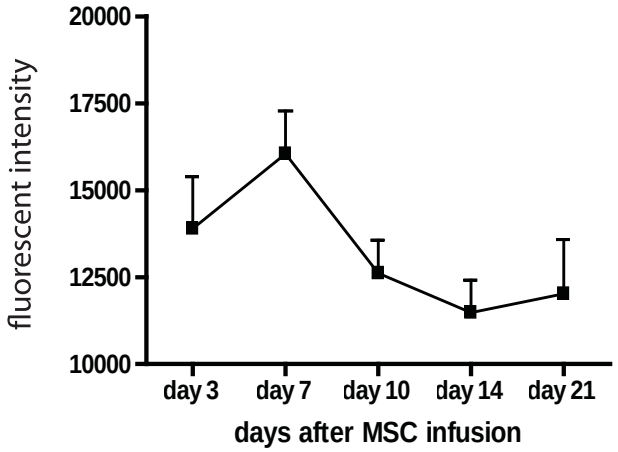

A
CD45
TER119
CD3
B220
CD11b
Gr-1
CD29
CD49e
CD90
CD105
Sca-1
B
C
D
1
2
3
4
5
Col2a1IIa
Col2aIIb
Sox5
Sox6
Col9
Agc
β-actin

Figure S2.

A


20000
17500
15000
12500
10000
fluorescent intensity
day 3
day 7
day 10
day 14
day 21
days after MSC infusion

B

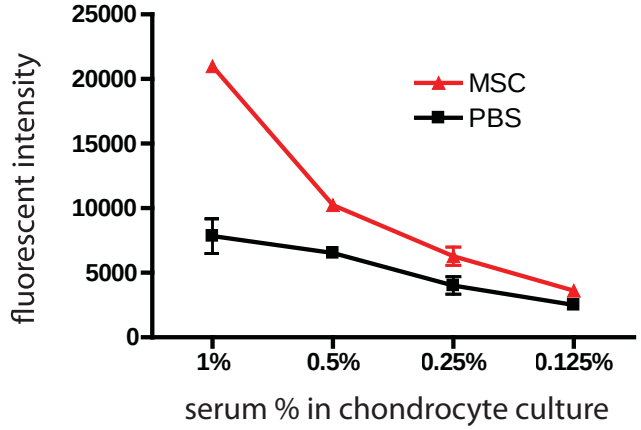
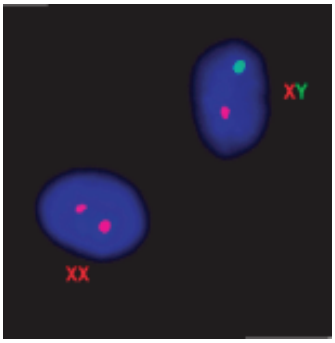

25000
20000
15000
10000
5000
0
fluorescent intensity
MSC
PBS
1%
0.5%
0.25%
0.125%
serum % in chondrocyte culture

Figure S3.

A


XY
XX

B

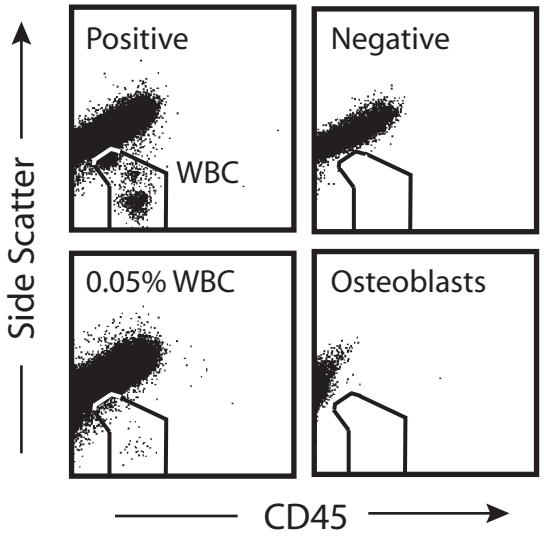

Positive
Negative
WBC
0.05% WBC
Osteoblasts
Side Scatter
CD45

Figure S4.

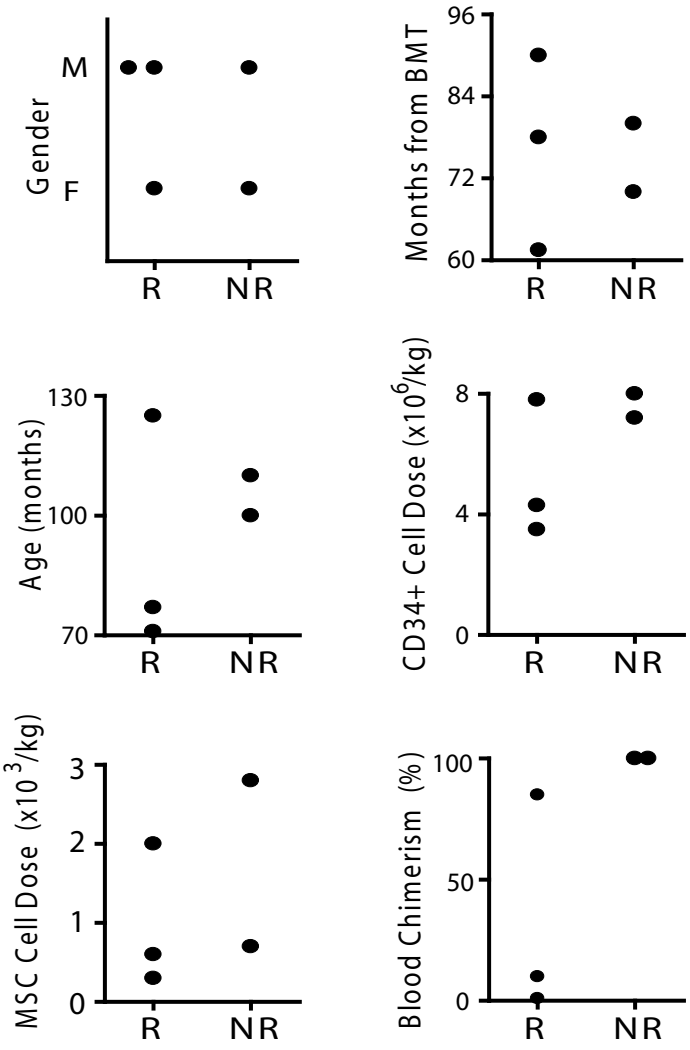
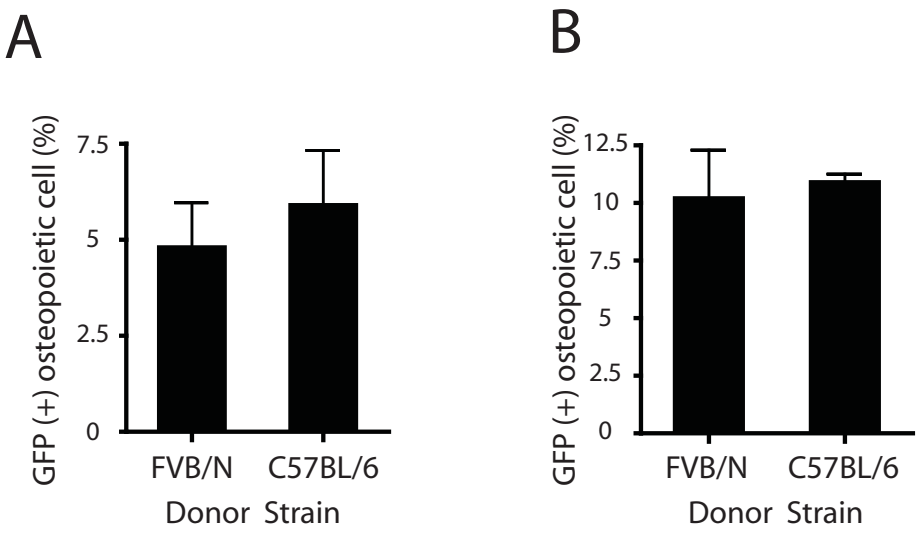

Gender
M
F
R
NR
Months from BMT
96
84
72
60
Age (months)
130
100
70
CD34+ Cell Dose (x10^6/kg)
8
4
0
MSC Cell Dose (x10^3/kg)
3
2
1
0
Blood Chimerism (%)
100
50
0

Figure S5.


A
B
GFP (+) osteopoietic cell (%)
7.5
5
2.5
0
FVB/N
C57BL/6
Donor Strain
GFP (+) osteopoietic cell (%)
12.5
10
7.5
5
2.5
0
FVB/N
C57BL/6
Donor Strain