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Circulating Osteogenic Cells: Characterization and Relationship to Rates of Bone Loss in Postmenopausal Women

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Abstract

There is increasing evidence that osteogenic cells are present not only in bone marrow (BM) but also in peripheral blood (PB). Since staining for alkaline phosphatase (AP) identifies osteoprogenitor cells in BM, we sought to further characterize BM versus PB hematopoietic lineage negative (lin[−])/AP⁺ cells and to compare gene expression in PB lin[−]/AP⁺ cells from postmenopausal women undergoing rapid versus slow bone loss. PB lin[−]/AP⁺ cells were smaller than their BM counterparts, and both were negative for the pan-hematopoietic marker, CD45. BM and PB lin[−]/AP⁺ cells were capable of mineralization *in vitro*. Using whole genome linear amplification followed by quantitative polymerase chain reaction (QPCR) analysis, we found that relative to the BM cells, PB lin[−]/AP⁺ cells expressed similar levels of a number of key osteoblast marker genes (runx2, osterix, osteopontin, OPG, periostin), consistent with the PB cells being in the osteoblastic lineage. Importantly, however, compared to the BM cells, PB lin[−]/AP⁺ cells expressed lower levels of mRNAs for AP, type I collagen, and for a panel of proliferation markers, but higher levels of osteocalcin, osteonectin, and PTHR1 mRNAs, as well as those for RANKL and ICAM-1, both of which are important in supporting osteoclastogenesis. Using microarray followed by QPCR analysis, we further demonstrated that, compared to postmenopausal women undergoing slow bone loss, PB lin[−]/AP⁺ cells from women undergoing rapid bone loss expressed lower levels of mRNAs for hydroxyprostaglandin dehydrogenase, interferon regulator factor 3, Wnt1-induced secreted protein 1, and TGFβ2, but higher levels of the Smad3 interacting protein, zinc finger DHHC-type containing 4 and coll1α2. These data thus demonstrate that while PB lin[−]/AP⁺ cells express a number of osteoblastic genes and are capable of mineralization, they are a relatively quiescent cell population, both in terms of cell proliferation and matrix synthesis. However, their higher expression of RANKL and ICAM-1 mRNAs as compared to BM lin[−]/AP⁺ cells suggests a role for the PB lin[−]/AP⁺ cells in regulating osteoclastogenesis that warrants further investigation. Our study also provides “proof-of-concept” for the use of PB lin[−]/AP⁺ cells in clinical-investigative studies, and identifies several pathways that could potentially regulate rates of bone loss in postmenopausal women.

Keywords

osteoporosis; bone loss; menopause; osteoblast; progenitors

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INTRODUCTION

A number of potential markers for the identification of multipotent bone marrow stromal cells have been developed in recent years [1]. While alkaline phosphatase (AP) is expressed by mature osteoblasts, recent studies have demonstrated that the AP⁺ fraction of human bone marrow (BM) contains virtually all of the osteoprogenitor population in the marrow [2], indicating that AP expression is associated not only with mature osteoblasts, but likely also with osteoblastic cells at varying stages of differentiation, including relatively early mesenchymal cells.

Although the presence of early mesenchymal and osteoprogenitor cells in BM is well established, the issue of whether there are similar, or at least related, populations in peripheral blood (PB) remains controversial. Based largely on work by Long and colleagues [3-6] who used cell sorting with antibodies to osteocalcin (OCN) and AP to identify non-adherent BM cells with osteogenic potential, we used flow cytometry following staining with antibodies to these markers to identify OCN⁺ and AP⁺ cells in human PB [7,8]. Subsequent studies by others have provided further support for the existence of circulating osteoprogenitor cells, although the precise relationship of these circulating cells to their BM counterparts remains to be fully defined. Thus, Otsuru et al. [9] showed that BM cells were recruited via the circulation into BMP-2 containing collagen pellets and contributed to ectopic bone formation. Subsequently, this group demonstrated that the PB cells mobilized in response to BMP-2 were negative for the pan-hematopoietic marker, CD45, and that a major chemokine for recruitment of these cells was stromal cell-derived factor-1 (SDF-1), which was induced locally by BMP-2 [10]. Interestingly, the ability to form bone may not strictly be limited to mesenchymal cells. For example, Suda et al. [11] have recently demonstrated that a population of circulating CD45⁺ cells that express type I collagen can also form bone both *in vitro* and *in vivo*. These cells are present in the circulation of normal subjects, but increased in PB of subjects with fibrodysplasia ossificans progressiva.

Recent studies have also demonstrated that BM cells expressing the smooth muscle cell marker, α -smooth muscle actin (α SMA) [12], and pericytes surrounding marrow vessels [13] may represent true mesenchymal cells with osteoblastic potential. Thus, Kalajzic et al. [12] found that in a mouse model of osteoblast ablation, repopulation of bone surfaces by osteoblasts was preceded by expansion of an α SMA⁺ population of cells that subsequently differentiated into osteoblasts. In addition, Sacchetti and colleagues [13] showed that subendothelial (pericytic) cells expressing MCAM/CD146 were capable of forming bone and providing a niche for hematopoiesis in heterotopic sites.

In the present study, we focused on hematopoietic lineage negative (lin⁻) cells (which should be enriched for mesenchymal cells) that also expressed AP and sought to identify possible similarities or differences between BM versus PB lin⁻/AP⁺ populations. We first characterized these cells for their ability to mineralize, followed by a systematic comparison of expression of key osteoblastic, proliferation, smooth muscle cell, pericytic, and other genes. Finally, to begin to apply these methods in clinical investigation for bone diseases, we performed a study to test whether postmenopausal women undergoing rapid trabecular bone loss had differences in the expression of specific genes, including candidate genes related to bone metabolism, in PB lin⁻/AP⁺ cells as compared to cells from similar aged women undergoing slow bone loss.

METHODS

Study subjects

All studies were approved by the Mayo Clinic's Institutional Review Board, and the study subjects provided written, informed consent. BM and PB samples were initially obtained from

normal healthy female volunteers for flow cytometry analysis and mineralization assays ($n = 7$, mean age, 61 years). Following local anesthesia, 30 ml of BM aspirate was collected in an anticoagulant (EDTA) from the iliac crest using a standard procedure, and 250 ml of blood from each subject was also collected in EDTA. For the clinical study, we selected 20 postmenopausal subjects between 50 and 80 years of age from an ongoing longitudinal study [14]. The study subjects were identified based on the rate of bone loss determined by quantitative computed tomography (QCT) of the lumbar spine done at baseline, 3, and 6 years. Details regarding this cohort and methods for the QCT analysis have previously been published [14,15]. None of the subjects were on bisphosphonates, estrogen, calcitonin, teriparatide, glucocorticoids, or other drugs known to affect bone metabolism. None had any significant medical comorbidities (e.g., underlying malignancy, congestive heart failure, renal failure, etc). Subjects were grouped into either the rapid or slow cohort by selecting individuals meeting the above criteria and at the two extremes for rates of vertebral trabecular volumetric bone mineral density (vBMD) bone loss. The subjects underwent a fasting blood draw of 250 ml in the outpatient Clinical Research Unit, following which the blood samples were processed as described below for obtaining the relevant cell populations and serum assays.

For the comparison of gene expression in PB versus BM lin^-/AP^+ cells, we selected 6 subjects (3 from the rapid loss group with the lowest rates of vertebral bone loss and 3 from the slow loss with the highest rates of vertebral bone loss, so as to avoid the extremes of bone loss for this comparison of PB versus BM lin^-/AP^+ cells) and performed the detailed quantitative polymerase chain reaction (QPCR) assays described below. Since we did not obtain BM samples in these subjects, we used BM samples from untreated control subjects obtained as part of an ongoing study examining effects of PTH 1-34 on gene expression in BM lin^-/AP^+ cells. From this untreated control group, we selected 6 subjects in a similar age range to those on whom we had processed the PB lin^-/AP^+ cells.

Mononuclear cell (MNC) isolation and flow cytometry analysis

Following Ficoll extraction, MNCs from BM and PB were labeled with a biotinylated anti-human AP monoclonal antibody (Ab) (B4-78) [16] and an isotype matched control, both from R & D Systems (Minneapolis, MN). Subsequently, the cells were stained with a PE-conjugated Streptavidin antibody (eBioscience, San Diego, CA) and a FITC-conjugated lineage (lin) cocktail of monoclonal Abs (BD Biosciences, San Jose, CA). The labeled samples were immediately analyzed on the Becton Dickinson FACScan flow cytometer (San Jose, CA). On flow analysis, the gate was determined on a forward versus side scatter dot plot on the live cell population in the lymphocyte/monocyte-enriched area. Using this specific gate and based on the coexpression of AP and lin markers, the lin^-/AP^+ population was defined. The percentage of lin^-/AP^+ cells was compared against the isotype control, and the percent positive lin^-/AP^+ fraction was defined by subtraction. In some analyses, we also costained for AP and the pan-hematopoietic marker, CD45, labeled with FITC (BD Biosciences, San Jose, CA).

Magnetic Activated Cell Sorting (MACS)

For cell separation using MACS, post-Ficoll BM and PB cells were magnetically labeled with a human hematopoietic lineage cell depletion kit (Miltenyi Biotec GmbH, Germany) containing Abs to CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and glycophorin A, according to the manufacturer's instructions. After one wash, the cell suspension was loaded onto an autoMACS cell sorter (Miltenyi Biotec GmbH, Germany). Subsequently, lin^- BM and PB cells were MACS-sorted using a biotinylated anti-human AP monoclonal Ab (R & D Systems, Minneapolis, MN) and anti-biotin beads (Miltenyi Biotec GmbH, Germany). MACS sorted lin^-/AP^+ cells from BM and PB were then stored in RLT buffer at -80°C for later extraction of RNA, as described below.

Osteogenic cultures

MACS-sorted lin⁻/AP⁺ BM cells were cultured in plastic plates at a plating density of 2.5×10^5 per cm² in medium containing phenol red-free α MEM, 10% FBS and 1% penicillin-streptomycin mixture, all obtained from Invitrogen (Carlsbad, CA). After one week, the medium was replaced with osteogenic differentiation medium containing phenol red-free α MEM, 10% FBS, 50 μ M ascorbic acid, 10^{-8} dexamethasone and 10 mM β -glycerolphosphate. The cultures were maintained for 21 days, fixed in 10% formalin, and then stained for calcium deposits using 2% alizarin red. MACS-sorted lin⁻/AP⁺ PB cells were suspended in growth medium (MesenCult Basal Medium, Stem Cell Technologies, Vancouver, BC, Canada) containing 10% FBS and 1% penicillin-streptomycin mixture and plated in fibronectin-coated plates (Becton Dickinson, San Jose, CA) at a plating density of 3.5×10^5 per cm². On day 21 the medium was changed to differentiation medium containing MesenCult Basal Medium with 15 percent osteogenic stimulatory supplements, 3.5 mM β -glycerolphosphate, 10^{-8} M dexamethasone, and 50 μ g/ml ascorbic acid, all purchased from StemCell Technologies (Vancouver, BC, Canada). After three weeks of differentiation, the cells were stained for alizarin red as described above. Throughout the culture of the PB cells, the total media with the non-adherent cell fraction from each well was aspirated once a week, spun down at 1300 rpm for 8 minutes at 4°C, and the non-adherent cells with fresh media were added back to the respective wells.

RNA extraction and cDNA preparation

Total RNA was isolated using spin columns (Micro columns, Qiagen, Valencia, CA). DNase treatment to digest all genomic DNA that could lead to false positive gene expression results was done following RNA isolation using Turbo DNA-free DNase (Ambion, Austin, TX). The RNA quantity and purity was measured with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and the RNA integrity was determined using the Agilent 2100 Bioanalyzer (Santa Clara, CA). Because the overall number of the lin⁻/AP⁺ cells was low and therefore the yield of total RNA was limited to run in-depth gene expression analyses, we used the WT-OvationTM Pico RNA amplification system (NuGEN, Technologies, Inc, San Carlos, CA) to synthesize g quantities of amplified cDNA starting with total RNA input amounts of ~20 ng. In this linear amplification system, the relative representation of each transcript species in the original sample is maintained during and after amplification [17,18].

Microarray analysis

Microarray analysis was done using the Affymetrix HG-U133 Plus2 GeneChip Array platform (Affymetrix, Santa Clara, CA), per the manufacturer's instructions.

QPCR analysis

For the QPCR analyses, we designed primers using the Primer Express program (Applied Biosystems, Foster City, CA). Primer sequences for any of the genes analyzed in this report are available on request. The primers (1.8 pmol/reaction, MWG-Biotech AG, Ebersberg, Germany) were applied to 384-well PCR-plates in a sucrose solution (30% sucrose, 1mM Tris pH 8.5) and dried onto the plates for 1 hour in a heated speed vacuum. The PCR reactions was then run in the ABI Prism 7900HT Real time System (Applied Biosystems, Foster City, CA) using SYBR Green (BioRad, Hercules, CA) as the detection method. 5 ng of the amplified cDNA was used per reaction and each gene was run in triplicate in a total volume of 10 μ l. Negative Reverse Transcriptase controls were included to check for genomic DNA contamination. Normalization for variations in input RNA was performed using a panel of 10 housekeeping genes (18S, G6PDH, GAPDH, HPRT, L13a, RPII, TBP, α -tubulin, β 2-microglobulin, β -actin) with the geNorm algorithm (<http://medgen.ugent.be/~jvdesomp/genorm/>) [19,20] used to select the 3-4 most stable

housekeeping genes from the 10 on the plate. The PCR Miner algorithm [21] was used to correct for variations in amplification efficiencies.

Serum analysis

All serum analyses were performed on the same blood sample as obtained for isolation of the PB lin⁻/AP⁺ cells. The serum bone resorption markers, C-telopeptide of type I collagen (CTX) and tartrate-resistant acid phosphatase 5b (TRACP5b) were measured by ELISA (Immunodiagnostic Systems, Fountain Hills, AZ) with interassay coefficients of variation (CVs) <10% and 4%, respectively. The bone formation marker, amino-terminal propeptide of type I procollagen (PINP), was measured using a radioimmunoassay (Immunodiagnostic Systems, Fountain Hills, AZ) with an interassay CV of 9%. Serum estradiol (E₂) was measured using mass spectroscopy, as previously described [22], with an interassay CV of < 15%. Serum prostaglandin E₂ was measured using a high sensitivity competitive binding immunoassay (R & D Systems, Minneapolis, MN, (interassay CV < 11%) following extraction using C₁₈ reverse phase column extraction. Serum interferon (IFN) α and IFN β were quantified using sandwich immunoassays (PBL Interferon Source, Piscataway, NJ, interassay CVs < 8%). Serum IFN γ and TGF β ₂ were measured using quantitative sandwich enzyme immunoassays (R & D Systems, Minneapolis, MN, interassay CVs < 8%). Serum OPG was measured using a quantitative immunoassay (Quidel Corporation, San Diego, CA, interassay CV, 6%).

Statistical methods

For analysis of the microarray data, initial pre-processing and normalization was followed by an assessment for the adequacy of normalization and chip quality. Low or unexpressed genes were then filtered and a comparison between groups for differentially expressed genes was made using t-tests following a log₂ transformation. Using the criteria for a log₂ fold difference of > 3 standard deviations (SDs) and P < 0.05, 30 genes were selected for further validation using QPCR. Since much of the QPCR data was not normally distributed, for consistency, we present all data as the median, 25th-75th percentile (Interquartile Range [IQR]). Comparisons between the slow and rapid bone loss groups were made using the Wilcoxon rank-sum test. Spearman correlations were used for the regression analysis, and P < 0.05 was considered significant.

RESULTS

Expression of AP on BM and PB lin⁻ cells

Figure 1A shows a representative example of a flow analysis using the isotype control and AP antibodies. In addition, Figure 1B shows that MACS-sorted lin⁻/AP⁺ cells were markedly enriched (by over 50-fold) for the AP mRNA as compared to lin⁻/AP⁻ cells. These findings thus indicate that using this well established AP antibody (B4-78) [16], we are able to identify cells expressing AP.

We next examined the coexpression of AP and hematopoietic lineage markers using a lineage cocktail that contained antibodies to identify hematopoietic cells (anti-CD3, CD14, CD16, CD19, CD20, and CD56) on freshly isolated BM MNCs by flow cytometry. As is evident from Figure 2A, a significant proportion of AP⁺ cells in BM also expressed one or more hematopoietic lineage markers recognized by the lineage cocktail. Thus, we used MACS to deplete the hematopoietic cell population from the freshly isolated BM MNCs. In order to confirm the depletion of lin⁺ cells by this method, we labeled the lin⁻ and lin⁺ BM fractions with a streptavidin-PerCP conjugated antibody (which will bind to any cell labeled with one of the biotinylated hematopoietic lin Abs). As expected, compared to the lin⁺ fraction, the presence of streptavidin-PerCP in the lin⁻ fraction was negligible (Fig. 2B), indicating that the MACS was effectively depleting cells expressing hematopoietic lineage markers.

We next analyzed AP expression on MACS-sorted lin⁻ cells obtained from BM and PB. On average, from ~100 million BM or PB MNCs, we obtained 4.5 and 1.1 million lin⁻ cells, respectively; following sorting for AP, typical yields were 240,000 and 150,000 lin⁻/AP⁺ cells from BM and PB, respectively. However, as shown in Figures 3A and B, based on forward versus side scatter characteristics (higher forward scatter is associated with larger cells), while the BM lin⁻/AP⁺ cells had a spectrum of forward scatter, PB cells tended to have very low forward scatter, consistent with a small cell size, which was also evident by light microscopy (data not shown).

To further verify that the lin⁻/AP⁺ fraction obtained from BM and PB was depleted of hematopoietic cells, we assessed coexpression of CD45, a pan-hematopoietic cell marker, on lin⁻/AP⁺ cells. As shown in Figure 3, only 0.2% of BM lin⁻ cells (Figure 3C) and 0.3% of PB lin⁻ cells (Figure 3D), which represents 12.5% and 10% of lin⁻/AP⁺ cells in BM and PB, respectively, were positive for CD45 staining.

Mineralization potential of lin⁻/AP⁺ cells

To determine the potential of BM and PB lin⁻/AP⁺ cells to mineralize *in vitro*, we cultured these cells under osteogenic conditions. For this, MACS-sorted lin⁻/AP⁻ and lin⁻/AP⁺ cells from BM were cultured on plastic plates in growth medium for a week followed by culture in osteoblast differentiation medium. At the end of four weeks, the cells were stained with alizarin red to assess calcium deposition. While BM lin⁻/AP⁻ cells failed to survive *in vitro* beyond a few days under these conditions (Figure 3A), the BM lin⁻/AP⁺ cells were fully capable of mineralizing (Figures 3B, C, and D). PB lin⁻/AP⁻ or lin⁻/AP⁺ cells did not grow on standard plastic plates, but when placed on fibronectin-coated plates for 3 weeks in growth medium followed by osteoblast differentiation medium, both cell populations remained viable. As shown in Figures 3E and F, PB lin⁻/AP⁻ cells showed minimal staining with alizarin red; by contrast, PB lin⁻/AP⁺ cells showed clear alizarin red staining (Figures 3G and H). These data thus demonstrate that within the PB lin⁻ population, selection for AP⁺ cells enriches for an osteogenic population capable of mineralization. There may, however, be some low level of mineralization by PB lin⁻/AP⁻ cells, since the lin⁻/AP⁻ fraction of PB may well be enriched for mesenchymal cells not expressing significant amounts of AP at the time of harvesting, but nonetheless capable of minimal mineral deposition following *in vitro* culture. It is also important to note that the extent of mineralization even by the PB lin⁻/AP⁺ cells was clearly less than that present in the BM lin⁻/AP⁺ cultures (compare Figures 3C, D with 3G, H).

Comparison of gene expression between BM and PB lin⁻/AP⁺ cells

Table 1 shows a detailed comparison of the expression of key genes by PB lin⁻/AP⁺ cells, normalized to the BM lin⁻/AP⁺ cells. As is evident, while a number of osteoblast marker genes (runx2, osterix, osteopontin, OPG, periostin) were expressed at similar levels between the 2 populations, there were important differences. Thus, AP, col1 α 1, and col1 α 2 mRNA levels were markedly lower in PB as compared to BM cells; by contrast, the PB cells expressed higher levels of OCN, osteonectin, PTHR1, and RANKL. Interestingly, virtually all of the proliferation marker genes assayed were expressed at significantly lower levels in PB as compared to BM cells, consistent with the PB cells being a quiescent cell type. PB cells also expressed significantly higher levels of a number of smooth muscle cell marker genes (α SMA, cald1, calponin1), but generally lower levels of pericyte markers. ICAM-1, which may be critical for the support of osteoclastogenesis and is expressed by quiescent cells lining bone surfaces [23,24], was expressed at significantly higher levels by the PB cells. PB cells also expressed higher levels of a number of integrins, particularly beta 3 and beta 5. The PB cells expressed significantly lower levels of the adipocytic marker genes, adiponectin and PPAR γ 2. The cartilage marker, sox9, was also expressed at lower levels in the PB cells,

although this was not statistically significant. Expression of the muscle marker, myoD, was similar in the two populations.

Assessment of gene expression in PB lin⁻/AP⁺ cells in postmenopausal women

In order to assess the utility of our methods for quantifying gene expression in circulating osteogenic cells, we next sought to evaluate whether expression of specific genes by PB lin⁻/AP⁺ cells differed between postmenopausal women undergoing rapid loss versus slow loss of trabecular bone at the lumbar spine. Table 2 provides a summary of the clinical characteristics of these subjects. The two groups were similar in age, but based on the selection of the subjects, rates of loss in vertebral trabecular, femoral neck total, and femoral neck trabecular vBMD were significantly greater in the rapid loss as compared to the slow loss group. Bone turnover makers were higher and serum E₂ levels were lower in the rapid loss as compared to the slow loss group, but none of these differences achieved statistical significance.

From the initial microarray analysis, we identified 30 genes that were differentially expressed by lin⁻/AP⁺ cells in the two groups, using the criteria noted earlier of a > 3 SD-fold difference and $P < 0.05$. These 30 genes, along with a panel of candidate genes that we hypothesized might differ between the groups, including genes related to osteoblast differentiation (runx2, osterix, AP, osteocalcin, osteonectin, osteopontin, coll α 1, coll α 2), potential Wnt targets (lef1, tcf7, BMP4, IGF-1, IGF-2, EphB4, CCN1/Cyr61, connexin43, survivin, versican, WISP1), BMP targets (id1, id2, areb6, hes-1, lysyl oxidase, sox4, smad1, smad5, tieg), TGF β family (TGF β 1, 2, and 3), and cytokines potentially regulating bone turnover (OPG, RANKL, IL1 α , IL1 β , IL-1ra, IL6, interferony, TNF α , M-CSF) were compared using QPCR in the lin⁻/AP⁺ cells from the slow loss and rapid loss groups. Using QPCR, we were able to independently validate 3 genes identified in the initial microarray (Table 3): hydroxyprostaglandin dehydrogenase (HPGD) and interferon (IFN) regulatory factor 3 (IRF3) were expressed at significantly lower levels, and zinc finger, DHHC-type containing 4 (ZDHHC4) was expressed at significantly higher levels in the rapid loss as compared to the slow loss subjects. In addition, from our candidate genes, we identified Wnt1-induced secreted protein 1 (WISP1) as being expressed at markedly lower levels (at ~1%), TGF β 2 as being expressed at significantly lower levels, and coll α 2 as being expressed at significantly higher levels in the rapid loss as compared to the slow loss subjects. Coll α 1 mRNA levels were also higher in lin⁻/AP⁺ PB cells in the rapid loss (median [IQR], 122.4 [5.64-232.9]) versus the slow loss subjects (1.00 [0.55-54.66]), but due the large variability between subjects, this did not achieve statistical significance ($P = 0.090$). As also shown in Table 3, OPG mRNA levels were over 3-fold higher in cells from the rapid as compared to the slow loss group, with a P-value (0.052) that was just at the level of statistical significance. By contrast, RANKL mRNA levels in the slow loss group (1.00 [0.81, 1.14]) were virtually identical to those in the rapid loss group (1.05 [0.97, 1.89]) ($P = 0.364$).

To further validate the gene expression data, we tested for possible correlations between the levels of these 6 genes and rates of change in vBMD at the 3 skeletal sites in all 20 subjects combined. As shown in Table 4, HPGD mRNA levels were significant associated with rates of change in femoral neck trabecular vBMD; IRF3 mRNA levels were significantly associated with rates of change in vertebral trabecular, femoral neck total, and femoral neck trabecular vBMD; ZDHHC4 mRNA levels were inversely associated with femoral neck trabecular vBMD changes; WISP1 mRNA levels were positively associated with vertebral and femoral neck trabecular vBMD changes; and TGF β 2 mRNA levels were positively associated with femoral neck total and trabecular vBMD changes. As an example, Figure 4 shows the plots of the relationship between IRF3 mRNA levels and rates of change in vertebral trabecular (Figure 4A), femoral neck total (Figure 4B), and femoral neck trabecular (Figure 4C) vBMD. None of the mRNA levels were correlated with any of the bone turnover markers (data not shown).

We next tested whether, based on several of the genes differentially expressed in the rapid versus slow bone loss groups, there were differences in serum levels of proteins related to each of the genes/pathways identified. For example, the higher *collα2* (and trend for higher *collα1*) mRNA levels in the rapid as compared to the slow loss groups were associated with ~20% higher serum PINP levels in the rapid bone loss group (Table 2). Based on the mRNA data in Table 3, we measured serum levels of prostaglandin E₂ (PGE₂, since HPGD is involved in its metabolism, see Discussion), IFNα, β, and γ (since IRF3 modulates IFN signaling, see Discussion), TGFβ₂, and OPG. We could not measure serum WISP1 levels since there is currently no assay available for this. As shown in Table 5, however, despite the fact that each of these genes was differentially expressed in PB lin⁻/AP⁺ cells from the rapid versus the slow bone loss groups, serum levels of PGE₂, IFNα, IFNβ, IFNγ, or TGFβ₂ did not differ between the two groups. Consistent with the gene expression data, serum OPG levels were ~25% higher in the rapid as compared to the slow bone losers, but this difference did not achieve statistical significance. We also correlated, using data from all 20 subjects, serum levels of these protein markers with mRNA levels in the PB lin⁻/AP⁺ cells. As shown in Table 6, IRF3 were significantly negatively correlated with serum IFNγ levels; there were trends for associations between mRNA levels for TGFβ₂ and OPG and the corresponding serum measurements, but these were not statistically significant.

DISCUSSION

We demonstrate in the current study that both BM and PB harbor lin⁻/CD45⁻ cells that express AP. The PB cells are smaller in size, and while both BM and PB lin⁻/AP⁺ cells can mineralize *in vitro*, mineralization is more robust in the BM cultures. Moreover, we were unable to culture the PB lin⁻/AP⁺ cells on plastic plates, and they required fibronectin-coated plates for survival *in vitro*. We should also note that using fairly standard conditions for culturing BM stromal cells [2], we were unable to culture osteogenic cells in the BM lin⁻/AP⁻ fraction, consistent with recent work by Gronthos and colleagues [2] demonstrating that selection for AP expression pulls out virtually all of the stromal cells from BM aspirates. However, it is possible that using different culture conditions than those used in the present study, multipotent stromal cells could be cultured from the BM lin⁻/AP⁻ population.

The detailed comparison of gene expression between the BM and PB lin⁻/AP⁺ cells was instructive in terms of providing insights into possible similarities and differences between the two cell populations. A number of key osteoblast marker genes (*runx2*, *osterix*, *osteopontin*, *OPG*, *periostin*) were expressed at approximately similar levels in the PB and BM cells, consistent with the PB cells being in the osteoblastic lineage. Further support for this is the fact that a number of osteoblastic genes (*OCN*, *osteonectin*, *PTHr1*, and *RANKL*) were, in fact, expressed at significantly higher levels in the PB as compared to the BM cells. Interestingly, genes critical for active bone formation and mineralization (*collα1*, *collα2* and *AP*), while present in the PB lin⁻/AP⁺ cells, were expressed at much lower levels in these cells as compared to their BM counterparts. Virtually all of the proliferation markers examined were suppressed in the PB cells compared to the BM cells. Collectively, these data indicate that the PB lin⁻/AP⁺ cells, while in the osteoblast lineage, are a relatively quiescent cell population, both in terms of cell proliferation and matrix synthesis. These findings may explain the difficulty others have had in culturing osteogenic cells from PB under conditions used to culture BM osteoblastic cells [25].

Of interest, the PB lin⁻/AP⁺ cells did express higher levels of *RANKL* and *ICAM-1* mRNA as compared to the BM cells. While the role of *RANKL* in supporting osteoclast development is well recognized [26], previous studies have found that *ICAM-1* may also be a critical molecule for osteoblastic support of osteoclastogenesis. In fact, our data are remarkably consistent with previous work by Tanaka and colleagues [23] who demonstrated that

osteoblastic cells from human trabecular bone samples could be sorted into ICAM-1+ and ICAM-1- fractions and that the ICAM-1+, but not ICAM-1-, cells expressed RANKL and could support osteoclast development. Moreover, similar to our findings with the PB lin⁻/AP⁺ cells, the ICAM-1+ cells from trabecular bone were growth-arrested. In subsequent studies, Everts et al. [24] have shown that, like the PB lin⁻/AP⁺ cells, the ICAM-1+ osteoblastic cells express low levels of type I collagen, and that these cells are present on quiescent bone surfaces; by contrast, matrix-synthesizing osteoblasts are ICAM-1- and produce large amounts of collagen. Of interest, recent studies by Xie et al. [27] have also found that osteoblastic cells forming the hematopoietic niche *in vivo* appear to be distinct from cells that express GFP driven by a collagen promoter (col 2.3), suggesting that osteoblastic cells that support hematopoiesis may also be a different population from matrix-synthesizing osteoblasts. Taken together, these data indicate that there likely are sub-populations of osteoblastic cells: active, proliferating, matrix synthesizing osteoblasts, and quiescent RANKL+ and ICAM-1+ cells that may be involved in supporting osteoclastogenesis and/or hematopoiesis. Our gene expression data would suggest that the PB lin⁻/AP⁺ cells are more related to the latter cell type, and point to the need for further studies testing the ability of these cells to support osteoclastogenesis and/or hematopoiesis. A caveat to be noted in terms of the gene expression data comparing BM versus PB lin⁻/AP⁺ cells, however, is that the specific microenvironment of the marrow or circulation may be having a significant impact on the gene expression profile of BM versus PB lin⁻/AP⁺ cells, so the differences in gene expression we observed may or may not be cell autonomous.

Relative to the BM lin⁻/AP⁺ cells, the PB cells also expressed higher levels of mRNAs for a number of smooth muscle cell markers, but lower levels of pericyte markers. Interestingly, a number of integrins, particularly beta 3 and beta 5, were expressed at much higher levels in the PB relative to the BM cells. PB cells also expressed lower levels of adipocytic (adiponectin, PPAR γ 2) and cartilage (sox9) markers. The significance of these findings is currently unclear, but they do point to some further differences between the PB and BM lin⁻/AP⁺ cell populations.

While clearly much remains to be learned about the biology of circulating cells expressing osteoblastic markers, we sought nonetheless to begin to apply our methods in clinical-investigative studies. For this, we selected 2 groups of women who, over the previous 6 years, had exhibited markedly different rates of bone loss. Using a combination of microarray and QPCR approaches, we identified 7 potential candidate genes whose expression differed in lin⁻/AP⁺ cells harvested from the PB of these subjects. In addition, the mRNA levels for a number of these genes showed significant correlations with rates of bone loss in the study subjects. The potential significance of each of these genes to bone metabolism is reviewed briefly below.

HPGD (15-hydroxyprostaglandin dehydrogenase), which was expressed at lower levels in the rapid as compared to the slow losers, is a key prostaglandin catabolic enzyme that catalyzes the initial oxidation of the prostaglandin E2 15(S)-hydroxyl group to generate a 15-keto metabolite, which has greatly reduced prostaglandin activity [28]. There is an extensive literature on effects of prostaglandins on bone metabolism (reviewed in [29]), and inactivating mutations in the HPGD gene are known to result in primary hypertrophic osteoarthropathy (or pachydermoperiostosis) [28,30]. This disorder affects the skeleton and the skin and manifests with digital clubbing, periostosis, acro-osteolysis, cranial suture defects, coarse facial features, and congenital heart disease.

Also expressed at lower levels in PB lin⁻/AP⁺ cells from the rapid losers was IRF3 (IFN regulatory factor 3), which is a member of the IFN regulatory transcription factor family. IRF3 forms a complex with the cyclic-AMP response element binding protein (CREBBP), which activates the transcription of IFNs α and β , as well as the IFN-stimulated response element

(ISRE) of some IFN-stimulated genes [31]. IFN γ has been shown to regulate osteoclastogenesis [32], and Duque et al. [33] recently demonstrated that IFN γ plays an important role during the commitment of BM stromal cells into the osteoblastic lineage both *in vitro* and *in vivo*.

Expression of TGF β 2 was also lower in PB lin $^{-}$ /AP $^{+}$ cells from the rapid losers. Members of the TGF β family are known to be key regulators of bone metabolism (for review, see [34]) and of interest, recent studies suggest an important role for TGF β in estrogen-deficiency mediated bone loss [35]. By contrast, mRNA levels for the Smad3 (a component of the TGF β signaling pathway [36]) binding protein, ZDHHC4 [37], were higher in cells from the women undergoing rapid bone loss. While it is of interest that the protein encoded by this gene may be involved in TGF β signaling, little is known regarding how ZDHHC4 may modulate cellular responses to TGF β .

WISP1 (Wnt1-induced secreted protein 1) is a member of the CYR61/CTGF family of growth factors and is known to play a role in mediating cell growth, transformation and survival [38]. We found lower levels of WISP1 in the rapid loss as compared to the slow loss group, which is of interest since French et al. [39] have shown that WISP1 expression promotes mesenchymal cell proliferation as well as BMP-2-induced osteoblastic differentiation *in vitro*. WISP1 may also play an important role during bone development and fracture repair [39].

We also found that the mRNA for coll1 α 2 was significantly higher in lin $^{-}$ /AP $^{+}$ cells from PB of women undergoing rapid as compared to slow bone loss, with a similar trend for the coll1 α 1 mRNA levels. The rapid losers also tended to have higher levels of serum PINP levels, consistent with their increased level of bone turnover. While these findings perhaps do not provide additional mechanistic insights, they do support the biological relevance of the changes in gene expression between the two groups that we observed. OPG mRNA levels were also higher in the rapid as compared to the slow bone loss group, although this difference was just at the level of statistical significance. Nonetheless, these findings are consistent with previous data indicating an increase in OPG production associated with increased bone turnover and bone loss [40], perhaps as a compensatory mechanism.

Interestingly, despite the significant differences in mRNA levels we found in PB lin $^{-}$ /AP $^{+}$ cells from women in the rapid versus slow bone loss groups, none of the serum protein measures (PGE $_2$, IFN α , β , γ , TGF β 2, OPG) differed significantly between groups. These findings suggest that assessing gene expression in PB lin $^{-}$ /AP $^{+}$ cells may represent a more sensitive tool as compared to serum protein biomarkers for evaluating the pathogenesis of osteoporosis and other metabolic bone diseases, although further studies addressing this clearly need to be done.

As noted earlier, recent work by Suda et al. [11] has demonstrated the presence of circulating CD45 $^{+}$ cells that express type I collagen and can also form bone, consistent with an osteogenic potential of these cells. Since our hematopoietic lineage depletion effectively depleted CD45 $^{+}$ cells, additional studies are needed to examine the possible changes in numbers and/or gene expression by PB CD45 $^{+}$ /collagen I $^{+}$ cells in women undergoing rapid versus slow bone loss or in patients with osteoporosis.

We do recognize several limitations of our work. First, using our approach of MACS sorting, it is difficult to accurately quantify the numbers of lin $^{-}$ /AP $^{+}$ cells in PB, so we cannot provide data on whether the absolute numbers of these cells differed in postmenopausal women undergoing rapid versus slow bone loss. Thus, additional studies addressing this issue need to be done; of interest, however, a recent study by Pirro et al. [41] found that the number of CD15- (a granulocyte marker)/AP $^{+}$ cells were reduced in PB of women with postmenopausal osteoporosis as compared to control women. Second, while we provide an improved understanding of the characteristics of PB lin $^{-}$ /AP $^{+}$ cells in relation to their BM counterparts,

which are generally accepted as containing authentic osteoblast precursors [2], clearly additional work needs to be done to evaluate the functional properties of the PB cells. This work is currently ongoing in our laboratory. In addition, our findings regarding the differentially expressed genes in PB lin⁻/AP⁺ cells from the women undergoing rapid versus slow bone loss need to be independently replicated in larger groups of women. Nonetheless, each differentially regulated gene identified does have potential relevance to bone metabolism, and mRNA levels for several of these genes (IRF3, ZDHHC4, WISP1, TGFβ2) not only differed between the slow and rapid loss groups, but also showed significant correlations with rates of bone loss at multiple skeletal sites. Our data thus support further human and animal studies evaluating the role of these genes in mediating estrogen deficiency and/or age-related bone loss.

In summary, our work provides further insights into the relationship between BM and PB osteogenic populations. These data demonstrate that while PB lin⁻/AP⁺ cells express a number of key osteoblastic genes and are capable of mineralization, they are a relatively quiescent cell population, both in terms of cell proliferation and matrix synthesis. However, their higher expression of RANKL and ICAM-1 mRNAs as compared to BM lin⁻/AP⁺ cells suggests a role for the PB lin⁻/AP⁺ cells in regulating osteoclastogenesis that warrants further investigation. Our study also provides a “proof-of-concept” regarding the use of the PB cell populations in clinical-investigative studies of metabolic bone diseases and identifies several pathways that could potentially regulate rates of bone loss in postmenopausal women.

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REFERENCES

- [1]. Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* 2007;25:2739–2749. [PubMed: 17656645]
- [2]. Gronthos S, Fitter S, Diamond P, Simmons PJ, Itescu S, Zannettino ACW. A novel monoclonal antibody (STRO-3) identifies an isoform of tissue nonspecific alkaline phosphatase expressed by multipotent bone marrow stromal stem cells. *Stem Cells Dev* 2007;16:953–963. [PubMed: 18158854]
- [3]. Long MW, Williams JL, Mann KG. Expression of human bone-related proteins in the hematopoietic microenvironment. *J Clin Invest* 1990;86:1387–1395. [PubMed: 2243119]
- [4]. Long MW, Robinson JA, Ashcraft EA, Mann KG. Regulation of human bone marrow-derived osteoprogenitor cells by osteogenic growth factors. *J Clin Invest* 1995;95:881–887. [PubMed: 7860771]
- [5]. Long MW, Ashcraft EK, Normalle D, Mann KG. Age-related phenotypic alterations in populations of purified human bone precursor cells. *J Gerontol* 1999;54A:B54–B62.
- [6]. Eipers PG, Kale S, Taichman RS, Pipia GG, Swords NA, Mann KG, Long MW. Bone marrow accessory cells regulate human bone precursor cell development. *Exp Hematol* 2000;28:815–825. [PubMed: 10907643]
- [7]. Eghbali-Fatourehchi GZ, Lamsam J, Fraser D, Nagel DA, Riggs BL, Khosla S. Circulating osteoblast-lineage cells in humans. *N Engl J Med* 2005;352:1959–1966. [PubMed: 15888696]
- [8]. Eghbali-Fatourehchi GZ, Moedder UI, Charatcharoenwitthaya N, Sanyal A, Undale AH, Clowes JA, Tarara JE, Khosla S. Characterization of circulating osteoblast lineage cells in humans. *Bone* 2007;40:1370–1377. [PubMed: 17320497]
- [9]. Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, Kaneda Y. Bone marrow-derived osteoblast progenitor cells in circulating blood contribute to ectopic bone formation in mice. *Biochem Biophys Res Commun* 2007;354:453–458. [PubMed: 17239347]

- [10]. Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, Kaneda Y. Circulating bone marrow-derived osteoblast progenitor cells are recruited to the bone-forming site by CXCR4/stromal cell-derived factor-1 pathway. *Stem Cells* 2008;26:223–234. [PubMed: 17932420]
- [11]. Suda RK, Billings PC, Egan KP, Kim JH, McCarrick-Walmsley R, Glaser DL, Porter DL, Shore EM, Pignolo RJ. Circulating osteogenic precursor cells in heterotopic bone formation. *Stem Cells* 2009;27:2209–2219. [PubMed: 19522009]
- [12]. Kalajzic Z, Li H, Wang L-P, Jiang X, Lamothe K, Adams DJ, Aguila HL, Rowe DW, Kalajzic I. Use of an alpha-smooth muscle actin GFP reporter to identify an osteoprogenitor population. *Bone* 2008;43:501–510. [PubMed: 18571490]
- [13]. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Gehron Robey P, Riminucci M, Bianco P. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 2007;131:324–336. [PubMed: 17956733]
- [14]. Riggs BL, Melton LJ, Robb RA, Camp JJ, Atkinson EJ, McDaniel L, Amin S, Rouleau PA, Khosla S. A population-based assessment of rates of bone loss at multiple skeletal sites: evidence for substantial trabecular bone loss in young adult women and men. *J Bone Miner Res* 2008;23:205–214. [PubMed: 17937534]
- [15]. Riggs BL, Melton LJ III, Robb RA, Camp JJ, Atkinson EJ, Peterson JM, Rouleau PA, McCollough CH, Bouxsein ML, Khosla S. Population-based study of age and sex differences in bone volumetric density, size, geometry, and structure at different skeletal sites. *J Bone Miner Res* 2004;19:1945–1954. [PubMed: 15537436]
- [16]. Lawson GM, Katzmann JA, Kimlinger TK, O'Brien JF. Isolation and preliminary characterization of a monoclonal antibody which interacts preferentially with the liver isoenzymes of human alkaline phosphatase. *Clin Chem* 1985;31:381–385. [PubMed: 3971557]
- [17]. Dafforn A, Chen P, Deng G, Herrler M, Iglehart D, Koritala S, Lato S, Pillarsietty S, Purohit R, Wang M, Wang S, Kurn N. Linear mRNA amplification from as little as 5ng total RNA for global gene expression. *Biotechniques* 2004;37:854–857. [PubMed: 15560142]
- [18]. Nygaard V, Hovig E. Options available for profiling small samples: a review of sample amplification technology when combined with microarray profiling. *Nucleic Acids Res* 2006;34:996–1014. [PubMed: 16473852]
- [19]. Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun* 2004;313:856–862. [PubMed: 14706621]
- [20]. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3 research0034.1-0-34.11.
- [21]. Zhao S, Fernald RD. Comprehensive algorithm for quantitative real-time polymerase chain reaction. *J Comput Biol* 2005;12:1047–1064. [PubMed: 16241897]
- [22]. Khosla S, Amin S, Singh RJ, Atkinson EJ, Melton LJ, Riggs BL. Comparison of sex steroid measurements in men by immunoassay versus mass spectroscopy and relationships with cortical and trabecular volumetric bone mineral density. *Osteoporos Int* 2008;19:1465–1471. [PubMed: 18338096]
- [23]. Tanaka Y, Maruo A, Fujii K, Nomi M, Nakamura T, Eto S, Minami Y. Intercellular adhesion molecule 1 discriminates functionally different populations of human osteoblasts: characteristic involvement of cell cycle regulators. *J Bone Miner Res* 2000;15:1912–1923. [PubMed: 11028443]
- [24]. Everts V, Delaisse JM, Korper W, Jansen DC, Tigchelaar-Gutter W, Saftig P, Beertsen W. The bone lining cell: its role in cleaning howship's lacunae and initiating bone formation. *J Bone Miner Res* 2002;17:77–90. [PubMed: 11771672]
- [25]. Kuznetsov SA, Mankani MH, Leet AI, Ziran N, Gronthos S, Robey PG. Circulating connective tissue precursors: extreme rarity in humans and chondrogenic potential in guinea pigs. *Stem Cells* 2007;25:1830–1839. [PubMed: 17464083]
- [26]. Kearns AE, Khosla S, Kostenuik PJ. Receptor activator of nuclear factor kappaB ligand and osteoprotegerin regulation of bone remodeling in health and disease. *Endocr Rev* 2008;29:155–192. [PubMed: 18057140]

- [27]. Xie Y, Yin T, Wiegraebe W, He XC, Miller D, Stark D, Perko K, Alexander R, Schwartz J, Grindley JC, Park J, Haug JS, Wunderlich JP, Li H, Zhang S, Johnson T, Feldman RA, Li L. Detection of functional haematopoietic stem cell niche using real-time imaging. *Nature* 2009;457:97–101. [PubMed: 19052548]
- [28]. Uppal S, Diggle CP, Carr IM, Fishwick CW, Ahmed M, Ibrahim GH, Helliwell PS, Lotos-Bielenska A, Phillips SE, Markham AF, Bennet CP, Bonthon DT. Mutations in 15-hydroxyprostaglandin dehydrogenase cause primary hypertrophic osteoarthropathy. *Nat Genet* 2008;40:789–793. [PubMed: 18500342]
- [29]. Hikiji H, Takato T, Shimizu T, Ishii S. The roles of prostanoids, leukotrienes, and platelet-activating factor in bone metabolism and disease. *Prog Lipid Res* 2008;47:107–126. [PubMed: 18187046]
- [30]. Yuksel-Konuk B, Sirmaci A, Ayten GE, Ozdemir M, Asian I, Yilmaz-Turay U, Erdogan Y, Tekin M. Homozygous mutations in the 15-hydroxyprostaglandin dehydrogenase gene in patients with primary hypertrophic osteoarthropathy. *Rheumatol Int.* in press DOI: 10.1007/s00296-009-0895-6.
- [31]. Yoneyama M, Suhara W, Fujita T. Control of IRF-3 activation by phosphorylation. *J Interferon Cytokine Res* 2002;22:73–76. [PubMed: 11846977]
- [32]. Gao Y, Grassi F, Ryan MR, Terauchi M, Page K, Yang X, Weitzmann MN, Pacifici R. IFN-gamma stimulates osteoclast formation and bone loss in vivo via antigen-driven T cell activation. *J Clin Invest* 2007;117:122–132. [PubMed: 17173138]
- [33]. Duque G, Huang DC, Macoritto M, Rivas D, Yang XF, Ste-Marie LG, Kremer R. Autocrine regulation of interferon gamma in mesenchymal stem cells plays a role in early osteoblastogenesis. *Stem Cells* 2009;27:550–558. [PubMed: 19096039]
- [34]. Fox SW, Lovibond AC. Current insights into the role of transforming growth factor-beta in bone resorption. *Mol Cell Endocrinol* 2005;243:19–26. [PubMed: 16219413]
- [35]. Weitzmann MN, Pacifici R. Estrogen deficiency and bone loss: an inflammatory tale. *J Clin Invest* 2006;116:1186–1194. [PubMed: 16670759]
- [36]. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signaling. *Nature* 2003;425:577–584. [PubMed: 14534577]
- [37]. Grimsby S, Jaensson H, Dubrovskaya A, Lomnytska M, Hellman U, Souchelnyskyi S. Proteomics-based identification of proteins interacting with Smad3: SREBP-2 forms a complex with Smad3 and inhibits its transcriptional activity. *FEBS Lett* 2004;577:93–100. [PubMed: 15527767]
- [38]. Xu L, Corcoran RB, Welsh JW, Pennica D, Levine AJ. WISP-1 is a Wnt-1- and beta-catenin-responsive oncogene. *Genes Dev* 2000;14:585–595. [PubMed: 10716946]
- [39]. French DM, Kaul RJ, D' Souza AL, Crowley CW, Bao M, Frantz GD, Filvaroff EH, Desnoyers L. WISP-1 is an osteoblastic regulator expressed during skeletal development and fracture repair. *Am J Pathol* 2004;165:855–867. [PubMed: 15331410]
- [40]. Rogers A, Eastell R. Review: Circulating osteoprotegerin and receptor activator for nuclear factor kappaB ligand: clinical utility in metabolic bone disease assessment. *J Clin Endocrinol Metab* 2005;90:6323–6331. [PubMed: 16105967]
- [41]. Pirro M, Leli C, Fabbriani G, Manfredelli MR, Callarelli L, Bagaglia F, Scarponi AM, Mannarino E. Association between circulating osteoprogenitor cell numbers and bone mineral density in postmenopausal osteoporosis. *Osteoporos Int* 2010;21:297–306. [PubMed: 19484167]

Figure 1.

(A) Example of a flow cytometry analysis using the isotype control (top panel) and AP antibody (bottom panel). As noted in the Methods, the percentage of cells staining with the isotype control was subtracted from the percentage staining with the AP antibody; (B) Expression of AP mRNA in MACS-sorted lin-/AP- and lin-/AP+ cells (normalized to tata binding protein [TBP]).

Figure 2.

BM MNCs were labeled with a biotinylated anti-human AP antibody and subsequently stained with PE-conjugated streptavidin and FITC-conjugated hematopoietic lineage cocktail monoclonal Abs, followed by FACS analysis. (A) Density plots with lin/AP coexpression; thresholds were set using the appropriate isotype control antibody, and the percentages are the percentage of positive cells with the AP antibody minus the percentage positive with the isotype control (see Methods). (B) The MACS sorted lin⁻ and lin⁺ BM cells were labeled with PerCP conjugated streptavidin to validate hematopoietic lineage depletion. The data is representative of six individual experiments.

Figure 3.

Lin⁻ cells obtained from BM and PB were labeled with a biotinylated anti-human AP antibody and subsequently stained with PE-conjugated streptavidin and FITC-labeled anti-human CD45. FACS analysis by density plots shows expression of AP on lin⁻ BM (A) and PB cells (B) and coexpression of AP/CD45 on lin⁻ BM (C) and PB cells (D).

Figure 4.

BM lin-/AP- (A) and lin-/AP+ (B, C, D) cells cultured under osteogenic conditions and PB lin-/AP- (E, F) and lin-/AP+ (G, H) cells cultured under osteogenic conditions, followed by alizarin red staining.

Figure 5.

Relationship of IRF3 mRNA levels in PB lin⁻/AP⁺ cells to rates of change in (A) vertebral trabecular, (B) total femoral neck, and (C) femoral neck trabecular vBMD. Shown are the Spearman correlation coefficients and corresponding P-values. Since non-parametric correlations were used, removal of the single outlier point in each panel did not significantly alter the correlation coefficients ($R = 0.45$, $P = 0.054$; $R = 0.62$, $P = 0.005$; and $R = 0.57$, $P = 0.010$ for vertebral trabecular, total femoral neck, and femoral neck trabecular vBMD changes, respectively, following removal of the outlier).

Table 1

Comparison of gene expression by BM and PB lin⁻/AP⁺ cells (n = 6 each). All data are expressed relative to BM cells and the values represent median (25th-75th percentiles, IQRs). Light shading indicates genes expressed at significantly lower levels, no shading indicates genes expressed at similar levels, and dark shading indicates genes expressed at significantly higher levels in PB as compared to BM cells

Gene	BM	PB	P-value
Osteoblast Markers			
AP	1.00 (0.66, 1.45)	0.003 (0.001, 0.036)	0.037
Col1 α 1	1.00 (0.90, 3.99)	0.065 (0.001, 0.218)	0.004
Col1 α 2	1.00 (0.66, 2.04)	0.019 (0.005, 0.030)	0.004
Runx2	1.00 (0.64, 1.24)	1.95 (1.09, 2.90)	0.109
Osterix	1.00 (0.10, 11.26)	2.61 (0.57, 5.09)	0.873
OPG	1.00 (0.82, 1.36)	2.14 (0.90, 3.63)	0.423
Osteopontin	1.00 (0.22, 1.52)	0.26 (0.06, 0.40)	0.200
Periostin	1.00 (0.85, 1.07)	1.10 (1.10, 5.36)	0.109
OCN	1.00 (0.76, 1.33)	2.57 (2.08, 3.98)	0.025
Osteonectin	1.00 (0.60, 1.22)	9.61 (6.34, 10.98)	0.004
PTHr1	1.00 (0.23, 2.83)	12.80 (7.60, 28.26)	0.010
RANKL	1.00 (0.27, 2.13)	23.29 (13.34, 29.69)	0.004
Proliferation Markers			
Ki67	1.00 (0.86, 1.19)	0.17 (0.09, 0.27)	< 0.004
CDK2	1.00 (0.85, 1.35)	0.57 (0.40, 0.68)	0.037
CyclinA	1.00 (0.64, 1.33)	0.36 (0.25, 0.58)	0.037
CyclinB1	1.00 (0.89, 1.37)	0.11 (0.07, 0.15)	0.004
CyclinB2	1.00 (0.87, 1.16)	0.06 (0.03, 0.09)	0.004
CyclinE1	1.00 (0.83, 1.12)	0.09 (0.07, 0.11)	0.004
E2F1	1.00 (0.95, 2.04)	0.25 (0.08, 0.37)	0.025
CyclinC	1.00 (0.78, 1.18)	0.82 (0.37, 0.96)	0.150
CDK6	1.00 (0.86, 1.14)	0.79 (0.68, 1.22)	0.423
Smooth Muscle Cell Markers			
α SMA	1.00 (0.95, 1.40)	2.88 (1.82, 5.07)	0.016
Cald1, variant1	1.00 (0.43, 2.42)	15.67 (10.35, 42.12)	0.025
Calponin1	1.00 (0.89, 1.09)	1658 (1472, 2947)	0.004
Pericyte Markers			
Desmin	1.00 (0.40, 2.19)	0.010 (0.003, 0.216)	0.055
PDGFR-A	1.00 (0.80, 1.70)	0.000 (0.000, 0.002)	0.004
PDGFR-B	1.00 (0.23, 1.59)	0.51 (0.13, 1.83)	0.631
CSPG4	1.00 (0.89, 1.09)	1.12 (0.98, 1.15)	0.262
Adhesion Molecules			
ICAM-1	1.00 (0.40, 1.38)	4.65 (4.10, 5.42)	0.006
Integrin alpha 2	1.00 (0.65, 2.64)	7.70 (5.15, 11.69)	0.004

Gene	BM	PB	P-value
Integrin alpha 5	1.00 (0.80, 1.27)	1.79 (1.35, 2.47)	0.025
Integrin beta 1	1.00 (0.62, 1.11)	5.59 (3.68, 11.02)	0.016
Integrin beta 3	1.00 (0.59, 3.36)	121.6 (75.34, 166.5)	0.004
Integrin beta 5	1.00 (0.81, 1.72)	77.9 (57.04, 81.89)	0.004
N-cadherin	1.00 (0.79, 1.67)	1.02 (0.41, 1.57)	0.522
Integrin alpha 1	1.00 (0.41, 2.11)	1.03 (0.29, 1.68)	0.522
Integrin alpha 3,variant 1	1.00 (0.22, 1.24)	0.66 (0.42, 4.86)	0.749
Integrin alpha 4	1.00 (0.64, 1.38)	1.66 (1.13, 2.78)	0.150
Integrin alpha M	1.00 (0.48, 2.21)	1.15 (0.43, 1.52)	0.522
Integrin alpha v	1.00 (0.96, 2.28)	1.17 (0.92, 2.85)	0.873
VCAM-1	1.00 (0.63, 1.77)	0.14 (0.11, 0.24)	0.010
Related Lineage Cell			
Adiponectin	1.00 (0.39, 1.16)	0.002 (0.002, 0.002)	0.004
PPAR γ 2	1.00 (0.52, 1.21)	0.25 (0.14, 0.33)	0.016
Sox9	1.00 (0.10, 51.12)	0.10 (0.09, 0.10)	0.262
MyoD	1.00 (0.89, 1.09)	1.12 (0.98, 1.15)	0.262

Table 2

Clinical and biochemical data of postmenopausal women undergoing slow versus rapid bone loss over 6 years of follow up. Data are median (25th-75th percentiles, IQRs)

	Slow Loss	Rapid Loss	P-value
N	10	10	0.352
Age, yrs	67.9 (58.8, 73.6)	71.2 (59.2, 76.0)	
vBMD rates of bone loss, %/year			
Vertebral trabecular	-0.7 (-1.3, -0.3)	-2.8 (-4.2, -2.0)	<0.001
Total Femoral neck	-0.4 (-1.7, -0.1)	-1.9 (-2.4, -1.3)	0.018
Femoral neck trabecular	-1.6 (-2.1, -1.3)	-3.4 (-4.0, -2.4)	<0.001
Serum measurements			
CTX (ng/ml)	0.45 (0.39, 0.49)	0.61 (0.42, 0.69)	0.124
TRACP 5b (U/L)	4.19 (3.07, 4.43)	4.59 (4.11, 5.16)	0.106
P1NP (ug/L)	36.6 (31.6, 46.5)	43.9 (37.5, 63.5)	0.144
E ₂ (pg/ml)	8.09 (3.03, 14.9)	3.57 (2.45, 4.29)	0.112

Table 3

QPCR analysis of differentially expressed genes in lin⁻/AP⁺ cells from women undergoing slow versus rapid loss of vBMD at multiple skeletal sites. All data are expressed relative to the slow loss group and are shown as median (25th-75th percentile [IQR]) mRNA levels. Shown are the genes initially identified by microarray and subsequently validated using QPCR, as well as additional genes from our pre-specified candidate genes, as described in the text

	Slow Loss	Rapid Loss	P-value
Genes identified by microarray and subsequently validated by QPCR			
HPGD	1.00 (0.70, 1.80)	0.58 (0.33, 1.11)	0.044
IRF3	1.00 (0.84, 1.19)	0.64 (0.55, 0.91)	0.044
ZDHHC4	1.00 (0.92, 1.23)	1.25 (1.12, 1.32)	0.028
Genes identified by QPCR analysis from candidate genes			
WISP1	1.00 (0.12, 2.29)	0.01 (0.01, 0.10)	0.036
TGFβ2	1.00 (0.49, 1.68)	0.31 (0.23, 0.86)	0.024
Col1α2	1.00 (0.41, 7.54)	17.03 (1.74, 23.15)	0.014
OPG	1.00 (0.62, 3.62)	3.65 (1.30, 6.34)	0.052

Table 4

Spearman correlation coefficients between rates of change in vBMD at the vertebrae and femoral neck and mRNA levels for the genes identified as being differentially expressed in the slow loss versus rapid loss groups using the QPCR analysis

Genes	Vertebral Trabecular vBMD	Femoral neck Total vBMD	Femoral neck trabecular vBMD
HPGD	0.34	0.43	0.49 [*]
IRF3	0.48 [*]	0.65 ^{**}	0.60 ^{**}
ZDHHC4	-0.43	-0.20	-0.51 [*]
WISP1	0.58 [*]	0.32	0.52 [*]
TGFβ2	0.40	0.45 [*]	0.55 [*]
Col 1α2	-0.43	-0.03	-0.29
OPG	-0.29	-0.11	-0.24

^{*} P < 0.05

^{**} P < 0.01

Table 5

Serum protein assays for PGE₂, IFN α , β , γ , TGF β ₂, and OPG in the two groups. Data are median (25th-75th percentile [IQR])

	Slow Loss	Rapid Loss	P-value
PGE ₂ (pg/ml)	270 (181, 557)	217 (139, 288)	0.241
IFN α (pg/ml)	5.22 (4.27, 24.7)	4.62 (4.40, 45.7)	0.903
IFN β (pg/ml)	46.8 (12.3, 2028)	15.1 (10.7, 1217)	0.466
IFN γ (pg/ml)	6.83 (6.51, 7.41)	6.88 (6.40, 7.37)	0.821
TGF β ₂ (pg/ml)	300 (244, 530)	255 (200, 271)	0.257
OPG (pmol/L)	3.24 (2.94, 4.19)	4.32 (3.94, 4.62)	0.212

Table 6

Spearman correlation coefficients between selected mRNA levels in PB lin[−]/AP⁺ cells and corresponding serum protein markers related to each of the pathways associated with the particular mRNA

mRNA	Serum protein marker						
	PGE ₂	IFN α	IFN β	IFN γ	TGF β ₂	PINP	OPG
HPGD	0.12	-	-	-	-	-	-
IRF3	-	-0.10	0.12	-0.50*	-	-	-
TGF β ₂	-	-	-	-	0.30	-	-
Coll α 2	-	-	-	-	-	0.12	-
OPG	-	-	-	-	-	-	0.24

* P < 0.05