

HIGH THROUGHPUT SCREEN FOR GENETIC VARIANTS ASSOCIATED WITH SHORT STATURE

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] The present application claims the priority benefit of U.S. Provisional Patent Application No. 63/471,634, filed June 7, 2023, U.S. Provisional Patent Application No. 63/540,792, filed September 27, 2023, and U.S. Provisional Patent Application No. 63/564,071, filed March 12, 2024, herein incorporated by reference in their entireties.

INCORPORATION BY REFERENCE TO THE SEQUENCE LISTING

[002] This application includes a sequence listing submitted electronically, in a file entitled: 58818_Seqlisting.xml created on June 3, 2024 and having a size of 56,620 bytes, which is incorporated by reference herein.

FIELD OF THE DISCLOSURE

[003] The present disclosure, relates, in general, to use of a high throughput screening method for identifying genetic mutations in genes, e.g., the NPR2 gene, associated with CNP dysfunction and short stature disorders.

BACKGROUND

[004] Methods of measuring the function of thousands of candidate regulatory sequences (CRSs) can be carried out using massively parallel reporter assays (MPRAs) (Gordon et al., Nat Protoc. 2020 15(8): 2387–2412).

[005] Many different genes are potentially associated with skeletal disorders such as achondroplasia and short stature. A C-type natriuretic peptide analog (CNP) is now approved by the FDA for achondroplasia - a common form of short stature. Binding of CNP to its receptor natriuretic peptide receptor 2 (*NPR2*), triggers endochondral and skeletal growth via cGMP production. Loss-of-function mutations in *NPR2* are responsible for dwarfism in mice and a lack of an intracellular cGMP response to CNP in cultured chondrocytes.

[006] *NPR2* is a bidirectional therapeutic target that is associated with various forms of short and tall stature. *NPR2* possesses guanylyl cyclase activity that leads to synthesis of cyclic guanosine monophosphate (cGMP), and down-regulation of this pathway is responsible for short stature phenotypes.

SUMMARY

[007] The high throughput characterization of natriuretic peptide receptor 2 (*NPR2*) variants described herein enables better prediction of novel *NPR2* variants associated with

CNP function, and for those which occur more commonly, could benefit diagnosis and clinical trial enrollment for eligible patients having short stature.

[008] The disclosure provides a method of identifying a variant gene associated with short stature that is a gain of function (GoF) or loss of function (LoF) variant comprising:

- transfecting cells comprising a cGMP-GFP reporter expression construct with a lentiviral vector expressing a polynucleotide encoding a variant protein associated with short stature operably linked to one or more unique barcode sequences;
- contacting the cells in culture with c-type natriuretic peptide (CNP) or a variant thereof having CNP activity;
- sorting the cells from the culture based on the level of expression of GFP produced by the cell; and
- identifying the variant protein associated with short stature as a GoF variant or a LoF variant, wherein a GoF variant has a higher level of cGMP production compared to a control, and wherein a LoF variant has a lower level of cGMP production compared to a control.

[009] In various embodiments, the variant gene associated with short stature is collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, insulin growth factor 1 receptor (IGF1R), DTL, PAPP2, or combinations thereof. In various embodiments, the variant gene associated with short stature is selected from the group consisting of natriuretic peptide receptor 2 (NPR2), Natriuretic peptide precursor C (NPPC), fibroblast growth factor receptor 3 (FGFR3) or combinations thereof. In various embodiments, the variant gene associated with short stature is NPR2.

[0010] Also contemplated herein is a method of identifying a variant of NPR2 as a gain of function (GoF) or loss of function (LoF) variant comprising:

- transfecting cells comprising a cGMP-GFP reporter expression construct with a lentiviral vector expressing a polynucleotide encoding a NPR2 variant protein operably linked to one or more unique barcode sequences;
- contacting the cells in culture with c-type natriuretic peptide (CNP) or a variant thereof having CNP activity;
- sorting the cells from the culture based on the level of expression of GFP produced by the cell; and

-identifying the NPR2 variant as a GoF variant or a LoF variant, wherein a GoF variant has a higher level of cGMP production compared to a control, and wherein a LoF variant has a lower level of cGMP production compared to a control.

[0011] In various embodiments, the cells are a mammalian cell line. In certain embodiments, the cells are HEK293 cells.

[0012] In various embodiments, the cells are sorted by flow cytometry.

[0013] In various embodiments, the lentiviral vector comprises a polynucleotide encoding a variant gene associated with short stature, a polynucleotide encoding a puromycin resistance gene and a polynucleotide encoding T2A-BFP promoter.

[0014] In various embodiments, the lentiviral vector further comprises between 20 and 60 barcode sequences. In various embodiments, the barcode sequences are from 15 to 30 basepairs.

[0015] In various embodiments, the barcode sequences are 3' to the variant gene polynucleotide.

[0016] In various embodiments, the expression construct comprises a polynucleotide encoding a GFP protein operably linked to a cGMP binding domain. In various embodiments, the cGMP binding domain is from mouse or human phosphodiesterase.

[0017] In various embodiments, the expression construct further comprises a CMV promoter operably linked to cGull and a PGK promoter operably linked to a blastocidin resistance gene.

[0018] In various embodiments, the CNP variant is selected from the group consisting of:
PGQEHPNARKYKGANKKGLSKGCFGLKLDRI GMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1); GQEHPNARKYKGANKKGLSKGCFGLKLDRI GMSGLGC (Gly-CNP-37) (SEQ ID NO: 2);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI GMSGLGC (Gly-CNP53) (SEQ ID NO: 3);
PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI GMSGLGC (Pro-CNP53) (SEQ ID NO: 4);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI GMSGLGC (Met-CNP53) (SEQ ID NO: 5);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI GSN SGLGC [CNP-53(M48N)] (SEQ ID NO: 6);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI GMSGLGC (CNP-52) (SEQ ID NO: 7);

RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-51)
(SEQ ID NO: 8);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP- 50)
(SEQ ID NO: 9);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-49) (SEQ
ID NO: 10);
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-48) (SEQ
ID NO: 11);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-47) (SEQ ID
NO: 12);
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-46) (SEQ ID
NO: 13);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-45) (SEQ ID
NO: 14);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-44) (SEQ ID NO:
15);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-43) (SEQ ID NO:
16); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-42) (SEQ ID
NO: 17); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-41) (SEQ ID
NO: 18); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-40) (SEQ ID
NO: 19); LLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-39) (SEQ ID NO:
20); LQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-38) (SEQ ID NO: 21);
QEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-37) (SEQ ID NO: 22);
EHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-36) (SEQ ID NO: 23);
HPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-35) (SEQ ID NO: 24);
PNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-34) (SEQ ID NO: 25);
NARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-33) (SEQ ID NO: 26);
ARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-32) (SEQ ID NO: 27);
RKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-31) (SEQ ID NO: 28);
KYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-30) (SEQ ID NO: 29);
YKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-29) (SEQ ID NO: 30);
KGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-28) (SEQ ID NO: 31);
GANKKGLSKGCFGLKLDRI SMSGLGC (CNP-27) (SEQ ID NO: 32);
ANKKGLSKGCFGLKLDRI SMSGLGC (CNP-26) (SEQ ID NO: 33);
NKKGLSKGCFGLKLDRI SMSGLGC (CNP-25) (SEQ ID NO: 34);
KKGLSKGCFGLKLDRI SMSGLGC (CNP-24) (SEQ ID NO: 35);
KGLSKGCFGLKLDRI SMSGLGC (CNP-23) (SEQ ID NO: 36);

LSKGCFLKLDRI SMSGLGC (CNP-21) (SEQ ID NO: 37);
SKGCFGLKLDRI SMSGLGC (CNP-20) (SEQ ID NO: 38);
KGCFGLKLDRI SMSGLGC (CNP-19) (SEQ ID NO: 39);
GCFGLKLDRI SMSGLGC (CNP-18) (SEQ ID NO: 40);
QEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC [CNP-37(M32N)] (SEQ ID NO: 41);
PQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Pro-CNP-37) (SEQ ID NO: 42);
MQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Met-CNP-37) (SEQ ID NO: 43);
GQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC [Gly-CNP-37(M32N)] (SEQ ID NO: 44);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Met-Gly-CNP-37) (SEQ ID NO: 45);
PGQEHPQARRYRGAQRRGLSRGCFGLKLDRI SMSGLGC (SEQ ID NO: 46);
PGQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 47);
PGQEHPNARRYRGANRRGLSRGCFGLKLDRI SMSGLGC (SEQ ID NO: 48);
PGQEHPQARKYKGAQKKGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 49);
GQEHPNARKYKGANPKGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 50);
GQEHPNARKYKGANQKGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 51);
GQEHPNARKYKGANQQGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 52); and
GQEHPNARKYKGANKPGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 53).

[0019] In various embodiments, the CNP is contacted with the cells at a dose between about 1 to about 100 nM. In various embodiments, the CNP is contacted with the cells at a dose of about 1, about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 55, about 60, about 65, about 70, about 75, about 80, about 85, about 90, about 95 or about 100 nM.

[0020] In various embodiments, the lentivirus is transfected into the cells at a multiplicity of infection of MOI between about 0.1 and about 0.5. In various embodiments, the lentivirus is transfected into the cells at a multiplicity of infection of MOI about 0.1, about 0.2, about 0.3, about 0.4 or about 0.5.

[0021] In various embodiments, the disclosure provides a lentiviral vector comprising a polynucleotide encoding a variant gene associated with short stature, a polynucleotide encoding a puromycin resistance gene and a polynucleotide encoding T2A-BFP promoter.

[0022] In various embodiments, the variant gene associated with short stature is selected from the group consisting of natriuretic peptide receptor 2 (NPR2), natriuretic peptide precursor C (NPPC), fibroblast growth factor receptor 3 (FGFR3) or combinations thereof. In various embodiments, the variant gene associated with short stature is NPR2.

[0023] Further contemplated is a lentiviral vector comprising a polynucleotide encoding a NPR2 variant, a polynucleotide encoding a puromycin resistance gene and a polynucleotide encoding T2A-BFP promoter. In various embodiments, the lentiviral vector further comprises between 20 and 60 barcode sequences. In various embodiments, the barcode sequences are from 15 to 30 basepairs. In various embodiments, the barcode sequences are 3' to the variant gene polynucleotide.

[0024] In various embodiments, the disclosure provides a method of making a lentiviral library comprising a variant gene associated with short stature, the method comprising:

- amplifying variant genes associated with short stature from a mammalian genome or genomic DNA database;
- cloning the amplified variants into a lentiviral vector, wherein the lentiviral vector comprises between 20-60 unique barcodes per vector;
- sequencing the variant associated with the barcodes in the vector;
- aligning the variant sequences with a control gene sequence to generate a read structure;
- extracting the barcodes from the variant read structure;
- identifying the barcodes for each variant; and
- isolating the lentiviral vectors expressing variant genes.

[0025] In various embodiments, the variant gene associated with short stature is selected from the group consisting of NPR2, NPPC, FGFR3 or combinations thereof.

[0026] Also provided is a method of making an NPR2 variant lentiviral library comprising

- amplifying NPR2 variants from a mammalian genome or genomic DNA database;
- cloning the amplified NPR2 variants into a lentiviral vector, wherein the lentiviral vector comprises between 20-60 unique barcodes per vector;
- sequencing the NPR2 variant associated with the barcodes in the vector;
- aligning the NPR2 variant sequences with a control NPR2 gene sequence to generate a read structure;
- extracting the barcodes from the NPR2 variant read structure;
- identifying the barcodes for each NPR2 variant; and
- isolating the lentiviral vectors expressing NPR2 variants.

[0027] The disclosure also contemplates a method for treating a subject with a short stature disorder comprising administering a CNP variant to a subject identified as having a loss of

function variant of a gene associated with short stature identified using a method of described herein.

[0028] In various embodiments, the disclosure provides a method of improving and/or maintaining bone strength in a subject in need thereof comprising administering a C-type natriuretic peptide (CNP) to the subject. In various embodiments, the subject has a short stature disorder.

[0029] In various embodiments, the short stature disorder is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, idiopathic short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis congenita, achondrogenesis, homozygous achondroplasia, chondrodysplasia congenita, campomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis congenita, short-rib polydactyly syndromes, rhizomelic type of chondrodysplasia congenita, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia, disorders related to NPR2 mutation, SHOX mutations, Turner's syndrome/Leri Weill, PTPN11 mutations, Noonan's syndrome, and IGF1R mutation.

[0030] The disclosure also contemplates a population PK model for dosing of CNP variant to a subject. In various embodiments, the disclosure provides a method for treating a CNP-responsive bone-related disorder, skeletal dysplasia or short stature disorder comprising administering to a subject in need thereof a CNP variant, wherein the CNP variant is administered according to a weight-band dosing regimen, wherein

- i) a subject between 10–11 kg receives between about 22–24 µg/kg CNP variant;
- ii) a subject between 12–16 kg receives between about 18–23 µg/kg CNP variant;
- iii) a subject between 17–21 kg receives between about 15–19 µg/kg CNP variant;
- iv) a subject between 22–32 kg receives between about 13–18 µg/kg CNP variant;
- v) a subject between 33–43 kg receives between about 12–15 µg/kg CNP variant;
- vi) a subject between 44–59 kg receives between about 10–14 µg/kg CNP variant;
- vii) a subject between 60–89 kg receives between about 8–12 µg/kg CNP variant; or
- viii) a subject of weight of ≥ 90 kg receives about ≤ 9 µg/kg CNP variant.

[0031] In various embodiments,

- i) a subject between 10–11 kg receives about 0.24 mg CNP variant;
- ii) a subject between 12–16 kg receives about 0.28 mg CNP variant;
- iii) a subject between 17–21 kg receives about 0.32 mg CNP variant;
- iv) a subject between 22–32 kg receives about 0.40 mg CNP variant;
- v) a subject between 33–43 kg receives about 0.50 mg CNP variant;
- vi) a subject between 44–59 kg receives about 0.60 mg CNP variant;
- vii) a subject between 60–89 kg receives about 0.7 mg CNP variant; or
- viii) a subject having a weight of ≥ 90 kg receives about 0.80 mg CNP variant.

[0032] In various embodiments, the CNP-responsive bone-related disorder, skeletal dysplasia or short stature disorder is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, idiopathic short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis congenita, achondrogenesis, homozygous achondroplasia, chondrodysplasia congenita, campomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis congenita, short-rib polydactyly syndromes, rhizomelic type of chondrodysplasia congenita, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenital, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia, disorders related to NPR2 mutation, SHOX mutations, Turner's syndrome/Leri Weill, PTPN11 mutations, Noonan's syndrome, and IGF1R mutation.

[0033] In various embodiments, the CNP variant is set out in SEQ ID NOs: 1-53. In various embodiments, the CNP variant is
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP-37) (SEQ ID NO: 2); or LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 21).

[0034] In various embodiments, the CNP variant further comprises a hydrophilic moiety. In various embodiments, the hydrophilic moiety is PEG.

[0035] Also contemplated is an expression construct comprising a polynucleotide encoding a GFP protein operably linked to a cGMP binding domain as described herein useful in a

reporter assay as described herein. The cGMP binding domain is from mouse or human phosphodiesterase. In various embodiments, the expression construct further comprises a CMV promoter operably linked to cGull and PGK promoter operably linked to a blastocidin resistance gene.

[0036] Further aspects and advantages will be apparent to those of ordinary skill in the art from a review of the following detailed description, taken in conjunction with the drawings. While the compositions, articles, and methods are susceptible of embodiments in various forms, the description hereafter includes specific embodiments with the understanding that the disclosure is illustrative, and is not intended to limit the invention to the specific embodiments described herein. For the compositions, articles, and methods described herein, optional features, including but not limited to components, compositional ranges thereof, substituents, conditions, and steps, are contemplated to be selected from the various aspects, embodiments, and examples provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037] Figures 1A-1D depict a schematic of a high throughput screening process using an NPR2 expressing lentivirus and a cGMP reporter construct. Figure 1A. Construct of bidirectional lentivirus that expresses NPR2 and puro-T2A-BFP. Figure 1B. cGMP-responsive GFP-fluorescent reporter reconstituted into HEK293s cells. Figure 1C. Barcoding strategy and PacBio (long-read) and Illumina (short-read) based methods for associating barcode to variant. Figure 1D. GFP-sorting-based screening strategy.

[0038] Figure 2 illustrates a method of generating a NPR2 variant lentiviral library.

[0039] Figures 3A-3B show levels of cGMP detected by catchpoint assay (Figure 3A) or cGMP-GFP reporter construct of the disclosure (Figure 3B).

[0040] Figures 4A-4C show the process of screening samples using PCR generation of NPR2 variants (Figure 4A), screening where LoF or GoF functions will have different levels of cGMP production (Figure 4B), and results of a cGMP-GFP reporter screen on a GoF or LoF NPR2 variant (Figure 4C).

[0041] Figure 5 shows a representative sample sort from flow cytometric analysis of a GFP high or GFP low screen, and demonstrates the screen is highly correlative with the function of the NPR2 variant and cGMP-GFP expression.

[0042] Figures 6A-6B show that the high throughput screen using the cGMP-GFP construct (Figure 6A) correlates with previous published results of NPR2 variant function (Figure 6B).

[0043] Figure 7A-7C show variant functional activity by genetic consequences and phenotypic effects. Figure 7A. Measured cGMP levels by variant functional consequence.

Figure 7B. Varsity ER LOO predictions can partially discriminate between missense variants that alter NPR2 function. Figure 7C. Comparison of library screen cGMP measurements vs. effect on human adult height in UKBiobank.

[0044] Figures 8A-8B show low CNP stimulation and identified 7 GoF variants that were not present in the high CNP screen (Figure 8A). Additionally, a constitutively active variant was observed within the no CNP stimulation screen study (Figure 8B).

[0045] Figures 9A-9B show polygenic scores for height modify the effect of NPR2 variants. Figure 9A shows the how adult height varies by polygenic score in people who reported being short at age 10. Figure 9B. A logistic regression model was trained to predict adult short stature in people who reported being short at age 10. Independent variables included the presence of an NPR2 variant with reduced activity and the polygenic score only. This simple model can predict 2/3 of true positives while maintaining a false positive rate below 20%.

[0046] Figure 10 shows the sequence of human NPR2 protein.

[0047] Figure 11 provides a table of NPR2 variants screened.

[0048] Figure 12 shows goodness of fit plots for the final PPK model.

[0049] Figure 13 shows dose-normalized VPC results for the PPK model; dose-normalized observed and simulated vosoritide concentrations versus time after first dose.

[0050] Figure 14 shows simulated vosoritide AUC values compared with observed AUC values at 15 µg/kg from study 111-301.

[0051] Figure 15 shows simulated vosoritide C_{max} values compared with observed C_{max} values at 15 µg/kg from study 111-301.

[0052] Figure 16A shows metacarpal cortical area (mm²) at each time point in treatment. Figure 16B shows metacarpal robustness (mm) at each time point in treatment.

DETAILED DESCRIPTION

[0053] CNP targets the NPR2 receptor and simulates a signal transduction that results in signals that stimulate bone growth. This high throughput characterization of NPR2 variants described herein enables better prediction of novel variants. For those mutations which occur more commonly, the method could benefit diagnosis and clinical trial enrollment for eligible patients.

[0054] Previous screening methods (e.g., catchpoint assay) required over a year of effort to characterize ~160 variants. The present library-based screening strategy has several

advantages including higher-throughput (e.g., simultaneous screening of >400-1000 variants in months and higher quality and reproducibility of data (e.g., increase replicates).

[0055] As used in the specification and the appended claims, the indefinite articles “a” and “an” and the definite article “the” include plural as well as singular referents unless the context clearly dictates otherwise.

[0056] The term “about” or “approximately” means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. In certain embodiments, the term “about” or “approximately” means within 1, 2, 3, or 4 standard deviations. In certain embodiments, the term “about” or “approximately” means within 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.05% of a given value or range. Whenever the term “about” or “approximately” precedes the first numerical value in a series of two or more numerical values, it is understood that the term “about” or “approximately” applies to each one of the numerical values in that series.

[0057] “Amplification” refers to any means by which a polynucleotide sequence is copied and thus expanded into a larger number of polynucleotide molecules, e.g., by reverse transcription, polymerase chain reaction, and ligase chain reaction.

[0058] “cDNA” refers to a DNA that is complementary or identical to an mRNA, in either single stranded or double stranded form.

[0059] Conventional notation is used herein to describe polynucleotide sequences: the left-hand end of a single-stranded polynucleotide sequence is the 5'-end; the left-hand direction of a double-stranded polynucleotide sequence is referred to as the 5'-direction. The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the same sequence as an mRNA is referred to as the “coding strand”; sequences on the DNA strand having the same sequence as an mRNA transcribed from that DNA and which are located 5' to the 5'-end of the RNA transcript are referred to as “upstream sequences”; sequences on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the coding RNA transcript are referred to as “downstream sequences.”

[0060] “Complementary” refers to the topological compatibility or matching together of interacting surfaces of two polynucleotides. Thus, the two molecules can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other. A first polynucleotide is complementary to a second polynucleotide if the nucleotide sequence of the first polynucleotide is identical to the nucleotide sequence of the polynucleotide binding partner of the second polynucleotide. Thus, the polynucleotide

whose sequence 5'-TATAC-3' is complementary to a polynucleotide whose sequence is 5'-GTATA-3'. A nucleotide sequence is "substantially complementary" to a reference nucleotide sequence if the sequence complementary to the subject nucleotide sequence is substantially identical to the reference nucleotide sequence.

[0061] "Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA produced by that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and non-coding strand, used as the template for transcription, of a gene or cDNA can be referred to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

[0062] "Expression control sequence" refers to a nucleotide sequence in a polynucleotide that regulates the expression (transcription and/or translation) of a nucleotide sequence operatively linked thereto. "Operatively linked" or "operably linked" refers to a functional relationship between two parts in which the activity of one part (*e.g.*, the ability to regulate transcription) results in an action on the other part (*e.g.*, transcription of the sequence). Expression control sequences can include, for example and without limitation, sequences of promoters (*e.g.*, inducible or constitutive), enhancers, transcription terminators, a start codon (*i.e.*, ATG), splicing signals for introns, and stop codons.

[0063] The term "promoter" as used herein refers to a region of DNA that functions to control the transcription of one or more DNA sequences, and that is structurally identified by the presence of a binding site for DNA-dependent RNA-polymerase and of other DNA sequences, which interact to regulate promoter function. A functional expression promoting fragment of a promoter is a shortened or truncated promoter sequence retaining the activity as a promoter. Promoter activity may be measured in any of the assays known in the art *e.g.*, in a reporter assay using luciferase or green fluorescent protein (GFP) as reporter, or in commercially available reporters.

[0064] The term "vector" refers to any carrier of exogenous DNA or RNA that is useful for transferring exogenous DNA to a host cell for replication and/or appropriate expression of

the exogenous DNA by the host cell. "Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in vitro expression system. Expression vectors include all those known in the art, such as viral vectors, cosmids, plasmids (e.g., naked or contained in liposomes), that incorporate the recombinant polynucleotide.

[0065] "Viral vector" refers to a vector that uses a viral backbone for carrying a polynucleotide expression cassette. Viral vectors include lentiviral vectors, adenoviral vectors or adeno-associated vectors (AAV).

[0066] "Expression cassette" or "cassette" refers to a component of vector or plasmid DNA that controls expression of a gene or protein, and may be interchangeable and easily inserted or removed from a vector. Expression cassettes often comprise a promoter sequence, an open reading frame, and a 3' untranslated region that contains a polyadenylation site.

[0067] "Polynucleotide" refers to a polymer composed of nucleotide units. Polynucleotides include naturally occurring nucleic acids, such as deoxyribonucleic acid ("DNA"), including cDNA, and ribonucleic acid ("RNA") as well as nucleic acid analogs. Nucleic acid analogs include those which include non-naturally occurring bases, nucleotides that engage in linkages with other nucleotides other than the naturally occurring phosphodiester bond or which include bases attached through linkages other than phosphodiester bonds. Thus, nucleotide analogs include, for example and without limitation, phosphorothioates, phosphorodithioates, phosphotriesters, phosphoramidates, boranophosphates, methylphosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs), and the like. Such polynucleotides can be synthesized, for example, using an automated DNA synthesizer. The term "nucleic acid" typically refers to large polynucleotides. The term "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (*i.e.*, A, T, G, C), this also includes an RNA sequence (*i.e.*, A, U, G, C) in which "U" replaces "T."

[0068] "Polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer. The term "protein" typically refers to large

polypeptides. The term "peptide" typically refers to short polypeptides. Conventional notation is used herein to portray polypeptide sequences: the left-hand end of a polypeptide sequence is the amino-terminus; the right-hand end of a polypeptide sequence is the carboxyl-terminus.

[0069] "Recombinant polynucleotide" refers to a polynucleotide having sequences that are not naturally joined together. An amplified or assembled recombinant polynucleotide may be included in a suitable vector, and the vector can be used to transform a suitable host cell. A host cell that comprises the recombinant polynucleotide is referred to as a "recombinant host cell." The gene is then expressed in the recombinant host cell to produce, *e.g.*, a "recombinant polypeptide." A recombinant polynucleotide may serve a non-coding function (*e.g.*, promoter, origin of replication, ribosome-binding site, *etc.*) as well. Recombinant protein refers to a protein encoded by a recombinant polynucleotide.

[0070] The term "barcode" refers to a short nucleotide tag appended to a polynucleotide sequence of interest during preparation of a DNA library to provide information about a specific polynucleotide to which the barcode is appended or cell in which the polynucleotide of interest may be expressed. A barcode can be between 10 to 30 basepairs (bp) in length, or 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 basepairs. Multiple barcodes can be appended to a polynucleotide of interest.

[0071] The term "polynucleotide library" or "library" refers to a set of polynucleotide fragments that have been cloned into expression vectors in order to identify the polynucleotide fragments and isolate a gene or genes of interest. The polynucleotide library can be RNA or DNA, including genomic DNA or cDNA.

[0072] The term "C-type natriuretic peptide" or "CNP" refers to a small, single chain peptide having a 17-amino acid loop structure at the C-terminal end (GenBank Accession No. NP_077720, for the CNP precursor protein, NPPC) and variants thereof. The 17-mer CNP loop structure, is also referred to as CNP 17, the CNP ring, or CNP cyclic domain. CNP includes the active 53-amino acid peptide (CNP-53) and the mature 22-amino acid peptide (CNP-22), and peptides of varying lengths between the two peptides.

[0073] In various embodiments, a "CNP variant" is at least about 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% homologous to the wild type NPPC over the same number of amino acid residues. It is further contemplated that a CNP variant peptide may comprise from about 1 to about 53, or 1 to 39, or 1 to 38, or 1 to 37, or 1 to 35, or 1 to 34, or 1 to 31, or 1 to 27, or 1 to 22, or 10 to 35, or about 15 to about 37 residues of the NPPC polypeptide. In one embodiment, a CNP variant may comprise a sequence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34,

35,36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, or 53 amino acids derived from the NPPC polypeptide. CNP variant also includes conjugates, salts or prodrugs of CNP variants described herein. "CNP therapy" refers to administration of a CNP variant to treat a subject having a bone-related disorder, skeletal dysplasia or short stature as described herein.

[0074] The term "conjugate moiety" refers to a moiety that is conjugated to the variant peptide. Conjugate moieties include a lipid, fatty acid, hydrophilic spacer, synthetic polymer, linker, or optionally, combinations thereof.

[0075] "Treatment" refers to prophylactic treatment or therapeutic treatment or diagnostic treatment. In certain embodiments, "treatment" refers to administration of a compound or composition to a subject for therapeutic, prophylactic or diagnostic purposes.

[0076] A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs of the disease, for the purpose of decreasing the risk of developing pathology. The compounds or compositions of the disclosure may be given as a prophylactic treatment to reduce the likelihood of developing a pathology or to minimize the severity of the pathology, if developed.

[0077] A "therapeutic" treatment is a treatment administered to a subject who exhibits signs or symptoms of pathology for the purpose of diminishing or eliminating those signs or symptoms. The signs or symptoms may be biochemical, cellular, histological, functional or physical, subjective or objective. The compounds of the disclosure may also be given as a therapeutic treatment or for diagnosis.

[0078] "Diagnostic" means identifying the presence, extent and/or nature of a pathologic condition. Diagnostic methods differ in their specificity and selectivity. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

[0079] "Pharmaceutical composition" or "formulation" refers to a composition suitable for pharmaceutical use in subject animal, including humans and mammals. A pharmaceutical composition comprises a therapeutically effective amount of CNP variant, optionally another biologically active agent, and optionally a pharmaceutically acceptable excipient, carrier or diluent. In an embodiment, a pharmaceutical composition encompasses a composition comprising the active ingredient(s), and the inert ingredient(s) that make up the carrier, as well as any product that results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients.

[0080] Accordingly, the pharmaceutical compositions of the present disclosure encompass any composition made by admixing a compound of the disclosure and a pharmaceutically acceptable excipient, carrier or diluent.

[0081] "Pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, buffers, and the like, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions (e.g., an oil/water or water/oil emulsion). Non-limiting examples of excipients include adjuvants, binders, fillers, diluents, disintegrants, emulsifying agents, wetting agents, lubricants, glidants, sweetening agents, flavoring agents, and coloring agents. Suitable pharmaceutical carriers, excipients and diluents are described in Remington's Pharmaceutical Sciences, 19th Ed. (Mack Publishing Co., Easton, 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent. Typical modes of administration include enteral (e.g., oral) or parenteral (e.g., subcutaneous, intramuscular, intravenous or intraperitoneal injection; or topical, transdermal, or transmucosal administration).

[0082] A "pharmaceutically acceptable salt" is a salt that can be formulated into a compound for pharmaceutical use, including but not limited to metal salts (e.g., sodium, potassium, magnesium, calcium, *etc.*) and salts of ammonia or organic amines.

[0083] By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to an individual without causing any undesirable biological effects or without interacting in a deleterious manner with any of the components of the composition in which it is contained or with any components present on or in the body of the individual.

[0084] "Physiological conditions" refer to conditions in the body of an animal (e.g., a human). Physiological conditions include, but are not limited to, body temperature and an aqueous environment of physiologic ionic strength, pH and enzymes. Physiological conditions also encompass conditions in the body of a particular subject which differ from the "normal" conditions present in the majority of subjects, e.g., which differ from the normal human body temperature of approximately 37 °C or differ from the normal human blood pH of approximately 7.4.

[0085] By "physiological pH" or a "pH in a physiological range" is meant a pH in the range of approximately 7.0 to 8.0 inclusive, more typically in the range of approximately 7.2 to 7.6 inclusive.

C-type natriuretic peptide variants

[0086] C-type natriuretic peptide (CNP) (Biochem. Biophys. Res. Commun., 168: 863-870 (1990) (GenBank Accession No. NP_077720, for the CNP precursor protein, NPPC) (J. Hypertens., 10: 907-912 (1992)) is a small, single chain peptide in a family of peptides (ANP, BNP, CNP) having a 17-amino acid loop structure (Levin et al., N. Engl. J. Med., 339: 863-870 (1998)) and have important roles in multiple biological processes. CNP interacts with natriuretic peptide receptor-B (NPR-B, GC-B, NPR2) to stimulate the generation of cyclic-guanosine monophosphate (cGMP) (J. Hypertens., 10: 1111-1114 (1992)). CNP is expressed more widely, including in the central nervous system, reproductive tract, bone and endothelium of blood vessels (Hypertension, 49: 419-426 (2007)).

[0087] In humans, CNP is initially produced from the natriuretic peptide precursor C (NPPC) gene as a single chain 126-amino acid pre-pro polypeptide (Sudoh et al., Biochem. Biophys. Res. Commun., 168: 863-870 (1990)). Removal of the signal peptide yields pro-CNP, and further cleavage by the endoprotease furin generates an active 53-amino acid peptide (CNP-53), which is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide (CNP-22) (Wu, J. Biol. Chem. 278: 25847-852 (2003)). CNP-53 and CNP-22 differ in their distribution, with CNP-53 predominating in tissues, while CNP-22 is mainly found in plasma and cerebrospinal fluid (J. Alfonzo, Recept. Signal. Transduct. Res., 26: 269-297 (2006)). Both CNP-53 and CNP-22 bind similarly to NPR-B.

[0088] Downstream signaling mediated by cGMP generation influences a diverse array of biological processes that include endochondral ossification. For example, knockout of either CNP or NPR-B in mouse models results in animals having a dwarfed phenotype with shorter long bones and vertebrae. Mutations in human NPR-B that block proper CNP signaling have been identified and result in dwarfism (Olney, *et al.*, J. Clin. Endocrinol. Metab. 91(4): 1229-1232 (2006); Bartels, *et al.*, Am. J. Hum. Genet. 75: 27-34 (2004)). In contrast, mice engineered to produce elevated levels of CNP display elongated long bones and vertebrae.

[0089] Natural CNP gene and polypeptide have been previously described. U.S. Patent No. 5,352,770 discloses isolated and purified CNP-22 from porcine brain identical in sequence to human CNP and its use in treating cardiovascular indications. U.S. Patent No. 6,034,231 discloses the human gene and polypeptide of pre-proCNP (126 amino acids) and the human CNP-53 gene and polypeptide. The mature CNP is a 22-amino acid peptide (CNP-22). Certain CNP variants are disclosed in US Patent 8,198,242, incorporated by reference herein.

[0090] In various embodiments, CNP of the disclosure includes truncated CNP ranging from human CNP-17 (hCNP-17) to human CNP-53 (hCNP-53), and having wild-type amino acid

sequences derived from hCNP-53 and also variants thereof. Such truncated CNP peptides include:

PGQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1); GQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Gly-CNP-37) (SEQ ID NO: 2);

GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Gly-CNP53) (SEQ ID NO: 3);

PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Pro-CNP53) (SEQ ID NO: 4);

MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Met-CNP53) (SEQ ID NO: 5);

DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SNSGLGC [CNP-53(M48N)] (SEQ ID NO: 6);

LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-52) (SEQ ID NO: 7);

RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-51) (SEQ ID NO: 8);

VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP- 50) (SEQ ID NO: 9);

DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-49) (SEQ ID NO: 10);

TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-48) (SEQ ID NO: 11);

KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-47) (SEQ ID NO: 12);

SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-46) (SEQ ID NO: 13);

RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-45) (SEQ ID NO: 14);

AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-44) (SEQ ID NO: 15);

AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-43) (SEQ ID NO: 16); WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-42) (SEQ ID

NO: 17); ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-41) (SEQ ID NO: 18); RLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-40) (SEQ ID NO: 19); LLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-39) (SEQ ID NO: 20); LQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-38) (SEQ ID NO: 21); QEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-37) (SEQ ID NO: 22); EHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-36) (SEQ ID NO: 23); HPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-35) (SEQ ID NO: 24); PNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-34) (SEQ ID NO: 25); NARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-33) (SEQ ID NO: 26); ARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-32) (SEQ ID NO: 27); RKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-31) (SEQ ID NO: 28); KYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-30) (SEQ ID NO: 29); YKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-29) (SEQ ID NO: 30); KGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-28) (SEQ ID NO: 31); GANKKGLSKGCFGLKLDRI SMSGLGC (CNP-27) (SEQ ID NO: 32); ANKKGGLSKGCFGLKLDRI SMSGLGC (CNP-26) (SEQ ID NO: 33); NKKGLSKGCFGLKLDRI SMSGLGC (CNP-25) (SEQ ID NO: 34); KKGLSKGCFGLKLDRI SMSGLGC (CNP-24) (SEQ ID NO: 35); KGLSKGCFGLKLDRI SMSGLGC (CNP-23) (SEQ ID NO: 36); LSKGCFGLKLDRI SMSGLGC (CNP-21) (SEQ ID NO: 37); SKGCFGLKLDRI SMSGLGC (CNP-20) (SEQ ID NO: 38); KGCFGLKLDRI SMSGLGC (CNP-19) (SEQ ID NO: 39); GCFGLKLDRI SMSGLGC (CNP-18) (SEQ ID NO: 40); QEHPNARKYKGANKKGLSKGCFGLKLDRI SNSGLGC [CNP-37(M32N)] (SEQ ID NO: 41); PQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Pro-CNP-37) (SEQ ID NO: 42); MQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Met-CNP-37) (SEQ ID NO: 43); GQEHPNARKYKGANKKGLSKGCFGLKLDRI SNSGLGC [Gly-CNP-37(M32N)] (SEQ ID NO: 44); MGQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Met-Gly-CNP-37) (SEQ ID NO: 45); PGQEHPQARRYRGAQRRGLSRGCFGLKLDRI SMSGLGC (SEQ ID NO: 46); PGQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 47);

PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 48); and
PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 49).

[0091] In various embodiments, the CNP variant peptides are modified CNP-37 or CNP-38 peptides, optionally having mutation(s)/substitution(s) at the furin cleavage site, and/or containing glycine or proline-glycine at the N-terminus. Exemplary CNP-37 variants include but are not limited to:

QEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [CNP-37(M32N); SEQ ID NO: 41];
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-CNP-37; SEQ ID NO: 43);
PQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP-37; SEQ ID NO: 42);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP-37 (M32N); SEQ ID NO: 44];
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP-37; SEQ ID NO: 1);
MQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP-37; SEQ ID NO: 45);
GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP-37; SEQ ID NO: 2)
GQEHPNARKYKGANPKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 50);
GQEHPNARKYKGANQKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 51);
GQEHPNARKYKGANQQGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 52); and
GQEHPNARKYKGANKPGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 53);

[0092] In various embodiments, CNP variants of the disclosure include
PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 46);
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 47);
PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 48); or
PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 49).

[0093] The variant peptide may further comprise an acetyl group. In various embodiments, the acetyl group is on the N-terminus of the peptide. In various embodiments, the peptide further comprises an OH or an NH₂ group at the C-terminus.

[0094] The variant peptide may comprise a conjugate moiety. In various embodiments, the conjugate moiety is on a residue of the CNP cyclic domain or at a site other than the CNP cyclic domain. In various embodiments, the conjugate moiety is on a lysine residue. In various embodiments, the conjugate moiety comprises one or more acid moieties. In various embodiments, the acid moiety is a hydrophobic acid.

[0095] In various embodiments, the variant has the structure:
PGQEHPQARRYRGAQRRGLSRGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC

(SEQ ID NO: 46), or Ac-PGQEHPQARRYRGAQRRGLSRGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO: 46).

[0096] In various embodiments, the variant is selected from the group consisting of Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 46); Ac-PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC-NH₂ (SEQ ID NO: 47); Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 48); Ac-PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC-NH₂ (SEQ ID NO: 48); Ac-PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC-NH₂ (SEQ ID NO: 46); Ac-PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC-NH₂ (SEQ ID NO: 49); and Ac-PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC-OH (SEQ ID NO: 49).

[0097] In various embodiments, the CNP variant is Ac-PGQEHPQARRYRGAQRRGLSRGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO: 46). In various embodiments, the CNP variant is Ac-PGQEHPNARKYKGANKKGLSKGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO: 47). In various embodiments, the CNP variant is Ac-PGQEHPNARKYKGANKKGLSKGCFGLK(AEEA-AEEA-γGlu-C18DA)LDRIGSMSGLGC-OH (SEQ ID NO: 47).

[0098] It is further contemplated that the CNP variant is conjugated to or is complexed to a moiety, e.g., a conjugate moiety, that confers increased stability or half-life. In various embodiments, the conjugate moiety is complexed via a non-covalent bond or is attached by a covalent bond. The moiety may be non-covalently attached with the peptide via electrostatic interactions. Alternatively, the moiety may be covalently associated to the peptide via one or more linker moieties. Linkers can be cleavable and non-cleavable linkers. Cleavable linkers may be cleaved via enzymes, nucleophilic/basic reagents, reducing agents, photo-irradiation, electrophilic/acidic reagents, organometallic and metal reagents, or oxidizing reagents. Linkers may also be self-immolative linkers. Exemplary linkers include, but are not limited to, N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene), beta alanine, 4-aminobutyric acid (GABA), 2-aminoethoxy acid (AEA), aminoethoxy-2-ethoxy

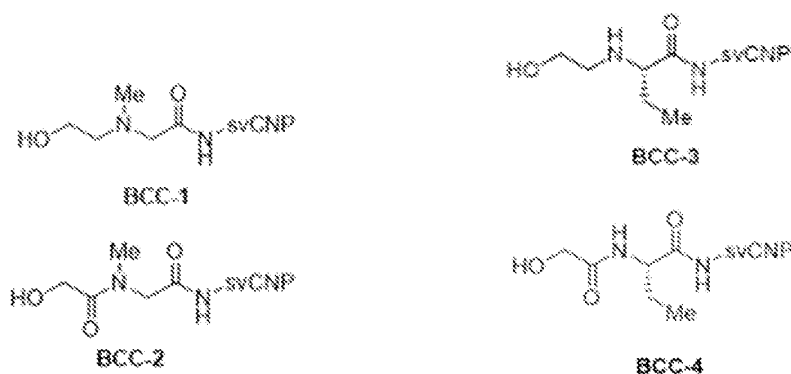
acetic acid (AEEA), 5 aminovaleric acid (AVA), 6-aminocaproic acid (Abx), a vicinal diol cleavable linker, Trimethyl Lock Lactonization, p-alkoxyphenyl carbamate, bicin, peptoid or bicin-type linkers, and electronic linkers as described herein.

[0099] It is contemplated that the linker is attached to a residue of the CNP variant within the CNP cyclic domain or at a site other than the CNP cyclic domain. In various embodiments, the linker is attached to a lysine residue. In various embodiments, the linker is attached to a lysine residue in the CNP cyclic domain.

[00100] In various embodiments, the CNP variant is attached to the conjugate moiety via the linker. In various embodiments, the linker is attached to the conjugate moiety via the hydrophilic spacer of the conjugate moiety.

[00101] In various embodiments, the linker is a hydrolysable linker.

[00102] In various embodiments the linker is a peptoid or electronic linker. In various embodiments the linker is a peptoid linker. In various embodiments the linker is an electronic linker. In various embodiments, the linker comprises an SO₂ moiety. Exemplary linkers are illustrated in Figure 7. It is further contemplated that linkers in Figure 7 are modified by substitution on the R groups. For example, bicin-type linkers include the structures as set out below:



[00103] In various embodiments, the moiety conjugated to the peptide is a synthetic polymer such as polyethylene glycol, a linker, a lipid moiety or fatty acid, or a combination thereof. In various embodiments, the CNP variant is conjugated with a fatty acid, an amino acid, a spacer and a linker. In various embodiments, the CNP variant is conjugated with a fatty acid, an amino acid, a polyethylene glycol spacer or a polyethylene glycol derivative spacer, and a linker. In various embodiments, the CNP variant is conjugated with a fatty acid, an amino acid, a spacer, and a linker, wherein the spacer comprises a substituted C-6 to C-20 alkyl chain or any amino acid, or a combination of both, wherein the carbon atoms of

the alkyl chain can be replaced by one or more of O, NH, N(C-1 to C-6 alkyl), or carbonyl groups.

[00104] In various embodiments, the CNP variant is conjugated with a fatty acid. It is hypothesized that the lipid technology increases the serum half-life of the CNP variant allowing for less frequent injections and/or improved oral delivery. In various embodiments, the fatty acid is a short chain, medium chain, long chain fatty acid, or a dicarboxylic fatty acid. In various embodiments, the fatty acid is saturated or unsaturated. In various embodiments, the fatty acid is a C-6 to C-20 fatty acid. In various embodiments, the fatty acid is a C-6, C-8, C-10, C-12, C-14, C-16, C-18 or C-20 fatty acid. In various embodiments, the fatty acid is decanoic acid, dodecanoic acid, myristic acid, palmitic acid, stearic acid, arachidic acid, or diacids of the same. In various embodiments, the fatty acid is conjugated to a lysine residue.

[00105] In various embodiments, it is contemplated that the CNP variants described herein comprise a conjugate moiety as described herein. It is contemplated that the conjugate moiety is on a residue of the CNP cyclic domain or at a site other than the CNP cyclic domain. In various embodiments, the conjugate moiety is on a lysine residue. In various embodiments, the conjugate moiety comprises one or more acid moieties. In various embodiments, the acid moiety is a fatty acid. Exemplary CNP variants and peptide conjugates are described in International Patent Application No. PCT/US2020/051100 and USSN 17/642,150, incorporated by reference herein in their entirety. Variants, conjugates and salts of CNP are disclosed in USSN 17/634,034, herein incorporated by reference.

[00106] In various embodiments, the conjugate moiety comprises an acid moiety linked to a hydrophilic spacer. In various embodiments, the hydrophilic spacer is a substituted C-6 to C-20 alkyl chain or any amino acid, or a combination of both, wherein the carbon atoms of the alkyl chain can be replaced by one or more of O, NH, N(C-1 to C-6 alkyl), or carbonyl groups. In various embodiments, the hydrophilic spacer is any amino acid. In various embodiments, the hydrophilic spacer is gamma glutamic acid (γ Glu). In various embodiments, the hydrophilic spacer is a substituted C-6 to C-20 alkyl chain. In various embodiments, the hydrophilic spacer is a substituted C-6, C-8, C-10, C-12, C-14, C-16, C-18 or C-20 alkyl chain. In various embodiments, the hydrophilic spacer is a substituted C-9 to C-18 alkyl chain. In various embodiments, the hydrophilic spacer is a substituted C-18 alkyl chain. In various embodiments, the hydrophilic spacer is a substituted C-9 alkyl chain. In various embodiments, the hydrophilic spacer is one or more OEG (8-amino-3,6-dioxaoctanoic acid) groups. In various embodiments, the hydrophilic spacer is one or two OEG (8-amino-3,6-dioxaoctanoic acid) groups. In various embodiments, the hydrophilic spacer is OEG (8-amino-3,6-dioxaoctanoic acid). In various embodiments, the spacer is

OEG (8-amino-3,6-dioxaoctanoic acid) or γ Glu. In various embodiments, the hydrophilic spacer is gamma glutamic acid (γ Glu) linked to one or more OEG (8-amino-3,6-dioxaoctanoic acid) groups. In various embodiments, the hydrophilic spacer is gamma glutamic acid (γ Glu) linked to one or two OEG (8-amino-3,6-dioxaoctanoic acid) groups (diEG). In various embodiments, the acid moiety and the hydrophilic spacer have the structure AEEA-AEEA- γ Glu-C18DA.

[00107] In various embodiments, the disclosure contemplates use of CNP variants comprising hydrophilic or water-soluble polymers (e.g., oxygenated alkyl chains, wherein the carbon atoms can be replaced with one or more oxygen atoms, such as polyethylene glycol (PEG) or polyethylene oxide (PEO) and the like). In various embodiments, the water soluble polymers can vary in type (e.g., homopolymer or copolymer; random, alternating or block copolymer; linear or branched; monodispersed or polydispersed), linkage (e.g., hydrolysable or stable linkage such as, e.g., amide, imine, aminal, alkylene, or ester bond), conjugation site (e.g., at the N-terminus, internal, and/or C-terminus), and length (e.g., from about 0.2, 0.4 or 0.6 kDa to about 2, 5, 10, 25, 50 or 100 kDa). The hydrophilic or water-soluble polymer can be conjugated to the CNP variant by means of N-hydroxy succinimide (NHS)- or aldehyde-based chemistry or other chemistry, as is known in the art. In various embodiments, negatively charged PEG-CNP variants can be designed for reduced renal clearance, including but not limited to use of carboxylated, sulfated and phosphorylated compounds (Caliceti, *Adv. Drug Deliv. Rev.*, 55: 1261-77 (2003); Perlman, *J. Clin. Endo. Metab.*, 88: 3227-35 (2003); Pitkin, *Antimicrob. Ag. Chemo.*, 29: 440-444 (1986); Vehaskari, *Kidney Int'l*, 22: 127-135 (1982)). In one embodiment, the PEG (or PEO) moiety contains carboxyl group(s), sulfate group(s), and/or phosphate group(s).

[00108] In another embodiment, the hydrophilic polymer (e.g., PEG or PEO) moieties conjugated to the N-terminus, C-terminus and/or internal site(s) of CNP variants described herein contain one or more functional groups that are positively charged under physiological conditions. Such moieties are designed, *inter alia*, to improve distribution of such conjugated CNP variants to cartilage tissues. In one embodiment, PEG moieties contain one or more primary, secondary or tertiary amino groups, quaternary ammonium groups, and/or other amine-containing (e.g., urea) groups.

[00109] In additional embodiments, for any of the CNP variants described herein that have lysine (Lys/K) residue(s), whether they have a wild-type sequence or a non-natural amino acid sequence, any Lys residue(s) can independently be substituted with any other natural or unnatural amino acids, including substitutions such as Lys to Arg. In various embodiments, all lysine residues are independently substituted with any other natural or unnatural amino acids, including substitutions such as Lys to Arg, except the Lys residue in

the CNP variant cyclic domain is not substituted with any other natural or unnatural amino acids.

C-type natriuretic peptide variants

[00110] Contemplated herein is a method of making a library of a variant gene associated with short stature using a lentiviral vector expression construct. Massively parallel reporter assays (MPRAs) have been disclosed previously (Gordon et al., Nat Protoc. 2020 15(8): 2387–2412). These assays utilized a library of regulatory genes (e.g., promoters and enhancers) to determine the effects of genetic mutations on the ability of the regulatory regions to carry out regulatory function. In another assay, a library of T cell open reading frames was generated to identify genes that positively regulate T cell activity (Daniloski, Nature 2022 603(7902):1-8).

[00111] However, these parallel reporter assays have not been used to identify variants of non-regulatory genes, when coupled with a functional reporter system. The present disclosure provides a method for making a lentiviral library of cell-surface receptor variants that are then introduced into a cell expressing a reporter construct having a readout that correlates with the cell surface receptor function.

[00112] Lentiviral vectors are known in the field of genetic engineering. Commercially available lentiviral vectors can be adapted as needed to express a variant gene associated with short stature or the wild type protein. For example, the lentiviral vector can be derived from human immunodeficiency virus, feline immunodeficiency virus, equine immunodeficiency virus, a pseudotyped lentivirus, a VSVg-pseudotype lentiviral vector, pLS-Scel vector (ADDGENE), other commercially available customizable vectors (e.g., VectorBuilder Inc.).

[00113] Other commercially available viral vectors are also contemplated.

[00114] In various embodiments, the method is useful to make a library of a variant gene associated with short stature. In various embodiments, the variant gene associated with short stature is collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, insulin growth factor 1 receptor (IGF1R), DTL, PAPP2, or combinations thereof. In various embodiments, the variant gene associated with short stature is NPR2, NPPC, or FGFR3. In various embodiments, the variant gene associated with short stature is NPR2.

[00115] In various embodiments, the lentiviral vector comprises NPR2 variants. NPR2 protein-altering variants have been identified in the UK Biobank study and described in Estrada et al. (Nat Commun. 2021, 12(1):2224) and in International Patent Publication

WO 2021/055497, herein incorporated by reference. The sequence of NPR2 is set out in Figure 10 and NPR2 variants identified for screening in the library include those set out in Figure 11. Approximately 160 NPR2 genetic variants were recently phenotypically characterized using a previously described cGMP-quantifying “catchpoint” assay (Estrada et al., *supra*). The present library preparation and screening is improved over the previous catchpoint assay.

[00116] In one embodiment, the lentiviral vector comprises a promoter region. In various embodiments, the promoter is a CMV promoter, an EFGR promoter, a MND promoter, a CAG promoter, a PGK promoter, an EF1A promoter.

[00117] In various embodiments, the lentiviral vector comprises a selection gene. In various embodiments, the selection gene includes a puromycin, kanamycin, blasticidin G418, or neomycin.

[00118] In one embodiment, a bidirectional lentivirus construct that expresses NPR2 and puromycin-T2A-BFP was used. In one embodiment, the lentivirus NPR2-expressing construct was constructed by subcloning into a bidirectional lentiviral vector which expressed puro-T2A-BFP in the other direction (Robinson et al., PLoS One 2021 Apr 9;16(4):e0249117).

[00119] In various embodiments, the lentiviral vector further comprises unique barcodes associated with the variant gene. In various embodiments, the variant in the vector comprises between 10 and 60 barcodes. In various embodiments, the variant in the vector comprises between 20 and 50 barcodes, between 20 and 40 barcodes, or between 30 and 45 barcodes.

[00120] In various embodiments, the barcodes are between 15 to 30 basepairs. In various embodiments, the barcodes are between 18 to 25 basepairs. In various embodiments the barcodes are 20 basepairs. An exemplary barcode library comprises approximately 20 basepair random sequences with $>10^9$ complexity. A barcode library is constructed, for example, as described by Azenta (Burlington, MA), and cloned into a lentiviral vector comprising a variant gene of interest. Primers and next generation sequencing pipeline for sequencing barcodes was designed and executed by Collecta using a similar protocol as described by the manufacturer (manuals.collecta.com/ngs-prep-kit-for-sgrna-shrna-dna-barcode).

Methods of Screening

[00121] The existence of a LoF or GoF variant in a gene related to short stature is determined by a biological activity assay. In various embodiments, the biological assay is a cGMP reporter assay.

[00122] In various embodiments, the reporter assay uses an expression construct comprising a cGMP binding domain linked to a reporter construct. In various embodiments the reporter is green fluorescent protein, red fluorescent protein, luciferase, beta galactosidase, and the like. In various embodiments, the cGMP binding domain is from human or mouse phosphodiesterase.

[00123] In various embodiments, the expression construct is a lentiviral vector.

[00124] In various embodiments, the lentiviral vector comprises a promoter region. In various embodiments, the promoter is a CMV promoter, an EFGR promoter, a MND promoter, a CAG promoter, a PGK promoter, or an EF1A promoter.

[00125] In various embodiments, the lentiviral vector comprises a selection gene. In various embodiments, the selection gene includes a puromycin, kanamycin, blasticidin G418, or neomycin.

[00126] In various embodiments, the expression construct is transfected or transduced into a host cell. In various embodiments the host cell is a mammalian cell. Exemplary mammalian cell lines include, but are not limited to, HEK293, CHO, MDCK, BHK, NIH/3T3, COS, A549, MEF, or HeLa cells.

[00127] In various embodiments, the expression construct comprises cGMP binding domain and a GFP reporter. In various embodiments, the expression construct comprises a CMV promoter operably linked to the cGMP-GFP reporter and PGK promoter operably linked to a blastocidin resistance gene. In various embodiments, the expression construct is transfected into HEK293 cells.

[00128] In various embodiments, cells transfected with a cGMP expression construct as described herein are contacted with CNP in order to induce cGMP production. In various embodiments, the cells are contacted with from about 1 to 60 nM CNP. In various embodiments, the cells are contacted with about 1 to 5 mM CNP, about 5 to 50 mM CNP, about 10 to 40 mM CNP, or about 15 to 50 mM CNP.

[00129] In various embodiments, the level of reporter expressed by the expression construct is measured by ELISA, flow cytometry, enzyme substrate assay, or other appropriate assay for the selected reporter.

[00130] In various embodiments, a LoF or GoF variant may be predicted based on mapping the change in the variant sequence to the predicted 3D structure and activity

domain of a protein encoded by the gene, e.g., using AlphaForm 3D mapping or other protein mapping tools.

Genes Associated with Short Stature

[00131] Achondroplasia is a result of an autosomal dominant mutation in the gene for fibroblast growth factor receptor 3 (FGFR-3), which causes an abnormality of cartilage formation. FGFR-3 normally has a negative regulatory effect on chondrocyte growth, and hence bone growth. In achondroplasia, the mutated form of FGFR-3 is constitutively active, which leads to severely shortened bones. In humans activating mutations of FGFR-3 are the primary cause of genetic dwarfism. Mice having activated FGFR-3 serve as a model of achondroplasia, the most common form of the skeletal dysplasias, and overexpression of CNP rescues these animals from dwarfism. Accordingly, functional variants of CNP are potential therapeutics for treatment of the various skeletal dysplasias.

[00132] By stimulating matrix production, proliferation and differentiation of chondrocytes and increasing long bone growth, the CNP variants of the disclosure are useful for treating mammals, including humans, suffering from a bone-related disorder, such as a skeletal dysplasia or short stature. Non-limiting examples of CNP-responsive bone-related disorders skeletal dysplasias and short stature disorders include achondroplasia, hypochondroplasia, short stature, idiopathic short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis congenita, achondrogenesis, homozygous achondroplasia, chondrodysplasia congenita, campomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis congenita, short-rib polydactyly syndromes, rhizomelic type of chondrodysplasia congenita, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia, disorders related to NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), PTPN11 mutations (Noonan's syndrome) and IGF1R mutation.

[00133] Additional short stature and growth plate disorders contemplated by the methods include disorders related to mutations in collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, or IGF1R. In various embodiments, the short stature gene is NPR2, NPPC or FGFR3.

[00134] Growth plate disorders include disorders that result in short stature or abnormal bone growth and that may be the result of a genetic mutation in a gene involved in bone growth, including collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3 or IGF1R. In various embodiments, the growth plate disorder or short stature is associated with one or more mutations in a gene associated with a RASopathy. In various embodiments, a subject with a growth plate disorder is heterozygous for a mutation in a growth plate gene. In various embodiments, the mutation is a loss-of-function mutation. In various embodiments, the mutation is a gain-of-function mutation. Growth plate disorders include, but are not limited to, familial short stature, dominant familial short stature which is also known as dominant inherited short stature, or idiopathic short stature. See, e.g., Plachy et al., J Clin Endocrinol Metab 104: 4273-4281, 2019.

[00135] Mutations in ACAN can give rise to familial osteochondritis dissecans and short stature and eventually osteoarthritis, characterized by areas of bone damage (or lesions) caused by the detachment of cartilage and sometimes bone from the end of the bone at a joint. It has been suggested that the disorganized cartilage network in growing bones impairs their growth, leading to short stature. A mutation associated with ACAN and short stature includes Val2303Met. See Stattin et al., Am J Hum Genet 86(2):126-37, 2010. It is contemplated that patients with a mutation in ACAN resulting in short stature would benefit from treatment with CNP as administration may be able to increase height in these patients by the known interaction of CNP with FGFR3.

[00136] The natriuretic peptide system, including receptor NPR2, has been shown to be involved in regulation of endochondral bone growth (Vasques et al., Horm Res Pediat 82:222-229, 2014). Studies have shown that homozygous or compound heterozygous loss-of-function mutations in NPR2 cause acromesomelic dysplasia type Maroteaux (AMDM), which is a skeletal dysplasia having extremely short stature (Vasquez et al., 2014, *supra*). There are reports implicating heterozygous loss-of-function (such as dominant negative) NPR2 mutations as a cause of short stature, whereas gain-of-function NPR2 heterozygous mutations have been found to be responsible for tall stature (Vasquez et al., 2014, *supra*). In view of CNP's interaction with NPR2 to stimulate cGMP generation, increasing cGMP levels is desirable in these conditions and would have therapeutic benefit in the management of the complications from these diseases and conditions.

[00137] Heterozygous mutations of NPR2 are believed to result in idiopathic short stature and other forms of short stature. Mutations in the NPR2 gene are set out below and described in Amano et al., J Clin Endocrinol Metab 99:E713-718, 2014, Hisado-Oliva et al., J Clin Endocrinol Metab 100:E1133-1142, 2015 and Vasques et al., J Clin Endocrinol Metab

98:E1636-1644, 2013, hereby incorporated by reference. It is contemplated that a subject having short stature to be treated with a CNP variant as described herein has a height SDS of less than -1.0, -1.5, -2.0, -2.5, or -3.0, and has at least one parent with a height SDS of less than -1.0, -1.5, -2.0 or -2.5, optionally wherein the second parent has height within the normal range. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -3.0. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -2.5. However, because de novo mutations in NPR2 can result in short stature as defined by a height SDS of less than -1.5, -2.0, -2.5, or -3.0, treatment of individuals who are heterozygous carriers of a deleterious mutation in NPR2 with neither parent having short stature is also contemplated. Further contemplated is treatment of individuals who are heterozygous for deleterious mutations in other growth plate genes with CNP to improve stature and/or enhance bone growth.

[00138] Exemplary mutations in NPR2 are disclosed in International Patent Publication WO 2021/055497, incorporated herein by reference.

[00139] NPPC's role in skeletal growth is well documented (Hisado-Oliva et al., Genetics Medicine 20:91-97, 2018). The NPPC knock out mouse showed severe disproportionate form of dwarfism including shortening of limbs and endochondral ossification (Hisado-Oliva et al., 2018, *supra*). Human genome wide studies have shown a link between NPPC and height (Hisado-Oliva et al., 2018, *supra*). Although CNP haploinsufficiency has been believed to be a cause of short stature in humans, a recent study identified heterozygous mutations in families with short stature and hands (Hisado-Oliva et al., 2018, *supra*). These studies observed significant reduction in cGMP production as measured in heterozygous state (Hisado-Oliva et al., 2018, *supra*). Mutations in NPPC include a 355G>T missense mutation causing a Gly119Cys change and a 349C>G missense mutation causing a Arg117Gly change. A CNP variant rescuing cGMP production may provide therapeutic benefit in the management of a disorder in patients having heterozygous loss-of-function NPPC mutations.

[00140] Leri-Weill dyschondrosteosis (LWD) is a rare genetic disorder characterized by shortening of the forearms and lower legs, abnormal misalignment of the wrist (Madelung deformity of the wrist), and associated short stature. LWD is caused by a heterozygous mutation in the short stature homeobox-containing (SHOX) gene or its regulatory elements located on the pseudoautosomal region 1 (PAR1) of the sex chromosomes. (See the Rare Disease Database and Carmona et al., Hum Mol Genet 20:1547-1559, 2011). The disorder Langer mesomelic dysplasia arises when there are two SHOX mutations, and may result from a mutation on each chromosome, either a homozygous or compound heterozygous

mutations. A subset of SHOX mutations give rise to idiopathic short stature. Turner syndrome results due to a deletion on the X chromosome that can include the SHOX gene. SHOX has been identified as involved in the regulation of *FGFR3* transcription and contributes to control of bone growth (Marchini et al., *Endocr Rev.* 37: 417–448, 2016). SHOX deficiency leads to increased FGFR3 signaling, and there is some evidence to support that SHOX has direct interactions with CNP/NPR2 as well (Marchini, *supra*). Given the association of SHOX with FGFR3 and bone growth, it is contemplated that a subject having a homozygous or heterozygous SHOX mutation would benefit from treatment with CNP variants as described herein.

[00141] RASopathies are a group of rare genetic conditions caused by mutations in genes of the Ras/mitogen-activated protein kinase (MAPK) pathway. RASopathies are a group of disorders characterized by increased signaling through RAS/MAPK pathway. This pathway leads to downstream activation of the RAF/MEK/ERK pathway. Short stature is a characteristic feature of certain RASopathies. For example, CNP signaling inhibits RAF and leads to decreased MEK and ERK activation.

[00142] RASopathies associated with short stature include Noonan syndrome, Costello syndrome, Cardiofaciocutaneous syndrome, Neurofibromatosis Type 1, and LEOPARD syndrome. Hereditary gingival fibromatosis type 1 is also a RASopathy contemplated herein. RASopathy patients (including Noonan syndrome, Costello syndrome, Cardiofaciocutaneous syndrome, Neurofibromatosis Type 1, LEOPARD syndrome, hereditary gingival fibromatosis type 1) include patients with heterozygous variants in one or more of the following genes: BRAF, CBL, HRAS, KRAS, LZTR1, MAP2K1, MAP2K2, MRAS, NF1, NRAS, PPP1CB, PTPN11, RAF1, RRAS, RIT1, SHOC2, SOS1, or SOS2 (Tajan et al. *Endocr. Rev.* 2018;39(5):676–700).

[00143] CFC is caused by mutations in several genes in the Ras/MAPK signaling pathway, including K-Ras, B-Raf, Mek1 and Mek2. Costello syndrome, also called faciocutaneoskeletal (FCS) syndrome is caused by activating mutations in the H-Ras gene. Hereditary gingival fibromatosis type I (HGF) is caused by dominant mutations in the SOS1 gene (*Son of Seven/less* homolog 1), which encodes a guanine nucleotide exchange factor (SOS) that acts on the Ras subfamily of small GTPases. Neurofibromatosis type I (NF1) is caused by mutations in the neurofibromin 1 gene, which encodes a negative regulator of the Ras/MAPK signaling pathway. Noonan syndrome (NS) is caused by mutations in one of several genes, including PTPN11, which encodes SHP2, and SOS1, as well as K-Ras and Raf-1.

[00144] CNP has been demonstrated to be an effective therapy in RASopathy models. Ono et al. generated mice deficient in *Nf1* in type II collagen producing cells (Ono et al., Hum. Mol. Genet. 2013;22(15):3048–62). These mice demonstrated constitutive ERK1/2 activation, and decreased chondrocyte proliferation, and maturation. Daily injections of CNP in these mice led to decreased ERK phosphorylation and corrected the short stature. A mouse model of Cardiofaciocutaneous syndrome using a *Braf* mutation (p.Q241R) (Inoue et al. Hum. Mol. Genet. 2019;28(1):74–83). exhibited decreased body length and reduced growth plate width with smaller proliferative and hypertrophic zones compared to wild type, and CNP administration led to increases in body length in these animals.

[00145] Mutations in multiple genes can cause Noonan syndrome, which is characterized by short stature, heart defects, bleeding problems, and skeletal malformations. Mutations in the PTPN11 gene cause about half of all cases of Noonan's syndrome. SOS1 gene mutations cause an additional 10 to 15 percent, and RAF1 and RIT1 genes each account for about 5 percent of cases. Mutations in other genes each account for a small number of cases. The cause of Noonan syndrome in 15 to 20 percent of people with this disorder is unknown.

[00146] The PTPN11, SOS1, RAF1, and RIT1 genes all encode for proteins that are important in the RAS/MAPK cell signaling pathway, which is needed for cell division and growth (proliferation), differentiation, and cell migration. Many of the mutations in the genes associated with Noonan syndrome cause the resulting protein to be turned on (active) and this prolonged activation alters normal RAS/MAPK signaling, which disrupts the regulation of cell growth and division, leading to the characteristic features of Noonan syndrome. See, e.g., Chen et al., Proc Natl Acad Sci U S A. 111(31):11473-8, 2014, Romano et al., Pediatrics. 126(4):746-59, 2010, and Milosavljević et al., Am J Med Genet 170(7):1874-80, 2016. It is contemplated that a subject having mutations that activate the MAPK pathway would benefit from treatment with CNP variants as described herein to improve bone growth and short stature. It is also contemplated that a subject having mutations that activate the MAPK pathway would benefit from treatment with CNP variants to improve other comorbidities associated with an overactive MAPK pathway in other cells throughout the body where the NPR2 receptor is expressed on its surface.

[00147] Mutations in the PTPN11 gene, which encodes the non-receptor protein tyrosine phosphatase SHP-2, lead to disorders characterized by short stature such as Noonan's Syndrome (Musente et al., Eur J Hum Genet 11:201–206 (2003). Musente (*supra*) identifies numerous mutations in the PTPN11 gene that lead to short stature. Gain of function mutations lead to overactive signaling through SHP2 and inhibit Growth Hormone-induced IGF-1 release, thereby contributing to a decrease in bone growth (Rocca

Serra-Nédélec, PNAS 109:4257-4262, 2012). It is contemplated that a subject having a homozygous or heterozygous PTPN11 mutation would benefit from treatment with CNP variants to improve bone growth and short stature.

[00148] Mutations in the Indian hedgehog (IHH) gene, which is related to regulation of endochondral ossification, have also been associated with short stature syndromes (Vasques et al., J Clin Endocrinol Metab. 103:604-614, 2018). Many IHH mutations identified segregate with short stature in a dominant inheritance pattern. Given the association of IHH with bone growth and ossification, it is contemplated that subjects having a homozygous or heterozygous IHH mutation will benefit from treatment with a CNP variant as described herein.

[00149] Mutations in FGFR3, including N540K and K650N, lead to short stature and hypochondroplasia.

[00150] Insulin-like growth factor 1 receptor (IGF1R) is a heterotetrameric ($\alpha 2\beta 2$) transmembrane glycoprotein with an intrinsic kinase activity. IGF1R has been shown to have a role in prenatal and postnatal growth. Heterozygous mutations in IGF1R have been identified in Small for gestational age children (SGA) and individuals with familial short stature (Kawashima et al., Endocrine J. 59:179-185, 2012). Mutations in IGF1R associated with short stature include R108Q/K115N, R59T, R709Q, G1050K, R481Q, V599E, and G1125A (Kawashima, *supra*).

[00151] Height is a highly heritable trait that can be influenced by the combined effect of hundreds or thousands of genes (Wood et al, 2014, Nature Genetics, 46:1173-1189). Short stature in an individual can be the result of the combined effect of these genes, without a single gene being the primary contributor. It is contemplated that such individuals with short stature defined by a height SDS of less than -1.0, -1.5, -2.0, -2.5, or -3.0, can be beneficially treated with a CNP variant given the ability of CNP to increase the length of normal animals, for example, enhance bone growth and length of bones.

[00152] In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of less than -1.0, -1.5, -2.0, -2.5, or -3.0, and having at least one parent with a height SDS of less than -1.0, -1.5, -2.0 or -2.5, optionally wherein the second parent has height within the normal range. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -3.0. In various embodiments, the CNP variants are useful to treat a subject with short stature having a height SDS of between -2.0 to -2.5. In various embodiments, the short stature is associated with one or more mutations in a gene associated with short stature, such as, collagen (COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian

hedgehog (IHH), PTPN11, NPR2, NPPC, FGFR3, insulin growth factor 1 receptor (IGF1R), DTL, PAPP2, or combinations thereof.

[00153] In various embodiments, the growth plate disorder or short stature is associated with one or more mutations in a gene associated with a RASopathy.

[00154] In various embodiments, the short stature is a result of mutations in multiple genes as determined by polygenic risk score (PRS). Polygenic risk scores (PRS) are calculated for height using the largest published genome-wide association study (GWAS) meta-analysis for height that do not include any samples from the UK Biobank project as described in WO 2021/055497. The cohort may be divided into five PRS quintiles (PRS 1 being the lowest height, PRS 5 the tallest height). In various embodiments, the subject has a mutation in NPR2 and a low PRS. In various embodiments, the subject has a mutation in FGFR3 and a low PRS. In various embodiments, the subject has a mutation in NPR2 and a low PRS. In various embodiments, the subject has a mutation in IGF1R and a low PRS. In various embodiments, the subject has a mutation in NPPC and a low PRS. In various embodiments, the subject has a mutation in SHOX and a low PRS. In various embodiments, the subject has one or more mutation in one or more of FGFR3, IGF1R, NPPC, NPR2 and SHOX, and a low PRS. In various embodiments, the PRS is 1 or 2. In various embodiments, the PRS is 1. In various embodiments, the PRS is 2.

[00155] In addition, CNP variants are useful for treating other bone-related conditions and disorders, such as rickets, hypophosphatemic rickets [including X-linked hypophosphatemic rickets (also called vitamin D-resistant rickets) and autosomal dominant hypophosphatemic rickets], and osteomalacia [including tumor-induced osteomalacia (also called oncogenic osteomalacia or oncogenic hypophosphatemic osteomalacia)].

[00156] Exemplary genes related to skeletal dysplasia or short stature, include but are not limited to, NPR2, SHOX, PTPN11, COL2A1, COL11A1, COL9A2, COL10), aggrecan (ACAN), indian hedgehog (IHH), NPPC, FGFR3, IGF1R, DTL, and pregnancy-associated plasma protein A2 (PAPP2).

[00157] It is reported herein that CNP also improves bone strength of subjects with achondroplasia that receive long term CNP therapy. Provided herein is a method of improving and/or maintaining bone strength in a subject in need thereof comprising administering a C-type natriuretic peptide (CNP) to the subject. In various embodiments, the subject has a bone-related disorder, such as a skeletal dysplasia or short stature. Non-limiting examples of CNP-responsive bone-related disorders skeletal dysplasias and short stature disorders include achondroplasia, hypochondroplasia, short stature, idiopathic short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis

imperfecta, osteogenesis congenita, achondrogenesis, homozygous achondroplasia, chondrodysplasia congenita, campomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis congenita, short-rib polydactyly syndromes, rhizomelic type of chondrodysplasia congenita, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenital, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, spondyloepimetaphyseal dysplasia, disorders related to NPR2 mutation, SHOX mutation (Turner's syndrome/Leri Weill), PTPN11 mutations (Noonan's syndrome), IGF1R mutation, rickets, hypophosphatemic rickets [including X-linked hypophosphatemic rickets (also called vitamin D-resistant rickets) and autosomal dominant hypophosphatemic rickets], and osteomalacia [including tumor-induced osteomalacia (also called oncogenic osteomalacia or oncogenic hypophosphatemic osteomalacia).

[00158] In certain embodiments, the CNP variants and compositions and formulations comprising the same of the present disclosure are useful for improving one or more of the symptom(s) or physiological consequences of a skeletal dysplasia, wherein the improvement may be increased absolute growth, increased growth velocity, increased qualitative computed tomography (QCT) bone mineral density, improvement in growth plate morphology, increased long bone growth, improvement in spinal morphology, improved elbow joint range of motion and/or decreased sleep apnea. In this regard, it is noted that the terms "improved", "improvement", "increase", "decrease" and grammatical equivalents thereof are all relative terms that when used in relation to a symptom or physiological consequence of a disease state, refer to the state of the symptom or physiological consequence of the disease after treatment with a CNP variant (or composition or formulation comprising the same) of the present invention as compared to the same symptom or physiological consequence of the disease before treatment with a CNP variant (or composition or formulation comprising the same) of the present invention (i.e., as compared to "baseline"). As described above, a "baseline" state can be determined either through measurement of the state in the subject prior to treatment (which can subsequently be compared to the state in the same subject after treatment), or through measurement of that state in a population of subjects suffering from the same affliction that share the same or similar characteristics (e.g., age, sex and/or disease state or progression).

[00159] In yet another embodiment, the disclosure provides CNP variants that *in vitro* or *in vivo* stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%,

110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 μ M). In a still further embodiment, the CNP variants of the disclosure *in vitro* or *in vivo* stimulate the production of at least about 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140% or 150% of the cGMP level produced under the same concentration of wtCNP22 (e.g., 1 μ M).

[00160] It is contemplated that any of the CNP variants described herein are useful in the methods.

[00161] Additional aspects and details of the disclosure will be apparent from the following examples, which are intended to be illustrative rather than limiting.

[00162] Further aspects and advantages will be apparent to those of ordinary skill in the art from a review of the following detailed description, taken in conjunction with the drawings. While the compositions, articles, and methods are susceptible of embodiments in various forms, the description hereafter includes specific embodiments with the understanding that the disclosure is illustrative, and is not intended to limit the invention to the specific embodiments described herein. For the compositions, articles, and methods described herein, optional features, including but not limited to components, compositional ranges thereof, substituents, conditions, and steps, are contemplated to be selected from the various aspects, embodiments, and examples provided herein.

EXAMPLES

Example 1-Construction of lentivirus construct that expresses NPR2

Materials and Methods

[00163] *Vector and Cell line construction.* A cGMP-GFP-on reporter was designed by subcloning the cGull insert from (Matsuda et al, ACS Sens 2017 2(1):46-51) and cloning into the VectorBuilder (VectorBuilder Inc., Chicago, IL) custom lentiviral vector with CMV promoter driving cGull and mouse PGK driving blastocidin resistance. cGull comprises the cGMP binding domain from mouse phosphodiesterase 5 α . The HEK293 cGMP-GFP-on reporter line was constructed by transducing HEK293T p11 cells with MOI=0.2 cGMP-GFP reporter virus and blasticidin selection (10 μ g/ml) for 4 days. Cells were tested by transfecting WT NPR-expressing vector and stimulating cells with 50nM CNP.

[00164] A custom bidirectional lentivirus construct that expresses NPR2 and puro-T2A-BFP was used. The lenti NPR2-expressing construct was constructed by subcloning (PspXI/NotI) into a custom bidirectional vector which expressed puro-T2A-BFP in the other direction (Robinson et al., PLoS One 2021 Apr 9;16(4):e0249117).

[00165] *Barcode synthesis and barcode-seq NGS primers.* A 20bp randomer sequence with $>10^9$ complexity was constructed and cloned (Genewiz/Azenta) into the PmeI site of the NPR2 vector. Primers and NGS pipeline for sequencing barcodes was designed and executed by Collecta using a similar protocol as described by the manufacturer (manuals.collecta.com/ngs-prep-kit-for-sgrna-shrna-dna-barcode-libraries).

Table 1

Fwd-NPR2-98539	TGTCTGGATCTCAAATCCCTCGGA (SEQ ID NO: 54)
Rev-NPR2-98539	AGGAAAGATGCGAACATACTGGCT (SEQ ID NO: 55)
P5-NFwd-NPR2-98539	AATGATACGGCGACCACCGAGATCTACACGTCATCGAATTCCTGCAGCC (SEQ ID NO: 56)
P7-NRev-NPR2-98539-IND-A	CAAGCAGAAGACGGCATACGAGATGCACGACGAGACGCAGACGAATACGACAGAGCTGGCTCTTAGGAGAGCGGA (SEQ ID NO: 57)
FSeq-NPR2-98539-BC	AGCTCAGTTAGCCTCCCCATCTCTCGGTTTAAAC (SEQ ID NO: 58)

[00166] *Cloning of NPR2 variants and PacBio association of barcodes to variants.* 160 NPR2 variants (Table 2) were selected from the UKBB and were amplified as a pool and cloned via PspXI/NotI into the barcoded NPR2 vector. 25 unique barcodes were selected per variant yielding a final library of ~4K elements. PacBio sequencing was performed using a no-amplification (HpaI/BstEII digestion) method (library-pre info) and sequencing on the Smart-seq Sequel I resulting in X aligned reads. Analysis to associate variant to barcode involved long-read alignment. The reference sequence used for alignment was comprised of a single fasta entry containing the NPR2 gene sequence followed by a 54bp spacer sequence (AGCGGCCGCGTTGGTCAGGCTTGGATTTCTATAACTTCGTATAGCAGTTTAAAC) (SEQ ID NO: 59), a 20bp barcode sequence and finally a 20bp 3' barcode flanking sequence (GTTTAAACCGAGAGATGGGG) (SEQ ID NO: 60). To account for the semi-degenerate structure of the barcode, Ns were used to fill this portion of the reference. Alignment was performed with a PacBio specific implementation of *minimap2*, namely *pbmm2 align*, where all default parameters were kept. All reads that did not map to the reference sequence were excluded from further analysis. Assembly of read structure and barcode extraction was done using the *python* api to *samtools*, namely *pysam*, was used to iterate over aligned reads and extract the reference positions aligned to, the aligned bases of the read (excluding soft clipped* bases) and the cigar* string. Reads that did not cover the start of the NPR2 gene as well as the barcode sequence were excluded from further analysis. The barcode sequence was extracted by a simple regular expression using *python's* *regex* library. A 5' barcode flanking sequence, comprised of the last 20bp of the spacer sequence mentioned above, was used to identify the position of the barcode, where *regex* allowed for a 1bp error

(insertion, deletion or substitution) in searching for this sequence. The following 20bp after the flanking sequence was then assumed to be the barcode. After barcode extraction, the aligned read was recreated with the use of the cigar string to account for insertions and deletions. Where deletions were identified in the read, a dash (-) was inserted, and bases that were inserted were removed. After the read was reconstructed the known variant positions could be simply extracted by indexing. Barcode polishing was used to correct for potential sequencing error in the barcode. Here barcodes with identical sequences were grouped into clusters. If a specific barcode sequence was observed more than 5 times it was assumed to be a genuine barcode. If it was observed 5 times or less it could have resulted from sequencing error. Barcodes with potential sequencing error were compared to all the barcodes in the 'genuine' list by Levenstein distance*, with the use of the *python* library *pylev*. If a barcode had a Levenstein distance of 2 or less to a 'genuine' barcode it was placed into this cluster.

[00167] *Library Packaging, Infection and Screen.* HEK293T cells were seeded at 65,000 cells per ccm in T225 flask in 40 mL media (DMEM, 10% fetal bovine serum) and incubated overnight at 37C, 5% CO₂. The next morning, 10.5 µg sgRNA library plasmid and 12ug LV-MAX Lentiviral Packaging Mix (ThermoFisher) and 22.5 µL Lipofectamine 2000 (ThermoFisher) were mixed into 2 mL serum free OptiMEM (Gibco), vortexed and incubated for 10 min at RT and added to the cells. 24 h later, 40 U DNaseI (NEB) were added to each plate in order to remove untransfected plasmid and at 72 h post-transfection, supernatant was harvested, passed through 0.45 µm filters (Millipore, Stericup) and aliquots were stored at -80C. HEK293-cGMP-GFP reporter cells were infected with library at MOI=0.2 to yield in initial coverage of ~500X. Library infected cells were puro-selected (~2ug/ml) for 4 days and coverage was always maintained above 1000X for the duration of the screen. The screen involved resuspending cells (~150M cells per replicate) and stimulating with 0, 1 or 50nM CNP for 1h with gentle shaking. Replicate sorts were performed on the Aria2, collecting ~250X coverage (4M cells) per replicate for both GFP-high (top 15%) and GFP-low (bottom 15%). Cell pellets were frozen and later processed for barcode sequencing using primer sequences described above. Mageck and Mann-Whitney analysis was performed comparing 4 GFP-high replicates to 4 GFP-low sorted samples.

Example 2-Screening strategy

[00168] It is contemplated that multiple rounds of screening may be necessary to reach the high throughput method. Library#1 is used to establish screening strategy using a cGMP-responsive GFP-on reporter system to phenotypically characterize NPR2 genetic variants; Library #2 (~60X larger) is useful as a similar screening strategy to phenotypically

characterize larger scale (e.g., ~160 NPR2) variants in parallel. Library#3 is able to phenotypically characterize ~450 NPR2 variants in parallel. Library generation requires initial long read alignment of the sequences, assembly of the read structures and barcode extraction, using the 5' and 3' flanking sequences). The barcodes for each variant are identified and determined whether they are unique barcodes that identify a variant.

[00169] A high number of barcodes per variant is desired (e.g., about 30) in the method since it provides for increased number of replicates, increases the statistical power of the method, controls for integration, provides cell heterogeneity, and allows for increased coverage of the gene. In the Library 1 prep, approximately 20 barcodes per variant were observed, giving a 93.3% unique barcode association.

Example 3. Establishment of Lenti-NPR2 and the cGMP-GFP-on reporter

[00170] To determine if the lentiviral vector comprising the NPR2 gene is detectable when transduced into cells, a lentiviral reporter system was developed.

[00171] Cells and vector were generated as described in Example 1. Stimulation of HEK cells comprising the lenti-NPR2 vector with cGMP led to detectable GFP levels in the cell. The levels compare favorably with that of the Catchpoint assay which had previously been used to characterize NPR2 variants (Figure 3).

[00172] Next, the variant libraries are screened for the effect of the variant on NPR2 function using the cGMP-GFP reporter assay using HEK293 cells expressing the GFP reporter construct and transfected with the NPR2 variant library. The library positive cells were isolated based on puromycin selectivity, and then sorted based on high to low GFP expression. DNA from the cells was extracted and the DNA linked to the library barcodes is amplified by next gen sequencing. The sequenced variants were then associated with their previously identified GFP high or low profile, and the effect of the NPR2 variant on function, e.g., gain of function, or loss of function, assessed. For example, in the screen, LoFs will have more counts in GFP low samples (relative to WT) (Figure 4B).

[00173] Using controls of known function, variants were screened in the present assay, and it was observed that sorted samples are highly correlated with function. Additionally, a Wilcoxon-Mann-Whitney test can be used to analyze the data since it is a nonparametric test of the null hypothesis that, for randomly selected values X and Y from two populations, the probability of X being greater than Y is equal to the probability of Y being greater than X . The analysis is robust and provides phenotypic directionality and successfully identified positive controls and other significant variants.

[00174] Screening of Library 2, which comprises approximately 160 variants, showed that the method provides a screening tool which is highly correlative. NPR2 variants identified previously in Estrada et al (*supra*) were screened using the present method and the present screening method produced results consistent with the previously identified NPR2 phenotype, identifying both LoF and GoF variants (Figure 6).

[00175] Functional activity of the variants was categorized by genetic consequences and phenotypic effects (Figure 7A-7B). cGMP levels were measured by variant functional consequence. For missense variants, Varsity ER LOO predictions can partially discriminate between missense variants that alter NPR2 function. A comparison of the present library screen cGMP measurements vs. effect on human adult height s determined by the UKBiobank is shown in Figure 7C.

[00176] The method is also useful to identify mis-characterized mutations. For example, variant Gln744del was previously thought to be an in-frame 3 nucleotide deletion that results in no change of NPR2 function. However, in the present screen a discrepancy was identified, characterizing the variant as a 4 nucleotide deletion that results in a LoF variant rather than a neutral variant.

[00177] It was hypothesized that the present method with its higher sensitivity than previous methods may be able to screen variants using lower concentrations of CNP. It has been shown that some GoF mutations (e.g., R562Q) responses saturate at high doses of CNP. GoFs may be easier to identify in conditions where CNP concentrations are limiting.

[00178] A CNP Stimulation curve was generated using 8 concentrations of CNP ranging from 50nM to 0nM Pro-Gly-CNP37 using a cGMP catchpoint assay. cGULL GFP ON were transiently transfected using Lipo 3000 in a 6-well plate – 2ug plasmid. Quadruplicates were tested for better accuracy. The screen identified 7 more GoFs at low CNP concentration (only 3 GoF identified from 1st screen, at high CNP) and yielded non-overlapping GoFs (Arg804) (Figure 8).

[00179] It was also confirmed that screening with low CNP, e.g., 1 nM, did not compromise the isolation of LoF variants.

[00180] Polygenic scores for height modify the effect of NPR2 variants on polygenic risk score for short stature can be used in combination with the screening method to help predict individuals who are at a higher risk of having idiopathic short stature. Figure 9A shows how adult height varies by polygenic score in people who reported being short at age 10. While only 29% of these individuals had short stature as adults (height Z-score < -2.25 SDs), 98% were below average height. Polygenic scores summarize the combined effects of thousands of common variants with small effects on height. These scores capture 43% of

the total population variation in adult human height but have limited ability to predict at the extreme ends of the distribution. Figure 9B illustrates a logistic regression model was trained to predict adult short stature in people who reported being short at age 10. Independent variables included the presence of an NPR2 variant with reduced activity and the polygenic score only. This simple model can predict 2/3 of true positives while maintaining a false positive rate below 20%. A model with only a polygenic score but blind to the presence of a reduced activity NPR2 variant had AUC-ROC = .66. In this sample, 20% of individuals reported being short at age 10. In the clinic, children with short stature are in the bottom 3% so a similar model would have a much better performance.

[00181] The results herein show that a novel pooled NPR2 variant screening system has been developed that uses lentiviral-expression of the variant genes, a cGMP-GFP reporter, a barcoding system and a sorting-based screening strategy. The screen successfully identifies both LoF and GoF variants. The screen allowed for re-classification of the 160 missense variants into 31 LoF, 6 GoF and 123 variants with neutral activity when compared to WT NPR2. This functional data indicates that at least 34/100,000 individuals carry an NPR2 variant with low activity that reduces stature. By incorporating polygenic scores, the method will allow for prediction of adult short stature in short children who carry these variants. The high-throughput method is advantageous because it provides scalability (screening a high number of variants), complexity (identifies a high number of replicates), and certainty (providing higher confidence in variant phenotypes).

Example 4. CNP Weight-Based Dosing Model

[00182] A PPK model was developed using data from 5 clinical trials in children with achondroplasia (aged 0.95–15 years) who received daily per-kg doses of vosoritide. The model was used to simulate expected exposures in children with a refined weight-band dosing regimen. Simulated exposure was compared with the observed exposure from the pivotal clinical trial to evaluate appropriateness of the weight-band dosing regimen.

[00183] Sampling for pharmacokinetic (PK) analysis of vosoritide was conducted as part of 3 phase II studies and 2 phase III studies that included the use of a daily subcutaneous dose of 15 µg/kg. In an analysis of 2 of these studies (Chan et al., Clin Pharmacokinet. 2022 61:263–80), the mean maximum observed plasma concentration (C_{max}) ranged from 4750 to 7180 pg/mL, the area under the plasma concentration–time curve (AUC) from time 0 to the time of the last measurable concentration ranged from 175,000 to 290,000 pg-min/mL, and the mean time to maximal plasma concentration ranged from 13.8 to 16.8 min after a single subcutaneous 15-µg/kg dose of vosoritide (Chan, *supra*). The PK data also demonstrated a positive correlation between plasma exposure to

vosoritide (AUC) and body weight in patients treated daily with a per-kg dose of vosoritide, which suggests that an alternative to weight-based dosing with vosoritide may yield more consistent exposure across the patient weight range (Chan, *supra*).

[00184] The current study was designed to develop a population PK (PPK) model for vosoritide in children with achondroplasia, as well as to evaluate the influence of clinically relevant covariates on the PK of vosoritide to better understand the sources of variability following subcutaneous administration. The original weight-based dosing regimen (15 µg/kg) utilized in clinical trials for vosoritide required multiple different dose levels for children weighing between 10 and 83 kg. A weight-band dosing regimen for vosoritide was developed to account for the characterized effect of body weight on vosoritide clearance and volume of distribution and to ensure more consistent exposure of the drug over the duration of a patient's treatment. This regimen would also allow for fewer required dose levels and fewer dose changes, as a new dose would only be needed when a child progresses from one weight band to the next, and therefore may simplify dosing for children with achondroplasia and their caregivers. The PPK model was used to perform simulations to develop the dosing recommendations.

[00185] PK, laboratory, and demographic data from children with achondroplasia were included in this PPK analysis. The data were collected from 5 clinical trials: study 111-202 (NCT02055157), a phase II, non-randomized, open-label, sequential-cohort, dose-finding trial of vosoritide (2.5, 7.5, 15, or 30 µg/kg) administered for 24 months in 35 children (5–14 years of age) with achondroplasia [(Chan, *supra*), Savarirayan et al., N Engl J Med. 2019;381:25–35]; study 111-205 (NCT02724228), an ongoing, phase II, open-label extension trial of vosoritide (15 or 30 µg/kg) administered in 30 children with achondroplasia who completed 24 months of treatment in study 111-202 (Savarirayan, *supra*); study 111-301 (NCT03197766), a phase III, randomized, double-blind, parallel-assignment, efficacy and safety trial of vosoritide (15 µg/kg) administered for 12 months in 121 children (5–18 years of age) with achondroplasia (Chan, *supra*); study 111-302 (NCT03424018), an ongoing, phase III, open-label extension, safety and efficacy trial of vosoritide (15 µg/kg) administered in children (≥ 6 years of age) with achondroplasia who completed 12 months of treatment in study 111-301; and study 111-206 (NCT03583697), a phase II, randomized, double-blind, parallel-assignment, safety and efficacy trial of vosoritide (15 or 30 µg/kg) administered for 12 months in 75 children (≤ 5 years of age) with achondroplasia; at the time of construction of the PPK model, the study was ongoing, so only interim data from sentinel patients were available for inclusion in model development.

[00186] Data were pooled into a single Nonlinear Mixed Effects Modeling (NONMEM™, version 7.4.4, ICON Development Solutions, Dublin, Ireland) database. A log

transform both sides approach and stochastic approximation expectation maximization (SAEM) followed by importance sampling (IMP) method was used for the PPK modeling of vosoritide.

[00187] The PPK model was developed in a series of steps. The base model was created with no consideration of covariate effects and was used to describe the structural and stochastic components of the model and to conduct a graphical evaluation of the covariates. The single-covariate model was used to test pre-specified covariate–parameter relationships graphically using covariates that were known to influence the PK of vosoritide or that were physiologically plausible. Once all single-covariate evaluations were completed, a full model was constructed using all single-covariate models that were statistically significant ($p < 0.01$) and were well estimated. Backward elimination was then carried out on the full covariate model, with 1 covariate being removed from the model at a time. Covariates that increased the objective function ($p < 0.001$) were retained in the final model.

[00188] The final PPK model parameters were used for the simulation without consideration for parameter precision. Five hundred replicates were run using the PPK model to generate intensive concentration–time profiles over the first 5 h (at 5-min intervals) following subcutaneous administration of varying stratified doses of vosoritide in pediatric patients weighing 10 to 90 kg. The simulations were conducted using doses from stock keeping units (0.8 mg/mL [0.5 mL]; 0.8 mg/mL [0.70 mL]; 2 mg/mL [0.60 mL]). The highest withdrawal doses (0.32, 0.48, 1, and 1.2 mg) were included in the simulation. PK non-compartmental analysis (PKNCA) to calculate AUC and C_{max} values was performed on simulated data using the PKNCA package in R, version 0.9.3. The simulated exposures were compared with the observed exposure data from study 111-301. The median, 5th percentile, and 95th percentile of PK parameters from the simulation were compared with the PK parameters calculated from the observed data evaluated at 15-μg/kg daily doses.

[00189] The final database used contained 4741 observations from 158 patients aged 0.95 to 15 years, with a mean age of 8.43. The weight of patients ranged from 9 to 74.5 kg, with a mean baseline weight of 23.8 kg. Actual doses administered during the study to patients whose data were included in the PPK model included 2.5 μg/kg/day (6 patients), 7.5 μg/kg/day (12 patients), 15 μg/kg/day (151 patients), and 30 μg/kg/day (11 patients).

[00190] The final PPK model consisted of a 1-compartment system with first-order elimination and a change-point first-order absorption that allowed a time-dependent change in absorption rate coefficient. Body weight was found to be a predictive factor for CL/F and V/F of the drug (Table 2).

Table 2. Average clearance (CL/F) and volume of distribution (V/F) across body weight range

WT (kg)	CL/F (L/h)	% of reference	V/F (L)	% of reference
9	35.72	75.26	7.54	41.88
20 (reference)	47.47	100.00	17.99	100.00
40	60.75	127.99	38.30	212.87
60	70.18	147.86	59.59	331.18
74.5	75.80	159.71	75.45	419.30

CL/F apparent clearance, *F* relative bioavailability, V/F apparent volume of distribution, *WT* body weight

[00191] Additionally, the SOLNC of 0.2 mg/mL, which was only used in study 111-202, and the duration of treatment were found to be predictive for the relative bioavailability (*F*) of vosoritide. Separate residual errors for the 2 assay types were estimated. The model also accounted for the IIV in CL/F, V/F, and change-point. An additional secondary study identity number (SIDN) was used to represent the clinical trial in which each patient was enrolled, but allowed for the fact that patients may have been enrolled in > 1 study. The effects of SIDN on the IIV of CL/F and V/F were modeled by an additional hierarchical level of effect (StudyCL and StudyV). The parameter estimates' typical values and parameter precision (% standard error) of the PPK model are presented in Table 3. The parameter precision was < 30%, with the exception of the terms IIV StudyCL and IIV StudyV, because there were only 3 SIDN values in the present database. The estimated typical parameter values were consistent with the median bootstrap parameter estimates, and the confidence intervals were reassuringly narrow and did not include the null.

Table 3. Parameter estimates of the PPK model

Parameter (units)	Typical value	Standard error (%)	Bootstrap lower 2.5th percentile	Median	Bootstrap upper 97.5th percentile
CL/F (L/h)	47.47	1.8	40.45	47.7	57.97
V/F (L)	17.99	3.3	15.18	18.36	23.09
Ka1 (1/h)	2.21	14.7	1.8	2.3	3.19
Ka2 (1/h)	0.06	3.8	0.04	0.06	0.09
Change-point (h)	0.31	7.3	0.27	0.3	0.33
Residual error 1 ^a (CV, %)	66.5	1.6	61.71	66.3	72
Residual error 2 ^b (CV, %)	61	1.5	56.61	60.85	65.4
Effect of SOLNC (0.2 mg/mL)	1.56	15.3	1.18	1.54	2.03
Effect of weight on CL/F	0.356	25.1	0.02	0.35	0.68
Effect of weight on V/F	1.09	8.1	0.85	1.1	1.31
Effect of time on <i>F</i>	0.21	8.1	0.16	0.21	0.26
IIV CL (CV, %)	33.6	8.3	27.3	33	40.49
IIV V (CV, %)	24.2	13.6	17.7	23.9	29.99

IIV StudyCL (CV, %)	25.7	47.4	18.51	26.15	34.6
IIV StudyV (CV, %)	1.2	51.8	0.4	2.05	5.7
IIV change-point (fixed)	22.4	0	NE	NE	NE

CL/F apparent clearance, *CV* coefficient of variation, *ECL* electrochemiluminescence, *ELISA* enzyme-linked immunosorbent assay, *F* relative bioavailability, *IIV* inter-individual variability, *Ka1* absorption rate constant 1, *Ka2* absorption rate constant 2, *NE* not estimated, *PPK* population pharmacokinetics, *SOLNC* solution concentration, *StudyCL* nested variability based on study identifier number, *StudyV* nested variability based on study identifier number, *V/F* apparent volume of distribution

Residual error 1 = residual error for concentrations arising from the ELISA assay; residual error 2 = residual error for concentrations arising from the ECL assay

[00192] The performance of the PPK model for vosoritide was evaluated using diagnostic goodness-of-fit plots and VPCs (Fig. 12 and 13). These showed that the model was able to accurately describe the data. The model's predictive distributions of median concentrations within various time intervals were compared with the observed medians using VPCs (Fig. 13). The lower and upper bounds of the observed and simulated data were generally similar, with the observed concentrations falling within the 5th and 95th percentiles of the predictive distribution for the final model across time intervals.

[00193] The PPK model for vosoritide was used to develop improved dosing recommendations for pediatric patients with achondroplasia. Simulations were conducted for various weight strata. An initial regimen of 4 weight bands was identified: 0.32 mg for a weight of 10–19 kg, 0.48 mg for a weight of 20–34 kg, 0.7 mg for a weight of 35–64 kg, and 1 mg for a weight of ≥ 65 kg. The simulated AUCs for patients weighing < 65 kg were within or slightly beyond the upper limit of the AUCs observed in studies 111-301, 111-202, and 111-205. However, the simulated AUCs for patients weighing ≥ 65 kg exceeded the upper limit. Given the results obtained with the initial regimen of 4 weight bands, a revised dosing regimen was tested. The new dosing regimen included more weight bands (8 compared with 4) to generate simulated exposure that better aligned with the observed exposure. The best weight-band dosing regimen identified was 0.24 mg for a weight of 10–11 kg, 0.28 mg for a weight of 12–16 kg, 0.32 mg for a weight of 17–21 kg, 0.40 mg for a weight of 22–32 kg, 0.50 mg for a weight of 33–43 kg, 0.60 mg for a weight of 44–59 kg, 0.70 mg for a weight of 60–89 kg, and 0.80 mg for a weight of ≥ 90 kg (Table 4).

Table 4. Proposed weight-band dosing for vosoritide

Body weight (kg)	SKU 1 concentration: 0.8 mg/mL (0.50 mL)	SKU 2 concentration: 0.8 mg/mL (0.70 mL)	SKU 3 concentration: 2 mg/mL (0.60 mL)
10–11	0.24 mg/0.30 mL (22–24 µg/kg)		

12–16	0.28 mg/0.35 mL (18–23 µg/kg)	
17–21	0.32 mg/0.40 mL (15–19 µg/kg)	
22–32	0.40 mg/0.50 mL (13–18 µg/kg)	
33–43		0.50 mg/0.25 mL (12–15 µg/kg)
44–59		0.60 mg/0.30 mL (10–14 µg/kg)
60–89		0.70 mg/0.35 mL (8–12 µg/kg)
≥ 90		0.80 mg/0.40 mL (≤ 9 µg/kg)

[00194] The proposed regimen includes doses < 15 µg/kg for patients weighing ≥ 44 kg, and doses > 15 µg/kg for patients weighing 10–16 kg. The new weight-band dosing regimen was found to yield more consistent exposure across the body weight range. The 5th to 95th percentiles of the simulated AUCs were within the range of the observed AUCs at 15 µg/kg, and the median simulated AUC values were distributed around the median observed AUC (Fig. 14). Additionally, the median values of simulated C_{max} were generally consistent with the observed C_{max} at 15 µg/kg, but the 5th and 95th percentiles of the simulated C_{max} were lower than the 5th and 95th percentiles of the observed C_{max} (Fig. 15). This discrepancy can be attributed to the model underestimating C_{max} as shown in VPC plots, and it could also be a result of the simulation being conducted with only 1 SIDN instead of the 3 SIDNs present in the model.

[00195] The PPK model was used to simulate drug concentrations and exposures with the goal of developing a more refined weight-band dosing regimen. Although a regimen of 4 weight bands was initially proposed, greater consistency between simulated and observed exposures was achieved with 8 weight bands. The weight-band regimen provided more consistent drug exposure across the body weight range. Specifically, for patients weighing > 44 kg, doses < 15 µg/kg were proposed to account for the correlation between observed non-linear relationship exposure and patient body weight. Similarly, for children aged 2–5 years and/or patients weighing 10–16 kg, doses > 15 µg/kg were proposed to avoid suboptimal exposure and to take into consideration an extrapolation approach. The 30-µg/kg dose has been tested in the phase II studies (studies 111-202, 111-205 [(Chan,

supra), Savarirayan *supra*], and 111-206) and demonstrated a similar safety profile as the 15-μg/kg dose.

[00196] The simulated exposure from the proposed 8 weight-band dosing regimen fell within the range demonstrated to be well tolerated and effective in previous studies (Chan, *supra*). Importantly, the proposed weight-band regimen will ensure more consistent vosoritide exposure, both within the patient population and over the duration of treatment for an individual patient, while simplifying dosing for children with achondroplasia and their caregivers.

Example 5: Improvement of Bone Strength using CNP

[00197] The primary objective of this study was to determine whether CNP variant affects both length and development of bone strength in children with achondroplasia using measurements of the second metacarpal.

[00198] This study included 103 deidentified AP hand/wrist radiographs from 30 children with achondroplasia (13M, 17F; ages 7.8-16 years). Proprietary data included hand films collected at four time points: at week 104 (at rollover into the phase II extension study), week 156, week 208 and week 260 on treatment. Second metacarpal length and midshaft width, cortical thickness, robustness (total area/length), and cortical area (correlated with strength) were measured. Measurements were compared to 378 radiographs from 114 average-stature controls (61M, 53F; ages 6-16 years) via non-parametric Kruskal-Wallis tests ($p < 0.05$).

[00199] Children with achondroplasia at week 208 and week 260 of treatment demonstrated longer metacarpals with increased cortical area and cortical thickness compared to week 104 (all $p < 0.05$). There was no significant change in RCA and robustness across the treatment time points (Table 5). Furthermore, no differences were seen between males and females with achondroplasia at any of the timepoints. Compared to the controls, children with achondroplasia had higher robustness and cortical area throughout treatment ($p < 0.001$).

Table 5: Measurements of the second metacarpal (mean±SD) at each time point in treatment.

	Baseline (n=29)	Week 52 (n=29)	Week 104 (n=28)	Week 156 (n=17)	Controls (n=378)
Le (mm)	39.75±4.49 ^{‡^°}	42.13 ± 4.36 [°]	44.12±4.56 ^{*°}	45.00±4.98 ^{*°}	56.83±9.40 ^{*#‡^}
T.Ar (mm²)	42.84±11.82 [^]	45.79±12.49 [°]	49.80±14.63 [°]	54.29±17.64 ^{*°}	39.01±11.79 ^{#‡^}
Ct.Ar (mm²)	25.60±6.59 ^{‡^°}	28.12±6.72 [^]	32.06±7.93 [*]	35.60±9.59 ^{*#°}	29.64±9.57 ^{*^}
Marrow Area (mm²)	17.24±9.38 [°]	17.67±10.54 [°]	17.73±12.15 [°]	18.68±12.06 [°]	9.37±4.81 ^{*#‡^}
Cortical Thickness (mm)	1.39±0.35 ^{‡^°}	1.49±0.37 [°]	1.67±0.43 ^{*°}	1.77±0.41 ^{*°}	1.10±0.35 ^{*#‡^}

RCA	0.61±0.12°	0.63±0.12°	0.66±0.12°	0.67±0.12°	0.76±0.10*#‡^
T.Ar/Le (mm)	1.08±0.30°	1.09±0.29°	1.13±0.32°	1.20±0.37°	0.68±0.13*#‡^

*significantly different (p<0.05) than baseline, #significantly different (p<0.05) than w52, ‡significantly different (p<0.05) than w104, ^significantly different (p<0.05) than w156, °significantly different (p<0.05) than controls°

[00200] Additional measurements were taken at weeks 208 and 260. Results are shown in Table 6 and Figures 16A-B.

Table 6. Measurements of the second metacarpal (mean±SD) at each time point in treatment.

	Baseline (n=17)	Week 104 (n=29)	Week 156 (n=20)	Week 208 (n=28)	Week 260 (n=17)	Controls (n=378)
Le (mm)	35.71±4.51°^	39.75±4.49°	42.13 ± 4.36°	44.12±4.56°*	45.00±4.98°*	56.83±9.40*#‡^
T.Ar (mm²)	39.30±12.04‡#	42.84±11.82°	45.79±12.49°*	49.80±14.63°*	54.29±17.64°*#	39.01±11.79‡^
Ct.Ar (mm²)	21.80±6.29‡#^	25.60±6.59°^	28.12±6.72°	32.06±7.93°#	35.60±9.59°*#‡	29.64±9.57°#
Marrow Area (mm²)	17.50±7.84°	17.24±9.38°	17.67±10.54°	17.73±12.15°	18.68±12.06°	9.37±4.81°#‡^
Cortical Thickness (mm)	1.20±0.27°	1.39±0.35°	1.49±0.37°	1.67±0.43°	1.77±0.41°*	1.10±0.35°#‡^
RCA	0.57±0.10°^	0.61±0.12°	0.63±0.12°	0.66±0.12°*	0.67±0.12°*	0.76±0.10°*#‡^
T.Ar/Le (mm)	1.10±0.30°	1.08±0.30°	1.09±0.29°	1.13±0.32°	1.20±0.37°	0.68±0.13°*#‡^

*significantly different (p<0.05) than screening,

#significantly different (p<0.05) than w104,

‡significantly different (p<0.05) than w156,

^significantly different (p<0.05) than w208,

°significantly different (p<0.05) than w260,

°significantly different (p<0.05) than controls

[00201] It was observed that an additional 2-3 years of vosoritide treatment was associated with increases in bone length compared to the initial timepoint (week 104) as well as increases in metacarpal cortical area, which is correlated with strength. This study suggested this bone lengthening treatment did not adversely affect bone strength in children with achondroplasia. The lack of a significant difference in robustness after treatment indicated that periosteal expansion continued outward at a pace which maintains robustness, allowing the bone to remain strong as it lengthened. Future work comparing treated and untreated children with achondroplasia is necessary to more rigorously confirm whether the treatment did not adversely affect the development of bone strength. Overall, this work may have important clinical implications in terms of treatment choices for children with achondroplasia.

[00202] Numerous modifications and variations in the disclosure as set forth in the above illustrative examples are expected to occur to those skilled in the art. Consequently, only such limitations as appear in the appended claims should be placed on the disclosure.

What is claimed is:

1. A method of identifying a variant gene associated with short stature that is a gain of function (GoF) or loss of function (LoF) variant comprising:

- transfecting cells comprising a cGMP-GFP reporter expression construct with a lentiviral vector expressing a polynucleotide encoding a variant protein associated with short stature operably linked to one or more unique barcode sequences;

- contacting the cells in culture with c-type natriuretic peptide (CNP) or a variant thereof having CNP activity;

- sorting the cells from the culture based on the level of expression of GFP produced by the cell; and

- identifying the variant protein associated with short stature as a GoF variant or a LoF variant, wherein a GoF variant has a higher level of cGMP production compared to a control, and wherein a LoF variant has a lower level of cGMP production compared to a control.

2. The method of claim 1 wherein the variant gene associated with short stature is selected from the group consisting of natriuretic peptide receptor 2 (NPR2), Natriuretic peptide precursor C (NPPC), fibroblast growth factor receptor 3 (FGFR3) or combinations thereof.

3. A method of identifying a variant of NPR2 as a gain of function (GoF) or loss of function (LoF) variant comprising:

- transfecting cells comprising a cGMP-GFP reporter expression construct with a lentiviral vector expressing a polynucleotide encoding a NPR2 variant protein operably linked to one or more unique barcode sequences;

- contacting the cells in culture with c-type natriuretic peptide (CNP) or a variant thereof having CNP activity;

- sorting the cells from the culture based on the level of expression of GFP produced by the cell; and

- identifying the NPR2 variant as a GoF variant or a LoF variant, wherein a GoF variant has a higher level of cGMP production compared to a control, and wherein a LoF variant has a lower level of cGMP production compared to a control.

4. The method of any one of claims 1 to 3, wherein the cells are a mammalian cell line.

5. The method of any one of claims 1 to 4, wherein the cells are HEK293 cells.
6. The method of any one of claims 1 to 5, wherein the cells are sorted by flow cytometry.
7. The method of any one of claims 1 to 6, wherein the lentiviral vector comprises a polynucleotide encoding a variant gene associated with short stature, a polynucleotide encoding a puromycin resistance gene and a polynucleotide encoding T2A-BFP promoter.
8. The method of any one of claims 1 to 7, wherein the lentiviral vector further comprising between 20 and 60 barcode sequences.
9. The method of claim 8, wherein the barcode sequences are from 15 to 30 basepairs.
10. The method of any one of claims 8 to 9, wherein the barcode sequences are 3' to the variant gene polynucleotide.
11. The method of any one of claims 1 to 10, wherein the expression construct comprises a polynucleotide encoding a GFP protein operably linked to a cGMP binding domain.
12. The method of claim 11, wherein the cGMP binding domain is from mouse phosphodiesterase 5 α .
13. The method of claim 11 or 12, wherein the expression construct further comprises a CMV promoter operably linked to cGull and a PGK promoter operably linked to a blastocidin resistance gene.
14. The method of any one of the preceding claims, wherein the CNP variant is selected from the group consisting of

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP-37) (SEQ ID NO: 2);

GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP53) (SEQ ID NO: 3);

PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-CNP53) (SEQ ID NO: 4);

MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-

CNP53) (SEQ ID NO: 5);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC [CNP-53(M48N)] (SEQ ID NO: 6);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-52) (SEQ ID NO: 7);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-51) (SEQ ID NO: 8);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP- 50) (SEQ ID NO: 9);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-49) (SEQ ID NO: 10);
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-48) (SEQ ID NO: 11);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-47) (SEQ ID NO: 12);
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-46) (SEQ ID NO: 13);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-45) (SEQ ID NO: 14);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-44) (SEQ ID NO: 15);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-43) (SEQ ID NO: 16);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-42) (SEQ ID NO: 17);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-41) (SEQ ID NO: 18);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-40) (SEQ ID NO: 19);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-39) (SEQ ID NO: 20);
LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 21);
QEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-37) (SEQ ID NO: 22);
EHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-36) (SEQ ID NO: 23);
HPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-35) (SEQ ID NO: 24);
PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-34) (SEQ ID NO: 25);
NARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-33) (SEQ ID NO: 26);
ARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-32) (SEQ ID NO: 27);
RKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-31) (SEQ ID NO: 28);
KYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-30) (SEQ ID NO: 29);
YKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-29) (SEQ ID NO: 30);
KGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-28) (SEQ ID NO: 31);

GANKKGLSKGCFGLKLDRI SMSGLGC (CNP-27) (SEQ ID NO: 32);
 ANKKGLSKGCFGLKLDRI SMSGLGC (CNP-26) (SEQ ID NO: 33);
 NKKGLSKGCFGLKLDRI SMSGLGC (CNP-25) (SEQ ID NO: 34);
 KKGLSKGCFGLKLDRI SMSGLGC (CNP-24) (SEQ ID NO: 35);
 KGLSKGCFGLKLDRI SMSGLGC (CNP-23) (SEQ ID NO: 36);
 LSKGCFGLKLDRI SMSGLGC (CNP-21) (SEQ ID NO: 37);
 SKGCFGLKLDRI SMSGLGC (CNP-20) (SEQ ID NO: 38);
 KGCFGLKLDRI SMSGLGC (CNP-19) (SEQ ID NO: 39);
 GCFGLKLDRI SMSGLGC (CNP-18) (SEQ ID NO: 40);
 QEHPNARKYKGANKKGLSKGCFGLKLDRI SNSGLGC [CNP-37(M32N)] (SEQ ID NO: 41);
 PQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Pro-CNP-37) (SEQ ID NO: 42);
 MQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Met-CNP-37) (SEQ ID NO: 43);
 GQEHPNARKYKGANKKGLSKGCFGLKLDRI SNSGLGC [Gly-CNP-37(M32N)] (SEQ ID NO: 44);
 MGQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Met-Gly-CNP-37) (SEQ ID NO: 45);
 PGQEHPQARRYRGAQRRGLSRGCFGLKLDRI SMSGLGC (SEQ ID NO: 46);
 PGQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 47);
 PGQEHPNARRYRGANRRGLSRGCFGLKLDRI SMSGLGC (SEQ ID NO: 48);
 PGQEHPQARKYKGAQKKGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 49);
GQEHPNARKYKGANPKGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 50);
GQEHPNARKYKGANQKGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 51);
GQEHPNARKYKGANQQGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 52); and
GQEHPNARKYKGANKPGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 53).

15. The method of any one of the preceding claims, wherein the CNP is contacted with the cells at a dose between 1 to 100 nM.

16. The method of any one of the preceding claims, wherein the lentivirus is transfected into the cells at a multiplicity of infection of MOI between about 0.1 and about 0.5.

17. A lentiviral vector comprising a polynucleotide encoding a variant gene associated with short stature, a polynucleotide encoding a puromycin resistance gene and a polynucleotide encoding T2A-BFP promoter.

18. The lentiviral vector of claim 17, wherein the variant gene associated with short stature is selected from the group consisting of natriuretic peptide receptor 2 (NPR2),

Natriuretic peptide precursor C (NPPC), fibroblast growth factor receptor 3 (FGFR3) or combinations thereof

19. A lentiviral vector comprising a polynucleotide encoding a NPR2 variant, a polynucleotide encoding a puromycin resistance gene and a polynucleotide encoding T2A-BFP promoter.

20. The lentiviral vector of any one of claims 17 to 19 further comprising between 20 and 60 barcode sequences.

21. The lentiviral vector of claim 20, wherein the barcode sequences are from 15 to 30 basepairs.

22. The lentiviral vector of claim 20 or 21, wherein the barcode sequences are 3' to the variant gene polynucleotide.

23. A method of making a lentiviral library comprising a variant gene associated with short stature, the method comprising

- amplifying variant genes associated with short stature from a mammalian genome or genomic DNA database;

- cloning the amplified variants into a lentiviral vector, wherein the lentiviral vector comprises between 20-60 unique barcodes per vector;

- sequencing the variant associated with the barcodes in the vector;

- aligning the variant sequences with a control gene sequence to generate a read structure;

- extracting the barcodes from the variant read structure;

- identifying the barcodes for each variant;

- isolating the lentiviral vectors expressing variant genes.

24. The method of claim 23, wherein the variant gene associated with short stature is selected from the group consisting of NPR2, NPPC, FGFR3 or combinations thereof

25. A method of making an NPR2 variant lentiviral library comprising

- amplifying NPR2 variants from a mammalian genome or genomic DNA database;

- cloning the amplified NPR2 variants into a lentiviral vector, wherein the lentiviral vector comprises between 20-60 unique barcodes per vector;
- sequencing the NPR2 variant associated with the barcodes in the vector;
- aligning the NPR2 variant sequences with a control NPR2 gene sequence to generate a read structure;
- extracting the barcodes from the NPR2 variant read structure;
- identifying the barcodes for each NPR2 variant;
- isolating the lentiviral vectors expressing NPR2 variants.

26. A method for treating a subject with a short stature disorder comprising administering a CNP variant to a subject identified as having a loss of function variant of a gene associated with short stature identified using a method of any one of claims 1-16.

27. The method of claim 26, wherein the short stature disorder is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, idiopathic short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis congenita, achondrogenesis, chondrodysplasia congenita, homozygous achondroplasia, chondrodysplasia congenita, campomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis congenita, short-rib polydactyly syndromes, rhizomelic type of chondrodysplasia congenita, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenita, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia, disorders related to NPR2 mutation, SHOX mutations, Turner's syndrome/Leri Weill, PTPN11 mutations, Noonan's syndrome, and IGF1R mutation.

28. The method of claim 26 or 27 wherein the CNP variant is selected from the group consisting of

PGQEHPNARKYKGANKKGLSKGCFGLKLDRI GMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1); GQEHPNARKYKGANKKGLSKGCFGLKLDRI GMSGLGC (Gly-CNP-37) (SEQ ID NO: 2);
GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI GMSGLGC (Gly-CNP53) (SEQ ID NO: 3);

PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Pro-CNP53) (SEQ ID NO: 4);
MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Met-CNP53) (SEQ ID NO: 5);
DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SNSGLGC [CNP-53(M48N)] (SEQ ID NO: 6);
LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-52) (SEQ ID NO: 7);
RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-51) (SEQ ID NO: 8);
VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP- 50) (SEQ ID NO: 9);
DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-49) (SEQ ID NO: 10);
TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-48) (SEQ ID NO: 11);
KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-47) (SEQ ID NO: 12);
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-46) (SEQ ID NO: 13);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-45) (SEQ ID NO: 14);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-44) (SEQ ID NO: 15);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-43) (SEQ ID NO: 16);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-42) (SEQ ID NO: 17);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-41) (SEQ ID NO: 18);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-40) (SEQ ID NO: 19);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-39) (SEQ ID NO: 20);
LQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-38) (SEQ ID NO: 21);
QEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-37) (SEQ ID NO: 22);
EHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-36) (SEQ ID NO: 23);
HPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-35) (SEQ ID NO: 24);
PNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-34) (SEQ ID NO: 25);
NARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-33) (SEQ ID NO: 26);
ARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-32) (SEQ ID NO: 27);
RKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-31) (SEQ ID NO: 28);

KYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-30) (SEQ ID NO: 29);
 YKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-29) (SEQ ID NO: 30);
 KGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-28) (SEQ ID NO: 31);
 GANKKGLSKGCFGLKLDRI SMSGLGC (CNP-27) (SEQ ID NO: 32);
 ANKKGLSKGCFGLKLDRI SMSGLGC (CNP-26) (SEQ ID NO: 33);
 NKKGLSKGCFGLKLDRI SMSGLGC (CNP-25) (SEQ ID NO: 34);
 KKGLSKGCFGLKLDRI SMSGLGC (CNP-24) (SEQ ID NO: 35);
 KGLSKGCFGLKLDRI SMSGLGC (CNP-23) (SEQ ID NO: 36);
 LSKGCFGLKLDRI SMSGLGC (CNP-21) (SEQ ID NO: 37);
 SKGCFGLKLDRI SMSGLGC (CNP-20) (SEQ ID NO: 38);
 KGCFGLKLDRI SMSGLGC (CNP-19) (SEQ ID NO: 39);
 GCFGLKLDRI SMSGLGC (CNP-18) (SEQ ID NO: 40);
 QEHPNARKYKGANKKGLSKGCFGLKLDRI SNSGLGC [CNP-37(M32N)] (SEQ ID NO: 41);
 PQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Pro-CNP-37) (SEQ ID NO: 42);
 MQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Met-CNP-37) (SEQ ID NO: 43);
 GQEHPNARKYKGANKKGLSKGCFGLKLDRI SNSGLGC [Gly-CNP-37(M32N)] (SEQ ID NO: 44);
 MGQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Met-Gly-CNP-37) (SEQ ID NO: 45);
 PGQEHPQARRYRGAQRRGLSRGCFGLKLDRI SMSGLGC (SEQ ID NO: 46);
 PGQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 47);
 PGQEHPNARRYRGANRRGLSRGCFGLKLDRI SMSGLGC (SEQ ID NO: 48);
 PGQEHPQARKYKGAQKKGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 49);
GQEHPNARKYKGANPKGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 50);
GQEHPNARKYKGANQKGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 51);
GQEHPNARKYKGANQQGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 52); and
GQEHPNARKYKGANKPGLSKGCFGLKLDRI SMSGLGC (SEQ ID NO: 53).

29. The method of any one of claims 26 to 28, wherein the CNP variant is
 PGQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Pro-Gly-CNP37) (SEQ ID NO:
 1); GQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Gly-CNP-37) (SEQ ID NO:
 2); or LQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-38) (SEQ ID NO: 21).

30. The method of any one of claims 26 to 29, wherein the CNP variant further
 comprises a hydrophilic moiety.

31. The method of claim 30, wherein the hydrophilic moiety is PEG.

32. A method for treating a CNP-responsive bone-related disorder, skeletal dysplasia or short stature disorder comprising administering to a subject in need thereof a CNP variant, wherein the CNP variant is administered according to a weight-band dosing regimen, wherein

- i) a subject between 10–11 kg receives between about 22–24 µg/kg CNP variant;
- ii) a subject between 12–16 kg receives between about 18–23 µg/kg CNP variant;
- iii) a subject between 17–21 kg receives between about 15–19 µg/kg CNP variant;
- iv) a subject between 22–32 kg receives between about 13–18 µg/kg CNP variant;
- v) a subject between 33–43 kg receives between about 12–15 µg/kg CNP variant;
- vi) a subject between 44–59 kg receives between about 10–14 µg/kg CNP variant;
- vii) a subject between 60–89 kg receives between about 8–12 µg/kg CNP variant; or
- viii) a subject of weight of ≥ 90 kg receives about ≤ 9 µg/kg CNP variant.

33. The method of claim 32 wherein

- i) a subject between 10–11 kg receives about 0.24 mg CNP variant;
- ii) a subject between 12–16 kg receives about 0.28 mg CNP variant;
- iii) a subject between 17–21 kg receives about 0.32 mg CNP variant;
- iv) a subject between 22–32 kg receives about 0.40 mg CNP variant;
- v) a subject between 33–43 kg receives about 0.50 mg CNP variant;
- vi) a subject between 44–59 kg receives about 0.60 mg CNP variant;
- vii) a subject between 60–89 kg receives about 0.7 mg CNP variant; or
- viii) a subject of weight of ≥ 90 kg receives about 0.80 mg CNP variant.

34. The method of claim 32 or 33, wherein the short stature disorder is selected from the group consisting of achondroplasia, hypochondroplasia, short stature, idiopathic short stature, dwarfism, osteochondrodysplasias, thanatophoric dysplasia, osteogenesis

congenita, achondrogenesis, chondrodysplasia congenita, homozygous achondroplasia, chondrodysplasia congenita, campomelic dysplasia, congenital lethal hypophosphatasia, perinatal lethal type of osteogenesis congenita, short-rib polydactyly syndromes, rhizomelic type of chondrodysplasia congenita, Jansen-type metaphyseal dysplasia, spondyloepiphyseal dysplasia congenital, atelosteogenesis, diastrophic dysplasia, congenital short femur, Langer-type mesomelic dysplasia, Nievergelt-type mesomelic dysplasia, Robinow syndrome, Reinhardt syndrome, acrodysostosis, peripheral dysostosis, Kniest dysplasia, fibrochondrogenesis, Roberts syndrome, acromesomelic dysplasia, micromelia, Morquio syndrome, Kniest syndrome, metatrophic dysplasia, and spondyloepimetaphyseal dysplasia, disorders related to NPR2 mutation, SHOX mutations, Turner's syndrome/Leri Weill, PTPN11 mutations, Noonan's syndrome, and IGF1R mutation.

35. The method of any one of claims 32-34 wherein the CNP variant is selected from the group consisting of

PGQEHNPARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1); GQEHNPARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Gly-CNP-37) (SEQ ID NO: 2);
 GDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Gly-CNP53) (SEQ ID NO: 3);
 PDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Pro-CNP53) (SEQ ID NO: 4);
 MDLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Met-CNP53) (SEQ ID NO: 5);
 DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SNSGLGC [CNP-53(M48N)] (SEQ ID NO: 6);
 LRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-52) (SEQ ID NO: 7);
 RVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-51) (SEQ ID NO: 8);
 VDTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP- 50) (SEQ ID NO: 9);
 DTKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-49) (SEQ ID NO: 10);
 TKSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-48) (SEQ ID NO: 11);

KSRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-47) (SEQ ID NO: 12);
SRAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-46) (SEQ ID NO: 13);
RAAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-45) (SEQ ID NO: 14);
AAWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-44) (SEQ ID NO: 15);
AWARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-43) (SEQ ID NO: 16);
WARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-42) (SEQ ID NO: 17);
ARLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-41) (SEQ ID NO: 18);
RLLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-40) (SEQ ID NO: 19);
LLQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-39) (SEQ ID NO: 20);
LQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-38) (SEQ ID NO: 21);
QEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-37) (SEQ ID NO: 22);
EHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-36) (SEQ ID NO: 23);
HPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-35) (SEQ ID NO: 24);
PNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-34) (SEQ ID NO: 25);
NARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-33) (SEQ ID NO: 26);
ARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-32) (SEQ ID NO: 27);
RKYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-31) (SEQ ID NO: 28);
KYKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-30) (SEQ ID NO: 29);
YKGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-29) (SEQ ID NO: 30);
KGANKKGLSKGCFGLKLDRI SMSGLGC (CNP-28) (SEQ ID NO: 31);
GANKKGLSKGCFGLKLDRI SMSGLGC (CNP-27) (SEQ ID NO: 32);
ANKKGLSKGCFGLKLDRI SMSGLGC (CNP-26) (SEQ ID NO: 33);
NKKGLSKGCFGLKLDRI SMSGLGC (CNP-25) (SEQ ID NO: 34);
KKGLSKGCFGLKLDRI SMSGLGC (CNP-24) (SEQ ID NO: 35);
KGLSKGCFGLKLDRI SMSGLGC (CNP-23) (SEQ ID NO: 36);
LSKGCFGLKLDRI SMSGLGC (CNP-21) (SEQ ID NO: 37);
SKGCFGLKLDRI SMSGLGC (CNP-20) (SEQ ID NO: 38);
KGCFGLKLDRI SMSGLGC (CNP-19) (SEQ ID NO: 39);
GCFGLKLDRI SMSGLGC (CNP-18) (SEQ ID NO: 40);
QEHPNARKYKGANKKGLSKGCFGLKLDRI SNSGLGC [CNP-37(M32N)] (SEQ ID NO: 41);
PQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Pro-CNP-37) (SEQ ID NO: 42);
MQEHPNARKYKGANKKGLSKGCFGLKLDRI SMSGLGC (Met-CNP-37) (SEQ ID NO: 43);

GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSNSGLGC [Gly-CNP-37(M32N)] (SEQ ID NO: 44);
MGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Met-Gly-CNP-37) (SEQ ID NO: 45);
PGQEHPQARRYRGAQRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 46);
PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 47);
PGQEHPNARRYRGANRRGLSRGCFGLKLDRIGSMSGLGC (SEQ ID NO: 48);
PGQEHPQARKYKGAQKKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 49);
GQEHPNARKYKGANPKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 50);
GQEHPNARKYKGANQKGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 51);
GQEHPNARKYKGANQQGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 52); and
GQEHPNARKYKGANKPGLSKGCFGLKLDRIGSMSGLGC (SEQ ID NO: 53).

36. The method of any one of claims 32 to 35, wherein the CNP variant is PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Pro-Gly-CNP37) (SEQ ID NO: 1); GQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (Gly-CNP-37) (SEQ ID NO: 2); or LQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC (CNP-38) (SEQ ID NO: 21).

37. The method of any one of claims 32 to 36, wherein the CNP variant further comprises a hydrophilic moiety.

38. The method of claim 37, wherein the hydrophilic moiety is PEG.