Directing mesenchymal stem cells to bone to augment bone formation and increase bone mass

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Aging reduces the number of mesenchymal stem cells (MSCs) that can differentiate into osteoblasts in the bone marrow, which leads to impairment of osteogenesis. However, if MSCs could be directed toward osteogenic differentiation, they could be a viable therapeutic option for bone regeneration. We have developed a method to direct MSCs to the bone surface by attaching a synthetic high-affinity and specific peptidomimetic ligand (LLP2A) against integrin α4β1 on the MSC surface to a bisphosphonate (alendronate, Ale) that has a high affinity for bone. LLP2A-Ale induced MSC migration and osteogenic differentiation in vitro. A single intravenous injection of LLP2A-Ale increased trabecular bone formation and bone mass in both xenotransplantation studies and in immunocompetent mice. Additionally, LLP2A-Ale prevented trabecular bone loss after peak bone acquisition was achieved or as a result of estrogen deficiency. These results provide proof of principle that LLP2A-Ale can direct MSCs to the bone to form new bone and increase bone strength.

A decrease in the number of MSCs in the bone marrow with aging leads to reduced osteogenesis and bone formation1–3. Bone regeneration through induction of MSCs to the bone surface by attaching a specific peptidomimetic ligand, LLP2A, against integrin α4β1 on the MSC surface to a bisphosphonate (alendronate, Ale) that has a high affinity for bone. LLP2A-Ale induced MSC migration and osteogenic differentiation in vitro. A single intravenous injection of LLP2A-Ale increased trabecular bone formation and bone mass in both xenotransplantation studies and in immunocompetent mice. Additionally, LLP2A-Ale prevented trabecular bone loss after peak bone acquisition was achieved or as a result of estrogen deficiency. These results provide proof of principle that LLP2A-Ale can direct MSCs to the bone to form new bone and increase bone strength.

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We used color-coded peptide beads (rainbow beads) to semi-quantitatively determine the integrin profiles of MSCs undergoing osteogenic differentiation. We found that the α4β1 integrin was highly expressed in the osteoprogenitor cells and had a high affinity for LLP2A (Supplementary Fig. 2). Treatment with LLP2A-Ale increased both the number of MSCs that differentiated into osteoblasts (Fig. 1a–c) as well as the migration of the MSCs (Fig. 1d,e), but did not affect their chondrogenic or adipogenic potentials.

**LLP2A-Ale increases the bone homing and retention of MSCs**

To determine whether LLP2A-Ale could direct transplanted MSCs to bone, we performed a xenotransplantation study. We intravenously (i.v.) injected human MSCs (huMSCs) or huMSCs plus LLP2A-Ale into nonobese diabetic (NOD)/severe combined immunodeficiency (SCID)/mucopolysaccharidosis type VII (MPS VII) mice. Twenty-four hours after the i.v. injections, we observed a number of huMSCs adjacent to the periosteal and trabecular bone surfaces in the lumbar vertebral bodies (LVB) of the mice co-injected with LLP2A-Ale and huMSCs but not of the mice injected with huMSCs only (Fig. 2a).

At 3 weeks after injection, we observed huMSC cells adjacent to the bone surface and embedded within the bone matrix in the mice treated with huMSC and LLP2A-Ale but not in those treated with huMSCs only, suggesting that there was retention of the transplanted huMSCs (Supplementary Fig. 3a) in the bone of the former group. We observed higher concentrations of procollagen type I amino-terminal propeptide (P1NP), an early osteoblast differentiation marker, in the mice treated with LLP2A-Ale + huMSCs compared to...
mice treated with huMSCs only. The concentrations of osteocalcin, an osteoblast maturation marker, and the bone formation parameters were higher in mice treated with LLP2A-Ale and those treated with huMSCs + LLP2A-Ale compared to the mice treated with PBS or huMSCs only (Fig. 2b).

In mice that we treated with GFP-labeled mouse MSCs or with LLP2A-Ale, the mice given the GFP-labeled mouse MSCs + LLP2A-Ale combination treatment showed higher numbers of the GFP-positive osteoblasts and osteocytes in both their trabecular (Fig. 2c) and cortical bone regions (Supplementary Fig. 3b) of the LVB of these mice at

Figure 3 Treatment with LLP2A-Ale increases trabecular bone mass in immunocompetent mice. (a) Trabecular bone volume compared to the total tissue volume (BV/TV) measured by repeated in vivo microCT in 8-week-old female 129/SvJ mice. L, low dose; H, high dose. (b,c) Representative three-dimensional thickness maps from micro computed tomography (microCT) scans of trabecular bone from the distal femur metaphyses of the mice at baseline (8 weeks of age) (b) and from the same mice and at the same bone sites 4 weeks after a single injection of PBS or a high dose of LLP2A-Ale (LLP2A-Ale-H) (12 weeks of age) (c). The width of the trabecular is color coded, with the blue-green color indicating thin trabeculae and the yellow-red color indicating thick trabeculae. (d) Maximum load and maximum stress of the sixth lumbar vertebral bodies in mice at 12 weeks of age, 4 weeks after one injection of the agents indicated on the x-axis. LLP2A-Ale-L, low dose of LLP2A-Ale; N, newton; MPa, megapascal. (e) Bone turnover markers measured from the serum of the mice treated with each indicated agent. (f) Surface-based bone histomorphometry was performed on the right distal femurs of mice from each indicated treatment group. Ob/BS, osteoblast surface. (g) Representative images from the trabecular bone of the distal femurs of the mice from the indicated treatment groups. The yellow arrowheads point to the osteoblastic bridges between the trabeculae in an LLP2A-Ale–treated mouse. (h) Surface-based bone histomorphometry was performed at the endosteal surface (Ec) and the periosteal surface (Ps) 4 weeks after the injections. Measurements of the maximum stress of the femurs were taken 4 weeks after the injections. (i) Representative cross sections of the tibial shafts from mice treated with PBS, LLP2A-Ale, a high dose of LLP2A-Ale or a low dose of LLP2A-Ale. The yellow arrows point to double-labeled bone surfaces. *P < 0.05 compared to the PBS group; **P < 0.05 compared to both the PBS and huMSC groups. Repeated measures analysis of variance followed by Bonferroni post hoc testing was used to analyze the in vivo microCT measurement in a. A nonparametric Kruskal-Wallis test was used for the parameters presented in d, e, f and h. Data are mean ± s.d.
Figure 4  Treatment with LLP2A-Ale prevents age-related trabecular bone loss. (a) Diagram of the study and treatment methods. (b) Trabecular bone area, osteoblast surface, mineralizing surface and bone formation rate over the total bone surface determined at the distal femoral metaphyses of mice treated with either PBS or LLP2A-Ale. (c) Representative distal femur sections from mice treated with PBS or LLP2A-Ale at 16 weeks after treatment. The white squares indicate the selected areas shown under higher magnification in the inset fluorescence images that indicate mineral apposition. The white arrowheads in the insets indicate the distances between the double labels. (d) Trabecular bone area, osteoblast surface, mineralizing surface and bone formation rate compared to total bone surface performed at the fifth LVB sections from mice treated with PBS or LLP2A-Ale at 16 weeks of age (8 weeks after treatment). (e) Representative images taken from the trabecular bone at the fifth LVB. The yellow arrowheads point to the osteoblastic bridges in a mouse treated with LLP2A-Ale. *P < 0.05 compared to treatment with PBS at the same time point (either 8 or 16 weeks of age). **P < 0.05 compared to baseline. Data are mean ± s.d.

3 weeks after injection. Collectively, these data show that LLP2A-Ale can direct transplanted MSCs to bone, increase the homing and retention of MSCs to bone and enhance endosteal and periosteal bone formation in a xenotransplantation model.

LLP2A-Ale augments bone formation in immunocompetent mice

To determine whether LLP2A-Ale could augment endogenous bone formation in immunocompetent mice without MSC transplantation, we used 2-month-old female 129/SvJ mice that received two doses of LLP2A-Ale, representing the effective dose compared to the placebo, LLP2A or Ale treatments and the maximum anabolic dose in our dose-finding studies. Two days after the i.v. injections, the cell populations expressing LLP2A, runt related transcription factor 2 (Runx2) (a gene marker for osteoblast maturation) and bromodeoxyuridine (Brdu) (a thymidine analog that is used in the detection of cell proliferation) were primarily located within the bone marrow in the LLP2A-Ale–treated mice but were located at the bone surface in the mice treated with LLP2A-Ale (Supplementary Fig. 4). The LLP2A and Brdu-positive cell populations were not detectable in the LVB at 21 d after injection in mice either injected with LLP2A or LLP2A-Ale.

Treatment with both doses of LLP2A-Ale induced a higher distal femoral trabecular bone volume (measured as trabecular bone volume (BV)/tissue volume (TV)) and thickness compared to baseline (Fig. 3a–c), with a corresponding greater maximum load and strength of the LVB in mice treated with LLP2A-Ale compared to mice treated with PBS, alendronate or LLP2A alone at 12 weeks after treatment (Fig. 3d). At 12 weeks after treatment, LLP2A-Ale dose-dependently resulted in a higher concentration of osteocalcin (Fig. 3e) and the surface-based bone-formation parameters at the distal femur in the LLP2A-Ale treated mice as compared to the groups treated with PBS or Ale only (Fig. 3f). More notably, LLP2A-Ale treatment increased the parameter of the osteoblast surface and formed bridges between adjacent trabeculae (Fig. 3g). Bone-formation rates on the endocortical surfaces of the tibial shafts were higher in mice that received LLP2A or LLP2A-Ale as compared to mice that received PBS or Ale (Fig. 3h,i).

LLP2A-Ale prevents trabecular bone loss in osteopenia mice

C57BL/6 mice usually achieve their peak bone mass by 6–8 weeks of age, and this peak is followed by an approximately 50% decline in both bone formation and bone mass at between 2 and 4 months of age. Treatment with LLP2A-Ale in C57BL/6 mice prevented age-related trabecular bone loss after peak bone acquisition was achieved (Fig. 4a), with higher amounts of bone-formation parameters being present at the distal femur (Fig. 4b,c) as well as at the LVB in these mice compared to mice treated with PBS (Fig. 4d,e). We observed osteoblast bridges at both of these trabecular bone sites (Fig. 4e) in LLP2A-Ale–treated mice. These data suggest that one i.v. injection of LLP2A-Ale prevented age-related reductions in trabecular bone mass and bone formation for up to 8 weeks after injection in C57BL/6 mice.

To determine whether LLP2A-Ale could prevent bone loss in a disease state, we treated 10-week-old ovariecetomized (OVX) mice with PBS, Ale, LLP2A, LLP2A-Ale or parathyroid hormone (PTH) 2 weeks after OVX (Fig. 5a). LLP2A-Ale treatment induced a larger osteoblast surface and a larger mineralizing surface, as well as a higher bone-formation rate per total bone surface at the LVB in
Figure 5 Treatment with LLP2A-Ale partially prevents trabecular bone loss and increases endosteal bone formation in OVX mice.

(a) Diagram of the study and treatment methods. OVX/sham, mice were either ovariectomized or sham treated at this time point. n = 6–8 mice per group. (b) Histomorphometric analyses of the fifth LVBs from mice in each indicated treatment group, including analyses of the trabecular bone area, osteoblast surface, mineralizing surface and bone formation rate compared to the total bone surface. (c) Representative images from the trabecular bone at the fifth LVBs of the mice in each indicated treatment group. The white arrowheads point to the osteoblasts. (d) Representative fluorescent images from the trabecular bone area, osteoblast surface, mineralizing surface and bone formation rate compared to the total bone surface. (e) Representative images from the trabecular bone at the fifth lumbar vertebral trabeculae of the mice from each indicated treatment group. The yellow arrows point to double-labeled trabecular bone surfaces. (e) Histomorphometry analyses of the right mid-femurs of mice from the indicated treatment groups, including analyses of bone formation at the endosteal or periosteal bone surfaces and cortical bone thickness. (f) Representative images from the mid-femur sections from mice in each indicated treatment group. The yellow arrows point to double-labeled endocortical bone surfaces. *P < 0.05 compared to the PBS group; **P < 0.05 compared to both the PBS and LLP2A groups; ***P < 0.05 compared to both the PBS and Ale groups. A nonparametric Kruskal-Wallis test was used for all comparisons. Data are mean ± s.d.

DISCUSSION

MSCs are precursors of osteoblasts. MSCs do not readily migrate to the bone, and this creates a major obstacle for the use of MSCs for bone regeneration. We have developed a ligand that targets integrin αvβ1, a protein that is highly expressed by MSCs undergoing osteoblast differentiation. Instead of using genetically modified MSCs, we attached LLP2A to a bisphosphonate (Ale) to guide the MSCs to the bone surface. Bisphosphonates are prescribed to reduce bone resorption and improve bone strength. Because we used approximately one-tenth of the therapeutic dose of Ale in our compound, we did not observe any anti-resorptive effects as a result of treatment. We observed an uncoupling of bone remodeling with bone formation and no marked changes in bone resorption during this short-term study period. This uncoupling of bone remodeling in favor of bone formation is also observed in short-term treatment with the anabolic agent human PTH (hPTH) (1-34)34. We hypothesized that we would also see a return to a coupling of bone turnover with this intervention after a longer treatment period. Additionally, the Ale concentrations that we used in these studies did not suppress TGF-β1 secretion (Supplementary Fig. 5), a growth factor that is crucial for coupling bone resorption and endogenous stem-cell recruitment to bone35.

In our xenotransplantation model, LLP2A-Ale increased homing and retention of the transplanted MSCs to bone, which indicates a breakthrough in using transplanted MSCs to augment bone formation. We found the transplanted human or mouse MSCs embedded in the bone matrix as osteocytes or adjacent to the bone surface as osteoblasts. In addition to increasing bone formation rates at both the endocortical and trabecular surfaces, the periosteal bone formation rate also increased after MSC transplantation and after treatment with LLP2A-Ale. This is notable, as the total cross-sectional area increase as a result of periosteal expansion is the most important determinant of

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bone strength. Our finding that LLP2A-Ale can directly transplanted human MSCs to the bone is of major importance. This approach provides a means to overcome a major obstacle of using MSCs in the treatment of bone degenerative diseases and, as such, may be a new treatment option for osteoporosis.

Further, LLP2A-Ale might also increase endogenous MSC osteoblast differentiation and augment bone formation. We could not directly track the endogenous MSCs lineage commitment to osteoblasts, as there is no single marker that would allow us to define or track the migration of the endogenous MSCs to bone or their osteoblast differentiation in vivo. However, we were able to partially overcome this limitation by using control groups that we administered equivalent doses of Ale and LLP2A. Because LLP2A is a specific ligand for activated αβ1 integrin, our findings support the previous report that targeting α4 alone could increase the homing of MSCs to bone. However, LLP2A by itself did not induce any marked changes in the bone architectures. In contrast, LLP2A-Ale enhanced osteoblast activities, as evidenced by the increased osteocalcin concentrations and increased bone formation in response to treatment that were primarily seen at the trabecular and endocortical bone surfaces that are in close contact with the bone marrow. Treatment with LLP2A-Ale not only increased the vertebral maximum load but also increased maximum bone stress, a parameter that is independent of bone shape, suggesting LLP2A-Ale treatment increased bone quality in addition to the observed increase in bone mass. Similarly, treatment with LLP2A-Ale prevented trabecular bone loss after peak bone mass had been achieved in C57BL/6 mice and partially prevented the rapid trabecular bone loss that is induced by OVX in these mice. Collectively, our findings show that LLP2A-Ale might be able to increase the migration of endogenous MSCs to bone, stimulate osteoblastic differentiation, augment bone formation and increase bone mass in young mice, as well as prevent trabecular bone losses associated with aging or estrogen deficiency. These results differ from what we observed in NOD/SCID/MPS VII mice that we treated with huMSCs and LLP2A-Ale, in which the combination treatment increased periosteal bone formation. This may have been a result of the lack of periosteal effects by LLP2A-Ale itself, the combination treatment may require more than one injection or a longer treatment period to achieve cortical bone responses.

In summary, we have shown that LLP2A-Ale augments endogenous bone formation and directs the transplanted MSCs to the bone to augment bone formation and bone mass. This new approach to increase the homing and retention of the MSCs to bone should now be examined in both preclinical and clinical studies for the treatment of osteoporosis and fracture repair.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Synthesis of LLP2A-Ale. The LLP2A-Ale was synthesized by Michael addition of the sulphydryl group of LLP2A-Lys(D-Cys) to alendronate-maleimide (Ale-Mal). The synthetic scheme is shown in Supplementary Figure 1. Specifically, the peptidomimetic section, LLP2A-Lys(D-Cys), was synthesized to have a D-cysteine (D-Cys) attached to the side chain amino group of lysine (Lys) and to have a pair of identical hydrophilic linkers in series between the peptidomimetic ligand, LLP2A and the dipeptide Lys(D-Cys). This synthesis was performed on resin amid 4-methyl benzhydramine (MBHA) resin using similar approaches as we previously reported. Ale-Mal was prepared in situ from Ale and sulfoalginic acid 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC). In brief, Ale disodium salt (1.0 eq.) (powder from lyophilization of aqueous solution of alendronic acid and 2 eq. NaOH) was dissolved in 0.1 M PBS (with 10 mM EDTA), pH 7.5. The aqueous solution was then cooled in an ice water bath, and a solution of Sulfo-SMCC (1.1 eq.) in water was added dropwise. After the completion of this addition, the resulting solution was allowed to warm to 20–25 °C while being stirred for 2 h. This solution was cooled before the dropwise addition of a solution of LLP2A-Lys(D-Cys) (1.0 eq.) in a small amount of 50% acetonitrile/water. The pH was adjusted to between 6 and 7 with aqueous NaHCO3. The resulting mixture was stirred for 1 h and then allowed to warm to room temperature 20–22 °C. After a negative Ellman test, the solution was lyophilized. The resulting powder was redisolved in a small amount of 50% acetonitrile/water and purified by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) (C18 column). Buffer A was 0.5% acetic acid/H2O. Buffer B was 0.5% acetic acid/acetonitrile. The collected eluent was lyophilized to give a white powder. The identity was confirmed with matrix-assisted laser desorption/ionization mass spectrometry: [M+H]+ (molecular weight value): 1970.78 (calculated: 1970.88).

In vitro cell differentiation arrays of MSCs into osteogenic, chondrogenic or adipogenic lineages. Mouse MSCs were obtained under a material transfer agreement between the University of California Davis and the Texas A&M Institute for Regenerative Medicine. These cells were a relatively pure population of stromal cells that were negative for CD11b, CD45 and CD34 markers of bone turnover, bone histomorphometry, immunohistochemistry and biomechanical testing. All mice were treated according to the US Department of Agriculture animal care guidelines with the approval of the University of California Davis Committee on Animal Research. We previously published the methods for microCT, biochemical markers of bone turnover, bone histomorphometry, immunohistochemistry and biomechanical testing.

Statistics. The group means and s.d. were calculated for all outcome variables. Repeated measures analysis of variance was used to evaluate parameters derived from repeated in vivo microCT scans, such as the trabecular bone volume (BV/TV), and Bonferroni post hoc tests were used to compare time (age)-dependent changes within the same treatment group or between treatment groups at the same time point. The nonparametric Kruskal-Wallis test was used to determine differences between the groups for the other outcome measures obtained at the end of the studies (SPSS Version 12, SPSS). Differences were considered significant at P < 0.05.

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