Title: PHARMACEUTICAL COMPOSITIONS FOR TREATING OR PREVENTING BONE CONDITIONS

Abstract: Provided herein is a pharmaceutical composition for treating, preventing or ameliorating a bone or cartilage condition and methods of making and using the same.
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PHARMACEUTICAL COMPOSITIONS FOR TREATING OR PREVENTING
BONE CONDITIONS
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BACKGROUND OF THE INVENTION

The present invention is generally related to pharmaceutical compositions
for treating or preventing bone condition. These pharmaceutical compositions may
be used to induce bone and/or cartilage formation in wound healing and tissue
repair.

The costs of treatment for orthopedic and craniofacial bone conditions
represent a significant biomedical burden. According to the 2002 US Health Cost
& Utilization Project, hospital costs for cranial surgery (craniotomies and
craniectomies) and facial trauma reconstruction alone were estimated to be
approximately $549 million and $400 million, respectively (Steiner, C., A.
costs for orthopedic surgeries (both trauma and nontrauma) are likely even higher
as the figure for orthopedic industry sales alone was estimated to be $13 billion in

Overall, the major problem encountered in the treatment of orthopedic and
craniofacial bone conditions concerns the modulation of bone and/or cartilage
formation. Preferably, bone formation can be increased under conditions in which
it would be desirable to have more or accelerated bone formation as part of the
treatment of certain conditions (e.g., orthopedic or craniofacial fracture repair,
spinal fusion surgery, joint fusion surgery, injured osteoporotic bone) or as part of
the prevention of certain conditions (e.g., fracture prevention in osteoporotic bone).
For long bone fracture, it would be desirable to have accelerated endochondral
bone formation by accelerating the cartilage to hypertrophy and replaced by bone.
Even more preferably, bone formation can also be decreased under conditions in
which it would be desirable to have decreased or inhibited bone formation as part
of the treatment or prevention of certain conditions (e.g., craniosynostosis, a
condition of premature calvarial overgrowth across sutures leading to premature
suture fusion; heterotopic ossification, a condition of abnormal bone formation in ectopic locations). Similarly, it would be preferred to increase cartilage formation under conditions in which it would be desirable to have more or accelerated cartilage formation (e.g., joint resurfacing, temporomandibular joint reconstruction, articular disc repair, intervertebral disc repair and regeneration).

Many compositions have been described for the treatment of bone conditions (Table 1). Most, if not all, describe compositions that promote bone formation through osteoconductive and/or osteoinductive properties. It is well established in the art that compositions with osteoinductive properties are generally more efficacious at forming bone than those with osteoconductive properties; however, both are necessary for optimal bone formation (Table 1). The current "gold standard" composition for treatment of many bone conditions is autologous bone graft, which has both osteoinductive and osteoconductive properties. However, autograft harvest can be associated with significant donor site morbidity including pain, gait disturbance, thigh paresthesia for iliac crest donor sites (Laurie, S.W., et al. Plast Reconstr Surg, 1984. 73(6): p. 933-8.). Thus, there is a critical need for better autograft alternatives. Of compounds with osteoinductive ability, the bone morphogenetic proteins (BMPs) have been extensively described. When coupled with an osteoconductive carrier, BMPs offer the greatest promise of equaling or even surpassing autograft for treatment of many bone conditions (Valentín-Opran, A., et al. Clin Orthop, 2002(395): p. 110-20).

However, the known functional heterogeneity of the BMPs (Ducy, P. and G. Karsenty, Kidney Int, 2000. 57(6): p. 2207-14; Wang, S., et al., Kidney Int, 2003. 63(6): p. 2037-49) and the high dose of BMPs required for osteoinduction may limit their use due to cost considerations and to unpredictable side effects such as maxillary sinus cyst formation (van den Bergh, J.P., et al., J Clin Periodontol, 2000. 27(9): p. 627-36). Consequently, there is an ongoing clinical and commercial need for alternative or complementary osteoinductive molecules to the BMPs to promote bone and/or cartilage formation. In addition, there is an ongoing clinical and commercial need for inhibiting bone and/or cartilage formation under specific conditions that is not addressed by the osteoinductive BMPs.

The embodiments described below address the above-identified problems and needs.
SUMMARY OF THE INVENTION

In one aspect of the present invention, provided herein is a pharmaceutical composition containing one or more agents such as one or more NELL peptides or NELL RNA. In one embodiment, the pharmaceutical composition contains an effective amount of one or more NELL peptides for treating bone conditions through promoting bone generation after injury, e.g., long bone fracture healing, spinal fusion, and craniofacial bone repair. In another embodiment, the pharmaceutical composition contains an effective amount of one or more NELL peptides for treating or preventing bone conditions through promoting bone generation without necessarily evidence of overt bone injury (e.g., osteoporosis, hip necrosis, and alveolar ridge bone resorption).

In some embodiments, the composition described herein is effective and can be used to treat, prevent, ameliorate, mitigate or reduce the symptoms of diseases/conditions that involve multiple symptoms where bone metabolism is a secondary effect. Examples of such diseases or conditions include, but are not limited to, chronic kidney diseases which can cause many systemic effects including renal osteodystrophy and vascular calcification. Nell can increase bone formation without stimulating undesirable bone formation, and thus it can stimulate the formation of bone only in bone compartments without stimulating proliferation of non-bone cells in the body (e.g. pre-cancerous cells), and as a result the targeted bone formation alleviates bone loss due to kidney damage. The NELL-induced mineralization also consumes the calcium and phosphate ions that otherwise form pathological calcification in normally non-calcifying tissues such as blood vessels. Other forms of pathological calcifications have multi-factorial origin (bacterial, paracrine, autocrine, etc.). The ability of Nell to favor the balance between bone deposition and bone resorption makes the composition described herein an effective composition to maintain the essential ions in the bone compartment and decrease their bioavailability in non-bone tissues, thereby reducing the risk for ectopic soft tissue calcification, gall stone, kidney stones,
pineal gland calcification, cataracts, salivary stones, cardiac valves, and/or prostate stones.

In another aspect of the present invention, the present invention provides a pharmaceutical composition that contains an effective amount of an inhibitor of NELL peptides for inhibiting bone generation (e.g., craniosynostosis or heterotopic ossification, osteopetrosis). In yet another aspect of the present invention, the invention provides a pharmaceutical composition that contains a sufficiently high enough dose of NELL peptides for inhibiting bone generation. In still a further aspect of the present invention, the present invention provides for a pharmaceutical composition that contains an effective amount of a modulator of the receptor of NELL1 or NELL2 peptides for promoting bone generation, e.g., craniofacial or long bone generation. The modulator can be an agonist of receptor of NELL1 or NELL2 peptides. In another embodiment, the pharmaceutical composition contains an effective amount of a modulator of the receptor of NELL1 or NELL2 peptides for promoting bone generation for treating or preventing a bone condition that decreases bone mass such as osteoporosis and alveolar ridge bone resorption. The modulator can be an agonist of receptor of NELL1 or NELL2 peptides.

In yet a further aspect of the present invention, the present invention provides a pharmaceutical composition that contains an effective amount of a modulator of the receptor of NELL1 or NELL2 peptides for inhibiting bone generation, e.g., craniofacial or long bone generation. The modulator can be an antagonist of receptor of NELL1 or NELL2 peptides. In one embodiment, the pharmaceutical composition contains an effective amount of a modulator of the receptor of NELL1 or NELL2 peptides for inhibiting bone generation for treating or preventing a bone condition that increases bone mass such as osteopetrosis. The modulator can be an antagonist of receptor of NELL1 or NELL2 peptides.

In a further aspect of the present invention, the present invention provides a pharmaceutical composition for bone generation that includes one or more enhancers for a NELL peptide.

In a further aspect of the present invention, the present invention provides a pharmaceutical composition that contains an effective amount of at least one agent for either directly or indirectly promoting the generation of cartilage for treating or preventing a cartilage related bone condition (e.g., joint resurfacing.
temporomandibular joint reconstruction, arthritis repair, or intervertebral disc repair). One of the agents for direct promotion of cartilage generation can be NELL peptides applied to chondrogenic cells such as, but not limited to, chondroblasts, chondrocytes, or chondroprogenitor cells, stem cells, bone marrow cells, a bone marrow stromal cells, a fibroblast, or adipose derived cells. The agent for indirect promotion of cartilage generation (e.g., through inducing chondroblast/chondrocyte differentiation) can be, e.g., one of NELL peptide, or agonists of NELL peptide receptors.

Under certain specific condition when inhibition of endochondral bone formation is desired to prevent further cartilage replacement by bone, the pharmaceutical composition can include, e.g., one or more inhibitors or antagonists of NELL peptide receptors, high dose NELL peptides, or combinations thereof. Such a composition is effective for inhibition of osteoblastic differentiation by inhibiting potential or committed osteogenic cells such as, but not limited to, osteoblasts, osteoprogenitor cells, stem cells, bone marrow cells, fibroblastic cells, dural cells, periosteal cells, pericytes, and/or muscle cells.

In a further aspect of the present invention, bone formation can be induced through small molecules regulating NELL promoter.

The above described pharmaceutical composition can optionally include a pharmaceutically acceptable carrier for a suitable mode of delivery for systemic or local delivery. For example, the pharmaceutically acceptable carrier can be a carrier for oral delivery, pulmonary delivery, parenteral delivery or implantation.

In a further aspect of the present invention, the present invention provides a method of treating or preventing bone conditions. The method generally includes administering to a mammal a pharmaceutical composition described herein.

The pharmaceutical composition can be formulated into various formulations for a suitable mode of delivery.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows schematic structures of rat NELL1 protein and mouse thrombospondin (TSP)-1. Signal peptide region (solid black box), TSP-N modules (TSP-N, shaded box), cysteine-rich (CR) domains (CR, solid white boxes), epidermal growth factor (EGF)-like domains (E, hatched boxes), coiled-coil
regions (CC, bars), Ca2+-binding type EGF-like domains (*), and RGD peptide domains (RGD, solid white box) are indicated.

Figure 2A shows the skeletal pattern of a wild-type mouse demonstrating normal skeletal pattern on skeletal staining (top, middle) and micro-computed tomography (CT) (bottom). Figure 2A also shows the typical borders of mineralization (dotted light blue line) as well as the location of the anterior (red asterisk) and posterior (blue asterisk) fontanelles. A faint outline of the right coronal suture (green arrows) can be seen. The coronal sutures are usually less visible because they are overlap rather than butt sutures. On the middle picture, note also the normal size of the clavicles (black arrows). The micro-CT reveals the typical craniofacial bone morphology. The coronal sutures (green arrows) and the anterior fontanelle (red asterisk) are highlighted. Figure 2B shows the skeletal pattern of a heterozygous core-binding factor \( \Delta1 \) knockout animal (\( Cbfal^{+/-} \)). Cbfal deficient animals have bone forming defects. These mice demonstrate widely patent midline sutures and fontanelles. Defective mineralization and bone formation is present in the poorly stained tissue (between yellow and light blue dotted lines) lateral to the midline calvarial defect. Lucency can also be seen in the area of the coronal suture (green arrows, top and bottom pictures). On the middle picture, note the significant degree of clavicular hypoplasia (black arrows). Figure 2C shows the skeletal pattern of progeny from \( Cbfal^{\Delta1} \) animals mated with NELL1 overexpressing animals (\( NELL1^{\text{overexp}} \)). The \( Cbfal^{\Delta1}, NELL1^{\text{overexp}} \) animal demonstrated significantly increased calvarial bone formation relative to the \( Cbfal^{\Delta1} \) haploid deficient animal on skeletal staining and micro-CT. On the middle picture, there is a lesser degree of clavicular hypoplasia (black arrows).

The figure also shows the restoration of bony overlap at the coronal sutures (green arrows, top and bottom pictures). Figure 2D shows calvarial bone overgrowth and ectopic bone formation in \( \text{ex vivo} \) calvarial bone organ culture when \( NELL1 \) is over-expressed or when the NELL1 protein was added. Figure 2E shows normal mouse calvarial explant with NELL1 protein added. Green fluorescent represents new bone growth. NELL1 protein induce bone over-growth (red arrows), and orthotopic bone formation (yellow arrow). Collectively, Figure 2 demonstrates increased bone growth in as a result of NELL1 overexpression in both craniofacial areas (e.g., calvaria) and axial skeletal areas (e.g., clavical).
Figure 3A shows Von Kossa’s staining of adenoviral NELL1 (AdNELL1) transduced bone marrow stromal cell (BMSCs) derived from long bones. Cells were cultured to 80% confluence and then infected with 50 plaque forming units (pfu)/cell AdNELL1 (right). Controls were infected with 50 pfu/cell Adß-Gal (left). At day 11, von Kossa’s stained bone nodules were counted and bone nodule numbers are presented at the mean ± SEM. Each experiment was performed in triplicate. Representative samples of stained bone nodules are indicated (green arrows). AdNELL1 transduced BMSCs had significantly more mineralization and bone formation. Figures 3B, 3C and 3D show transduced BMSCs injected into muscle of nude mice. Figure 3B shows histology of relatively more mature bone with lamellar pattern. Figure 3C shows AdNELL1 transduced BMSCs with more mature bone pattern radiographically. Figure 3D shows AdNELL1 transduced BMSCs with more bone formation radiographically. Collectively, Figures 3A-3D demonstrates increased bone mineralization and bone formation in as a result of NELL1 overexpression in cells derived from non-calvarial sources. In this case, NELL1 induced stem cells to form bone.

Figure 4 is volume analyses of NELL1 or BMP2 treated calvarial defects, showing significantly increased bone formation above control (blue line) for NELL1 (green line) and BMP2 (red line). This demonstrates that NELL1 can regenerate/repair bone.

Figures 5A-D are micro-CT images of treated calvaria at 4 weeks. Figure 5A represents a 4 week calvarial section treated with NELL1-loaded membrane (outline of original defect in green), and BMP2-loaded PLGA membrane (outline of original defect in red) (endocranial view). Figure 5B represents the same specimen as Figure 5A (exocranial view). Figure 5C represents a 4 week calvarial section treated with NELL1-loaded PLGA membrane (outline of original defect in green) (endocranial view). The contralateral untreated control is also shown (outline of original defect in yellow). Figure 5D represents the same specimen as Figure 5C (exocranial view). Bar scale: 3 mm (in yellow, bottom left).

Collectively, Figures 5A-5D demonstrates similarly increased bone mineralization and bone formation from NELL1 and BMP2 treatment.

Figures 6A-C show histology sections of treated calvaria at 4 weeks. Sections were stained using Masson’s trichome. Figure 6A represents a 4 week
calvarial section treated with NELL1-loaded membrane. Complete bone regeneration across the defect is seen. Figure 6B represents a 4 week calvarial section treated with BMP2-loaded membrane. There is also complete bone regeneration across the defect. Figure 6C represents a 4 week calvarial section treated with non-loaded membrane. There is minimal bone regeneration across the defect. Collectively, Figures 6A-C also demonstrates similarly increased bone formation from NELL1 and BMP2 treatment.

Figures 7A-D show NELL1 induce cartilage formation and endochondral bone formation under different microenvironment conditions. Figure 7A shows that NELL1 is expressed throughout the tibia including both articular cartilage region (Upper panel) and also the endochondral long bone formation region (lower panel). Upper panel demonstrate that NELL1 can modulate and increase cartilage differentiation in the articular cartilage region. Accordingly, these data show that increased NELL peptide activity directly (e.g., through addition of NELL peptides or increased NELL peptide expression) or indirectly (e.g., through addition of NELL peptide enhancers and/or NELL peptide receptor agonists and/or activators) promotes cartilage formation. In the lower panel, in the long bone shaft region where endochondral bone formation originated, increased NELL1 causes cartilage formation and then hypertrophy and increased endochondral bone formation, while absence of NELL1 allows maintenance of less differentiated articular chondroblast/chondrocyte phenotype without endochondral bone formation in the Cbfa1 knock out model. Accordingly, these data show that increased NELL peptide activity directly (e.g., through addition of NELL peptides or increased NELL peptide expression) or indirectly (e.g., through addition of NELL peptide enhancers and/or NELL peptide receptor agonists and/or activators) promotes cartilage formation, cartilage hypertrophy and endochondral ossification. It is useful in endochondral bone formation such as bone fracture. The absence of exogenously NELL1 associates with controlled articular chondroblast/chondrocyte phenotype and suppression of hypertrophy which is important to prevent articular cartilage replaced by bone. Accordingly, the inhibition of NELL peptide activity directly (through decreased NELL peptide expression or use of NELL peptide inhibitors) or indirectly (through NELL peptide receptor antagonists and/or inhibitors) can prevent cartilage hypertrophy and endochondral ossification and
promote maintenance of articular cartilage phenotype. Overall, these data not
intended to be limiting, but rather to show that NELL has broad effects on
osteochondroprogenitor cell types and that the exact phenotype induced by NELL
depends on a complex interplay between the amount and timing of NELL
application, the exact cell type, cell differentiation state, and the microenvironment.

Figure 7B shows that, in a palatal distraction model, NELL1 protein induce
cartilage to from (blue staining). Figure 7C shows that NELL1 increases
chondroblast proliferation indicated by increase Sox 9 staining. Sox 9 is the
marker for chondrogenic cell proliferation. Figure 7D shows NELL1 induces the
cartilage to further differentiate as indicated by increased type X collagen staining.
Again, Figure 7D demonstrates that NELL1 can accelerate cartilage
differentiation/formation and also cartilage based endochondral bone formation.
Collectively, Figures 7A-D demonstrate that NELL1 can modulate cartilage
differentiation and hypertrophy. Increased NELL1 causes cartilage formation and
hypertrophy and increased endochondral bone formation under different
microenvironment, while absent NELL1 allows maintenance of less articular
chondroblast/chondrocyte phenotype.

Figure 8 shows synergistic effect of NELL1 with BMP2 in vitro (A) and in
vivo (B, C). These data demonstrate that NELL1 and BMPs are synergistic in
inducing osteoblastic differentiation marker expression and in inducing bone
formation.

Figure 9 shows spinal fusion of NELL1 with demineralized bone matrix as
carrier. Radiographic and MicroCT three dimensional reconstruction images on 6-
week samples of Nell1 treated spine with fusion (A, B and C) and control samples
with nonunion (D, E and F). (A) The red arrows identify the radio-opaque tissue
masses on both side of spine at L4 and L5 segments. The medial edge (green
arrows) of each mass displayed the highest density similar to cortical bone; (B)
This microCT 3D image displayed a well defined tissue mass (red arrows) with
density similar to bone was packed on the dorsal surface of two transverse
processes and the spaces between them (green arrows); (C) The bridging bones
(green arrows) clearly connected with both transverse processes (yellow arrows) as
shown in this coronal cutting plane image of 3D microCT; (D) Smaller tissue mass
(red arrows) with lower radio-opaque seen in this radiograph; (E) Tissue mass (red
arrows) over the L4 and L5 region without close contact with transverse processes. In coronal cutting plane of 3D microCT, clefs (pink arrows) were identified. This data demonstrate that NELL1 can induce spinal fusion through one bridge formation.

Figure 10 show histology of 6-week samples of fusion by NELL1 (A, B, C, G and H) and nonunion with control (D, E, F, I and J). (A) Green arrows indicate cortical bone like bridging bone connecting two transverse processes denoted with dotted lines on H&E stained sections. (B and C) High power views of lamellar bones in defined area of the bridging bone from A. (D) H&E staining showed smaller bone mass close to a transverse process denoted with dotted line; (E and F) High power views of premature bones in defined area from D. (G and H) New bone growth as indicated with osteocytes forming cement lines on Masson trichrome staining section. (I and J) More cartilaginous tissues emerging from remodeling DBM particles (arrows). Original magnification for A and D: 9.8 X; B, E, G and I: 100X; C, F, H and J: 200X. This data demonstrate that NELL1 can induce spinal fusion through bone bridge formation.

Figures 11A and 11B show the human, mouse and rat NELL1 promoters contain multiple OSE2 consensus motifs. Figure 11A shows putative OSE2 binding sites, A, B and C are shown along with sequence and position relative to the transcription start site. Cryptic OSE2 site is depicted by striped box. Figure 11B is a comparative schematic of the human, mouse and rat NELL1 promoters (not drawn to scale). Two of the OSE2 sites in the mouse and rat promoter (sites m1 and 2, and sites r1 and 3, respectively) are located in a region that is 81% homologous. Cryptic sites are indicated by striped boxes. This data shows the sequence of the promoter of Nell can be used for drug screening to induce Nell expression.

DETAILED DESCRIPTION

In one aspect of the present invention, provided herein is a pharmaceutical composition containing one or more agents such as one or more NELL peptides to treat or prevent bone conditions. In one embodiment, the pharmaceutical composition contains an effective amount of one or more NELL peptides for modulating (e.g., promoting bone generation, e.g., craniofacial bone generation, dental implant integration, periodontal bone generation, dental or orthopedic
implant integration, long bone fracture healing, spinal fusion or combinations thereof. In another embodiment, the pharmaceutical composition contains an effective amount of one or more NELL peptides for treating or preventing a bone condition such as osteoporosis.

In another aspect of the present invention, the present invention provides a pharmaceutical composition that contains an effective amount of an inhibitor of NELL1 or NELL2 peptides for treating or preventing bony overgrowth across cranial sutures.

In still a further aspect of the present invention, the present invention provides for a pharmaceutical composition that contains an effective amount of a modulator of the receptor of NELL1 or NELL2 peptides for promoting bone generation, e.g., craniofacial bone generation. The modulator can be an agonist or antagonist of receptor of NELL1 or NELL2 peptides. The modulator can activate or inhibit the receptors by itself. In another embodiment, the pharmaceutical composition contains an effective amount of a modulator of the receptor of NELL1 or NELL2 peptides for treating or preventing a bone condition such as osteoporosis.

In a further aspect of the present invention, the present invention provides a pharmaceutical composition that contains an effective amount of at least agent for promoting the generation of cartilage for treating or preventing a cartilage related bone condition. The agent can be one of NELL peptide inhibitors, antagonists of NELL peptide receptors, and combinations thereof.

The above described pharmaceutical composition can optionally include a pharmaceutically acceptable carrier for a suitable mode of delivery for systemic or local delivery. For example, the pharmaceutically acceptable carrier can be a carrier for oral delivery, parenteral delivery or implantation.

In a further aspect of the present invention, the present invention provides a method of treating or preventing a bone condition. The method generally includes administering to a mammal a pharmaceutical composition described herein.

In a further aspect of the present invention, the composition described herein can be used to induce bone formation in conjuncture with a bone matrix. The bone matrix can be a demineralized bone matrix or mineralized bone matrix.

In a further aspect of the present invention, a pharmaceutical composition provided herein can be used to induce a stem cell to differentiate into osteoblast by
contact the stem cell with the composition. The stem cell can be an embryonic stem cell or an adult stem cell. Further, the pharmaceutical composition can be used to induce bone marrow stromal cell to form bone by contacting the bone marrow stromal cell with the composition described herein.

In a further aspect of the present invention, the composition described herein is effective and can be used to treat, prevent, ameliorate, mitigate, or reduce the symptoms of conditions related to, for example, bone loss due to microgravity, disuse atrophy, prolonged bed-rest, etc.

In some embodiments, the composition described herein is effective and can be used to treat, prevent, ameliorate, mitigate or reduce the symptoms of diseases/conditions that involve multiple symptoms where bone metabolism is a secondary effect. Examples of such diseases or conditions include, but are not limited to, chronic kidney diseases which can cause many systemic effects including renal osteodystrophy and vascular calcification. Nell can increase bone formation without stimulating undesirable bone formation, and thus it can stimulate the formation of bone only in bone compartments without stimulating proliferation of non-bone cells in the body (e.g. pre-cancerous cells), and as a result the targeted bone formation alleviates bone loss due to kidney damage. The NELL-induced mineralization also consumes the calcium and phosphate ions that otherwise form pathological calcification in normally non-calcifying tissues such as blood vessels. Other forms of pathological calcifications have multi-factorial origin (bacterial, paracrine, autocrine, etc.). The ability of Nell to favor the balance between bone deposition and bone resorption makes the composition described herein an effective composition to maintain the essential ions in the bone compartment and decrease their bioavailability in non-bone tissues, thereby reducing the risk for ectopic soft tissue calcification, gall stone, kidney stones, pineal gland calcification, cataracts, salivary stones, cardiac valves, and/or prostate stones.

In some embodiments, the present inventions provide a pharmaceutical composition for promoting bone formation in a mammalian cell. Examples of such a mammalian cell includes, but is not related to, a stem cell, a bone marrow stromal cell, a fibroblast, or an adipose derived cell.
As used herein, the term "NELL (Nel-like molecule-1; Nel (a protein strongly expressed in neural tissue encoding epidermal growth factor like domain)) peptides" can be NELL1 or NELL2 polypeptide, or a fragment thereof; a NELL1 or NELL2 related polypeptide, or a fragment thereof; any polypeptide with significant homology to "NELL peptides" or a fragment thereof. Significant homology can be construed to mean >50% homology to "NELL peptides", e.g., >60% homology to "NELL peptides", >70% homology to "NELL peptides," or >80% homology to "NELL peptides." The NELL peptides can be natural and/or recombinant NELL peptides with a non-mutated wild-type sequence or recombinant NELL peptides with a mutated wild-type sequence that still contains significant homology to NELL peptides. In addition, NELL peptides can be derived from, but not limited to, an organism such as human cells, bacteria, yeast, or insect or plant cells. In some embodiments, the term "NELL peptide" includes structural, functional or conformational equivalents of NELL peptide. As used herein, a structural equivalent of a NELL peptide refers to a protein or peptide including a structure equivalent or substantially similar to that of a NELL peptide or of a functional domain of a NELL peptide. A functional equivalent of a NELL peptide refers to a protein or peptide having a function equivalent or substantially similar to that of a NELL peptide or of a functional domain of a NELL peptide. A conformational equivalent of a NELL peptide refers to a protein or peptide having a conformation equivalent or substantially similar to that of a NELL peptide or of a functional domain of a NELL peptide.

In some embodiments, the NELL peptide described herein can be a derivative of the NELL peptide. The term "derivative" as used herein, refers to any chemical or biological compounds or materials derived from a NELL peptide, structural equivalents thereof, or conformational equivalents thereof. For example, such a derivative can include any pro-drug form, PEGylated form, or any other form of a NELL peptide that renders the NELL peptide more stable or to have a better osteo philicity or lipophilicity. In some embodiments, the derivative can be a NELL peptide attached to poly(ethylene glycol), a poly(amine acid), a hydrocarbyl short chain having C1-C20 carbons, or a biocompatible polymer. In some embodiments, the term "derivative" can include a NELL peptide mimetics. Synthesis of mimetics of a peptide is well document in the art. The following
describes an example of the basic procedure for the synthesis of a peptide, including a peptide mimetics:

Before the peptide synthesis starts, the amine terminus of the amino acid (starting material) can protected with FMOC (9-fluoromethyl carbamate) or other protective groups, and a solid support such as a Merrifield resin (free amines) is used as an initiator. Then, step (1) through step (3) reactions are performed and repeated until the desired peptide is obtained: (1) a free-amine is reacted with carboxyl terminus using carbodiimide chemistry, (2) the amino acid sequence is purified, and (3) the protecting group, e.g., the FMOC protecting group, is removed under mildly acidic conditions to yield a free amine. The peptide can then be cleaved from the resin to yield a free standing peptide or peptide mimetics.

In some embodiments, the peptide derivative described herein includes a physically or chemically modified NELL peptide. Physically modified peptide can be modification by, for example, modification by ionic force such as forming an ionic pair with a counterion, modification by hydrogen bonding, modification by modulation of pH, modulation by solvent selection, or modification by using different protein folding/unfolding procedures, which can involve selection of folding/unfolding temperature, pH, solvent, and duration at different stage of folding/unfolding.

In some embodiments, the peptide derivative can include a chemically modified NELL peptide. For example, a short hydrocarbon group(s) (e.g. methyl or ethyl) can be selectively attached to one or multiple sites on the NELL peptide molecule to modify the chemical and/or physical properties of the peptide. In some embodiments, a mono-, oligo- or poly(ethylene glycol) (PEG) group(s) can be selectively attached to one or multiple sites on the NELL peptide molecule to modify the chemical and/or physical properties of the peptide by commonly known protein PEGylation procedures (see, e.g., Mok, H., et al., Mol. Ther., 11(1):66-79 (2005)).

The term “inhibitor of NELL peptides” refers to a chemical or biological compound capable of inhibiting the activity of NELL peptides. The term also includes a chemical or biological compound capable of suppressing the expression of NELL peptides. Inhibitors of NELL peptides can interact directly or indirectly with NELL peptide transcripts or translational products. As examples, methods of
interactions can include but are not limited to decreased transcription or translation of NELL peptides, decreased stability of NELL peptide transcripts or protein products, decreased activity of NELL peptide transcripts or protein products, and increased degradation of NELL peptide transcript or protein products. The term "enhancer of NELL peptides" refers to a chemical or biological compound capable of enhancing the activity of NELL peptides. The term also includes a chemical or biological compound capable of enhancing the expression of NELL peptides. As examples, methods of interactions can include but are not limited to increased transcription or translation of NELL peptides, increased stability of NELL peptide transcripts or protein products, increased activity of NELL peptide transcripts or protein products, and decreased degradation of NELL peptide transcript or protein products.

The term "modulator of NELL peptide receptors" refers to a chemical or biological compound capable of facilitating or inhibiting the binding of NELL peptide receptors to or by NELL peptides or to a chemical or biological compound capable of modulating NELL peptide receptor activity irrespective of the presence or the absence of NELL peptide. The modulator that facilitates the binding and/or activation of NELL peptide receptors to or by NELL peptides is referred to as an "agonist" of the receptor, and the modulator that inhibits the binding and/or activation of NELL peptide receptors to or by NELL peptides is referred to as an "antagonist" of the receptor. The modulator that facilitates the activation of NELL peptide receptors irrespective of NELL peptides is referred to as an "activator" of the receptor, and the modulator that inhibits activation of NELL peptide receptors irrespective of NELL peptides is referred to as an "inhibitor" of the receptor.

The term "NELL peptide", "inhibitor of NELL peptide" or "modulator of NELL peptide receptor(s)" is also referred to as an "agent" throughout the specification.

The term "bone conditions" can involve, but are not limited to: 1) modulation of bone healing and regeneration by increasing or decreasing bone formation such as after accidental or iatrogenic orthopedic injury [e.g., from trauma (e.g., long bone fractures) (see Figures 7B-D), or surgery (e.g., spinal fusion)] see Figures 2, 9 and 10) modulation of bone mass by increasing or decreasing bone formation without evidence of overt orthopedic injury [e.g., hip
osteonecrosis, osteoporosis (decreased bone mass), osteopetrosis (increased bone mass); 3) modulation of bone healing and regeneration by increasing or decreasing bone formation such after accidental or iatrogenic craniofacial bone and/or periodontal injury [e.g., from trauma (e.g., craniofacial fractures), surgery (e.g., cleft lip/palate repair, cranial defect repairs) (see Figures 4, 5, and 6) or dental procedures (e.g., tooth extraction, dental implant placement)]; 4) modulation of bone mass by increasing or decreasing bone formation without evidence of overt craniofacial bone and/or periodontal injury (e.g., restoration and/or preservation of maxillary and mandibular alveolar dental ridges; inhibition of premature calvarial overgrowth across sutures); 5) modulation of bone healing and regeneration by increasing bone formation at sites of hardware implantation to facilitate osseous integration (e.g., total knee implants, dental implants, spinal implants) 6) modulation of cartilage healing and regeneration by modulating hypertrophic cartilage formation (e.g., prevent cartilage hypertrophy in bone conditions where non-hypertrophied cartilage is desirable such as intraarticular fractures causing severe joint injury, severe osteoarthritis or rheumatoid arthritis with progressive joint surface loss; promote cartilage hypertrophy in bone conditions where hypertrophied cartilage is desirable such as acceleration of endochondral ossification; prevent cartilage hypertrophy in bone conditions where hypertrophied cartilage is not desirable such as intramembranous ossification).

The term “stem cells” can involve, but are not limited to adult stem cells, fetal stem cells, embryonic stem cells, mesenchymal stem cells, and bone marrow stem cells.

**Osteoblast formation and function**

Osteoblast formation and function encompass two important aspects of bone biology (Aubin, J.E., Rev Endocr Metab Disord, 2001. 2(1): p. 81-94; Ducy, P., et al., Genes Dev, 1999. 13(8): p. 1025-36). Both concepts are central to osteoinduction and bone regeneration. According to Aubin (Aubin, 2001), osteoblast formation involves several differentiation stages consisting of initial mesenchymal stem cell (MSC) commitment to an osteoprogenitor lineage with eventual differentiation into osteoblasts and finally osteocytes and apoptotic cells. Osteoblast function, on the other hand, involves the activity of already differentiated osteoblasts in matrix deposition and bone formation. Bone
formation, which requires both osteoblast formation and function, can occur during embryonic development, growth, remodeling, fracture repair, and experimentally by implanting decalcified bone matrix or adding purified BMP (I.d.). Thus, osteoblast differentiation and function are two, but not necessarily distinct processes in so far as proper osteoblast function can only occur within the context of proper osteoblast differentiation.


<table>
<thead>
<tr>
<th>Graft Type</th>
<th>Description</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autograft</td>
<td>Bone graft taken from the patient</td>
<td>1. Second surgical site</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Prolonged anesthesia time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Short and long term donor site morbidity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Limited supply</td>
</tr>
<tr>
<td>Allograft based</td>
<td>Cadaveric bone graft. Can be deproteinized or demineralized.</td>
<td>1. Risk for infection, disease transmission</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Limited osteoinductive ability (in demineralized grafts only)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Limited supply</td>
</tr>
<tr>
<td>Xenograft based</td>
<td>Deproteinized (but not demineralized) bone graft from non-human species (i.e., BioOss- a bovine graft)</td>
<td>1. No osteoinductive ability</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Somewhat limited supply</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Risk for infection, disease transmission</td>
</tr>
<tr>
<td>Cell based</td>
<td>Seed patient's own cells into porous scaffolds</td>
<td>1. Second surgical site</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. May require additional culture time and manipulation</td>
</tr>
<tr>
<td>Ceramic based</td>
<td>Examples include calcium phosphate, calcium sulfate, and bioglass</td>
<td>Only minor osteoinductive ability</td>
</tr>
<tr>
<td>Polymer based</td>
<td>Both degradable and nondegradable polymers</td>
<td>No osteoinductive ability</td>
</tr>
<tr>
<td>Growth Factor based</td>
<td>BMPs, non-BMPs (e.g., FGF, TGF-β, IGF, VEGF, PDGF, PTH/PTHr)*, and gene therapy</td>
<td>1. Need appropriate delivery or osteoconductive vehicle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Pleiotropic effects on multiple cells types</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Unpredictable <em>in vivo</em> osteoinductive effects (i.e., for many, specificity depends on carrier)</td>
</tr>
</tbody>
</table>

*Abbreviations: FGF (fibroblast growth factors), TGF-β (transforming growth factor-β),
The osteoinductive properties of Cbfa1 have also been studied. Bone marrow stromal cells (BMSCs) transduced with an adenoviral \textit{Cbfa1 (Ad Cbfa1)} demonstrated increased mineralization \textit{in vitro} and increased bone formation \textit{in vivo} when the transduced C3H10T1/2 cells were subcutaneously implanted into immunodeficient mice (Franceschi, R.T., et al., Cells Tissues Organs, 2004. 176(1-3): p. 95-108; Yang, S., et al., J Bone Miner Res, 2003. 18(4): p. 705-15). These results show that the responsiveness of osteoprogenitor cell populations to BMPs can be enhanced \textit{in vitro} and \textit{in vivo} by Cbfa1, a major regulator of the osteoprogenitor lineage (Franceschi, R.T., et al., Cells Tissues Organs, 2004. 176(1-3): p. 95-108).

\textbf{NELL peptides as downstream targets of Cbfa1}

NELL peptides can be downstream targets of Cbfa1 (Kuroda, S., et al., Biochem. Biophys Res Commun, 1999. 265:79-86; Ting, K., et al., J Bone Miner Res, 1999. 14:80-89). Cbfa1 is known to promote transcription of many downstream osteoblastic genes such as \textit{al type I collagen (Col1-\alpha)}, \textit{Bone sialoprotein (Bsp)}, \textit{Osteopontin (Op)}, and \textit{Oc} by binding to the osteoblast-specific \textit{cis}-acting element 2 (OSE2) response elements in their promoter regions (Ducy, P., et al., Genes Dev, 1999. 13(8): p. 1025-36). Studies have shown the presence of three functional OSE2 response elements on the human \textit{NELL1} gene, confirming that \textit{NELL1} is a Cbfa1 regulated gene (see Figure 11).

\textbf{NELL1 was first noted to associate with bone formation when adenoviral \textit{NELL1} overexpression \textit{in vitro} significantly increased differentiation and mineralization \textit{selectively} in osteoblastic cells, but not in non-osteoblastic cells such as NIH3T3 fibroblasts (Zhang, X., et al., J Clin Invest, 2002. 110(6): p. 861-}
and when NELL1 overexpression in vivo significantly increased premature bone formation and bony calvarial overgrowth across cranial sutures of transgenic animals. As stated in the previous paragraph, NELL1 expression is downstream of and directly regulated by Cbfa1/Runx2, a critical mediator of osteoblast formation and function, indicating that NELL1 can act more specifically or preferentially on further differentiated osteogenic lineage cells (i.e., committed osteoblasts).


In vivo, endogenous NELL1 expression has been identified to correspond temporally and spatially with advancing osteogenic fronts of fusing sutures. Transgenic NELL1 overexpression mice also demonstrated pathological bony calvarial overgrowth across cranial sutures (Zhang, X., et al., J Clin Invest, 2002. 110(6): p. 861-70).

NELL1 as a downstream mediator of Cbfa1 in osteoblast differentiation and function is further established by functional compensation of some aspects of Cbfa1 deficiency by NELL1. In one study, F2 progeny from intercrossed NELL1 overexpression mice (NELL1overexp) and Cbfa1-/- mice were examined. Minimal rescue of the osteoblastic phenotype was observed in NELL1overexp+Cbfa1-/- mice, which presumably lack committed osteoblasts. In addition, NELL1overexp+Cbfa1-/- mice demonstrated increased chondrocyte hypertrophy (see Figure 7A) indicating that NELL1 is also important processes related to endochondral ossification. Nine
of the eleven NELL1\textsuperscript{overexp} Chfai\textsuperscript{+/+} mice, which should contain committed, but imperfectly functioning osteoblasts, showed definitive rescue from the usual CCD-like phenotype (Otto, F., et al., Cell, 1997. 89(5): p. 765-71). Alizarin red and Alcian blue staining along with micro-CT analyses confirmed that fontanelle size and suture width were considerably smaller along with less hypoplastic clavicles in the NELL1\textsuperscript{overexp} Chfai\textsuperscript{+/+} mice compared to the non-rescued Chfai\textsuperscript{+/+} mice (Figure 2).

The studies have shown, among others, that: 1) Chfai upregulates NELL1 expression; 2) NELL1 overexpression selectively increases osteoblastic-type differentiation (i.e., increased ALP activity, OP and OC expression) in susceptible cell types; 3) NELL1 overexpression acts on further differentiated osteogenic lineage cells (i.e., committed osteoblasts); and 4) NELL1 overexpression increases bony overgrowth across cranial sutures; 5) NELL1 overexpression can functionally compensate for some aspects of Chfai deficiency; and 6) NELL1 overexpression selectively increases processes associated with endochondral bone formation (e.g., chondrocyte hypertrophy).

NELL peptides are also effective for non-craniofacial bone generation. For example, the in vitro effects of transduced AdNELL1 on bone marrow stromal cells (BMSC) isolated from long bones and the in vivo effects of AdNELL1 injection into nude mice were investigated. This study demonstrated that AdNELL1 transduced BMSC showed significantly increased mineralized bone nodule formation above Adβ-Galactosidase (Adβ-Gal) controls (Figure 3), while AdNELL1 injection resulted in ectopic calcified nodule formation in muscle, showing that NELL1 can enhance non-craniofacial osteoblast differentiation and bone formation.

Furthermore, NELL peptides can also up-regulate osteoblast differentiation markers and work synergistically with a BMP protein, a TGFβ protein, FGF, IGF (insulin like growth factors), VEGF, or a combination thereof to increase expression of bone differentiation markers in vitro (e.g., ALP, OC) and bone formation in vivo (see Figure 8). For example, NELL2 and BMP2 can be synergistic in inducing osteoblast differentiation. Examples as shown in Figure 8 demonstrate that NELL peptides, such as NELL2 and NELL1, can modulate osteoblast differentiation to promote bone formation.
Accordingly, in some embodiments, the present invention provides a method for identifying a molecule that induces expression of a NELL peptide. The method includes: (1) contacting a NELL1 promoter gene with a test compound, (2) detecting the level of expression of the NELL1 promoter gene, (3) comparing the level of expression of the NELL1 promoter gene to the level of expression of the NELL1 promoter gene without the test compound, and (4) designating the test compound as a modulator of the expression of the NELL peptide if the level of expression of the NELL1 promoter gene with the test compound is different from the level of expression of the NELL1 promoter gene without the test compound. In some embodiments, the method step further comprises: (5) designating the modulator as an inhibitor of the expression of the NELL peptide if the level of expression of the NELL1 promoter gene with the test compound is lower than the level of expression of the NELL1 promoter gene without the test compound, or (6) designating the modulator as an enhancer of the expression of the NELL peptide if the level of expression of the NELL1 promoter gene with the test compound is higher than the level of expression of the NELL1 promoter gene without the test compound. A modulator identified according to the method can be used to modulate the expression of a NELL peptide in a mammal.

**Systems expressing NELL peptides**

A NELL1 peptide is a protein which can be expressed by the NELL1 gene or cDNA or RNA or any fragments thereof. Such NELL1 gene, cDNA, RNA or fragments thereof includes SEQ ID NO: 1-11, which encode human NELL1 peptide or a fragment thereof, SEQ ID NO: 17-71, which encode mouse NELL1 peptide or a fragment thereof, and SEQ ID NO: 75 and 76, which encode rat NELL1 peptide or a fragment thereof. The NELL1 peptide can include a NELL1 peptide fragment that retains the ability to induce osteogenic cell differentiation, osteoblast differentiation bone formation, or cartilage regeneration.

A NELL2 peptide is a protein which can be expressed by the NELL2 gene, cDNA or RNA or any fragments thereof. Such NELL2 gene, cDNA or RNA or any fragments thereof includes SEQ ID NO: 12-16, which encode human NELL2 peptide or a fragment thereof, SEQ ID NO: 72-74, which encode mouse NELL2 peptide or a fragment thereof, and SEQ ID NO: 77-81, which encode rat NELL2
peptide or a fragment thereof. The NELL2 peptide can include NELL2 peptide
fragments that retain similar activity to the NELL2 peptide described herein.

The NELL1 or NELL2 peptide can be expressed in a nucleic acid construct
that includes any of the above described NELL1 or NELL2 genes. In one
embodiment, the invention includes a method of expressing a functional NELL
peptide, such as NELL1 or NELL2 peptide, using an insect cell line. In one
embodiment, the insect cell can be a high five cell, Sf9 and other Sf cells.

In one embodiment, the method can include providing a nucleic acid
sequence encoding a NELL1 or NELL2 peptide described herein. The nucleic acid
sequence can also include sequences such as those with substantial sequence
similarity, such as sequences having at least about 75% sequence similarity with
any portion of the sequences listed above.

In some embodiments, the nucleic acid can include an expression vector for
expressing the nucleic acid sequence encoding a NELL peptide, such as NELL1 or
NELL2 peptide. For example, the expression vector can be pLZT/V5-His
(Invitrogen), and selective markers can also include blastcidin and neomycin.

In some embodiments, the nucleic acid sequence can also include
additional nucleic acids which encode reporter products to monitor levels of gene
expression, or encode peptide tags which can be visualized using known methods
in the art to monitor levels of peptide expression. Additional sequences can be
selected so as to not interfere with the expression of the nucleic acid, or the
functionality of the expressed peptide product.

The nucleic acid construct can include a nucleic acid sequence encoding a
signal peptide. Such a signal peptide can be any NELL signal peptide. Some
examples of such signal peptide human, rat, mouse or dog NELL signal peptides.
Some other examples of NELL signal peptides include, but are not limited, human
NELL2 signal peptide SEQ ID NO: 89, which is encoded by nucleic acid SEQ ID
NO: 88, rat NELL2 signal peptide SEQ ID NO: 91, which is encoded by nucleic
acid SEQ ID NO: 90, mouse NELL2 signal peptide SEQ ID NO: 93, which is
encoded by nucleic acid SEQ ID NO: 92, and dog NELL2 signal peptide SEQ ID
NO: 95, which is encoded by nucleic acid SEQ ID NO: 94. The nucleic acid can
include an expression vector for expressing the nucleic acid sequence encoding a
NELL peptide. Further, the nucleic acid sequence can include additional nucleic
acids which encode reporter products to monitor levels of gene expression, or encode peptide tags which can be visualized using known methods in the art to monitor levels of peptide expression.

Nucleic acid constructs can comprise expression and cloning vectors should containing a selection gene, also termed a selectable marker, such as a gene that encodes a protein necessary for the survival or growth of a host cell transformed with the vector. The presence of this gene ensures that any host cell which deletes the vector will not obtain an advantage in growth or reproduction over transformed hosts. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate or tetracycline, (b) complement auxotrophic deficiencies.

Nucleic acid constructs can also include a promoter which is recognized by the host organism and is operably linked to the NELL encoding nucleic acid. Promoters are untranslated sequences located upstream from the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of nucleic acid under their control, including inducible and constitutive promoters. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. Some examples of NELL1 promoter nucleic acid sequence include, but are not limited to, SEQ ID NO: 82, 84, and 86, which encode a human NELL1 promoter, a mouse NELL1 promoter, and a rat NELL1 promoter, respectively. Some examples of NELL2 promoter nucleic acid sequence include, but are not limited to, SEQ ID NO: 83, 85, and 87, which encode a human NELL2 promoter, a mouse NELL2 promoter, and a rat NELL2 promoter, respectively.

A nucleic acid can be operably linked when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein which participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the

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transcription of the sequence; or a ribosome binding site is operably linked to a
coding sequence if it is positioned so as to facilitate translation.

In one embodiment, the invention can include a nucleic acid construct for
expressing a NELL peptide, such as NELL1 and/or NELL2 peptide in a
mammalian cell such as a Chinese hamster ovary cell (CHO cell). The nucleic
acid sequence can be a cDNA, genomic DNA, or RNA, encoding at least a
functional portion of a NELL peptide. For example, the nucleic acid sequence can
include a NELL1 or NELL2 gene described above. In some embodiments, the
nucleic acid sequence can also include sequences such as those with substantial
sequence similarity, such as sequences having at least about 75% sequence
similarity with any portion of the sequences listed above.

In some embodiments, for production of NELL1 and/or NELL2 peptides in
mammalian cells (e.g., CHO cells), the expressing system for NELL1 and/or
NELL2 can include the nucleic acid or cDNA that expresses the endogenous signal
peptide. In some embodiments, the expressing system for NELL1 and/or NELL2
peptides can include the nucleic acid or cDNA that expresses NELL2 signal
peptide. The incorporation of the NELL2 signal nucleic acid or cDNA into the
system expressing NELL1 peptide allows the production of the NELL1 peptide
more efficiently.

In one embodiment, the invention can include cells that express functional
NELL peptides. In one embodiment, the cell can be an insect cell. In one
embodiment, the insect cell can be a high five cell.

In one embodiment, the cell can be transfected with a nucleic acid construct
encoding a NELL peptide. For example, the cell line can be transfected transiently
or stably with the nucleic acid construct encoding a NELL peptide. In one
embodiment, NELL expressing nucleic acids (e.g., cDNA(s)) can be cloned into
gene expression vector or viral particles that are competent to transfect cells (such
as insect cells or Chinese hamster ovary cells (CHO cells)). In some embodiments,
the construct can include a vector such as pTB701 using a signal peptide, which
can be any of Preprotrypsin, human tPA, immunoglobulin light chain of Ig, and Fc
fragment, interleukin. The pTB701 vector was reported in Kuroda et al.,
The nucleic acid sequence can also include a nucleic acid sequence encoding a NELL peptide, such as NELL1 or NELL2 peptide, in frame with a nucleic acid sequence encoding an insect secretory signal peptide.

In one embodiment, the invention can include cells that express functional NELL peptides, and can secrete functional proteins. In one embodiment, the invention can include a polypeptide (amino acid sequence) comprising a NELL peptide, such as NELL1 or NELL2 peptide, and can include secretory signal peptide.

In some embodiments, gene sequences expressing NELL peptide or proteins can include any gene sequences that express the whole NELL molecule or a fragment thereof. Such gene sequences can optionally include noncoding sequences. Generally, genome sequences can be roughly classified as: (1) genome sequences that code for functional proteins (this can include different mRNA splice variants), (2) noncoding, non-transcribed genome sequences that may modulate expression of functional proteins (promoter regions, other non-transcribed DNA regions, etc), and (3) noncoding, transcribed genome sequences that may modulate expression of functional proteins (e.g., tRNA and rRNA, but also introns, 5' and 3'-UTR, transposable elements, intergenic regions and interestingly thousands of different small stable RNA with or without antisense capabilities.

Each DNA molecule contains many genes — the basic physical and functional units of heredity. A gene is a specific sequence of nucleotide bases, whose sequences carry the information required for constructing proteins, which provide the structural components of cells and tissues as well as enzymes for essential biochemical reactions. The human genome is estimated to comprise more than 30,000 genes.

Human genes vary widely in length, often extending over thousands of bases, but only about 10% of the genome is known to include the protein-coding sequences (exons) of genes. Interspersed within many genes are intron sequences, which have no coding function. The balance of the genome is thought to consist of other noncoding regions (such as control sequences and intergenic regions), whose functions are obscure. All living organisms are composed largely of proteins; humans can synthesize at least 100,000 different kinds. Proteins are large, complex molecules made up of long chains of subunits called amino acids. Twenty different
kinds of amino acids are usually found in proteins. Within the gene, each specific sequence of three DNA bases (codons) directs the cell's protein-synthesizing machinery to add specific amino acids. For example, the base sequence ATG codes for the amino acid methionine. Since 3 bases code for 1 amino acid, the protein coded by an average-sized gene (3000 bp) will contain 1000 amino acids. The genetic code is thus a series of codons that specify which amino acids are required to make up specific proteins.

The protein-coding instructions from the genes are transmitted indirectly through messenger ribonucleic acid (mRNA), a transient intermediary molecule similar to a single strand of DNA. For the information within a gene to be expressed, a complementary RNA strand is produced (a process called transcription) from the DNA template in the nucleus. This mRNA is moved from the nucleus to the cellular cytoplasm, where it serves as the template for protein synthesis. The cell's protein-synthesizing machinery then translates the codons into a string of amino acids that will constitute the protein molecule for which it codes. In the laboratory, the mRNA molecule can be isolated and used as a template to synthesize a complementary DNA (cDNA) strand, which can then be used to locate the corresponding genes on a chromosome map.

In some embodiments, the composition described can be stabilized by binding with other chemicals or by incorporating in nanocage or biomaterial until successful delivery. There are more noncoding regions than coding regions in humans. The noncoding part of genomes plays an important regulatory role. At least half of the human genome is transcribed. Around 95% of this transcriptional output is non-coding RNA (ncRNA) encompassing not only tRNA and rRNA, but also introns, 5' and 3'-UTR, transposable elements, intergenic regions and interestingly thousands of different small stable RNA. The nanocage or biomaterial can be a carrier or scaffold described below.

A number of these transcribed regions are evolutionarily conserved between human and rodents (up to 95% conservation between man and mouse), suggesting preserved functions. An essential characteristic of a wide fraction of these noncoding RNA is their antisense capabilities: they can target another RNA through more or less extended base pairing complementarities. This has been demonstrated for snoRNA and miRNA. ncRNA are fulfilling some unexpected
functions. They play an important role in regulating cellular processes including development, heterochromatin formation, transcription, alternative splicing and editing, chemical modification of nucleic acids and genomic stability in eukaryotes. While most ncRNA with precisely described functions are ubiquitous, most newly identified ncRNA have been found to be developmentally regulated, i.e., expressed in a gender-, tissue- or cell-specific manner. Among the antisense ncRNA, a large family is rapidly emerging: the micro-RNA (miRNA). They are highly conserved among higher organisms, are involved in temporal cell lineage decision and tissue-specific gene regulation and regulate various developmental and physiological processes. Their common mode of action is to target mRNA for destruction or inhibition of translation.

Inhibitors of NELL Peptide

In one aspect of the present invention, the pharmaceutical composition disclosed herein can include an agent that inhibits the activity of a NELL peptide for treating, preventing or ameliorating a bone condition associated with premature or excessive bone generation. The agent can be, but not limited to, a NELL1 inhibitor or NELL2 inhibitor or a combination thereof. The term “inhibitor of NELL peptides” has been previously described in the Summary section.

Any assay methods of screening for an inhibitor of a bioactive compound such as a protein can be used to screen for inhibitors of NELL peptides. Some assay methods are described in PCT/2003/029281 (WO 2004/024893).

Representative NELL1 or NELL2 inhibitors include any agents that can specifically inhibit NELL1 or NELL2 at the transcriptional stage (e.g., Cbfa1 specific siRNA, antibodies, since NELL1 or NELL2 contains Cbfa1 binding sites in the promoter) and/or translational stage (e.g., NELL -1 specific siRNA, NELL2 specific siRNA, or receptors binding NELL1 or NELL2 such as NELL -1 or NELL2 specific antibodies).

Enhancers of NELL peptides

In another embodiment, it is provided a pharmaceutical composition that includes one or more enhancers of NELL peptides.

Modulators of receptors of NELL Peptides

In a further aspect of the present invention, the pharmaceutical composition provided herein can include a modulator of a receptor of NELL peptide. NELL1

Modulators of the receptors of NELL peptides can be identified by any established method for screening for modulators of a receptor. In one embodiment, the modulators of the receptors of NELL peptides can be screened for by competitive binding. For example, one method of screening for such modulators can include the following steps: (1) contacting a receptor molecule of a NELL peptide with a test compound, (2) contacting the NELL peptide with the receptor molecule and the test compound, (3) detecting the extent of binding of the NELL peptide to the receptor molecule with the test compound, (4) comparing the extent of binding of the NELL peptide to the receptor molecule with the test compound with the extent of binding of a control wherein the control is obtained by detecting the extent of binding of the NELL peptide to the receptor molecule without the test compound, and (5) designating the test compound as a modulator of the receptor of the NELL peptide if the extent of binding of the NELL peptide to the receptor molecule with the test compound is different from the extent of binding of the control. The modulators can be designated as an antagonist or an agonist of the receptor. If the extent of binding of the NELL peptide to the receptor molecule with the test compound is lower than the extent of binding of the control, the modulator is an antagonist of the receptor of the NELL peptide. If the extent of binding of the NELL peptide to the receptor molecule with the test compound is higher than the extent of binding of the control, the modulator is designated as an agonist of the receptor of the NELL peptide.

In some embodiments, the NELL modulators described herein can include molecules that stabilize or degrade NELL and/or NELL receptors, as well as molecules that are involved in the stabilization and phosphorylation of the NELL-receptor complex after initial receptor ligation. In some embodiments, the modulators described herein can include agonists and antagonists of the aforementioned agonists and antagonists. For example, a composition including inhibitors of NELL-agonists can increase bone metabolism. In all cases, please expand the clinical applications to include those discussed in previous paragraph.
Modulators of a receptor of a NELL peptide can be screened for by manual testing or by an automated system such as a system based on combinatorial chemistry. One example of the screening system based on combinatorial chemistry is described in PCT/2003/029281 (WO 2004/024893).

5 Cartilage regeneration

Articular cartilage is comprised of mostly water (60-80 wt%) and the remaining ECM comprises mostly type II collagen (50-90% dry mass) and proteoglycans (5-10%). Other collagens and minor ECM molecules have been identified in small quantities. It is organization of the ECM into distinct zones, and the interaction between water and the ECM in the various zones that provide the toughness that is required for the absorption and transmission of biomechanical forces across joints, and simultaneously the frictionless articulating surfaces that are needed for joint motion. Stresses as high as 4 and 20 MPa have been reported in human hip joints during routine walking and jumping, respectively! As amazing as the articular cartilage is, it exhibits unfortunately minimal capacity for repair.


Accordingly, in a further aspect of the present invention, the pharmaceutical composition provided herein includes at least a NELL peptide or an agonist of the receptor of NELL peptides in an amount effective for inducing chondroblast and chondrocyte to form cartilage. NELL proteins, peptides, DNA, RNA, and NELL agonists, and antagonist inhibitors can be used alone or in conjunction with scaffolds with and without cells, with or without mechanical stimulation, in the presence or absence of additional growth factors. For example,
in one embodiment, the pharmaceutical composition can be effective in regenerate cartilage in intervertebral disc, articular cartilage repair and regeneration. In another embodiment, the pharmaceutical composition can be effective in forming cartilage via ex vivo gene therapy and protein application to cells with or without scaffold in tissue engineering.

Depending on the delivery method and the local environment, a composition including a NELL peptide (e.g., a NELL1 peptide) can be used to induce an osteogenic cell, as such as a chondrocyte or chondroblast, to differentiate and form cartilage only. For example, in an articular cartilage defect, the composition described herein can induce an chondrogenic cell such as chondrocyte/blast to form cartilage only. The composition can be applied to the defected cartilage area as a scaffold/carrier. In some embodiments, the composition can optionally include cells (stem cells, chondroblast etc). In some embodiments, the composition can be applied as gene therapy.

In some yet embodiments, the composition can be used in cartilage tissue engineering. For example, when chondroblasts are cultured on an "oscillating", intermittent stress tension environment, NELL1 peptide can include the chondroblast cells to differentiate and form cartilage. In these embodiments, the duration of application of the oscillating stress also plays an important role. For example, if the oscillating force is applied continuously, the composition having a NELL1 peptide can induce endochondral bone formation. Therefore, in the application of the oscillating stress shall be intermittently such that the differentiation of an osteogenic cell (e.g., chondrocyte/blast) can stop at the cartilage stage and thus prevent the cell from differentiating into endochondral bone formation.

Therefore, in some embodiments, the composition described herein can be used to regenerate/repair cartilage, e.g., for disc repair in articular cartilage and intervertebral disc.

Other exemplary cartilage conditions that can be treated, prevented, or ameliorated by a pharmaceutical composition disclosed herein include, but are not limited to, chondrocalcinosis, osteoarthritis, and/or other diseases characterized by pathological cartilage degeneration.

Other Agents

In one embodiment, the pharmaceutical composition contains a NELL1 peptide and a BMP peptide. As an example, a human osteosarcoma cell line, Saos-2 (McQuillan, D.J., et al., Bone, 1995. 16(4): p. 415-26; Fedde, K.N., Bone Miner, 1992. 17(2): p. 145-51), is cultured with recombinant NELL1 and BMP2 proteins at 100 ng/ml and 200 ng/ml, respectively. The test results demonstrated up to 5-fold increase in ALP activity in combined NELL1/BMP2 cultures relative to
BMP2 cultures, showing that NELL1 can enhance the responsiveness of osteoblast-like cell populations to BMPs.

In some embodiments, the composition described herein can optionally include a LIM protein.

In some embodiments, the composition described herein can specifically exclude one or more the above described agents.

**Dosages**

Dosages of NELL peptides and other agents can be determined according to methods known in the art based on type of agent, the disease, and other factors such as age and gender.

In one embodiment, the dosage of NELL peptide for bone formation generally ranges from 0.001 pg/mm² to 1 pg/mm², or more preferably from 0.001 ng/mm² to 1 ng/mm², or more preferably from 0.001 μg/mm² to 1 μg/mm², or more preferably from 0.001 mg/mm² to 1 mg/mm², or more preferably from 0.001 g/mm² to 1 g/mm², with or without a particular carrier or scaffold. In another embodiment, the dosage of NELL peptide for bone formation generally ranges from 0.001 pg/ml to 1 pg/ml, or more preferably from 0.001 ng/ml to 1 ng/ml, or more preferably from 0.001 μg/ml to 1 μg/ml, or more preferably from 0.001 mg/ml to 1 mg/ml, or more preferably from 0.001 g/ml to 100 g/ml, with or without a particular carrier or scaffold. In yet another embodiment, the dosage of NELL peptide for bone formation generally ranges from 0.001 pg/kg to 1 pg/kg, or more preferably from 0.001 ng/kg to 1 ng/kg, or more preferably from 0.001 μg/kg to 1 μg/kg, or more preferably from 0.001 mg/kg to 1 mg/kg, or more preferably from 0.001 gm/kg to 1 gm/kg, more preferably from 0.001 kg/kg to 1 kg/kg with or without a particular carrier or scaffold. Furthermore, it is understood that all dosages may be continuously given or divided into dosages given per a given timeframe. Examples of timeframes include but are not limited to every 1 hour, 2 hour, 4 hour, 6 hour, 8 hour, 12 hour, 24 hour, 48 hour, or 72 hour, or every week, 2 weeks, 4 weeks, or every month, 2 months, 4 months, and so forth.

However, because NELL peptides may have effects on *in vitro* osteoblast apoptosis (Zhang, X., et al., J Bone Miner Res, 2003. 18(12): p. 2126-34), NELL dosages (e.g., NELL1 dosages) that are significantly above an optimal range may
not increase bone formation. Accordingly, even more preferable dosages of NELL peptide shall not be significantly above the optimal dosage range. The even more preferable optimal dosage ranges of NELL peptides may vary according to factors such as the type, the age, the location, and the gender of a mammalian subject; the carrier or scaffold material employed; and the purity and potency of different NELL peptides. In one embodiment, the even more preferable optimal dosage ranges of NELL peptides includes but are not limited to 1 ng/mm² to 100 ng/mm², or even more preferably from 100 ng/mm² to 1000 ng/mm², or even more preferably from 1 μg/mm² to 100 μg/mm², or even more preferably from 100 μg/mm² to 1000 μg/mm². In another embodiment, the even more preferable optimal dosage ranges of NELL peptides includes but are not limited to 1 ng/ml to 100 ng/ml, or even more preferably from 100 ng/ml to 1000 ng/ml, or even more preferably from 1 μg/ml to 100 μg/ml, or even more preferably from 100 μg/ml to 1000 μg/ml. In yet another embodiment, even more preferable optimal dosage ranges of NELL peptide for bone formation generally ranges from 1 μg/kg to 100 μg/kg, or even more preferably from 100 μg/kg to 1000 μg/kg, or even more preferably from 1 mg/kg to 100 mg/kg with or without a particular carrier or scaffold. Furthermore, it is understood that all dosages may be continuously given or divided into dosages given per a given timeframe. Examples of timeframes include but are not limited to every 1 hour, 2 hour, 4 hour, 6 hour, 8 hour, 12 hour, 24 hour, 48 hour, or 72 hour, or every week, 2 weeks, 4 weeks, or every month, 2 months, 4 months, and so forth. As used herein, the term “significantly above the optimal range” means, e.g., about 1% to about 50%, about 5% to about 50%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, or about 40% to 50% over the optimal range.

The dosage for inhibitors of NELL peptides varies according to the type of the inhibitor, the bone or cartilage condition to be treated, prevented, or ameliorated, and the age, the location, and the gender of the mammalian subject receiving the pharmaceutical composition containing the inhibitor. Generally, the dosage for inhibitors of NELL peptides ranges from but not limited to: 0.001 pg/mm² to 1 pg/mm², or more preferably from 0.001 ng/mm² to 1 ng/mm², or more preferably from 0.001 μg/mm² to 1 μg/mm², or more preferably from 0.001 mg/mm² to 1 mg/mm², or more preferably from 0.001 g/mm² to 1 g/mm²; with or
without a particular carrier or scaffold. In another embodiment, the dosage for inhibitors of NELL peptides generally ranges from 0.001 pg/ml to 1 pg/ml, or more preferably from 0.001 ng/ml to 1 ng/ml, or more preferably from 0.001 μg/ml to 1 μg/ml, or more preferably from 0.001 mg/ml to 1 mg/ml, or more preferably from 0.001 g/ml to 100 g/ml, with or without a particular carrier or scaffold. In yet another embodiment, the dosage for inhibitors of NELL peptides generally ranges from 0.001 pg/kg to 1 pg/kg, or more preferably from 0.001 ng/kg to 1 ng/kg, or more preferably from 0.001 μg/kg to 1 μg/kg, or more preferably from 0.001 mg/kg to 1 mg/kg, or more preferably from 0.001 gm/kg to 1 gm/kg, more preferably from 0.001 kg/kg to 1 kg/kg with or without a particular carrier or scaffold.

Furthermore, it is understood that all dosages may be continuously given or divided into dosages given per a given timeframe. Examples of timeframes include but are not limited to every 1 hour, 2 hour, 4 hour, 6 hour, 8 hour, 12 hour, 24 hour, 48 hour, or 72 hour, or every week, 2 weeks, 4 weeks, or every month, 2 months, 4 months, and so forth.

The dosage for modulators of receptors of NELL peptides varies according to the type of the inhibitor, the type of receptor, the bone or cartilage condition to be treated, prevented, or ameliorated, and the age, the location, and the gender of the mammalian subject receiving the pharmaceutical composition containing the modulators of receptors of NELL peptides. Generally, the dosage for modulators of receptors of NELL peptides ranges from but at not limited to: 0.001 pg/mm² to 1 pg/mm², or more preferably from 0.001 ng/mm² to 1 ng/mm², or more preferably from 0.001 μg/mm² to 1 μg/mm², or more preferably from 0.001 mg/mm² to 1 mg/mm², or more preferably from 0.001 g/mm² to 1 g/mm², with or without a particular carrier or scaffold. In another embodiment, the dosage for modulators of receptors of NELL peptides generally ranges from 0.001 pg/ml to 1 pg/ml, or more preferably from 0.001 ng/ml to 1 ng/ml, or more preferably from 0.001 μg/ml to 1 μg/ml, or more preferably from 0.001 mg/ml to 1 mg/ml, or more preferably from 0.001 g/ml to 100 g/ml, with or without a particular carrier or scaffold. In yet another embodiment, the dosage for modulators of receptors of NELL peptides generally ranges from 0.001 pg/kg to 1 pg/kg, or more preferably from 0.001 ng/kg to 1 ng/kg, or more preferably from 0.001 μg/kg to 1 μg/kg, or more preferably from 0.001 mg/kg to 1 mg/kg, or more preferably from 0.001 gm/kg to
1 gm/kg, more preferably from 0.001 kg/kg to 1 kg/kg with or without a particular carrier or scaffold. Furthermore, it is understood that all dosages may be continuously given or divided into dosages given per a given timeframe. Examples of timeframes include but are not limited to every 1 hour, 2 hour, 4 hour, 6 hour, 8 hour, 12 hour, 24 hour, 48 hour, or 72 hour, or every week, 2 weeks, 4 weeks, or every month, 2 months, 4 months, and so forth.

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, (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.

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 virucidal and immunostimulants which do not impair the desired action and/or supplement the desired action.

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, 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 µM. A preferred concentration range is from 0.2 to 20 µM and most preferably about 1 to 10 µM. However, the concentration of active ingredient in the 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 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, alginate, karya gum, methylcellulose, carboxymethylcellulose, methylethylcellulose, and hydroxypropylcellulose. Representative synthetic polymers include, for example, poly(acrylic acid), tragacanth, poly(methyl vinylether-co-maleic anhydride), poly(ethylene oxide), carbopol, poly(vinyl pyrrolidone), poly(ethylene glycol), poly(vinyl alcohol), poly(hydroxyethylmethylacrylate), and polycarbophil. Other bioadhesive materials available 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 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.

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, corn 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 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 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 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 formulations with pharmaceutically acceptable carriers. Preferred are those carriers that will 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.

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 (1983). These may also be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent 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 stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic 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.


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 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 are then readily administered in a variety of dosage forms such as tablets, powders, lozenges, 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 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 tabletting purposes. Solid compositions of a similar type may also be employed as fillers in soft and hard filled gelatin capsules. Preferred materials for this 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.
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 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 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 cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be 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 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 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, the invention may include a method of incorporating a NELL peptide in carriers or substrates, and the resulting substrates.

In one embodiment, a composition for inducing bone formation may include an effective amount of a first agent to induce bone formation selected from the group including but not limited to a NELL peptide, e.g., NELL1 peptide, a NELL2 peptide, an agent that alters expression of NELL1 peptide, an agent that alters the activity of a NELL1 peptide, an agent that alters expression of NELL2 peptide, an agent that alters the activity of a NELL2 peptide; and optionally a carrier.

The composition may include a second agent including, but not limited to TGF-beta, BMP2, BMP4, BMP7, bFGF, FGF, IGF (insulin like growth factors), VEGF, collagen, bone, bone matrix, tendon matrix or ligament matrix, osteogenic and/or osteoblastic cells.

In one embodiment, the carrier may be biodegradable, such as degradable by enzymatic or hydrolytic mechanisms. Examples of carriers include, but are not limited to synthetic absorbable polymers such as such as but not limited to poly(α-hydroxy acids) such as poly (L-lactide) (PLLA), poly (D, L-lactide) (PDLLA), polyglycolide (PGA), poly (lactide-co-glycolide (PLGA), poly (-caprolactone), poly (trimethylene carbonate), poly (p-dioxanone), poly (-caprolactone-co-glycolide), poly (glycolide-co-trimethylene carbonate) poly (D, L-lactide-co-trimethylene carbonate), polyanhydrides, polyhydroxybutyrate (PHB), poly(lactides), poly(lactide-co-imide), propylene-co-fumarates, poly(α-caprolactone), poly(anhydride-co-imide), polyanhydrides, polyesters, polycarbonates, polyanionic polymers, polyphosphazenes, poly(amino-acids), homopolypeptides, poly(phosphazenes), poly (glaxanone), polysaccharides, and poly(orthoesters), polyglactin, polyglycolic acid, poly(lactides), poly(lactide-co-glycolide), poly(acrylic acids), polyalkanoates; copolymers and admixtures thereof, and any derivatives and modifications. See for example, U. S. Patent 4,563,489, and PCT Int. Appl. # WO/03024316, herein incorporated by reference. Other examples of carriers include celluloseic polymers such as, but not limited to alkylcellulose, hydroxyalkylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, 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. See for example U.S. Patent Application 2002187104; PCT Int. Appl. WO/9731661; and PCT Int. Appl. WO/0071083, herein incorporated by reference.

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. See, for example, U.S. Patents 6,426,114 and 6,013,591; and PCT Int. Appl. WO/9117965 herein incorporated by reference.

Other examples of carriers include collagen (e.g. Collastat, Helistat collagen sponges), hyaluronan, fibrin, chitosan, alginate, and gelatin, or a mixture thereof. See for example, PCT Int. Appls. WO/9505846; WO/02085422, the teachings of which are incorporated herein by reference.

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), p. 43229-43235, the teachings of which are incorporated herein by reference.

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

In one embodiment, the substrate may include 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 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, 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, the carrier may be photopolymerizable, such as by electromagnetic radiation with wavelength of at least about 250 nm. Example of photopolymerizable polymers include polyethylene (PEG) acrylate derivatives, PEG 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. See for example, U.S. Patent 5,410,016, herein incorporated by reference.

In one embodiment, the substrate may include a carrier that is temperature sensitive. Examples include carriers made from N-isopropylacrylamide (NiPAM), or modified NiPAM with lowered lower critical solution temperature (LCST) and enhanced peptide (e.g. NELL1) binding by incorporation of ethyl methacrylate and N-acryloxy succinimide; or alkyl methacrylates such as butylmethacrylate, hexylmethacrylate and dodecylmethacrylate (PCT Int. Appl. WO/2001070288; U.S. Patent No. 5,124,151, the teachings of which are incorporated herein by reference).

In one embodiment, 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, Melp-class adhesive 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, the carrier may include comprised of sequestering 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 example, United States Patent 6,620,406, herein incorporated by reference.

In one embodiment, the carrier may include surfactants to promote NELL1 or NELL2 stability and/or distribution within the carrier materials such as, but not limited to polyoxyester (e.g. polysorbate 80, polysorbate 20 or Pluronic F-68).

In one embodiment, 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. Patent 5,385,887, herein incorporated by reference. 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. The membrane may be soaked in a solution of an agent including for example, NELL1 peptide, NELL2 peptide, or a mixture thereof.

In one embodiment, an implant for use in the human body may include a substrate that includes one or more agents described above, including for example NELL1 peptide, NELL2 peptide, or a mixture thereof in an amount sufficient to induce bone formation proximate to the implant.

In one embodiment, an implant for use in the human body may include a substrate having a surface that includes an agent such as NELL1 peptide, NELL2 peptide, or a mixture thereof in an amount sufficient to induce bone formation proximate to the implant.

In one embodiment, an implant for use in the human body may include a substrate having a surface including osteogenic cells, and for example NELL1 or NELL2 in an amount sufficient to induce bone formation. In one embodiment, the implant may be seeded with cells, including but not limited to autologous cells, osteogenic or osteoblastic cells, cells expressing a NELL peptide such as NELL1 peptide, NELL2 peptide, or a mixture thereof or another osteogenic molecule.
An implant may include a substrate formed into the shape of a mesh, pin, screw, plate, or prosthetic joint. By way of example, a substrate may be in a form of a dental or orthopedic implant and may include agent such as for example NELL1 peptide, NELL2 peptide, or a mixture thereof may be used to enhance integration in bone in proximity to the implant. An implant may include a substrate that is resorbable, such as a substrate including collagen.

In one example, a composition according to this invention may be contained within a time release tablet.

An agent such as a NELL peptide, e.g., the NELL1 peptide, NELL2 peptide, or a mixture thereof peptide may be combined 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 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 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.

The compositions 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.

The compositions of this invention may comprise a solution of an agent such as a NELL peptide such as the NELL1 peptide, NELL2 peptide, or a mixture thereof peptide dissolved in a pharmaceutically acceptable carrier, such as an aqueous carrier for water-soluble peptides. A variety of carriers can be used, e.g., buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically
acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like.

The concentration of an agent such as a NELL peptide, e.g., NELL1 peptide, NELL2 peptide, or a mixture thereof peptide in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

In some embodiments, the scaffold can include 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. See for example U.S. Patent Application 2002187104; PCT Int. Appl. WO/9731661; and PCT Int. Appl. WO/0071083, the teachings of which are incorporated herein by reference. Additional examples of bioceramic carriers include autologous, allogeneic, and xenogenic bone grafts, which may be intact or de-proteinized or de-mineralized. Other examples of carriers include synthetic and natural bioceramics and polymers and composites thereof, that increase the osteopontin gene expression of osteoblasts or their progenitors by at least 1.5 fold when the local calcium and phosphate concentrations of the local microenvironment are between 0.01-10 mM (Calcium) and 0.01-3 mM (Phosphate), respectively; and synthetic and natural bioceramics and polymers and composites thereof, that increase the osteopontin gene expression of osteoblasts or their progenitors by at least 1.5 fold when the local phosphate and calcium concentrations of the local microenvironment are between 0.01-10 mM (Phosphate) and 0.01-3 mM (Calcium).

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 0.1 to 10-fold the natural serum concentration and adjusted by various means to solutions with pH range of about 2.8-9.8 at temperature from about 15-65 °C, depending on carrier material. See, for example, U.S. Patents 6,426,114 and 6,013,591; and
International Application WO/9117965 incorporated herein by reference. Other examples of coating materials include synthetic and natural bioceramics and polymers and composites thereof, that increase the osteopontin gene expression of osteoblasts or their progenitors by at least 1.5 fold when the local calcium and phosphate concentrations of the local microenvironment are between 0.01-10 mM (Calcium) and 0.01-3 mM (Phosphate), respectively; and synthetic and natural bioceramics and polymers and composites thereof, that increase the osteopontin gene expression of osteoblasts or their progenitors by at least 1.5 fold when the local phosphate and calcium concentrations of the local microenvironment are between 0.01-10 mM (Phosphate) and 0.01-3 mM (Calcium).

Use of the Pharmaceutical Composition

In accordance with embodiments of the invention, a pharmaceutical composition of the various described embodiments can be administered to a mammal for treating or preventing a bone condition or bone related conditions. As used herein, the term "mammal" encompasses all mammalian subjects including human beings and animals.

In one embodiment, the pharmaceutical composition can be administered to a mammal for treating, preventing, or ameliorating a bone condition where bone generation is desirable.

In another embodiment, the pharmaceutical composition provided herein can be administered to a mammal for treating, preventing or ameliorating a bone condition where bone generation is excessive or undesirable. In a further embodiment, the pharmaceutical composition provided herein can be administered to a mammal for treating, preventing or ameliorating a bone condition.

The various bone conditions that can be treated, prevented, and/or ameliorated by the pharmaceutical composition described herein are described above.

EXAMPLES

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. Bone formation using a Calvarial Wound Model

General procedures
In one embodiment, the critical size defect in a non-union model can be used to determine the proper concentration for NELL1 in calvarial repair. Calvarial defects have been used as models to test bone regeneration under a non-load bearing conditions (Hollinger, J.O. and J.C. Kleinschmidt, J Craniofac Surg, 1990. 1(1): p. 60-8). An exemplary procedure is described below.

**Standardization.** To standardize bone repair characteristics, skeletally mature 5 month old male Sprague-Dawley rats rather than growing (skeletally immature animals) animals will be used for the survival surgeries (Allen, M.R. and S.A. Bloomfield, J Appl Physiol, 2003. 94(2): p. 642-50). After induction of anesthesia, the scalp area of adult rats will be shaved, prepped 3x with alcohol and betadine, and then draped with sterile drapes. A full-thickness scalp incision will be made and the periosteum reflected to expose bilateral parietal bones. A trephine drill will be used under constant irrigation with sterile saline to prevent overheating the bone edges. A full-thickness craniotomy defect will be created in each parietal bone with care to avoid injury to the underlying dura [i.e., two parietal defects per rat; each defect diameter = 5 mm (critical size)]. The estimated healing rate of bilateral, untreated 5 mm defects were provided in Table 2.
<table>
<thead>
<tr>
<th>Intervention Groups</th>
<th>$\text{Diameter (Critical) = } 19.6 \text{ mm}^2 \text{ Total Area}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I – Final Concentration</strong></td>
<td><strong>Total NELL1</strong>&lt;br&gt;Applied (ng)</td>
</tr>
<tr>
<td>= Total NELL1 (ng) / Total Area (mm$^2$)</td>
<td></td>
</tr>
<tr>
<td>ceramic/collagen carrier only (control)</td>
<td>--</td>
</tr>
<tr>
<td>ceramic/collagen + 5 ng/mm$^2$ NELL1</td>
<td>98</td>
</tr>
<tr>
<td>ceramic/collagen + 15 ng/mm$^2$ NELL1</td>
<td>294</td>
</tr>
<tr>
<td>ceramic/collagen + 30 ng/mm$^2$ NELL1</td>
<td>589</td>
</tr>
<tr>
<td>ceramic/collagen + 60 ng/mm$^2$ NELL1</td>
<td>1178</td>
</tr>
<tr>
<td>ceramic/collagen + 120 ng/mm$^2$ NELL1</td>
<td>2356</td>
</tr>
<tr>
<td>ceramic/collagen + 240 ng/mm$^2$ NELL1</td>
<td>4712</td>
</tr>
<tr>
<td><strong># Animals Used – Group I</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Group II – Final Concentration</strong></th>
<th><strong>Total BMP</strong>&lt;br&gt;Applied (ng)</th>
<th># Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>= Total BMP (ng) / Total Area (mm$^2$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ceramic/collagen carrier only (control)</td>
<td>--</td>
<td>5 rats</td>
</tr>
<tr>
<td>ceramic/collagen + 30 ng/mm$^2$ BMP2</td>
<td>589</td>
<td>5 rats</td>
</tr>
<tr>
<td>ceramic/collagen + 60 ng/mm$^2$ BMP2</td>
<td>1178</td>
<td>5 rats</td>
</tr>
<tr>
<td>ceramic/collagen + 120 ng/mm$^2$ BMP2</td>
<td>2356</td>
<td>5 rats</td>
</tr>
<tr>
<td>ceramic/collagen + 240 ng/mm$^2$ BMP2</td>
<td>4712</td>
<td>5 rats</td>
</tr>
<tr>
<td>ceramic/collagen + 30 ng/mm$^2$ BMP7</td>
<td>589</td>
<td>5 rats</td>
</tr>
<tr>
<td>ceramic/collagen + 60 ng/mm$^2$ BMP7</td>
<td>1178</td>
<td>5 rats</td>
</tr>
<tr>
<td>ceramic/collagen + 120 ng/mm$^2$ BMP7</td>
<td>2356</td>
<td>5 rats</td>
</tr>
<tr>
<td>ceramic/collagen + 240 ng/mm$^2$ BMP7</td>
<td>4712</td>
<td>5 rats</td>
</tr>
<tr>
<td><strong># Animals Used – Group II</strong></td>
<td></td>
<td>45 rats</td>
</tr>
</tbody>
</table>

Following creation, each defect will be flushed with saline to remove bone debris and then grafted with either control ceramic carrier mixed with sterile saline or with ceramic carrier mixed with differential NELL1 doses to determine the optimal NELL1 treatment concentration. NELL1 concentration will be standardized according to defect area (i.e., amount of NELL1 protein (ng) per mm$^2$) (Table 2, Group I). Five rats (N = 10 defects) will be used for each intervention.
subgroup in Group I (N = 35 rats total) (Table 2). In addition, different concentrations of BMP2 and BMP7 will be applied in an identical fashion to the 5 mm defect models (Table 2, Group II) (N = 45 rats). The concentrations for NELL1 testing are based on preliminary studies. The concentrations for BMP testing are based on published studies for 8 mm diameter rat calvarial defects in which BMP concentrations (by area) ranged from ~20 ng/mm² to ~600 ng/mm² (Table 3).
Table 3. BMP2 Dosages in Published Rat Calvarial Critical-Sized Defect Models

<table>
<thead>
<tr>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total BMP2 Applied</td>
</tr>
<tr>
<td>Defect Diameter</td>
</tr>
<tr>
<td>Defect Area</td>
</tr>
<tr>
<td>Strain</td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Delivery System</td>
</tr>
<tr>
<td>Ref.</td>
</tr>
</tbody>
</table>

200 and 600 ng/mm²
10 and 30 ng/BMP2
8 mm
50 Long Evans
28-35 M PLGA microparticles

20, 100, and 400 ng/mm²
1, 5, and 20 ng/BMP2
8 mm
50 Sprague Dawley
84-92 F Fibrin matrix

44 and 130 ng/mm²
2.2 and 6.5 ng/BMP2
8 mm
50 Long Evans
28-35 M ICBM

100 ng/mm²
5 rhBMP2
8 mm
50 NA NA PEG-based hydrogel

*Indicates application dosage with the most bone formation.
Abbreviations: F (female); ICBM (insoluble collagenous bone matrix); M (male); NA (not available); ngBMP2 (nonglycosylated BMP2); PEG (polyethylene glycol); PLGA (microparticles of poly (D,L-lactide-co-glycolide); rhBMP2 (recombinant BMP2)


The five rats in each intervention subgroup from Groups I and II (Table 2) will undergo live serial weekly imaging with micro-CT for 8 consecutive weeks using established protocols (Cowan, C.M., et al., *Adipose-derived adult stromal cells heal critical-size mouse calvarial defects*. Nat Biotechnol, 2004. 22(5): p. 560-7). The live micro-CT will provide accurate real-time quantitative data on bone density and bone regeneration area and volume as well as some qualitative data on bone morphology in the different intervention subgroups. At eight weeks, the animals will be sacrificed for high resolution, cadaveric micro-CT analyses and histology. Calvarial sections will undergo hematoxylin and eosin (H&E) staining to histologically assess the quality of bone formation. If cartilage is observed,
Alcian blue will be used to verify the finding. Optimal concentrations for NELL1, BMP2, and BMP7 will be defined as the concentration inducing the largest CT-derived area of histologically confirmed bone at 8 weeks. If a plateau in bone formation is observed beyond a certain concentration, the lowest concentration at which the plateau is reached will be termed “optimal.”

*Equipment.* High resolution micro-CT will utilize the latest 9-20 µm resolution technology from µCT40 (Scanco, PA) as previously published (Zhang, X., et al., J Clin Invest, 2002. 110(6): p. 861-70). Micro-CT data can be collected at 50 kVp and 160µA and reconstructed using the cone-beam algorithm supplied with the micro-CT scanner by Scanco. Both 2D and 3D data will be acquired and analyzed to ensure the optimal characterization of biologic behavior. Visualization and reconstruction of the data can be performed using the MetaMorph® Imaging System (for 2-D) (Universal Imaging Corporation, Downingtown, PA), Image Pro Plus version 5.0 (Media Cybernetics, Carlsbad, CA) (for 2D), and Amira™ (for 3-D) (Visual Concepts GmbH, Berlin, Germany).

CT-based morphometric analyses of a number of known bone-specific 3-D structural parameters including: 1) bone volume/tissue volume - number of bone voxels in the volume of interest (VOI) divided by the total number of tissue voxels in the VOI; 2) mineralization density - radiopacity of the bone mass divided by the volume of bone mass; and 3) trabecular thickness, trabecular number, and trabecular separation (derived from bone volumes and surface areas) can be performed (Borah, B.D., T.E. et al., JBMR, 2000. 15(9): p. 1786-1797).

*Scaffold fabrication.* The ceramic carrier supplied by MTF is marketed under the name Synthacer. Synthacer is available in block cylindrical forms (95% hydroxyapatite; 200-800 micron pore size, 65-80% porous), and in loose powder form. The experiments will utilize block Synthacer disks that are made to fit the corresponding defects; as supplied by MTF. Initial studies on apatite carriers demonstrated faster osteoinductive response times in collagen/growth factor-coated apatites relative to collagen-free controls, and thus, all growth factors will be incorporated in a type I collagen solution. The solution can be prepared by adding growth factors at 0°C to pH-adjusted collagen solution. Pre-determined amounts of collagen/growth factor solution will then be applied onto each disk and the formulated scaffolds will be brought to 20°C for gelation and then air dried to form
a thin layer of collagen/growth factor. If necessary, other biomaterials (hyaluronan, fibrin, or alginate) may be employed to replace the collagen component.

*Analyses of craniofacial bone formation using optimized NELL1, BMP2, and BMP7 concentrations.* Animal surgery will be performed as described above for the critical defect (non-union) model. Optimized NELL1, BMP2, and BMP7 concentrations will be applied using the ceramic carrier (Table 4). Controls will consist of ceramic carrier and sterile saline. To temporally delineate the newly formed bone on histology, animals will undergo sequential *in vivo* fluorescent labeling with a single intraperitoneal injection of Calcein blue (30 mg/kg body weight) at day 0 (immediately after surgery), a single intraperitoneal injection of Xylenol orange (90 mg/kg body weight) at day 14, and a single intraperitoneal injection of Calcein (10 mg/kg body weight) at day 28. Calcein blue (emits blue), Xylenol orange (emits orange), and Calcein (emits green) are chelating fluorochromes with similar distribution patterns to radiolabelled calcium that deposit in sites of active bone or cartilage matrix mineralization (reviewed in Lee, T.C., et al., J Anat, 2003. 203(2): p. 161-72). Measurement of the distance between the different fluorochrome bands divided by the administration interval will allow for calculation of the mineral apposition rate (MAR) as described by Iwamoto et al. (Iwamoto, J., J.K. Yeh, and J.F. Aloia, J Bone Miner Res, 2000. 15(9): p. 1842-9).

In addition, each animal will undergo live serial weekly imaging with micro-CT and micro-PET using established protocols until sacrifice (Cowan, C.M., et al., Nat Biotechnol, 2004. 22(5): p. 560-7; Berger, F., et al., Eur J Nucl Med Mol Imaging, 2002. 29(9): p. 1225-36). Micro-PET will facilitate quantitative analysis of how NELL1, BMP2, or BMP7 addition may affect bone metabolic activities in a temporally and spatially distinct fashion. Subgroup animals will be sacrificed at 1,
2, 4, and 8 weeks. Identically treated, paired calvarial specimens from each animal will be differentially harvested and processed. One specimen will be harvested with a large rim of normal tissue and fixed in 4% paraformaldehyde for histology and subsequent Phase II cellular analyses. The other specimen will be harvested with a small rim (<2 mm) of normal tissue and immediately frozen in liquid nitrogen and stored at −70°C in anticipation of more detailed Phase II molecular analyses.

The fixed specimens will first undergo morphologic analyses using high resolution micro-CT and imaging software as described above. Following this, the fixed specimen will be bisected. Half of the specimen will be demineralized, dehydrated, embedded in paraffin, sectioned (5 μm thickness), and H&E stained; the other half will be processed undecalcified and embedded in either methyl methacrylate. For visualization of in vivo labeling, four unstained, non-decalcified, non-consecutive sections (10 μm thickness) will be examined using fluorescence microscopy. Additional sections (4 μm thickness) will be stained with Masson-Goldner's trichrome for histomorphometric measurements such as trabecular bony volume and surface density as described by Hollinger et al (Hollinger, J.O., D. Buck, and J.P. Schmitz, Clin Plast Surg, 21(3): p. 463-75) (1994).

The micro-PET will provide detailed metabolic information on whether activity is most intense at the trephine rim or at the defect center and how optimized NELL1 or BMP addition will influence this activity. Both NELL1 and BMP will increase bone metabolic activity at the trephine rim and defect center. In addition, the use of different chelating fluorochromes will allow the correlation of the calculated MAR with the observed bone formation on micro-CT and metabolic activity on micro-PET as well as determine more exactly the temporal and spatial sequence of newly deposited bone (e.g., rate of central vs. rim bone deposition, dural vs. periosteal bone deposition).

Regeneration of calvarial bone by NELL1

Creation of calvarial defect. The critical size calvarial defect represents a non-osseous union model [which was defined as < 10% healing on 3 dimensional (D) volume measurement by 3 months], while the subcritical size calvarial defect represents a delayed osseous union model (which can be defined as ≤15% healing on 3D volume measurement by 3 months). Estimated healing rate of bilateral,
untreated critical size (5 mm diameter) and subcritical size (3 mm diameter) calvarial defects in the rat model are shown in Table 5. The less than 10% healing for the 5 mm defects concur with other reports in the literature (Bosch, C., et al., J Craniofac Surg, 1998. 9(4): p. 310-6). Although some rat critical size defect models involve a single 8 mm diameter defect centered over the sagittal suture (Kenley, R., et al., J Biomed Mater Res, 1994. 28(10): p. 1139-47; Schmoekel, H., et al., J Orthop Res, 2004. 22(2): p. 376-81), the bilateral model (which can accommodate dual defects up to 5 mm) was chosen for the following reasons: 1) to specifically avoid inclusion of the fibrous tissue within the sagittal suture; 2) to minimize injury to the midsagittal sinus; and 3) to allow for paired experimental design (Bosch, C., et al., J Craniofac Surg, 1998. 9(4): p. 310-6).


PLGA scaffolds were prepared as previously described (Cowan, C.M., et al., Nat Biotechnol, 2004. 22(5): p. 560-7). After scaffold fabrication, scaffolds were coated with type I collagen (Vitrogen®; Cohesion, Palo Alto, CA) in which were premixed with the appropriate amounts of either NELL1, BMP2, or sterile saline controls. The total dose of NELL1 and BMP2 for the pilot studies was 200 ng per each 3 mm diameter PLGA scaffolds to provide snug fit in the trephined defect. Controls consisted of PLGA membrane alone. Animals were sacrificed at 0, 1, 2, 3, and 4 weeks for micro-CT and histological analyses of the calvariae.

In these studies, the initial dose of 200 ng was derived empirically from in vitro NELL1 cell culture data and in vivo BMP2 critical size defect data (Table 3, supra). In vitro NELL1 concentrations in the range of 5 to 50 ng/ml concomitantly
increased apoptosis and bone nodule formation, while concentrations above 100 ng/ml increased apoptosis but decreased bone nodule formation, and concentrations above 200 ng/ml were associated with increased apoptosis and minimal bone nodule formation (Zhang, X., et al., J Bone Miner Res, 2003. 18(12): p. 2126-34). Thus, this indicates that excessive NELL1 dosages will reduce bone formation. In the published BMP2 studies, a relatively low 1 μg total applied dose effectively closed 46% to 74% of an 8 mm diameter defect in 3 weeks (Schmoekel, H., et al., J Orthop Res, 2004. 22(2): p. 376-81). When normalized to total defect area, the 1 μg dose was equivalent to 20 ng/mm² (Standardization to defect area rather than volume was to facilitate comparison of the dosages in this example to published studies in which the calvarial thickness was not always available for volume calculations). The 200 ng total dose used herein, divided by total area for a 3 mm diameter defect (i.e., 7 mm² = Total Area), corresponds to 28 ng/mm².

*Induction of Calvarial Bone Regeneration by NELL1.* Studies utilizing 200 ng total dose (28 ng/mm²) NELL1 and BMP2 loaded onto PLGA membranes demonstrated significant bone formation for both NELL1 and BMP2 treated specimens over non-loaded PLGA controls (N = 4 to 6 defects per treatment subgroup, per time point). Volume analysis demonstrated significantly increased bone formation for both NELL1 and BMP-2 relative to control over the 4 week study period (Figure 4). As shown in Figure 4, NELL1 induced significantly more bone than BMP2 at week 1.

Overall, not more than 15% bone volume regeneration is estimated at 3 months (Table 5). At 2 weeks, NELL1 and BMP2 demonstrated approximately 70-80% defect closure by surface analysis and 35-45% volume regeneration by volume analysis (Figure 4). Histological sections confirmed the presence of bone. Osteoid deposition and trabecular branching patterns were not markedly different between NELL1 and BMP2. Figure 5 demonstrated near 90-100% defect closure by surface analysis in the NELL1 and BMP2 treated specimens at 4 weeks which corresponded to 45-50% volume regeneration by 3D volume analysis. Histological sections confirmed the presence of bone (Figures 6A-6C). Again, there were no marked histological differences between NELL1 and BMP2 induced bone (Figures 6A-6C).
Of note, the standard deviation on surface analyses was significantly higher than that for volume analyses. This serves to highlight that 2D-based linear

<table>
<thead>
<tr>
<th>Table 5. Estimated Healing Rate</th>
<th>Volume of New Bone (mm³)</th>
<th>Defect Volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defect Diameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mm (Subcritical)</td>
<td>7 mm²</td>
<td>~10%</td>
</tr>
<tr>
<td>5 mm (Critical)</td>
<td>19.6 mm²</td>
<td>&lt;10%</td>
</tr>
</tbody>
</table>

*Volume was calculated by Area x Calvarial Thickness. Mean calvarial thickness measured from histology sections was 0.6 mm

measurements may not necessarily reflect all tissue events in 3D organisms.

Indeed, it can be seen from the histological specimens (Figures 6A-6C) that although 2D-based “defect closure” has occurred for the NELL1 and BMP2 treated calvariae, the cross-sectional thickness of regenerated bone in the defect is not as thick as non-wounded bone. Thus, 2D-based defect “closure” does not necessarily correspond to 3D-based defect “reconstitution.” Gosain et al. have also noted the importance of cross-sectional or more “3D” based data acquisition and analysis parameters in the evaluation of critical, and especially subcritical size defects (Gosain, A.K., et al., Plast Reconstr Surg. 2000. 106(2): p. 360-71; discussion 372).

These studies demonstrate that recombinant NELL1 is osteoinductive in vivo and that NELL1 induced bone is indistinguishable from BMP2 induced bone at 4 weeks.

Example 2. Mammalian system for expression of recombinant human NELL (rhNELL1)

In order to study the function of NELL1 and NELL2 protein/peptides, attempts were successfully made to produce and purify the peptide. The mammalian expression system used for production of rhNELL1 by non-viral DNA delivery in this invention can include, but not limit to these commonly used stable expression systems listed in Table 6. The detailed protocols including vector design, host cell line culture, transfection and selection of stable cell line as well as purification of rhNELL1 in HEK 293 and CHO system are described below for reference.
Table 6. Mammalian Expression System for production of rhNELL1

<table>
<thead>
<tr>
<th>System</th>
<th>Parental vector</th>
<th>Leader sequence</th>
<th>Gene amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>p3Xflag-CMV</td>
<td>preprotrypsin</td>
<td>No/optimal</td>
</tr>
<tr>
<td>DXB11</td>
<td>mp19-Lp</td>
<td>human tPA</td>
<td>DHFR/MTX</td>
</tr>
<tr>
<td>HEK293</td>
<td>pSecTag</td>
<td>immunoglobulin</td>
<td>No/optimal</td>
</tr>
<tr>
<td>NS/0 or Sp2/0</td>
<td>pdCs-Fe-X</td>
<td>light chain of Ig and Fc fragment</td>
<td>DHFR/MTX</td>
</tr>
<tr>
<td>pEE12</td>
<td>N/A</td>
<td>GS/MSX</td>
<td></td>
</tr>
</tbody>
</table>

DHFR: dihydrofolate reductase; MTX: methotrexate; GS: glutamine synthetase
MSX: methionine sulphoximine.

A. **CHO system**

*Vector design:* A cDNA fragment was ligated into the expression vector p3XFlag-CMV (Sigma). The resulting expression construct, pCMV-rhNELL-3Xflag, includes a preprotrypsin leading sequence, cDNA fragment of the mature human NELL1 coding region and a 3Xflag sequences at c-terminal.

*Host Cell line:* The CHO-K1 was an adherent cell line and can be adapted to suspension culture in serum-free medium. The construct of pCMV-rhNELL1-3Xflag was transfected by either lipofectamine (Invitrogen) or calcium phosphates treatment. The stable cell lines were selected by adding G418 (400-600ug/ml) into the cell culture medium for about two weeks. The stable transformants were further screened for single clones with high productivity of rhNELL1 by limiting dilution.

The selected stable cell lines can be used in laboratory or industrial scale bioreactors for rhNELL1 production.

*Purification procedure:* rhNELL1 peptide containing media or cell lysate was purified through anti-flag antibody M2 (Sigma) affinity column at its native condition and eluted with 3Xflag peptide.

B. **HEK293 system**

*Vector design:* A cDNA fragment was ligated into the expression vector pSecTagA (Invitrogen). The resulting expression construct, pSec-hNELL1-Tag, includes a murine immunoglobulin k-chain leader sequence, cDNA fragment of the mature human NELL1 coding region and dual tag of Myc and His sequences at c-terminal.

*Host Cell line:* The human embryo kidney cell line, HEK-293 which was adapted to serum-free medium and grown in suspension format, was transfected
with the NELL1 peptide expression vector, pSec-hNELL1-Tag. Cells were either cultured for a couple of days as transient transfection before collecting conditioned medium for purification of rhNELL1 or treated with Zeocin (250 ug/ml) for selection of stable expression cell line. The stable transformants were further screened for single clones with high productivity of rhNELL1 by limiting dilution. The selected stable cell lines can be used in laboratory or industrial scale bioreactors for rhNELL1 production.

Purification procedure: rhNELL1 peptide containing media were purified through Ni\(^{2+}\) affinity column at its native condition and eluted with 1M imidazole. The rhNELL1 was tested for its integrity, purity and bioactivity after extensively dialysis against at least 1000 volumes of PBS (pH 7.4) at 4\(^{\circ}\)C for 20hrs.

In addition, the modifications of parental vectors for replacing existing leader sequence with a new one such as rat serum albumin, CD33, tPA and human interleukin-2 leader sequence or adding gene amplification target such as DHFR or GS into the backbone sequence will result in new expression vectors and systems. In this invention, the native signal peptide of human NELL1 is not effective enough to guide the protein secretion and sometimes even the external leading sequence didn’t work well, either. Thus, the construction of expression vector with in frame fusion of a small natural secretory protein such as human granulocyte-macrophage colony stimulating factor (GM-CSF) by a spacer containing intraprotein His tag and proteolytic cleavage site as “MPHHHHHHHGGGGDDDDDKDPM” can be needed. The epitope tags used for purification of NELL1 can be one of the following: 6XHistidines, 3XFlag, Myc, GST (glutathione S-transferase), EGFP or CTHS (C-terminal half of SUMO which stands for small ubiquitin modifying protein) etc, but also can be dual of His plus Myc as listed plasmid pSecTag in Table 6.

Furthermore, the dicistronic or multicistronic vectors using IRES can be constructed for regulatory or inducible expression of rhNELL1 under certain circumstances. The genetic modifications of host cell lines for gaining longer lasting proliferation and delayed apoptosis or compatible with special requests such as Tetracycline inducible system and Flp-In specific site integration system can be considered for improvement of rhNELL1 production.
Besides the stable expression of system for production of rhNELL1 mentioned above, a large-scale transient transfection (LST) approach using multi-milligram purified plasmid vector (pREP4) can be used to transfec HEK 293 or BHK suspension cells with cationic polymer PEI as backup alternative or complimentary to stable system.

While particular embodiments of the present invention have been shown and described, it will be obvious to those skilled in the art that changes and modifications can be made without departing from this invention in its broader aspects. Therefore, the appended claims are to encompass within their scope all such changes and modifications as fall within the true spirit and scope of this invention.
CLAIMS
What is claimed is:

1. A pharmaceutical composition for treating, preventing, or ameliorating a bone related condition in a mammal, comprising an effective amount of a NELL peptide, or a NELL RNA.

2. The composition of claim 1, wherein the NELL peptide is in a dosage not substantially exceeding an optimal dosage range of the NELL peptide.

3. The pharmaceutical composition of claim 1, wherein the NELL peptide is selected from the group consisting of NELL1, NELL2, a fragment of NELL1 peptide, a fragment of NELL2 peptide, and combinations thereof.

4. The pharmaceutical composition of claim 1, wherein the bone related condition is selected from the group consisting of craniofacial bone generation, non-craniofacial bone generation, long bone generation, periodontal bone generation, intramembranous bone generation, endochondral bone generation, cartilage regeneration, cartilage hypertrophy and combinations thereof.

5. The pharmaceutical composition of claim 1, wherein the bone related condition is selected from the group consisting of osteoporosis, bone loss due to microgravity, disuse atrophy, prolonged bed-rest, and a disease that involves multiple symptoms where bone metabolism is a secondary effect.

6. The pharmaceutical composition of claim 5, wherein the disease that involves multiple symptoms results in pathological calcification.

7. The pharmaceutical composition of claim 6, wherein the disease that involves multiple symptoms is a chronic kidney disease which causes renal osteodystrophy and/or vascular calcification.

8. The pharmaceutical composition of claim 5, wherein the bone related condition is ectopic soft tissue calcification, gall stone, kidney stones, pineal gland calcification, cataracts, salivary stones, cardiac valves, or prostate stones.

9. The pharmaceutical composition of claim 1, wherein the NELL RNA is mRNA, noncoding RNA, microRNA, dsRNA, or combinations thereof.

10. The composition of claim 1, wherein the NELL RNA is stabilized by a chemical or by incorporating in NELL RNA in a nanocage or biomaterial.
11. A pharmaceutical composition, comprising an effective amount of an agent for treating, preventing, or ameliorating bony overgrowth across cranial sutures in a mammal, wherein the agent is selected from the group consisting of NELL peptides, inhibitors of NELL peptides, antagonists of a receptor of a NELL peptide, and combinations thereof.

12. A pharmaceutical composition for treating or preventing a cartilage related bone condition in a mammal, comprising an effective amount of at least an agent effective for regeneration of cartilage, wherein the agent is selected from the group consisting of a NELL peptide, inhibitors of the NELL peptide, antagonists of an receptor of a NELL peptide, an enhancer of NELL peptides, a NELL RNA and combinations thereof.

13. The pharmaceutical composition of claim 12, wherein the NELL peptide is selected from the group consisting of NELL1, NELL2, a fragment of NELL1 peptide, a fragment of NELL2 peptide, and combinations thereof.

14. The pharmaceutical composition of claim 12, wherein the NELL RNA is mRNA, noncoding RNA, microRNA, dsRNA, or combinations thereof.

15. The pharmaceutical composition of claim 12, wherein the NELL RNA is stabilized by a chemical or by incorporating in NELL RNA in a nanocage or biomaterial.

16. The pharmaceutical composition of claim 12, wherein the agent is effective for joint resurfacing, temporomandibular joint reconstruction, arthritis repair, or intervertebral disc repair.

17. A pharmaceutical composition, comprising an effective amount of a modulator of a receptor of a NELL peptide for treating, preventing or ameliorating a bone related condition.

18. The pharmaceutical composition of claim 17, wherein the modulator is selected from the group consisting of an agonist or antagonist of a NELL peptide receptor, a molecule that stabilizes or degrades a NELL peptide, a molecule that stabilizes or degrades the NELL peptide receptor, a molecule that is involved in the stabilization and phosphorylation of a complex of the NELL peptide and the receptor after initial receptor ligation, an agonist of the agonist or antagonist of the NELL peptide receptor, an antagonist of the agonist or antagonist of the NELL peptide receptor, and combinations thereof.
19. The pharmaceutical composition of claim 17, wherein the bone related condition is selected from the group consisting of osteoporosis, bone loss due to microgravity, disuse atrophy, prolonged bed-rest, and a disease that involves multiple symptoms where bone metabolism is a secondary effect.

20. The pharmaceutical composition of claim 17, wherein the disease that involves multiple symptoms results in pathological calcification.

21. The pharmaceutical composition of claim 17, wherein the disease that involves multiple symptoms is a chronic kidney disease which causes renal osteodystrophy and/or vascular calcification.

22. The pharmaceutical composition of claim 17, wherein the bone related condition is ectopic soft tissue calcification, gall stone, kidney stones, pineal gland calcification, cataracts, salivary stones, cardiac valves, or prostate stones.

23. A pharmaceutical composition for treating, preventing, or ameliorating a bone related condition in a mammal, comprising an effective amount of a derivative of a NELL peptide.

24. The pharmaceutical composition of claim 23, further comprising a pharmaceutically acceptable carrier.

25. The pharmaceutical composition of claim 24, wherein the pharmaceutically acceptable carrier is a carrier for a mode of delivery selected from the group consisting of oral administration, topical administration, in situ implant, intravenous administration, parenteral administration, local administration, intra-arterial injection, injection into a fracture site, and delivery in a biodegradable matrix.

26. The pharmaceutical composition of claim 23, wherein the bone related condition is selected from the group consisting of bone loss due to microgravity, disuse atrophy, prolonged bed-rest, and a disease that involves multiple symptoms where bone metabolism is a secondary effect.

27. The pharmaceutical composition of claim 26, wherein the disease that involves multiple symptoms results in pathological calcification.

28. The pharmaceutical composition of claim 26, wherein the disease that involves multiple symptoms is a chronic kidney disease which causes renal osteodystrophy and/or vascular calcification.
29. The pharmaceutical composition of claim 23, wherein the bone related condition is ectopic soft tissue calcification, gall stone, kidney stones, pineal gland calcification, cataracts, salivary stones, cardiac valves, or prostate stones.

30. The pharmaceutical composition of claim 23, wherein the derivative of a NELL peptide is a physically modified NELL1 peptide or NELL2 peptide.

31. The pharmaceutical composition of claim 23, wherein the derivative of a NELL peptide is a chemically modified NELL1 peptide or NELL2 peptide.

32. The pharmaceutical composition of claim 31, wherein the derivative of a NELL peptide is selected from a PEGylated NELL1 peptide, a PEGylated NELL2 peptide, a NELL1 peptide comprising at least one short hydrocarbon group, or a NELL2 peptide comprising at least one short hydrocarbon group.

33. The pharmaceutical composition of claim 31, wherein the chemically modified NELL peptide is a NELL1 peptide mimetics or a NELL2 peptide mimetics.

34. The pharmaceutical composition of any of claims 1-33, further comprising a pharmaceutically acceptable carrier.

35. The pharmaceutical composition of any of claims 1-33, further comprising a second agent, wherein the composition is effective for bone generation or treating, preventing, or ameliorating a bone related condition.

36. The pharmaceutical composition of claim 35, wherein the second agent is selected from the group consisting of a BMP protein, a TGFβ protein, a FGF protein, IGF (insulin like growth factors), VEGF, and a combination thereof.

37. The pharmaceutical composition of claim 36, wherein the bone condition is selected from the group consisting of bone fracture, spinal fusion, long bone fracture, craniofacial bone healing or formation, dental or orthopedic implant integration, dental implant integration, or combinations thereof.

38. The pharmaceutical composition of claim 35, further comprising a pharmaceutically acceptable carrier.

39. The pharmaceutical composition of any of claims 1-33 in a formulation suitable for a mode of delivery selected from the group consisting of oral delivery, parenteral delivery, pulmonary delivery, and implantation.
40. A method of treating, preventing, or ameliorating a bone related condition, comprising administering to a mammal a pharmaceutical composition according to any of claims 1-33.

41. A method of inducing osteoblast or bone formation, comprising:
contacting a cell a composition comprising a NELL peptide, a
NELL RNA and optionally a second agent.

42. The method of claim 41, wherein the cell is a mammalian cell.

43. The method of claim 41, wherein the NELL peptide is selected from the group consisting of NELL1, NELL2, a fragment of NELL1 peptide, a fragment of NELL2 peptide, and combinations thereof,
wherein the second agent is selected from the group consisting of a BMP protein, a TGFβ protein, a FGF protein, an IGF, a VEGF, and a combination thereof, and
wherein the cell is a stem cell, a bone marrow stromal cell, a fibroblast, or an adipose derived cell.

44. The pharmaceutical composition of claim 41, wherein the NELL RNA is mRNA, noncoding RNA, microRNA, dsRNA, or combinations thereof.

45. The pharmaceutical composition of claim 41, wherein the NELL RNA is stabilized by a chemical or by incorporating in NELL RNA in a nanocage or biomaterial.

46. A method of inducing bone formation, comprising:
contacting a bone matrix a composition comprising a NELL peptide a NELL RNA and optionally a second agent.

47. The method of claim 46, wherein the NELL peptide is selected from the group consisting of NELL1, NELL2, a fragment of NELL1 peptide, a fragment of NELL2 peptide, and combinations thereof,
wherein the second agent is selected from the group consisting of a BMP protein, a TGFβ protein, a FGF protein, IGF (insulin like growth factors), VEGF, and a combination thereof, and
wherein the bone matrix is demineralized bone matrix or mineralized bone matrix.

48. The pharmaceutical composition of claim 46, wherein the NELL RNA is mRNA, noncoding RNA, microRNA, dsRNA, or combinations thereof.
49. The pharmaceutical composition of claim 46, wherein the NELL RNA is stabilized by a chemical or by incorporating in NELL RNA in a nanocage or biomaterial.

50. A method of identifying a modulator of a receptor of a NELL related peptide, comprising:

contacting a receptor molecule of a NELL peptide with a test compound,

contacting the NELL peptide with the receptor molecule and the test compound,

detecting the extent of binding of the NELL peptide to the receptor molecule with the test compound,

comparing the extent of binding of the NELL peptide to the receptor molecule with the extent of binding of a control wherein the control is obtained by detecting the extent of binding of the NELL peptide to the receptor molecule without the test compound, and

designating the test compound as a modulator of the receptor of the NELL peptide if the extent of binding of the NELL peptide to the receptor molecule with the test compound is different from the extent of binding of the control.

51. The method of claim 50, wherein the designating step further comprises:

designating the modulator as an antagonist of the receptor of the NELL peptide if the extent of binding of the NELL peptide to the receptor molecule with the test compound is lower than the extent of binding of the control, or

designating the modulator as an agonist of the receptor of the NELL peptide if the extent of binding of the NELL peptide to the receptor molecule with the test compound is higher than the extent of binding of the control.

52. A method of producing a NELL peptide, comprising expressing a nucleic acid construct encoding a NELL gene in a host cell.

53. The method of claim 52, wherein the nucleic acid construct further comprises a gene of a signal peptide.

54. The method of claim 52, wherein the construct comprises a gene having a sequence selected from SEQ ID NO: 1-81.

55. The method of claim 54, wherein the construct further comprises a promoter gene selected from SEQ ID NO: 82-87 and/or a gene that encodes a signal peptide selected from SEQ ID NO: 88-95.
56. The method of claim 54, wherein the nucleic acid construct further comprises a noncoding gene in the genomic DNA of a NELL peptide.

57. The method of claim 56, wherein the NELL peptide is NELL1.

58. The method of claim 52, wherein the host cell is a Chinese hamster ovary cell.

59. The method of claim 54 where the construct further comprises a gene that expresses peptide to facilitate secretion.

60. A recombinant NELL peptide produced according to any of claims 52-59.

61. A method of identifying a molecule that induces expression of a NELL peptide, comprising:
   contacting a NELL1 promoter gene with a test compound,
   detecting the level of expression of the NELL1 promoter gene,
   comparing the level of expression of the NELL1 promoter gene to the level of expression of the NELL1 promoter gene without the test compound, and
   designating the test compound as a modulator of the expression of the NELL peptide if the level of expression of the NELL1 promoter gene with the test compound is different from the level of expression of the NELL1 promoter gene without the test compound.

62. The method of claim 50, wherein the designating step further comprises:
   designating the modulator as an inhibitor of the expression of the NELL peptide if the level of expression of the NELL1 promoter gene with the test compound is lower than the level of expression of the NELL1 promoter gene without the test compound, or
   designating the modulator as an enhancer of the expression of the NELL peptide if the level of expression of the NELL1 promoter gene with the test compound is higher than the level of expression of the NELL1 promoter gene without the test compound.

63. A method of modulating the expression of a NELL peptide in a mammal, comprising administering to the mammal a modulator identified according to any of claims 61 and 62.

64. A pharmaceutical composition comprising the modulator identified according to any of claims 61-62.
65. A scaffold comprising an effective amount of a NELL peptide, or a NELL RNA.

66. The scaffold of claim 65, wherein the NELL peptide is in a dosage not substantially exceeding an optimal dosage range of the NELL peptide.

67. The scaffold of claim 65, wherein the NELL peptide is selected from the group consisting of NELL1, NELL2, a fragment of NELL1 peptide, a fragment of NELL2 peptide, and combinations thereof.

68. The scaffold of claim 65, wherein the bone related condition is selected from the group consisting of craniofacial bone generation, non-craniofacial bone generation, long bone generation, periodontal bone generation, intramembranous bone generation, endochondral bone generation, cartilage regeneration, cartilage hypertrophy and combinations thereof.

69. The scaffold of claim 65, wherein the bone related condition is selected from the group consisting of osteoporosis, bone loss due to microgravity, disuse atrophy, prolonged bed-rest, and a disease that involves multiple symptoms where bone metabolism is a secondary effect.

70. The scaffold of claim 69, wherein the disease that involves multiple symptoms results in pathological calcification.

71. The scaffold of claim 70, wherein the disease that involves multiple symptoms is a chronic kidney disease which causes renal osteodystrophy and/or vascular calcification.

72. The scaffold of claim 69, wherein the bone related condition is ectopic soft tissue calcification, gall stone, kidney stones, pineal gland calcification, cataracts, salivary stones, cardiac valves, or prostate stones.

73. The scaffold of claim 65, wherein the NELL RNA is mRNA, noncoding RNA, microRNA, dsRNA, or combinations thereof.

74. The scaffold of claim 65, wherein the NELL RNA is stabilized by a chemical or by incorporating in NELL RNA in a nanocage or biomaterial.

75. A method of treating, preventing, or ameliorating a bone related condition, comprising administering to a mammal a scaffold according to any of claims 1-33.
Figure 1

![Diagram showing Nell-1 and TSP-1](image)

Figures 2A-C

![Images of wild-type and different genetic modifications](image)

Figure 2D

NELL Protein

Figure 2E

Vehicle control
Figures 3A-C show that Nell-1 can differentiate bone marrow stem cells (BMSCs) into an osteoblastic phenotype. (A) After 2 weeks of infection with Nell-1 or LacZ, a significant increase was also seen in the formation of calcium nodules in the AdNell-1 BMSCs compared to AdLacZ (n=3; p=0.0018). Figure 3B shows AdNell-1 transduced BMSCs with more bone formation radiographically. Figure 3C, Nell-1 transduced BMSCs shows histology of relatively more mature bone with lamellar pattern (upper panel) than the control (lower panel).
(A) Real time PCR showing that Nell-1 and BMP2 synergistically induced osteopontin, a osteoblast differentiation marker. Nell-1 and BMP2 also synergistically induced osteopontin, another osteoblast differentiation specific marker (data not shown). (B) Arrows indicate Nell-1 and BMP2 co-transduction induced much more bone than either one of them individually. "L" indicates left; "R" indicates right. (C) Excised femur specimen from BMP2+Nell-1 treated (L) and BMP2 treated (R) animals showing significantly increased bone formation the BMP2+Nell-1 treated animals.
Figure 9
Figure 11
SEQUENCE LISTING

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TING, KANG
KURODA, SHUNICHI
WU, BEN

PHARMACEUTICAL COMPOSITIONS FOR TREATING OR PREVENTING
BONE CONDITIONS

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PatentIn Ver. 3.3

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<400> 29
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a, c, g, t, unknown or other

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<211> 31
<212> DNA
<213> Mus musculus

<400> 31
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tagtcagct atacacagat acattttaag gattccatga taatgttaaa agtacctttt 180
tgttatttgg tgttaccaacaa taataagagct tcggaccat ctcatttttt tttttgtttt 240
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<210> 32
<211> 298
<212> DNA
<213> Mus musculus

<400> 32
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<210> 33
<211> 284
<212> DNA
<213> Mus musculus

<220>
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<222> (24)
<223> a, c, g, t, unknown or other

<400> 33
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tctgtttttt gccatataat ctatataaaa agttcctgtg tgg 284

<210> 34
<211> 280
<212> DNA
<213> Mus musculus

<400> 34
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tgttttatttt gttgaccaac atataagaga cttgccacca ttatttttt ttatttttt 240
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<210> 35
<211> 280
<212> DNA
<213> Mus musculus

<400> 35
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<211> 293
<212> DNA
<213> Mus musculus

<400> 36
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<210> 37
<211> 297
<212> DNA
<213> Mus musculus

<400> 37
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<210> 38
<211> 294
<212> DNA
<213> Mus musculus

<400> 38
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<210> 39
<211> 283
<212> DNA
<213> Mus musculus

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<210> 40
<211> 243
<212> DNA
<213> Mus musculus

<400> 40
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<210> 41
<211> 293
<212> DNA
<213> Mus musculus

<400> 41
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<210> 42
<211> 234
<212> DNA
<213> Mus musculus

<220>
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<223> a, c, g, t, unknown or other

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<210> 43
<211> 236
<212> DNA
<213> Mus musculus

<400> 43
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Mus musculus

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ccttattt 247

ctatccc

Mus musculus

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tcctttcgtg gacttccc 310

Mus musculus

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gacaacctggg accatttatt tatattttct tcatttttattt gatttttggga tcaaatctta taataaagtt 180
cctctggctg cttttttccc 200

Mus musculus

Mus musculus
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<210> 49
<211> 294
<212> DNA
<213> Mus musculus

<400> 49
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<210> 50
<211> 214
<212> DNA
<213> Mus musculus

<400> 50
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accacacttac tagagacattgc gccacttcac tttatatattcg ttgattttttt gatcaacttc 180
taaaataaaa gttcctgttt ctcgactttcata ctc 214

<210> 51
<211> 323
<212> DNA
<213> Mus musculus

<400> 51
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<210> 52
<211> 302
<212> DNA
<213> Mus musculus

<400> 52
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<210> 53
<211> 309
<212> DNA
<213> Mus musculus

<400> 53
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gttcgacctt

<210> 54
<211> 287
<212> DNA
<213> Mus musculus

<400> 54
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gattttttgga tcaaaattctta aaatataaagtt cccctgtggt gacctctcttt 287

<210> 55
<211> 299
<212> DNA
<213> Mus musculus

<400> 55
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<210> 56
<211> 287
<212> DNA
<213> Mus musculus

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<210> 57
<211> 288
<212> DNA
<213> Mus musculus

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cataacctt gttcagaagt gcacaaagct ggaataacg cggagaatgc 288

<210> 58
<211> 233
<212> DNA
<213> Mus musculus

<400> 58
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<210> 59
<211> 281
<212> DNA
<213> Mus musculus

<400> 59
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<210> 60
<211> 281
<212> DNA
<213> Mus musculus

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<210> 61
<211> 278
<212> DNA
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<210> 62
<211> 229
<212> DNA
<213> Mus musculus

<400> 62
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<210> 63
<211> 245
<212> DNA
<213> Mus musculus

<400> 63
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<210> 64
<211> 289
<212> DNA
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<210> 65
<211> 294
<212> DNA
<213> Mus musculus

<400> 65
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<210> 71
<211> 265
<212> DNA
<213> Mus musculus

<400> 71
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<222> (684)
<223> a, c, g, t, unknown or other

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