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Dysregulated *in vitro* Hematopoiesis, Radiosensitivity, Proliferation, and Osteoblastogenesis with Marrow from SAMP6 Mice

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Abstract

The Senescence Accelerated-Prone mouse variant 6 (SAMP6) shows normal growth followed by rapid aging, development of osteopenia, and shortened lifespan, compared with control R1 mice. Because oxidative stress is a fundamental mechanism of tissue aging, we tested whether cellular parameters that are associated with oxidative stress are impaired with marrow from SAMP6 mice. We compared *in vitro* hematopoiesis, irradiation sensitivity, proliferative potential, and osteoblastogenesis with marrow cells from SAMP6 and R1 mice. Marrow cells from SAMP6 mice showed shortened *in vitro* hematopoiesis; their stromal cells showed greater radiation sensitivity and decreased proliferation. Consistent with those properties, there was constitutive upregulation of TGF- β_1 , an inhibitor of hematopoiesis, and of cell cycle inhibitory genes, *p16^{INK4A}* and *p19^{ARF}*. Paradoxically, there was constitutive expression of osteoblast genes in stromal cells from SAMP6 mice, but *in vitro* matrix mineralization was impaired. These studies and data included in other reports indicate that impaired proliferation of osteoblast progenitors in SAMP6 marrow may be a major factor contributing to accelerated loss of bone mass. In sum, marrow from SAMP6 mice had diminished capacity for long-term hematopoiesis, increased radiosensitivity, and reduced proliferative capacity.

Keywords

SAMP6 mice; long-term bone marrow culture; hematopoiesis; radiosensitivity; marrow stromal cells

Human skeletal aging is associated with a loss in bone mass, development of osteoporosis, and increased risk of fracture. Skeletal homeostasis depends on multiple factors regulating bone remodeling, the cycle that consists of bone resorption by osteoclasts and new bone formation by osteoblasts. Emerging evidence from *in vitro* studies with human marrow

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stromal cells from young and old subjects shows an age-related decline in osteoblast differentiation potential *in vitro* [1, 2] and an age-related increase in ability to support osteoclastogenesis *in vitro* [3]. Little is known about the effects of age on *in vitro* hematopoiesis.

The Senescence-Accelerated Mouse (SAM Prone) strains arose spontaneously from the AKR/J background and display shortened life span and an array of signs of accelerated aging, compared with control SAMR strains [4]. In particular, the SAMP6 variant displays approximately 4 months of normal skeletal development and growth, followed by an age-related accelerated decrease in bone mass [5, 6] with increased marrow adiposity [7]. It is notable that development of osteopenia in SAMP6 mice was prevented by transplantation of bone marrow from normal allogeneic mice [8]; this suggests that the skeletal defect may be due to dysregulated bone progenitor cells, their products, or other components of marrow. There has been some research comparing *in vitro* osteoblast potential in SAMP6 and SAMR1 marrow cells, with evidence for no differences, and for less, or for more with SAMP6 marrow depending on the bone and the age of the mice [9–11].

It has been proposed that increased sensitivity to oxidative stress accounts for accelerated aging in SAM-prone mice [12–14]. Indices of oxidative damage have been shown in cells from aging C57BL/6 mice [15], from SAMP strains of mice [16], as well as in human marrow-derived mesenchymal stem cells from older subjects [17]. Sensitivity of marrow cells to oxidative stress can also be monitored by longevity of hematopoiesis in long-term bone marrow cultures and by cellular sensitivity to irradiation [18, 19]. Thus, we tested the hypothesis that bone marrow from SAMP6 mice would demonstrate diminished functionality in three assay systems: 1) maintenance of hematopoiesis in long-term marrow culture, 2) resistance to ionizing irradiation, and 3) growth and differentiation potential.

Materials and Methods

SAMR1 and SAMP6 mice

The SAMP6 and SAMR1 mice and breeding pairs were obtained through Harlan Laboratories (Indianapolis, IN). All animals were bred, maintained, and studied in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

Long-term bone marrow cultures from SAMR1 and SAMP6 mice

Long-term bone marrow cultures were established from four SAMP6 and four SAMR1 mice, 5–9 weeks old [20, 21]. Cultures were maintained in McCoy's 5A medium (Gibco, Gaithersburg, MD), supplemented with 25% horse serum (Cambrex, Rockland, ME), 1% penicillin/streptomycin (Cellgro, Manassas, VA), 1% L-glutamine (Gibco), and 10^{-5} M hydrocortisone sodium hemisuccinate at 33°C in 7% CO₂. After 4 weeks, horse serum was replaced with 25% fetal bovine serum (FBS, Gibco). Cultures were monitored weekly for percent confluence, cobblestone island formation, hematopoietic cell production, and formation of hematopoietic colonies as previously described [20, 21]. Data analysis used a two-sided two-sample t-test comparing the number of cobblestone islands from the SAMP6 and SAMR1 cultures each week.

Marrow stromal cell lines and clonal sub-lines

Adherent cell layers from 12-week-old LTBMcs from SAMP6 and SAMR1 mice were expanded in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 10% FBS at 37°C in 5% CO₂. Clonal sub-lines were established from each of the parent lines by culture of individual single cells, as published [22].

Irradiation survival curves for stromal cell lines and clonal cell sub-lines

SAMP6 and SAMR1 cell suspensions were irradiated using a ^{137}Cs g-ray source (JL Shepherd, San Fernando, CA, USA) with doses ranging from 0 to 8 Gy and plated in Linbro 4-well tissue culture dishes. Cultures were incubated at 37°C in 5% CO_2 . Seven days later, cells were stained with crystal violet; colonies 50 cells were counted (GelCount colony counter, Oxford Optronix, Oxford, UK). Data were analyzed with linear quadratic and single-hit multitarget models [23].

Proliferation assays

Stromal cell lines and clonal sub-lines were seeded at 1×10^4 cells per well (n=4 to 6) of a 6-well plate. At intervals, cells were re-suspended with 0.5 mL of 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA), and the viable cell number was determined by hemacytometer with 0.1% trypan blue (Gibco).

Osteoblast differentiation

Reagents were obtained from Sigma-Aldrich, St. Louis, MO unless indicated otherwise. Cell lines were seeded at a seeding density of 2×10^5 per well/6 well plate and cultured in DMEM with 10% FBS-HI at 37°C and 5% CO_2 until confluence. Thereafter the cultures were changed to osteoblastogenic medium (Iscove's Modified Dulbecco's Medium (IMDM, Gibco) with 1% Fetal Bovine Serum-Heat Inactivated (FBS-HI), 100 U/ml penicillin, 100 µg/ml streptomycin, 10 nM dexamethasone, 5mM β -glycerophosphate, and 50 µg/ml ascorbate-2-phosphate [24]. RNA was isolated at days 0, 3, 5, 7, and 10 with Trizol reagent (Invitrogen). At day 7, alkaline phosphatase (ALP) activity was assayed, with data presented as p-nitrophenyl phosphate (µmole) hydrolysed per minute at 37° C per gram of total cellular protein [2]. After 16 days in osteogenic media other plates were used to determine mineralization using Alizarin Red [25].

Primary adherent marrow cell (AMC) cultures from SAMR1 and SAMP6 mice

For *in vitro* experiments with primary adherent marrow cells (AMCs), bone marrow was isolated from femurs of SAMR1 and SAMP6 mice (5–7 months old), as described previously [26, 27]. The marrow cells were seeded at a density of 2.5×10^6 cells per 35-mm tissue culture dish (BD Falcon, Franklin Lakes, NJ) and cultured in phenol red-free α -MEM medium, 10% FBS-HI, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). After 72 h, non-adherent cells were removed with the first medium change; thereafter medium was changed twice each week.

At day 14, the number of colonies (colony-forming unit-fibroblast, CFU-F) was determined after staining with 0.5% toluidine blue; the number of cells per dish was determined by hemacytometer after treatment with 0.5 ml of 0.25% trypsin-EDTA (Invitrogen); the average number of cells per colony was calculated for triplicate dishes for each mouse strain. Total RNA was isolated from other dishes with Trizol reagent (Invitrogen).

For osteoblast differentiation, medium was changed on day 3 to an osteoblastogenic medium (phenol red-free α -MEM with 10% FBS-HI, 100 U/mL penicillin, 100 µg/mL streptomycin, 5 mM β -glycerophosphate, 10 nM dexamethasone, and 50 µg/mL ascorbate-2-phosphate). After 14 days, alkaline phosphatase (ALP) activity was measured [2].

RNA isolation and RT-PCR

For RT-PCR, 2 µg of total RNA was reverse transcribed into cDNA with M-MLV reverse transcriptase (Promega, Madison, WI). Concentrations of cDNA and amplification conditions were optimized for each gene to reflect the exponential phase of amplification.

One-twentieth of the cDNA was used in each 50 μ l PCR reaction. The gene-specific primers for p16, p21, p53 [28]; p19 [29]; ALP [27]; Runx 2 and Oc [30]; SOST [31], PPAR γ and LPL [32]; TGF- β_1 [33], PTHR1 [34], and VDR [35] were used for amplification (30–40 cycles at 94°C for 1 min, 55–60°C for 1 min, and 72°C for 2 min). Following amplification, the reaction products were separated by 2% agarose gel electrophoresis; the gel images were captured with KODAK Gel logic 200 imaging system and were quantified by densitometry with Image J software. Data are expressed by normalizing densitometric units to those for GAPDH (internal control).

Statistical analysis

Experiments were performed at least in triplicate. Group data are presented as Mean $\pm$ SEM. Quantitative data were analyzed with non-parametric tools, either the Mann-Whitney test or Spearman correlation test. If data allowed, parametric tools were used, either t-test for two groups, ANOVA for multiple group comparisons, or Pearson correlation test.

Results

Diminished longevity of hematopoiesis in long-term bone marrow cultures derived from SAMP6 mice

Long-term bone marrow cultures established from SAMP6 and SAMR1 mice showed a time-dependent increase in confluent adherent cells (Figure 1A). At weeks 4 and 5, SAMR1 cultures were significantly more confluent (91% and 95%, respectively) when compared with 83% and 87% confluency for SAMP6 cultures ($p=0.0011$ and $p=0.002$, respectively). From week 6 through week 42 the amount and stability of the adherent layers were indistinguishable.

The adherent layer of each flask was scored weekly for the number of cobblestone islands per flask, as an index of hematopoietic foci (Figure 1B). There were significantly more cobblestone islands in SAMR1 cultures at weeks 4 and 5 ($p=0.018$ and <0.0001 , respectively). This trend was reversed by week 8, with significantly more cobblestone islands in SAMP6 cultures (weeks 8 through 14, p value range 0.001 to 0.015). Thereafter the number of cobblestone islands per flask decreased, particularly at later time points (Figure 1B). Generation of cobblestone islands persisted in SAMR1 long-term bone marrow cultures longer than in SAMP6 cultures (Figure 1B, C). The SAMP6 cultures maintained the adherent stromal layer but cobblestone islands were absent by week 30 (Figure 1C:a,b). In contrast, SAMR1 long-term bone marrow cultures continued to generate cobblestone islands (Figure 1C:c,d).

Proliferative and clonogenic potential of the non-adherent cells generated in long-term bone marrow cultures was assessed by counting the number of GM-CFUs that formed after transfer to semi-solid medium (Figure 1D). Between weeks 5 and 19, SAMP6 long-term marrow cultures generated significantly more colonies compared with those derived from SAMR1 mice (p value range <0.0001 to 0.004). After 24 weeks the non-adherent cells from the SAMR1 cultures gave rise to significantly more colonies than did those from the SAMP6 cultures (p value range <0.0001 to 0.012).

Greater radiosensitivity of SAMP6 bone marrow stromal cell lines and clonal sub-lines

Permanent bone marrow stromal cell lines were established from representative long-term bone marrow cultures at week 12, a time when hematopoiesis was maintained in both SAMP6 and SAMR1 cultures. The clonogenic survival curve assay revealed that the SAMP6 stromal cell line ($D_0 = 2.1 \pm 0.1$ Gy) was more radiosensitive than the SAMR1 stromal cell line ($D_0 = 2.8 \pm 0.1$, $p = 0.016$) (Figure 2A, Table 1). Analysis of the clonal sub-

lines demonstrated a similar pattern of greater radiosensitivity of the SAMP6 clonal sub-lines ($D_0 = 1.5 \pm 0.1$ Gy) compared with the SAMR1 clonal sub-lines ($D_0 = 1.9 \pm 0.1$, $p = 0.032$) (Figures 2B and 2C, Table 1).

Decreased proliferative capacity in vitro of SAMP6 bone marrow stromal cell lines and clonal sub-lines

Short-term assays were used to evaluate the proliferative capacity of the SAMP6 and SAMR1 stromal cell lines and several clonal sub-lines (Figure 3). Decreased proliferation was demonstrated in SAMP6 stromal cell lines compared with SAMR1 cells ($p < 0.0001$, Figure 3A). There was significantly decreased proliferation in the SAMP6 clonal sublines.

Comparison of constitutive gene expression in SAMP6 and SAMR1 stromal cell lines and clonal sub-lines

We evaluated stromal cell lines and several clonal sub-lines for constitutive expression of selected genes, including inhibitors of proliferation and markers associated with the known differentiation capacity of bone marrow stromal cells (also known as mesenchymal stem cells) (Figure 4A). The parent SAMP6 stromal cell line demonstrated constitutive upregulation of cell cycle inhibitors p16 and p19, the inhibitor of hematopoiesis TGF- β , and VDR and LDL, compared with the parent SAMR1 stromal cell line. There was also greater constitutive expression of osteoblast signature genes RUNX2, ALP, OC, and PTHR1 in SAMP6 stromal cells compared with SAMR1 stromal cell line. Constitutive gene expression analysis of clonal sub-lines showed results similar to the parent stromal cell lines in most respects (Figure 4B).

Comparison of osteoblastogenic potential of SAMP6 and SAMR1 stromal cell lines

Time-course of osteoblast differentiation in osteoblastogenic medium was monitored by gene expression analysis (Figure 5). For SAMP6 stromal cells, there was a time-dependent upregulation of RUNX2, OC, PTHR1, and SOST expression. For SAMR1 stromal cells, there was also upregulation of ALP and OC, with induction of SOST detectable only at day 7. At day 7, ALP activity was greater in both the SAMP6 and SAMR1 stromal cells in osteoblastogenic medium. There was higher ALP activity in SAMP6 in both basal and osteogenic medium (Figure 6A). These results are consistent with the gene expression analysis; matrix mineralization, however was impaired for SAMP6 stromal cells, compared to SAMR1 stromal cells ($p = 0.05$).

Properties of primary adherent marrow cells (AMCs) from SAMP6 and SAMR1 mice

Proliferative capacity of primary AMCs was assessed by CFU-F assays with bone marrow pooled from SAMR1 ($n = 7$) or SAMP6 ($n = 11$) female mice (7-months old). There were differences in colony size (Figure 7A), but the mean numbers of colonies per dish were similar from SAMP6 (23 ± 2) and SAMR1 mice (27 ± 1) (Figure 7B). There were significantly more cells per dish for AMC-SAMR1 (Figure 7C, $171.6 \pm 17.5 \times 10^3$, $p < 0.0001$) than for AMC-SAMP6 ($11.4 \pm 1.6 \times 10^3$) and there were significantly more cells per colony for AMC-SAMR1 (Figure 7D, $6.4 \pm 0.6 \times 10^3$, $p < 0.0001$) than for AMC-SAMP6 ($0.5 \pm 0.1 \times 10^3$).

Because of the constitutive expression of SOST in a SAMP6 clonal sub-line (Figure 4) and the early induction of SOST in the SAMP6 stromal cell line (Figure 5), constitutive expression of SOST was evaluated in AMCs from both mouse strains (5-month old male mice). There was constitutive expression of SOST in AMC-SAMP6 with none detectable in AMC-SAMR1 (Figure 8A). Osteoblast differentiation of AMCs was measured by alkaline phosphatase activity 14-days after transfer of half the cultures to osteoblastogenic medium

(Figure 8B). In basal medium, there was a trend for more ALP activity in AMC-SAMP6. Both AMC-SAMR1 and AMC-SAMP6 showed significantly increased ALP activity when cultured in osteoblastogenic medium ($p < 0.05$).

Discussion

The SAMP6 mouse model represents an opportunity to compare the process of aging with the biology of age-associated changes in marrow-derived cells. The results from this research show dysregulation of many properties of marrow-derived cells, including longevity of *in vitro* hematopoiesis, sensitivity to irradiation, proliferation potential, and constitutive expression of differentiation genes.

The experiments with long-term bone marrow cultures demonstrated that SAMP6 had reduced longevity of hematopoiesis compared with SAMR1 cultures. Initially, SAMP6 cultures had significantly higher production of cobblestone islands than the SAMR1 cultures. Those findings confirm and extend a study by Kajkenova *et al.* that demonstrated greater production of non-adherent GM-CFUs in long-term bone marrow cultures from SAMP6 mice than in cultures from SAMR1 mice, monitored for 10 weeks [7]. Although we too observed greater hematopoiesis in cultures from SAMP6 mice initially, by limiting use of horse serum to the first 4 weeks it was possible to extend the stability of the cells [36]; longer culture revealed cessation of hematopoiesis in SAMP6 cultures after approximately 22 weeks, but continuation of hematopoiesis in SAMR1 cultures for 40 weeks. Thus, there was reduced longevity of hematopoiesis in SAMP6 cultures, compared with SAMR1 cultures. It is notable that a previous comparison of longevity of hematopoiesis with marrow from 28 inbred strains and outbred stocks of mice revealed that the strain of mice with marrow showing the longest duration of *in vitro* hematopoiesis was AKR/J [21], the background strain from which the SAM lineages were derived [4]. We tested for constitutive expression of TGF- β_1 in the stromal cells because we had previously shown significantly increased longevity of *in vitro* hematopoiesis in the absence of TGF- β signaling [37]. Accordingly, the demonstrated upregulation of TGF- β_1 in SAMP6 stromal cells, compared with SAMR1 stromal cells, may contribute to the decreased longevity of hematopoiesis in SAMP6 long-term bone marrow cultures.

Reduced longevity of hematopoiesis in long-term bone marrow culture has been associated with diminished intrinsic antioxidant capacity [38], production of inhibitors of hematopoiesis, such as TGF- β [37], or consumption of antioxidants in response to external oxidative stress [19, 39]. Irradiation survival assays showed that the SAMP6 stromal cell line and clonal sub-lines were more radiosensitive than the SAMR1 cells. Both the diminished longevity of hematopoiesis in long-term bone marrow cultures and greater sensitivity to ionizing radiation of SAMP6 bone marrow stromal cells were consistent with reduced capacity to respond to oxidative stress.

Evidence with murine and human marrow cultures shows that irradiation induces increases in *p53*, *p21*, SA- β -gal, and apoptosis [40–43]. Irradiated bone marrow stromal cells, maintained in contact inhibition, continue to produce cytokines [22] and support hematopoiesis and, although there is no requirement for further cell division, the cells may then undergo delayed apoptosis [42]. Irradiated stromal cells show late effects of irradiation, in particular delayed proliferative capacity and reduced clonogenic capacity [19]. The common factor in both marrow stromal aging and the cellular response to ionizing irradiation is oxidative stress [44].

Upregulation of *p16^{INK4A}* and *p19^{ARF}* in SAMP6 stromal cells and clonal sub-lines is consistent with reduced proliferation of those cells compared with SAMR1-derived cells. In

mice, p16^{INK4A} and p19^{ARF} are tumor suppressor proteins that block cell cycle progression [45], the former by inhibiting cyclin D-dependent kinases, and the latter by co-regulation of p53 and Retinoblastoma protein (Rb) pathways [46]. We quantified proliferation of primary adherent marrow cells with colony assays because of their heterogeneity and resistance to passaging. Primary AMCs from SAMP6 marrow generated colonies but they contained far fewer cells (8%) than colonies from SAMR1 mice. This is inconsistent with Kajkenova *et al.*, who found for SAMR1 and SAMP6 marrow similar numbers of colonies with more than 50 cells, but the range of cellularity per colony was not reported [15]. These findings are consistent with Jilka *et al.* who determined by different methods that marrow from SAMR1 mice generated colonies that contained more cells [17].

Bone marrow stromal cells are often called mesenchymal stem cells because of multipotentiality when cultured in different pro-differentiation media [47, 48]. We expected to see little or no constitutive expression of the osteoblast genes, but there was expression of osteoblast signature genes, alkaline phosphatase, osteocalcin, the PTH receptor, and the vitamin D receptor in SAMP6 stromal cells and some clonal sub-lines. One clonal sub-line, SAMP6-1, and the AMCs from SAMP6 mice showed constitutive expression of sclerostin (*sost*), which is typically expressed in mature osteoblasts/osteocytes and functions as a negative regulator of bone formation by inhibition of the Wnt signaling pathway [31]. Although osteoblast markers were expressed in SAMP6-derived cells, production of sclerostin protein may result in inhibition of bone matrix production. Epigenetic dysregulation of osteoblast genes may contribute to their upregulation in *in vitro* senescence, a model in which late passage and early passage cells are compared. It was reported that with increased passage number, human marrow-derived mesenchymal stem cells showed upregulation of several osteoblast signature genes, including alkaline phosphatase and osteocalcin [49]. In that study, dramatic alterations in histone H3 acetylation coincided with osteoblast gene expression levels and are likely to play key roles in stem cell aging and differentiation.

In this study, ALP enzymatic activity was used as an index of the osteoblast phenotype. Consistent with ALP gene expression, the SAMP6 stromal line had ALP activity in basal medium, and both SAMP6 and SAMR1 lines had greater activity after culture in osteoblastogenic medium. Although there was constitutive dysregulation of osteoblast markers, SAMP6 stromal cells showed impaired matrix mineralization, as indicated by the alizarin red assay. This indicates that *in vitro* capacity for bone formation by SAMP6 stromal cells was diminished. Others reported that SAMP6 bone marrow had reduced capacity for bone formation [9]. Reduced proliferation of marrow stromal cells could give the appearance of fewer colonies; slower growth of SAMP6 colonies may have occurred in that study inasmuch as more than half of the SAMP6 colonies were small, in contrast to large colonies from SAMR1 marrow. Jilka *et al.* reported that marrow from SAMR1 had 3-fold more osteoblast potential than marrow from SAMP6 mice; they also indicated, however, that on a per-cell basis, there was no difference between the two mouse strains. Thus, the major influence was the difference in proliferation. Other studies with colony and monolayer assays [18, 19, 50] may have underestimated the effects of different proliferation rates of SAMP6 and SAMR1 stromal cells.

The most prominent age-related decrease in SAMP6 bone is the reduction of vertebral bone mass, with less striking differences for long bones [51]. Some studies attributed age-related bone loss in SAMP6 mice to reduced bone formation rates, but in fact, differences in measured bone formation rates *in vivo* between SAMP6 and SAMR1 mice were detected only in certain bones and only at certain ages [9, 11]. For both SAMP6 and SAMR1 mice, there was a general decrease in histomorphometric markers of endocortical bone formation in femurs from 2 to 4 to 6 months, but not for 12 months; although not statistically

significant, there was a trend for lower mineral apposition rates in SAMP6 than SAMR1 femurs, but it was the opposite for 6 month-old mice [19]. Silva *et al.* described those correlations as extremely weak and highly dependent on site and age [19]. Those observations and our data raise concerns about extrapolating osteogenesis *in vitro* to skeletal mass *in vivo*. We conclude that decreased proliferative capacity of SAMP6 osteoprogenitors may be the major factor contributing to osteopenia *in vivo*. It is also possible that upregulation of sclerostin, an inhibitor of bone formation, may contribute to osteopenia *in vivo*.

Diminished response to oxidative stress, which is accompanied by reduction in cellular antioxidant pools, principally glutathione, has been demonstrated in experiments that replicate the process of aging, including the negative effects of irradiation on *in vitro* hematopoiesis [18, 19]. Further, there is decreased capacity to respond to oxidative stress in animal models of defective aging of intrinsic defects in antioxidant production such as MnSOD [52, 53]. Anti-oxidant therapies ameliorate the acute and chronic toxicity of ionizing radiation exposure both *in vivo* [38, 54–56] and *in vitro* [39]. In addition, disruption of TGF- β signaling results in *in vivo* protection against ionizing radiation [57] and in increased *in vitro* hematopoiesis [37]. Anti-oxidant therapies were also shown to restore osteogenesis in a mouse model of impaired osseous wound healing subjected to local irradiation [58].

In conclusion, this report adds evidence from long-term bone marrow cultures, from radiation sensitivity studies, and from proliferation and gene expression assays with marrow from SAMP6 mice that are consistent with the oxidative stress mechanism of aging. Further, these studies and data included in other reports indicate that impaired proliferation of osteoblast progenitors in SAMP6 marrow may be the major factor contributing to low bone mass.

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Fig. 1.

Comparison of long-term bone marrow cultures from SAMP6 and SAMR1 mice. A) Confluence of the adherent layer was estimated weekly. Each point represents the mean of cultures from four SAMP6 and four SAMR1 mice. B) The number of cobblestone islands, which is indicative of adherent hematopoietic foci, was measured in each flask at weekly intervals. Each point represents the mean of cultures from four SAMP6 and four SAMR1 mice. C) Photomicrographs of long-term bone marrow cultures at week 30 shows the relative lack of cobblestone islands in cultures from (a,b) SAMP6 mice and persistence of cobblestone islands in cultures from (c,d) SAMR1 mice. At high magnification (d), the cobblestones appear as collections of small dark cells (black arrow) attached to the stromal cells, and cells being released into the medium appear as white refractile cells (white arrow). Scale bars indicate (a,c) 50 μm and (b,d) 25 μm . D) The number of colonies (groups of more than 50 cells) that formed in semi-solid medium was counted 14 days after weekly transfer of non-adherent cells from long-term bone marrow cultures. Each point represents the mean of triplicate cultures.

Fig. 2.

Radiosensitivity of SAMP6 and SAMR1 stromal cell lines and clonal sub-lines, determined with clonogenic radiation survival assays. A) Radiation survival curves of stromal cell lines derived from long-term bone marrow cultures show greater radiosensitivity of SAMP6 than SAMR1 cells. B) Radiation survival curves show individual radiation sensitivity of five clonal sub-lines derived from parent SAMP6 stromal cell line. C) Radiation survival curves show individual radiation sensitivity of five clonal sub-lines derived from parent SAMR1 stromal cell line.

Fig. 3.

Proliferation of bone marrow stromal cell lines and clonal sub-lines derived from SAMP6 and SAMR1 long-term bone marrow cultures. A) Short-term proliferation assays of parent stromal cell lines shows less expansion of SAMP6 than SAMR1 cells (n=6 wells each). B) Proliferation of five individual clonal sub-lines (n=4 wells) of SAMP6 stromal cell line shows a range, with bars smaller than the symbols for these samples. C) Proliferation of five individual clonal sub-lines (n=4 wells) of SAMR1 stromal cell line shows a range.

Fig. 4. Constitutive gene expression in SAMP6 and SAMR1 bone marrow stromal cell lines and clonal sublines, A) SAMP6 and SAMR1 bone marrow stromal cell lines and B) SAMP6-clone 1 through clone 5, and SAMR1-clone 5. Gel electrophoretogram shows RT-PCR products associated with cell cycle (p16, p19, p21, p53); osteoblast phenotype (Runx2, Alkaline Phosphatase, Osteocalcin, SOST); adipocyte phenotype (PPAR γ , Lipoprotein Lipase), and cytokines and receptors (TGF β ₁, PTHR1, VDR). GAPDH was included as an internal control.

Fig. 5.

Timecourse of osteoblast differentiation in SAMP6 and SAMR1 stromal cell lines. Lanes indicate the number of days after transfer to osteoblastogenic medium; NC = negative control, i.e. PCR reactions without cDNA. Gel electrophoretogram shows expression of osteoblast signature genes (Runx 2, ALP, OC, and SOST), adipocyte signature genes (PPAR γ , LPL), the receptors PTHR1 and VDR, and the housekeeping gene, GAPDH.

Fig. 6. Alkaline phosphatase Enzymatic activity and Alizarin Red in SAMP6 and SAMR1 stromal cell lines. A) At day 7 the ALP activity was measured in SAMP6 and SAMR1. There was an increase in ALP activity after 7 days in osteogenic medium with significantly higher expression of ALP evidenced in the SAMP6 stromal cells relative to SAMR1. B) The Alizarin Red assay was carried out at 16 days and shows impaired Matrix Mineralization in SAMP6 stromal cells relative to SAMR1. *** $p < 0.0001$, * $p < 0.05$

Fig. 7.

CFU-F assay and proliferative potential of AMCs. A) Photograph shows representative AMC cultures that were stained with toluidine blue (arrow: large colony; arrowhead: small colony). B) The mean number of CFU-F colonies per 35-mm dish was similar for AMCs from SAMR1 and SAMP6 mice (7 months old). C) The mean number of cells per dish was significantly lower for AMCs from SAMP6 mice than from SAMR1 mice. D) The mean number of cells per colony for AMCs from SAMP6 mice was significantly lower than from SAMR1 mice. *** $p < 0.0001$

Fig. 8.

Constitutive expression of SOST in AMCs and induction of alkaline phosphatase (ALP) activity in osteoblastogenic medium. A) AMCs from SAMP6, but not SAMR1 mice (5 months old) expressed SOST gene. Total RNA obtained from tibiae of SAMR1 and SAMP6 mice was used as positive controls. B) ALP activity in AMCs from both SAMR1 and SAMP6 mice was stimulated after 14 days in osteoblastogenic medium. There was significantly more ALP activity in AMCs from SAMP6 (n=3 dishes) than from SAMR1 mice (n=3). *p<0.05; **p<0.01

TABLE 1

Comparison of radiosensitivity of stromal cells lines and clonal sub-lines derived from long-term bone marrow cultures from SAMP6 and SAMR1 mice. Results were calculated with linear quadratic and single-hit, multi-target models and presented as D_0 (Gy, the dose required to reduce the fraction of surviving cells to 37%) and $\tilde{n}$ (extrapolation number derived by extension of the linear portion of the radiation survival curve to the ordinate of the graph utilizing a log/linear plot).

		D_0 (Gy)	$\tilde{n}$
Stromal Cell Lines	SAMP6	2.1 ± 0.1	3.3 ± 0.5
	SAMR1	2.8 ± 0.1	3.0 ± 0.8
	p (P6 vs. R1)	0.016	0.797
Clonal Sub-lines	SAMP6 (1–5)	1.5 ± 0.1	6.6 ± 1.4
	SAMR1 (1–5)	1.9 ± 0.1	9.4 ± 2.6
	p (P6 vs. R1)	0.032	0.352