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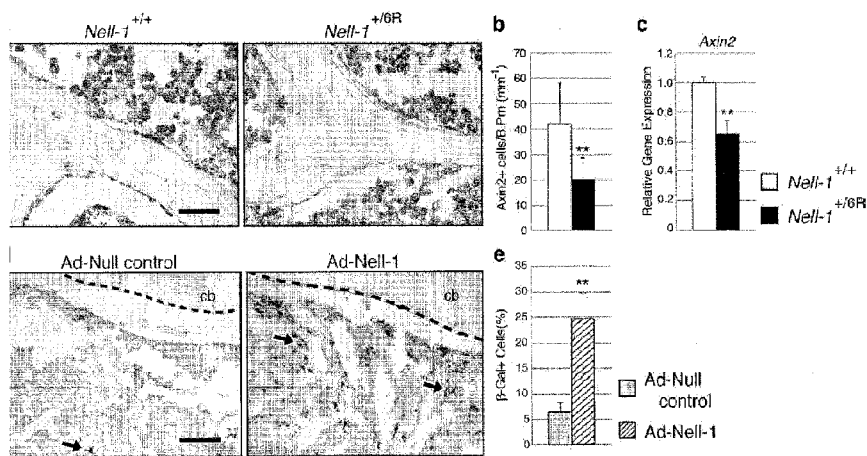
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(54) Title: COMPOSITION FOR BONE AND METHODS OF MAKING AND USING THE SAME



Figures 3a-e

(57) **Abstract:** Disclosed herein is a composition for bone condition and methods of making and using the same. The composition comprises an osteoinductive agent, and the osteoinductive agent is effective to increase the cell number and activity of osteoblast (OB) and decrease the cell number and activity of osteoclast (OC).

5 COMPOSITION FOR BONE AND METHODS OF MAKING AND USING THE SAME

10 STATEMENT OF GOVERNMENT SUPPORT

This invention was made with Government support under Grant Nos. AR061399 and DE016107 awarded by the National Institutes of Health. The Government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

15 The present application claim the benefit of U.S. provisional application Nos. 62/171,167, filed on June 4, 2015, and 62/175,026, filed on June 12, 2015, the teaching of which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

20 Bone disorders such as osteoporosis is a disease of severe bone loss affecting an estimated 10 million Americans and causing two million pathological fractures per year (1). Osteoporosis results from an imbalance between bone formation and resorption. This balance depends on both the number of osteoblastic (OB) and osteoclastic (OC) cells as well as their cellular activity within the bone metabolic unit (2-4). Typically, bone anabolic agents produce a secondary osteoclastogenic response, as is the case for PTH, BMP2, and retinoic acid (5-9), and as such, increasing the level of osteoblasts or the activities thereof would also increase the osteoclasts or their activities. The interest in therapies with the potential to uncouple OB and OC activity has led to intense focus on activation of the Wnt/ β -catenin signaling pathway, as this pathway has predominantly anabolic effects on bone. However, high lipophilicity and insolubility make recombinant Wnt proteins challenging for bioactive delivery (10-12).

30 An alternative approach is to increase levels of Wnt signaling via inactivation of endogenous Wnt inhibitors (Sclerostin (SOST) and DKK-1). In fact, treatment with anti-SOST and anti-DKK-1 antibodies results in increased OB number and activity, reduced OC number and activity, and a consequent increase in bone mineral density (BMD) (13,14),

5 observed in rat (15, 16), non-human primate (17), and human studies (18). In aggregate, positive regulation of the Wnt/ β -catenin signaling pathway has emerged as a promising new field for anabolic, anti-osteoclastic therapies in osteoporosis. However, antibody therapies have inherent limitations such as, among others, high production cost and low tissue penetration.

10 Therefore, there is a continuing need for methods and compositions for treating, ameliorating or preventing a bone disorder.

The embodiments described below address the above-identified problems and needs.

SUMMARY OF THE INVENTION

15 In one aspect of the present invention, it is provided a composition for a bone condition, the composition comprising an effective amount of a first component that is effective to increase the cell number and activity of osteoblast (OB) and an effective amount of a second component that is effective to decrease the cell number and activity of osteoclast (OC) such that the composition is effective to cause the increased cell number of OB (“increased OB”) and the decreased cell number of OC (“decreased OC”) to have a ratio that
20 decreased OC / increased OB is from about 100:1 to about 1:100, wherein the first component and/or the second component are not one of anti-SOST and anti-DKK-1 antibodies.

In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the first component and the second component comprise an osteoinductive agent.

25 In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the first component or the second component comprise a bisphosphonate.

In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the composition comprises a scaffold
30 impregnated with the bisphosphonate.

In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the osteoinductive agent comprises NELL-1.

5 In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the osteoinductive agent is effective for activation of the Wnt/ β -catenin signaling pathway.

 In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the increased OB and the decreased OC
10 have a ratio that decreased OC / increased OB is from about 10:1 to about 26:1.

 In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the bone condition is osteopenia.

 In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the bone condition is osteoporosis.

15 In a second aspect of the present invention, it is provide a method of treating or preventing a bone condition, comprising administering to a subject:

 an effective amount of a first component of a composition that is effective to increase the cell number and activity of osteoblast (OB) and

 an effective amount of a second component of the composition that is effective to
20 decrease the cell number and activity of osteoclast (OC), and thereby

 causing the increased cell number of OB ("increased OB") and the decreased cell number of OC ("decreased OC") to have a ratio that decreased OC / increased OB is from about 100:1 to about 1:100,

 wherien the first component and/or the second component are not one of anti-SOST
25 and anti-DKK-1 antibodies.

 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the the first component and the second component comprise an osteoinductive agent.

 In some embodiments of the invention method, optionally in combination with any or
30 all the various embodiments disclosed herein, the first component or the second component comprise a bisphosphonate.

 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the composition comprises a scaffold impregnated with the bisphosphonate.

5 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the osteoinductive agent comprises NELL-1.

 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the osteoinductive agent is effective for activation of the Wnt/ β -catenin signaling pathway.

10 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the increased OB and the decreased OC have a ratio that decreased OC / increased OB is from about 10:1 to about 26:1.

 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the bone condition is osteopenia.

15 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the bone condition is osteoporosis.

 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the subject is a human being.

 In a further aspect of the present invention, it is provide a method of fabricating a
20 composition, comprising:

 providing an effective amount of a first component that is effective to increase the cell number and activity of osteoblast (OB),

 providing an effective amount of a second component that is effective to decrease the cell number and activity of osteoclast (OC), and

25 forming the composition,

 wherein the composition is effective to cause the increased cell number of OB (“increased OB”) and the decreased cell number of OC (“decreased OC”) to have a ratio that decreased OC / increased OB is from about 100:1 to about 1:100, and

 wherein the first component and/or the second component are not one of anti-SOST
30 and anti-DKK-1 antibodies.

 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the the first component and the second component comprise an osteoinductive agent.

5 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the first component or the second component comprise a bisphosphonate.

In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the composition comprises a scaffold

10 impregnated with the bisphosphonate.

In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the osteoinductive agent comprises NELL-1.

In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the osteoinductive agent is effective for

15 activation of the Wnt/ β -catenin signaling pathway.

In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the increased OB and the decreased OC have a ratio that decreased OC / increased OB is from about 10:1 to about 26:1.

In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the bone condition is osteopenia.

In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the bone condition is osteoporosis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a-1t show Nell-1 expression with aging, and the osteoporotic phenotype of aged *Nell-1* haploinsufficient Mice, (a) Immunohistochemical staining for Nell-1 protein in rat spines, at 1 week, 3 and 9 months, (b) Semi-quantification of Nell-1 immunohistochemical staining, expressed as Nell-1⁺ bone lining cells per B.Pm (N=20, 39, 25 images, respectively). (c) Relative *Nell-1* gene expression in mouse spines at 1 week, 3 and 9 months, by qRT-PCR (N=3 samples per time point). (d) 3D image of live CT and ¹⁸F radioisotope incorporation of aged (18 mo. old) wild type (*Nell-1*^{+/+}) and *Nell-1* haploinsufficient (*Nell-1*^{+/-}) mice, (e) Quantification of BMD stratified by lumbar vertebral level (L1-L6) (N=11 and 12 mice, respectively). (f) Quantification of ¹⁸F incorporation (N=6 mice per genotype). (g) 3D coronal reconstructions of lumbar vertebrae of aged *Nell-1*^{+/+} and *Nell-1*^{+/-} mice, (h-j) Trabecular analyses of lumbar vertebrae, including (h) Trabecular bone Thickness (Tb.Th), (i) Number (Tb.N), and Q Spacing (Tb.Sp) (N=12 and 19 vertebrae, respectively). (k-m) Serum studies

among *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} aged mice including (k) serum PINP (Procollagen I N-terminal Propeptide, N=19 and 17 mice), (l) serum TRAP5b (Tartrate-Resistant Acid Phosphatase Isoform 5b, N=10 and 9 mice), and (m) serum CTX (C-Terminal Telopeptide, N=12 and 22 mice). (n) Masson's Trichrome staining of lumbar vertebral bodies of *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} mice, coronal section, (o-q) Histomorphometric quantifications of lumbar vertebral bodies. Measurements include (o) Bone Area (B.Ar) (N=29 and 26 images), (p) Percentage (%) B.Ar (N=29 and 26 images), and (q) Bone Perimeter (B.Pm) (N=30 and 29 images). (r) TRAP (Tartrate Resistant Acid Phosphatase) staining of lumbar vertebral bodies, indicating osteoclasts. Quantification of (s) Osteoblast Number (Ob.N) per B.Pm (N=20 images per genotype), and (t) Osteoclast Number (Oc.N) per B.Pm (N=26 and 39 images). Black scale bar: 25 (μm; Yellow scale bar: 100 (μm. HU: Hounsfield Units; pCi (pico Curie). Data points indicate means, while error bars represent one SEM. **P*<0.05, ***P*<0.01.

Figures 2a-j show *Nell-1* haploinsufficient mice exhibit impaired osteoblastic and excessive osteoclastic activity. OB or OC precursors were derived from the bone marrow of aged (18 mo. old) wildtype (*Nell-1*^{+/+}) and *Nell-1* haploinsufficient (*Nell-1*^{+/^{6R}}) mice. (a-d) *In vitro* osteogenic differentiation assays of OB precursors from *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} mice. (a) Alkaline phosphatase (ALP) staining, at 5 days osteogenic differentiation. (b) Alizarin red (AR) staining of bone nodules, at 10 days osteogenic differentiation. (c) Photometric quantification of ALP and AR activity, normalized to total protein content (N=3 mice and 9 wells per genotype). (d) Relative gene expression among undifferentiated *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} OB precursors by qRT-PCR including *Runx2*, *Alkaline phosphatase (Alp)*, *Osteocalcin (Ocn)*, *Osteopontin (Opn)*, *Bmp (Bone Morphogenetic Protein) 2, 4 and 7* (N=3 mice and 3 wells per genotype). (e) Bromodeoxyuridine (BrdU) incorporation assays of OB precursors after 72 hours growth. (f-j) *In vitro* bone resorption assays of OC precursors derived from aged *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} mice, performed in the presence of M-CSF and soluble RANKL (N=3 mice and 6 wells per genotype), (f) Calvarial disc bone resorption assays with OC precursors from *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} mice, as shown by Toluidine blue staining after 5 days resorption. Red arrowheads indicate resorption pits. (g) Photographic quantification of bone resorption, as determined by Toluidine blue staining (N=3 mice per genotype and 9 and 11 resorption discs, respectively). (h,i) Quantification of calvarial resorption as assessed by SEM of (h) mean bone surface roughness (N=36 and 29 measurements) and (i) mean

resorption pit depth (N=9 measurements per genotype). (j) Reconstructions of representative resorption assays based on SEM. Red arrows indicate representative resorption pits. Colorized SEM highlights resorption pits in purple. Converse experiments in which recombinant NELL-1 is added are presented in Supplementary Figure 3. Black scale bar: 55 μ m. Data points indicate means, while error bars represent one SEM. ** $P<0.01$.

Figures 3a-3e show *Nell-1* signaling activates Wnt/ β -catenin signaling activity *in vivo* through evaluation of Wnt/ β -catenin signaling in *Nell-1*^{+/^{6R}} animals (a-e). (a) *Axin2* immunohistochemistry in aged (18 mo. old) lumbar spine specimens of *Nell-1*^{+/⁺} and *Nell-1*^{+/^{6R}} animals, appearing brown. Haemotoxylin counterstain appears purple. (b) Semi-quantification of *Axin2* immunohistochemical staining among trabecular bone-lining cells (N=32 and 22 images). (c) *Axin2* mRNA expression in bone marrow flush of aged (18 mo. old) *Nell-1*^{+/⁺} and *Nell-1*^{+/^{6R}} mice, evaluated by quantitative RT-PCR (N=4 samples per genotype). (d,e) Evaluation of Wnt/ β -catenin signaling in TOPGAL reporter mice in the context of *Nell-1* overexpression. Wnt/ β -catenin signaling activity was assessed after intrafemoral injection of either *Nell-1* expressing adenovirus (Ad-*Nell-1* or Ad-Null control, 5x10¹² pt/mL). (d) Xgal staining in TOPgal femurs at 1 week post-injection. Blue staining indicates Wnt responsive cells, indicated by black arrows, cb: cortical bone (N=3 mice per treatment group). (e) FACS analysis of femoral TOPgal bone marrow flush for β -gal positivity, indicating Wnt responsive cells. Percentage of β -gal+ cells expressed as a portion of CD45-marrow cells (non-hematopoietic cells); analysis performed 2 weeks post Ad-*Nell-1* injection (N=4 mice per treatment group). Black scale bar: 25 μ m. Data points indicate means, while error bars represent one SEM. ** $P<0.01$.

Figures 4a-4p show *Nell-1* requires integrin β 1 to activate Wnt/ β -catenin signaling in OB and OC precursors. (a-d) RhNELL-1 increases Wnt signaling in the M2-10B4 BMSC line. (a) Active β -catenin immunocytochemistry in M2-10B4 cells, treated with rhNELL-1, WNT3A or control (PBS). (b,c) Western blotting and quantification of cytoplasmic and nuclear β -catenin (N=3 wells per treatment). (d) M2-10B4 cells were transfected with TOPFLASH reporter (N=4 wells per treatment). (e,f) RhNELL-1 requires intact Wnt/ β -catenin Signaling for induction of OB differentiation (N=4 wells per treatment). (e) M2-10B4 cells were treated with PBS or rhNELL-1 with or without DKK-1 for 3 days. *Runx2* expression measured by qRT-PCR. (f) M2-10B4 cells were transduced with *Runx2*-EGFP

reporter lentivirus and treated with PBS or rhNELL-1 with or without XAV939. *Runx2* reporter assay was performed after 3 days. (g-i) RhNELL-1 increases Wnt signaling in the RAW264.7 osteoclast cell line (N=3 wells per treatment). (g) Gene expression after 2 days with or without rhNELL-1. (h) Active β -catenin immunocytochemistry in RAW264.7 cells, treated with rhNELL-1, WNT3A or control (PBS). (i,j) Western blot and quantification with or without rhNELL-1. (k-p) RhNELL-1 requires integrin β 1 to activate Wnt/ β -catenin signaling (N=3 wells per treatment). (k,l) SiRNA mediated knockdown of the known NELL-1 receptor integrin β 1 was performed in M2-10B4 cells, confirmed by Western blot and quantification. (m,n) Similar siRNA mediated knockdown of integrin β 1 was performed in RAW264.7 OC cells. (o,p) After two days rhNELL-1 (300 ng/ml) treatment, Wnt signaling gene expression was evaluated in either scramble or integrin β 1 siRNA treated M2-10B4 or RAW264.7 cells. Quantitative RT-PCR for *CyclinD* and *Axin2* was performed. Black scale bars: 100 μ m. Data points indicate means, while error bars represent one SEM. * P <0.05, ** P <0.01.

Figures 5a-5j show rhNELL-1 protein intravertebral injection increases bone formation in an osteoporotic sheep model. Recombinant human (rh)NELL-1 protein or vehicle control was injected into the lumbar vertebral bodies of osteoporotic sheep. RhNELL-1 was lyophilized onto β -TCP particles, which has been previously shown to increase the stability of rhNELL-1 *in vivo* (25). Composition of the injected materials can be found in Table 3. (a) Absolute change in trabecular Bone Mineral Density (BMD) among control- and rhNELL-1-treated vertebrae by CT quantification of individual vertebral bodies (N=9 control-treated vertebrae, N=3 vertebrae per treatment dosage). (b) Percent change in Bone Volume / Tissue Volume (BV/TV) among control- and rhNELL-1-treated vertebrae by CT quantification of individual vertebral bodies. All quantified values are normalized to month 1, control treatment data (N=9 control-treated vertebrae, N=3 vertebrae per treatment dose). (c) Axial sections of high-resolution CT images. Yellow dashed boxes indicate area of high magnification on right. Red outlines indicate region of interest (ROI) for trabecular analyses. (d) Cortical Thickness (Ct.Th) as determined by microCT quantification, using the cortex ipsilateral to rhNELL-1 injection (N=162 control-treated measurements, N=54 measurements per treatment dose). (e-g) Trabecular analyses, including Trabecular bone Thickness (Tb.Th), Trabecular bone Number (Tb.N), and Trabecular bone Spacing (Tb.Sp)

5 (N=9 control-treated vertebrae, N=3 vertebrae per treatment dose). (h) Representative images of Von Kossa MacNeal's Tetrachrome (VKMT) staining trabecular bone adjacent to the injection tract. Yellow dashed boxes indicate area of high magnification on right. (i) Osteoblast Number per Bone Perimeter (Ob.N / B.Pm) (N=10, 9, 9 and 9 images, respectively). (j) Osteoclast Number (Oc.N) per 200x field (N=9, 10, 9 and 9 images, respectively). Black scale bars: 0.5 mm. Data points indicate means, while error bars represent one SEM. * $P < 0.05$, ** $P < 0.01$ in comparison to vehicle control. See Supplementary Figure 6 for additional details of sheep experimentation and analyses.

Figures 6a-6n show intravenous injection of rhNELL-1 increases bone formation in an osteoporotic mouse model. (a) Induction of osteoporosis was performed by ovariectomy (OVX) and ensuing bone loss over a five-week period, confirmed by DXA analysis of the lumbar vertebrae (N=13 and 15 mice, respectively). Confirmation of OVX was also performed postmortem by demonstration of uterine atrophy, (Figures 13b,c). (b,c) RhNELL-1 was next administered by tail vein injection (1.25 mg/kg q48hr) over a period of four weeks, and compared to PBS control treatment (N=6, 7, 6 and 9 mice, respectively). DXA analysis of the lumbar vertebrae was performed weekly. (d) Representative images after four weeks rhNELL-1 treatment, including live CT, 18F-PET, and microCT. MicroCT indices have been compared to published norms to ensure accuracy of analysis and reporting(59-61). (e-i) MicroCT quantification after four weeks treatment, including (e) BMD, (f) BV, (g) BV/TV, (h) Tb.Th, (i) Tb.N, and (j) Tb.Sp (N=6, 7, 6 and 9 mice, respectively). (k) Osteocalcin (OCN) immunohistochemical staining, and quantification of OCN+ bone-lining cells per B.Pm (N=18, 23, 25 and 30 images, respectively). (l) TRAP staining and quantification of TRAP+, multinucleated, bone-lining cells per B.Pm (N=28, 34, 28, and 40 images, respectively). (m) Calcein / Alizarin red complexon bone labeling and quantification of mineral apposition rate (MAR) and bone formation rate (BFR). Red and green arrows highlight the space between fluorochrome labels (N=3 mice and 6 measurement fields per treatment group). (n) Finite element analysis (FEA) and quantification of von Mises stress. Red color indicates areas of high stress (N=6 per treatment group). Black scale bars: 25 μ m. Data points indicate means, while error bars represent one SEM. * $P < 0.05$, ** $P < 0.01$ in comparison to PBS control, unless otherwise indicated.

Figures 7a-7h show skeletal phenotype of juvenile *Nell-1* haploinsufficient mice. (a) Representative post-mortem, 3D, micro Computed Tomography (microCT) reconstructions at birth of wildtype (*Nell-1*^{+/+}) and *Nell-1* haploinsufficient (*Nell-1*^{+/^{6R}}) mice. The axial and appendicular skeletons were grossly identical. (b) Quantification of Bone Mineral Density (BMD) and Bone Volume (BV) of the entire lumbar spine at birth. N=10 mice per genotype. (c) Histomorphometric analysis of newborn mice spines showed no statistical difference, presented in Table 1. In addition, cellular proliferation and cell death showed no observed differences, as assessed by PCNA and TUNEL staining, respectively (*data not shown*). (d) Representative microCT and ¹⁸F radioisotope incorporation images at 1 month of age. (e) BMD quantification at 1 month of age, stratified by lumbar vertebral level (L1-L6). N=8 mice per genotype. (f) ¹⁸F-PET radioisotope quantification at 1 month, stratified by lumbar vertebral level (L1-L6). N=8 mice per genotype. (g,h) Quantification of microCT and ¹⁸F-PET, stratified by lumbar vertebral level (L1-L6). N=8 mice per genotype. Data points indicate means, while error bars represent one standard errors of the mean (SEM). ***P*<0.01 in comparison to *Nell-1*^{+/+} values.

Figures 8a-8x show skeletal phenotype and body habitus of aged *Nell-1* haploinsufficient mice. Mice were analyzed at 18 months of life. (a) Representative DXA images of wildtype (*Nell-1*^{+/+}) and *Nell-1*^{6R} heterozygote (*Nell-1*^{+/^{6R}}) littermates. N=12 *Nell-1*^{+/+} and 16 *Nell-1*^{+/^{6R}} mice were used for DXA analyses. (b,c) Mean Bone Mineral Density (BMD) and Bone Mineral Content (BMC) of the lumbar vertebrae among *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} mice, assessed by DXA. (d) MicroCT quantification of BV/TV in the lumbar spine of aged *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} mice. N=12 *Nell-1*^{+/+} and 19 *Nell-1*^{+/^{6R}} individual vertebrae per genotype. (e) MicroCT reconstructions of the distal femur of aged *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} mice. (f-j) Trabecular microCT quantifications of the distal femur, including (f) BMD, (g) BV/TV, (h) Tb.Th, (i) Tb.N, and (j) Tb.Sp. N=10 individual vertebrae per genotype. (k,l) Finite element analysis (FEA) and quantification of von Mises stress of aged *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} lumbar spines. N=6 *Nell-1*^{+/+} and 8 *Nell-1*^{+/^{6R}} mice. (m,n) Finite element analysis (FEA) and quantification of von Mises stress of aged *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} distal femurs. Red color indicates areas of high stress. N=6 *Nell-1*^{+/+} and 8 *Nell-1*^{+/^{6R}} mice. (o-q) BioDent™ Mechanical Testing in aged *Nell-1* haploinsufficient mice. Mechanical properties

5 were examined between the sacral (S1) vertebral body of *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} aged littermates. N=3 mice per genotype, with N=6 measurements per mouse performed. (o) Total Indentation Distance, calculated by measuring the maximum indentation distance achieved during a measurement. (p) Indentation Distance Increase, calculated by measuring the difference between the depths reached at peak force during the first indentation cycle and last
 10 indentation cycle. (q) Unloading Stiffness, as calculated by evaluating the top portion of the unloading section of the force displacement curve. (r-t) Trabecular analyses of histological sections of lumbar vertebrae of aged *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} mice, including (r) Trabecular Width (Tb.Wi), (s) Number (Tb.N), and (t) Spacing (Tb.Sp). (u) Mean weight among *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} mice, showing a significant reduction in overall body weight among *Nell-1*^{+/^{6R}} mice. N=12 *Nell-1*^{+/+} and 16 *Nell-1*^{+/^{6R}} mice. (v) Nuclear Magnetic Resonance (NMR) analysis of % fat body mass and % lean body mass, showing no significant difference between *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} mice. N=12 *Nell-1*^{+/+} and 16 *Nell-1*^{+/^{6R}} mice were used for NMR analyses. (w,x) RANKL and OPG immunohistochemistry in aged *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} mouse spines. Semi-quantification is expressed as relative RANKL⁺ to OPG⁺ bone
 15 lining cells. Black scale bar: 25 um. Data points indicate means, while error bars represent one SEM. **P*<0.05, ***P*<0.01 in comparison to *Nell-1*^{+/+} values.

Figures 9a-9g show rhNELL-1 *In Vitro* Effects in OB precursors and OC precursors. Cells were derived from the marrow of wildtype mice for either OB precursor or OC precursor assays. (a-c) OB precursor differentiation assays. (a) Alkaline Phosphatase (ALP)
 25 staining among wildtype OB precursors with or without rhNELL-1 (100 & 300 ng/mL) at 7 days of osteogenic differentiation. (b) Alizarin Red (AR) staining of bone nodule formation at 14 days of osteogenic differentiation with or without rhNELL-1 (100 & 300 ng/mL). (c) Photometric quantification of ALP activity and AR staining, normalized to total protein content (N=4 wells per group). (d-g) Calvarial disc bone resorption assays among wildtype
 30 OC precursors treated with or without rhNELL-1 (0-1200 ng/mL), after 5 days resorption (N=4 resorption discs per group). (d) Photographic quantification of bone resorption as determined by Toluidine Blue staining. (e,f) Quantification of calvarial resorption as assessed by SEM of (e) average surface roughness (N=12 measurements per group) and (f) average pit depth (N=30 measurements per group). (g) Reconstructions of calvarial disc resorption assays
 35 based on scanning electron microscopy (SEM). Red arrows indicate representative resorption

5 pits. Colorized SEM highlights resorption pits in purple. Data points indicate means, while error bars represent one SEM. * $P < 0.05$, ** $P < 0.01$.

Figures 10a-10d show evidence of NELL-1 - Integrin β 1 binding. Please note that evidence for NELL-1 binding to Integrin β 1 by co-immunoprecipitation assays has been previously published^[1]. (a) Thermal shift assays of NELL-1 and integrin α 3 β 1, shown as the first derivative. The integrin α 3 β 1 heterodimer was used so as to maintain protein bioactivity (R&D Systems, 2840-A3-050). Two T_m from integrin α 3 β 1 were shifted upon addition of NELL-1. Assays were performed in triplicate with a representative assay shown, with means and standard deviations of T_m shown in the chart below. (b-d) Docking stimulation of NELL-1 interaction. The three top-scoring models of NELL-1 - integrin β 1 interaction predicted by docking simulation by using RosettaDock are shown. The structure of integrin β 1 was taken from the crystal structure of α 5 β 1 integrin headpiece (PDB entry 3VI3). The I-like domain and hybrid domain of integrin β 1 (in a green color) are highlighted. The model of the EGF-like domain of NELL-1 was built by using Robetta protein structure prediction server (amino acid 549-582 of NELL-1 protein). (b) The model predicts that the EGF-like domain of NELL-1 binds to the I-like domain of the integrin β 1, the same binding pocket as previously reported^[2]. Its predicted total energy is -230kcal/mol. (c) The model predicts that the EGF-like domain of NELL-1 binds to the I-like domain of the integrin β 1 at an alternative binding pocket. Its predicted total energy is -235kcal/mol. (d) The model predicts that the EGF-like domain of NELL-1 binds to the hybrid domain of the integrin β 1. Its predicted total energy is -231kcal/mol.

Figures 11a-11e show NELL-1 signaling increases Wnt/ β -catenin activity in human BMSCs derived from osteoporotic and non-osteoporotic patients. See Table 2 for details of patient samples. (a) Alizarin red staining among osteoporotic and non-osteoporotic hBMSCs, assessed at 11 days. (b) Photographic quantification of Alizarin red staining. (c) *Axin2* and *CyclinD* mRNA expression 3 days after viral infection in non-osteoporotic hBMSCs. (d,e) Western blot of β -catenin of both cytoplasmic and nuclear fractions of hBMSCs overexpressing *Nell-1*. A GFP encoding adenovirus (ad-GFP) was used as control. (d) Western blot and quantification from non-osteoporotic hBMSCs. (e) Western blot and quantification from osteoporotic hBMSCs. Black scale bar: 100 μ m. All experiments using human BMSC were performed in triplicate wells. Data points indicate means, while error

5 bars represent one SEM. $**P<0.01$. Confirming efficacy of transection, Ad-Nell-1 resulted in a 17.5 fold increase in *Nell-1* mRNA transcripts (*data not shown*).

Figures 12a-12m show sheep study design, induction of osteoporosis and additional data. (a) Study design. Osteoporosis was induced over four months time by a combination of ovariectomy, steroid administration, and dietary deficiency in Vitamin D and Calcium. After
 10 successful induction of osteoporosis, rhNELL-1 was applied by intravertebral body injection. Postoperative analysis included monthly CT scans. Harvest at 3 months post-operative was followed by high-resolution microCT, FEA, and histological analysis. (b) Representative DXA images before and after osteoporotic induction. (c) Quantification of Bone Mineral Density (BMD) as determined by DXA pre- and post-osteoporotic induction, stratified by
 15 lumbar (L) vertebral level. (d-k) RhNELL-1 Protein Intravertebral Injection Increases Bone Formation in Osteoporotic Sheep by Histology. Focus of study included the side of the vertebral body most distant from rhNELL-1 injection. N=9 random images per treatment group. (d-j) Histomorphometric quantification of cortical and trabecular measurements, including (d) Cortical Width (Ct.Wi), (e) Trabecular bone Area (Tb.Ar), (f) Percentage
 20 Trabecular bone Area (% Tb.Ar), (g) Trabecular bone Perimeter (Tb.Pm), (h) Trabecular bone Width (Tb.Wi), (i) Trabecular bone Number (Tb.N) and (j) Trabecular bone Spacing (Tb.Sp). (k) Representative images of Von Kossa MacNeal's Tetrachrome (VKMT) staining of the area contralateral to the injection site. (l) Images and (m) quantification of biomechanical stress, determined by Finite Element Analysis (FEA). The region of interest is a rectangle
 25 adjacent to the injection tract. Black scale bar: 1mm; Yellow scale bar: 0.5 mm. N=9 control-treated vertebrae, N=3 vertebrae per treatment dose. Data points indicate means, while error bars represent one SEM. $*P<0.05$, $**P<0.01$ in comparison to control.

Figures 13a-13g show systemic NELL-1 treatment and additional data. (a) Serum concentration of rhNELL-1 after a single intravenous injection. N=3 samples per timepoint.
 30 (b,c) Induction of osteoporosis was performed by ovariectomy (OVX) and ensuing bone loss over a five week period. OVX was confirmed by post-mortem examination of uterine atrophy and mean weight (N=13 and 15 mice, respectively). (d-) RhNELL-1 was next administered by tail vein injection (1.25 mg/kg q48hr), sacrificed after four weeks. (d) H&E staining and quantification of percentage Bone Area (B. Ar). (e) Osteopontin (OPN) immunohistochemical
 35 staining, and quantification of OPN⁺ bone-lining cells per B.Pm. (f) RANKL

immunohistochemical staining and quantification of RANKL⁺ bone lining cells per B.Pm. (g) OPG immunohistochemical staining and quantification of OPG⁺ bone lining cells per B.Pm (N=18-30 images per group for each immunohistochemical quantification). Data points indicate means, while error bars represent one SEM. **P*<0.05, ***P*<0.01 in comparison to PBS control.

Figure 14 shows raw images for data presented in Figures 4b, 4i, 4k, 4m, and Figures 11d and 11e.

Figure 15 Effects on expression of Opg and Rankl by an embodiment of invention composition comprising rhNELL-1 as a component of the composition in primary mouse BMSC.

DETAILED DESCRIPTION

Definitions

As used herein, the term “agent” refers to osteoinductive agent effective for promoting bone formation. The term “agent” explicitly excludes anti-SOST and anti-DKK-1 antibodies. In some embodiments, the term “agent” is also referred to from time to time as “bioactive agent”, “compound”, “chemical”, “chemical compound”, peptide, polypeptide, or protein.

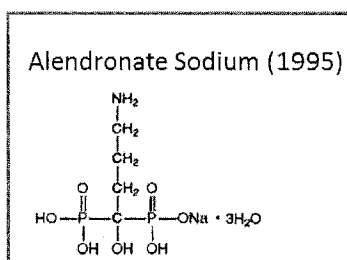
The term “effective” is also referred to as “therapeutically effective” refers to inducing statistically significant result of bone formation under clinical conditions in that it has the effect (1) to increase the cell number and activity of osteoblast (OB) and (2) to decrease the cell number and activity of osteoclast (OC). As such, the term “effective” as used herein is different from / does not encompass an agent that shows only of (1) and (2) effects. In this context, the term “therapeutically effective amount”, as used herein, is an amount of an osteoinductive agent effective for inducing statistically significant result of bone formation under clinical conditions in that it includes a sufficient amount of the osteoinductive agent (1) to increase the cell number and activity of osteoblast (OB) and (2) to decrease the cell number and activity of osteoclast (OC). As such, the term “effective amount” as used herein is different from / does not encompass an amount that shows only of (1) and (2) effects.

Whenever used herein, the term “safe and effective amount” refers to the quantity of a component which is sufficient to yield a desired therapeutic response without undue adverse side effects (such as toxicity, irritation, or allergic response) commensurate with a reasonable benefit/risk ratio when used in the manner of this invention. The specific safe and effective

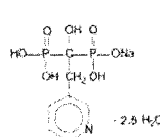
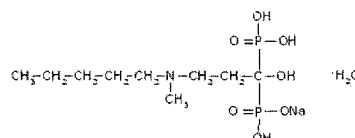
5 amount or therapeutically effective amount will vary with such factors as the particular condition being treated, the physical condition of the patient, the type of mammal or animal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives.

As used herein, the term “significantly” or “significant” shall mean statistically
 10 significant.

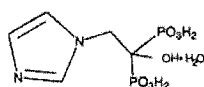
As used herein, the term bisphosphonate refers to a compound that has two phosphonate groupings. In some embodiments, the bisphosphonate is one of



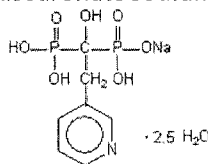
Risedronate Sodium (1998) Ibandronate Sodium (2003)



Zoledronic Acid (2007)



Risedronate Sodium (2010)



As used herein, the term “impregnated” or “embedded” refers to a state of physical
 15 mixture or blend where the parts of the mixture or blend have no or minimal interaction among/between components thereof so as not to involve conjugation or covalent chemical bonding. As such, the term “impregnated” or “embedded” explicitly exclude the state of conjugation or covalent chemical bonding.

The term “functional” when used in conjunction with “derivative” or “variant” refers
 20 to a compound or agent which possesses a biological activity that is substantially similar to a biological activity of the osteoinductive compound or agent of which it is a derivative or variant. By “substantially similar” in this context is meant that at least 50% of the relevant or desired biological activity of a corresponding osteoinductive compound or agent is retained, e.g., preferably the variant retains at least 60%, at least 70%, at least 80%, at least 90%, at
 25 least 95%, at least 100% or even higher (i.e., the variant or derivative has greater activity than

5 the original osteoinductive compound or agent), e.g., at least 110%), at least 120%, or more compared to a measurable activity of the osteoinductive compound or agent.

As used herein the term “comprising” or “comprises” is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the invention, yet open to the inclusion of unspecified elements, whether essential or not.

10 As used herein the term “consisting essentially of” refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

As used in this specification and the appended claims, the singular forms “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise. Thus for
15 example, references to “the method” includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

The term “about” or “approximately” means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on
20 how the value is measured or determined, i.e., the limitations of the measurement system. For example, “about” can mean within 1 or more than 1 standard deviation, per the practice in the art. Alternatively, “about” can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order
25 of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated the term “about” meaning within an acceptable error range for the particular value should be assumed.

The term “active fragment or variant” is meant a fragment that is 100% identical to a contiguous portion of the peptide, polypeptide or protein, or a variant that is at least 90%,
30 preferably 95% identical to a fragment up to and including the full length peptide, polypeptide or protein. A variant, for example, may include conservative amino acid substitutions, as defined in the art, or nonconservative substitutions, providing that at least e.g. 10%, 25%, 50%, 75% or 90% of the activity of the original peptide, polypeptide or protein is retained.

Unless otherwise indicated, the terms “peptide”, “polypeptide” or “protein” are used
35 interchangeably herein, although typically they refer to peptide sequences of varying sizes.

5 The term “expression vector” as used herein refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules, siRNA, ribozymes, and the like. Expression vectors can contain a variety of control sequences, which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operatively linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well.

15 By “encoding” or “encoded”, “encodes”, with respect to a specified nucleic acid, is meant comprising the information for translation into the specified protein. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated regions of the nucleic acid, or may lack such intervening non-translated sequences (e.g., as in cDNA). The information by which a protein is encoded is specified by the use of codons. Typically, the amino acid sequence is encoded by the nucleic acid using the “universal” genetic code.

20 As used herein, “heterologous” in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, one or both are substantially modified from their original form. A heterologous protein may originate from a foreign species or, if from the same species, is substantially modified from its original form by deliberate human intervention.

25 “Sample” is used herein in its broadest sense. A sample comprising polynucleotides, polypeptides, peptides, antibodies and the like may comprise a bodily fluid; a soluble fraction of a cell preparation, or media in which cells were grown; a chromosome, an organelle, or membrane isolated or extracted from a cell; genomic DNA, RNA, or cDNA, polypeptides, or peptides in solution or bound to a substrate; a cell; a tissue; a tissue print; a fingerprint, skin or hair; and the like.

5 The terms “patient”, “subject” or “individual” are used interchangeably herein, and refers to a mammalian subject to be treated, with human patients being preferred. In some cases, the methods of the invention find use in experimental animals, in veterinary application, and in the development of animal models for disease, including, but not limited to, rodents including mice, rats, and hamsters; and primates.

10 “Treatment” is an intervention performed with the intention of preventing the development or altering the pathology or symptoms of a disorder. Accordingly, “treatment” refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. As used herein, “ameliorated” or “treatment” refers to a symptom which is
15 approaches a normalized value (for example a value obtained in a healthy patient or individual), e.g., is less than 50% different from a normalized value, preferably is less than about 25% different from a normalized value, more preferably, is less than 10% different from a normalized value, and still more preferably, is not significantly different from a normalized value as determined using routine statistical tests. For example the term “treat” or
20 “treating” with respect to tumor cells refers to stopping the progression of said cells, slowing down growth, inducing regression, or amelioration of symptoms associated with the presence of said cells. Treatment of an individual suffering from an infectious disease organism refers to a decrease and elimination of the disease organism from an individual. For example, a decrease of viral particles as measured by plaque forming units or other automated diagnostic
25 methods such as ELISA etc.

Compositions

 In one aspect of the present invention, it is provided a composition for a bone condition, the composition comprising an effective amount of a first component that is effective to increase the cell number and activity of osteoblast (OB) and an effective amount
30 of a second component that is effective to decrease the cell number and activity of osteoclast (OC) such that the composition is effective to cause the increased cell number of OB (“increased OB”) and the decreased cell number of OC (“decreased OC”) to have a ratio that decreased OC / increased OB is from about 100:1 to about 1:100, wherein the first component and/or the second component are not one of anti-SOST and anti-DKK-1 antibodies.

5 In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the first component and the second component comprise an osteoinductive agent.

 In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the first component or the second
10 component comprise a bisphosphonate.

 In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the composition comprises a scaffold impregnated with the bisphosphonate.

 In some embodiments of the invention composition, optionally in combination with
15 any or all the various embodiments disclosed herein, the osteoinductive agent comprises NELL-1.

 In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the osteoinductive agent is effective for activation of the Wnt/ β -catenin signaling pathway.

20 In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the increased OB and the decreased OC have a ratio that decreased OC / increased OB is from about 10:1 to about 26:1.

 In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the bone condition is osteopenia.

25 In some embodiments of the invention composition, optionally in combination with any or all the various embodiments disclosed herein, the bone condition is osteoporosis.

Formulation Carriers

 The pharmaceutical composition described herein may be administered to a subject in need of treatment by a variety of routes of administration, including orally and parenterally,
30 (e.g., intravenously, subcutaneously or intramedullary), intranasally, as a suppository or using a “flash” formulation, i.e., allowing the medication to dissolve in the mouth without the need to use water, topically, intradermally, subcutaneously and/or administration via mucosal routes in liquid or solid form. The pharmaceutical composition can be formulated into a variety of dosage forms, e.g., extract, pills, tablets, microparticles, capsules, oral liquid.

5 There may also be included as part of the pharmaceutical composition pharmaceutically compatible binding agents, and/or adjuvant materials. The active materials can also be mixed with other active materials including antibiotics, antifungals, other virucidals and immunostimulants which do not impair the desired action and/or supplement the desired action.

10 In one embodiment, the mode of administration of the pharmaceutical composition described herein is oral. Oral compositions generally include an inert diluent or an edible carrier. They may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the aforesaid compounds may be incorporated with excipients and used in the form of tablets, troches, capsules, elixirs, suspensions, syrups,
15 wafers, chewing gums and the like. Some variation in dosage will necessarily occur, however, depending on the condition of the subject being treated. These preparations should produce a serum concentration of active ingredient of from about 0.01 nM to 1,000,000 nM, e.g., from about 0.2 to 40 μ M. A preferred concentration range is from 0.2 to 20 μ M and most preferably about 1 to 10 μ M. However, the concentration of active ingredient in the
20 drug composition itself depends on bioavailability of the drug and other factors known to those of skill in the art.

 In another embodiment, the mode of administration of the pharmaceutical compositions described herein is topical or mucosal administration. A specifically preferred mode of mucosal administration is administration via female genital tract. Another preferred
25 mode of mucosal administration is rectal administration.

 Various polymeric and/or non-polymeric materials can be used as adjuvants for enhancing mucoadhesiveness of the pharmaceutical composition disclosed herein. The polymeric material suitable as adjuvants can be natural or synthetic polymers. Representative natural polymers include, for example, starch, chitosan, collagen, sugar, gelatin, pectin,
30 alginate, kary gum, methylcellulose, carboxymethylcellulose, methylethylcellulose, and hydroxypropylcellulose. Representative synthetic polymers include, for example, poly(acrylic acid), tragacanth, poly(methyl vinyl ether-co-maleic anhydride), poly(ethylene oxide), carbopol, poly(vinyl pyrrolidone), poly(ethylene glycol), poly(vinyl alcohol), poly(hydroxyethylmethacrylate), and polycarbophil. Other bioadhesive materials available

5 in the art of drug formulation can also be used (see, for example, Bioadhesion--Possibilities and Future Trends, Gurny and Junginger, eds., 1990).

It is to be noted that dosage values also varies with the specific severity of the disease condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted to the individual need and the professional
10 judgment of the person administering or supervising the administration of the aforesaid compositions. It is to be further understood that the concentration ranges set forth herein are exemplary only and they do not limit the scope or practice of the invention. The active ingredient may be administered at once, or may be divided into a number of smaller doses to be administered at varying intervals of time.

15 The formulation may contain the following ingredients: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, com starch and the like; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; and a sweetening agent such as sucrose or saccharin or flavoring agent such as peppermint, methyl
20 salicylate, or orange flavoring may be added. When the dosage unit form is a capsule, it may contain, in addition to material of the above type, a liquid carrier such as a fatty oil. Other dosage unit forms may contain other various materials which modify the physical form of the dosage unit, for example, as coatings. Thus tablets or pills may be coated with sugar, shellac, or other enteric coating agents. Materials used in preparing these various compositions
25 should be pharmaceutically pure and non-toxic in the amounts used.

The solutions or suspensions may also include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methylparabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such
30 as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

The pharmaceutical compositions of the present invention are prepared as
35 formulations with pharmaceutically acceptable carriers. Preferred are those carriers that will

5 protect the active compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems.

Biodegradable, biocompatible polymers can be used, such as polyanhydrides, polyglycolic acid, collagen, and polylactic acid. Methods for preparation of such formulations can be readily performed by one skilled in the art.

10 Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) are also preferred as pharmaceutically acceptable carriers. Methods for encapsulation or incorporation of compounds into liposomes are described by Cozzani, I.; Jori, G.; Bertoloni, G.; Milanesi, C.; Sicuro, T. *Chem. Biol. Interact.* 53, 131-143 (1985) and by Jori, G.; Tomio, L.; Reddi, E.; Rossi, E. *Br. J. Cancer* 48, 307-309
15 (1983), for example. These may also be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811 (which is incorporated herein by reference in its entirety). For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearyl phosphatidyl ethanolamine, stearyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic
20 solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound is then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

Other methods for encapsulating compounds within liposomes and targeting areas of
25 the body are described by Sicuro, T.; Scarcelli, V.; Vigna, M. F.; Cozzani, I. *Med. Biol. Environ.* 15(1), 67-70 (1987) and Jori, G.; Reddi, E.; Cozzani, I.; Tomio, L. *Br. J. Cancer*, 53(5), 615-21 (1986), for example.

The pharmaceutical composition described herein may be administered in single (e.g., once daily) or multiple doses or via constant infusion. The compounds of this invention may
30 also be administered alone or in combination with pharmaceutically acceptable carriers, vehicles or diluents, in either single or multiple doses. Suitable pharmaceutical carriers, vehicles and diluents include inert solid diluents or fillers, sterile aqueous solutions and various organic solvents. The pharmaceutical compositions formed by combining the compounds of this invention and the pharmaceutically acceptable carriers, vehicles or diluents
35 are then readily administered in a variety of dosage forms such as tablets, powders, lozenges,

5 syrups, injectable solutions and the like. These pharmaceutical compositions can, if desired, contain additional ingredients such as flavorings, binders, excipients and the like according to a specific dosage form.

 Thus, for example, for purposes of oral administration, tablets containing various excipients such as sodium citrate, calcium carbonate and/or calcium phosphate may be
10 employed along with various disintegrants such as starch, alginic acid and/or certain complex silicates, together with binding agents such as polyvinylpyrrolidone, sucrose, gelatin and/or acacia. Additionally, lubricating agents such as magnesium stearate, sodium lauryl sulfate and talc are often useful for tableting purposes. Solid compositions of a similar type may also be employed as fillers in soft and hard filled gelatin capsules. Preferred materials for this
15 include lactose or milk sugar and high molecular weight polyethylene glycols. When aqueous suspensions or elixirs are desired for oral administration, the active pharmaceutical agent therein may be combined with various sweetening or flavoring agents, coloring matter or dyes and, if desired, emulsifying or suspending agents, together with diluents such as water, ethanol, propylene glycol, glycerin and/or combinations thereof.

20 For parenteral administration, solutions of the compounds of this invention in sesame or peanut oil, aqueous propylene glycol, or in sterile aqueous solutions may be employed. Such aqueous solutions should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal
25 administration. In this connection, the sterile aqueous media employed are all readily available by standard techniques known to those skilled in the art.

 For intranasal administration or administration by inhalation, the compounds of the invention are conveniently delivered in the form of a solution or suspension from a pump spray container that is squeezed or pumped by the patient or as an aerosol spray presentation
30 from a pressurized container or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurized container or nebulizer may contain a solution or suspension of a compound of this invention. Capsules and
35 cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be

5 formulated containing a powder mix of a compound or compounds of the invention and a suitable powder base such as lactose or starch.

The pharmaceutical composition provided herein can also be used with another pharmaceutically active agent effective for a disease such as neurodisorders, cardiovascular disorders, tumors, AIDS, depression, and/or type-1 and type-2 diabetes. Such additional
10 agents can be, for example, antiviral agent, antibiotics, anti-depression agent, anti-cancer agents, immunosuppressant, anti-fungal, and a combination thereof.

The pharmaceutical composition described herein can be formulated alone or together with the other agent in a single dosage form or in a separate dosage form. Methods of preparing various pharmaceutical formulations with a certain amount of active ingredient are
15 known, or will be apparent in light of this disclosure, to those skilled in this art. For examples of methods of preparing pharmaceutical formulations, see Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., 19th Edition (1995).

Scaffolds

In one embodiment, if desirable, the composition of invention can be formulated into a
20 scaffold. Such a scaffold can include a carrier, which can be biodegradable, such as degradable by enzymatic or hydrolytic mechanisms. Examples of carriers include, but are not limited to synthetic absorbable polymers such as but not limited to poly(.alpha.-hydroxy acids) such as poly (L-lactide) (PLLA), poly (D, L-lactide) (PDLLA), polyglycolide (PGA), poly (lactide-co-glycolide (PLGA), poly (-caprolactone), poly (trimethylene
25 carbonate), poly (p-dioxanone), poly (-caprolactone-co-glycolide), poly (glycolide-co-trimethylene carbonate) poly (D, L-lactide-co-trimethylene carbonate), polyarylates, polyhydroxybutyrate (PHB), polyanhydrides, poly (anhydride-co-imide), propylene-co-fumarates, polylactones, polyesters, polycarbonates, polyanionic polymers, polyanhydrides, polyester-amides, poly(amino-acids), homopolypeptides, poly(phosphazenes), poly
30 (glaxanone), polysaccharides, and poly(orthoesters), polyglactin, polyglactic acid, polyaldonic acid, polyacrylic acids, polyalkanoates; copolymers and admixtures thereof, and any derivatives and modifications. See for example, U.S. Pat. No. 4,563,489, and PCT Int. Appl. # WO/03024316, herein incorporated by reference. Other examples of carriers include
cellulosic polymers such as, but not limited to alkylcellulose, hydroxyalkylcellulose,
35 methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose,

5 hydroxypropyl-methylcellulose, carboxymethylcellulose, and their cationic salts. Other examples of carriers include synthetic and natural bioceramics such as, but not limited to calcium carbonates, calcium phosphates, apatites, bioactive glass materials, and coral-derived apatites.

10 In one embodiment, the carrier may further be coated by compositions, including bioglass and or apatites derived from sol-gel techniques, or from immersion techniques such as, but not limited to simulated body fluids with calcium and phosphate concentrations ranging from about 1.5 to 7-fold the natural serum concentration and adjusted by various means to solutions with pH range of about 2.8-7.8 at temperature from about 15-65 degrees C. Other examples of carriers include collagen (e.g. Collastat, Helistat collagen sponges),
15 hyaluronan, fibrin, chitosan, alginate, and gelatin, or a mixture thereof.

In one embodiment, the carrier may include heparin-binding agents; including but not limited to heparin-like polymers e.g. dextran sulfate, chondroitin sulfate, heparin sulfate, fucan, alginate, or their derivatives; and peptide fragments with amino acid modifications to increase heparin affinity. See for example, Journal of Biological Chemistry (2003), 278(44),
20 p. 43229-43235, the teachings of which are incorporated herein by reference.

In one embodiment, the scaffold may be in the form of a liquid, solid or gel.

In one embodiment, the scaffold can be a carrier that is in the form of a flowable gel. The gel may be selected so as to be injectable, such as via a syringe at the site where bone formation is desired. The gel may be a chemical gel which may be a chemical gel formed by
25 primary bonds, and controlled by pH, ionic groups, and/or solvent concentration. The gel may also be a physical gel which may be formed by secondary bonds and controlled by temperature and viscosity. Examples of gels include, but are not limited to, pluronics, gelatin, hyaluronan, collagen, polylactide-polyethylene glycol solutions and conjugates, chitosan, chitosan & b-glycerophosphate (BST-gel), alginates, agarose, hydroxypropyl cellulose,
30 methyl cellulose, polyethylene oxide, polylactides/glycolides in N-methyl-2-pyrrolidone. See for example, Anatomical Record (2001), 263(4), 342-349, the teachings of which are incorporated herein by reference.

In one embodiment of the scaffold, the carrier may be photopolymerizable, such as by electromagnetic radiation with wavelength of at least about 250 nm, Example of
35 photopolymerizable polymers include polyethylene (PEG) acrylate derivatives, PEG

5 methacrylate derivatives, propylene fumarate-co-ethylene glycol, polyvinyl alcohol derivatives, PEG-co-poly(-hydroxy acid) diacrylate macromers, and modified polysaccharides such as hyaluronic acid derivatives and dextran methacrylate.

In one embodiment, the scaffold may include a carrier that is temperature sensitive. Examples include carriers made from N-isopropylacrylamide (NiPAM), or modified NiPAM
10 with lowered lower critical solution temperature (LCST) and enhanced peptide (e.g. NELLI) binding by incorporation of ethyl methacrylate and N-acryloxysuccinimide; or alkyl methacrylates such as butylmethacrylate, hexylmethacrylate and dodecylmethacrylate (PCT Int. Appl. WO/2001070288; U.S. Pat. No. 5,124,151, the teachings of which are incorporated herein by reference).

15 In one embodiment of the scaffold, where the carrier may have a surface that is decorated and/or immobilized with cell adhesion molecules, adhesion peptides, and adhesion peptide analogs which may promote cell-matrix attachment via receptor mediated mechanisms, and/or molecular moieties which may promote adhesion via non-receptor mediated mechanisms binding such as, but not limited to polycationic polyamino-acid-peptides (e.g. poly-lysine), polyanionic polyamino-acid-peptides, Mefp-class adhesive
20 molecules and other DOPA-rich peptides (e.g. poly-lysine-DOPA), polysaccharides, and proteoglycans. See for example, PCT Int. Appl. WO/2004005421; WO/2003008376; WO/9734016, the teachings of which are incorporated herein by reference.

In one embodiment of the scaffold, the carrier may be comprised of sequestering
25 agents such as, but not limited to, collagen, gelatin, hyaluronic acid, alginate, poly(ethylene glycol), alkylcellulose (including hydroxyalkylcellulose), including methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropyl-methylcellulose, and carboxymethylcellulose, blood, fibrin, polyoxyethylene oxide, calcium sulfate hemihydrate, apatites, carboxyvinyl polymer, and poly(vinyl alcohol). See for
30 example, U.S. Pat. No. 6,620,406, herein incorporated by reference.

In one embodiment of the scaffold, the carrier may include buffering agents such as, but not limited to glycine, glutamic acid hydrochloride, sodium chloride, guanidine, heparin, glutamic acid hydrochloride, acetic acid, succinic acid, polysorbate, dextran sulfate, sucrose, and amino acids. See for example, U.S. Pat. No. 5,385,887, herein incorporated by reference.

5 In one embodiment, the carrier may include a combination of materials such as those listed above. By way of example, the carrier may be a PLGA/collagen carrier membrane.

In one embodiment, the scaffold can be an implant of the various embodiments described herein.

Time release formulation

10 In one embodiment, the composition according to this invention may be contained within a time release tablet. A bioactive agent described herein can be formulated with an acceptable carrier to form a pharmacological composition. Acceptable carriers can contain a physiologically acceptable compound that acts, for example, to stabilize the composition or to increase or decrease the absorption of the agent. Physiologically acceptable compounds can
15 include, for example, carbohydrates, such as glucose, sucrose, or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins, compositions that reduce the clearance or hydrolysis of the anti-mitotic agents, or excipients or other stabilizers and/or buffers. Other physiologically acceptable compounds include wetting agents, emulsifying agents, dispersing agents or preservatives which are particularly useful for
20 preventing the growth or action of microorganisms. Various preservatives are well known and include, for example, phenol and ascorbic acid. One skilled in the art would appreciate that the choice of a carrier, including a physiologically acceptable compound depends, for example, on the route of administration.

Dosage forms

25 Embodiments of the composition of invention can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable may include powder, tablets, pills, capsules.

Method of Making

In another aspect of the present invention, it is provide a method of fabricating a
30 composition, comprising:
providing an effective amount of a first component that is effective to increase the cell number and activity of osteoblast (OB),
providing an effective amount of a second component that is effective to decrease the cell number and activity of osteoclast (OC), and
35 forming the composition,

5 wherein the composition is effective to cause the increased cell number of OB (“increased OB”) and the decreased cell number of OC (“decreased OC”) to have a ratio that decreased OC / increased OB is from about 100:1 to about 1:100, and

 wherein the first component and/or the second component are not one of anti-SOST and anti-DKK-1 antibodies.

10 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the the first component and the second component comprise an osteoinductive agent.

 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the first component or the second component
15 comprise a bisphosphonate.

 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the composition comprises a scaffold impregnated with the bisphosphonate.

 In some embodiments of the invention method, optionally in combination with any or
20 all the various embodiments disclosed herein, the osteoinductive agent comprises NELL-1.

 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the osteoinductive agent is effective for activation of the Wnt/ β -catenin signaling pathway.

 In some embodiments of the invention method, optionally in combination with any or
25 all the various embodiments disclosed herein, the increased OB and the decreased OC have a ratio that decreased OC / increased OB is from about 10:1 to about 26:1.

 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the bone condition is osteopenia.

 In some embodiments of the invention method, optionally in combination with any or
30 all the various embodiments disclosed herein, the bone condition is osteoporosis.

Method of Using

 In a further aspect of the present invention, it is provide a method of treating or preventing a bone condition, comprising administering to a subject:

 an effective amount of a first component of a composition that is effective to increase
35 the cell number and activity of osteoblast (OB) and

5 an effective amount of a second component of the composition that is effective to decrease the cell number and activity of osteoclast (OC), and thereby

causing the increased cell number of OB ("increased OB") and the decreased cell number of OC ("decreased OC") to have a ratio that decreased OC / increased OB is from about 100:1 to about 1:100,

10 wherein the first component and/or the second component are not one of anti-SOST and anti-DKK-1 antibodies.

In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the the first component and the second component comprise an osteoinductive agent.

15 In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the first component or the second component comprise a bisphosphonate.

In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the composition comprises a scaffold
20 impregnated with the bisphosphonate.

In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the osteoinductive agent comprises NELL-1.

In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the osteoinductive agent is effective for
25 activation of the Wnt/ β -catenin signaling pathway.

In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the increased OB and the decreased OC have a ratio that decreased OC / increased OB is from about 10:1 to about 26:1.

In some embodiments of the invention method, optionally in combination with any or
30 all the various embodiments disclosed herein, the bone condition is osteopenia.

In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the bone condition is osteoporosis.

In some embodiments of the invention method, optionally in combination with any or all the various embodiments disclosed herein, the subject is a human being.

35 EXAMPLES

5 The embodiments of the present invention will be illustrated by the following set forth examples. All parameters and data are not to be construed to unduly limit the scope of the embodiments of the invention.

Example 1. Osteoinductive studies on invention composition embodiments comprising NELL-1 as a component.

10 Summary:

NELL-1 is a secreted, osteoinductive protein whose expression rheostatically controls skeletal ossification. Overexpression of NELL-1 results in craniosynostosis in humans and mice whereas lack of *Nell-1* expression is associated with skeletal under-mineralization. Herein, *Nell-1* haploinsufficient mice showed normal skeletal development but exhibited an
15 osteoporotic phenotype with age characterized by a reduction in osteoblast:osteoclast (OB:OC) ratio and increased bone fragility. As a consequence of binding to integrin $\beta 1$, recombinant NELL-1 induced Wnt/ β -catenin signaling, associated with increased OB differentiation and inhibition of OC-directed bone resorption. Systemic delivery of NELL-1 to mice rendered osteoporotic by gonadectomy resulted in improved bone mineral density.
20 When extended to a large animal model, local delivery of NELL-1 to osteoporotic sheep spine led to significantly increased bone formation. In aggregate, these findings suggest that NELL-1 deficiency plays a role in manifestation of an osteoporotic phenotype, and demonstrate the potential utility of NELL-1 as a combination anabolic/anti-osteoclastic therapeutic for osteoporosis.

25 Introduction

NELL-1 is a unique secreted protein of 810-amino acids first studied in the context of human craniofacial skeletal development, where NELL-1 was noted to be osteoinductive and its overexpression associated with human craniosynostosis (CS)(19). Since that time, transgenic *Nell-1* overexpressing mice have been observed to recapitulate a CS-like
30 phenotype(20). Conversely, *Nell-1* deficient mice (as developed by ENU-induced mutagenesis) exhibit cranial and vertebral bone defects with under-mineralization(21). Mechanistically, NELL-1 binds to the cell surface receptor integrin $\beta 1$ (22), and regulates activity of the master osteogenic transcription factor, Runt-related transcription factor-2 (Runx2)(23). Despite the well-demonstrated osteogenic effects of NELL-1(24-28), there has
35 been little understanding of the signaling pathway through which NELL-1's effects are

5 exerted, nor the potential role of NELL-1 in osteoporosis. However, a recent genome wide study of single nucleotide polymorphisms found a linkage between *NELL-1* and osteoporosis in human patients(29).

Here, we explore the causative and therapeutic possibilities of manipulating NELL-1 signaling in osteoporosis. First, the effects of *Nell-1* deficiency in skeletal aging were
 10 evaluated by studying the skeletons of *Nell-1* haploinsufficient mice. Next, the opposing effects of NELL-1 on OB and OC cells were uncovered, as well as mechanisms underlying NELL-1's activation of Wnt/ β -catenin signaling. Further, a large animal study was performed in which surgical delivery of NELL-1 was used in order to combat osteoporotic bone loss in sheep spine. Finally, systemic administration of NELL-1 was used to reverse gonadectomy-
 15 induced osteoporotic bone loss in mice. In sum, the present study describes a new role for NELL-1 as an etiological factor and treatment of osteoporosis.

Results

Osteoporotic Phenotype of Nell-1 Haploinsufficient Mouse

NELL-1 expression, known to be present during skeletal development, was evaluated
 20 with skeletal aging. *Nell-1* protein showed a significant reduction with age, as shown by immunohistochemistry of trabecular bone-lining osteoblasts in the rat lumbar spine (Fig. 1a). Semi-quantitative analysis demonstrated a significant decrease in *Nell-1*⁺ bone-lining cells with age (Fig. 1b). These results were further quantified by qRT-PCR, showing a progressive loss of *Nell-1* in bone with age (Fig. 1c). The consequences of *Nell-1* deficiency on skeletal
 25 aging were next assessed. Deficiency was induced by generation of a point mutation in the *Nell-1* gene (*Nell-1*^{6R/6R}) resulting in a premature stop codon and near complete loss of transcript levels. Complete deficiency (*Nell-1*^{6R/6R}) is neonatal lethal with major skeletal anomalies, while the heterozygote (*Nell-1*^{+6R}) mice are without gross abnormalities at birth(21). The *Nell-1*^{+6R} mouse skeleton was studied across its lifetime. The neonatal (P1
 30 days), young adult (1 month), and adult skeleton (6 months) showed no significant skeletal phenotype, as assessed by combined live CT / fluoride radioisotope (¹⁸F) incorporation studies, and histomorphometric analyses (Figures 7, Table 1). In contrast, by 18 months of life the *Nell-1*^{+6R} mouse developed significant osteoporosis (Fig. 1, Figures 8). This was apparent both in analysis of the lumbar spine (Fig. 1d-t), and the distal femur (Figures 8f-k).
 35 Analyses showed significantly reduced BMD and reduced ¹⁸F incorporation (Fig. 1d-f). Dual

energy X-ray absorptiometry (DXA), high-resolution microCT, and trabecular bone analyses confirmed an osteoporotic phenotype in the aged *Nell-1*^{+/-6R} mouse in both the lumbar spine and femur (Fig. 1g-j, Figures 8a-k). Further, the aged *Nell-1*^{+/-6R} bone showed increased bone fragility and decreased bone stiffness as shown by both computer simulated biomechanical compression testing as well as microindentation testing (Figures 8l-r). Next, serum biomarkers evaluated total OB and OC activity (Fig. 1k-m). Total bone formation activity (serum PINP) was reduced among *Nell-1*^{+/-6R} mice; while total bone resorption activity was increased in *Nell-1*^{+/-6R} mice, (serum TRAP5b and CTX). Histological analysis confirmed a significant reduction in vertebral body trabecular bone among *Nell-1*^{+/-6R} specimens (Fig. 1n-q, Figures 8s-y). Next, OB and OC prevalence was analyzed histologically (Fig. 1r-t). OB Number (Ob.N / B.Pm) was significantly reduced in *Nell-1*^{+/-6R} lumbar vertebrae. Next, OC numbers, evaluated by TRAP staining, showed a significant increase in Oc.N / B.Pm among *Nell-1*^{+/-6R} vertebrae. Thus, by radiographic, biomechanical and histologic analyses, *Nell-1* haploinsufficiency results in normal skeletogenesis and development, but results in an osteoporotic defect with age. This osteoporotic defect, manifested in both sexes, is characterized by increased cortical bone fragility, altered trabecular bone morphology, decreased OB and increased OC number.

NELL-1 has Inverse Effects on Bone Formation and Resorption

In vivo studies demonstrated marked alterations in both OB / OC number and activity. Next, the isolated cellular effects of *Nell-1* haploinsufficiency were determined by the culture of either OB or OC precursors. First, marrow-derived *Nell-1*^{+/-6R} OB precursors were examined (Fig. 2a-e). Consistent with *in vivo* findings, *Nell-1*^{+/-6R} OB precursors showed a significant reduction in expression across all markers of *in vitro* osteogenic differentiation, including alkaline phosphatase (ALP) positive cells, as well as alizarin red positive bone nodules. Specific gene expression showed a global reduction across both 'early' and 'later' markers of OB differentiation. In addition, OB precursor proliferation was reduced among *Nell-1*^{+/-6R} cells. Next, converse experiments were performed with OC precursors (Fig. 2f-j). OC precursors from *Nell-1*^{+/+} and *Nell-1*^{+/-6R} aged littermates were seeded on bone discs for resorption assays. By gross photographic and scanning electron microscopy (SEM) assessments, greater bone resorption was detected among *Nell-1*^{+/-6R} OC precursors. These results were quantified, showing an increase in resorption by *Nell-1*^{+/-6R} OC precursors

5 (including topographic roughness and resorption pit depth). In summary, *Nell-1*^{+6R} OB precursors showed a reduction in proliferation, differentiation, and bone nodule formation. Conversely, *Nell-1*^{+6R} OC precursors showed increased OC activity and bone resorption when compared to *Nell-1*^{+/+} cells.

10 Having demonstrated the effect of *Nell-1* deficiency in OB and OC precursors, we next inquired as to the effects of NELL-1 gain of function, via the addition of NELL-1 protein. To answer this, marrow-derived wildtype OB and OC precursors were harvested, and identical assays *in vitro* performed, now in the presence of recombinant human (rh)NELL-1 (Figures 9). As expected, rhNELL-1 dose-dependently increased OB precursor osteogenic differentiation by all markers. In contrast, culture of OC precursors with rhNELL-1 led to
15 reduced bone resorption. Thus, rhNELL-1 had contrary effects on OB and OC precursors: rhNELL-1 increased OB precursor differentiation, but inhibited OC precursor differentiation / bone resorption.

NELL-1 Increases Wnt/ β -catenin Signaling via Integrin β 1

The divergent effects of NELL-1 on OB and OC cells parallel the known effects of
20 Wnt/ β -catenin signaling(30). To assess this link, Wnt signaling activation in the aged *Nell-1*^{+6R} spine was compared to *Nell-1*^{+/+} littermates (Fig. 3a-c). Axin2 immunohistochemistry was first examined as a marker of Wnt signaling activation. A significant reduction in Axin2 expression was observed among bone lining OBs as well as in marrow cells in the *Nell-1*^{+6R} spine (Fig. 3a), confirmed by semi-quantitative analysis of bone-lining cells (Fig. 3b). This
25 reduction was further quantified using qRT-PCR, to reveal an approximate 35% reduction in *Axin2* expression (Fig. 3c). Next, gain-of-function experiments were performed, using a TOPGAL Wnt reporter mouse (Fig. 3d,e). Here, adenoviral *Nell-1* (Ad-Nell-1)(31) was injected into the femoral bone marrow cavity. In comparison to control virus, Ad-Nell-1 led to a significant increase in Wnt/ β -catenin signaling activity as shown by the number of β -gal⁺
30 marrow cells (Fig. 3d), confirmed by flow cytometry (Fig. 3e). Thus, NELL-1 loss- or gain-of-function led to reduced or increased intramarrow Wnt/ β -catenin signaling, respectively.

Next, we confirmed the association between NELL-1 and Wnt/ β -catenin signaling in both OB and OC cells (Fig. 4). First, the M2-10B4 mouse bone marrow stromal cell (BMSC) line was treated with rhNELL-1 or WNT3A as a positive control. By three independent
35 methods, rhNELL-1 induced a significant increase in Wnt/ β -catenin (Fig. 4a-c). First,

immunocytochemistry for active β -catenin showed increased staining (Fig. 4a). Western blotting for cytoplasmic and nuclear fractions demonstrated increased nuclear β -catenin (Fig. 4b,c). Finally, M2-10B4 cells expressing the TOPFLASH reporter system exhibited an increase in TCF/LEF1 activity (Fig. 4d). Next, we determined if interference with Wnt/ β -catenin signaling would impede rhNELL-1-induced OB differentiation. To answer this, two antagonists of Wnt signaling were used: DKK-1 (Fig. 4e) or XAV939 (Fig. 4f). Results showed that both Wnt antagonists inhibited rhNELL-1's induction of Runx2. We next determined whether rhNELL-1 induced Wnt/ β -catenin signaling in OC precursor cells, using the mouse OC line RAW264.7 (Fig. 4g-j). As assessed by immunocytochemistry, nuclear β -catenin accumulation, and gene markers of Wnt/ β -catenin signaling, rhNELL-1 showed a similar induction of Wnt signaling activity in RAW264.7 cells. In summary, rhNELL-1 protein activates Wnt/ β -catenin signaling in both OB and OC cell types *in vitro*. Moreover, NELL-1's induction of OB differentiation is dependent on intact Wnt/ β -catenin signaling.

We have previously determined that NELL-1 binds to the surface receptor integrin β 1, and that this interaction is necessary for NELL-1's positive regulation of OB proliferation and adhesion(22). Hasebe *et al.* subsequently verified that NELL-1 binds to integrin β 1 and integrin α 3(32). Here, we confirmed these findings by thermal shift assays using integrin α 3 β 1 with or without NELL-1 (Figures 10). In order to predict potential binding sites between NELL-1 and integrin β 1, we next employed computational modeling to determine likely sites of protein interaction (Figures 10b-d). Integrins are α/β heterodimeric cell surface receptors that control numerous cellular processes, including OB and OC proliferation, differentiation and activity(33). Furthermore, integrin receptor complexes containing integrin β 1 activate Wnt/ β -catenin signaling via integrin-linked kinase signaling and modulation of GSK3P phosphorylation(34). Therefore, we assayed whether integrin β 1 was required for NELL-1 activation of Wnt/ β -catenin signaling (Fig. 4k-p). SiRNA mediated knockdown of integrin β 1 inhibited rhNELL-1's activation of Wnt/ β -catenin signaling, both in OB and OC precursor cell lines. Thus, integrin β 1 is required for NELL-1 activation of Wnt/ β -catenin signaling in both OB and OC cell types.

Subsequently, we sought to extend these findings to the human patient. For this purpose, human (h)BMSC were harvested from patients with or without a clinical history of osteoporosis (Table 2, Figures 11). NELL-1 signaling was manipulated by adenoviral *Nell-1*

5 (Ad-Nell-1) or control (Ad-GFP). Ad-Nell-1 increased OB differentiation in hBMSC derived from either osteoporotic or non-osteoporotic samples. Further, Ad-Nell-1 treatment resulted in increased Wnt/ β -catenin signaling activity in non-osteoporotic and osteoporotic hBMSC, as shown by gene markers and nuclear accumulation of β -catenin.

10 In summary, NELL-1 activates Wnt/ β -catenin signaling in OB and OC precursor cells, in a process requiring integrin β 1. Moreover, NELL-1 signaling activates Wnt/ β -catenin signaling in human cells, from either non-osteoporotic or osteoporotic patients.

Local NELL-1 Increases Bone Formation in an Osteoporotic Sheep Model

To translate NELL-1's osteogenic function into a clinically relevant large animal model, local surgical delivery of rhNELL-1 was performed in sheep spine, which has similar
15 dimensions, mineral content, and collagen composition to that of humans(35-38). Induction of osteoporosis was achieved by ovariectomy (OVX), glucocorticoid administration, and a low calcium and low vitamin D diet (Figure 12a). Similar to human osteoporosis, lumbar spines are significantly compromised and prone to compression fracture. Local surgical delivery of rhNELL-1 was performed to L1, 3 and 5. rhNELL-1 protein was injected into the
20 cancellous bone of the vertebral body, after lyophilization onto β -tricalcium phosphate (β -TCP) and using a hyaluronic acid carrier (Table 3 for injection composition).

Live CT scans performed monthly after rhNELL-1 injection, showed a significant increase in BMD and bone volume in rhNELL-1 treated vertebrae (Fig. 5a,b). Moreover, high-resolution microCT imaging and quantification showed increased Cortical bone
25 Thickness (Ct.Th), and increased trabecular bone density in rhNELL-1 treated vertebrae (Fig. 5c-g). Histological examination confirmed a significant anabolic response to rhNELL-1 injection (Fig. 5h), quantified by histomorphometric analysis of cortical and trabecular bone measurements in the peri-injection area (Table 4). Bone distant from the injection site was analyzed, showing a similar anabolic response (Figures 12d-g). We next examined the effects
30 of rhNELL-1 on OB and OC number. Consistent with our *in vitro* observations, rhNELL-1 significantly increased Ob.N and either reduced or had no effect on Oc.N (Fig. 5i,j). In summary, local rhNELL-1 delivery had significant and sustained bone-forming effects in osteoporotic sheep, observed in both cortical and cancellous bone, and accompanied by an increased OB:OC ratio.

5 *Systemic NELL-1 Increases Bone Formation in an Osteoporotic Mouse Model*

While the local effects of rhNELL-1 on bone formation have been established in other models(24-28), the effects of systemic rhNELL-1 administration are entirely unknown and represent a broader impact for the treatment of osteoporosis. Systemic delivery was achieved by intravenous injection of rhNELL-1 in either non-osteoporotic or OVX-induced

10 osteoporotic mice (Fig. 6, Figures 13). As expected, OVX induced a loss in mean BMD, observed over a five-week period (Fig. 6a). Next we examined the pharmacokinetics of systemic rhNELL-1 (Table 5). The elimination half-life of systemic rhNELL-1 was found to be 5.52 hours (Figure 13a); thus, for sustained treatment rhNELL-1 was injected every 48 hours. In comparison to control and over a four-week period, results showed that systemic

15 rhNELL-1 treatment induced significant bone formation in both non-OVX and OVX mice (Fig. 6b-n). Analysis of the lumbar vertebrae showed rhNELL-1 induced bone formation by weekly DXA (Fig. 6b,c), live CT/¹⁸F-PET imaging (Fig. 6d), and high-resolution microCT reconstruction and quantification (Fig. 6d-j). Histologic analyses were performed, including assessment of OB and OC markers (Fig. 6k,l and Figures 13d-g). In agreement with our prior

20 *in vitro* and *in vivo* observations, systemic rhNELL-1 administration showed both pro-osteoblastic and anti-osteoclastic effects, with an increase in Ob.N and reduction in Oc.N. This was accompanied by reduced RANKL and increased OPG protein expression with rhNELL-1 administration, as observed by immunohistochemistry (Figures 13f,g). Bone labeling and dynamic histomorphometric analyses demonstrated a significant increase in

25 MAR and BFR with rhNELL-1 administration (Fig. 6m). Moreover, computer simulated biomechanical (FEA) testing demonstrated increased bone strength with rhNELL-1 treatment, in both sham and OVX conditions (Fig. 6n). Importantly, no adverse effects were observed with rhNELL-1 in animal morbidity or mortality across the study period. As an external validity, formal five day repeat intravenous toxicity testing in mice showed no adverse effects

30 (Tables 9,10) (performed by Pacific Bioloab, Hercules, CA). Thus, NELL-1, an osteoinductive protein that activates Wnt/ β -catenin signaling via integrin β 1, can be a safe, systemic therapy to improve osteoporotic bone quality, tipping the balance in favor of bone anabolism over bone resorption.

5 Discussion

In summary, we report for the first time that *Nell-1* deficiency in aged mice results in an osteoporotic phenotype, characterized by increased bone fragility, reduced Wnt/ β -catenin signaling, and reduced OB:OC ratio in terms of cell number and activity. On the cellular level, NELL-1 exerts its effects through interaction with integrin β 1, and subsequent positive
10 regulation of Wnt/ β -catenin signaling. Finally, rhNELL-1 increases endocortical and cancellous bone in osteoporotic animal models, via either local or systemic administration. We conclude that NELL-1 activates Wnt/ β -catenin signaling via integrin β 1, uncouples OB:OC activity, may play a protective role against bone loss, and represents a new anabolic and anti-osteoclastic pharmacotherapeutic agent.

15 First, we have found that NELL-1 differs from most other osteoinductive molecules in its negative effects on osteoclastic bone resorption. Typically, anabolic agents also produce a secondary osteoclastogenic response. In contrast, we observed NELL-1 to have inhibitory effects on OCs, with decreased OC number and activity - much like anti-Wnt inhibitor therapies. Our studies suggest that NELL-1's OC inhibition may be direct and/or indirect.
20 NELL-1 directly activates Wnt/ β -catenin signaling in both OB and OC cell types. Wnt/ β -catenin signaling in OBs is well described to induce OB-derived expression of the major osteoclast inhibitor Osteoprotegerin (OPG), and alteration of the balance between OPG and RANKL, thereby indirectly inhibiting OC activity(39-42). In addition, Wnt/ β -catenin signaling activation in OC precursors has anti-osteoclastogenic effects, independent of OB
25 elaborated OPG(43). Therefore, NELL-1's positive effects on bone formation and negative effects on bone resorption may be explained both by activation of Wnt/ β -catenin signaling in OB and OC precursors, respectively. The extent to which the anti-OC effects of NELL-1 are similar or different to anti-Wnt inhibitor therapies, such as anti-SOST antibodies, has yet to be determined.

30 Second, we have shown that NELL-1 activation of Wnt/ β -catenin in OB and OC cell types requires expression of integrin β 1. Our research group recently identified integrin β 1 as the only known cell surface receptor for NELL-1, and that NELL-1's effects on OB attachment required integrin β 1 expression(22). Another research group subsequently verified that the C terminal region of NELL-1 binds to integrin α 3 β 1(32). Integrins have
35 documented roles in OB cell attachment, proliferation, and differentiation(44, 45) and,

likewise, known functions in OC adhesion and regulation of cytoskeletal organization for OC resorptive function(46). In particular integrin $\alpha v \beta 3$ is highly expressed in OC and mediates bone extracellular matrix attachment(46). Although the specific effects of integrin $\beta 1$ agonists have not been examined, integrin $\beta 1$ ablation led to reduced OC resorptive capacity(47). Our findings of NELL-1 activation of Wnt/ β -catenin signaling in OCs via integrin $\beta 1$ may represent a new and unexplored function of integrins in OCs(34). In summary, our observations of NELL-1 interacting with integrin $\beta 1$ to exert its cellular and tissue level effects are overall in agreement with the known roles of integrin $\beta 1$ in bone biology.

Finally, we have found that NELL-1 has potential dual uses as both a local bone-forming growth factor as well as a systemic osteogenic factor for osteoporosis. The present study advances our knowledge of the local bone-forming effects of NELL-1 to a sheep osteoporotic model. Therapeutic options for bone graft in the osteoporotic patient are limited. For example, autograft bone is less effective(48) and donor site fracture is more common(49). With similar drawbacks, BMP2 based substitutes induce direct stimulation of bone resorption(50, 51), leading to vertebral subsidence or collapse(52). With these limitations of current bone graft substitutes in the osteoporotic population, NELL-1 may be a future bone graft substitute well suited for the osteoporotic patient.

On the other hand, for the first time we observed the potential therapeutic efficacy of systemic NELL-1 for the reversal of osteoporosis. NELL-1 possesses several practical and theoretical benefits over other systemic therapies for osteoporosis. First, as we observed, NELL-1 has dual anabolic and anti-osteoclastic properties. Second, NELL-1 has documented tumor suppressive properties and its expression is lost in several carcinomas(53, 54). This lies in contrast to PTH, whose clinical use is limited by the risk of osteosarcoma as suggested by rat studies(55). Finally, NELL-1 has an excellent safety profile. Mice with constitutive *Nell-1* overexpression have a normal lifespan, and are without abnormalities excepting the skeleton(20). Similarly, formal intravenous NELL-1 toxicity testing found no pathologic or biochemical abnormalities. Although the kinetics of unmodified NELL-1 protein involve rapid elimination, structural modification of NELL-1 may allow for sustained therapeutic serum levels. However, NELL-1 has known functions in neurogenesis(56),

5 chondrogenesis(57), and vasculogenesis(58) – and a thorough study of these off-bone effects must be instituted before consideration of NELL-1 as an osteoporotic therapy.

In summary, we demonstrate for the first time that NELL-1 deficiency induces age related osteoporosis in a rodent model. As biological insight, our data suggest that NELL-1 modulates OB and OC activities through Integrin β 1 binding and subsequent Wnt/ β -catenin
10 signaling activation. Mostly importantly, the therapeutic impact of our large and small osteoporotic animal studies demonstrates how NELL-1 may be used as a new anabolic, anti-osteoclastic treatment for osteoporosis in both local and systemic intervention. Finally, demonstration of the ability of systemic NELL-1 to activate Wnt/ β -catenin signaling will provide fundamental insights relevant to the development of NELL-1 based therapies for the
15 treatment of multiple bone pathologies.

General Methods

Animal Care

All animals were cared for according to institutional guidelines set by the Chancellor's Animal Research Committee of the Office for Protection of Research Subjects at the
20 University of California, Los Angeles as well as the UCLA Office of Animal Research Oversight. Matings were done overnight and females were examined for the presence of vaginal plugs, E 0.5 dpc (days postcoitum). Animal groups were of mixed gender unless otherwise stated. CD-1 wildtype mice were obtained from Charles River for bone marrow OB precursor, OC precursor, and calvarial bone isolation studies. B6 mice were obtained
25 from Charles River for ovariectomy studies. Sprague-Dawley (SD) rats were used for Nell-1 expression studies. Heterozygote carriers of the *Nell-1*^{+/^{6R}} gene were provided by the Mammalian Genetic Research Facility at Oak Ridge National Laboratory and were transferred with permission of the Chancellor's Animal Research Committee. The *Nell-1*^{6R/6R} mouse genotypes were identified from DNA extracted from clipped tails of mutant and wildtype
30 mice. The extracted DNA was amplified using micro satellite primers D7Mit 315-L; TGATA ACAAA ACAGT CAGTA TGAAGC (SEQ ID NO: 1), D7Mit 315; RCTGATCCATCTGTATGATGTTACTTG (SEQ ID NO: 2). All mice were housed in the light and temperature controlled UCLA vivarium, and provided water and feed *ad libitum*. Sheep were cared for at Colorado State University according to Veterinary Teaching Hospital
35 (VTH) institutional guidelines. All sheep were fed a grass/alfalfa mix hay, and provided

5 water and feed *ad libitum*. Whenever possible, animals were randomized with even distribution across treatment groups (including data presented in Figures 5 and 6).

Murine Radiographic Analyses

Murine samples were analyzed by DXA, live microCT / ^{18}F PET, and high-resolution microCT. DXA was performed as previously described on live *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} littermates as follows. Scans were performed under isoflurane sedation using a Lunar PIXImus II Densitometer (GE Healthcare, Piscataway, NJ). BMD and BMC were calculated using a rectangular ROIs encompassing the lumbar spine, or the distal left femur. Analyses were performed on 18 mo. old mice; N=12 *Nell-1*^{+/+} and 16 *Nell-1*^{+/^{6R}} mice were used for DXA analyses. Unless otherwise stated, for all studies utilizing *Nell-1*^{+/^{6R}} mice, the entire colony of the studied age and gender was included for analysis. No exclusion criteria were established. Live CT/PET studies were performed as follows. Fluoride ion was produced using ^{18}O -water and proton bombardment using an RDS cyclotron (Siemens Preclinical Solutions). ^{18}F -Fluoride ion was produced at specific activities of ~37 TBq/mmol. Using an ARC-approved isolated imaging chamber, all animals underwent PET/CT scanning. Mice were injected with ^{18}F -Fluoride ion via tail vein using a 27-gauge needle. Animals were positioned in a multimodality, portable isolated bed system. Whole-body scans were performed with a 10-min acquisition time using a microPET FOCUS 200 system (Siemens Preclinical Solutions). Immediately afterward, a noncontrast-enhanced microCT study using a microCAT II (Siemens Preclinical Solutions) imaging system was used to scan animals with a 20-min acquisition time. PET scan images were reconstructed using filtered backprojection and an iterative 3D reconstruction algorithm (maximum a posteriori [MAP]). MicroCT images were created using Fledkamp reconstruction at 200 μm resolution. ^{18}F -Fluoride and CT data were analyzed and quantified by AMIDE (A Medical Image Data Examiner). To quantify the BMD and ^{18}F uptake, a cylindrical region of interest (ROI) was drawn encompassing a single lumbar vertebrae of set dimensions. Data for CT/PET images were compiled and analyzed by 3 independent, blinded reviewers. N=8 mice per genotype were analyzed at 1 and 6 mo. of age, N=11 *Nell-1*^{+/+} mice and 12 *Nell-1*^{+/^{6R}} mice were analyzed at 18 mo. of age. For PET, N=6 mice per genotype for were analyzed at 18 mo. of age. High-resolution, post-mortem microCT scanning and analysis were performed on neonatal whole mice (N=10 mice per genotype), and aged mouse spines (N=12 *Nell-1*^{+/+} and 19 *Nell-1*^{+/^{6R}}

5 individual vertebrae per genotype). Samples were harvested, formalin-fixed and imaged using high-resolution micro-CT (Skyscan 1172F, Skyscan, Belgium) at an image resolution of 17.8 - 28.2 μm and analyzed using DataViewer, Recon, CTAn, and CTVol softwares provided by the manufacturer. For neonatal spine CT data analysis, ROI (region of interest) included the entire volume lumbar spine to quantify BMD and Bone Volume / Tissue Volume (BV/TV). Trabecular analysis was performed on aged mouse spine specimens, for which ROIs were drawn to include each individual lumbar vertebral body excluding cortical bone. Reconstructions were performed using Osirix software, using coronal cross sectional images with a 0.25 μm width. All quantitative and structural morphometric data use nomenclature described by the American Society for Bone and Mineral Research (ASBMR) Nomenclature Committee (62). MicroCT indices were compared to published norms to ensure accuracy of analysis and reporting(59-61). Whenever possible, all radiographic studies were performed and quantified in a blinded fashion. *Nell-1^{+/+}* and *Nell-1^{+6R}* littermates look nearly identical, with the exception of a variably lighter coat color among *Nell-1^{+6R}* mice. However, unblinding of radiographic technicians would have minimal potential for the introduction of bias, as all radiographic studies were highly routinized. ROI construction was performed in a completely blinded fashion. However, a visually osteopenic skeleton among *Nell-1^{+6R}* mice in theory presented a potential for unblinding.

Murine Additional Analyses

Additional *in vivo* analyses included nuclear magnetic resonance (NMR), histology and histomorphometry, serum studies, gene expression, and biomechanical testing. NMR was used to determine total body fat mass and lean mass on live *Nell-1^{+/+}* and *Nell-1^{+6R}* littermates as follows. A Bruker Minispec was used with software from Echo Medical Systems (Houston, TX). In total, 12 *Nell-1^{+/+}* and 16 *Nell-1^{+6R}* mice were used for NMR analyses. All mice were weighed prior to NMR analysis.

30 For histology, all tissues were fixed in 10% PBS-buffered formalin. Mouse samples were decalcified in 19% EDTA, and embedded in paraffin. Five micron thick sections were made and stained with H&E, Masson's Trichrome, Aniline Blue, and Tartrate Resistant Acid Phosphatase (TRAP) staining (Sigma-Aldrich) per standard protocols. Analysis of Masson's Trichrome and Aniline Blue staining was performed to demonstrate the degree of mineralization between samples. Histological specimens were analyzed using the Olympus

5 BX51 microscopes and images acquired using MicroFire digital camera with Picture Frame software (Optronics, Goleta, CA). Indirect immunohistochemistry was performed using AEC as chromogen, with primary antibodies listed in Table 6. Histomorphometric analysis was performed using Adobe Photoshop. Measurements included Bone Area (B.Ar), percentage Bone Area (% B.Ar), Bone Perimeter (B.Pm), Cortical Width (Ct.Wi), and Trabecular
 10 analyses (Tb.Wi, Tb.N, Tb.Sp). Numbers of quantified images for each analysis are as follows: B.Ar (N=29 *Nell-1*^{+/+} and 26 *Nell-1*^{+6R} images), % B.Ar (N=29 *Nell-1*^{+/+} and 26 *Nell-1*^{+6R} images), B.Pm (N=30 *Nell-1*^{+/+} and 29 *Nell-1*^{+6R} images). Osteoblast number (Ob.N) was assessed by per high power field of Masson's Trichrome staining (N=20 *Nell-1*^{+/+} and 20 *Nell-1*^{+6R} images analyzed; N=18-30 images per treatment group analyzed for Figure
 15 6), while osteoclast number (Oc.N) was assessed per high power field of TRAP staining (N=26 *Nell-1*^{+/+} and 39 *Nell-1*^{+6R} images analyzed; N=28-40 images per treatment group analyzed for Figure 6). Cytomorphologic definition of an osteoblast required bone-lining cells with a single round to ovoid nuclei and fairly abundant cytoplasm. Characteristic cell morphology with 3 or more nuclei was used to define Oc.N. Analyses of
 20 immunohistochemical staining are either reported as the number of immunoreactive cells per B.Pm, or semi-quantitative measurement of relative total immunoreactivity. Semi-quantitative measurements were performed using the magic wand tool in Adobe Photoshop.

ELISA based serum studies were performed using serum samples per manufacturer's
 25 instructions. Briefly, *Nell-1*^{+/+} and *Nell-1*^{+6R} littermates were bled via the retro-orbital sinus at 9am on the same day after overnight fasting. Serum was collected via centrifugation and stored prior to examination of serum PINP (Procollagen I N-terminal Propeptide), serum TRAP (Tartrate-Resistant Acid Phosphatase)-5b, and serum CTX (C-Terminal Telopeptide). All assays were obtained from ImmunoDiagnostic Systems, Inc. and performed according to
 30 manufacturer instructions. For serum PINP, a Rat/Mouse Enzymeimmunoassay was used (N=19 and 17 mice for *Nell-1*^{+/+} and *Nell-1*^{+6R} mice, respectively). For serum CTX, a RatLaps™ Enzymeimmunoassay was used (N=12 and 22 mice for *Nell-1*^{+/+} and *Nell-1*^{+6R} mice, respectively). For serum TRAP5b, a MouseTRAP™ solid phase immunofixed enzyme activity assay was used (N=10 and 9 mice for *Nell-1*^{+/+} and *Nell-1*^{+6R} mice, respectively),
 35 (kits listed in Table 7). Gene expression was performed after microdissection of lower lumbar

5 vertebral bodies, following by mRNA extraction and quantitative real time (RT)-PCR. N=3 samples (Fig. 1c) or 4 samples (Fig. 3c) per genotype were used for analysis. Primers are listed in Table 8.

For biomechanical testing, two methods were independently used: the BioDent™ Reference Point Indenter and computerized simulation (finite element analysis, FEA).

10 Analysis of the SI vertebral body was performed on all mice. Lumbar vertebrae could not be used, as the spines in the *Nell-1*^{+6R} mice were too fragile for analysis. The indenter was placed on the dorsal periosteal surface for measurement acquisition. The following settings were used: 2N indentation force, 2 indentations per second, 10 indentations per measurement. Total Indentation Distance (TID) was calculated by measuring the maximum indentation
15 distance achieved during a measurement. Indentation Distance Increase (IDI) was calculated by measuring the difference between the depths reached at peak force during the first indentation cycle and last indentation cycle. Unloading Stiffness is calculated by evaluating the top portion of the unloading section of the force displacement curve. N=3 mice per genotype, with N=6 measurements per mouse used for indentation studies.

20 Finite Element (biomechanical) Analysis (FEA) was performed using micro-CT images converted to DICOM files using SKyScan Dicom Converter software (DicomCT application, Skyscan 1172F, Skyscan). Tetrahedral three-dimensional mesh models were created using an VOI of either the lumbar spine (level 4) using ScanIP software (Simpleware Limited). A constant thickness of 0.54 mm was used for both VOIs. Finite element analyses
25 were performed using the ABAQUS software (Dassault Systèmes) with boundary conditions set as encastre, constrained in all directions. Next, we applied a uniform compressive pressure of 0.5 MPa on the superior surface of the VOL. The von Mises stress experienced and total strain energy of the samples were analyzed. N=8 mice per genotype for FEA analyses.

30 For bone fluorescent labeling studies, mice were injected intraperitoneally with calcein (20 mg/kg) and alizarin red complexon (50 mg/kg) at nine days and two days before sacrifice, respectively. Lumbar vertebrae were dissected, fixed in 70% ethanol, dehydrated, and embedded undecalcified in methyl methacrylate. Coronal sections at 5 µm thickness were analyzed using the OsteoMeasure morphometry system (Osteometries, Atlanta, GA, USA).

35 For dynamic histomorphometry, the mineral apposition rate (MAR, µm/d), the distance

5 between the midpoints of the two labels divided by the time between the midpoints of the interval, were measured in unstained sections under UV light and used to calculate bone formation rate with a bone surface referent (BFR/BS, $\mu\text{m}^3/\mu\text{m}^2/\text{y}$). Bone formation rate per bone surface (BFR/BS) is the volume of mineralized bone formed per unit time and per unit bone surface. The fixed area and location at 200x magnification of lumbar vertebral 4-5 (L4-10 5) was selected as the ROI (N=3 mice and 6 measurement fields per treatment group). All image acquisition and analysis was performed in a blinded fashion.

OB Precursor Culture and Experiments

Mouse osteoblast (OB) precursors were harvested by flushing the femoral marrow cavities and harvesting adherent cells on standard culture-treated plates. For isolation of *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} OB precursors, cells were isolated from 18 mo. old littermates (N=3 mice per genotype). For additional experiments using wildtype OB precursors, 3-month-old CD-1 animals were used. Proliferation and osteogenic differentiation and assessments were performed with or without recombinant human (rh)NELL-1 protein (100-300 ng/mL). Osteogenic differentiation medium was constituted with 10 mM β -glycerophosphate and 50 20 μM ascorbic acid (Fisher Scientific, Pittsburgh, PA) in high-glucose DMEM, 10% fetal bovine serum (FBS), 1% penicillin / streptomycin (GIBCO, Invitrogen, Carlsbad, CA). To assess early to intermediate OB differentiation, alkaline phosphatase staining and quantification was performed in each case normalized to total protein content in sister wells. To assess bone nodule formation, Alizarin red staining and quantification was performed by 25 CPC leaching and photometric quantification, normalized to total protein content. Quantitative real time PCR was performed in triplicate wells per RNA isolate. Primers are listed in Table 8. All experiments were performed in triplicate wells. Passage 2 OB precursors only were used for all assays. For all *in vitro* studies, technical execution of the experiments was performed in an unblinded fashion, unless otherwise noted.

OC Precursor Resorption Experiments

30 Calvarial discs were obtained using a 2 mm punch biopsy from the mid-parietal bone of postnatal day 7, CD-1, wildtype mice. Discs were processed by gentle removal of periosteal and dural tissues and stored in 100% ethanol to decellularize the tissues at 4°C. Mouse osteoclast (OC) precursors were obtained by femoral and tibial marrow flushing. For

isolation of *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} OC precursors were isolated from 18 mo. old littermates (N=3 mice per genotype used). For additional experiments using wildtype OC precursors, 3-month-old CD-1 animals were used. The product of total bone marrow flush was cultured in 25 ng/mL recombinant mouse M-CSF (R&D Systems, Minneapolis, MN) overnight. The non-adherent cells were then cultured on calvarial discs for resorption assays in phenol red-free α MEM media, 10% FBS, 25 ng/mL rmM-CSF and 100 ng/mL recombinant soluble RANKL (R&D Systems), with or without rhNELL-1 (0-1200 ng/mL). After 5 days, calvarial discs were harvested, washed in 1x PBS and stained with 1% Toluidine Blue dye in dilute Sodium Borate (Fisher Scientific). Calvarial discs were photographed and quantification of intensity blue staining (indicating presence of bone) was performed using the magic wand tool in Adobe Photoshop CS5 (N=9 and 11 samples for *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} samples, respectively). Next, scanning electron microscopy (SEM) was performed as using a Nova 230 microscope (FEI, Hillsboro, Oregon) in a low vacuum mode operated at 40 Pascal. Accelerating voltage was used at 10 kV with a working difference varying between 4.8 and 5.7 mm. Samples were analyzed at a magnification of 200x. The MeX 3D software version 5.1, (Alicona Imaging Corporation, Austria), was used to analyze mean resorption pit depth and mean roughness of the calvarial discs. In order to measure the depth of the pits several lines were drawn through each suspected pit at 30° intervals; the line which provided the largest difference between peak and valley was used, determining the distance in mm between the peak and valley measurements. Resorption pits were defined as having a minimum depth of 1.5 μ m (N=9 measurements per genotype). Average roughness was measured by drawing six lines across each sample at 100 μ m intervals using MeX software (N=36 and 29 measurements for *Nell-1*^{+/+} and *Nell-1*^{+/^{6R}} samples, respectively). The calculated roughness of each line was then used to calculate mean roughness per treatment group. SEM measurements were performed in a blinded fashion.

30 *M2-10B4 and RAW264.7 Cell Culture*

The M2-10B4 cell line, a clone derived from BMSCs from a (C57BL/6J X C3H/HeJ) F1 mouse, was purchased from American Type Culture Collection (ATCC # CRL-1972, Lot # 58696031, Manassas, VA) and used for experiments within 6 months of mycoplasma contamination testing. RAW264.7 cells were a kind gift from the laboratory of Dr. Tintut.

Cells were maintained in growth medium (RPMI 1640 supplemented with 10% FBS, 1 mM

5 sodium pyruvate, and 100 U/ml penicillin/streptomycin). RAW264.7 cells were cultured in α MEM + 10%) FBS. Wnt/ β -catenin signaling was assayed by four methods: immunocytochemistry for active β -catenin, Western blotting for nuclear β -catenin, TCF/LEF1 reporter activity (TOPFLASH), and qRT-PCR. Experiments used recombinant WNT3A as a positive control. For immunocytochemistry, M2-10B4 cells were seeded on Millicell EX
 10 Slides (PEZGS0816, Millipore) at 5×10^4 cells/well in RPMI 1640+10% FBS for 24 hours and serum starved in RPMI 1640+1% FBS overnight. After 2 hours treatment, cells were fixed using ice-cold acetone for 10 min. Anti-active β -catenin antibody (Millipore) was applied at a dilution of 1:200. ABC complex (Vector Laboratories, Burlingame, CA) was applied to the sections following incubation with biotinylated secondary antibody (Dako). AEC substrate
 15 (Dako) was used as a chromogen, and the sections were lightly counterstained with hematoxylin. Photomicrographs were acquired using Olympus BX51 (200 $\times$ magnification lens, UPLanFL, Olympus) (N=4 wells per treatment group). For Western blotting, nuclear and cytoplasmic protein was isolated by NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL). Western blotting was performed using an antibody against
 20 β -catenin at a dilution of 1:1000 (N=3 wells per treatment group). For TCF/LEF1 reporter activity, cells were transfected with 20 mg Super(16 $\times$) TOPFLASH (TCF/LEF1 Reporter Plasmid) and 1 mg *Renilla* Luciferase plasmids for 24 hours and then seeded at 4.0×10^3 cells/well in 96-well plates. Cells were starved in RPMI 1640+1 %FBS overnight and then treated with rhNELL-1. Luciferase activity was measured 48 hours after treatment using Dual
 25 Luciferase Reporter Assay System (Promega, Madison, WI) per the manufacturer's instructions (N=4 wells per treatment group).

Lentiviral Vector for Runx2 Reporter Assay

The transduction plasmid for preparing lentiviral vector carrying the *Runx2* P1-EGFP expression cassette (*Runx2*-EGFP reporter) was prepared by substituting the CMV promoter
 30 in the pRRL-cPPT-CMV-X-PRE-SIN plasmid with the mouse *Runx2* P1 promoter. The mouse *Runx2* P1 promoter was obtained by PCR of mouse genomic DNA (forward primer: GCGAATTACTCGAGAGCAGCACTGTTGCTCAGAA (SEQ ID NO: 3); reverse primer: GCGAATGCCCGGGTCACACAATCCAAAAAAGC (SEQ ID NO: 4)). 293T cells were cotransfected with the transduction plasmids, the package plasmid pCMV-dR8.2-vprX and
 35 the envelope plasmid pCMV-VSVG. The viral vectors were collected at 2 to 4 days post-

5 transfection, filtered and concentrated, and the concentrations of viral vector were quantified by counting the core protein p24 by ELISA assay. For cell transfection, M2-10B4 cells were seeded in 24-well cell culture plates at 2×10^4 cells/well 16 hours prior to infection. Viral vectors with p24 counts of 0.4 μ g were added to each well in 24-well plates. 3 hours post-infection, the viral vectors were washed away and fresh medium was added to the cultures.

10 At 24 hours post-infection, rhNELL-1 was added into the culture media. Cells were trypsinized and collected three days post-infection. Flow cytometry was performed to quantify GFP expression in the collected cells using a Cytomics FC500 cell sorter (Beckman Coulter, Brea, CA). Cells infected with mock vector were used as the negative control to establish gates. The percentages of GFP-positive cells were counted to quantify the

15 expression of GFP in the infected cultures (N=4 wells per treatment). For select experiments, DKK-1 (100 ng/mL) or XAV939 (1 μ M) were used (N=4 wells per treatment group).

Small interfering RNA experiments

RNA knockdown experiments were performed in M2-10B4 and RAW264.7 cells using chemically synthesized and annealed small interfering RNA (siRNA) specific to

20 integrin β 1 (Santa Cruz, with N=3 wells per treatment group. When cells reached 30% confluence, cells were transfected with 50 nM integrin β 1 siRNA or nontarget negative control siRNA (Santa Cruz) using Lipofectamine RNAiMax (Invitrogen). Efficiency of knockdown was validated using western blot.

Human BMSC Culture

25 Human BMSCs were obtained from N=5 patients, with or without a history of osteoporosis (see Table 2 for a description of patient samples). Samples were obtained during a surgical procedure, predominantly for fracture or joint replacement surgery (see Table 2), and with UCLA IRB exemption (IRB #11-000724). Samples were de-identified to protect the privacy of the patient. As no direct contact and no risk were poised to the patient, neither

30 informed consent nor authorization was obtained, as per University guidelines. Both nonosteoporotic and osteoporotic patients were without major medical comorbidities. Bone marrow tissues with bone chips were obtained and stored on ice during transport. These tissues were next digested with Type II collagenase (2 mg/ml) under agitation, and followed by Ficoll centrifugation. The mononuclear cells were separated and seeded into T-75 flasks

5 for cell expansion. hBMSCs were obtained by marrow flush and harvesting adherent cells on standard culture-treated plates. Cells were expanded in α MEM + 20% FBS on 10 mm cell culture plates. All experiments were performed in triplicate for each patient sample. To manipulate Nell-1 signaling, hBMSCs were transduced with Ad-Nell-1 or Ad-GFP at MOI 50 pfu/cells. First, cells were either seeded for osteogenic differentiation assays (2×10^4 cells/well) in 24 well plates. Osteogenic differentiation medium (ODM) consisted of 50 μ g/ml ascorbic acid and 10 mM β -glycerophosphate. Staining for AR was performed at 11 days of differentiation. Next, Wnt/ β -catenin signaling activity was assayed on the gene and protein level. mRNA analysis was performed 3 days after transduction. For Western blot analysis, cell lysate was collected 3 days after transduction. Nuclear and cytoplasmic fractions were extracted using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL). Western blot was performed with anti- β -catenin (610153, BD Biosciences) and anti- β -actin (sc-1616, Santa Cruz). Quantification was performed using Image Pro. Taqman primers were used for human *NELL-1* (Hs00196243_ml) and human *GAPDH* (Hs99999905_ml). All experiments using human BMSC were performed in triplicate wells.

Assessments of NELL-1/integrin β 1 interaction

Confirmation of NELL-1 binding to integrin β 1pi was performed in two methods, including thermal shift assays and computational modeling. The thermal shift assay was performed using the 7300 Real Time PCR System (Applied Biosystems, CA). Prior to use, the environmentally sensitive fluorescent dye SYPRO Orange stock solution in DMSO (5,000 $\times$, Sigma) was diluted 1:125 in PBS. The samples were prepared in a 96-well plate in triplicate containing 2 μ g NELL-1 and/or 1 μ g integrin α 3 β 1 (R&D Systems, 2840-A3-050), and freshly diluted SYPRO orange in PBS buffer (0.01 M, pH 7.4) with the total volume of 15 μ l, and then the plate was sealed with optical quality sealing tape and centrifuged at 4,000 rpm for 2 min. The fluorescent intensity was monitored as the plate was heated from 25 to 89 $^{\circ}$ C in an increment of 1 $^{\circ}$ C/min. Second, computational modeling was performed between NELL-1 and the extracellular domain of integrin β 1 based on RosettaDock. The three top-scoring models of NELL-1 to integrin β 1 predicted by docking simulation are depicted in Figure 10.

5 *TOPGAL Mouse Intrafemoral Ad-Nell-1 Injection*

Wnt reporter TOPGAL mice were injected by intrafemoral injection with Ad-Nell-1 (5x10¹² pfu/mL) or Ad-CMV-Null control (Vector BioLabs, Philadelphia, PA). Mice were randomized to each treatment group. Block randomization was used to ensure equal sample sizes. A percutaneous, infrapatellar approach was used, with a 26 gauge needle, and a 10-μl total injection volume. Animals were sacrificed one and two weeks post-injection for analysis of β-galactosidase expression within the marrow space. For histological analysis, whole mount X-gal staining was performed 1 week post-injection followed by paraffin embedding and examination of serial sections. N=3 mice per treatment group. For quantification, flow cytometry was performed two weeks post-injection. Bone marrow cells were flushed from the femur. Next, the femoral shaft was finely minced and the bone chips were digested with collagenase II (3 mg/ml) for 2 hours. The resultant cells from both bone marrow flush and cell digestion were combined, pelleted, and washed twice with PBS + 1% FBS. β-galactosidase substrate FDG (Invitrogen F1179) was then added to cells under hypotonic shock for 1 min and run for flow cytometry (LSRII, BD Bioscience). N=4 mice per treatment group.

Mouse Ovariectomy and rhNELL-1 Intravenous Injection:

Ovariectomy was performed on B6 mice with age-matched Sham controls at 12 weeks of age, using a 5mm dorsal incision. Anesthesia was performed with Isoflurane (3-5% for induction, 1.5-2% for maintenance). Pre-operative analgesia (Buprenorphine) was given once before surgery and twice daily for two days postoperative. Five weeks post ovariectomy (OVX), rhNELL-1 delivery by intravenous injection was instituted, by lateral tail vein injection using a 25 gauge needle. Animals were assigned to treatment groups by simple randomization. Drug administrators were completely blinded as to treatment groups. The dosage of rhNELL-1 (1.25 mg/kg) was obtained from pilot studies examining a wide range of doses. PBS served as vehicle control. Each injection consisted of a total 0.1 mL solution, and injection was performed every 48 hours for the study period. Analyses were as per above, including DXA, live CT/PET, high-resolution microCT and quantification, histology and immunohistochemical staining. Animal numbers for each analysis were as follows (Sham Control, Sham rhNELL-1, OVX Control, OVX rhNELL-1): DXA (6, 7, 6, 9 mice), high-resolution microCT (5, 5, 6, 9 mice), Osteocalcin quantification (18, 23, 25, 30 images),

5 TRAP quantification (28, 34, 28, 40 images), and FEA (N=6, 7, 6, 9 mice). Regions of
interest for analysis included the lumbar vertebrae. MicroCT indices were compared to
published norms to ensure accuracy of analysis and reporting (59-61). Additionally,
postmortem analysis of uteri weight was obtained to confirm OVX. In select experiments,
FITC conjugation was employed to determine the pharmacokinetics of rhNELL-1 systemic
10 delivery.

Sheep Experiment Preparation and Surgery

Osteoporosis was induced through ovariectomy (OVX), controlled diet, and steroid
induction in eight adult ewes. Post-ovariectomy, three intramuscular injections of 500 mg
methylprednisolone acetate (Depomedrol) were administered at three-week intervals starting
15 two weeks post-operation. Special low calcium and low vitamin D osteoporosis diets were
formulated in cooperation with Purina LabDiet and were fed to the sheep for eight months
post-ovariectomy. Based on pre-established inclusion criteria, of eight sheep in total, the six
sheep with highest response to osteoporotic induction were used for further study. Successful
induction of osteoporosis was confirmed using DXA imaging and quantification. The
20 contents of material for sheep intrabody vertebral injection are in Table 3, and included
hyaluronic acid, β -tricalcium phosphate (P-TCP), with or without rhNELL-1 protein. Block
randomization was used to ensure equal treatment group sizes. Surgeons were completely
blinded to treatment group. As injection contents appeared visually identical between groups,
no unblinding of the operating surgeons was observed. Surgery was performed four months
25 post-ovariectomy on N=6 osteoporotic sheep. A ventrolateral incision was made for a
retroperitoneal approach. Induction of anesthesia was performed by Valium or Midazolam or
propofol along with ketamine through a venous catheter placed in an ear vein. Following
induction, animals were intubated and transferred to Isoflurane in oxygen inhalant titrated to
maintain a plane of surgical anesthesia. Dissection was carried out to expose the cranial
30 aspect of the L1-L5 vertebral bodies. A 3.2 mm unicortical drill hole was created on the left
lateral aspect of the vertebral body. An introducer with stylet that perfectly fitted the drillhole
was then inserted, ensuring an airtight, watertight seal. The stylet was then removed, and
using a luer lock syringe the implant material injected into the introducer, and the stylet
reinserted so as to push all materials into the cancellous bone of the vertebral body. No
35 leakage of injection contents was observed. Bone wax was used to seal the drill hole. Three

5 different vertebral levels were instrumented per sheep (L1, L3 and L5). Three sheep were injected with the control vehicle and three sheep were injected with the treatment. Thus, final group numbers included N=9 control-treated vertebrae, and N=3 vertebrae per treatment dose. Postoperative analgesia consisted of phenylbutazone for three days postoperatively, with close indoor monitoring for two weeks postoperatively.

10 *Sheep Experiment Analyses*

Analysis was performed by DXA, CT and microCT, Finite Element Analysis (FEA), histology, and histomorphometry. DXA scans were performed pre- and post-induction (Konica Minolta mc5430DL). Next, a Philip's GEMINI TF Big Bore CT machine (0.8 mm thickness) was used to analyze bone formation post-operative, monthly until sacrifice at 15 months. Although serial live animal imaging was performed, data evaluation was performed at the study endpoint (no interim evaluation of the results performed). Post-mortem, high-resolution microCT scanning of individual vertebrae was performed as per mouse studies. Cortical and trabecular analysis were performed on post-mortem individual sheep vertebrae. Cortical measurements were obtained per vertebral body either assessing the surface of 20 injection or the opposing cortical surface. Serial measurements along the cortex were obtained (N=162 control-treated measurements, N=55 measurements per treatment dose). Trabecular measurements were made using an ROI excluding cortical bone. Finite Element (biomechanical) Analysis (FEA) was performed using micro-CT images converted to DICOM files using SKyScan Dicom Converter software (DicomCT application, Skyscan 1172F, 25 Skyscan). Tetrahedral three-dimensional mesh models were created by drawing a rectangular VOI directly underneath injection tract, using ScanIP software (Simpleware Limited). Finite element analyses were performed using the ABAQUS software (Dassault Systèmes) with boundary conditions set as encastre, constrained in all directions. Next, we applied a uniform compressive pressure of 0.5 MPa on the superior surface of the spine, to reproduce human 30 intradiscal pressure experienced in relaxed standing. The von Mises stress experienced and total strain energy of the samples were analyzed. All radiographic analyses, including ROI construction, were performed in a blinded fashion. The significant and visually observed anabolic effect of rhNELL-1 in theory presented a potential for unblinding. For histology, tissues were formalin-fixed, resin-embedded and stained with Hematoxylin and Eosin (H&E), 35 Goldner's Modified Trichrome (GMT), and Von Kossa-MacNeal's Tetrachrome.

5 Histological specimens were analyzed using the Olympus BX51 microscopes and images
 acquired using MicroFire digital camera with Picture Frame software (Optronics, Goleta,
 CA). Histomorphometry was performed as per mouse studies. Osteoblast number (Ob.N)
 was assessed by 400x images of Von Kossa MacNeal's Tetrachrome (N=10, 9, 9 and 9
 images per treatment group, respectively), while osteoclast number (Oc.N) was assessed by
 10 200x images of GMT staining (N=9, 10, 9 and 9 images per treatment group, respectively).
 Characteristic cell morphology with 3 or more nuclei was used to define Oc.N.

Statistical Analysis

Quantitative data are expressed at mean $\pm$ SEM unless otherwise described, with $*P < 0.05$ and $**P < 0.01$ considered significant. A Shapiro-Wilk test for normality was
 15 performed on all datasets. Homogeneity was confirmed by a comparison of variances test. Parametric data was analyzed using an appropriate Student's *t*-test when two groups were
 being compared, or a one-way ANOVA was used when more than two groups were
 compared, followed by a post-hoc Tukey's test to compare two groups. Nonparametric data
 was analyzed with a Mann-Whitney U test when two groups were being compared or a
 20 Kruskal-Wallis one-way analysis when more than two groups were compared. As
 appropriate, adjustments for multiple comparisons were performed using a Bonferroni
 correction (see Tables 9 and 10). Sample size calculations were performed for experiments
 presented in Figures 5 and 6 and Figures 12, 13 and 14 as follows: for experiments presented
 in Fig. 5 and Figures 12 initial animal numbers were based on an $\alpha=0.05$, power=0.8, and an
 25 anticipated effect size of 3.73 (based on our previously published data in sheep spinal fusion)
 (27). For experiments presented in Fig. 6 and Figures 13 and 14, initial animal numbers were
 based on an $\alpha=0.05$, power=0.8, and an anticipated effect size of 2.69 (based on our
 previously published data in local rhNELL-1 injection in ovariectomized rats) (63). No
 sample size calculations were performed for animal studies in Figure 1, Figures 7 and 8, as all
 30 available transgenic animals were examined. *In vitro* experiments were performed in
 biological triplicate, unless otherwise described. *In vivo* experiments were performed without
 replicate, unless otherwise described. Figure 14 shows raw images for data presented in
 Figures 4b, 4i, 4k, 4m, and Figures 11d and 11e.

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- 5 Table 1. Spine histomorphometric measurements at birth with *Nell-1* haploinsufficiency. Material is based on histomorphometric analysis of N=5 samples per genotype. Data is presented as mean $\pm$ one standard error of the mean (SEM).

	<i>Nell-1</i> ^{+/+} (Mean $\pm$ SEM)	<i>Nell-1</i> ^{+/^{6R}} (Mean $\pm$ SEM)	<i>p</i> -value
Percent Tb.Ar (%)	21.71 $\pm$ 2.16	21.07 $\pm$ 0.814	0.405
Tb.Wi (μ m)	1.321 $\pm$ 0.208	0.963 $\pm$ 0.114	0.084
Tb.N (μ m ⁻¹)	0.252 $\pm$ 0.092	0.276 $\pm$ 0.030	0.495
Tb.Sp (μ m)	4.38 $\pm$ 0.930	3.01 $\pm$ 0.335	0.065

Table 2. Demographics of samples for human BMSC derivation. AVN: Avascular necrosis.

Patient #	Age	Gender	H/o Osteoporosis	Site	Indication
1	23	F	N	Femur	Fracture
2	47	M	N	Femur	AVN
3	54	F	N	Femur	Joint replacement
4	47	F	N	Femur	Joint replacement
5	32	F	N	Femur	Joint replacement
6	83	F	Y	Femur	Fracture
7	91	F	Y	Femur	Fracture
8	71	F	Y	Femur	Fracture

Table 3. Treatment composition for sheep intervertebral body injections.

	Control	rhNELL-1 (low)	rhNELL-1 (mid)	rhNELL-1 (high)
Volume Hyaluronic Acid	500 μ L	500 μ L	500 μ L	500 μ L
Mass β -TCP	50 mg	50 mg	50 mg	50 mg
Concentration rhNELL-1	0 mg/ml	0.6 mg/ml	1.5 mg/ml	3.0 mg/ml
Total Dose rhNELL-1	0 mg	0.3 mg	0.75 mg	1.5 mg
Vertebral body N	9	3	3	3

- 5 Table 4. Histomorphometric measurements after sheep intervertebral body injections. Data presented as mean $\pm$ one standard error of the mean (SEM).

		Control	rhNELL-1 (low)	rhNELL-1 (mid)	rhNELL-1 (high)
Contra. Ct.Wi	Mean $\pm$ SE M <i>p-value</i>	0.645 $\pm$ 0.0 12	0.751 $\pm$ 0.028 <i>0.020</i>	0.747 $\pm$ 0.036 <i>0.028</i>	0.749 $\pm$ 0.032 <i>0.023</i>
Ipsi. Ct.Wi	Mean $\pm$ SE M <i>p-value</i>	0.660 $\pm$ 0.0 12	0.819 $\pm$ 0.028 <i>< 0.001</i>	0.830 $\pm$ 0.032 <i>< 0.001</i>	0.778 $\pm$ 0.028 <i>0.002</i>
Tb.Th	Mean $\pm$ SE M <i>p-value</i>	0.238 $\pm$ 0.0 02	0.263 $\pm$ 0.002 <i>0.002</i>	0.259 $\pm$ 0.003 <i>0.007</i>	0.263 $\pm$ 0.001 <i>0.002</i>
Tb.N	Mean $\pm$ SE M <i>p-value</i>	0.973 $\pm$ 0.0 15	1.267 $\pm$ 0.253 <i>< 0.001</i>	1.032 $\pm$ 0.050 <i>0.529</i>	1.006 $\pm$ 0.039 <i>0.859</i>
Tb.Sp	Mean $\pm$ SE M <i>p-value</i>	0.615 $\pm$ 0.0 06	0.563 $\pm$ 0.001 <i>0.002</i>	0.582 $\pm$ 0.003 <i>0.022</i>	0.577 $\pm$ 0.004 <i>0.011</i>

Table 5. Pharmacokinetics of intravenous rhNELL-1 in mice.

	A (μ g/m L)	α	$T_{1/2\alpha}$ (h)	B (μ g/m L)	β	$T_{1/2\beta}$ (h)	AUC (μ g/m L)	Cmax calc (μ g/mL)
FITC- rhNELL-1	8.620	1.60 2	0.433	1.000	0.12 6	5.520	13.34 6	9.620

Table 6.

Antibody	Vendor	Cat. No.	References	Validation Profile URL
Anti-Axin2	Abeam	AB32197	PMID: 22928951	http://1degreebio.org/reagents/product/104112/?qid=749839
Anti- β -actin	Santa Cruz	sc-1616	PMID: 23283970	http://1degreebio.org/reagents/product/1309/?qid=749854

Antibody	Vendor	Cat. No.	References	Validation Profile URL
Anti- β -catenin, active	Millipore	05665		http://1degreebio.org/reagents/product/869393/?qid=750604
Anti- β -catenin	BD Biosciences	610153	PMID: 17438527	http://1degreebio.org/reagents/product/862646/?qid=750591
Anti-H3	Abeam	AB8898	PMID: 24169366	http://1degreebio.org/search/?search=AB8898&in=antibodies
Anti-Nell-1	Genetex, Inc.	GTX 111493	PMID: 25376942	http://1degreebio.org/reagents/product/1934501/?qid=750581
Anti-Nell-1	Allele Biotechnology	PAB-11648	PMID: 25376942	
Anti-PCNA	Dako	M0879	PMID: 1981239	
Anti-RANKL	Novus Biologicals	NB100-80849		http://www.antibodypedia.com/gene/3366/TNFSF11/antibody/71905/NB100-80849
Anti-Integrin β 1	Abeam	AB95623	PMID: 10194438	http://1degreebio.org/reagents/product/327726/?qid=750547
Anti-OPG	Abeam	AB73400		http://1degreebio.org/reagents/product/333174/?qid=749915
Anti-Osteocalcin	Santa Cruz	Sc-30044	PMID: 22238668	
Anti-Osteopontin	Abeam	AB8448	PMID: 24586387	http://1degreebio.org/reagents/product/1162507/?qid=749910

5 Table 7. List of ELISA assays used.

Protein	Cat. No.	Manufacturer
PINP (Procollagen I N-terminal Propeptide)	AC-33F1	ImmunoDiagnostic Systems, Inc
TRAP (Tartrate-Resistant Acid Phosphatase)-5b	SB-TR103	ImmunoDiagnostic Systems, Inc
CTX (C-Terminal Telopeptide)	AC-07F1	ImmunoDiagnostic Systems, Inc

Table 8. List of primers and sequences used.

Gene	Species	Forward Sequence	Reverse Sequence
<i>Alp</i>	Mouse	TGCCACTGTGAGAAGACCTG (SEQ ID NO: 5)	TGCACAGGAAGTGAGTCTGG (SEQ ID NO: 6)
<i>Axin2</i>	Mouse	TCCAGTCCACCAAACCTATGC	CTGGTCAAAGGGGAATCGGG

<i>Bmp2</i>	Mouse	(SEQ ID NO: 7) TCTTCCGGGAACAGATACAGG	(SEQ ID NO: 8) TGGTGTCCAATAGTCTGGTCA
<i>Bmp4</i>	Mouse	(SEQ ID NO: 9) TTCCTGGTAACCGAATGCTGA	(SEQ ID NO: 10) CCTGAATCTCGGCGACTTTTT
<i>Bmp7</i>	Mouse	(SEQ ID NO: 11) ACGGACAGGGCTTCTCCTAC	(SEQ ID NO: 12) ATGGTGGTATCGAGGGTGGA
<i>C-Myc</i>	Mouse	(SEQ ID NO: 13) TCTCCATCCTATGTTGCGGTC	(SEQ ID NO: 14) TCCAAGTAACTCGGTCATCAT
<i>Cyclin D</i>	Mouse	(SEQ ID NO: 15) CTGGAAGAAGTCTGCGTCGG	CT (SEQ ID NO: 16) GTCTTGCCAAAGCGGTTTCAG
<i>Gapdh</i>	Mouse	(SEQ ID NO: 17) TGCACCACCAACTGCTTAGC	(SEQ ID NO: 18) CCACCACCCTGTTGCTGTAG
<i>Nell-1</i>	Mouse	(SEQ ID NO: 19) TCCTGGGTAGATGGTGACAA	(SEQ ID NO: 20) CATTGGCCAGAAATATGCAC
<i>Ocn</i>	Mouse	(SEQ ID NO: 21) GCAATAAGGTAGTGAACAGAC	(SEQ ID NO: 22) AGCAGGGTTAAGCTCACACTG
		TCC	((SEQ ID NO: 24)
<i>Opn</i>	Mouse	(SEQ ID NO: 23) ATCTCACCATTTCGGATGAGTCT	TCAGTCCATAAGCCAAGCTAT
		(SEQ ID NO: 25)	CA (SEQ ID NO: 26)
<i>Runx2</i>	Mouse	CGGTGCAAACCTTCTCCAGGA	GCACTCACTGACTCGGTTGG
		((SEQ ID NO: 27)	(SEQ ID NO: 28)
<i>Axin2</i>	Human	CTCCCCACCTTGAATGAAGA	ACTGGGTCGCTTCTCTTGAA
		(SEQ ID NO: 29)	(SEQ ID NO: 30)
<i>Cyclin D</i>	Human	ATGGAGGGCGGATTGGAAATG	TCGGTGTCTTACTTCAAAGTG
		A (SEQ ID NO: 31)	TG (SEQ ID NO: 32)
<i>Gapdh</i>	Human	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAAT
		(SEQ ID NO: 33)	A (SEQ ID NO: 34)

- 5 Table 9. Results of 5 day intravenous toxicity testing of rhNELL-1, including mean animal and organ body weights. N=4 animals per treatment group. No statistically significant differences were found with rhNELL-1 treatment, although a non-significant trend toward reduced stomach weight and increased bone weight was observed. Data is presented as mean $\pm$ standard error of the mean (SEM). * $P < 0.0014$ in comparison to 0 mg/kg/day group.

		0 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	6.25 mg/kg/day
Body Weight (g)	Mean $\pm$ SEM	30.75 $\pm$ 0.48	31.25 $\pm$ 0.48	31.25 $\pm$ 1.03	30.75 $\pm$ 1.03
	<i>p</i> -value		0.244	0.341	0.500

Heart Weight (g)	Mean $\pm$ SEM	0.161 $\pm$ 0.009	0.162 $\pm$ 0.002 <i>0.454</i>	0.173 $\pm$ 0.01 <i>0.190</i>	0.157 $\pm$ 0.005 <i>0.374</i>
	<i>p-value</i>				
Lungs Weight (g)	Mean $\pm$ SEM	0.211 $\pm$ 0.01	0.21 $\pm$ 0.008 <i>0.447</i>	0.233 $\pm$ 0.019 <i>0.188</i>	0.218 $\pm$ 0.015 <i>0.369</i>
	<i>p-value</i>				
Thymus Weight (g)	Mean $\pm$ SEM	0.078 $\pm$ 0.003	0.077 $\pm$ 0.013 <i>0.464</i>	0.087 $\pm$ 0.004 <i>0.073</i>	0.069 $\pm$ 0.004 <i>0.060</i>
	<i>p-value</i>				
Stomach Weight (g)	Mean $\pm$ SEM	0.426 $\pm$ 0.02	0.402 $\pm$ 0.022 <i>0.226</i>	0.329 $\pm$ 0.017 <i>0.006</i>	0.346 $\pm$ 0.019 <i>0.014</i>
	<i>p-value</i>				
Duodenum Weight (g)	Mean $\pm$ SEM	0.134 $\pm$ 0.026	0.137 $\pm$ 0.026 <i>0.474</i>	0.115 $\pm$ 0.022 <i>0.292</i>	0.15 $\pm$ 0.019 <i>0.326</i>
	<i>p-value</i>				
Liver Weight (g)	Mean $\pm$ SEM	1.987 $\pm$ 0.121	2.109 $\pm$ 0.041 <i>0.199</i>	2.022 $\pm$ 0.126 <i>0.426</i>	1.937 $\pm$ 0.047 <i>0.359</i>
	<i>p-value</i>				
Spleen Weight (g)	Mean $\pm$ SEM	0.119 $\pm$ 0.005	0.147 $\pm$ 0.029 <i>0.204</i>	0.128 $\pm$ 0.009 <i>0.226</i>	0.13 $\pm$ 0.014 <i>0.241</i>
	<i>p-value</i>				
Kidneys Weight (g)	Mean $\pm$ SEM	0.479 $\pm$ 0.017	0.530 $\pm$ 0.020 <i>0.046</i>	0.5242 $\pm$ 0.027 <i>0.102</i>	0.533 $\pm$ 0.017 <i>0.081</i>
	<i>p-value</i>				
Brain Weight (g)	Mean $\pm$ SEM	0.479 $\pm$ 0.010	0.477 $\pm$ 0.023 <i>0.462</i>	0.478 $\pm$ 0.009 <i>0.457</i>	0.446 $\pm$ 0.011 <i>0.076</i>
	<i>p-value</i>				
Testes Weight (g)	Mean $\pm$ SEM	0.218 $\pm$ 0.012	0.213 $\pm$ 0.011 <i>0.386</i>	0.213 $\pm$ 0.021 <i>0.414</i>	0.204 $\pm$ 0.09 <i>0.187</i>
	<i>p-value</i>				

Femur Weight (g)	Mean $\pm$ SEM	0.069 $\pm$ 0.002	0.093 $\pm$ 0.006 <i>0.008</i>	0.094 $\pm$ 0.01 <i>0.052</i>	0.087 $\pm$ 0.004 <i>0.006</i>
	<i>p-value</i>				

- 5 Table 10. Results of 5 day intravenous toxicity testing of rhNELL-1, including hematology and chemistry panels. N=4 animals per treatment group. No statistically significant differences were found with rhNELL-1 treatment, although a non-significant trend toward reduced BUN (Blood Urea Nitrogen) was observed. Data is presented as mean $\pm$ standard error of the mean. * $P < 0.0008$ in comparison to 0 mg/kg/day group.

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		0 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	6.25 mg/kg/day
WBC ($\times 10^3$)	Mean $\pm$ SEM	4.1 $\pm$ 0.85	4.6 $\pm$ 0.95	4.9 $\pm$ 0.7	4.3 $\pm$ 0.35
	<i>p-value</i>		0.370	0.257	0.450
RBC ($\times 10^6$)	Mean $\pm$ SEM	8.7 $\pm$ 0.25	8.8 $\pm$ 0.2	8.6 $\pm$ 0.25	8.8 $\pm$ 0.35
	<i>p-value</i>		0.437	0.401	0.455
Hgb (gm/dl)	Mean $\pm$ SEM	13.6 $\pm$ 0.3	14.1 $\pm$ 0.3	14.4 $\pm$ 0.4	14.6 $\pm$ 0.5
	<i>p-value</i>		0.139	0.082	0.167
HCT (%)	Mean $\pm$ SEM	49.7 $\pm$ 1.0	50.1 $\pm$ 1.15	51.1 $\pm$ 1.6	52 $\pm$ 2.2
	<i>p-value</i>		0.401	0.256	0.200
Platelet Count ($\times 10^3$)	Mean $\pm$ SEM	1293 $\pm$ 145	1078 $\pm$ 208	1043 $\pm$ 173	1098 $\pm$ 139
	<i>p-value</i>		0.217	0.156	0.185
Sodium (mEq/L)	Mean $\pm$ SEM	151.2 $\pm$ 0.9	150.7 $\pm$ 0.25	149.2 $\pm$ 1.1	150.5 $\pm$ 1.15
	<i>p-value</i>		0.320	0.110	0.320
Potassium (mEq/L)	Mean $\pm$ SEM	7.5 $\pm$ 0.25	7.1 $\pm$ 0.45	6.9 $\pm$ 0.4	7 $\pm$ 0.25
	<i>p-value</i>		0.268	0.166	0.149
Chloride (mEq/L)	Mean $\pm$ SEM	107.7 $\pm$ 0.45	108.5 $\pm$ 1.65	107 $\pm$ 0.55	107 $\pm$ 0.4
	<i>p-value</i>		0.345	0.179	0.140
Bicarbonate (mEq/L)	Mean $\pm$ SEM	9.5 $\pm$ 1.3	7.7 $\pm$ 1.4	9 $\pm$ 2.45	9.5 $\pm$ 1.9
	<i>p-value</i>		0.202	0.433	0.500
BUN (mg/dL)	Mean $\pm$ SEM	33 $\pm$ 2	40 $\pm$ 1.5	26 $\pm$ 1	27 $\pm$ 0.8
	<i>p-value</i>		0.131	0.014	0.021

Creatine (mg/dL)	Mean $\pm$ SEM <i>p-value</i>	0.2 $\pm$ 0	0.2 $\pm$ 0 <i>0.146</i>	0.2 $\pm$ 0 <i>0.999</i>	0.2 $\pm$ 0 <i>0.999</i>
Glucose (mg/dL)	Mean $\pm$ SEM <i>p-value</i>	230.2 $\pm$ 16.4	207.5 $\pm$ 8.25 <i>0.139</i>	243.7 $\pm$ 23.8 <i>0.330</i>	211.5 $\pm$ 3.45 <i>0.170</i>
Calcium (mg/dL)	Mean $\pm$ SEM <i>p-value</i>	11.2 $\pm$ 0.05	11.1 $\pm$ 0.075 <i>0.262</i>	11 $\pm$ 0.18 <i>0.249</i>	10.9 $\pm$ 0.09 <i>0.102</i>
Phosphorus (mg/dL)	Mean $\pm$ SEM <i>p-value</i>	13.7 $\pm$ 0.65	14.5 $\pm$ 0.6 <i>0.196</i>	13.3 $\pm$ 0.7 <i>0.379</i>	12.8 $\pm$ 0.4 <i>0.160</i>
AST (IU/L)	Mean $\pm$ SEM <i>p-value</i>	211 $\pm$ 40	428 $\pm$ 102 <i>0.060</i>	303 $\pm$ 206 <i>0.344</i>	121 $\pm$ 41 <i>0.083</i>
ALT (IU/L)	Mean $\pm$ SEM <i>p-value</i>	110 $\pm$ 44	114 $\pm$ 25 <i>0.472</i>	124 $\pm$ 85.5 <i>0.447</i>	44 $\pm$ 5 <i>0.115</i>
Total Bilirubin	Mean $\pm$ SEM <i>p-value</i>	0.1 $\pm$ 0	0.1 $\pm$ 0 <i>0.999</i>	0.12 $\pm$ 0.025 <i>0.196</i>	0.1 $\pm$ 0 <i>0.999</i>
ALP (IU/L)	Mean $\pm$ SEM <i>p-value</i>	155 $\pm$ 6	126 $\pm$ 8 <i>0.065</i>	138 $\pm$ 10 <i>0.115</i>	140 $\pm$ 9.5 <i>0.133</i>
Total Protein (g/dL)	Mean $\pm$ SEM <i>p-value</i>	5.2 $\pm$ 0.1	5.1 $\pm$ 0.05 <i>0.320</i>	4.9 $\pm$ 0.05 <i>0.084</i>	5.2 $\pm$ 0.1 <i>0.450</i>
Albumin (g/dL)	Mean $\pm$ SEM <i>p-value</i>	3 $\pm$ 0.05	3 $\pm$ 0.05 <i>0.248</i>	2.8 $\pm$ 0.05 <i>0.023</i>	3 $\pm$ 0.05 <i>0.248</i>
Globulin (g/dL)	Mean $\pm$ SEM <i>p-value</i>	2.1 $\pm$ 0.05	2.1 $\pm$ 0.05 <i>0.500</i>	2 $\pm$ 0.025 <i>0.122</i>	2.2 $\pm$ 0.05 <i>0.347</i>

	Mean $\pm$ SEM	827 $\pm$ 156.5	3582 $\pm$ 1698	1108 $\pm$ 693	613 $\pm$ 313.5
CPK (U/L)	<i>p-value</i>		0.102	0.358	0.286

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Acronyme List:

Ad-Nell-1	Adenoviral Nell-1
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
ASBMR	American Society for Bone and Mineral Research
AR	Alizarin Red
AST	Aspartate Aminotransferase
ALT	Alanine Aminotransferase
AVN	Avascular Necrosis
β -TCP	P-Tricalcium Phosphate
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMP	Bone Morphogenetic Protein
BMSC	Bone Marrow Mesenchymal Stem Cells
B.Ar	Bone Area
B.Pm	Bone Perimeter
BrdU	Bromodeoxyuridine
BUN	Blood Urea Nitrogen
BV	Bone Volume
BV/TV	Bone Volume / Tissue Volume
CPC	Cetylpyridinium Chloride
CPK	Creatine Phosphokinase
Ct.Wi	Cortical Width
Ct.Th	Cortical Thickness
CT	Computed Tomography
CTX	C-Terminal Telopeptide
DKK-1	Dickkopf related protein 1
DXA	Dual Energy X-ray Absorptiometry
EDTA	Ethylenediamine Tetra-acetic Acid

ERK1/2	Extracellular Signal-Related Kinase 1/2
FBS	Fetal Bovine Serum
FEA	Finite Element Analysis
GFP	Green Fluorescent Protein
GMT	Goldner's Modified Trichrome
H&E	Haemotoxylin and Eosin
HCT	Hematocrit
Hgb	Hemoglobin
HU	Hounsfield Units
IDI	Indentation Distance Increase
JNK	c-Jun N-terminal Kinase
MAPK	Mitogen-Activated Protein Kinase
M-CSF	Macrophage Colony-Stimulating Factor
NMR	Nuclear Magnetic Resonance
OB	Osteoblast
Ob.N	Osteoblast Number
OC	Osteoclast
Oc.N	Osteoclast Number
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
OVX	Ovariectomy
PBS	Phosphate Buffered Saline
pCi	Pico Curie
PET	Positron Emission Tomography
PINP	Procollagen I N-terminal Propeptide
PTH	Parathyroid Hormone
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RANKL	Receptor Activator of Nuclear Factor K B Ligand
RBC	Red Blood Cells
rhNELL-1	Recombinant human NELL-1
ROI	Region of Interest

RUNX2	Runt-related Transcription Factor 2
SEM	Scanning Electron Microscopy
SEM	Standard Errors of the Mean
SD	Sprague-Dawley
SOST	Sclerostin
TID	Total Indentation Distance
Tb.Ar	Trabecular bone Area
Tb.N	Trabecular bone Number
Tb.Pm	Trabecular bone Perimeter
Tb.Sp	Trabecular bone Spacing
Tb.Wi	Trabecular bone Width
TRAP	Tartrate Resistant Acid Phosphatase
VKMT	Von Kossa MacNeaPs Tetrachrome
VOI	Volume of Interest
WBC	White Blood Cells

5 References (cited in Figures 7-14 and Tables 1-10):

1. Shen, J. *et al.* NELL-1 promotes cell adhesion and differentiation via Integrin β 1. *J Cell Biochem* 113, 3620-8 (2012).
2. Ieguchi, K. *et al.* Direct binding of the EGF-like domain of neuregulin-1 to integrins (α v β 3 and α 6 β 4) is involved in neuregulin-1/ErbB signaling. *J Biol Chem* 285, 31388-98 (2010).
3. Klinck, J. & Boyd, S.K. The magnitude and rate of bone loss in ovariectomized mice differs among inbred strains as determined by longitudinal in vivo micro-computed tomography. *Calcif Tissue Int* 83, 70-9 (2008).
4. Buie, H.R., Moore, C.P. & Boyd, S.K. Postpubertal architectural developmental patterns differ between the L3 vertebra and proximal tibia in three inbred strains of mice. *J Bone Miner Res* 23, 2048-59 (2008).
5. Bouxsein, M.L. *et al.* Ovariectomy-induced bone loss varies among inbred strains of mice. *J Bone Miner Res* 20, 1085-92 (2005).

20 **Example 2. Studies on modification of OB:OC ratios by invention composition comprising NELL-1**

Effects on expression of Opg and Rankl by invention composition comprising rhNELL-1 as a component of the composition in primary mouse BMSC were studied. Cells were treated with or without an invention composition comprising rhNELL-1 (300 ng/mL) for

- 5 24 hours, followed by evaluating of gene expression by quantitative RT-PCR. Data presented as either relative *Rankl* expression (left), relative *Opg* expression (middle), or relative *Rankl* to *Opg* expression (right) between control and cells treated by the invention composition comprising rhNELL-1 (Figure 15). Experiments performed with N=3 replicates. *p<0.05, **p<0.01. Figure 15 clearly shows that the invention composition can modify OB:OC ratios
- 10 via changes in *Rankl/Opg* expression.

Those skilled in the art will know, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

We Claim

1. A composition for a bone condition, comprising an effective amount of a first component that is effective to increase the cell number and activity of osteoblast (OB) and an effective amount of a second component that is effective to decrease the cell number and activity of osteoclast (OC) such that the composition is effective to cause the increased cell number of OB ("increased OB") and the decreased cell number of OC ("decreased OC") to have a ratio that decreased OC / increased OB is from about 100:1 to about 1:100,

wherein the first component and/or the second component are not one of anti-SOST and anti-DKK-1 antibodies.

2. The composition of claim 1, wherein the first component and the second component comprise an osteoinductive agent.

3. The composition of claim 2, wherein the first component or the second component comprise a bisphosphonate.

4. The composition of claim 3, comprising a scaffold impregnated with the bisphosphonate.

5. The composition of claim 3, wherein the osteoinductive agent comprises NELL-1.

6. The composition of claim 2, wherein the osteoinductive agent is effective for activation of the Wnt/ β -catenin signaling pathway.

7. The composition of claim 1, wherein the increased OB and the decreased OC have a ratio that decreased OC / increased OB is from about 10:1 to about 26:1.

8. The composition of claim 1, wherein the bone condition is osteopenia.

9. The composition of claim 1, wherein the bone condition is osteoporosis.

10. A method of treating or preventing a bone condition, comprising administering to a subject:

an effective amount of a first component of a composition where the first component is effective to increase the cell number and activity of osteoblast (OB), and

an effective amount of a second component of the composition where the first component is effective to decrease the cell number and activity of osteoclast (OC);
to cause the increased cell number of OB ("increased OB") and the decreased cell number of OC ("decreased OC") to have a ratio that decreased OC / increased OB is from about 100:1 to about 1:100,

wherein the first component and/or the second component are not one of anti-SOST and anti-DKK-1 antibodies.

11. The method of claim 10, wherein the composition is according to any one of claims 2-9.

13. The method of claim 10, wherein the bone condition is osteopenia.

14. The method of claim 10, wherein the bone condition is osteoporosis.

15. The method of claim 10, wherein the subject is a human being.

16. A method of fabricating a composition, comprising:

providing an effective amount of a first component that is effective to increase the cell number and activity of osteoblast (OB),

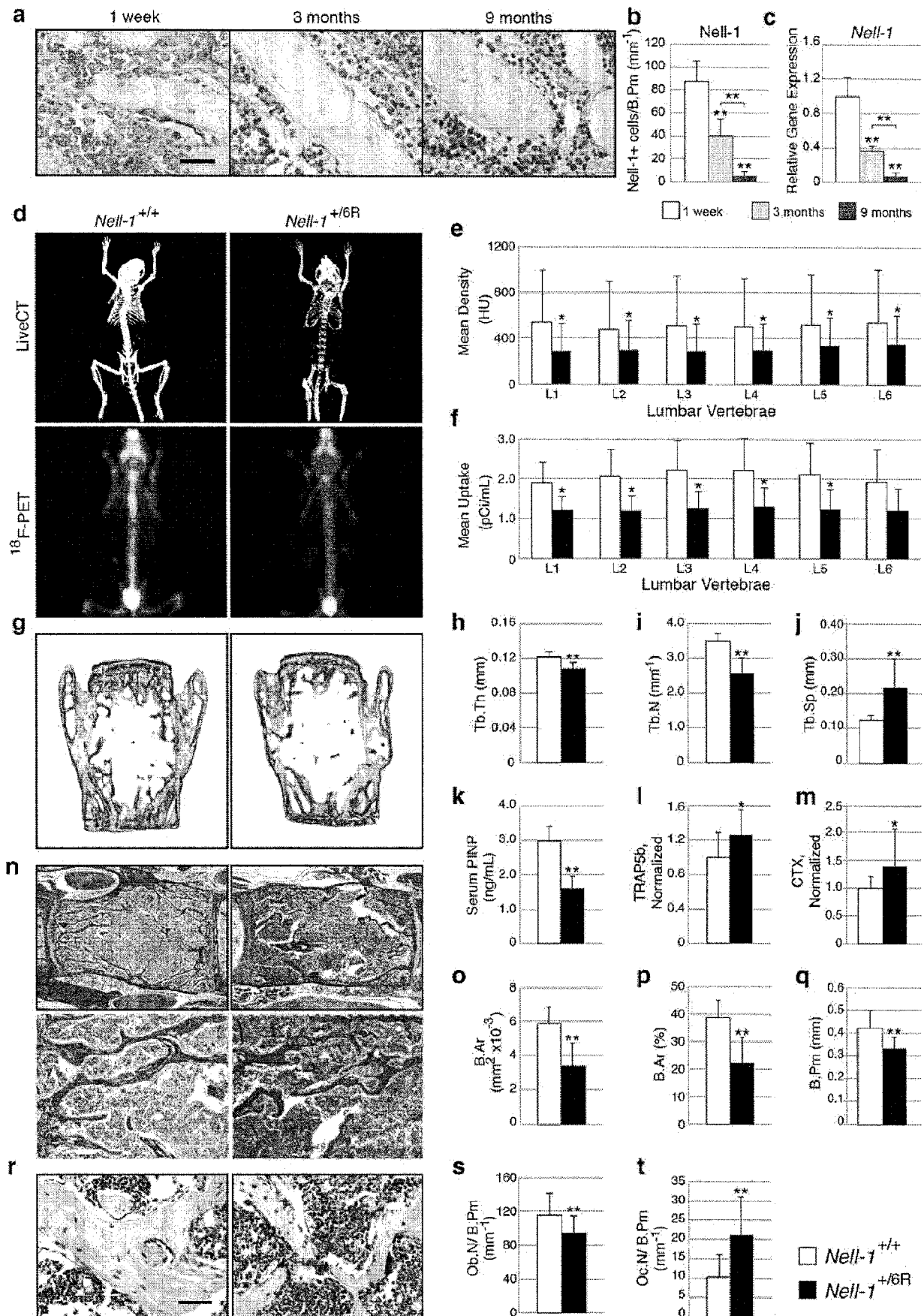
providing an effective amount of a second component that is effective to decrease the cell number and activity of osteoclast (OC), and

forming the composition,

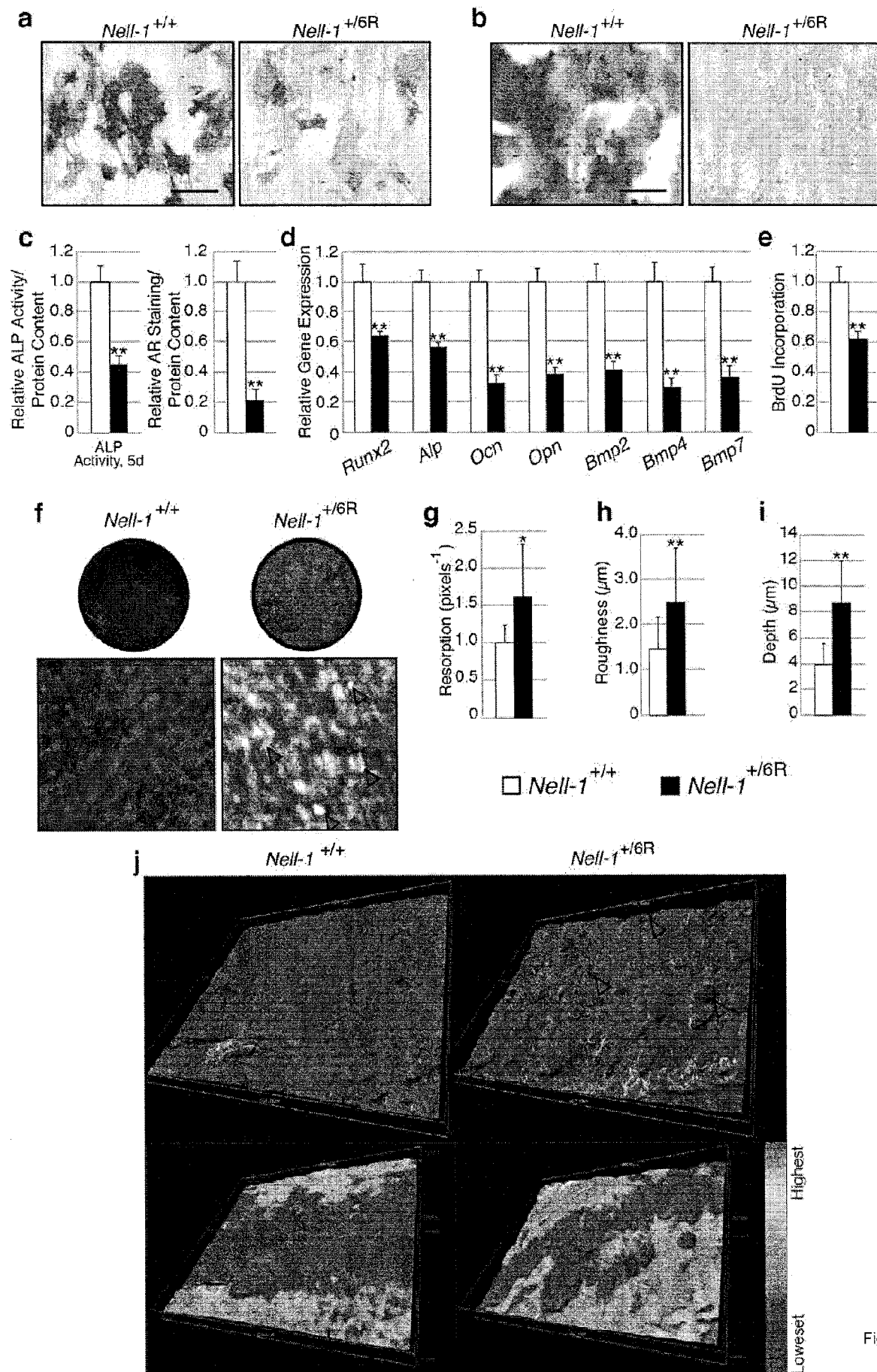
wherein the composition is effective to cause the increased cell number of OB ("increased OB") and the decreased cell number of OC ("decreased OC") to have a ratio that decreased OC / increased OB is from about 100:1 to about 1:100, and

wherein the first component and/or the second component are not one of anti-SOST and anti-DKK-1 antibodies.

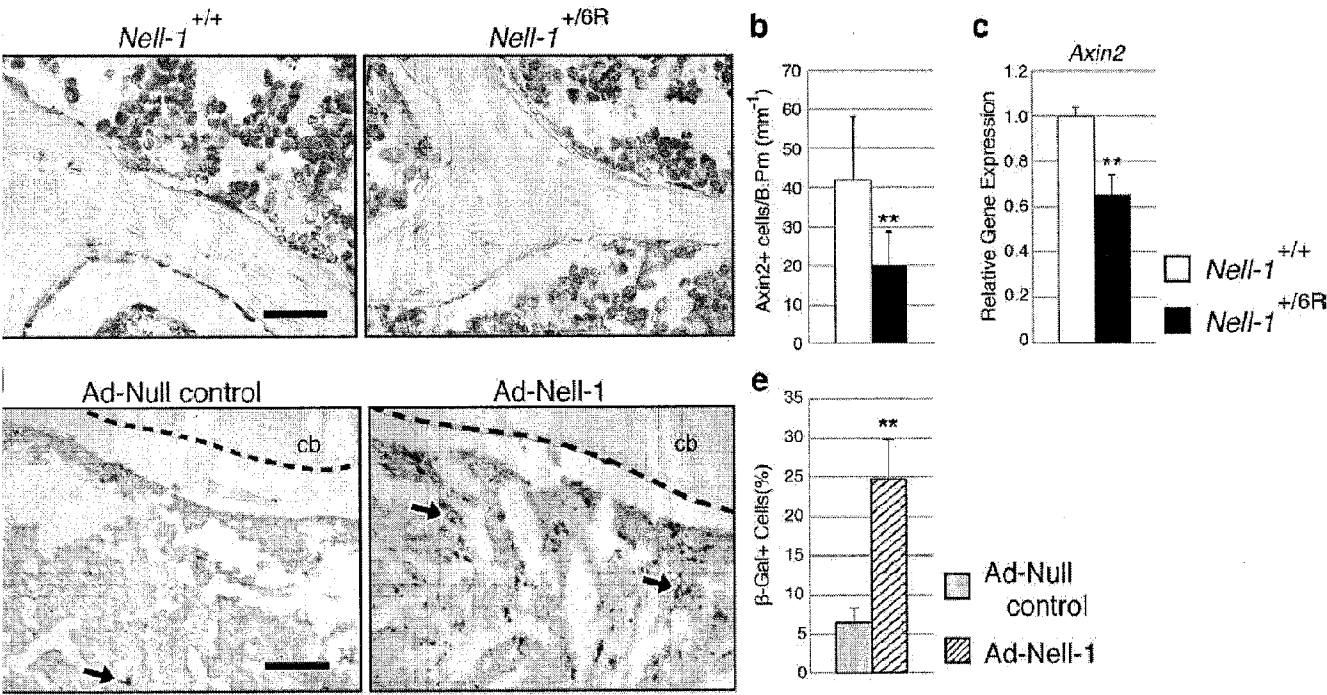
17. The method of claim 15, wherein the composition is according to any one of claims 2-9.



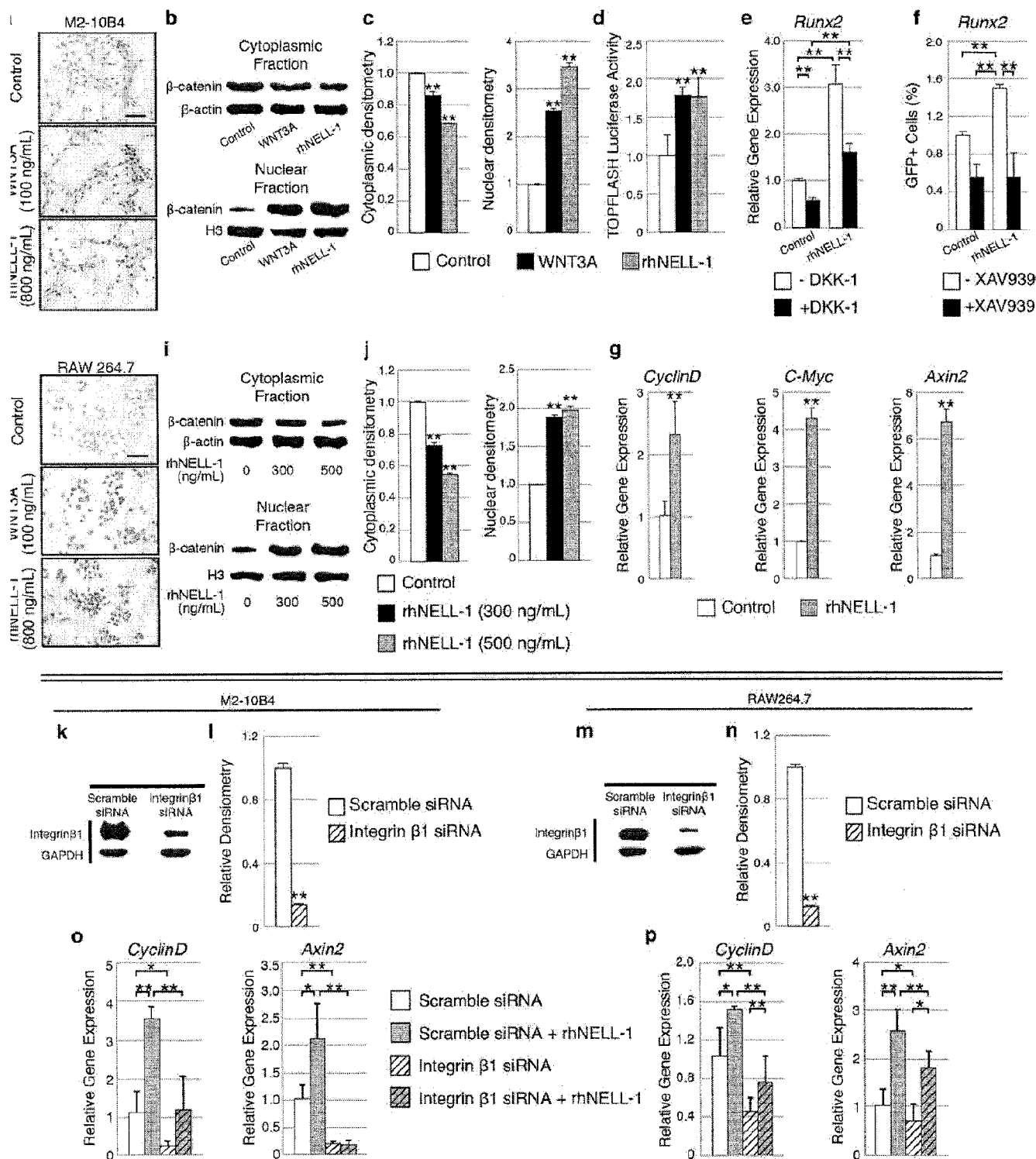
Figures 1a-t



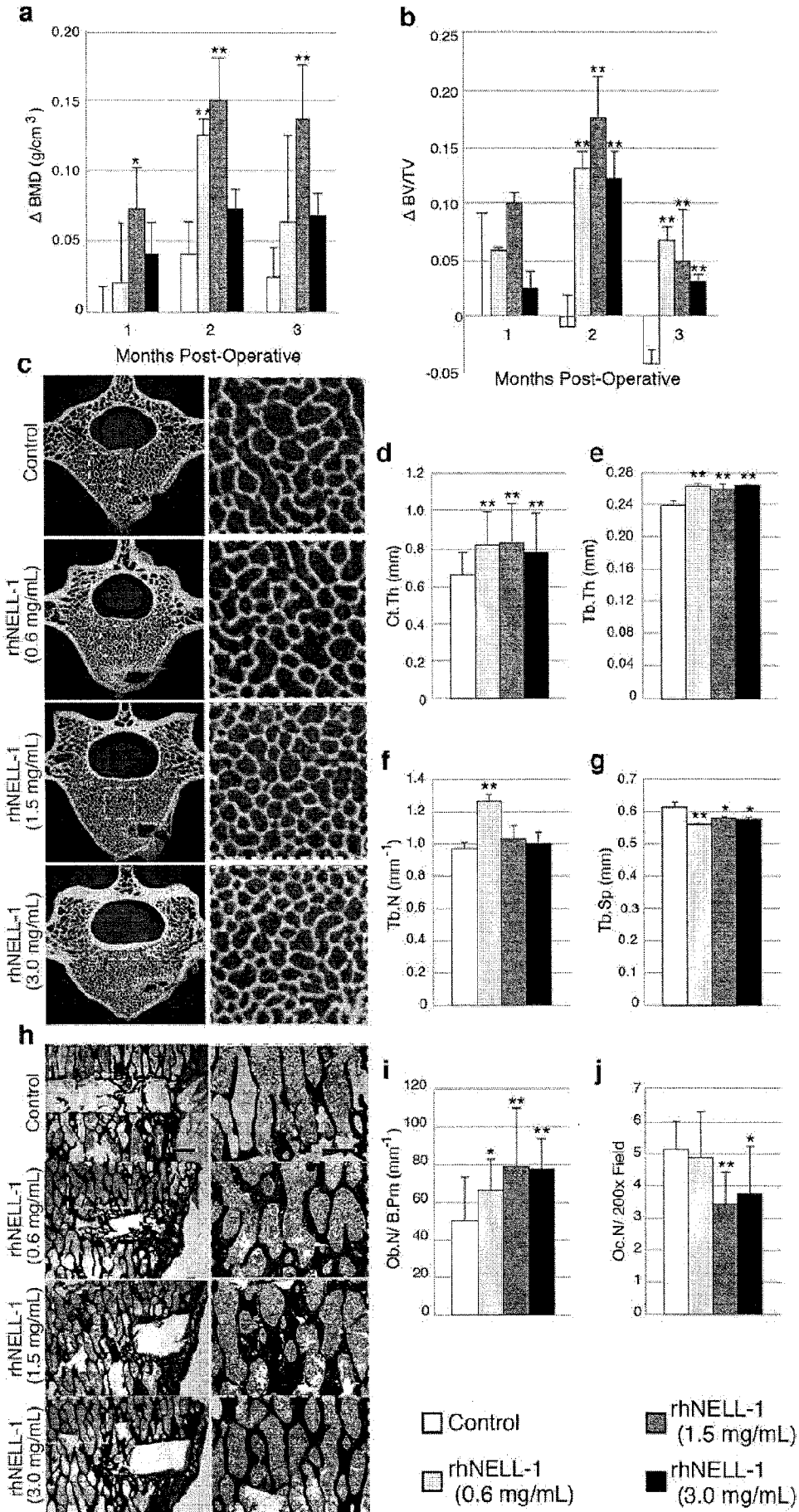
Figures 2a-j



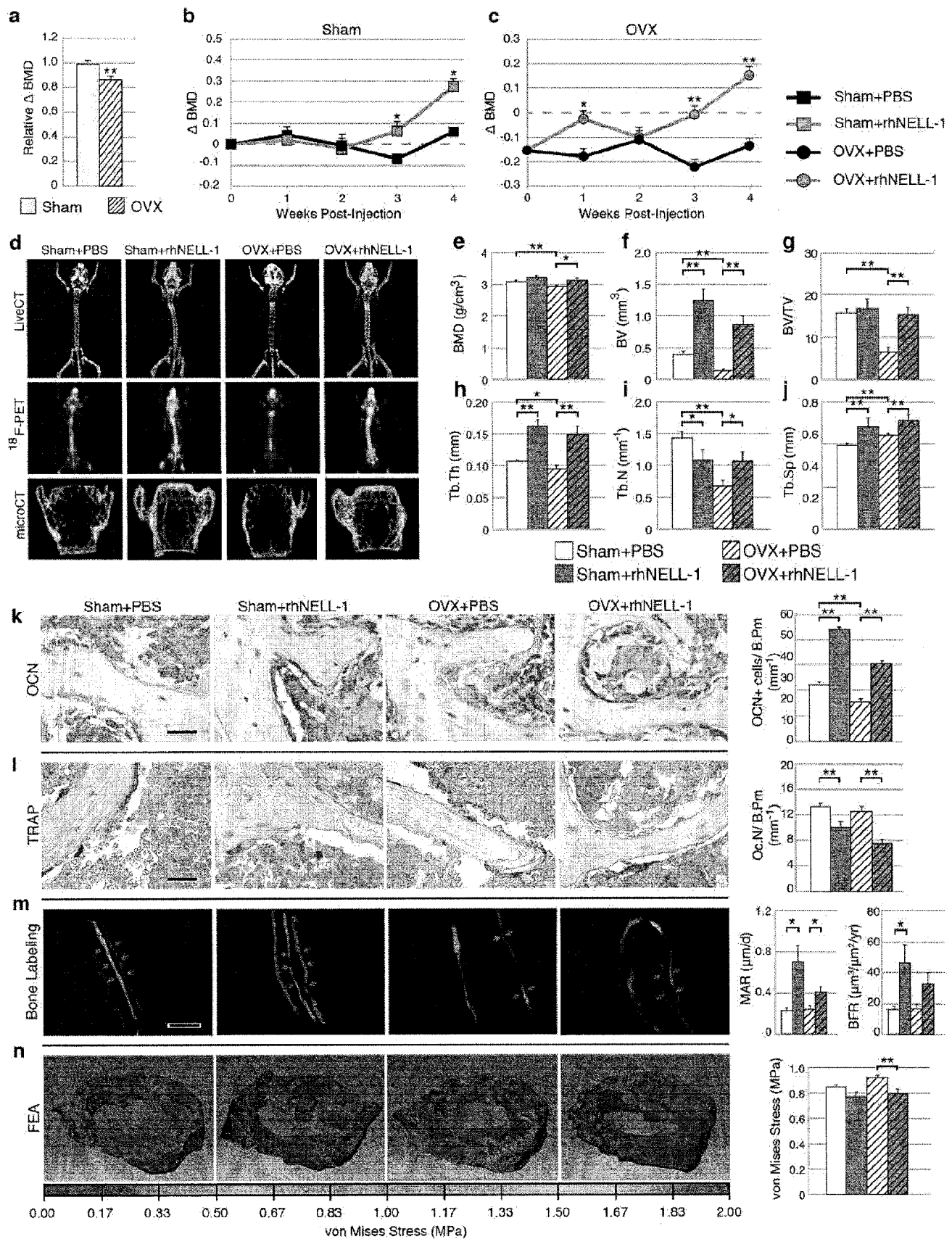
Figures 3a-e



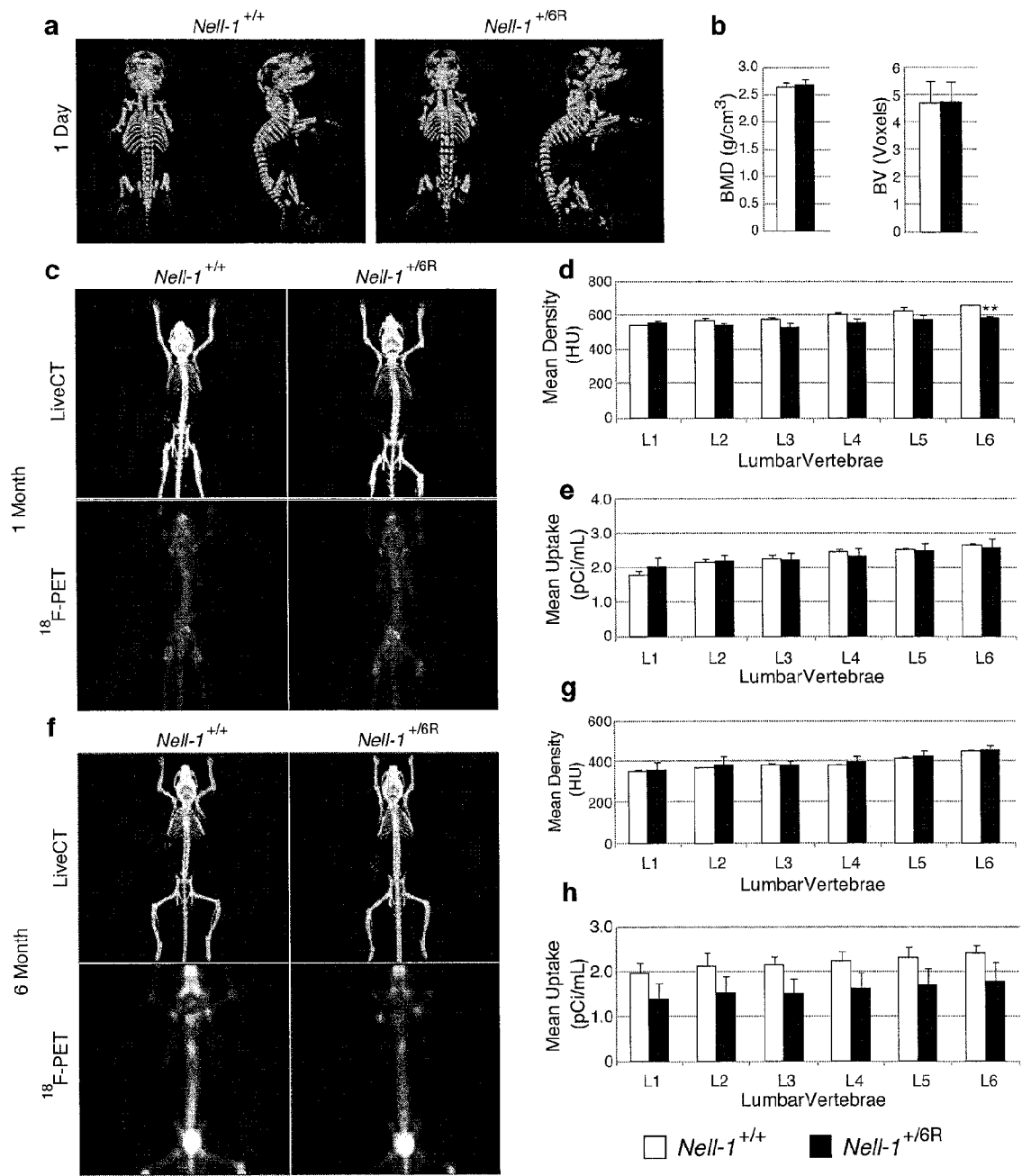
Figures 4a-p



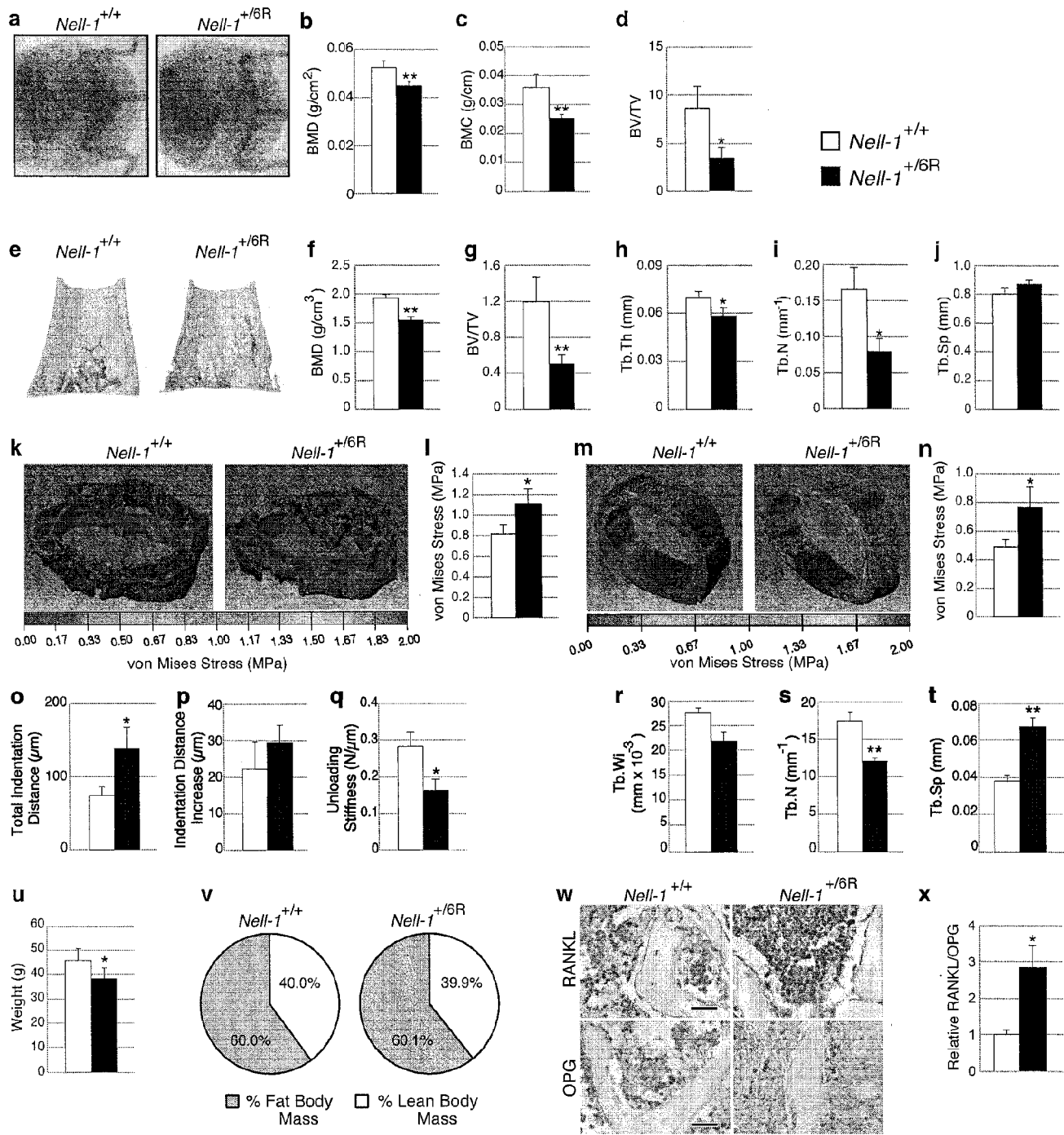
Figures 5a-j



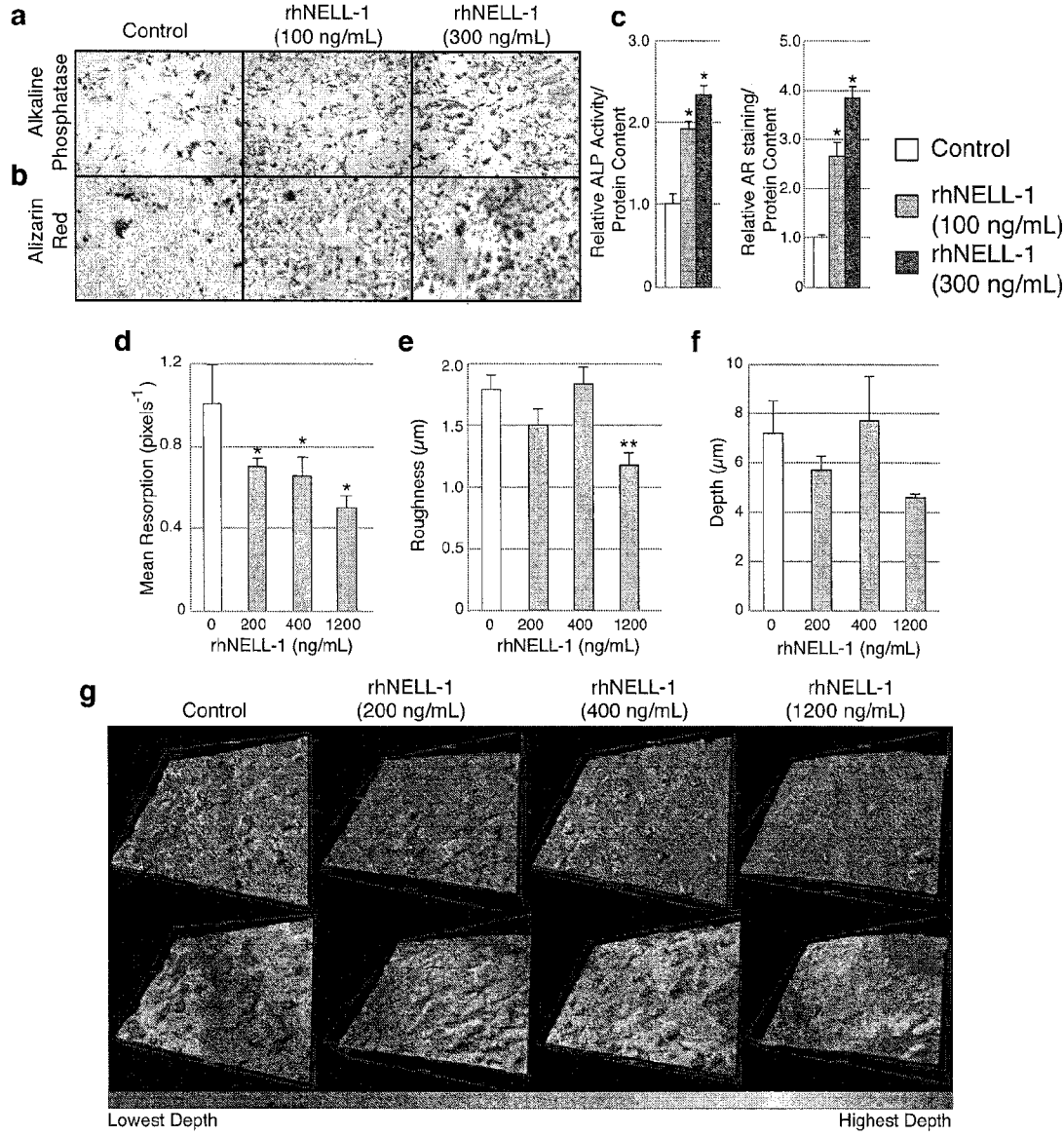
Figures 6a-n



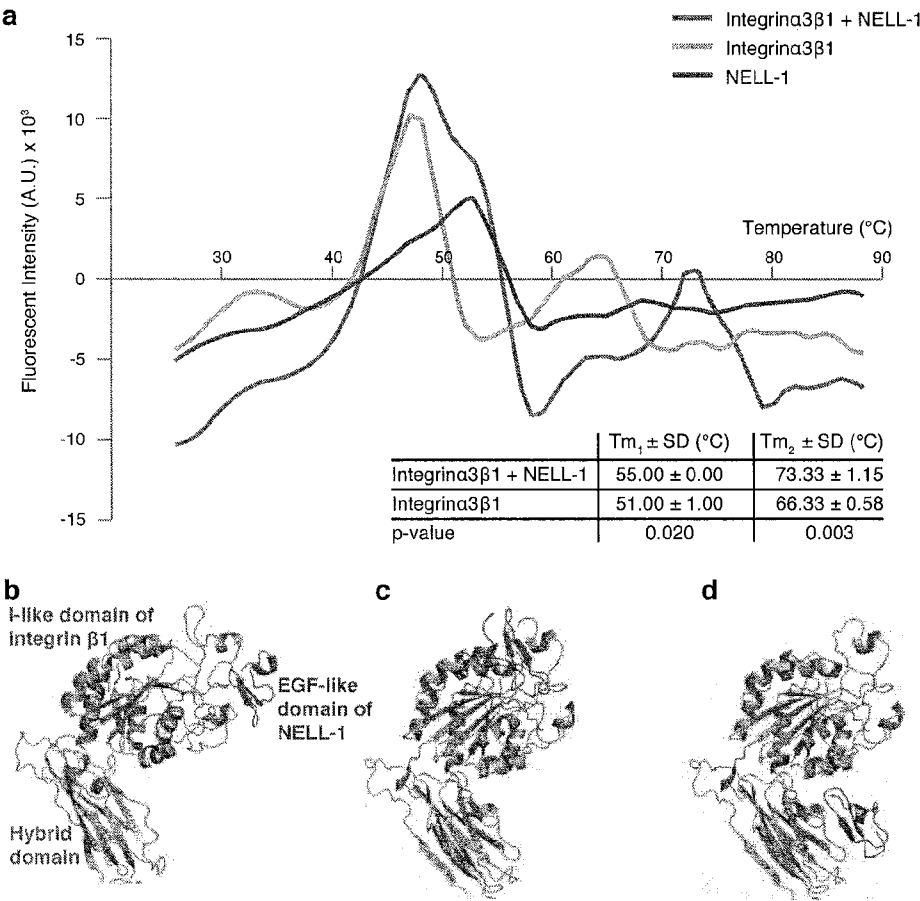
Figures 7a-h



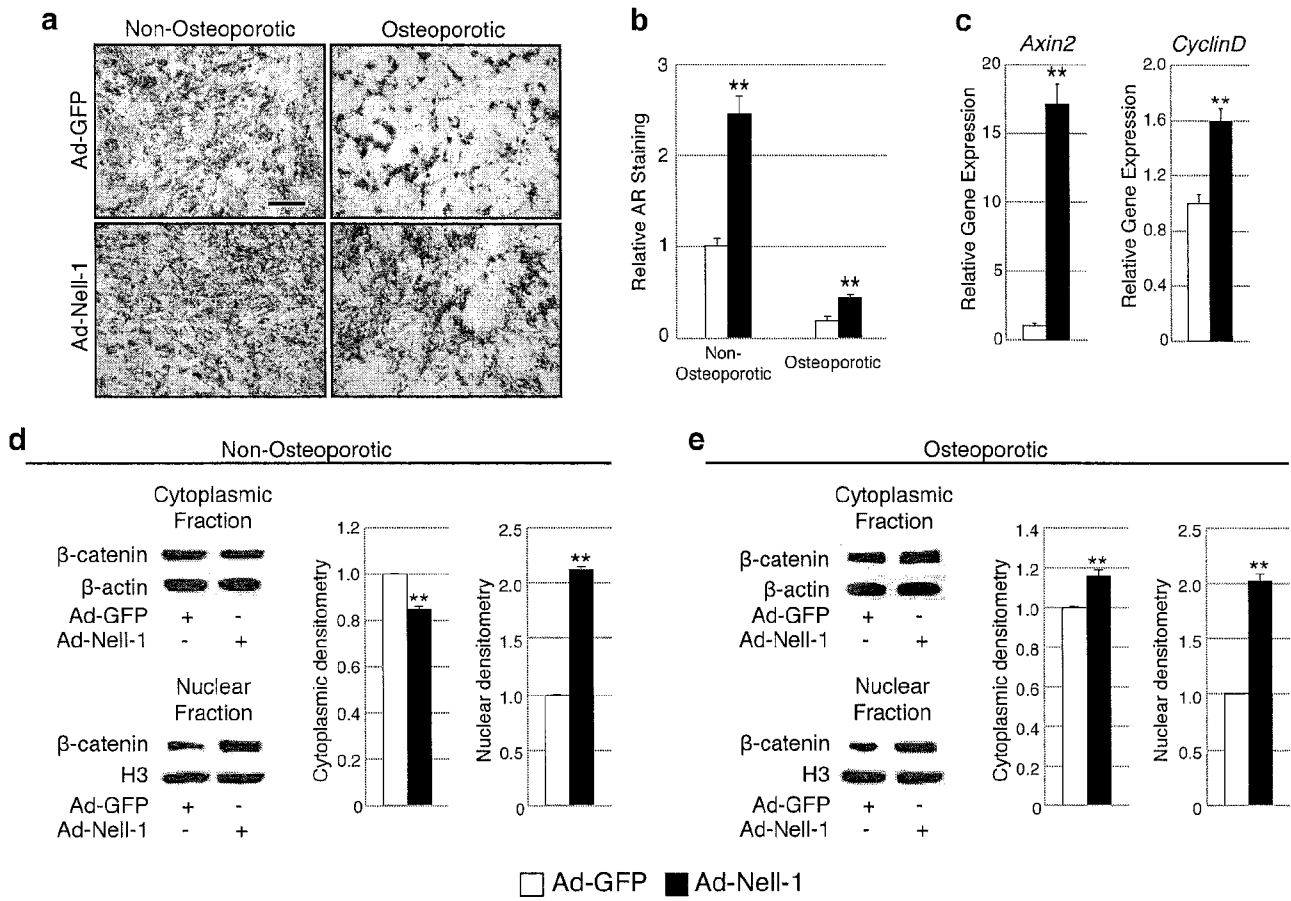
Figures 8a-x



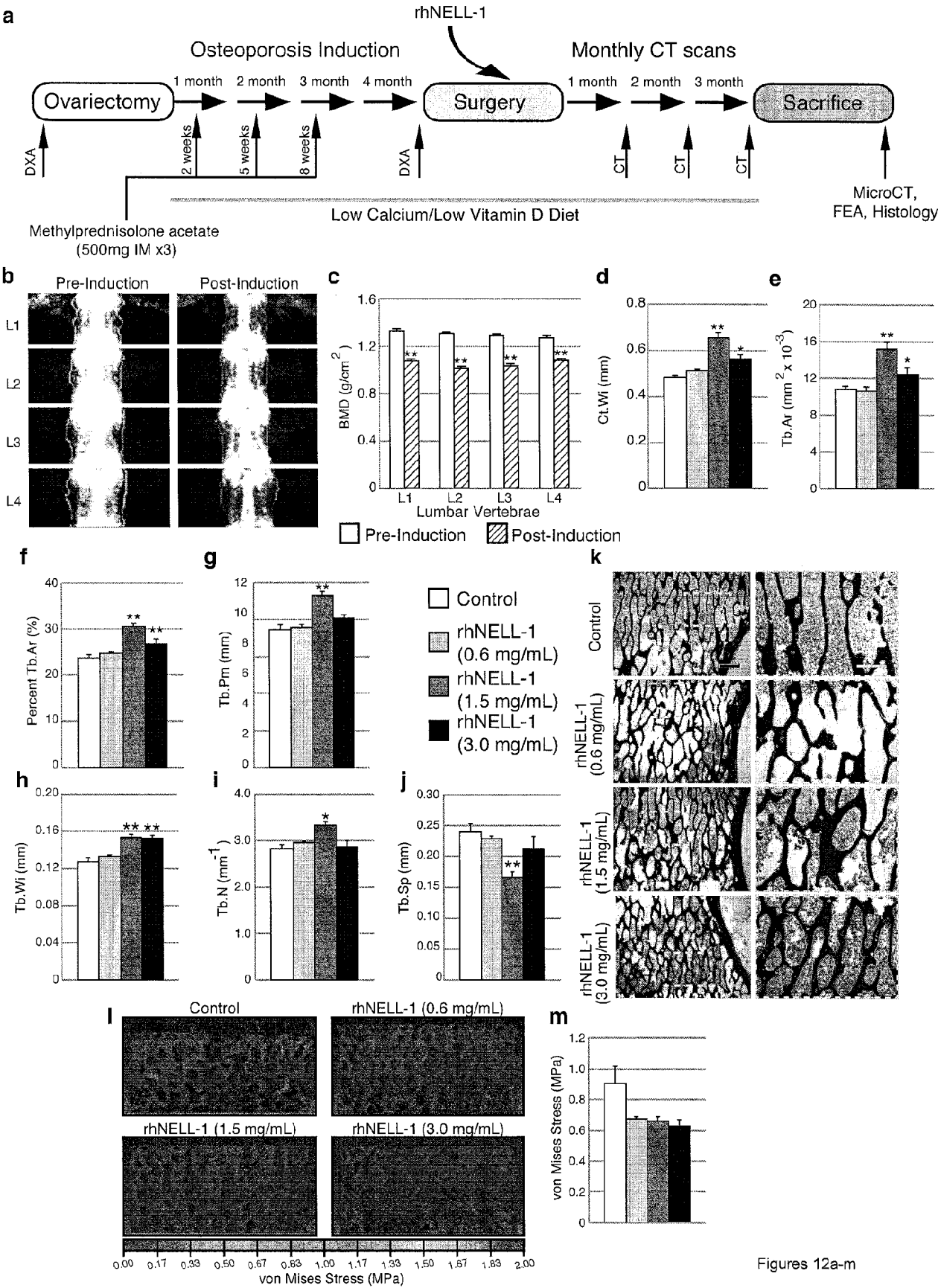
Figures 9a-g



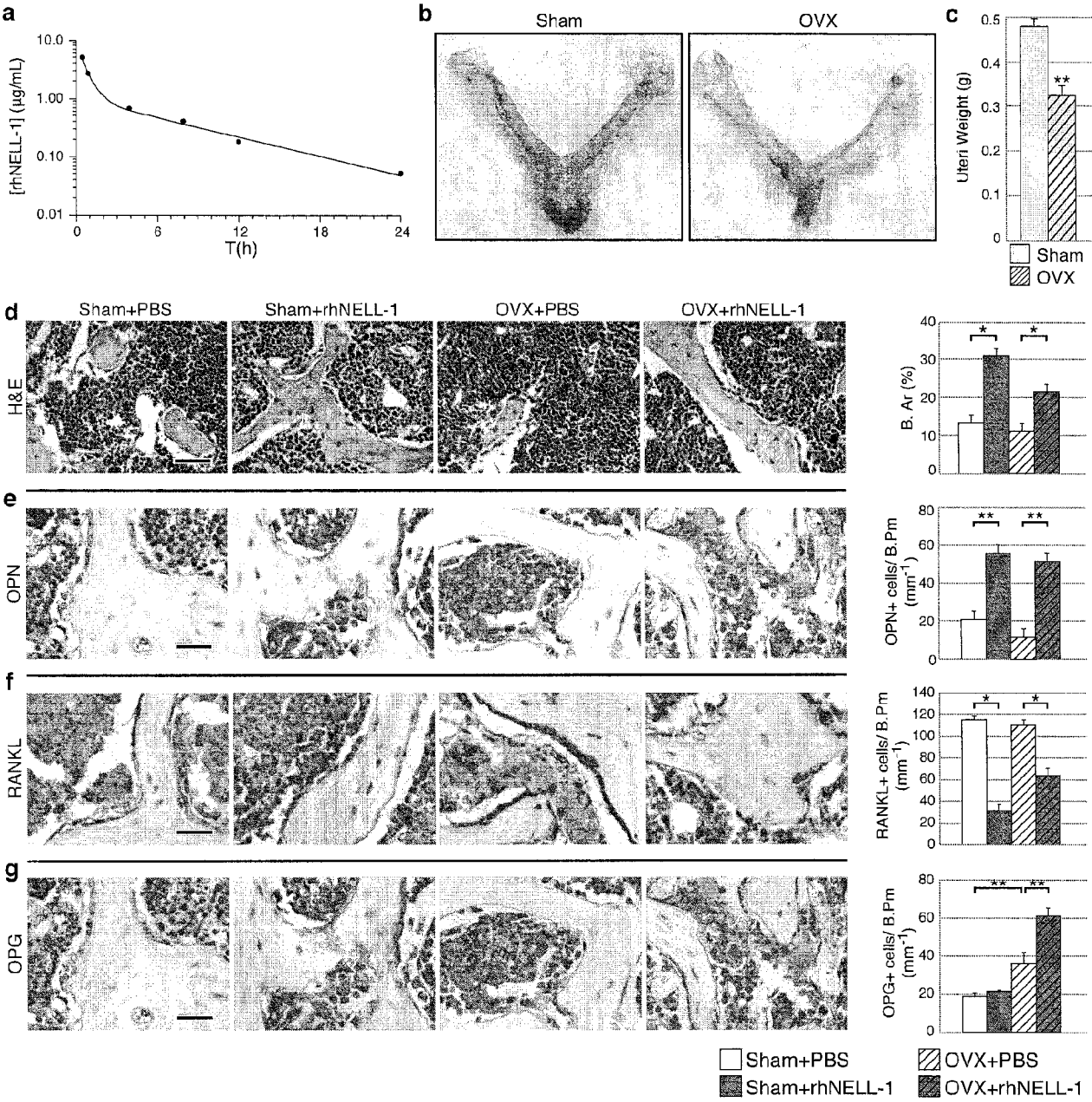
Figures 10a-d



Figures 11a-e



Figures 12a-m



Figures 13a-g

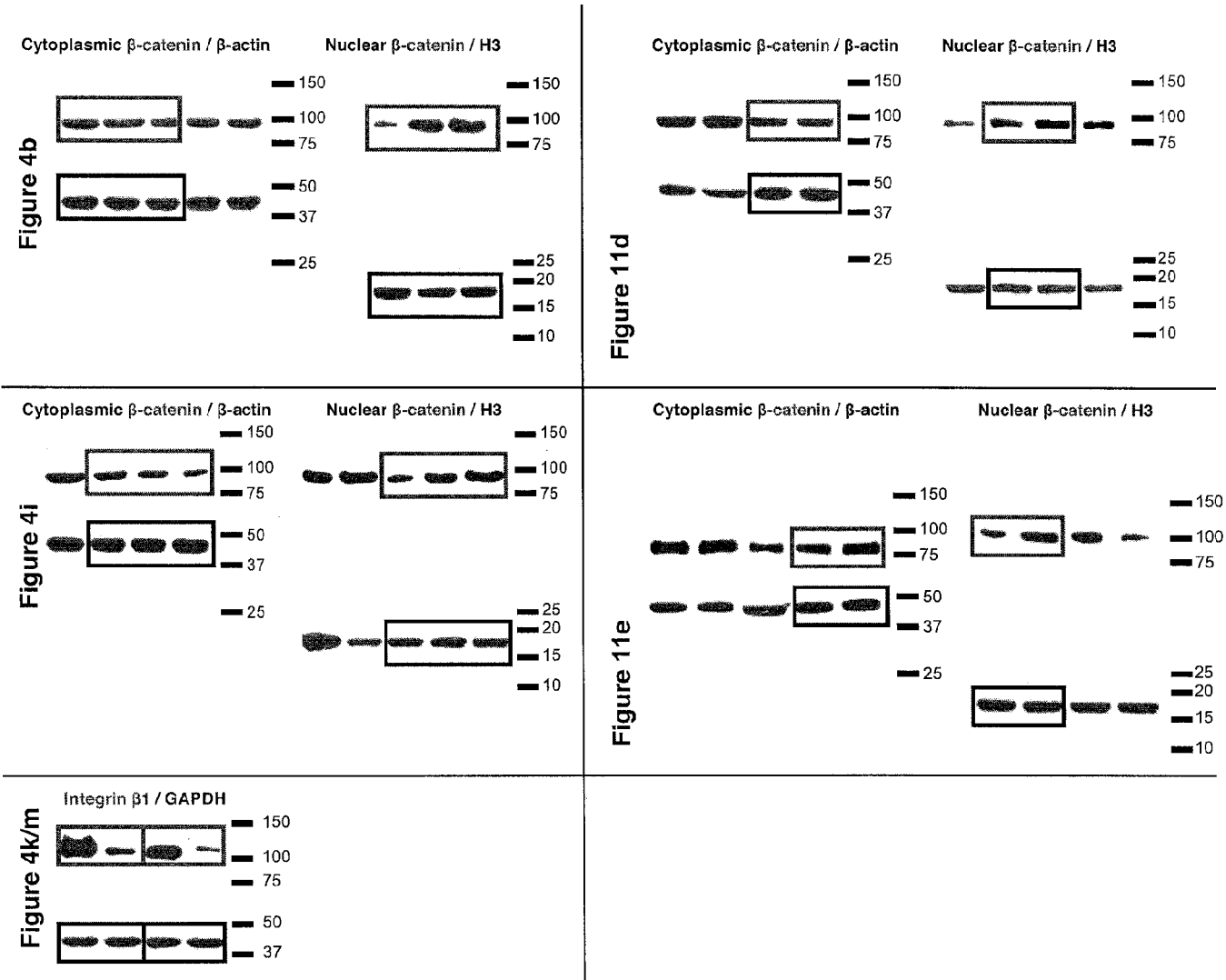
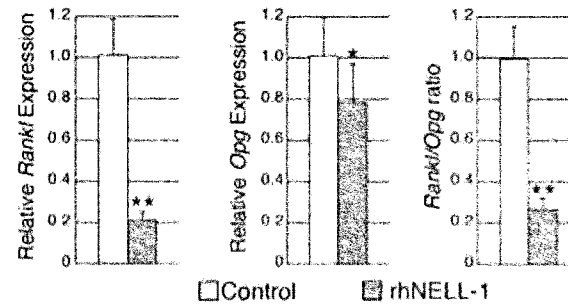


Figure 14



Response Letter Fig. 19: Expression of *Opg* and *Rankl* after rhNELL-1 treatment in primary mouse BMSC. Cells were treated with or without rhNELL-1 (300 ng/mL) for 24 hours, followed by evaluation of gene expression by quantitative RT-PCR. Data presented as either relative *Rankl* expression (left), relative *Opg* expression (middle), or relative *Rankl* to *Opg* expression (right) between control and rhNELL-1 treated cells. Experiments performed with N=3 replicates. * $P < 0.05$, ** $P < 0.01$.

Figure 15.

A. CLASSIFICATION OF SUBJECT MATTER**A61K 35/32(2006.01)i, A61K 31/663(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 35/32; A61K 35/12; A61P 19/00; A61K 38/17; A61P 19/08; A61K 31/663

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: bond condition, cell number, osteoblast, ratio, osteoinductive, bisphosphonate, anti-SOST, anti-DKK-1, osteopenia, osteoporosis**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015-009991 A2 (CAYMAN CHEMICAL COMPANY, INC. et al.) 22 January 2015 See abstract; paragraphs [0001], [0007]-[0014], [0103], [0105]-[0106], [0115]-[0117], [0443].	1-4, 6-9, 16
Y		5
Y	JAMES, A. W., "Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation", Scientifica, 2013, Vol. 2013, Article ID 684736, Internal pages 1-17. See internal pages 6-7; figure 5.	5
A	US 2012-0244128 A1 (SOO, B. C. et al.) 27 September 2012 See abstract; claims 1, 9.	1-9, 16
A	US 2008-0193515 A1 (SHAUGHNESSY, J. D. et al.) 14 August 2008 See abstract; claim 16.	1-9, 16
PX	JAMES, A. W. et al., "NELL-1 in the treatment of osteoporotic bone loss", Nature communications, [Epub.] 2015.06.17, Vol. 6, Internal pages 1-14. See the whole document. ※ Note: Claim 12 is found missing in this application.	1-9, 16



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 November 2016 (18.11.2016)

Date of mailing of the international search report

18 November 2016 (18.11.2016)

Name and mailing address of the ISA/KR

International Application Division

Korean Intellectual Property Office

189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea



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Telephone No. +82-42-481-3516



INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2016/035705**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 10-11, 13-15, 17
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 10-11, 13-15 and 17 pertain to methods for treatment of the human body by surgery or therapy, and thus relate to a subject matter which this International Searching Authority is not required to search (PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv)).
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2016/035705

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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