Cells Derived from Young Bone Marrow Alleviate Renal Aging

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ABSTRACT

Bone marrow-derived stem cells may modulate renal injury, but the effects may depend on the age of the stem cells. Here we investigated whether bone marrow from young mice attenuates renal aging in old mice. We radiated female 12-mo-old 129SvJ mice and reconstituted them with bone marrow cells (BMC) from either 8-wk-old (young-to-old) or 12-mo-old (old-to-old) male mice. Transfer of young BMC resulted in markedly decreased deposition of collagen IV in the mesangium and less β-galactosidase staining, an indicator of cell senescence. These changes paralleled reduced expression of plasminogen activator inhibitor-1 (PAI-1), PDGF-B (PDGF-B), the transdifferentiation marker fibroblast-specific protein-1 (FSP-1), and senescence-associated p16 and p21. Tubulointerstitial and glomerular cells derived from the transplanted BMC did not show β-galactosidase activity, but after 6 mo, there were more FSP-1-expressing bone marrow-derived cells in old-to-old mice compared with young-to-old mice. Young-to-old mice also exhibited higher expression of the anti-aging gene Klotho and less phosphorylation of IGF-1 receptor β. Taken together, these data suggest that young bone marrow-derived cells can alleviate renal aging in old mice. Direct parenchymal reconstitution by stem cells, paracrine effects from adjacent cells, and circulating anti-aging molecules may mediate the aging of the kidney.

Aging is defined as impaired adaptive susceptibility to environmental or internal stresses with increasing rates of disease and death.[1] Kidney mass decreases by approximately 20 to 25% between the age of 30 and 80 yr, with reduced GFR and increased glomerulosclerosis and interstitial fibrosis.[2] Numerous injurious mechanisms contribute to age-related organ dysfunction, including increased profibrotic mediators, oxygen radicals,[3] mitochondrial injury, loss of telomeres, and imbalance of cell repair and proliferation *versus* apoptosis and cell death.[4] These mechanisms are, however, not unique to aging but common to many progressive-injury settings.[5] Cellular senescence is more specifically linked to aging-related diseases and describes the cessation of cell division after extended serial passage in culture.[6] Senescence is accompanied by specific functional and morphologic changes and is believed to be irreversible. Increased expression of

the cell cycle regulator p16INK4a was strongly associated with aging and histology changes while inversely correlated with cell replication in mice.[7] Once senescent, the cell appears more sensitive to injury and less likely to recover, which may be attributed to excretion of more cellular factors and deterioration of age-related changes in adaptive responses. In normal adults, senescent renal resident cells can be replaced by proliferating adjacent cells

or differentiated stem cells, the process of which is delayed in aging.

Infusion of putative stem cells indicates these cells can differentiate into a variety of renal cell types, including tubular epithelial cells, endothelial cells, mesangial cells, and podocytes.[8–10] It still remains unclear whether these effects are due to transdifferentiation of the infused cells, fusion of the infused cells with endogenous kidney cells, or a combination of both events. Bone marrow transplantation is a suitable way to observe the contribution of bone marrow-derived (BMD) cells, including stem cells, to renal injury. The group of Striker found that bone marrow from normal young female donors reduced glomerular hypertrophy and sclerosis in postmenopausal recipients.[11] However, cell-specific mechanisms of these effects remain to be more fully elucidated. We therefore further explored the mechanisms of modulation of the aging kidney by young *versus* old BMD cells and investigated possible roles of cellular senescence, transdifferentiation, and the anti-aging gene Klotho.

RESULTS

Reconstitution of Female Mice with Male Bone Marrow

The Y chromosome was detected in 80% of renal cells in positive-control male kidneys by *in situ* hybridization and was negative in females. At the age of 15 mo (3 mo after male bone marrow transplantation to female), around 8% of renal cells were Y-chromosome positive in the recipient female mice, similar to previous data (5 to 15%).[12,13] These bone marrow-derived cells were found in the mesangial area, anatomical location of podocytes, tubular epithelial cells, and interstitium. By quantitative PCR, similar amounts of Y chromosome in the kidneys of young-to-old (Y2O) and old-to-old (O2O) bone-marrow-transplant mice were found in both 15-mo-old (Y2O 7.79 ± 2.94% *versus* O2O 7.70 ± 2.55%, $P = 0.982$) and 18-mo-old (Y2O 5.19 ± 1.07% *versus* O2O 4.13 ± 0.41%, $P = 0.318$) mice (Figure 1), indicating similar amounts of BMD cells were present in both.

Compared with the sham group, bone marrow transplantation decreased plasma estrogen level in both 15-mo-old (Sham 20.83 ± 0.72, *versus* Y2O 15.20 ± 0.84, O2O 11.30 ± 1.10 ng/ml, $P < 0.05$) and 18-mo-old (Sham 20.83 ± 0.72, *versus* Y2O 15.20 ± 0.84, O2O 11.30 ± 1.10 ng/ml, $P < 0.05$) mice. However, no differences were found between Y2O and O2O. Systolic BP was not changed by bone marrow trans-

plantation (18-mo-old, Sham 102.50 ± 2.22, Y2O 96.00 ± 7.54, O2O 97.00 ± 3.79, $P = 0.440$).

Renal Aging Was Alleviated by Young BMT

There was a trend to increase proteinuria in O2O *versus* Y2O mice at 18 mo old (urine protein/creatinine Y2O 6.99 ± 3.41 *versus* O2O 34.76 ± 12.95, $P = 0.09$). Mild interstitial fibrosis and glomerular mesangial expansion were observed in aging kidneys. Glomerular collagen IV deposition in O2O mice increased from age 15 to 18 mo, with no difference compared with sham nontransplanted age-matched control. Young bone marrow transplant resulted in significantly less collagen IV deposition in glomeruli than old bone marrow both in 15-mo-old (Y2O 21.60 ± 1.07% *versus* O2O 26.52 ± 1.02%, $P < 0.05$) and 18-mo-old (Y2O 23.25 ± 2.04% *versus* O2O 30.61 ± 2.34%, $P < 0.05$) mice (Figure 2). Renal interstitial capillary bed density, stained by CD31, was not different among groups at 15 mo (Sham 1.44 ± 0.33, Y2O 1.34 ± 0.12, O2O 1.39 ± 0.26%, $P = 0.96$) and 18 mo (Sham 1.31 ± 0.21, Y2O 1.31 ± 0.03, O2O 1.21 ± 0.19, $P = 0.801$).

Changes in Fibrosis-related Factors and Senescence-related Protein after BMT

We next determined expressions of some factors related to aging, fibrosis, and transdifferentiation. There was no significant difference in TGFβ1 gene expression but decreased PAI-1 expression at 18 mo (Y2O 0.70 ± 0.15 *versus* O2O 1.49 ± 0.29, $P < 0.05$) and increased PDGF-B at 15 mo (Y2O 1.00 ± 0.11 *versus* O2O 0.58 ± 0.10, $P < 0.05$) in Y2O
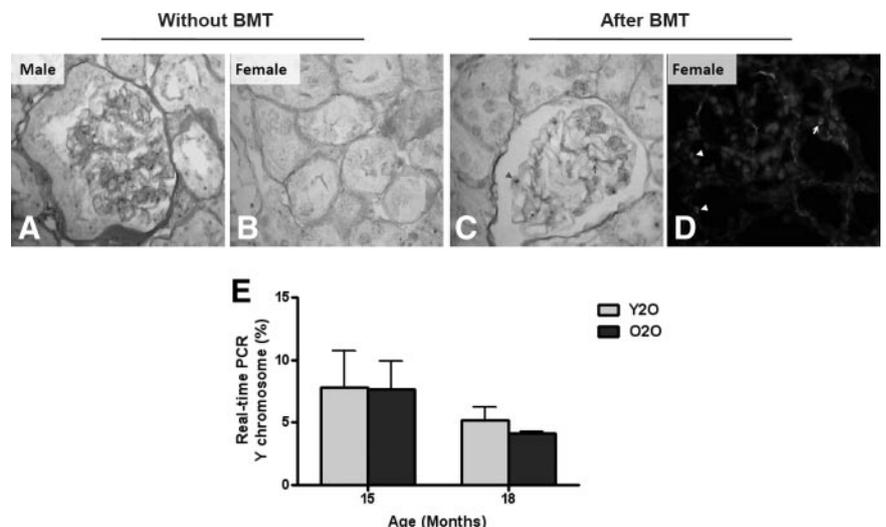

Figure 1. Reconstitution of female mice with male bone marrow. Y chromosome was detected in 80% of cells in positive control male kidneys (A) and was negative in females (B). After bone marrow transplantation (BMT), around 8% of cells were Y-chromosome positive. These bone marrow-derived cells were mostly present in mesangial areas (arrow in C) and in the interstitium (arrow in D). Some podocyte-like cells (triangle in C) and tubular epithelial cells (triangle in D) were also Y-chromosome positive. Real-time PCR showed similar amounts of Y chromosome in the kidneys of young-to-old (Y2O) and old-to-old (O2O) bone-marrow-transplant mice (E).
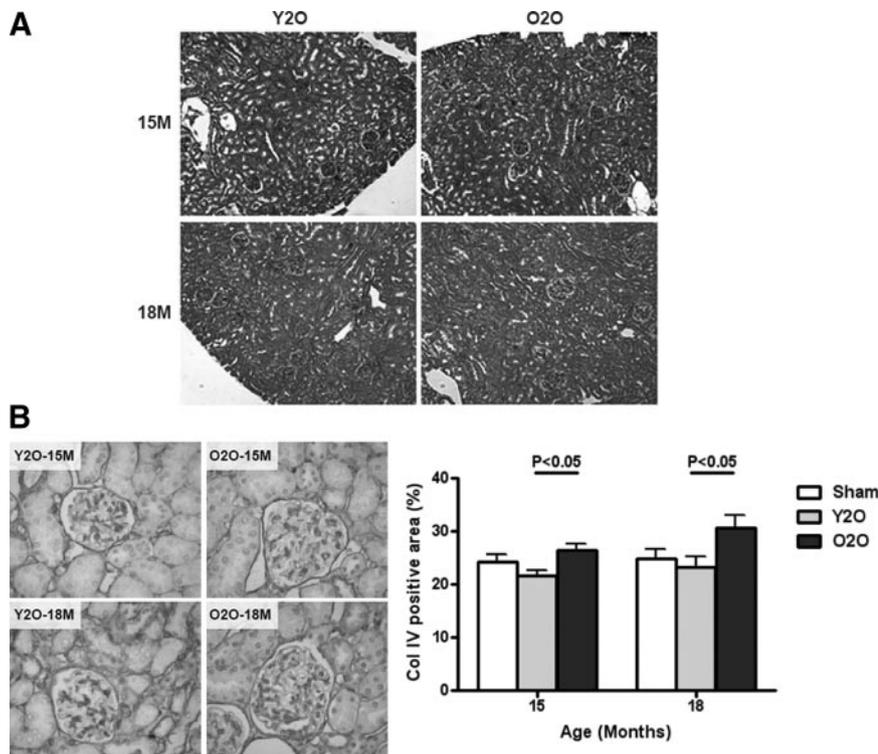
Figure 2. Renal injury in aging. There was more interstitial fibrosis and glomerular mesangial expansion in old-to-old (O2O) compared with young-to-old (Y2O) bone-marrow-transplant mice (A, Masson trichrome staining, 40X). Glomerular collagen IV deposition in O2O mice increased from 15 to 18 mo and was slightly more than in sham nontransplanted age-matched controls. Young bone marrow resulted in significantly less collagen IV deposition than old bone marrow (B, 200X).

kidneys (Figure 3). PAI-1 was expressed in podocytes, mesangial, and tubular epithelial cells (Figure 3A). PDGF-B was expressed in podocytes, mesangial, and tubular epithelial cells as well as endothelial cells (Figure 3B).

Fibroblast-specific protein-1, FSP-1, a cell transdifferentiation marker related to aging and fibrosis, was expressed in the interstitium and some tubular epithelial and glomerular cells (Figure 4A). Although some macrophages expressed FSP-1, most FSP-1-positive cells are F4/80 negative (Figure 4B). Y2O mice had less FSP-1 in glomeruli (Y2O 0.13 ± 0.02 *versus* O2O 0.58 ± 0.09 per glomerulus, $P < 0.05$) and the interstitium (Y2O 1.39 ± 0.29 *versus* O2O 3.69 ± 0.43 /HPF, $P < 0.05$) at 15 mo, and in the interstitium (Y2O 2.28 ± 0.37 *versus* O2O 4.40 ± 0.75 /HPF, $P < 0.05$) at 18 mo, when compared with O2O mice (Figure 4B).

Cell senescence is one postulated mechanism for aging that can be visualized by SA-β-gal staining. Sham mice had no significant increase in senescence from 15 to 18 mo. In contrast, renal β-gal positivity was markedly increased in 15-mo-old O2O and decreased in Y2O (Sham $5.62 \pm 1.25\%$, O2O $9.10 \pm 1.19\%$, Y2O $4.50 \pm 0.69\%$, $P < 0.05$ Y2O *versus* O2O) (Figure 5A). Cell senescence in mice is regulated by the p16/Rb pathway and ARF/p53 pathway. Compared with the young-bone-marrow mice and sham mice at 15 mo,

mice reconstituted with old bone marrow had more renal p16 (O2O 3.28 ± 0.45 *versus* Y2O 1.07 ± 0.21, Sham 1.00 ± 0.16, $P < 0.05$) and p21 expression (O2O 2.84 ± 0.83 *versus* Y2O 1.02 ± 0.11, Sham 0.35 ± 0.18, $P < 0.05$) (Figure 5B).

Phenotypical and Functional Changes of Bone Marrow-derived Cells in the Kidney

Compared with aging bone marrow, mice with young bone marrow had less macrophage infiltration in the kidneys at 15 mo (Y2O 3.28 ± 0.61 *versus* O2O 6.94 ± 0.91/ HPF, $P < 0.05$). Of note, macrophages showed a similar percentage of Y-chromosome positivity as control-male tissues in all groups, suggesting a nearly complete replacement of bone marrow cells from the donor (Figure 6).

In situ hybridization for the Y chromosome with costaining for FSP-1 showed that $9.3 \pm 1.9\%$ of young bone marrow-derived cells expressed FSP-1, compared with $17.4 \pm 2.6\%$ FSP-1 staining of old bone marrow-derived cells at 15 mo ($P < 0.05$). A trend for decreased FSP-1 in young BMD cells was seen at 18 mo (Y2O $19.3 \pm 3.7\%$ *versus* O2O $28.2 \pm 3.0\%$, $P = 0.09$) (Figure 7).

Interestingly, the SA-β-gal-positive cells did not colocalize with the Y-chromosome-positive cells, indicating that no or few bone marrow-derived cells showed senescence. However, there were fewer β-gal-positive cells adjacent to Y-chromosome-positive cells in Y2O than in O2O kidneys (Figure 8).

Preserved Klotho Expression by Transplantation with Young Bone Marrow

Klotho is a molecule that has recently been linked to aging. It is predominantly expressed in the kidney and can be secreted into urine and plasma. As a circulating factor, Klotho can antagonize aging by inhibition of insulin/IGF 1 (IGF-1) signaling and activation of FoxO. In our study, Klotho was expressed in distal tubules, not only at the apical side but also in the cytoplasm. Transplantation with young bone marrow preserved Klotho in aging kidneys both at 15 mo (Y2O $17.30 \pm 0.67\%$ *versus* O2O $11.89 \pm 2.05\%$, $P < 0.05$) and at 18 mo (Y2O $16.24 \pm 1.77\%$ *versus* O2O $10.29 \pm 1.50\%$, $P < 0.05$) (Figure 9A). Real-time PCR mirrored these changes among groups at 15 mo (Y2O 1.00 ± 0.07 *versus* O2O 0.32 ± 005, $P < 0.05$) and 18 mo (Y2O 0.71 ± 0.08 *versus* O2O 0.30 ± 0.06, $P < 0.05$). Accordingly, IGF-1 receptor β phosphorylation decreased in Y2O compared with O2O ($P < 0.05$) (Figure 9B).
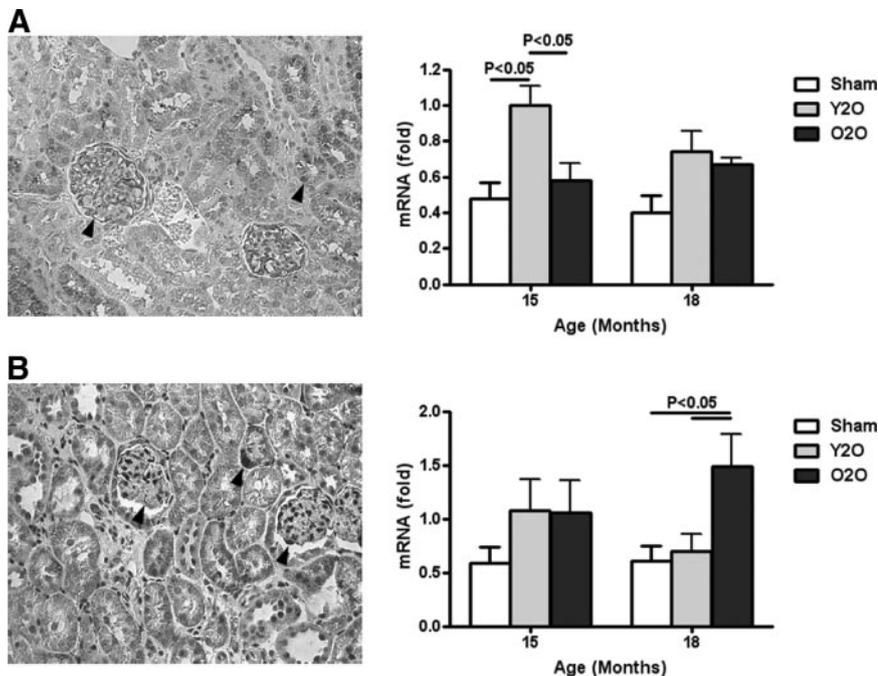
Figure 3. Fibrosis-related factors after bone marrow transplant. PDGF-B was expressed in podocytes, mesangial, and tubular epithelial cells, as well as endothelial cells (A, arrow head, 200X). PAI-1 was expressed in podocytes, mesangial, and tubular epithelial cells (B, arrow head, 200X). PAI-1 expression was decreased at 18 mo and PDGF-B was increased at 15 mo in young-to-old (Y2O) kidneys compared with old-to-old (O2O) bone-marrow-transplant kidneys.
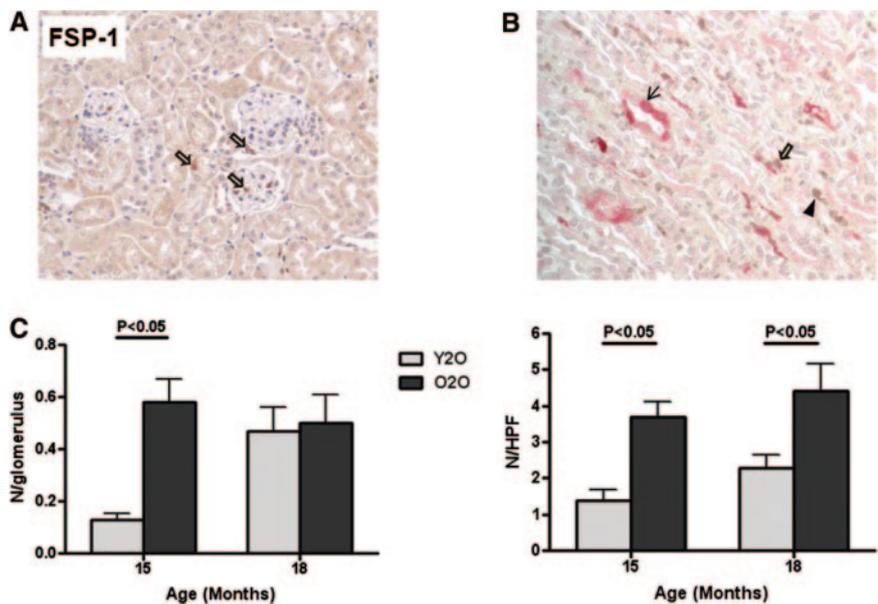

Figure 4. Renal FSP-1 expression after bone marrow transplant (BMT). Fibroblastic specific protein-1 (FSP-1) was expressed in the interstitium and in some tubular epithelial and glomerular cells (A, open arrow, anti-FSP-1, 400X). Some macrophages just expressed F4/80 (brown, arrowhead), while some macrophages expressed both F4/80 and FSP-1 (brown and red, open arrow). Some tubular epithelial cells just expressed FSP-1 (red, solid arrow; B, 400X). Young-to-old (Y2O) bone-marrow-transplant mice had less FSP-1 in glomeruli and interstitium at 15 mo, and in the interstitium at 18 mo, when compared with old to old (O2O) (C).

DISCUSSION

Similar to the previous findings from the group of Striker, our study also shows that young bone marrow alleviates renal aging, including decreasing mesangial expansion with decreased collagen IV.[11] We have now determined that the mechanism of these effects of cells derived from young bone marrow include less fibroblast transdifferentiation, paracrine effects of bone marrow-derived cells on aging in nearby cells, and increased maintenance of Klotho. Thus, both local and systemic, and direct and indirect pathways, mediate these processes of age-related fibrosis and are impacted by bone marrow-derived cells.

Kidney-aging changes vary greatly in different human populations and are also strain-dependent and gender-dependent in mice. Thus, aging-related glomerulosclerosis in Bolivian and Japanese populations is minimal compared with the US Caucasian population, and a great excess of sclerosis with aging occurs in both male and female African American populations.[14] In mice, the C57BL6 strain is more resistant to renal aging than other strains, and morphologic changes of aging are milder in female mice *versus* males.[15,16] Female C57BL6 mice begin to have irregular, lengthened estrous cycles around 10 to 14 mo of age, and cycles usually cease at 13 to 16 mo of age.[17] Dr. Striker's group has shown glomerular hypertrophy, vascular pole sclerosis, and mesangial cell proliferation in 18-mo-old C57BL6 female mice,[18] while we used renal-sclerosis-prone 129/Sv mice. The estrogen level of our 129/Sv 18-mo-old mice was significantly lower than at 15 mo, and mesangial expansion and mild interstitial fibrosis were found at both 15 and 18 mo, indicating an early aging stage in our studies.

Impaired adaptive response and increased susceptibility to stress are characteristics of the aging process. Cell senescence includes replicative senescence, due to a lack of response to mitogenic stimuli, and stress-induced premature senescence (SIPS), which is induced rapidly in response to various physiologic stresses, independently of the number of cell divisions.[19] In our bone-marrow-transplant model, radiation-related damage accelerated aging-like inju-
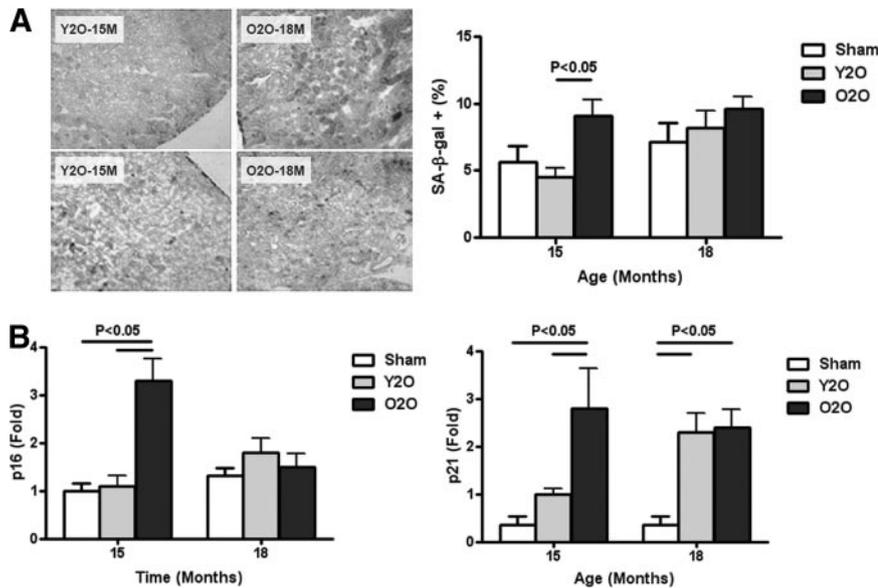
Figure 5. Senescence-related proteins after bone marrow transplant. Renal SA-β-gal positivity was significantly decreased in Y2O kidneys at 15 mo *versus* O2O kidneys (A, 100X). Cell senescence is regulated in mice by the p16/Rb pathway and ARF/p53 pathway. Mice with young bone marrow (Y2O) had less p16 and p21 mRNA expression compared with old bone marrow (O2O) (B).
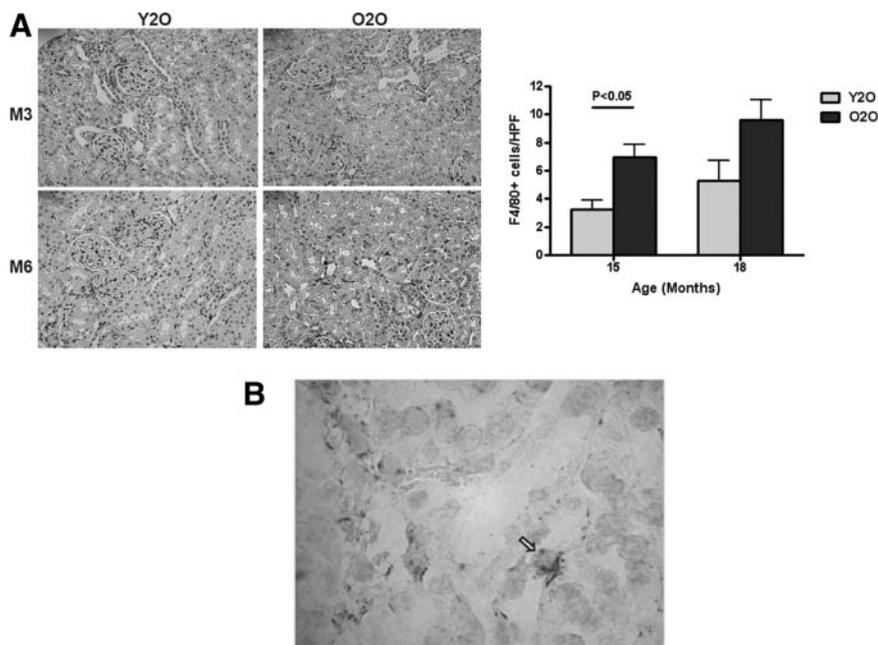

Figure 6. Renal macrophage infiltration after bone marrow transplant. Compared with aging bone marrow (O2O), mice with young bone marrow (Y2O) had less macrophage infiltration in the kidneys (A, 100X). Macrophages showed similar percentages of Y-chromosome positivity (arrow) as control male tissues in all groups, suggesting a nearly complete replacement of bone marrow cells from the donor (B, 400X).

ries, especially cell senescence after 3 mo, which was at similar levels as the control after 6 mo. Replicative senescence in humans is accompanied by loss of telomeres, and DNA repeats at the ends of chromosomes due to lack of telomerase,[20] while it is mainly regulated by the p16/Rb pathway and ARF/p53 path-

way in mice.[21] p16INK4a inhibits the activity of the cyclin-dependent kinases (CDKs) 4 and 6, thereby leading to hypophosphorylation of the retinoblastoma (Rb) gene and irreversible cell-cycle arrest. p19ARF, by binding to MDM2, prevents ubiquitination and degradation of p53, which results in cell-cycle arrest mediated by p53 through p21CIP1/WAF1.[7] In our study, senescence-associated β-galactosidase staining was reduced in the young BMT group, with reduced p16 INK4a and p21CIP1/WAF1, indicating young progenitor cells may contribute to decreased cell senescence through inhibition of both p16/Rb and ARF/p53 pathways.

The mechanisms of BMD cells modulating the aging kidney phenotype are complex. We did observe occasional apparent replacement of resident renal cells by BMD, such as mesangial area cells, podocyte like cells, tubular epithelial cells, and some interstitial cells. Similar findings have also been observed in human heart and kidney transplants and other experimental models.[9,10,12,22] However, we also found fewer β-gal-positive cells adjacent to young BMD cells than to old ones, suggesting BMD cells may affect resident cells through paracrine mechanisms. BMD cells include two major unique cell types: the hematopoietic stem cell (HSC) and the marrow stromal cell (MSC). MSCs are known to secrete various growth factors and cytokines, including IGF-1, vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF), which could be effective in renal repair.[23] In our study, reduced PAI-1 and increased PDGF-B in the young-to-old BM transplant mice could be partly contributed by such infiltrating BMD cells and their adjacent cells. Of note, PDGF-B may reflect early fibrosis and/or proliferation, whereas other fibrotic mechanisms may be more prominent in later stages. In addition to this local, paracrine effect, the increased Klotho observed in Y2O mice suggests BMD cells also may modulate systemic aging-related factors. Klotho is a newly identified anti-aging factor, with effects on insulin/IGF-1 signaling and phosphate/calcium homeostasis.[24] It is highly expressed in the kidney and plays a major role in renal mineral metabolism. Mice with genetic deficiency of Klotho develop accelerated aging-related diseases, including stroke, arteriosclerosis, and osteoporosis. In contrast, overexpression
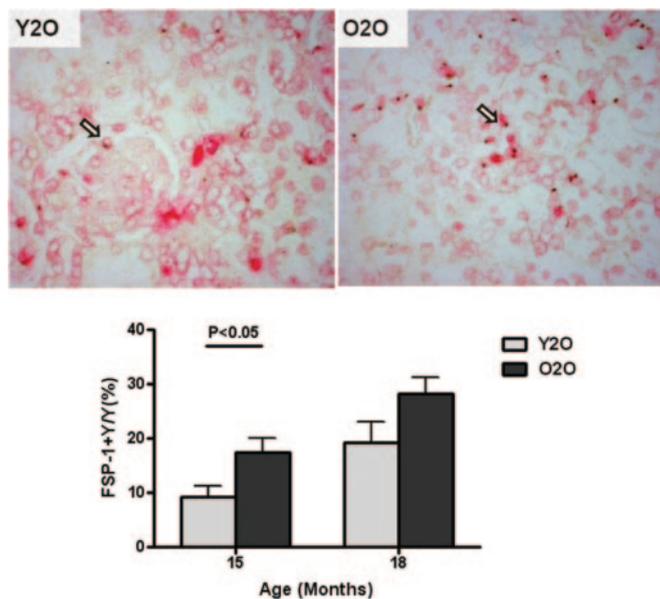
Figure 7. FSP-1 expression in bone marrow-derived cells. *In situ* hybridization for the Y chromosome (brown) with costaining for FSP-1 (red) showed that young bone marrow-derived cells (Y2O) in glomeruli expressed less FSP-1 than old bone marrow-derived cells (O2O) in aging kidneys (200X).
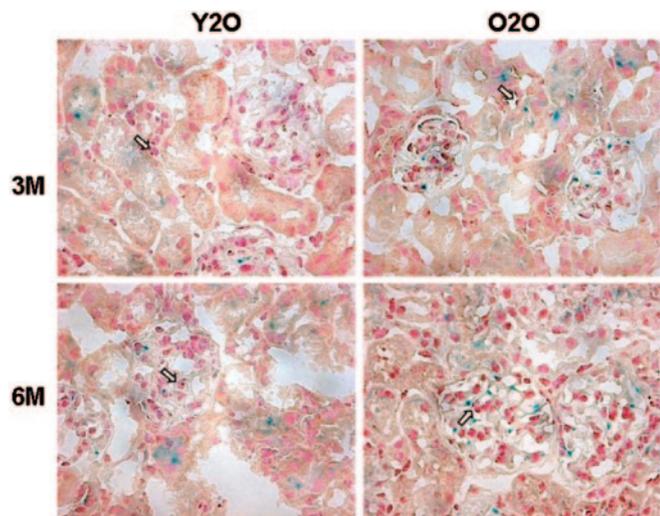

Figure 8. Cell senescence and bone marrow-derived cells. The SA-β-gal-positive cells (blue) did not colocalize with the Y-chromosome-positive cells (brown), indicating that no or few bone marrow-derived cells showed senescence. Moreover, there were less β-gal-positive cells adjacent to Y-chromosome-positive cells in young-to-old (Y2O) than in old-to-old (O2O) bone-marrow-transplant kidneys (200X).

of the Klotho gene extended life span in the mouse. We have previously shown that PPAR gamma agonist ameliorated aging-related renal injury, in part by increasing Klotho expression.[25,26] In the current study, transplantation of young bone marrow significantly preserved Klotho expression and inhibited its downstream IGF-1 receptor β phosphorylation in ag-

ing kidneys. Klotho may also regulate age-dependent stem cell regenerative capacity through the Wnt pathway. In addition, since we transplanted male bone marrow into female marrow, it is possible that BMD cells influence renal aging through some gender-related mechanisms, although the female recipient mice in our study have similar estrogen levels regardless of the young or old bone marrow received.

Similar Y-chromosome positivity in young-to-old and old-to-old bone-marrow-transplant mice suggests the function of BMD cells is more important than the number of cells. Most previous data indicate the number of MSCs remains relatively stable with aging but that changes in their morphology and function occur. This coincides with a decreased capacity for proliferation and differentiation.[27] In our study, more of the aging BMD cells expressed FSP-1 than the young BMD cells, indicating divergent differentiation capacity. Based on their epigenetically fixed differentiation potential and self-renewal capacities, three classes of hematopoietic stem cells have been distinguished, namely, so-called balanced, lymphoid-biased, and myeloid-biased classes.[28] New data show that the proportions of these subsets change during aging.[29] Hematopoietic stem cells that generate lymphocyte-rich progeny are depleted, while myeloid-biased cells are enriched in the aged hematopoietic stem cell compartment. Thus, the age-related changes in the hematopoietic stem cell compartment derive from a changing composition of the different classes of these cells.[30] Our study does not establish whether different cell function in young *versus* old bone marrow is due to different cell compartment proportions or different responsibility to old environments. Of note, there were fewer macrophages in young-to-old than old-to-old bone-marrow-transplant mice kidneys, although there was similar Y-chromosome positivity in these two groups.

In summary, young BMD cells affect fibrosis by complex mechanisms. These include not only direct replacement of renal cells but also paracrine and systemic effects on senescence, through modulation of various factors such as p16 and Klotho, respectively.

CONCISE METHODS

Experimental Design and Animals

Aging female 129/Sv mice (Jackson Laboratories, Bar Harbor, ME) were housed under controlled conditions with a 12-h light/dark cycle at 70°F, with 40% humidity and 12 air exchanges per hour. All animal protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee. At the age of 12 mo, female mice were radiated and reconstituted with bone marrow transplantation (BMT) from 12-mo-old (old-to-old, O2O) or 8-wk-old (young-to-old, Y2O) adult male mice. Bone marrow transplantation was performed as described previously.[31] Recipients were lethally irradiated with 9 Gy using a cesium gamma source. Bone marrow cells were harvested from the femurs and tibias of donor mice by flushing with RPMI-1640 media (Life Technologies BRL, Gaithersburg, MD), and
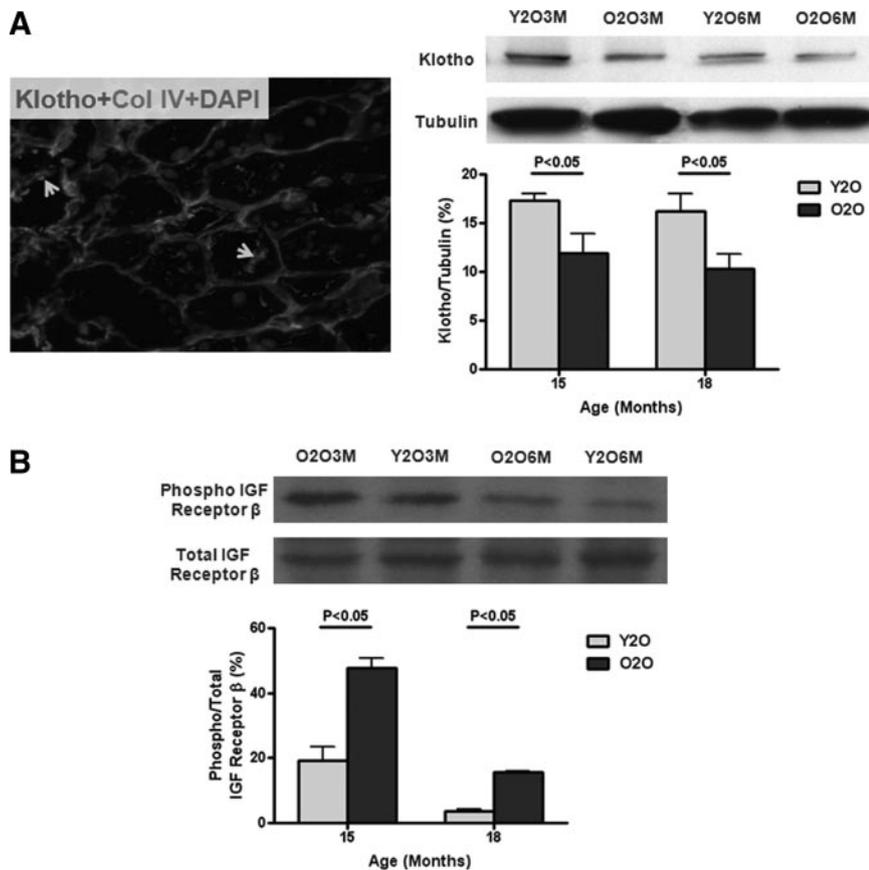
Figure 9. Renal Klotho expression after bone marrow transplant. Klotho was expressed in distal tubules at the apical side and also in the cytoplasm (A, left panel, 200X). Compared with old bone marrow (O2O), transplantation with young bone marrow (Y2O) preserved Klotho protein in aging kidneys at age 15 mo and 18 mo (A), while IGF-1 receptor β phosphorylation decreased (B).

recipients received 5×10^6 bone marrow cells per mouse in 0.2 ml of medium intravenously through the retro orbital sinus. The recipient mice were then sacrificed at 3 or 6 mo after BMT ($n = 5$ for each group and time point). Age-matched nontransplanted female mice were used as control (Sham).

Blood and Urinary Measurements

Systolic BP (SBP) and 24-h urinary protein were assessed at baseline and 3 and 6 mo after BMT. SBP was measured using tail-cuff plethysmography in unanesthetized prewarmed trained mice at ambient temperature of 29 °C. Plasma estrogen was measured by ELISA kit (TEZ ElisaTM, Framingham, MA). Urine protein was measured by Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Urine creatinine was measured by Vitros CREA slides (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY).

Senescence-associated β-galactosidase Activity

Senescence-associated β-galactosidase (SA-β-gal) activity was examined as described previously[32] Briefly, frozen sections were washed and incubated at 37 °C with freshly prepared SA-β-gal stain solution: 1 mg of 5-bromo-4-chloro-3-indolyl β-d-galactoside (X-gal)/mL (stock solution 20 mg/ml of dimethylformamide), 40 mM citric acid,

sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl2. Staining was evident in 16 h. The percentage of β-galactosidase-positive area was measured by point counting on 20 consecutive 100X magnification fields.

Immunohistochemistry

Kidney tissue was immersion-fixed, routinely processed, and paraffin-embedded sections were deparaffinized and hydrated. For staining of FSP-1, sections were microwaved in 0.01 M sodium citrate (pH 6.0) 3 × 5 min. For staining of collagen IV, F4/80, and Klotho, sections were trypsinized (1mg/ml, Sigma, St. Louis, MO) 20 min at 37 °C. Endogenous peroxidase was quenched with 3% hydrogen peroxidase for 10 min and slides exposed to Power Block (Bio-Genex Laboratories, San Ramon, CA) for 30 min. The primary antibodies used were rabbit anti-mouse FSP-1 (1:400, gift from Dr. Eric G. Neilson), rabbit anti-mouse collagen IV (1: 1000, Chemicon, Billerica, MA), rat anti-mouse F4/80 (1:800, AbD Serotec, Raleigh, NC), and rat anti-mouse Klotho (1:500, R&D systems, Minneapolis, MN). Sections were incubated overnight at 4 °C. Immunoperoxidase staining was performed with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA), with diaminobenzidine (DAB) as a chromogen. Hematoxylin was used as a counterstain. The results were quantitated by point counting on 40 consecutive 400X magnification fields or forty glomeruli. For double staining, F4/80 was stained in brown by DAB, followed by FSP-1, shown in red with VECTOR Red Alkaline Phosphatase Substrate Kit.

Double staining for Klotho and collagen IV was done as follows: Frozen sections were air dried and fixed in 4% PFA for 5 min, incubated with rabbit anti-mouse collagen IV (1:500) at 37 °C for 1 h, and Alexa Fluor® 488 goat anti-rabbit IgG (1:800, Invitrogen, Carlsbad, CA) at RT for 30 min and Power Block for 1 h. Klotho immunostaining was performed with primary rat anti-mouse Klotho (1:200), followed by Alexa Fluor® 594 chicken anti-rat IgG (1:800, Invitrogen).

CD31 (1:50, Pharmingen, San Diego, CA) was stained on frozen section and calculated by ImageJ software.

In Situ Hybridization

Sections were digested in 0.4% wt/vol pepsin in 0.1 M HCl for 10 min at 37 °C. The protease was quenched in 0.2% glycine (G4392; Sigma) in double concentration PBS and sections were then rinsed in PBS, postfixed in 4% paraformaldehyde in PBS, dehydrated through graded ethanols, and air dried. Sections were then denatured at 65 °C for 2 min in preheated 70% deionized formamide/2X SSC (SSC) buffer and quenched with ice-cold 70% ethanol for 1.5 min. The biotin-labeled mouse Y-chromosome probe (STAR*FISH; Cambio, Cam-

bridge, England) was denatured at 65 °C for 10 min and applied to the sections at 37 °C (15ul/slide). The sections were coverslipped and sealed with rubber cement for incubation overnight in a hydrated slide box at 37 °C. The next day, all slides were washed in deionized formamide/2x SSC at 45 °C, washed with 2x SSC, and incubated in 4x SSC/0.05% Tween-20 for 10 min at 37 °C. To visualize the Y signals, slides were washed with PBS and incubated with ABC complex (Vector Laboratories) for 30 min at RT before being developed in DAB, washed with PBS, and stained with periodic acid-Schiff.

For double staining, *in situ* hybridization for the Y chromosome was done before β-galactosidase staining, or followed by FSP-1, F4/80, or collagen IV staining.

Western Blot
Frozen kidney samples were homogenized in RIPA buffer containing proteinase inhibitor (Roche Diagnostics GmbH, Mannheim, Germany), 1:100 phosphatase inhibitor cocktail I, and 1:100 phosphatase inhibitor cocktail II (Sigma, St. Louis, MO). Tissue lysates were centrifuged at 13,000 rpm for 15 min at 4 °C. The protein concentration was measured using Dc Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Sixty μg of protein from samples were loaded and separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a 0.2 μmol/L nitrocellulose membrane. P21 (1:200, Santa Cruz, CA), P16 (1:200, Santa Cruz), Klotho (1:250, R&D Systems), and total and phospho IGF-1 receptor β (1:1000, Cell Signaling Technology, Danvers, MA) were detected by using the corresponding antibody overnight at 4 °C. After washing in Tris-buffered saline with 0.1% Tween 20 (TBS-T), horseradish peroxidase-labeled donkey anti-rabbit or anti-mouse IgG secondary antibody (1:2500 in 5% non-fat milk TBS-T) was added and incubated at room temperature for 1 h. Protein bands on Western blots were visualized by ECL Plus (Amersham, Arlington Heights, IL), according to the manufacturer's instructions, and developed on film. The membranes were stripped with 100 mmol/L β-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mmol/L Tris-HCL, pH 6.7. The amount of loading was detected by using mouse anti-α-tubulin polyclonal antibody (Abcam, Cambridge, MA). The ratio of specific message to tubulin was used to quantify expression for each tissue sample.

PCR and Quantitative Real-time PCR
Reverse transcription was performed using TaqMan Reverse Transcription Kit (Applied Biosystems, Branchburg, NJ) in a total reaction volume of 80 μL (2 μg total RNA, 5 mM MgCl2, 1 mM dNTPs, 1 U/μl RNase inhibitor, 2.5 μM random hexamers, and 2.5 U/μl MuLV-RT) for 30 min at 42 °C. Quantitative real-time PCR was performed in a total reaction volume of 25 μL using iQ™ SYBR Green Supermix (Bio-rad, Hercules, CA), 1 μL cDNA, and 500 nM forward and reverse primers [Y chromosome forward (5′-TGGAGAGCCACAAGCTAACCA), reverse (5′-TCCCAGCATGAGAAAGATTCTTC); PDGF forward (5′-TGTAACACCAGCAGCGTCAAG), reverse (5′-CTGGACCTCT-TTCAATTTTGGC); TGFβ forward (5′-GCAACATGTGGAACTCTAC-CAGAA), reverse (5′-GACGTCAAAAGACAGCCACTCA); PAI-1 forward (5′-CCGATGGGCTCGAGTATG), reverse (5′-TTGTCTGATGAGTT-CAGCATC)]. P16, p21, and Klotho primers were bought from SABiosciences Corporation (Frederick, MD). Quantitative real-time PCR

was carried out using the iCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad), with the following cycling parameters: polymerase activation for 3 min at 95 °C and amplification for 40 cycles of 20 s at 95 °C, 20 s at 60 °C, and 40 s at 72 °C. Following amplification, a final melting curve was obtained for each sample by cooling the reaction mixture to 55 °C and then slowly heating it to 95 °C at 0.2 °C/s. Experimental cycle threshold (Ct) values were normalized to GAPDH, measured on the same plate, and fold differences in gene expression were determined by using the $2-\triangle\triangle Ct$ method. [33]

Statistical Analysis
Results are expressed as mean $\pm$ SEM. Statistical difference was assessed by a single-factor variance (ANOVA) followed by unpaired t test, as appropriate. Nonparametric data were compared by the Mann-Whitney U test. A P value <0.05 was considered to be significant.

DISCLOSURES
None.

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