# METHODS, COMPOSITIONS, AND KITS FOR THE TREATMENT OF MATRIX MINERALIZATION DISORDERS

## Background of the Invention

The present invention relates to the treatment of matrix mineralization disorders such as hypophosphatasia (HPP). HPP is a rare, heritable disease caused by one or more loss-of-function mutation in the gene *ALPL*, which encodes tissue-nonspecific alkaline phosphatase (TNALP; a.k.a liver/bone/kidney type ALP). Alkaline phosphatase deficiency in osteoblasts and chondrocytes impairs skeletal mineralization, leading to rickets or osteomalacia. The severity of HPP depends on its age of onset. Perinatal HPP, the most severe form of HPP, results in an almost complete absence of bone mineralization *in utero* and can cause stillbirth. Infantile HPP, diagnosed before the age of six months, results in impaired skeletal mineralization that can lead to fractures and deformities. Childhood HPP can cause the premature loss of deciduous teeth and rickets. Finally, adult HPP can cause stress fractures and attacks of arthritis and pyrophosphate arthropathy.

Given the devastating immediate and long-term impact of matrix mineralization disorders such as HPP on affected patients, particularly infants and children, there is a need to develop methods and compositions for treating matrix mineralization disorders.

## Summary of the Invention

In the present invention, it has surprisingly been found that an alkaline phosphatase-Fc fusion protein lacking a polyaspartic acid or polyglutamic acid region is effective in treating HPP in an HPP mouse model. Accordingly, the present invention provides compositions including an sALP-Fc or Fc-sALP polypeptide and compositions including a nucleic acid that encodes an sALP-Fc or Fc-sALP polypeptide. The invention also provides methods and kits for using such polypeptides and nucleic acids to treat matrix mineralization disorders such as HPP and its associated phenotypes.

Accordingly, in one aspect, the invention features a method of treating a matrix mineralization disorder in a subject, the method including administering to the

subject a therapeutically effective amount of a pharmaceutical composition including: (a) a polypeptide including the structure Z-sALP-Y-Fc-X or the structure Z-Fc-Y-sALP-X; and (b) a pharmaceutically acceptable excipient, where sALP is the extracellular domain of an alkaline phosphatase; each of X, Y, and Z is absent or is an amino acid sequence of at least one amino acid; and the polypeptide does not include a polyaspartic acid or polyglutamic acid region longer than three consecutive aspartic acid or glutamic acid residues.

In another aspect, the invention features a pharmaceutical composition including: (a) a polypeptide including the structure Z-sALP-Y-Fc-X or the structure Z-Fc-Y-sALP-X; and (b) a pharmaceutically acceptable excipient, where sALP is the extracellular domain of an alkaline phosphatase; each of X, Y, and Z is absent or is an amino acid sequence of at least one amino acid; and the polypeptide does not include a polyaspartic acid or polyglutamic acid region longer than three consecutive aspartic acid or glutamic acid residues.

In yet another aspect, the invention features a method of treating a matrix mineralization disorder in a subject, the method including administering to the subject a therapeutically effective amount of a pharmaceutical composition including: (a) an isolated nucleic acid encoding a polypeptide including the structure Z-sALP-Y-Fc-X or the structure Z-Fc-Y-sALP-X; and (b) a pharmaceutically acceptable excipient, where sALP is the extracellular domain of an alkaline phosphatase; each of X, Y, and Z is absent or is an amino acid sequence of at least one amino acid; and the isolated nucleic acid does not encode an amino acid sequence including a polyaspartic acid or polyglutamic acid region longer than three consecutive aspartic acid or glutamic acid residues.

In another aspect, the invention features a pharmaceutical composition formulated for the treatment of a matrix mineralization disorder in a subject, the pharmaceutical composition including: (a) an isolated nucleic acid encoding a polypeptide including the structure Z-sALP-Y-Fc-X or the structure Z-Fc-Y-sALP-X; and (b) a pharmaceutically acceptable excipient, where sALP is the extracellular domain of an alkaline phosphatase; each of X, Y, and Z is absent or is an amino acid sequence of at least one amino acid; and the isolated nucleic acid does not encode an amino acid sequence including a polyaspartic acid or polyglutamic acid region longer than three consecutive aspartic acid or glutamic acid residues.

In any embodiment of the invention, the polypeptide may optionally include the structure Z-sALP-Y-Fc-X.

In any embodiment, the polypeptide optionally does not include a polyaspartic acid or polyglutamic acid region longer than two consecutive aspartic acid or glutamic acid residues.

In any embodiment, the polypeptide optionally does not include a bone-targeting moiety.

In any embodiment, the amino acid sequence of the sALP optionally includes or consists of amino acid residues 23-508 of SEQ ID NO: 15. For example, the amino acid sequence of the sALP optionally includes or consists of amino acid residues 23-512 of SEQ ID NO: 15.

In any embodiment, the amino acid sequence of the sALP optionally includes or consists of amino acid residues 18-498 of SEQ ID NO: 16. For example, the amino acid sequence of the sALP may optionally include or consist of amino acid residues 18-502 of SEQ ID NO: 16.

In any embodiment, the amino acid sequence of the sALP optionally includes an amino acid sequence having at least 85% (e.g., at least 95% or 99%) sequence identity to SEQ ID NO: 5. For example, the amino acid sequence of the sALP optionally includes SEQ ID NO: 5 or consists of SEQ ID NO: 5.

In any embodiment, the Fc may optionally include a $C_{H2}$ domain, a $C_{H3}$ domain and a hinge region. For example, the Fc optionally is a constant domain of an immunoglobulin selected from the group consisting of IgG-1, IgG-2, IgG-3, IgG-3 and IgG-4. In particular embodiments, the immunoglobulin is IgG-1. In other particular embodiments, the Fc includes an amino acid sequence having at least 85% (e.g., at least 95% or 99%) sequence identity to SEQ ID NO: 3. For example, the amino acid sequence of the Fc optionally includes SEQ ID NO: 3 or consists of SEQ ID NO: 3.

In any embodiment, Z is optionally absent.

In any embodiment, Y is optionally two amino acid residues (e.g., leucine-lysine).

In any embodiment, X is optionally absent.

In any embodiment, Z and X may both be absent, and Y may be absent or may be an amino acid sequence of at least one amino acid. For example, the polypeptide

may consist of the structure sALP-Y-Fc or the structure Fc-Y-sALP. In some embodiments of polypeptides consisting of the structure sALP-Y-Fc or Fc-Y-sALP, Y may consist of two amino acid residues, e.g., leucine-lysine. For example, the polypeptide may consist of the structure sALP-Y-Fc. Optionally, the amino acid sequence of sALP is the amino acid sequence of SEQ ID NO: 5, the amino acid sequence of Y is leucine-lysine, and/or the amino acid sequence of Fc is the amino acid sequence of SEQ ID NO: 3. For example, the amino acid sequence of the polypeptide may consist of the amino acid sequence of SEQ ID NO: 4.

In any embodiment, the amino acid sequence of the polypeptide optionally includes an amino acid sequence having at least 85% (e.g., at least 95% or 99%) sequence identity to SEQ ID NO: 4. For example, the amino acid sequence of the polypeptide may optionally include SEQ ID NO: 4 or consist of SEQ ID NO: 4.

In any embodiment, the polypeptide optionally is pegylated or glycosylated.

In any embodiment, the pharmaceutical composition optionally includes a dimer of the polypeptide.

In any embodiment, the pharmaceutically acceptable excipient optionally includes saline.

In any embodiment, the pharmaceutical composition is optionally lyophilized.

In any of the methods of the invention, the pharmaceutical composition is optionally administered subcutaneously, intravenously, orally, nasally, intramuscularly, sublingually, intrathecally, or intradermally. In certain embodiments, the pharmaceutical composition is administered subcutaneously. For example, the methods of the invention may optionally include administering a pharmaceutical composition including the polypeptides of the invention to the subject in a dosage of about 0.5 mg/kg/day to about 10 mg/kg/day (e.g., about 2 mg/kg/day to about 3 mg/kg/day). In other examples, the methods include administering the pharmaceutical composition to the subject between one and seven times a week (e.g., three times a week).

In any of the methods of the invention, the matrix mineralization disorder is optionally hypophosphatasia (e.g., infantile HPP, childhood HPP, perinatal HPP, adult HPP, or odontohypophosphatasia).

In any of the methods of the invention, the pharmaceutical composition is optionally administered in an amount that is therapeutically effective to treat a HPP

phenotype selected from the group consisting of HPP-related seizure, premature loss of deciduous teeth, incomplete bone mineralization, elevated blood and/or urine levels of inorganic pyrophosphate ($PP_i$), elevated blood and/or urine levels of phosphoethanolamine (PEA), elevated blood and/or urine levels of pyridoxal 5'-phosphate (PLP), inadequate weight gain, rickets, bone pain, calcium pyrophosphate dihydrate crystal deposition, aplasia, hypoplasia, and dysplasia of the dental cementum. In some embodiments, the incomplete bone mineralization is incomplete femoral bone mineralization, incomplete tibial bone mineralization, incomplete metatarsal bone mineralization, or incomplete rib bone mineralization.

In any embodiment, the subject may be human.

Any of the pharmaceutical compositions of the invention may optionally be formulated for treating a matrix mineralization disorder in a subject.

In any of the methods of the invention featuring the administration of an isolated nucleic acid, the isolated nucleic acid is optionally administered to the subject in a lentiviral vector. In some embodiments, the isolated nucleic acid is optionally administered to the subject at a dosage of from about 0.1 mg to about 10 mg.

Any of the pharmaceutical composition of the invention featuring an isolated nucleic acid may optionally include a recombinant expression vector (e.g., a lentiviral vector) including the isolated nucleic acid.

The invention also features an isolated recombinant host cell transformed or transfected with such a vector.

The invention also features methods of producing any polypeptide of the invention, including culturing such a host cell in a culture medium under conditions suitable to effect expression of the polypeptide and recovering the polypeptide from the culture medium. For example, the host cell is optionally an L cell, a C127 cell, a 3T3 cell, a CHO cell, a BHK cell, or a COS-7 cell. In some embodiments, the host cell is a CHO cell (e.g., a CHO-DG44 cell).

The invention also features kits. For example, the invention features a kit including: (a) any of the pharmaceutical compositions of the invention and (b) instructions for administering the pharmaceutical composition to a subject to treat a matrix mineralization disorder.

By "about" is meant ±10% of the recited value.

By "bone-targeting moiety" is meant an amino acid sequence of between 6 and 20 amino acid residues in length having a sufficient affinity to the bone matrix such that the bone-targeting moiety has an *in vivo* binding affinity to the bone matrix that is at least $10^{-6}$ M or better (e.g., $10^{-7}$ M, $10^{-8}$ M, $10^{-9}$ M, or better).

By "Fc" is meant a fragment crystallizable region of an immunoglobulin, e.g., IgG-1, IgG-2, IgG-3, IgG-3 or IgG-4, including the $C_{H2}$ and $C_{H3}$ domains of the immunoglobulin heavy chain. Fc may also include any portion of the hinge region joining the Fab and Fc regions. The Fc can be of any mammal, including human, and may be post-translationally modified (e.g., by glycosylation). In a non-limiting example, Fc can be the fragment crystallizable region of human IgG-1 having the amino acid sequence of SEQ ID NO: 3.

By "matrix mineralization disorder" is meant a disorder affecting mineralization of the bone matrix or any phenotype associated with the disorder. Matrix mineralization disorders and their associated phenotypes include, for example, rickets (defects in growth plate cartilage), osteomalacia, osteogenesis imperfecta, severe osteoporosis, and hypophosphatasia (HPP) (e.g., infantile HPP, childhood HPP, perinatal HPP, adult HPP, or odontohypophosphatasia), HPP-related seizure, premature loss of deciduous teeth, incomplete bone mineralization, elevated blood and/or urine levels of inorganic pyrophosphate ($PP_i$), elevated blood and/or urine levels of phosphoethanolamine (PEA), elevated blood and/or urine levels of pyridoxal 5'-phosphate (PLP), inadequate weight gain, bone pain, calcium pyrophosphate dihydrate (CPPD) crystal deposition, and aplasia, hypoplasia or dysplasia of the dental cementum. Matrix mineralization disorders can be diagnosed, for example, by growth retardation with a decrease of long bone length (such as femur, tibia, humerus, radius, ulna), a decrease of the mean density of total bone and a decrease of bone mineralization in bones such as femur, tibia, ribs and metatarsi, and phalange, a decrease in teeth mineralization, and premature loss of deciduous teeth (e.g., aplasia, hypoplasia or dysplasia of dental cementum). Without being so limited, treatment of matrix mineralization disorders may be observed by one or more of the following: an increase of long bone length, an increase of mineralization in bone and/or teeth, a correction of bowing of the legs, a reduction of bone pain and a reduction of CPPD crystal deposition in joints.

By "pharmaceutical composition" is meant a composition containing a polypeptide or nucleic acid described herein, formulated with a pharmaceutically acceptable excipient, and manufactured or sold with the approval of a governmental regulatory agency as part of a therapeutic regimen for the treatment of disease in a mammal. Pharmaceutical compositions can be formulated, for example, for oral administration in unit dosage form (e.g., a tablet, capsule, caplet, gelcap, or syrup); for topical administration (e.g., as a cream, gel, lotion, or ointment); for intravenous administration (e.g., as a sterile solution free of particulate emboli and in a solvent system suitable for intravenous use); for subcutaneous administration; or any other formulation described herein.

By "pharmaceutically acceptable excipient" is meant a carrier that is physiologically acceptable to the treated subject while retaining the therapeutic properties of the compound with which it is administered. One exemplary pharmaceutically acceptable excipient is physiological saline. Other physiologically acceptable excipients and their formulations are known to one skilled in the art and described, for example, in "Remington: The Science and Practice of Pharmacy" (20th ed., ed. A.R. Gennaro, 2000, Lippincott Williams & Wilkins).

By "polypeptide" is meant any natural or synthetic chain of amino acids at least two amino acids in length, including those having post-translational modification (e.g, glycosylation or phosphorylation).

As used herein, when a polypeptide or nucleic acid sequence is referred to as having "at least X% sequence identity" to a reference sequence, it is meant that at least X percent of the amino acids or nucleotides in the polypeptide or nucleic acid are identical to those of the reference sequence when the sequences are optimally aligned. An optimal alignment of sequences can be determined in various ways that are within the skill in the art, for instance, the Smith Waterman alignment algorithm (Smith et al., *J. Mol. Biol.* 147:195-7, 1981) and BLAST (Basic Local Alignment Search Tool; Altschul et al. *J. Mol. Biol.* 215: 403-10, 1990). These and other alignment algorithms are accessible using publicly available computer software such as "Best Fit" (Smith and Waterman, Advances in Applied Mathematics, 482-489, 1981) as incorporated into GeneMatcher Plus™ (Schwarz and Dayhof, Atlas of Protein Sequence and Structure, Dayhoff, M.O., Ed pp 353-358, 1979), BLAST, BLAST-2, BLAST-P, BLAST-N, BLAST-X, WU-BLAST-2, ALIGN, ALIGN-2, CLUSTAL, or

Megalign (DNASTAR). In addition, those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve optimal alignment over the length of the sequences being compared. In general, for polypeptides, the length of comparison sequences can be at least five amino acids, preferably 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250, 300, 400, 500, 600, 700, or more amino acids, up to the entire length of the polypeptide. For nucleic acids, the length of comparison sequences can generally be at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, or more nucleotides, up to the entire length of the nucleic acid molecule. It is understood that for the purposes of determining sequence identity when comparing a DNA sequence to an RNA sequence, a thymine nucleotide is equivalent to a uracil nucleotide.

The terms "sALP," "soluble alkaline phosphatase," and "extracellular domain of an alkaline phosphatase" are used interchangeably and mean a soluble, non-membrane-bound alkaline phosphatase or a domain, biologically active fragment, or biologically active variant thereof. sALPs include, for example, an alkaline phosphatase lacking a C-terminal GPI signal sequence, e.g., a polypeptide including or consisting of the amino acids 18-502 of human TNALP (SEQ ID NO: 6). sALPs further include, for example, soluble, non-membrane-bound forms of mammalian orthologs of human TNALP (e.g., polypeptides including or consisting of amino acids 16-502 or 18-502 of SEQ ID NO: 7, amino acids 18-502 of SEQ ID NO: 8, amino acids 18-502 of SEQ ID NO: 9, amino acids 18-502 of SEQ ID NO: 10, or amino acids 1-480 of SEQ ID NO: 11), soluble, non-membrane-bound forms of human IALP, GCALP, and PLALP (e.g., polypeptides including or consisting of amino acids 20-503 of SEQ ID NO: 12, amino acids 20-503 of SEQ ID NO: 13, or amino acids 23-506 of SEQ ID NO: 14), and additional variants and analogs thereof which retain alkaline phosphatase activity, e.g., the ability to hydrolyze $PP_i$.

By "subject" is meant any mammal, e.g., a human.

By "therapeutically effective amount" is meant an amount of a nucleic acid or polypeptide of the invention that is sufficient to substantially treat, prevent, delay, suppress, or arrest any symptom of a matrix mineralization disorder. A therapeutically effective amount of a compound of the invention may depend on the

severity of the matrix mineralization disorder and the condition, weight and general state of the subject and can be determined by ordinarily-skilled artisan with consideration of such factors. A therapeutically effective amount of a compound of the invention can be administered to a mammal in a single dose or in multiple doses administered over a period of time.

By "treating" is meant administering a pharmaceutical composition for prophylactic and/or therapeutic purposes. To "prevent disease" refers to prophylactic treatment of a subject who is not yet ill, but who is susceptible to, or otherwise at risk of, a particular disease. To "treat disease" or use for "therapeutic treatment" refers to administering treatment to a subject already suffering from a disease to improve or stabilize the subject's condition. Thus, in the claims and embodiments, treating is the administration to a subject either for therapeutic or prophylactic purposes.

## Brief Description of the Drawings

Figure 1 shows schematic representations of polypeptides of the present invention. (a) The complete primary translation product of the human tissue non-specific alkaline phosphatase gene (hTNALP), including the N-terminal signal peptide and the transient membrane-anchored signal for GPI addition. (b) The primary translation product of the hsTNALP-Fc fusion protein. (c) The secreted hsTNALP-Fc, which lacks the N-terminal signal peptide.

Figure 2 shows the amino acid sequence of hsTNALP-Fc (SEQ ID NO: 1), including the N-terminal signal peptide (the first 17 amino acid residues, underlined and italicized; position 2, a valine, differs from the wild-type residue in that position, isoleucine). Amino acids of the hsTNALP portion of the polypeptide (SEQ ID NO: 2), which correspond to amino acids 1-502 of hTNALP (SEQ ID NO: 6), with the exception of position 2 as noted above, are italicized. The Fc portion of this polypeptide (SEQ ID NO: 3) is underlined. A two amino acid leucine-lysine linker between the hsTNALP and Fc portions is shown in bold.

Figure 3 shows the amino acid sequence of secreted hsTNALP-Fc (SEQ ID NO: 4), which lacks the N-terminal signal peptide. Amino acids of the hsTNALP portion of the polypeptide (SEQ ID NO: 5), which correspond to amino acids 18-502 of hTNALP, are italicized. The Fc portion of this polypeptide (SEQ ID NO: 3) is

underlined. A two amino acid leucine-lysine linker between the hsTNALP and Fc portions is shown in bold.

Figures 4(a)-(d) show a CLUSTAL™ W (1.82) multiple sequence alignment of mammalian TNALP orthologs and human ALP isozymes. Mammalian TNALP orthologs include human TNALP ("sp|P05186|TNALP_HUMAN"; Accession No. P05186; SEQ ID NO: 6), cow TNALP ("sp|P09487|TNALP_BOVINE"; Accession No. P09487; SEQ ID NO: 7); cat TNALP ("sp|Q29486|TNALP_FELCA"; Accession No. Q29486; SEQ ID NO: 8), mouse TNALP ("sp|P09242|TNALP_MOUSE"; Accession No. P09242; SEQ ID NO: 9), rat TNALP ("sp|P08289|TNALP_RAT"; Accession No. P08289; SEQ ID NO: 10), and a partial sequence of dog TNALP ("tr|Q9N0V0|TNALP_CANFA"; Accession No. Q9N0V0; SEQ ID NO: 11). Human ALP isozymes include a human IALP ("sp|P09923|IALP_HUMAN"; Accession No. P09923; SEQ ID NO: 12), a human GCALP ("sp|P10696|GCALP_HUMAN"; Accession No. P10696; SEQ ID NO: 13), and a human PLALP ("sp|P05187|PLALP_HUMAN"; Accession No. 05187; SEQ ID NO: 14). "*" denotes that the residues in that column are identical in all sequences of the alignment, ":" denotes that conserved substitutions are observed, and "." denotes that semi-conserved substitutions are observed. A consensus sequence is derived from this alignment ("Consensus"; SEQ ID NO: 15), where X denotes degenerate positions.

Figures 5(a)-(c) show a CLUSTAL™ W (1.82) multiple sequence alignment of mammalian TNALP orthologs, including human TNALP ("sp|P05186|TNALP_HUMAN"; Accession No. P05186; SEQ ID NO: 6), cow TNALP ("sp|P09487|TNALP_BOVINE"; Accession No. P09487; SEQ ID NO: 7); cat TNALP ("sp|Q29486|TNALP_FELCA"; Accession No. Q29486; SEQ ID NO: 8), mouse TNALP ("sp|P09242|TNALP_MOUSE"; Accession No. P09242; SEQ ID NO: 9), rat TNALP ("sp|P08289|TNALP_RAT"; Accession No. P08289; SEQ ID NO: 10), and a partial sequence of dog TNALP ("tr|Q9N0V0|TNALP_CANFA"; Accession No. Q9N0V0; SEQ ID NO: 11). "*" denotes that the residues in that column are identical in all sequences of the alignment, ":" denotes that conserved substitutions are observed, and "." denotes that semi-conserved substitutions are observed. A consensus sequence is derived from this alignment ("Consensus"; SEQ ID NO: 16), where X denotes degenerate positions.

Figure 6 shows a nucleic acid sequence (SEQ ID NO: 17) encoding the hsTNALP-Fc polypeptide depicted in Figure 2.

Figure 7 shows the mean (±SD) concentration profile in serum following single dose IV and SC administration of (a) sALP and (b) sALP-Fc.

Figure 8 shows (a) percentage survival and (b) median survival of *Akp2*$^{-/-}$ mice receiving either Vehicle or escalating doses of sALP-Fc, i.e., Tx-1, Tx-3, and Tx-6.

Figure 9 shows changes in body weight with age as a function of treatment dose. Average daily body weights from Day 1 to the end of the study of *Akp2*$^{-/-}$ mice treated with Vehicle, Tx-1, Tx-3, Tx-6 or untreated WT mice.

Figure 10 shows tibiae/femora lengths as a function of treatment dose. Individual lengths of the left tibia and femora at the end of the study for two treatment doses compared to WT.

Figure 11 shows radiographs of representative left foot (radiographic magnification 3X), rib cage (radiographic magnification (2X), and right lower limb specimens (radiographic magnification 2X) in *Akp2*$^{-/-}$ and normal mice.

Figure 12 shows the percentage of normal individuals as a function of sALP-Fc dose in relation to mineralization improvement in feet.

## Detailed Description

The present invention features soluble alkaline phosphatase (sALP) fused to Fc, nucleic acid encoding such, and their uses to treat matrix mineralization disorders such as hypophosphatasia. Additional details of the invention are provided below.

### Alkaline phosphatase

Alkaline phosphatases encompass a group of enzymes that share the property of being able to cleave phosphate in a variety of contexts (e.g., hydrolysis of pyrophosphate, $PP_i$). There are four known mammalian alkaline phosphatase (ALP) isozymes: tissue non-specific alkaline phosphatase (TNALP; described further below), placental alkaline phosphatase (PLALP) (e.g., Accession Nos. P05187, NP_112603, and NP_001623), germ cell alkaline phosphatase (GCALP) (e.g., Accession No. P10696), and intestinal alkaline phosphatase (IALP) (e.g., Accession Nos. P09923 and NP_001622). These isozymes possess very similar three dimensional structures. Each of their catalytic sites contains four metal binding

domains that bind to metal ions necessary for enzymatic activity, including two zinc ions and one magnesium ion. These enzymes catalyze the hydrolysis of monoesters of phosphoric acid and also catalyze a transphosphorylation reaction in the presence of high concentrations of phosphate acceptors. It has been shown that PALP is physiologically active toward phosphoethanolamine (PEA), inorganic pyrophosphate ($PP_i$), and pyridoxal 5'-phosphate (PLP), all three being known natural substrate for TNALP (Whyte, 1995). An alignment between these isozymes is shown in Figures 4(a)-4(d). Additional alkaline phosphatases are described, e.g., in WO 2008/138131 and in U.S. Publication No. 2006/0014687, which are hereby incorporated by reference.

Tissue non-specific phosphatases are a family of proteins, encoded by a single genes, that differ from each other by post-translational modification. TNALPs are present predominantly in the liver, kidneys, and bone, but can occur throughout the body. Known TNALPs in mammals include, e.g., human TNALP (Accession Nos. NP-000469, AAI10910, AAH90861, AAH66116, AAH21289, and AAI26166); rhesus TNALP (Accession No. XP-001109717); rat TNALP (Accession No. NP_037191); dog TNALP (Accession No. AAF64516); pig TNALP (Accession No. AAN64273), mouse (Accession No. NP_031457), cow TNALP (Accession Nos. NP_789828, NP_776412, AAM 8209, AAC33858), and cat TNALP (Accession No. NP_001036028), in addition to other examples provided herein.

**Soluble alkaline phosphatase**

The soluble alkaline phosphatases (sALP) of the invention include, for example, soluble (e.g., extracellular or non membrane-bound) forms of any of the alkaline phosphatases described herein. The soluble alkaline phosphatase of the invention can be, for example, a soluble form of human TNALP. A schematic representation of the domains of human TNALP (hTNALP) is shown in Figure 1. TNALP is a membrane-bound protein anchored through a glycolipid bound to its C-terminal (Swiss-Prot, P05186). This glycolipid anchor (GPI) is added post translationally after the removal of a hydrophobic C-terminal end, which serves both as a temporary membrane anchor and as a signal for the addition of the GPI. This GPI anchor is buried in the cell membrane, and the remaining portions of the protein are extracellular. TNALP, including hTNALP, can be engineered to replace the first

amino acid of the hydrophobic C-terminal sequence (an alanine) with a stop codon. The engineered hTNALP so formed contains all amino acids of the native anchored form of TNALP but lacks the GPI membrane anchor. An hTNALP which is soluble is herein referred to as "hsTNALP". One skilled in the art will appreciate that the position of the GPI membrane anchor will vary in different alkaline phosphatases and may include, for example, the last 10, 12, 14, 16, 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 34, 36, 38, 40, 45, 50, or more amino acid residues on the C-terminus of the polypeptide. For example, the GPI membrane anchor of the hTNALP of SEQ ID NO: 6 is amino acids 503-524. The amino acid sequence of this hsTNALP (with one variation at position 2), as fused to Fc, is shown in Figure 2. The sequence of a nucleic acid encoding this hsTNALP-Fc fusion polypeptide is shown in Figure 6.

In addition to the C-terminal GPI anchor, TNALP also has an N-terminal signal peptide sequence. The N-terminal signal peptide is initially present on the protein when it is synthesized, but is cleaved after translocation into the ER. Thus, the N-terminal signal peptide is absent from the secreted form of TNALP. The sALPs of the invention include both secreted (i.e., lacking the N-terminal signal) and non-secreted (i.e., having the N-terminal signal) forms thereof. One skilled in the art will appreciate that the position of the N-terminal signal peptide will vary in different alkaline phosphatases and may include, for example, the first 5, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 27, 30, or more amino acid residues on the N-terminus of the polypeptide. For example, the N-terminal signal peptide of the hTNALP of SEQ ID NO: 6 is its first 17 amino acids. Thus, a secreted, soluble form of this hTNALP is amino acids 18-502 of SEQ ID NO: 6 (SEQ ID NO: 5). The amino acid sequence of this secreted hsTNALP, as fused to Fc, is shown in Figure 3. The sALPs of the invention include both secreted and non-secreted forms thereof. One of skill in the art can predict the position of a signal sequence cleavage site, e.g., by an appropriate computer algorithm such as that described in Bendtsen et al. (*J. Mol. Biol.* 340(4):783-795, 2004) and available on the Web at http://www.cbs.dtu.dk/services/SignalP/.

The sALPs of the invention also include, for example, polypeptide sequences satisfying a consensus sequence derived from the ALP extracellular domain of human ALP isozymes and of mammalian TNALP orthologs (human, mouse, rat, cow, cat, and dog) (SEQ ID NO:15), or a consensus derived from just the ALP extracellular

domain of human ALP isozymes (SEQ ID NO:16). The sALPs of the invention also include those which satisfy similar consensus sequences derived various combinations of these TNALP orthologs or human ALP isozymes. Such consensus sequences are given, for example, in WO 2008/138131, herein incorporated by reference.

Furthermore, it has been shown that recombinant hsTNALP retaining original amino acids 1 to 501 (18 to 501 when secreted) (Oda et al., *J. Biochem* 126: 694-699, 1999), amino acids 1 to 502 (18 to 502 when secreted) (WO 2008/138131), amino acids 1 to 504 (18 to 504 when secreted) (US 6,905,689, which is herein incorporated by reference) and amino acids 1 to 505 (18-505 when secreted) (US 2007/0081984, which is herein incorporated by reference), are enzymatically active. This indicates that certain amino acid residues can be truncated from the C-terminal end of the soluble hsTNALP polypeptide without affecting its enzymatic activity. This also indicates that certain amino acids residues of the GPI membrane anchor, when present, do not significantly affect the solubility of the polypeptide. Hence, the sALPs of the invention also include those where, e.g., up to five (e.g., one, two, three, four, or five) amino acid residues are truncated on its C-terminal end, and those where, e.g., up to five (e.g., one, two, three, four, or five) amino acid residues of the GPI membrane anchor are present. For example, non-secreted sALPs of the invention include those containing amino acid residues 1-497, 1-498, 1-499, 1-500, 1-501, 1-502, 1-503, 1-504, 1-505, 1-506 or 1-507 of SEQ ID NO: 6 as well as variants thereof where the amino acid at position 2 is a valine, and secreted sALPs of the invention include those containing amino acid residues 18-497, 18-498, 18-499, 18-500, 18-501, 18-502, 18-503, 18-504, 18-505, 18-506, or 18-507 of SEQ ID NO: 6.

One skilled in the art will appreciate that many mutations in the amino acid sequence of an enzyme will not significantly disrupt the catalytic function of the enzyme. In some cases, certain mutation may even benefit the catalytic function of the enzyme in the context of therapy for matrix mineralization disorders. Therefore, the sALPs of the invention include not only the wild type sequence of the alkaline phosphatases described above, but also include any polypeptide having at least 50% (e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more) sequence identity to these alkaline phosphatases. It is known, however, that specific mutations in TNALP are

known to cause HPP. Such pathogenic mutations are preferably absent in the sALPs of the invention. Examples of such mutations are listed below.

In particular, the sALPs of the invention, using the numbering of the consensus sequence of SEQ ID NO:15, the amino acid at position 22 is not a phenylalanine residue; the amino acid at position 33 (position 11 in the sequence without signal peptide) is not a cysteine residue; the amino acid at position 38 (position 16 in the sequence without signal peptide) is not a valine residue; the amino acid at position 42 (position 20 in the sequence without signal peptide) is not a proline residue; the amino acid at position 45 (position 23 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 56 (position 34 in the sequence without signal peptide) is not a serine or a valine residue; the amino acid residue at position 67 (position 45 in the sequence without signal peptide) is not a leucine, an isoleucine or a valine residue; the amino acid residue at position 68 (position 46 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 73 (position 51 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 76 (position 54 in the sequence without signal peptide) is not a cysteine, a serine, a proline or a histidine residue; the amino acid residue at position 77 (position 55 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 80 (position 58 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 81 (position 59 in the sequence without signal peptide) is not an asparagine residue; the amino acid residue at position 105 (position 83 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 113 (position 89 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 116 (position 94 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 117 (position 95 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 119 (position 97 in the sequence without signal peptide) is not a glycine residue; the amino acid residue at position 121 (position 99 in the sequence without signal peptide) is not a serine or a threonine residue; the amino acid residue at position 125 (position 103 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 128 (position 106 in the sequence without signal peptide) is not a aspartate residue; the amino acid residue at position 133

(position 111 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 134 (position 112 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 137 (position 115 in the sequence without signal peptide) is not a threonine or a valine residue; the amino acid residue at position 139 (position 117 in the sequence without signal peptide) is not a histidine or an asparagine residue; the amino acid residue at position 141 (position 119 in the sequence without signal peptide) is not a histidine residue; the amino acid residue at position 153 (position 131 in the sequence without signal peptide) is not an alanine or an isoleucine residue; the amino acid residue at position 167 (position 145 in the sequence without signal peptide) is not a serine or a valine residue; the amino acid residue at position 172 (position 150 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 175 (position 153 in the sequence without signal peptide) is not an aspartate residue; the amino acid residue at position 176 (position 154 in the sequence without signal peptide) is not a tyrosine or an arginine residue; the amino acid residue at position 181 (position 159 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 182 (position 160 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 184 (position 162 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 186 (position 164 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 189 (position 167 in the sequence without signal peptide) is not a tryptophan residue; the amino acid residue at position 194 (position 172 in the sequence without signal peptide) is not a glutamate residue; the amino acid residue at position 196 (position 174 in the sequence without signal peptide) is not a lysine or a glycine residue; the amino acid residue at position 197 (position 175 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 198 (position 176 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 206 (position 184 in the sequence without signal peptide) is not a tyrosine residue; the amino acid residue at position 208 (position 186 in the sequence without signal peptide) is not a glutamate residue; the amino acid residue at position 207 (position 190 in the sequence without signal peptide) is not a proline residue; the amino acid residue at position 216 (position 194 in the sequence without signal peptide) is not a aspartate

residue; the amino acid residue at position 217 (position 195 in the sequence without signal peptide) is not a phenylalanine residue; the amino acid residue at position 223 (position 201 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 225 (position 203 in the sequence without signal peptide) is not a valine or an alanine residue; the amino acid residue at position 226 (position 204 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 228 (position 206 in the sequence without signal peptide) is not a tryptophan or a glutamine residue; the amino acid residue at position 229 (position 207 in the sequence without signal peptide) is not a glutamate residue; the amino acid residue at position 231 (position 209 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 240 (position 218 in the sequence without signal peptide) is not a glycine residue; the amino acid residue at position 251 (position 229 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 254 (position 232 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 269 (position 247 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 277 (position 255 in the sequence without signal peptide) is not a cysteine, a leucine or a histidine residue; the amino acid residue at position 280 (position 258 in the sequence without signal peptide) is not a proline residue; the amino acid residue at position 295 (position 273 in the sequence without signal peptide) is not a phenylalanine residue; the amino acid residue at position 297 (position 275 in the sequence without signal peptide) is not a lysine residue; the amino acid residue at position 298 (position 276 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 300 (position 278 in the sequence without signal peptide) is not a tyrosine or an alanine residue; the amino acid residue at position 301 (position 279 in the sequence without signal peptide) is not a valine, a threonine or an isoleucine residue; the amino acid residue at position 303 (position 281 in the sequence without signal peptide) is not an aspirate residue; the amino acid residue at position 304 (position 282 in the sequence without signal peptide) is not a lysine residue; the amino acid residue at position 305 (position 283 in the sequence without signal peptide) is not a praline residue; the amino acid residue at position 312 (position 290 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 313 (position 291 in the sequence without

signal peptide) is not a serine or a leucine residue; the amino acid residue at position 317 (position 295 in the sequence without signal peptide) is not a lysine residue; the amino acid residue at position 332 (position 310 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 333 (position 311 in the sequence without signal peptide) is not a cysteine, a glycine or a leucine residue; the amino acid residue at position 334 (position 312 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 340 (position 318 in the sequence without signal peptide) is not an aspartate residue; the amino acid residue at position 345 (position 323 in the sequence without signal peptide) is not an arginine or a glutamate residue; the amino acid residue at position 354 (position 332 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 360 (position 338 in the sequence without signal peptide) is not an aspartate residue; the amino acid residue at position 361 (position 339 in the sequence without signal peptide) is not a threonine or an isoleucine residue; the amino acid residue at position 377 (position 355 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 380 (position 358 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 383 (position 361 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 384 (position 362 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 387 (position 365 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 388 (position 366 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 395 (position 373 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 397 (position 375 in the sequence without signal peptide) is not a cysteine or a histidine residue; the amino acid residue at position 398 (position 376 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 401 (position 379 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 405 (position 383 in the sequence without signal peptide) is not a serine or a valine residue; the amino acid residue at position 406 (position 384 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 412 (position 390 in the sequence without signal peptide) is not a glycine residue; the amino acid residue at position 416 (position 394

in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 417 (position 395 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 420 (position 398 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 423 (position 401 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 426 (position 404 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 429 (position 407 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 430 (position 408 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 432 (position 410 in the sequence without signal peptide) is not a cysteine or an aspartate residue; amino acid residue at position 434 (position 412 in the sequence without signal peptide) is not a proline residue; amino acid residue at position 435 (position 413 in the sequence without signal peptide) is not a lysine residue; amino acid residue at position 442 (position 420 in the sequence without signal peptide) is not a histidine residue; amino acid residue at position 451 (position 429 in the sequence without signal peptide) is not a proline residue; amino acid residue at position 456 (position 434 in the sequence without signal peptide) is not a histidine or a cysteine residue; amino acid residue at position 458 (position 436 in the sequence without signal peptide) is not a lysine residue; amino acid residue at position 460 (position 438 in the sequence without signal peptide) is not an arginine residue; amino acid residue at position 461 (position 439 in the sequence without signal peptide) is not a serine or an aspartate residue; amino acid residue at position 462 (position 440 in the sequence without signal peptide) is not a tryptophan or an arginine residue; amino acid residue at position 465 (position 443 in the sequence without signal peptide) is not a methionine or a leucine residue; amino acid residue at position 472 (position 450 in the sequence without signal peptide) is not a leucine residue; amino acid residue at position 473 (position 451 in the sequence without signal peptide) is not a threonine residue; amino acid residue at position 474 (position 452 in the sequence without signal peptide) is not a threonine residue; amino acid residue at position 479 (position 457 in the sequence without signal peptide) is not a serine residue; amino acid residue at position 482 (position 460 in the sequence without signal peptide) is not a lysine or a glycine residue; amino acid residue at position 484 (position 462 in the sequence without signal peptide) is not a leucine

residue; amino acid residue at position 495 (position 473 in the sequence without signal peptide) is not a serine residue; amino acid residue at position 496 (position 474 in the sequence without signal peptide) is not a phenylalanine residue; and amino acid residue at position 497 (position 475 in the sequence without signal peptide) is not an arginine residue.

Also more specifically, when a sTNALP is used in the bone targeted sALPs of the present invention, using the numbering of the human TNALP sequence, the amino acid at position 17 is not a phenylalanine residue; the amino acid at position 28 (position 11 in the sequence without signal peptide) is not a cysteine residue; the amino acid at position 33 (position 16 in the sequence without signal peptide) is not a valine residue; the amino acid at position 37 (position 20 in the sequence without signal peptide) is not a proline residue; the amino acid at position 40 (position 23 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 51 (position 34 in the sequence without signal peptide) is not a serine or a valine residue; the amino acid residue at position 62 (position 45 in the sequence without signal peptide) is not a leucine, an isoleucine or a valine residue; the amino acid residue at position 63 (position 46 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 68 (position 51 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 71 (position 54 in the sequence without signal peptide) is not a cysteine, a serine, a proline or a histidine residue; the amino acid residue at position 72 (position 55 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 75 (position 58 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 76 (position 59 in the sequence without signal peptide) is not an asparagine residue; the amino acid residue at position 100 (position 83 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 108 (position 89 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 111 (position 94 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 112 (position 95 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 114 (position 97 in the sequence without signal peptide) is not a glycine residue; the amino acid residue at position 116 (position 99 in the sequence without signal peptide) is not a serine or a threonine residue; the amino

acid residue at position 120 (position 103 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 123 (position 106 in the sequence without signal peptide) is not a aspartate residue; the amino acid residue at position 128 (position 111 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 129 (position 112 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 132 (position 115 in the sequence without signal peptide) is not a threonine or a valine residue; the amino acid residue at position 134 (position 117 in the sequence without signal peptide) is not a histidine or an asparagine residue; the amino acid residue at position 136 (position 119 in the sequence without signal peptide) is not a histidine residue; the amino acid residue at position 148 (position 131 in the sequence without signal peptide) is not an alanine or an isoleucine residue; the amino acid residue at position 162 (position 145 in the sequence without signal peptide) is not a serine or a valine residue; the amino acid residue at position 167 (position 150 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 170 (position 153 in the sequence without signal peptide) is not an aspartate residue; the amino acid residue at position 171 (position 154 in the sequence without signal peptide) is not a tyrosine or an arginine residue; the amino acid residue at position 176 (position 159 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 177 (position 160 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 179 (position 162 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 181 (position 164 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 184 (position 167 in the sequence without signal peptide) is not a tryptophane residue; the amino acid residue at position 189 (position 172 in the sequence without signal peptide) is not a glutamate residue; the amino acid residue at position 191 (position 174 in the sequence without signal peptide) is not a lysine or a glycine residue; the amino acid residue at position 192 (position 175 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 193 (position 176 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 201 (position 184 in the sequence without signal peptide) is not a tyrosine residue; the amino acid residue at position 203 (position 186 in the sequence without signal peptide) is not a

glutamate residue; the amino acid residue at position 207 (position 190 in the sequence without signal peptide) is not a praline residue; the amino acid residue at position 211 (position 194 in the sequence without signal peptide) is not a aspartate residue; the amino acid residue at position 212 (position 195 in the sequence without signal peptide) is not a phenylalanine residue; the amino acid residue at position 218 (position 201 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 220 (position 203 in the sequence without signal peptide) is not a valine or an alanine residue; the amino acid residue at position 221 (position 204 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 223 (position 206 in the sequence without signal peptide) is not a tryptophane or a glutamine residue; the amino acid residue at position 224 (position 207 in the sequence without signal peptide) is not a glutamate residue; the amino acid residue at position 226 (position 209 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 235 (position 218 in the sequence without signal peptide) is not a glycine residue; the amino acid residue at position 246 (position 229 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 249(position 232 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 264 (position 247 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 272 (position 255 in the sequence without signal peptide) is not a cysteine, a leucine or a histidine residue; the amino acid residue at position 275 (position 258 in the sequence without signal peptide) is not a proline residue; the amino acid residue at position 289 (position 272 in the sequence without signal peptide) is not a phenylalanine residue; the amino acid residue at position 291 (position 274 in the sequence without signal peptide) is not a lysine residue; the amino acid residue at position 292 (position 275 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 294 (position 277 in the sequence without signal peptide) is not a tyrosine or an alanine residue; the amino acid residue at position 295 (position 278 in the sequence without signal peptide) is not a valine, a threonine or an isoleucine residue; the amino acid residue at position 297 (position 280 in the sequence without signal peptide) is not an aspirate residue; the amino acid residue at position 298 (position 281 in the sequence without signal peptide) is not a lysine residue; the amino acid residue at position 299 (position 282 in

the sequence without signal peptide) is not a proline residue; the amino acid residue at position 306 (position 289 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 307 (position 290 in the sequence without signal peptide) is not a serine or a leucine residue; the amino acid residue at position 311 (position 294 in the sequence without signal peptide) is not a lysine residue; the amino acid residue at position 326 (position 309 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 327 (position 310 in the sequence without signal peptide) is not a cysteine, a glycine or a leucine residue; the amino acid residue at position 328 (position 311 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 334 (position 317 in the sequence without signal peptide) is not an aspartate residue; the amino acid residue at position 339 (position 322 in the sequence without signal peptide) is not an arginine or a glutamate residue; the amino acid residue at position 348 (position 331 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 354 (position 337 in the sequence without signal peptide) is not an aspartate residue; the amino acid residue at position 355 (position 338 in the sequence without signal peptide) is not a threonine or an isoleucine residue; the amino acid residue at position 371 (position 354 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 374 (position 357 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 377 (position 360 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 378 (position 361 in the sequence without signal peptide) is not a valine residue; the amino acid residue at position 381 (position 364 in the sequence without signal peptide) is not an arginine residue; the amino acid residue at position 382 (position 365 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 389 (position 372 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 391 (position 374 in the sequence without signal peptide) is not a cysteine or a histidine residue; the amino acid residue at position 392 (position 375 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 395 (position 378 in the sequence without signal peptide) is not a threonine residue; the amino acid residue at position 399 (position 382 in the sequence without signal peptide) is not a serine or a valine residue; the amino acid residue at position

400 (position 383 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 406 (position 389 in the sequence without signal peptide) is not a glycine residue; the amino acid residue at position 410 (position 393 in the sequence without signal peptide) is not a leucine residue; the amino acid residue at position 411 (position 394 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 414 (position 397 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 417 (position 400 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 420 (position 403 in the sequence without signal peptide) is not a serine residue; the amino acid residue at position 423 (position 406 in the sequence without signal peptide) is not an alanine residue; the amino acid residue at position 424 (position 407 in the sequence without signal peptide) is not a methionine residue; the amino acid residue at position 426 (position 409 in the sequence without signal peptide) is not a cysteine or an aspartate residue; amino acid residue at position 428 (position 411 in the sequence without signal peptide) is not a proline residue; amino acid residue at position 429 (position 412 in the sequence without signal peptide) is not a lysine residue; amino acid residue at position 436 (position 419 in the sequence without signal peptide) is not a histidine residue; amino acid residue at position 445 (position 428 in the sequence without signal peptide) is not a proline residue; amino acid residue at position 450 (position 433 in the sequence without signal peptide) is not a histidine or a cysteine residue; amino acid residue at position 452 (position 435 in the sequence without signal peptide) is not a lysine residue; amino acid residue at position 454 (position 437 in the sequence without signal peptide) is not an arginine residue; amino acid residue at position 455 (position 438 in the sequence without signal peptide) is not a serine or an aspartate residue; amino acid residue at position 456 (position 439 in the sequence without signal peptide) is not a tryptophane or an arginine residue; amino acid residue at position 459 (position 442 in the sequence without signal peptide) is not a methionine or a leucine residue; amino acid residue at position 466 (position 449 in the sequence without signal peptide) is not a leucine residue; amino acid residue at position 467 (position 450 in the sequence without signal peptide) is not a threonine residue; amino acid residue at position 468 (position 451 in the sequence without signal peptide) is not a threonine residue; amino acid residue at position 473 (position 456 in the sequence without signal peptide) is

not a serine residue; amino acid residue at position 476 (position 459 in the sequence without signal peptide) is not a lysine or a glycine residue; amino acid residue at position 478 (position 461 in the sequence without signal peptide) is not a leucine residue; amino acid residue at position 489 (position 472 in the sequence without signal peptide) is not a serine residue; amino acid residue at position 490 (position 473 in the sequence without signal peptide) is not a phenylalanine residue; and amino acid residue at position 491 (position 474 in the sequence without signal peptide) is not an arginine residue. In other specific embodiments, one or more Xs are defined as being any of the amino acids found at that position in the sequences of the alignment or a residue that constitutes a conserved or semi-conserved substitution of any of these amino acids. In other specific embodiments, Xs are defined as being any of the amino acids found at that position in the sequences of the alignment. For instance, the amino acid residue at position 51 (position 34 in the sequence without signal peptide) is an alanine or a valine residue; the amino acid residue at position 177 (position 160 in the sequence without signal peptide) is an alanine or a serine residue; the amino acid residue at position 212 (position 195 in the sequence without signal peptide) is an isoleucine or a valine residue; the amino acid residue at position 291 (position 274 in the sequence without signal peptide) is a glutamic acid or an aspartic acid residue; and the amino acid residue at position 374 (position 357 in the sequence without signal peptide) is a valine or an isoleucine residue.

**Fragment crystallizable region (Fc) fragments**

The fusion polypeptides of the invention may include a C-terminal domain such as Fc, a fragment crystallizable region of an immunoglobulin. An immunoglobulin molecule has a structure that is well known in the art. It includes two light chains (~23 kD each) and two heavy chains (~50-70 kD each) joined by inter-chain disulfide bonds. Immunoglobulins are readily cleaved proteolytically (e.g., by papain cleavage) into Fab (containing the light chain and the $V_H$ and $C_{H1}$ domains of the heavy chain) and Fc (containing the $C_{H2}$ and $C_{H3}$ domains of the heavy chain, along with adjoining sequences). Cleavage typically occurs in a flexible hinge region joining the Fab and Fc regions. For example, papain cleaves the hinge region immediately before the disulfide bonds joining the two heavy chains.

Useful Fc fragments of the invention include the Fc fragment of any immunoglobulin molecule, including IgG, IgM, IgA, IgD, or IgE, and their various subclasses (e.g., IgG-1, IgG-2, IgG-3, IgG-4, IgA-1, IgA-2), taken from any mammal (e.g., human). The Fc fragments of the invention include, for example, the $C_{H2}$ and $C_{H3}$ domains of the heavy chain, as well as any portion of the hinge region. Furthermore, the Fc region can be glycosylated at various amino acid residues known to those skilled in the art. In some embodiments, the Fc fragment is of human IgG-1. In particular embodiments, the Fc fragment of the fusion polypeptide has the amino acid sequence of SEQ ID NO: 3, or has at least 50% (e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more) sequence identity to SEQ ID NO: 3.

**Additional polypeptide features**

The polypeptides of the invention optionally include one or more additional amino acids 1) at the N-terminus of the polypeptide 2) between the sALP and Fc regions of the polypeptide, and 3) at the C-terminus of the polypeptide. Thus, the invention includes, for example, polypeptides of the form Z-sALP-Y-Fc-X or Z-Fc-Y-sALP-X, where Z is one or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 20, 25, 30, 35, 40, 45, 50, or more) additional amino acids at the N-terminus of the polypeptide, Y is one or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 20, 25, 30, 35, 40, 45, 50, or more) additional amino acids (i.e., a linker) between the sALP and Fc regions of the polypeptide, and X is one or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 20, 25, 30, 35, 40, 45, 50, or more) additional amino acids at the C-terminus of the polypeptide. In a particular example, Y is the dipeptide leucine-lysine. Alternatively, any combination of X, Y, and Z may be present or absent. For example, in some embodiments, Z and X are both absent, and Y is absent or is an amino acid sequence of at least one amino acid. For example, the polypeptide may consist of the structure sALP-Y-Fc or the structure Fc-Y-sALP. In some embodiments of polypeptides consisting of the structure sALP-Y-Fc or Fc-Y-sALP, Y may consist of two amino acid residues, e.g., leucine-lysine. For example, the polypeptide may consist of the structure sALP-Y-Fc. Optionally, the amino acid sequence of sALP is the amino acid sequence of SEQ ID NO: 5, the amino acid sequence of Y is leucine-lysine, and/or the amino acid sequence of Fc is the amino

acid sequence of SEQ ID NO: 3. In some embodiments, the amino acid sequence of the polypeptide consists of the amino acid sequence of SEQ ID NO: 4.

In some embodiments, additional amino acids can be introduced into the polypeptide according to the cloning strategy used to produce the fusion polypeptides. In some embodiments, the additional amino acids do not provide an additional GPI anchoring signal so as to maintain the polypeptide in a soluble form. Furthermore, in some embodiments, any such additional amino acids, when incorporated into the polypeptide of the invention, do not provide a cleavage site for endoproteases of the host cell. The likelihood that a designed sequence would be cleaved by the endoproteases of the host cell can be predicted as described, e.g., by Ikezawa (*Biol. Pharm. Bull.* 25:409-417, 2002).

The polypeptides of the invention also include any polypeptide having one or more post-translational modifications such as glycosylation (e.g., mannosylation and other forms of glycosylation discussed herein), acetylation, amidation, blockage, formylation, gamma-carboxyglutamic acid hydroxylation, methylation, ubiquitination, phosphorylation, pyrrolidone carboxylic acid modification, and sulfation.

In certain embodiments, the polypeptides of the invention are associated into dimers or tetramers. For example, two sALP-Fc monomers can covalently linked through two disulfide bonds located in the hinge regions of the Fc fragments.

**Production of nucleic acids and polypeptides**

The nucleic acids and polypeptides of the invention can be produced by any method known in the art. Typically, a nucleic acid encoding the desired fusion protein is generated using molecular cloning methods, and is generally placed within a vector, such as a plasmid or virus. The vector is used to transform the nucleic acid into a host cell appropriate for the expression of the fusion protein. Representative methods are disclosed, for example, in Maniatis, et al. (Cold Springs Harbor Laboratory, 1989). Many cell types can be used as appropriate host cells, although mammalian cells are preferable because they are able to confer appropriate post-translational modifications. For example, Chinese Hamster Ovary (CHO) cells have been used as a host for expressing the fusion proteins of the present invention, as described in more detail in the examples below. Other host cells useful for expressing

the fusion proteins of the invention include, for example, L cells, C127 cells, 3T3 cells, BHK cells, or COS-7 cells. Particular CHO cells of interest for expressing the fusion proteins of the present invention include CHO-DG44 and CHO/dhfr$^-$. This latter cell line is available through the American Type Culture Collection (ATCC number CRL-9096).

The polypeptides of the invention can be produced under any conditions suitable to effect expression of the polypeptide in the host cell. Such conditions include appropriate selection of a media prepared with components such as a buffer, bicarbonate and/or HEPES, ions like chloride, phosphate, calcium, sodium, potassium, magnesium, iron, carbon sources like simple sugars, amino acids, potentially lipids, nucleotides, vitamins and growth factors like insulin; regular commercially available media like alpha- MEM, DMEM, Ham's-F12 and IMDM supplemented with 2-4 mM L-glutamine and 5% Fetal bovine serum; regular commercially available animal protein free media like Hyclone™ SFM4CHO, Sigma CHO DHFR$^-$, Cambrex POWER™ CHO CD supplemented with 2-4 mM L-glutamine. These media are desirably prepared without thymidine, hypoxanthine and L-glycine to maintain selective pressure, allowing stable protein-product expression.

Additional details of the production of the polypeptides and nucleic acids of the invention are given in the Examples.

**Therapeutic applications**

The nucleic acids and polypeptides of the invention can have a wide variety of therapeutic applications, e.g., in the fields of congenital bone disease, fracture repair, bone and dental implants, vertebrae fusion, and others. In particular, the nucleic acids and polypeptides of the invention are useful for the treatment of matrix mineralization disorders, as described herein, such as HPP.

**Hypophosphatasia (HPP)**

HPP is historically classified according to age at diagnosis and includes (in order from most severe to least severe) perinatal, infantile, childhood, adult, and odontohypophosphatasia forms. The most severe form, perinatal (lethal) HPP, is manifest as an almost complete absence of bone mineralization *in utero* and can cause stillbirth. Some neonates with perinatal HPP may survive for several days, but suffer

increased respiratory compromise due to hypoplastic and rachitic disease of the chest. In infantile HPP, diagnosed before 6 months-of-age, postnatal development seems normal until the onset of poor feeding, inadequate weight gain, and appearance of rickets. Infantile HPP has characteristic radiological features showing impaired skeletal mineralization, sometimes with progressive skeletal demineralization leading to rib fractures and chest deformity. Childhood HPP has highly variable clinical expression. One symptom of childhood HPP is the premature loss of deciduous teeth resulting from aplasia, hypoplasia or dysplasia of dental cementum that connects the tooth root with the periodontal ligament. Another symptom of childhood HPP is rickets, which causes short stature and skeletal deformities such as bowed legs and enlarged wrists, knees and ankles as a result of flared metaphysis. Adult HPP usually presents during middle age, but is frequently preceded by a history of rickets and/or early loss of teeth followed by good health during adolescence and young adult life. In adult HPP, recurrent metatarsal stress fractures are common, and calcium pyrophosphate dihydrate deposition can cause attacks of arthritis and pyrophosphate arthropathy. Finally, odontohypophosphatasia is diagnosed when the only clinical abnormality is dental disease, and radiological studies and even bone biopsies reveal no signs of rickets or osteomalacia.

The more severe clinical forms of HPP are usually inherited as autosomal recessive traits, with parents of such patients showing subnormal levels of serum AP activity. For the milder forms of HPP, i.e., adult HPP and odontohypophosphatasia, an autosomal dominant pattern of inheritance has also been documented.

In the healthy skeleton, TNALP is an ectoenzyme present on the surface of the plasma membrane of osteoblasts and chondrocytes, and on the membranes of their shed matrix vesicles (MVs), where the enzyme is particularly enriched. Deposition of hydroxyapatite during bone mineralization normally initiates within the lumen of these MVs. Electron microscopy has shown that TNALP-deficient MVs from severely affected HPP patients and $Akp2^{-/-}$ mice (a TNALP null mouse model, see below) contain hydroxyapatite crystals, but that extravesicular crystal propagation appears retarded. This defect is attributed to the extracellular accumulation of $PP_i$, a potent inhibitor of calcification, due to a deficiency of TNALP activity.

At physiological concentrations (0.01-0.1 mM), $PP_i$ has the ability to stimulate mineralization. This has been demonstrated in organ-cultured chick femurs and in

isolated rat MVs. However, at concentrations above 1 mM, $PP_i$ inhibits calcium phosphate mineral formation by coating hydroxyapatite crystals, thus preventing mineral crystal growth and proliferative self-nucleation. Thus, $PP_i$ has a dual physiological role: it functions as a promoter of mineralization at low concentrations but as an inhibitor of mineralization at higher concentrations. TNALP has been shown to hydrolyze the mineralization inhibitor $PP_i$ to facilitate mineral precipitation and growth. Recent studies using the $Akp2^{-/-}$ mice have indicated that the primary role of TNALP *in vivo* is to restrict the size of the extracellular $PP_i$ pool to allow proper skeletal mineralization.

The severity of hypophosphatasia depends on the nature of the TNALP mutation. Missense mutations at a variety of positions in TNALP, including the enzyme's active site vicinity, homodimer interface, crown domain, amino-terminal arm, and calcium-binding site, have all been found to affect its catalytic activity. In addition, missense, nonsense, frame-shift, and splice site mutations have also been shown to lead to aberrant mutant proteins or intracellular trafficking defects that lead to subnormal activity on the cell surface. The multitude of mutations that cause HPP, and the fact that compound heterozygosity is a common occurrence in HPP, explain the variable expressivity and incomplete penetrance often observed in this disease.

Progress on the human form of HPP has benefited greatly from the existence of the TNALP null mice ($Akp2^{-/-}$), an animal model of HPP. $Akp2^{-/-}$ mice phenocopy infantile HPP remarkably well: they are born with a normally mineralized skeleton but develop radiographically apparent rickets at about 6 days of age, and die between days 12-16 suffering severe skeletal hypomineralization and episodes of apnea and epileptic seizures attributable to disturbances in PLP (vitamin $B_6$) metabolism.

Both $PP_i$ and PLP are confirmed natural substrates of TNALP, and some TNALP active site mutations have been shown to have different effects on the ability of the enzyme to metabolize $PP_i$ and PLP. Abnormalities in PLP metabolism explain the epileptic seizures observed in $Akp2^{-/-}$ mice, while abnormalities in $PP_i$ metabolism explain the skeletal phenotype in this mouse model of HPP.

**Formulation**

Formulation will depend on the route of administration, as well as on other therapeutic goals. The nucleic acids and polypeptides of the invention can be

administered by any route known in the art, e.g., subcutaneous (e.g., by subcutaneous injection), intravenously, orally, nasally, intramuscularly, sublingually, intrathecally, or intradermally. By way of example, pharmaceutical compositions of the invention can be in the form of a liquid, solution, suspension, pill, capsule, tablet, gelcap, powder, gel, ointment, cream, nebulae, mist, atomized vapor, aerosol, or phytosome.

In some embodiments of the invention, the compositions of the invention can be administered subcutaneously. Subcutaneous administration is advantageous because it is relatively non-invasive and offers desirable pharmacokinetic profiles. Suitable volumes are known to those skilled in the art, and are typically 5 mL or smaller (e.g., 4 mL, 3.5 mL, 3 mL, 2.7 mL, 2.5 mL, 2.3 mL, 2.2 mL, 2.1 mL, 2.0 mL, 1.9 mL, 1.8 mL, 1.7 mL, 1.5 mL, 1.3 mL, 1.0 mL, 0.7 mL, 0.5 mL, 0.3 mL, 0.1 mL, 0.05 mL, 0.01 mL, or smaller). The small volumes required for subcutaneous administration require a sufficiently high concentration of the composition to be administered. Thus, not all compositions may be suitable for subcutaneous adminstion, e.g., polypeptides which aggregate at high concentrations. It has been found that the compositions of the invention can be formulated in a sufficiently high concentration as to be suitable for subcutaneous administration. Typically, the compositions of the invention can be formulated at a concentration between, e.g., 1 mg/mL and 500 mg/mL (e.g., between 10 mg/mL and 300 mg/mL, 20 mg/mL and 120 mg/mL, 40 mg/mL and 200 mg/mL, 30 mg/mL and 150 mg/mL, 40 mg/mL and 100 mg/mL, 50 mg/mL and 80 mg/mL, or 60 mg/mL and 70 mg/mL) for subcutaneous administration.

For oral administration, tablets or capsules can be prepared by conventional means with pharmaceutically acceptable excipients such as binding agents, fillers, lubricants, disintegrants, or wetting agents. The tablets can be coated by methods known in the art. Liquid preparations for oral administration can take the form of, for example, solutions, syrups, or suspension, or they can be presented as a dry product for constitution with saline or other suitable liquid vehicle before use. Compositions of the invention for oral administration also can contain pharmaceutically acceptable excipients such as suspending agents, emulsifying agents, non-aqueous vehicles, preservatives, buffer salts, flavoring, coloring, and sweetening agents as appropriate. Preparations for oral administration also can be suitably formulated to give controlled release of the active ingredients.

Enteric coatings can further be used on tablets of the present invention to resist prolonged contact with the strongly acidic gastric fluid, but dissolve in the mildly acidic or neutral intestinal environment. Without being so limited, cellulose acetate phthalate, Eudragit™ and hydroxypropyl methylcellulose phthalate (HPMCP) can be used in enteric coatings of pharmaceutical compositions of the present invention. Cellulose acetate phthalate concentrations generally used are 0.5-9.0% of the core weight. The addition of plasticizers improves the water resistance of this coating material, and formulations using such plasticizers are more effective than when cellulose acetate phthalate is used alone. Cellulose acetate phthalate is compatible with many plasticizers, including acetylated monoglyceride; butyl phthalybutyl glycolate; dibutyl tartrate; diethyl phthalate; dimethyl phthalate; ethyl phthalylethyl glycolate; glycerin; propylene glycol; triacetin; triacetin citrate; and tripropionin. It is also used in combination with other coating agents such as ethyl cellulose, in drug controlled-release preparations.

The compounds of the invention may be administered in combination with pharmaceutically acceptable, sterile, aqueous or non-aqueous solvents, suspensions or emulsions. Examples of nonaqueous solvents are propylene glycol, polyethylene glycol, vegetable oil, fish oil, and injectable organic esters. Aqueous carriers include water, water-alcohol solutions, emulsions or suspensions, including saline and buffered medical parenteral vehicles including sodium chloride solution, Ringer's dextrose solution, dextrose plus sodium chloride solution, Ringer's solution containing lactose, or fixed oils. Intravenous vehicles may include fluid and nutrient replenishers, electrolyte replenishers, such as those based upon Ringer's dextrose, and the like.

In some embodiments, the pharmaceutical compositions of the present invention can be delivered in a controlled release system. In some embodiments, polymeric materials including polylactic acid, polyorthoesters, cross- linked amphipathic block copolymers and hydrogels, polyhydroxy butyric acid and polydihydropyrans can be used (see also Smolen and Ball, Controlled Drug Bioavailability, Drug product design and performance, 1984, John Wiley & Sons; Ranade and Hollinger, Drug Delivery Systems, pharmacology and toxicology series, 2003, 2nd edition, CRRC Press). In another embodiments, a pump may be used (Saudek et al., 1989, N. Engl. J. Med. 321 : 574).

The compositions of the invention could be formulated in the form of a lyophilized powder using appropriate excipient solutions (e.g., sucrose) as diluents.

Furthermore, cells (e.g., osteoblasts) can be isolated from an individual having a matrix mineralization disorder, transformed with a nucleic acid of the invention, and reintroduced to the afflicted individual (e.g., subcutaneous or intravenous injection). Alternatively, the nucleic acid can be administered directly to the afflicted individual, for example, by injection. The nucleic acid can also be delivered through a vehicle such as a liposome, which can be designed to be targeted to a specific cell type, and engineered to be administered through different routes.

The compositions of the present invention may also be used in combination with at least one other active ingredient to correct a bone mineralization defect or another detrimental symptom of HPP. For example, it may also be used in combination with at least one other active ingredient to correct a cementum defect.

**Gene therapy**

The fusion proteins of the present invention could also be advantageously delivered through gene therapy, where an exogenous nucleic acid encoding the proteins is delivered to tissues of interest and expressed *in vivo*. Gene therapy methods are discussed, e.g., in Verme et al. (*Nature* 389:239-242, 1997) and Yamamoto et al. (*Molecular Therapy* 17:S67-S68, 2009), which are hereby incorporated by reference. Both viral and non-viral vector systems can be used. The vectors may be, for example, plasmids, artificial chromosomes (e.g., bacterial, mammalian, or yeast artificial chromosomes), virus or phage vectors provided with an origin of replication, and optionally, a promoter for the expression of the nucleic acid encoding the viral polypeptide and optionally, a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example, an ampicillin or kanamycin resistance gene in the case of a bacterial plasmid or a restistance gene for a fungal vector. Vectors may be used in *in vitro*, for example, for the production of DNA, RNA, or the viral polypeptide, or may be used to transfect or transform a host cell, for example, a mammalian host cell, e.g., for the production of the viral polypeptide encoded by the vector. The vectors may also be adapted to be used *in vivo*, for example, in a method of vaccination or gene therapy.

Examples of suitable viral vectors include, retroviral, lentiviral, adenoviral, adeno-associated viral, herpes viral, including herpes simplex viral, alpha-viral, pox viral, such as Canarypox and vaccinia-viral based systems. Gene transfer techniques using these viruses are known in the art. Retrovirus vectors, for example, may be used to stably integrate the nucleic acids of the invention into the host genome. Replication-defective adenovirus vectors by contrast remain episomal and therefore allow transient expression. Vectors capable of driving expression in insect cells (e.g., baculovirus vectors), in human cells, yeast, or in bacteria may be employed in order to produce quantities of the viral polypeptide(s) encoded by the nucleic acids of the invention, for example, for use in subunit vaccines or in immunoassays. Useful gene therapy methods include those described in WO 06/060641, US 7,179,903 and WO 01/36620 (each of which is hereby incorporated by reference), which use an adenovirus vector to target a nucleic acid of interest to hepatocytes as protein producing cells.

In an additional example, a replication-deficient simian adenovirus vector may be used as a live vector. These viruses contain an E1 deletion and can be grown on cell lines that are transformed with an E1 gene. Examples of these replication-deficient simian adenovirus vectors are described in U.S. Patent No. 6,083,716 and WO 03/046124 (each of which is hereby incorporated by reference). These vectors can be manipulated to insert a nucleic acid of the invention, such that the encoded viral polypeptide(s) may be expressed.

Promoters and other expression regulatory signals may be selected to be compatible with the host cell for which expression is designed. For example, mammalian promoters include the metallothionein promoter, which can be induced in response to heavy metals such as cadmium, and the β-actin promoter. Viral promoters, such as the SV40 large T antigen promoter, human cytomegalovirus (CMV) immediate early (1E) promoter, rous sarcoma virus LTR promoter, adenovirus promoter, or a HPV promoter, particularly the HPV upstream regulatory region (URR) may also be used. All these promoters, as well as additional promoters, are well-described in the art.

The nucleic acid(s) of the invention may also be administered using non-viral based systems. For example, these administration systems include microsphere encapsulation, poly(lactide-co-glycolide), nanoparticle, and liposome-based systems.

Non-viral based systems also include techniques facilitating the delivery of "naked" polynucleotides (such as electroporation, "gene gun" delivery and various other techniques used for the introduction of polynucleotides).

The introduced polynucleotide can be stably or transiently maintained in the host cell. Stable maintenance typically requires that the introduced polynucleotide either contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosomal replicon (e.g., a plasmid) or a nuclear or mitochondrial chromosome.

**Dosage**

Any amount of a pharmaceutical composition of the invention can be administered to a subject. The dosages will depend on many factors including the mode of administration and the age of the subject. Typically, the amount the composition of the invention contained within a single dose will be an amount that is effective to treat a mineral mineralization disorder without inducing significant toxicity. For subcutaneous administration, the polypeptides of the invention can be administered to subjects in individual doses ranging from 0.01 mg/kg to 100 mg/kg (e.g., from 0.1 mg/kg to 50 mg/kg, 0.5 mg/kg to 25 mg/kg, 1.0 mg/kg to 10 mg/kg, 1.5 mg/kg to 5 mg/kg, or 2.0 mg/kg to 3.0 mg/kg). The doses can be administered hourly, bihourly, daily, bidaily, twice a week, three times a week, four times a week, five times a week, six times a week, weekly, biweekly, monthly, bimonthly, or yearly. The amount and frequency of dosage will be adapted by the clinician in accordance with conventional factors such as the extent of the disease and different parameters from the subject.

The nucleic acids of the invention can be administered according the formulations described herein to a patient in dosages suitable for gene therapy. The amount of the nucleic acids administered will depend on a number of factors known to those skilled in the art, including: the length and nature of the nucleic acid, the vector (e.g., viral or non-viral) used, the activity of the polypeptide encoded, the presence of excipients, the route and method of administration, and the general condition and fitness of the subject. Exemplary dosages and routes of administration are described, e.g., in Melman et al. (*Isr. Med. Assoc. J.* 9:143-146, 2007; describing the intrapenile injection of 0.5 mg to 7.5 mg of a human cDNA in a plasmid for

treating erectile dysfunction), Powell et al. (*Circulation* 118:58-65, 2008; describing the intramuscular injection of 0.4 mg to 4.0 mg of a hepatocyte growth factor plasmid to treat critical limb ischemia, Waddill et al. (*AJR Am. J. Roentgenol.* 169:63-67, 1997; describing the CT-guided intratumoral injection of 0.01 mg to 0.25 mg of plasmid DNA encoding an MHC antigen to treat melanoma), Kastrup et al. (*J. Am. Coll. Cardiol.* 45:982-988, 2005; describing the intramyocardial injection of 0.5 mg of a VEGF plasmid to treat severe angina pectoris), Romero et al. (*Hum. Gene. Ther.* 15:1065-1076, 2004; describing the intramuscular injection of 0.2 mg to 0.6 mg of a plasmid to treat Duchenne/Becker muscular dystrophy), each of which is hereby incorporated by reference.

In certain embodiments, the nucleic acids of the invention can be administered to the subject at a dose in the range from, e.g., 0.01 mg to 100 mg (e.g., from 0.05 mg to 50 mg, 0.1 mg to 10 mg, 0.3 mg to 3 mg, or about 1 mg) of nucleic acid. The total volume at which the nucleic acid can be administered will depend on its concentration, and can range from, e.g., 1 μL to 10 mL (e.g. from 10 μL to 1 mL, 50 μL to 500 μL, 70 μL to 200 μL, 90 μL to 150 μL, or 100 μL to 120 μL). The nucleic acids can be administered in single or multiple doses (e.g., hourly, bihourly, daily, bidaily, twice a week, three time per week, four times a week, five times a week, six times a week, weekly, biweekly, monthly, bimonthly, or yearly).

These are guidelines, since the actual dose should be carefully selected and titrated by an attending physician or nutritionist based upon clinical factors unique to each subject. The optimal daily dose will be determined by methods known in the art and will be influenced by factors such as the age of the subject, as indicated above, and other clinically relevant factors. In addition, subjects may be taking medications for other diseases or conditions. The other medications may be continued during the time that a polypeptide or nucleic acid of the invention is given to the subject, but it is advisable in such cases to begin with low doses to determine if adverse side effects are experienced.

**Examples**

The examples provided below describe the successful treatment of *Akp2*$^{-/-}$ mice using a novel, soluble recombinant protein that includes human tissue-nonspecific alkaline phosphatase that is fused at its carboxy-terminus to a crystalline

fragment (Fc) region of human lgG-1. These examples are intended to illustrate the invention, not limit it.

*Example 1: Expression and production of sALP-Fc*

sALP-Fc cDNA was generated by PCR amplification from pIRES-sALP-FcD10 plasmid, and cloned into the pSV(Hygromycin) expression vector (Selexis Inc.). CHO-K1S cells grown in Power CHO2 CD media (Lonza) supplemented with 4 mM L-glutamine (Wisent) were transfected with PvuI-linearized pSV-sALP-Fc plasmid using the manufacturer's protocol. Forty-eight hours after transfection, media was replaced with media containing 200 μg/mL hygromycin B (Invitrogen). Viability was monitored and media was replaced every 96 hours. Once viability attained 90%, culture was screened for high expression clones using Clonepix FL technology platform (Genetix Inc.) using a FITC-coupled anti-sALP antibody. Best clones were amplified and selected in 96-well plates using sALP enzymatic assay on culture supernatant. Selected clones were amplified and grown in 25L WAVE bioreactor. At the end of the culture, the concentration of sALP-Fc in the spent medium was 135 mg/L as assessed by TNSALP enzymatic activity. When viability dropped below 10%, WAVE motion was stopped to allow cells to settle, and supernatant was harvested by filtration through both Millistak+ Pod (Millipore) and 0.22 μm 1L sterile filter units (Corning). After 10-fold concentration and dialysis of the media in PBS to 2.5 L using tangential flow filtration through Kvick Lab SCU unit (GE Healthcare, 30 kDa cutoff), media was loaded on a Mab Select Sure column that was previously equilibrated with EQ buffer (50 mM $NaPO_4$, 100 mM NaCl, pH 7.5). Column was then washed with 3.5 volumes of EQ buffer, 5.5 volumes of Wash2 buffer (50 mM Tris-Cl, 1.5 M NaCl, pH 8.0), and 3.5 volumes of Wash3 buffer (50 mM Tris-Cl, pH 8.0). sALPFc was then eluted in 10 volumes of Elution buffer (50 mM Tris-Cl, pH 11.0). Eluted protein was further purified on Butyl-sepharose column using the following procedure: Protein was first mixed with an equal volume of 2X equilibration buffer (eq) (40 mM Hepes, 2.5 M ammonium sulfate, pH 7.5), and then loaded on the column which was previously equilibrated in 6 volumes of 1X equilibration buffer. After washing in 5 volumes of eq buffer and 3 volumes of wash2 buffer (20 mM Hepes, 0.95 ammonium sulfate, pH 7.5), protein was eluted in 5 volumes of elution buffer (25 mM $NaPO_4$, 0.5 M ammonium sulfate, pH 7.4). Purified

sALP-Fc was finally concentrated and dialysed against phosphate buffer (25 mM $NaPO_4$, 150 mM NaCl, pH 7.4) using 30 kDa cutoff Vivaspin units (Sartorius VS2022). The overall yield of the purification procedure was 30%, with purity surpassing 95% as assessed by Sypro ruby stained 4-12% SDS-PAGE (Invitrogen Inc.) and size exclusion HPLC. Purified sALP-Fc preparations were stored at 4°C and remained stable for several months.

*Example 2: Papain digestion to generate sALP*

sALP was generated from sALP-Fc using papain digestion as follows: sALP-Fc was first dialyzed against Digestion buffer (20 mM sodium phosphate, pH 7.0) using Vivaspin units (Sartorius VS2022), adjusted to 10 mg/mL, and added to Eppendorf tubes at 600 µL per tube. In parallel, papain-agarose (Sigma P4406) was resuspended in Wash buffer (20 mM sodium phosphate, 1 mM EDTA, pH 6.9) at 25 units in 1.5 mL. To digest 1 nmol of sALPFc, 42 mU of papain-agarose was transferred to a conical tube, centrifuged for 5 min at 1000 g and washed with 10 volumes of Digestion buffer. After another spin down, the beads were resuspended in Digestion buffer and adjusted to the same total volume as prepared sALPFc. Papain was then activated by adding 10 mM DTT to have a final concentration of 0.5 mM. 600 µL of papain-agarose was then immediately added to each Eppendorf containing sALPFc which was pre-heated at 37°C, and incubated for 2 hours at 37°C with mild agitation. The mix was then poured onto empty PD-10 columns (GE Healthcare 17-0435-01) and flow-through was collected. Gel was rinsed with 3 volumes of Digestion buffer and this volume was pooled with the flow-through. The Fc region was then separated from sALP by purification on Hitrap protein A Mab Select SuRe column (GE Healthcare 11-0034-9) connected to an AKTAfplc system. Briefly, column was equilibrated with 10 volumes of buffer A (10 mM sodium phosphate, 150 mM NaCl, pH 7.4), and protein was loaded at 1 mL/min for a 5 mL column. The flow-through containing sALP was collected in one fraction. Column was then washed (1 mL/min) with buffer A until 280 nm absorbance returned to baseline. This wash was collected in one fraction. Column was then washed at 1.5 mL/min with at least 3 column volumes of buffer B (50 mM Tris-Cl, pH 8.0), and eluted in buffer C (50 mM Tris-Cl, pH 11.0) until 280 nm absorbance returned to baseline. Flow-through and buffer A wash fractions were pooled and dialyzed against storage buffer

(25 mM sodium phosphate, 150 mM NaCl, pH 7.4) using Vivaspin units (Sartorius VS2022). Protein concentration was determined using 280 nm optical density assay, and efficiency of digestion and yield were determined using SDS-PAGE analysis, SEC-HPLC, and sALP enzymatic activity assay.

*Example 3: Pharmacokinetic analysis of sALP and sALP-Fc*

*In vivo* pharmacokinetic (PK) studies were performed on purified sALP and sALP-Fc, which were administered intravenously (IV) and subcutaneously (SC) to 5-6 week old C57BL/6 male mice.

Experimental design

Animals received a single IV or SC dose as described in Table 1.

Table 1: Experimental design of an *in vivo* PK study of sALP and sALP-Fc in mice

| Group No. | Group Description | Dose Level (U/kg) | PK Time points | Animals No. Males |
|---|---|---|---|---|
| 1 | sALP IV | 10000 | 0.5, 4 and 26 hr post dose | 101, 102, 103 |
| | | | 1, 8 and 32 hr post dose | 104, 105, 106 |
| | | | 2, 12 and 49 hr post dose | 107, 108, 109 |
| | | | 21 and 45 hr post dose | 110, 111, 112 |
| 2 | sALP SC | 10000 | 4, 6 and 26 hr post dose | 201, 202, 203 |
| | | | 1, 8 and 32 hr post dose | 204, 205, 206 |
| | | | 2, 12 and 49 hr post dose | 207, 208, 209 |
| | | | 21 and 45 hr post dose | 210, 211, 212 |
| 3 | sALP-Fc IV | 2000 | 0.5, 26 and 96 hr post dose | 101, 102, 103 |
| | | | 1, 8 and 49 hr post dose | 104, 105, 106 |
| | | | 3, 12 and 32 hr post dose | 107, 108, 109 |
| | | | 21, 45 and 72 hr post dose | 110, 111, 112 |
| 4 | sALP-Fc SC | 2000 | 6, 26 and 96 hr post dose | 201, 202, 203 |
| | | | 1, 8 and 49 hr post dose | 204, 205, 206 |
| | | | 3, 12 and 32 hr post dose | 207, 208, 209 |
| | | | 21, 45 and 72 hr post dose | 210, 211, 212 |

Administration of test article dose formulation

Dose administrations were performed between 7:00am and 10:30am. Each mouse was injected with test article subcutaneously into the scapular region or immobilized in a rodent restrainer and injected with the test article as an intravenous bolus via the caudal vein. The dose volume was set at 3.33-4.0 mL/kg. The animals received only one dose before scheduled blood collection and sacrifice. Individual body weights were assessed before scheduled test article injection and the volume of test article administered was based on this body weight determination.

Blood sampling and conditioning

Blood samples (0.1 to 0.12 mL) were collected via a jugular vein under isoflurane anesthesia at different time points. At the last time point, blood samples were collected by cardiac puncture under isoflurane anesthesia. Blood samples were collected into Microvette 200Z/gel tube (Sarstedt, serum/gel clotting activator, #20.1291), incubated at room temperature for 30 to 60 min and centrifuged at 10 000g for 5 min at 2-8°C. Serum was transferred into a fresh 0.5mL tube (Sarstedt, #72.699), snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Test article concentration in serum

The presence of sALP and sALP-Fc in serum samples was assessed upon completion of treatment using a colorimetric enzymatic assay. Enzymatic activity was determined using a chromogenic substrate where increase of absorbance is proportional to substrate conversion to products. Final volume was 200 µL. First, 20 µL of standard or 20 µL of PK serum sample was added. A blank was prepared in duplicate also. The reaction was carried out in 180 µL of buffer (20 mM Bis Tris Propane (HCl) pH 9.0, 50 mM NaCl, 0.5 mM $MgCl_2$ and 50 µM $ZnCl_2$) containing 1mM of substrate (pNPP: para-nitrophenyl phosphate disodium hexahydrate). The latter was added last to initiate the reaction. The absorbance was recorded at 405 nm every 30 seconds over a twenty minutes period on a spectrophotometric plate reader (Molecular Devices). Enzymatic activity expressed as an initial speed rate was assessed by fitting the steepest slope over 20 adjacent readings on the spectrophotometric plate reader.

Standards of known concentrations of sALP or sALP-Fc were prepared and the enzymatic activity was determined as described above. The standard curve was generated by plotting Log of the initial speed rate as a function of the Log of the standard quantities. The standard curve was used to qualify each assay run.

sALP or sALP-Fc concentration in U/L of PK serum samples was determined by the conversion of their respective Vmax into units. One unit is defined as the amount of sALP or sALP-Fc that hydrolyzes 1 μmol of p-nitrophenylphosphate to p-nitrophenol in 1 minute at 37°C. Due to the presence of endogenous forms of sALP or sALP-Fc in serum, the measured concentrations were corrected for the baseline value measured in spare animals.

Non compartmental pharmacokinetic analysis

A non-compartmental analysis (NCA) was used to calculate the pharmacokinetic parameters of testing compound. All measured serum concentrations of testing compound versus time profile were processed using WinNonlin™ Enterprise Edition version 5.2.1. NCA Model 200 was selected for SC input or Model 201 for IV-Bolus input using sparse sampling analysis module for both model.

The following non-compartmental PK parameters were calculated:

- area under the curve from time zero to infinity ($AUC_\infty$) calculated as $AUC_{last}+C_{last}/\lambda_z$ (where $\lambda_z$ = slope of the terminal phase of the serum concentration vs time curve);
- maximum observed serum concentration ($C_{max}$), time of maximum serum concentration ($T_{max}$);
- the terminal elimination half-life ($T_{½}$, calculated as $0.693/\lambda_z$);
- systemic clearance after intravenous administration (CL) and subcutaneous administration (CL_F) was calculated as Dose/$AUC_\infty$;

Absolute bioavailability (F%) between routes was calculated using the following formula:

[$AUC_\infty$ (sc)/Dose(sc)]/[ $AUC_\infty$ (iv)/Dose(iv)] x 100.

Nominal times were used in the calculations and times were set relative to start of dosing. Nominal doses were used for the calculation of CL and CL_F.

Results

The mean (±SD) concentration profile in serum following single dose IV and SC administration of sALP and sALP-Fc are shown in Figures 7(a) and 7(b), respectively. The calculated PK parameters are shown in Tables 2(a) and 2(b).

Table 2a: Pharmacokinetic parameters of sALP and sALP-Fc in serum (single dose IV administration).

| **PK Parameters (NCA)** | **Units** | **sALP** | **sALP-Fc** |
|---|---|---|---|
| Dose | U/kg | 10000 | 2000 |
| $C_{max}$ /Dose | U/L/U/kg | 4.32 | 8.54 |
| $AUC_{\infty}$/Dose | U/L*hr/U/kg | 16.0 | 70.6 |
| CL | L/hr/kg | 0.0627 | 0.0142 |
| Half life | hr | 17.6 | 46.9 |

Table 2b: Pharmacokinetic parameters of sALP and sALP-Fc in serum (single dose SC administration).

| **PK Parameters (NCA)** | **Units** | **sALP** | **sALP-Fc** |
|---|---|---|---|
| Dose | U/kg | 10000 | 2000 |
| $C_{max}$/Dose | U/L/U/kg | 0.0514 | 0.413 |
| $AUC_{\infty}$/Dose | U/L*hr/U/kg | 0.483 | 26.2 |
| CL_F | L/hr/kg | 2.07 | 0.0382 |
| Half life | hr | 24.5 | 43.7 |
| Bioavailability | % | 3.02 | 37.1 |

Conclusions

From the data, it can be seen that fusion of the IgG1 Fc domain to sALP increases its SC bioavailability by a factor of more than 10 and increases its systemic exposure following SC administration by a factor of more than 50.

*Example 4: Evaluation in Akp2$^{-/-}$ Mice of the Relationship between Dose and Response after 43 Days of Bolus Subcutaneous Injections of sALP-Fc*

Generation of *Akp2*$^{-/-}$ knockout mice

The *Akp2*$^{-/-}$ knockout mice, a mouse model of infantile HPP, was created by insertion of the Neo cassette into exon 6 of the mouse TNSALP gene (*Akp2*) via homologous recombination, functionally inactivates the *Akp2* gene resulting in no detectable TNSALP mRNA or protein. They are maintained as 12.5% C57Bl/6 – 87.5% 129J *Akp2*$^{-/-}$. Phenotypically, *Akp2*$^{-/-}$ mice closely mimic infantile HPP. Like these patients, *Akp2*$^{-/-}$ mice have global deficiency of TNSALP activity, extracellular accumulation of the ALP substrates $PP_i$, PLP, and phosphoethanolamine (PEA), and postnatally manifest an acquired defect in mineralization of skeletal matrix leading to radiographically and histologically obvious rickets or osteomalacia. They have stunted growth and also develop epileptic seizures and apnea, and die between postnatal days 10-12. Pyridoxine supplementation briefly suppresses their seizures and extends their lifespan, but only until postnatal days 18-22. Therefore, all animals (breeders, nursing mothers and their pups, and weanlings) in this study were given free access to modified laboratory rodent diet 5001 containing increased levels (325 ppm) of pyridoxine.

The *Akp2*$^{-/-}$ homozygotous mice were identified at birth (Day 0) by PCR of tissue biopsies using specific primers to exon VI of the mouse TNSALP gene: GTCCGTGGGCATTGTGACTACCAC (SEQ ID NO: 18) and TGCTGCTCCACTCACGTCG (SEQ ID NO: 19).

Experimental Design

*Akp2*$^{-/-}$ mice were divided into 4 treatment groups:

- Group 1 (Vehicle): *Akp2*$^{-/-}$ mice treated with vehicle SC daily (n=18);
- Group 2 (Tx-1): *Akp2*$^{-/-}$ mice treated with sALP-Fc at 1 mg/kg SC daily (n=17);
- Group 3 (Tx-3): *Akp2*$^{-/-}$ mice treated with sALP-Fc at 3 mg/kg SC daily (n=19);
- Group 4 (Tx-6): *Akp2*$^{-/-}$ mice treated with sALP-Fc at 6 mg/kg SC daily (n=17);

- Group 5 (WT): wild-type littermates of *Akp2*$^{-/-}$ mice served as reference animals and did not receive injections (n=35).

Injections were administered between 8:00 and 11:00am, and dose volume was set at 3.3 mL/kg body weight. The actual volume given was calculated and adjusted based on the daily body weight measured prior to injection. Vehicle or sALP-Fc was injected SC into the scapular region. All treatments began on postnatal Day 1, and repeated daily for up to 43 days or until the time of necropsy.

Terminal procedures

Necropsy was performed on Day 44, 24 hours after the final injection for those animals that completed the experimental protocol or sooner for those animals that appear terminally ill. All animals were euthanized by bilateral thoracotomy under isoflurane anesthesia. The necropsy consisted of a gross pathology check, with a piece of ear collected to reconfirm the *Akp2*$^{-/-}$ genotype. The bone samples were cleaned, fixed in 10% neutral buffered formalin for 3 days at 2 to 8°C, and then transferred to 70% ethanol for storage at 2 to 8°C. Femur and tibia lengths were measured using a caliper.

Radiographic analysis

Radiographic images were obtained with a Faxitron MX-20 DC4 (Faxitron X-ray Corporation, Wheeling, IL), using energy of 26 kV and an exposure time of 10 seconds. Defects in bone mineralization were classified in a blinded fashion by a veterinarian radiologist. Animals were "Abnormal" if at least one bone structure (including secondary ossification centers) was absent.

Statistical analyses

All numerical values are presented as mean values ± standard deviation. Non-parametric analysis was preferred for all parameters because of the small sample sizes. The Log-Rank test was used to compare survival curves. Chi-square test was used to compare the distribution of normal and abnormal radiographs between treatments. An Anova model was used to compare the average body weights between groups at each day, and the average bone lengths at the end of the study.

Dose-Response Analysis

The following conceptual pharmacodynamic (PD) models were used to fit dose-response data as describe in Table 3.

Table 3: Pharmacodynamic models used to fit dose-response data

| Model | Equations |
|---|---|
| Linear Model | $E= E_0 + S * C$ |
| Simple $E_{max}$ Model | $E= (E_{max} * C)/(C+ED_{50})$ |
| Simple $E_{max}$ Model with $E_0$ | $E=E_0+ (E_{max}-E_0)* (C/(C+ED_{50}))$ |
| Sigmoid $E_{max}$ Model | $E=(E_{max} * C^{\gamma})/(C^{\gamma}+ED_{50}^{\gamma})$ |
| Sigmoid $E_{max}$ Model with $E_0$ | $E=E_0 + (E_{max} -E0)*( C^{\gamma}/( C^{\gamma} + ED_{50}^{\gamma}))$ |
| Weibull | $E = E_{max} * (1-\exp(-(C/ ED_{50})^{S}))$ |
| Makoid-Banakar | $E = E_{max} *(C/Cmax)^{S}*\exp(S*(1-C/Cmax))$, when $C \leq Cmax$, $E = E_{max}$, when $C > C_{max}$ |

E= effects, C= Dose, S= Slope, $\gamma$= sigmoidicity factor, $C_{max}$ = maximal dose, $E_{max}$= maximal effects, $E_0$= baseline effects at C=0; $ED_{50}$= dose that induced 50% of $E_{max}$

Akaike Information (AIC) and Schwarz Bayesian (SBC) Criterions were used as a measure of goodness of fit to select the best model for each data set. When comparing several models for a given data set, the model associated with the smallest AIC and SBC was selected during the model discrimination process. The correlation coefficient ($r^2$) and visual assessments of fit (VAF), absolute residual distribution, coefficient of variation of PD parameters were also used for the model discrimination.

Once the appropriate model has been selected, the fit was re-run using predefined weighting schemes (1/Y, 1/Yhat, 1/Y.Y and 1/Yhat. Yhat) in WinNonlin 5.2. The same criteria as those used to select the model were again utilized for selecting the appropriate weighting scheme for each data set. The final PD parameters were generated from selected model using the best weighting scheme.

Pharmacodynamic (PD) analysis was performed using WinNonlin™ Enterprise v5.2. Tables and figures were generated using Microsoft Office (Excel) 2003, and SigmaPlot v9.0.1.

All analyses were performed using full precision. Whenever possible, individual data was reported to 3 significant figures. PD parameters and their 95% confidence intervals were determined using WinNonlin 5.2.

Results

The objective of the present study was to define the dose response relationship between increasing amounts of sALP-Fc using bolus SC injections and the therapeutic response after 43 days. Endpoints were survival, body weight, bone length of the tibiae and femora, and bone mineralization defects of feet.

Survival of mice representing each treatment group was significantly improved when compared to Vehicle ($p<0.0001$) (Figure 8(a)). Differences were also statistically significant when the survival curves of the treated groups were compared ($p<0.0001$). Median survival was 19, 25, and 41 days in the Vehicle, Tx-1, and Tx-3 groups, respectively (Figure 8(b)). Only four of 17 mice in Tx-6 died before the completion of the study; thus, median survival could not be calculated for this group. However, there was a clear relationship between the sALP-Fc daily dose and the survival of *Akp2*$^{-/-}$ mice.

Changes in body weight are depicted in Figure 9. At the beginning of treatment, average daily body weights (BWs) showed no statistical difference between the newborn *Akp2*$^{-/-}$ mice of each treatment group and newborn WT mice. At the end of the study, average daily BWs remained similar between the treated *Akp2*$^{-/-}$ mice and the WT mice.

Changes in femur/tibial lengths of the mice are depicted in Figure 10. These data show that, at the end of the study, there was no statistically significant difference between *Akp2*$^{-/-}$ mice of each treatment group and WT mice in the average lengths of the tibiae and femora. Deaths of the vehicle mice precluded age-matched bone length analysis.

Representative radiographs of "normal" and "abnormal" feet, rib cage, and lower limb specimens are depicted in Figure 11. The radiographic results for each treatment group, i.e., *Akp2*$^{-/-}$ receiving vehicle alone versus 1, 3, or 6 mg/kg/day sALP-Fc, are summarized in Table 4. Here, the distributions of normal and abnormal radiographic images are presented with the percentages given in parentheses. Individual radiographic image distribution values of every treatment group were corrected for the baseline number of animals with normal mineralization found in the vehicle group. The number of animals in the vehicle group having normal mineralization was considered as the baseline values. The baseline value was

subtracted from the corresponding number of normal and abnormal animals found in dosing groups.

Table 4: Distribution between normal and abnormal radiographic images of the feet of *Akp2*$^{-/-}$ mice receiving vehicle or 1, 3, or 6 mg/kg/day sALP-Fc.

| **Uncorrected for baseline value** | | **Corrected for baseline value** | |
|---|---|---|---|
| **Group** | **% Normal** | **Group** | **% Normal** |
| **Vehicle (N=17)** | 8 (47) | **Vehicle (N=9)** | 0 (0) |
| **Tx-1 (N=17)** | 12 (70) | **Tx-1 (N=9)** | 4 (44) |
| **Tx-3 (N=19)** | 17 (89) | **Tx-3 (N=11)** | 9 (82) |
| **Tx-6 (N=17)** | 16 (94) | **Tx-6 (N=9)** | 8 (89) |

The dose response to sALP-Fc treatment was examined by evaluating the effect of various doses on the radiographic image distribution (RID) pharmacodynamic endpoint. With the use of mathematical and graphical criteria, a sigmoid $E_{max}$ model was determined to be the best model for fitting the corrected RID feet dataset. A uniform error model was used throughout the model fit procedure.

There was a clear relationship between daily dose of sALP-Fc and percent of mice with normal bone of the foot. The 80% effective dose was estimated to be 3.0 mg/kg/day for feet (Figure 12).

These examples demonstrate that HPP mice treated with sALP-Fc experience therapeutic benefits that are significantly greater than those of sALP.

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## Other Embodiments

All publications, patent applications, and patents mentioned in this specification are herein incorporated by reference.

While the invention has been described in connection with specific embodiments, it will be understood that it is capable of further modifications. Therefore, this application is intended to cover any variations, uses, or adaptations of the invention that follow, in general, the principles of the invention, including departures from the present disclosure that come within known or customary practice within the art.

What is claimed is:

## Claims

1. A method of treating a matrix mineralization disorder in a subject, said method comprising administering to said subject a therapeutically effective amount of a pharmaceutical composition comprising:

(a) a polypeptide comprising the structure Z-sALP-Y-Fc-X or the structure Z-Fc-Y-sALP-X; and

(b) a pharmaceutically acceptable excipient,

wherein sALP is the extracellular domain of an alkaline phosphatase; each of X, Y, and Z is absent or is an amino acid sequence of at least one amino acid; and said polypeptide does not comprise a polyaspartic acid or polyglutamic acid region longer than three consecutive aspartic acid or glutamic acid residues.

2. The method of claim 1, wherein said polypeptide comprises the structure Z-sALP-Y-Fc-X.

3. The method of claim 1 or 2, wherein said polypeptide does not comprise a polyaspartic acid or polyglutamic acid region longer than two consecutive aspartic acid or glutamic acid residues.

4. The method of any one of claims 1-3, wherein said polypeptide does not comprise a bone-targeting moiety.

5. The method of any one of claims 1-4, wherein the amino acid sequence of said sALP comprises amino acid residues 23-508 of SEQ ID NO: 15.

6. The method of claim 5, wherein the amino acid sequence of said sALP consists of amino acid residues 23-512 of SEQ ID NO: 15.

7. The method of any one of claims 1-4, wherein the amino acid sequence of said sALP comprises amino acid residues 18-498 of SEQ ID NO: 16.

8. The method of claim 7, wherein the amino acid sequence of said sALP consists of amino acid residues 18-502 of SEQ ID NO: 16.

9. The method of any one of claims 1-4, wherein the amino acid sequence of said sALP comprises an amino acid sequence having at least 85% sequence identity to SEQ ID NO: 5.

10. The method of claim 9, wherein the amino acid sequence of said sALP comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 5.

11. The method of claim 10, wherein the amino acid sequence of said sALP comprises an amino acid sequence having at least 99% sequence identity to SEQ ID NO: 5.

12. The method of claim 11, wherein the amino acid sequence of said sALP comprises SEQ ID NO: 5.

13. The method of claim 12, wherein the amino acid sequence of said sALP consists of SEQ ID NO: 5.

14. The method of any one of claims 1-13, wherein said Fc comprises a $C_{H2}$ domain, a $C_{H3}$ domain and a hinge region.

15. The method of claim 14, wherein said Fc is a constant domain of an immunoglobulin selected from the group consisting of IgG-1, IgG-2, IgG-3, IgG-3 and IgG-4.

16. The method of claim 15, wherein said immunoglobulin is IgG-1.

17. The method of claim 16, wherein the amino acid sequence of said Fc comprises an amino acid sequence having at least 85% sequence identity to SEQ ID NO: 3.

18. The method of claim 17, wherein the amino acid sequence of said Fc comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 3.

19. The method of claim 18, wherein the amino acid sequence of said Fc comprises an amino acid sequence having at least 99% sequence identity to SEQ ID NO: 3.

20. The method of claim 19, wherein the amino acid sequence of said Fc comprises SEQ ID NO: 3.

21. The method of claim 20, wherein the amino acid sequence of said Fc consists of SEQ ID NO: 3.

22. The method of any one of claims 1-21, wherein Z is absent.

23. The method of any one of claims 1-22, wherein Y is two amino acid residues.

24. The method of claim 23, wherein Y is leucine-lysine.

25. The method of any one of claims 1-24, wherein X is absent.

26. The method of any one of claims 1-25, wherein the amino acid sequence of said polypeptide comprises an amino acid sequence having at least 85% sequence identity to SEQ ID NO: 4.

27. The method of claim 26, wherein the amino acid sequence of said polypeptide comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 4.

28. The method of claim 27, wherein the amino acid sequence of said polypeptide comprises an amino acid sequence having at least 99% sequence identity to SEQ ID NO: 4.

29. The method of claim 28, wherein the amino acid sequence of said polypeptide comprises SEQ ID NO: 4.

30. The method of claim 29, wherein the amino acid sequence of said polypeptide consists of SEQ ID NO: 4.

31. The method of any one of claims 1-30, wherein said polypeptide is pegylated.

32. The method of any one of claims 1-31, wherein said polypeptide is glycosylated.

33. The method of any one of claims 1-32, wherein said pharmaceutical composition comprises a dimer of said polypeptide.

34. The method of any one of claims 1-33, wherein said pharmaceutically acceptable excipient comprises saline.

35. The method of any one of claims 1-34, wherein said pharmaceutical composition is lyophilized.

36. The method of any one of claims 1-35, wherein said pharmaceutical composition is administered subcutaneously, intravenously, orally, nasally, intramuscularly, sublingually, intrathecally, or intradermally.

37. The method of claim 36, wherein said pharmaceutical composition is administered subcutaneously.

38. The method of claim 37, comprising administering said pharmaceutical composition to said subject in a dosage of about 0.5 mg/kg/day to about 10 mg/kg/day.

39. The method of claim 38, comprising administering said pharmaceutical composition to said subject in a dosage of about 2 mg/kg/day to about 3 mg/kg/day.

40. The method of any one of claims 37-39, comprising administering said pharmaceutical composition to said subject between one and seven times a week.

41. The method of claim 40, comprising administering said pharmaceutical composition to said subject three times a week.

42. The method of any one of claims 1-41, wherein said matrix mineralization disorder is hypophosphatasia.

43. The method of claim 42, wherein said matrix mineralization disorder is infantile hypophosphatasia (HPP), childhood HPP, perinatal HPP, adult HPP, or odontohypophosphatasia.

44. The method of any one of claims 1-43, wherein said pharmaceutical composition is administered in an amount that is therapeutically effective to treat a hypophosphatasia (HPP) phenotype selected from the group consisting of HPP-related seizure, premature loss of deciduous teeth, incomplete bone mineralization, elevated blood and/or urine levels of inorganic pyrophosphate ($PP_i$), elevated blood and/or urine levels of phosphoethanolamine (PEA), elevated blood and/or urine levels of pyridoxal 5'-phosphate (PLP), inadequate weight gain, rickets, bone pain, calcium pyrophosphate dihydrate crystal deposition, aplasia, hypoplasia, and dysplasia of the dental cementum.

45. The method of claim 44, wherein said incomplete bone mineralization is incomplete femoral bone mineralization, incomplete tibial bone mineralization, incomplete metatarsal bone mineralization, or incomplete rib bone mineralization.

46. The method of any one of claims 1-45, wherein said subject is human.

47. A pharmaceutical composition comprising:

(a) a polypeptide comprising the structure Z-sALP-Y-Fc-X or the structure Z-Fc-Y-sALP-X; and

(b) a pharmaceutically acceptable excipient,

wherein sALP is the extracellular domain of an alkaline phosphatase; each of X, Y, and Z is absent or is an amino acid sequence of at least one amino acid; and said polypeptide does not comprise a polyaspartic acid or polyglutamic acid region longer than three consecutive aspartic acid or glutamic acid residues.

48. The pharmaceutical composition of claim 47, wherein said pharmaceutical composition is formulated for treating a matrix mineralization disorder in a subject.

49. A method of treating a matrix mineralization disorder in a subject, said method comprising administering to said subject a therapeutically effective amount of a pharmaceutical composition comprising:

(a) an isolated nucleic acid encoding a polypeptide comprising the structure Z-sALP-Y-Fc-X or the structure Z-Fc-Y-sALP-X; and

(b) a pharmaceutically acceptable excipient,

wherein sALP is the extracellular domain of an alkaline phosphatase; each of X, Y, and Z is absent or is an amino acid sequence of at least one amino acid; and said isolated nucleic acid does not encode an amino acid sequence comprising a polyaspartic acid or polyglutamic acid region longer than three consecutive aspartic acid or glutamic acid residues.

50. The method of claim 49, wherein said isolated nucleic acid is administered to said subject in a lentiviral vector.

51. The method of claim 50, wherein said isolated nucleic acid is administered to said subject at a dosage of from about 0.1 mg to about 10 mg.

52. A pharmaceutical composition formulated for the treatment of a matrix mineralization disorder in a subject, said pharmaceutical composition comprising:

(a) an isolated nucleic acid encoding a polypeptide comprising the structure Z-sALP-Y-Fc-X or the structure Z-Fc-Y-sALP-X; and

(b) a pharmaceutically acceptable excipient,

wherein sALP is the extracellular domain of an alkaline phosphatase; each of X, Y, and Z is absent or is an amino acid sequence of at least one amino acid; and said isolated nucleic acid does not encode an amino acid sequence comprising a polyaspartic acid or polyglutamic acid region longer than three consecutive aspartic acid or glutamic acid residues.

53. The pharmaceutical composition of claim 52, comprising a recombinant expression vector comprising said isolated nucleic acid.

54. The pharmaceutical composition of claim 53, wherein said recombinant expression vector is a lentiviral vector.

55. An isolated recombinant host cell transformed or transfected with the vector of claim 54.

56. A method of producing the polypeptide of claim 47, comprising culturing the host cell of claim 55 in a culture medium under conditions suitable to effect expression of the polypeptide and recovering said polypeptide from said culture medium.

57. The method of claim 56, wherein said host cell is an L cell, a C127 cell, a 3T3 cell, a CHO cell, a BHK cell, or a COS-7 cell.

58. The method of claim 57, wherein said host cell is a CHO cell.

59. The method of claim 58 wherein said CHO cell is a CHO-DG44 cell.

60. A kit comprising:

(a) the pharmaceutical composition of any one of claims 47-48 or 52-54 and

(b) instructions for administering said pharmaceutical composition to a subject to treat a matrix mineralization disorder.