MESENCHYMAL STEM CELL DIFFERENTIATION

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. § 1.119(e) of U.S. provisional Application No. 61/225,293, filed July 14, 2009, the contents of which are incorporated by reference in the entirety.

BACKGROUND OF THE INVENTION

[0002] Osteoarthritis (OA) represents the most common musculoskeletal disorder. Approximately 40 million Americans are currently affected and this number is predicted to increase to 60 million within the next twenty years as a result of the aging population and an increase in life expectancy, making it the fourth leading cause of disability. OA is characterized by a slow degenerative breakdown of the joint including both the articular cartilage (containing the cells and matrix which produce lubrication and cushioning for the joint) and the subchondral bone underlying the articular cartilage. Current OA therapies include pain relief with oral NSAIDs or selective cyclooxygenase 2 (COX-2) inhibitors, intra-articular (IA) injection with agents such as corticorsteroids and hyaluronan, and surgical approaches.

[0003] Mesenchymal stem cells (MSCs) are present in adult articular cartilage and upon isolation can be programmed in vitro to undergo differentiation to chondrocytes and other mesenchymal cell lineages. In part it is regulated by growth factors (TGFβs, BMPs), serum conditions and cell-cell contact.

BRIEF SUMMARY OF THE INVENTION

[0004] The present invention provides methods of ameliorating or preventing arthritis or joint injury in a mammal. In some embodiments, the method comprises administering to a joint of the mammal a composition comprising an effective amount of a polypeptide comprising an amino acid sequence having at least 95% identity to SEQ ID NO:1 or 25, thereby ameliorating or preventing arthritis or joint injury in the mammal.

[0005] In some embodiments, the individual has arthritis or joint injury. In some embodiments, the individual does not have, but is at risk for, arthritis or joint injury.

[0006] In some embodiments, the polypeptide comprises SEQ ID NO:1 or 25.

[0007] In some embodiments, the amino acid sequence has at least 95% identity to SEQ ID NOs 2, 3, 4, 26, 27, or 28. In some embodiments, the amino acid sequence comprises SEQ ID NOs: 2, 3, 4, 26, 27, or 28.

[0008] In some embodiments, the amino acid sequence is at least 80% identical to any of SEQ ID NOs: 5-24. In some embodiments, the amino acid sequence comprises any of SEQ ID NOs: 5-24.

[0009] In some embodiments, the arthritis is selected from the group consisting of osteoarthritis, traumatic arthritis, and autoimmune arthritis.

[0010] In some embodiments, the mammal is a human.

[0011] In some embodiments, the composition further comprises hyaluronic acid.

[0012] The present invention also provides methods of inducing differentiation of mesenchymal stem cells into chondrocytes which form the cartilage matrix. In some embodiments, the method comprises contacting mesenchymal stem cells with a sufficient amount of a polypeptide comprising an amino acid sequence having at least 95% identity to SEQ ID NO:1 or 25 to induce differentiation of the stem cells into chondrocytes.

[0013] In some embodiments, the method is performed in vitro. In some embodiments, the method is performed in vivo and the stem cells are present in a mammal. In some embodiments, the mammal is a human.

[0014] In some embodiments, the polypeptide comprises SEQ ID NO:1 or 25.

[0015] In some embodiments, the amino acid sequence has at least 95% identity to SEQ ID NOs: 2, 3, 4, 26, 27, or 28. In some embodiments, the amino acid sequence comprises SEQ ID NOs: 2, 3, 4, 26, 27, or 28.

[0016] In some embodiments, the amino acid sequence is at least 80% identical to any of SEQ ID NOs: 5-24. In some embodiments, the amino acid sequence comprises any of SEQ ID NOs: 5-24.

[0017] The present invention also provides pharmaceutical compositions for intra-articular delivery and systemic delivery. In some embodiments, the composition comprising a pharmaceutically effective amount of a polypeptide comprising an amino acid sequence having at least 95% identity to SEQ ID NO:1 or 25.

[0018] In some embodiments, the composition further comprises hyaluronic acid.

[0019] In some embodiments, the polypeptide comprises SEQ ID NO:1 or 25.

[0020] In some embodiments, the amino acid sequence has at least 95% identity to SEQ ID NOs 2, 3, 4, 26, 27, or 28. In some embodiments, the amino acid sequence comprises SEQ ID NOs:2, 3, 4, 26, 27, or 28.

[0021] In some embodiments, the amino acid sequence is at least 80% identical to any of SEQ ID NOs: 5-24. In some embodiments, the amino acid sequence comprises any of SEQ ID NOs: 5-24.

[0022] Additional features, advantages, and embodiments of the invention may be set forth or apparent from consideration of the following detailed description, and claims. Moreover, it is to be understood that both the foregoing summary of the invention and the following detailed description are exemplary and intended to provide further explanation without limiting the scope of the invention as claimed.

DEFINITIONS

[0023] The terms "peptidomimetic" and "mimetic" refer to a synthetic chemical compound that has substantially the same structural and functional characteristics of a naturally or non-naturally occurring polypeptide (e.g., ANGPTL3). Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" (Fauchere, J. *Adv. Drug Res.* 15:29 (1986); Veber and Freidinger *TINS* p. 392 (1985); and Evans *et al. J. Med. Chem.* 30:1229 (1987), which are incorporated herein by reference). Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent or enhanced therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biological or pharmacological activity), such as found in a polypeptide of interest, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of, e.g., -CH2NH-, -CH2S-, -CH2-CH2-, -CH=CH- (cis and trans), -COCH2-, -CH(OH)CH2-, and -CH2SO-. The mimetic can be either entirely composed of synthetic, non-natural analogues of amino acids, or, is a chimeric molecule of partly natural peptide amino acids and partly non-natural analogs of amino acids. The mimetic can also incorporate any amount of natural amino acid conservative substitutions as long as such

substitutions also do not substantially alter the mimetic's structure and/or activity. For example, a mimetic composition is within the scope of the invention if it is capable of carrying out at least one activity of a polypeptide of interest.

[0024] The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers.

[0025] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Naturally encoded amino acids are the 20 common amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) and pyrrolysine and selenocysteine.

[0026] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every

possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

[0027] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[0028] The following eight groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);

2) Aspartic acid (D), Glutamic acid (E);

3) Asparagine (N), Glutamine (Q);

4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);

7) Serine (S), Threonine (T); and

8) Cysteine (C), Methionine (M) (*see, e.g.*, Creighton, *Proteins* (1984)).

[0029] "Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (e.g., a polypeptide of the invention), which does not comprise additions or deletions, for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[0030] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same sequences. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, 60% identity, optionally 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity over a specified region, or, when not specified, over the entire sequence), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. The invention provides polypeptides that are substantially identical to the polypeptides, respectively, exemplified herein (e.g., any of SEQ ID NOs: 1-28), as well as uses thereof including but no limited to use for treating or preventing arthritis or joint injury. Optionally, the identity exists over a region that is at least about 50 nucleotides in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides in length, or the entire length of the reference sequence.

[0031] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0032] A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman (1970) *Adv. Appl. Math.* 2:482c, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Nat'l. Acad. Sci. USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual

inspection (*see, e.g.,* Ausubel *et al.*, *Current Protocols in Molecular Biology* (1995 supplement)).

[0033] Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (1977) *Nuc. Acids Res.* 25:3389-3402, and Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[0034] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5787). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison

of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0035] The term "hyaluronic acid" are used herein to include derivatives of hyaluronic acid that include esters of hyaluronic acid, salts of hyaluronic acid and also includes the term hyaluronan. The designation also includes both low and high molecular weight forms of hyaluronans and crosslinked hyaluronans or hylans. Examples of such hyaluronans are Synvisc™ (Genzyme Corp. Cambridge, Mass.), ORTHOVISC™ (Anika Therapeutics, Woburn, Mass.), and HYALGAN™ (Sanofi-Synthelabo Inc., Malvern, Pa.).

BRIEF DESCRIPTION OF THE DRAWINGS

[0036] **Figure 1. Quantitative assessment of MSC-induced type II collagen production during *in vitro* chondrogenesis.** hMSCs (10,000 / 384 well) were plated for 24 hours in hMSC growth media (Millipore). The cells were treated with the proteins above for an additional 72 hours. The media was replaced with serum free DMEM and cultured an additional 14 days in the absence of any additional stimuli. Upon termination, the cells were fixed with formalin, washed, stained with an antibody for type II collagen and counterstained with DAPI. The amount of type II collagen staining was quantitated by high content imaging (Opera, Perkin Elmer). Data is pooled from 3 experiments in duplicate for each dose (n=6).

[0037] **Figure 2. Characterization of ANGTPL3-induced chondrogenesis.** (A) hMSCs were grown in pellet culture (1x106 cells/pellet) for 21 days in serum free DMEM, 1X ITS and ANGTPL3 (where indicated). The media was replaced every 3 days. Aggrecan mRNA expression was quantified using human Taqman specific probes according to the manufacturer's instructions (data pooled from 3 experiments in duplicate (n=6). (B) Bovine cartilage was isolated, punched into symmetric circles and put into organ culture. The slices were treated for 48 hours with 20ng/ml TNFa and 10ng/ml OSM (inflammatory mediators) to induce degradation of the cartilage matrix in the presence or absence of ANGPTL3 to identify the percent inhibition of glycosaminoglycan release, an indicator of matrix damage (data pooled from 4 donors,n=12). (C) Primary normal human chondrocytes and synoviocytes (2500/384 well in growth media) were plated and cultured for 24 hrs at 37°C. A moderate chondrocyte-specific cell growth increase was demonstrated in response to ANGTPL3 over the24 hr period (data pooled from 2 experiments, 2 replicates / dose (n=4)).

[0038] Figure 3. mANGTPTL3. (A) The primary protein structure and confirmed glycosylation sites. (B) The atomic structure (1.8 angstroms) of mANGTPL3 the C-terminal FLD (225-455).

[0039] Figure 4: Sequence alignment ANGPTL3 sequences. (A) Sequence alignment of the human, mouse, bovine, and canine native ANGPTL3 proteins; (B) Sequence alignment of the human, mouse, bovine, canine and equine native ANGPTL3 proteins.

[0040] Figure 5. *In vivo* efficacy of mANGPTL3 in a surgical osteoarthritis model. Surgical transection of the anterior cruciate ligament (ACL), medial meniscal tibial ligament (MMTL), and medial collateral ligament (MCL) of the right knee from C57BL/6 mice (n=12 / group) was performed to induce instability in the knee joint and thus lead to an OA phenotype. One week following surgery, the mice were dosed intra-articularly as indicated once / per for 3-4 weeks. (A). Peripheral blood was collected on day 28 following the surgery. The circulating type II collagen fragments (CTX-II) were quantified by an ELISA (Nordic biosciences). mANGPTL3 dose = 200ng / knee, 3 weekly injections(B) Quantitative assessments of the tibial plateau were made on a 0-4 scale, 0 being normal and 5 being severe OA (full thickness disruption of the cartilage). Two sections from each mouse were blindly graded by 2 observers. mANGPTL3 dose = 200ng / knee, 3 weekly injections (C)OA pain was measured by incapacitance testing, or determining the percentage of time the mouse stood on the surgical leg vs the other leg through the monitoring device. Readouts represent the pain response on day 36 after surgery (3 doses weekly doses of ANGPTL3 at the indicated concentration).

DETAILED DESCRIPTION

I. Introduction

[0041] The present invention is based, in part, on the discovery that Angiopoietin-like 3 (ANGPTL3) stimulates chondrocyte differentiation in mesenchymal stem cells. Accordingly, the present invention provides for methods of induction of mesenchymal stem cell differentiation into chondrocytes. Further, the present invention provides for administration of ANGPTL3 proteins to prevent or ameliorate arthritis or joint injury by administrating an ANGPTL3 protein into a joint, the vertebrae, vertebral disc or systemically.

II. Angiopoietin-like 3

[0042] Angiopoietin-like 3 is a member of the angiopoietin-like family of secreted factors. It is predominantly expressed in the liver, and has the characteristic structure of angiopoietins, consisting of a signal peptide, N-terminal coiled-coil domain (CCD) and the C-terminal fibrinogen (FBN)-like domain. The FBN-like domain in angiopoietin-like 3 was shown to bind αV/β3 integrins, and this binding induces endothelial cell adhesion and migration.

[0043] A variety of ANGPTL3 proteins can be used according to the present invention. As explained herein, native ANGPTL3 is generally cleaved in vivo into amino-terminal and carboxyl terminal fragments. The present invention contemplates use of various ANGPTL3 proteins having chondrogenic activity. In some embodiments, the invention provides for use of full-length native (or variants thereof) ANGPTL3 protein amino acid sequences. In some embodiments, the invention provides for ANGPTL3 proteins comprising a portion (not the full-length native sequence) of the ANGPTL3 sequence, or a variant thereof, that retains chondrogenic activity, i.e., not the amino-terminal end of the native protein. In some embodiments, the ANGPTL3 proteins of the invention do not have the CCD domain and/or do not have significant CCD activity. Thus, in some embodiments, the ANGPTL3 proteins of the invention comprise at least a fragment (e.g., at least 50, 100, 150, 200, 250 contiguous amino acids) of the native mouse (e.g., SEQ ID NO:12), human (e.g., SEQ ID NO:8), bovine (e.g., SEQ ID NO:16), dog (e.g., SEQ ID NO:20), or equine (e.g. SEQ ID NO:24) ANGPTL3 protein sequence or substantially identical sequences, but do not comprise at least 200 contiguous amino-terminal amino acids of a native ANGPTL3 protein.

[0044] In some embodiments, the ANGPTL3 proteins of the invention comprise a fibrinogen-like domain. In some embodiments, the ANGPTL3 proteins of the invention comprise contiguous amino acids corresponding to amino acids 207-455, 207-400, 207-350, 225-455, 225-400, 225-350, 241-455, 241-400, 241-350 of the native mouse (e.g., SEQ ID NO:12), human (e.g., SEQ ID NO:8), bovine (e.g., SEQ ID NO:16), dog (e.g., SEQ ID NO:20), or equine (e.g. SEQ ID NO:24) ANGPTL3 protein sequence or are substantially identical to such sequences, but do not include the flanking native ANGPTL3 protein amino acid sequence. In some embodiments, the ANGPTL3 proteins of the invention (including but not limited to any of SEQ ID NOs: 1-28) but lack at least a portion of the C-terminal sequence, e.g., lack 10, 20, 30, 40, 50 amino acids from the C-terminus.

[0045] While the ANGPTL3 proteins of the invention as described above may not include native ANGPTL3 protein sequences flanking the regions described above, the ANGPTL3 proteins of the invention can include non-native ANGPTL3 protein flanking sequences. For example, the chondrogenic active portion of an ANGPTL3 protein can be fused to one or more heterologous amino acids to form a fusion protein. Fusion partner sequences can include, but are not limited to, amino acid tags, non-L (e.g., D-) amino acids or other amino acid mimetics to extend *in vivo* half-life and/or protease resistance, targeting sequences or other sequences.

[0046] The ANGPTL3 proteins of the invention encompass variants and truncations of native ANGPTL3 proteins as well as variants and truncations of active fragments described herein. Active variants can be identified in any number of ways known to those of skill in the art. In some embodiments, amino acid alignments of active proteins can be established to identify those positions that are invariant or that are include conserved amino acid changes. SEQ ID NOs: 1, 2, 3, or 4 represent consensus sequences comprising the invariant amino acids between certain areas (position 241-455, 225-455 and 207-455, and native full-length, respectively) of the human, mouse, bovine, and canine native ANGPTL3 proteins. SEQ ID NOs: 25, 26, 27, or 28 represent consensus sequences comprising the invariant amino acids between certain areas (position 241-455, 225-455 and 207-455, and native full-length, respectively) of the human, mouse, bovine, canine, and equine native ANGPTL3 proteins. Thus, in some embodiments, the chondrogenic ANGPTL3 proteins of the invention comprise SEQ ID NOs: 1, 2, 3, 4, 25, 26, 27, or 28. In some embodiments, the chondrogenic ANGPTL3 proteins of the invention comprise an amino acid sequence substantially identical to any of SEQ ID NOs: 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 (as measured across the length of SEQ ID NOs).

[0047] The ANGPTL3 proteins of the invention have chondrogenic activity. As defined herein, chondrogenesis or chondrogenic activity refers to the development of chondrocytes from MSCs. Indicators of chondrogenic activity include, but are not limited to, cartilage matrix production. Cartilage matrix production may be measured by various markers, for example, such as Sox9, type II collagen, or glycosaminoglycan (GAG) production. In some embodiments, GAG production is measured as a marker for cartilage matrix production. In some embodiments, a 3-fold increase in GAG production with cartilage specific protein expression indicates positive cartilage matrix production.

[0048] In some embodiments, the ANGPTL3 polypeptides of the invention will comprise at least one non-naturally encoded amino acid. Methods of making and introducing a non-

naturally-occurring amino acid into a protein are known. *See*, e.g., US Patent Nos. 7,083,970; and 7,524,647. The general principles for the production of orthogonal translation systems that are suitable for making proteins that comprise one or more desired unnatural amino acid are known in the art, as are the general methods for producing orthogonal translation systems. For example, see International Publication Numbers WO 2002/086075, entitled "METHODS AND COMPOSITION FOR THE PRODUCTION OF ORTHOGONAL tRNA-AMINOACYL-tRNA SYNTHETASE PAIRS;" WO 2002/085923, entitled "IN VIVO INCORPORATION OF UNNATURAL AMINO ACIDS;" WO 2004/094593, entitled "EXPANDING THE EUKARYOTIC GENETIC CODE;" WO 2005/019415, filed Jul. 7, 2004; WO 2005/007870, filed Jul. 7, 2004; WO 2005/007624, filed Jul. 7, 2004; WO 2006/110182, filed Oct. 27, 2005, entitled "ORTHOGONAL TRANSLATION COMPONENTS FOR THE VIVO INCORPORATION OF UNNATURAL AMINO ACIDS" and WO 2007/103490, filed Mar. 7, 2007, entitled "SYSTEMS FOR THE EXPRESSION OF ORTHOGONAL TRANSLATION COMPONENTS IN EUBACTERIAL HOST CELLS." Each of these applications is incorporated herein by reference in its entirety. For discussion of orthogonal translation systems that incorporate unnatural amino acids, and methods for their production and use, see also, Wang and Schultz, (2005) "Expanding the Genetic Code." Angewandte Chemie Int Ed 44: 34-66; Xie and Schultz, (2005) "An Expanding Genetic Code." Methods 36: 227-238; Xie and Schultz, (2005) "Adding Amino Acids to the Genetic Repertoire." Curr Opinion in Chemical Biology 9: 548-554; and Wang, et al., (2006) "Expanding the Genetic Code." Annu Rev Biophys Biomol Struct 35: 225-249; Deiters, et al, (2005) "In vivo incorporation of an alkyne into proteins in Escherichia coli." Bioorganic & Medicinal Chemistry Letters 15:1521-1524; Chin, et al., (2002) "Addition of p-Azido-L-phenylalanine to the Genetic Code of Escherichia coli." J Am Chem Soc 124: 9026-9027; and International Publication No. WO2006/034332, filed on Sep. 20, 2005, the contents of each of which are incorporated by reference in their entirety. Additional details are found in U.S. Pat. No. 7,045,337; No. 7,083,970; No. 7,238,510; No. 7,129,333; No. 7,262,040; No. 7,183,082; No. 7,199,222; and No. 7,217,809.

[0049] A "non-naturally encoded amino acid" refers to an amino acid that is not one of the common amino acids or pyrolysine or selenocysteine. Other terms that may be used synonymously with the term "non-naturally encoded amino acid" are "non-natural amino acid," "unnatural amino acid," "non-naturally-occurring amino acid," and variously hyphenated and non-hyphenated versions thereof. The term "non-naturally encoded amino acid" also includes, but is not limited to, amino acids that occur by modification (e.g. post-

translational modifications) of a naturally encoded amino acid (including but not limited to, the 20 common amino acids or pyrrolysine and selenocysteine) but are not themselves naturally incorporated into a growing polypeptide chain by the translation complex. Examples of such non-naturally-occurring amino acids include, but are not limited to, N-acetylglucosaminyl-L-serine, N-acetylglucosaminyl-L-threonine, and O-phosphotyrosine.

[0050] A non-naturally encoded amino acid is typically any structure having any substituent side chain other than one used in the twenty natural amino acids. Because the non-naturally encoded amino acids of the invention typically differ from the natural amino acids only in the structure of the side chain, the non-naturally encoded amino acids form amide bonds with other amino acids, including but not limited to, natural or non-naturally encoded, in the same manner in which they are formed in naturally occurring polypeptides. However, the non-naturally encoded amino acids have side chain groups that distinguish them from the natural amino acids. For example, R optionally comprises an alkyl-, aryl-, acyl-, keto-, azido-, hydroxyl-, hydrazine, cyano-, halo-, hydrazide, alkenyl, alkynl, ether, thiol, seleno-, sulfonyl-, borate, boronate, phospho, phosphono, phosphine, heterocyclic, enone, imine, aldehyde, ester, thioacid, hydroxylamine, amino group, or the like or any combination thereof. Other non-naturally occurring amino acids of interest that may be suitable for use in the present invention include, but are not limited to, amino acids comprising a photoactivatable cross-linker, spin-labeled amino acids, fluorescent amino acids, metal binding amino acids, metal-containing amino acids, radioactive amino acids, amino acids with novel functional groups, amino acids that covalently or noncovalently interact with other molecules, photocaged and/or photoisomerizable amino acids, amino acids comprising biotin or a biotin analogue, glycosylated amino acids such as a sugar substituted serine, other carbohydrate modified amino acids, keto-containing amino acids, amino acids comprising polyethylene glycol or polyether, heavy atom substituted amino acids, chemically cleavable and/or photocleavable amino acids, amino acids with an elongated side chains as compared to natural amino acids, including but not limited to, polyethers or long chain hydrocarbons, including but not limited to, greater than about 5 or greater than about 10 carbons, carbon-linked sugar-containing amino acids, redox-active amino acids, amino thioacid containing amino acids, and amino acids comprising one or more toxic moiety.

[0051] Exemplary non-naturally encoded amino acids that may be suitable for use in the present invention and that are useful for reactions with water soluble polymers include, but are not limited to, those with carbonyl, aminooxy, hydrazine, hydrazide, semicarbazide, azide and alkyne reactive groups. In some embodiments, non-naturally encoded amino acids

comprise a saccharide moiety. Examples of such amino acids include N-acetyl-L-glucosaminyl-L-serine, N-acetyl-L-galactosaminyl-L-serine, N-acetyl-L-glucosaminyl-L-threonine, N-acetyl-L-glucosaminyl-L-asparagine and O-mannosaminyl-L-serine. Examples of such amino acids also include examples where the naturally-occurring N- or O-linkage between the amino acid and the saccharide is replaced by a covalent linkage not commonly found in nature--including but not limited to, an alkene, an oxime, a thioether, an amide and the like. Examples of such amino acids also include saccharides that are not commonly found in naturally-occurring proteins such as 2-deoxy-glucose, 2-deoxygalactose and the like.

[0052] Another type of modification that can optionally be introduced into the ANGPTL3 proteins of the invention (e.g. within the polypeptide chain or at either the N- or C-terminal), e.g., to extend in vivo half-life, is PEGylation or incorporation of long-chain polyethylene glycol polymers (PEG). Introduction of PEG or long chain polymers of PEG increases the effective molecular weight of the present polypeptides, for example, to prevent rapid filtration into the urine. In some embodiments, a Lysine residue in the ANGPTL3 sequence is conjugated to PEG directly or through a linker. Such linker can be, for example, a Glu residue or an acyl residue containing a thiol functional group for linkage to the appropriately modified PEG chain. An alternative method for introducing a PEG chain is to first introduce a Cys residue at the C-terminus or at solvent exposed residues such as replacements for Arg or Lys residues. This Cys residue is then site-specifically attached to a PEG chain containing, for example, a maleimide function. Methods for incorporating PEG or long chain polymers of PEG are well known in the art (described, for example, in Veronese, F. M., et al., *Drug Disc. Today* 10: 1451-8 (2005); Greenwald, R. B., et al., *Adv. Drug Deliv. Rev.* 55: 217-50 (2003); Roberts, M. J., et al., *Adv. Drug Deliv. Rev.*, 54: 459-76 (2002)), the contents of which is incorporated herein by reference. Other methods of polymer conjugations known in the art can also be used in the present invention. In some embodiments, poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) is introduced as a polymer conjugate with the ANGPTL3 proteins of the invention (*see*, e.g., WO2008/098930; Lewis, et al., *Bioconjug Chem.*, 19: 2144-55 (2008)). In some embodiments, a phosphorylcholine-containing polymer conjugate with the ANGPTL3 proteins can be used in the present invention. A person of skill would readily recognize that other biocompatible polymer conjugates can be utilized.

[0053] A more recently reported alternative approach for incorporating PEG or PEG polymers through incorporation of non-natural amino acids (as described above) can be performed with the present polypeptides. This approach utilizes an evolved tRNA/tRNA synthetase pair and is coded in the expression plasmid by the amber suppressor codon

(Deiters, A, et al. (2004). *Bio-org. Med. Chem. Lett.* 14, 5743-5). For example, p-azidophenylalanine can be incorporated into the present polypeptides and then reacted with a PEG polymer having an acetylene moiety in the presence of a reducing agent and copper ions to facilitate an organic reaction known as "Huisgen [3+2]cycloaddition."

[0054] In certain embodiments, the present invention contemplates specific mutations of the ANGPTL3 proteins so as to alter the glycosylation of the polypeptide. Such mutations may be selected so as to introduce or eliminate one or more glycosylation sites, including but not limited to, O-linked or N-linked glycosylation sites. In certain embodiments, the ANGPTL3 proteins of the present invention have glycosylation sites and patterns unaltered relative to the naturally-occurring ANGPTL3 proteins. In certain embodiments, a variant of ANGPTL3 proteins includes a glycosylation variant wherein the number and/or type of glycosylation sites have been altered relative to the naturally-occurring ANGPTL3 proteins. In certain embodiments, a variant of a polypeptide comprises a greater or a lesser number of N-linked glycosylation sites relative to a native polypeptide. An N-linked glycosylation site is characterized by the sequence: Asn-X-Ser or Asn-X-Thr, wherein the amino acid residue designated as X may be any amino acid residue except proline. The substitution of amino acid residues to create this sequence provides a potential new site for the addition of an N-linked carbohydrate chain. Alternatively, substitutions which eliminate this sequence will remove an existing N-linked carbohydrate chain. In certain embodiments, a rearrangement of N-linked carbohydrate chains is provided, wherein one or more N-linked glycosylation sites (typically those that are naturally occurring) are eliminated and one or more new N-linked sites are created.

[0055] Exemplary ANGPTL3 proteins variants include cysteine variants wherein one or more cysteine residues are deleted from or substituted for another amino acid (e.g., serine) relative to the amino acid sequence of the naturally-occurring ANGPTL3 proteins. In certain embodiments, cysteine variants may be useful when ANGPTL3 proteins must be refolded into a biologically active conformation such as after the isolation of insoluble inclusion bodies. In certain embodiments, cysteine variants have fewer cysteine residues than the native polypeptide. In certain embodiments, cysteine variants have an even number of cysteine residues to minimize interactions resulting from unpaired cysteines.

[0056] In some embodiments, functional variants or modified forms of the ANGPTL3 proteins include fusion proteins of an ANGPTL3 protein of the invention and one or more fusion domains. Well known examples of fusion domains include, but are not limited to, polyhistidine, Glu-Glu, glutathione S transferase (GST), thioredoxin, protein A, protein G, an

immunoglobulin heavy chain constant region (Fc), maltose binding protein (MBP), or human serum albumin. A fusion domain may be selected so as to confer a desired property. For example, some fusion domains are particularly useful for isolation of the fusion proteins by affinity chromatography. For the purpose of affinity purification, relevant matrices for affinity chromatography, such as glutathione-, amylase-, and nickel- or cobalt-conjugated resins are used. Many of such matrices are available in "kit" form, such as the Pharmacia GST purification system and the QLAexpress™ system (Qiagen) useful with (HIS_6) fusion partners. As another example, a fusion domain may be selected so as to facilitate detection of the ANGPTL3 proteins. Examples of such detection domains include the various fluorescent proteins (e.g., GFP) as well as "epitope tags," which are usually short peptide sequences for which a specific antibody is available. Well known epitope tags for which specific monoclonal antibodies are readily available include FLAG, influenza virus haemagglutinin (HA), and c-myc tags. In some cases, the fusion domains have a protease cleavage site, such as for Factor Xa or Thrombin, which allows the relevant protease to partially digest the fusion proteins and thereby liberate the recombinant proteins therefrom. The liberated proteins can then be isolated from the fusion domain by subsequent chromatographic separation. In certain embodiments, an ANGPTL3 protein is fused with a domain that stabilizes the ANGPTL3 protein in vivo (a "stabilizer" domain). By "stabilizing" is meant anything that increases serum half life, regardless of whether this is because of decreased destruction, decreased clearance by the kidney, or other pharmacokinetic effect. Fusions with the Fc portion of an immunoglobulin are known to confer desirable pharmacokinetic properties on a wide range of proteins. Likewise, fusions to human serum albumin can confer desirable properties. Other types of fusion domains that may be selected include multimerizing (e.g., dimerizing, tetramerizing) domains and functional domains (that confer an additional biological function, as desired).

III. Angiopoietin-like 3 Proteins Disease Indications

[0057] It is contemplated that the polypeptides, compositions, and methods of the present invention may be used to treat or prevent any type of arthritis or joint injury. It is further contemplated that the polypeptides, compositions, and methods of the present invention may be used to treat or prevent various cartilagenous disorders. In some embodiments, the proteins of the invention are administered to prevent arthritis or joint injury, for example where there is a genetic or family history of arthritis or joint injury or prior or during joint surgery. Exemplary conditions or disorders to be treated or prevented with the polypeptides, compositions, and methods of the invention, include, but are not limited to systemic lupus

erythematosis, rheumatoid arthritis, juvenile chronic arthritis, osteoarthritis, degenerative disc disease, spondyloarthropathies, systemic sclerosis (scleroderma), idiopathic inflammatory myopathies (dermatomyositis, polymyositis), Sjogren's syndrome, systemic vasculitis, sarcoidosis, autoimmune hemolytic anemia (immune pancytopenia, paroxysmal nocturnal hemoglobinuria), autoimmune thrombocytopenia (idiopathic thrombocytopenic purpura, immune-mediated thrombocytopenia), thyroiditis (Grave's disease, Hashimoto's thyroiditis, juvenile lymphocytic thyroiditis, atrophic thyroiditis), diabetes mellitus, immune-mediated renal disease (glomerulonephritis, tubulointerstitial nephritis), demyelinating diseases of the central and peripheral nervous systems such as multiple sclerosis, idiopathic demyelinating polyneuropathy or Guillain-Barr syndrome, and chronic inflammatory demyelinating polyneuropathy, hepatobiliary diseases such as infectious hepatitis (hepatitis A, B, C, D, E and other non-hepatotropic viruses), autoimmune chronic active hepatitis, primary biliary cirrhosis, granulomatous hepatitis, and sclerosing cholangitis, inflammatory bowel disease (ulcerative colitis: Crohn's disease), gluten-sensitive enteropathy, and Whipple's disease, autoimmune or immune-mediated skin diseases including bullous skin diseases, erythema multiforme and contact dermatitis, psoriasis, allergic diseases such as asthma, allergic rhinitis, atopic dermatitis, food hypersensitivity and urticaria, immunologic diseases of the lung such as eosinophilic pneumonias, idiopathic pulmonary fibrosis and hypersensitivity pneumonitis, transplantation associated diseases including graft rejection and graft-versus-host-disease. In some embodiments of the invention, the polypeptides, compositions, and methods of the present invention may be used to treat osteoarthritis.

[0058] In some embodiments, the polypeptides, compositions, and methods of the present invention provide a method for stimulating chondrocyte proliferation and cartilage production in cartilagenous tissues that have been damaged due to traumatic injury or chondropathy. Of particular importance for treatment are tissues that exhibit articulated surfaces, such as, spine, shoulder, elbow, wrist, joints of the fingers, hip, knee, ankle, and the joints of the feet. Examples of diseases that may benefit from treatment include osteoarthritis, rheumatoid arthritis, other autoimmune diseases, or osteochondritis dessicans. In addition, cartilage malformation is often seen in forms of dwarfism in humans suggesting that the polypeptides, compositions, and methods would be useful in these patients.

[0059] It is contemplated that the polypeptides, compositions, and methods of the present invention may be used to treat a mammal. As used herein a "mammal" to any mammal classified as a mammal, including humans, domestic and farm animals, and zoo, sports or pet

animals, such as cattle (e.g. cows), horses, dogs, sheep, pigs, rabbits, goats, cats, etc. In some embodiments of the invention, the mammal is a human.

[0060] In some embodiments, the polypeptides of the invention can be heterologous to the mammal to be treated. For example, a bovine ANGPTL3 protein or fragments thereof, a protein or peptide derived from a bovine ANGPTL3 protein (e.g., a modified bovine ANGPTL3 protein, a conservative variant of bovine ANGPTL3 protein, a peptidomimetic derived from a bovine ANGPTL3 protein) are used in the treatment of a human patient. In some embodiments, a heterologous ANGPTL3 protein can be used to expand chondrocyte populations in culture for transplantation. In some embodiments, the expanded cultures will then be admixed with polypeptides and compositions homologous to the mammal to be treated, and placed in the joint space or directly into the cartilage defect. Alternatively, the polypeptides of the invention are derived from the same species, i.e., a human ANGPTL3 protein or fragments thereof, a protein or peptide derived from a human ANGPTL3 protein (e.g., a modified human ANGPTL3 protein, a conservative variant of human ANGPTL3 protein, a peptidomimetic derived from a human ANGPTL3 protein) is used in the treatment of a human patient. By using a protein derived from the same species of mammal as is being treated, one may avoid inadvertent immune responses.

[0061] The polypeptides and compositions of the present invention can be applied by direct injection into the synovial fluid of the joint, systemic administratin (oral or intravenously) or directly into the cartilage defect, either alone or complexed with a suitable carrier for extended release of protein. The polypeptides, compositions, and methods of the present invention can also be used to expand chondrocyte populations in culture for autogenous or allogenic chondrocyte transplantation. The transplantation can be optionally administered with concurrent treatment consisting of administration of the polypeptides and compositions of the present invention. In these procedures, for example, chondrocytes can be harvested arthroscopically from an uninjured minor load-bearing area of the damaged joint, and can be cultured in the presence of the polypeptides and compositions of the present invention to increase the number of cells prior to transplantation. The expanded cultures will then be admixed with the polypeptides and compositions of the present invention, and placed in the joint space or directly into the defect. The polypeptides and compositions of the present invention can be used in combination with periosteal or perichondrial grafts that contain cells that can form cartilage and/or help to hold the transplanted chondrocytes or their precursor cells in place. The polypeptides and compositions of the present invention can be used to repair cartilage damage in conjunction with lavage of the joint, stimulation of bone marrow,

abrasion arthroplasty, subchondral drilling, or microfracture of the subchondral bone. Additionally, after the growth of cartilage due to the administration of the polypeptides and compositions of the present invention, additional surgical treatment may be necessary to suitably contour the newly formed cartilage surface.

IV. Pharmaceutical Compositions

[0062] The dose of a compound of the present invention for treating the above-mentioned diseases or disorders varies depending upon the manner of administration, the age and the body weight of the subject, and the condition of the subject to be treated, and ultimately will be decided by the attending physician or veterinarian. Such an amount of the compound as determined by the attending physician or veterinarian is referred to herein as an "effective amount."

[0063] Formulations suitable for administration include exipients, including but not limited to, aqueous and non-aqueous solutions, isotonic sterile solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives.

[0064] The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial response in the subject over time. The dose will be determined by the efficacy of the particular protein employed and the condition of the subject, as well as the body weight or surface area of the area to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular protein or vector in a particular subject. Administration can be accomplished via single or divided doses.

V. Methods of Administration

[0065] Any method for delivering the proteins of the invention to an affected joint can be used. In the practice of this invention, compositions can be administered, for example, intra-articularly (i.e., into a joint), orally, intravenously. The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials. Solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. The proteins of the present invention can also be used effectively in combination with one or more additional active agents (e.g., hyaluronic acid or a salt thereof) depending on the desired therapy or effect.

EXAMPLES

[0066] The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1: <u>High throughput screen for inducers of chondrogenesis</u>

[0067] To identify and define a new, non-invasive strategy for OA joint repair, we developed a non-biased high throughput screen of proteins capable of selectively directing the differentiation of human mesenchymal stem cells (MSCs) to chondrocytes. The assay system models the resident MSCs in cartilage and identifies mediators that stimulate the natural repair potential and enhance integrated cartilage regeneration. We choose to test a unique secreted protein library by a high throughput, cell-based screens of human and mouse MSCs. This approach provides a strategy that allows the rapid identification of uncharacterized and native protein ligands that affect chondrogenesis.

[0068] To study these secreted proteins, two approaches were taken: the production of conditioned media (CM) from a mammalian producer cell line (HEK293T) and the generation of purified proteins from free style HEK-F cells. Together these complementary approaches allowed for new target identification in a variety of applications.

[0069] Screening of proteins in the MSC assay identified the lead candidate, Angiopoeitin-like 3, abbreviated ANGPTL3. ANGPTL3 was identified in two parallel but orthogonal initial screens of both the CM and purified proteins. In the proof of concept CM screen, C3H10t1/2 cells were incubated for 7 days following transfer of the CM. Chondrogenesis was found to occur in wells containing ANGPTL3 -75 assayed by Alcian blue staining for detection of cartilage matrix production. ANGPTL3 -75 was independently identified by screening 531 purified proteins for chondrocytic differentiation of human MSCs.

[0070] Following the initial screening assays, ANGPTL3 was subsequently characterized in six secondary assays. These assays include: (1) monolayer culture of mouse mesenchymal C3H10t1/2 cells: induction of type II collagen and Sox9 protein expression (markers of chondrogenesis) but no induction of osteocalcin (markers of osteogenesis); (2) inhibition of TNFα / oncostatin M (OSM) – induced nitric oxide (NO) release in bovine chondrocytes; (3) inhibition of TNFα / OSM – induced glycosaminoglycan (GAG) release in bovine cartilage organ culture; (4) induction of cartilage matrix gene expression (aggrecan) in a human MSC

pellet culture system; (5) lack of toxicity in primary human chondrocytes, human synovial fibroblasts, and human MSCs; (6) stimulation of proliferation of human chondrocytes.

[0071] The mouse ANGPTL3 protein functions in mouse, human and bovine cell types and therefore was chosen for further characterization as it potently induced (0.5-5nM) chondrogenic differentiation although its human homolog retains a similar potency for chondrogenesis compared to other previously know inducers of chondrogenesis in the quantitative imaged based type II collagen assay (Figure 1). Both mouse and human MSCs differentiated in monolayer and pellet culture (respectively) for 18 to 21 days when treated with 0.5-5nM ANGPTL3 and express three cartilage specific proteins: type II collagen, Sox9 and aggrecan. This was assessed by both immunohistochemical staining and mRNA quantification by Taqman (Figure 2A). Additionally, data suggest after culturing mouse MSCs for 18 days, ANGPTL3 inhibits the spontaneous tendency towards a fibrotic repair response through reduced expression of alkaline phosphatase. To assess the potential ability to prevent tissue damage, bovine cartilage organ cultures were stimulated with TNFα and oncostatin M (OSM). The stimulated glycosaminoglycan release, an indicator of matrix degeneration, was significantly inhibited by treatment with ANGPTL3 (Figure 2B). Additionally, treatment of primary human chondrocytes but not human synoviocytes led to a 2 fold increase in cell growth within 24 hrs (Figure 2C) suggesting a specificity of its action on cartilage. The protein also had no obvious *in vitro* toxic effects (<100μM) on the viability of in primary human chondrocytes, synovial fibroblasts and MSCs (data not shown).

[0072] In a direct comparison between the two candidates presently in clinical trials, treatment with 100ng/ml of FGF18 or 100ng/ml BMP7 could induce chondrogenic nodules, but with less overall matrix production compared to ANGPTL3. FGF18 lacked the ability to increase Alcian Blue, Sox9 or Type II collagen staining, indicating a lack of specificity of a true cartilage matrix. 100ng/ml of BMP7 increased Alcian blue, Sox9 and Type II collagen staining, but the significance was not as great at similar concentrations.

Example 2: <u>Expression of recombinant full length ANGPTL3 and mutant protein and function analysis</u>

[0073] Mouse ANGPTL3 is predicted to be 51 kDa protein. It belongs to a family of 7 identified Angiopoietin-like (ANGPTL) proteins that have structural similarity to the angiopoiteins, but lack the ability to bind the Tie2 receptor and have distinct functions. They contain an N-terminal coiled-coil domain (CCD) and a C-terminal fibrinogen-like domain (FLD). ANGPTL proteins are tightly regulated by their microenvironment and interactions

with the extracellular matrix (ECM), yet the precise interaction sites nor partners have been elucidated in detail. ANGPTL3 is secreted by the liver and circulates systemically. It is controlled through liver X receptors (LXR), with evidence that LXR- induced hyper-triglyceridmedia is due to ANGPTL3 release. Interactions between the CCD and the ECM through a putative heparin binding motif may lead to inhibition of cleavage at the proprotein convertase recognition sequence (R221-R224), similar to that reported for ANGPTL4. Cleavage results in a significant increase in the CCD's ability to inhibit lipoprotein lipase activity (LPL), and thereby leads to increase of triglyceridemia (TG). This represents the major biological function of ANGPTL3 identified prior to the present invention. The C terminal FLD is sufficient to induce endothelial cell adhesion and mediate angiogenesis after direct implantation in the rat cornea *in vivo*. It was also demonstrated that recombinant ANGPTL3 could bind to purified αVβ3 integrin and lead to an increase in FAK, MAPK and AKT signaling in endothelial cells. These data suggest the FLD interaction with integrins to mediate angiogenesis.

[0074] No expression of ANGPTL3 has been reported nor observed in our studies using western blotting in human chondrocytes, hMSCs or human synovial fibroblasts, and no expression of it was found in mouse knee joints. Furthermore, there are no reported activities for either fragment in joint cells relating to this novel chondrogenic function identified in our screen.

[0075] Our data indicates that full length ANGPTL3 is a novel mediator of chondrogenesis and cartilage protection. We examined which domains of ANGPTL3 are critical for this novel chondrogenic function. A truncation series of ANGPTL3 and full length protein have been expressed and purified in an engineered HEK-S cell line that gives limited and homogeneous glycosylation for detailed biophysical characterization. Mass spectrometric approaches confirmed the occupancy of all four predicted N-linked glycosylations (N115, N232, N296, and N357, Figure 3A). From HEK endogenously processed fragments, the proteolytic cleavage site between the CCD and FLD domain was determind to be R224. The full length ANGPTL3 elutes by size exclusion chromatography with a mass >400 kDa, indicative of a trimer with heterogeneous glycosylation. The FLD (241-455), shown to be a monomer by size exclusion and static light scattering, was crystallized. The atomic structure of the FLD was determined at 1.8 angstroms resolution. The FLD structure revealed a core beta sheet configuration with helical stretches that orient three loops toward the C-terminus, typical of other FLD homologs (Figure 3B). The temperature factors in the C-terminal portion of the domain are higher than the rest of the structure suggesting a high degree of

flexibility in this region. Superposition with the ANGPTL2/Tie2 structure (PDB code: 2GY7) suggests that the C-terminal portion of the FLD may be involved in protein-protein interactions.

[0076] The five truncation products generated have been evaluated in multiple chondrogenic assays. The results suggests no activity retention in the CCD alone, but activity remains in the FLD. Additionally, SMAD1 phosphorylation is increased upon stimulation for 3 days with the full length or mutants G or H alone, suggesting activation of chondrogenic signaling cascade.

[0077] Although systemic exposure of a protein injected intra-articularly is limited due to the synovial fluid lymph drainage, minimizing systemic exposure can sometimes be desirable. If there is systemic exposure, the cleavage of ANGPTL3 might lead to the release the CCD domain. The increased inhibition of LPL activity by CCD could lead to alteration of the patients' TG levels. Our results indicate potential clinical advantages of ANGPTL3. For instance, by dissecting where the chondrogenic activity in ANGPTL3 is localized we would minimize or exclude the unwanted systemic effects on lipid metabolism or the angiogenic properties. The chondrogenic activity is localized primarily to the C terminus thus specific use of this domain would alleviate the concerns of TG regulation of the full length molecule or N terminus. As full length ANGPTL3 is normally present in serum, there is no expected immunogenicity. An additional advantage of using the C terminus is that it is cleaved and is a monomer (~29kDa) compared to the trimerized full length protein, thus possibly reducing systemic half life of any small percentage which might be present in circulation and limiting any potential effect from angiogenesis.

Example 3: *In vivo* analysis of ANGPTL3

[0078] We have performed several in vivo evaluations of the full length ANGPTL3 to address potential adverse events and intra-articular retention. Following intra-articular (IA) injection of the left knee joints of 8 week old C57BL/10 mice with 3.6μg of full length ANGPTL3, we evaluated immunohistochemically the expression in the presence and absence of exposure for 24 hours. By 24 hours, very little to no detectable levels of ANGPTL3 should remain in the synovial fluid as the typical turnover by trans-synovial flow into the synovial lymph vessels for proteins and water is approximately 2 hours. The results revealed no endogenous expression of ANGPTL3 in untreated joints, but significant detection of the protein in the pericellular matrix in the articular cartilage and in the menisci even at the 24 hour time point. Additionally no widespread cytotoxicity to the chondrocytes or cartilage

damage in vivo was detected. Following a series of 3 IA injections into the knee joints of rats (once / week for three weeks), clincially there was no toxicity (no joint swelling or alternations in gait) or evidence of an acute inflammatory reaction in the joint of the rat. Histologically, there was no increase in synovitis or uncontrolled proliferation in the joints of the five rats injected. As in the mouse, ANGPTL3 could be detected in the cartilage matrix and surrounding the chondrocytes. These results indicate that ANGPTL3 does not cause any undesired effects to the cartilage itself. and that ANGPTL3 does enter into and is retained in cartilage and menisci in vivo.

[0079] OA is not a single disease entity and can be considered the consequence of various etiologic factors. In humans it is often caused by abnormal biomechanical stress or genetic or acquired abnormalities of the articular cartilage or bone. Therefore, choosing the "best" small animal OA model is difficult and multiple models should be explored to determine the protective properties of any therapeutic. We have completed efficacy studies a chronic OA model (collagenase VII-induced based upon the research described by van der Kraan and colleagues) and an acute surgical model involving transection of the three of the major ligaments (ACL, MCL and MMTL) in the joint based upon the work of Glasson et al. Both models induced pathological changes commonly associated with OA: loss of proteoglycan staining, erosion of the cartilage and bone, osteophyte formation and metaplastic alterations in the synovium and ligaments can be evident 4-8 weeks after initiation of OA. Figure 5 depicts the regenerative capacity of ANGPTL3 surgical model of OA. To begin to examine potential biomarkers for OA, peripheral blood was collected during the surgical model to measure the type II collagen fragments released due to cartilage damage (Figure 5A). Histological analysis and subsequent grading of the medial tibial plateau revealed regeneration in the cartilage matrix after treatment with 200ng ANGTPL3 / knee once per week for 3 weeks (Figure 5B).

[0080] In a 8 week surgical OA subset of mice, three doses of ANGPTL3 were examined for alleviation of OA-induced pain through incapacitance measurements. This method measures the weight distribution between the surgical and non-surgical legs. On day 36 following surgery and 3 weekly treatments with PRO1, as low as 100ng / knee dosing demonstrated significant improvement compared to the vehicle treated surgical knees (Figure 5C). These combined data provide concrete evidence that ANGTPL3 has *in vivo* efficacy in two OA models (both pathological correction and pain diminishment) and supports the advancement of its development as a novel OA therapeutic.

[0081] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

SEQUENCE LISTING

X= any amino acid
Amino acids in parentheses indicate that the amino acid can be absent or present

SEQ ID NO:1
241-455 consensus
XXPAXCXXXYNXGXHTSGXYXIXPXNSQXFXVYCDXXSGSXWTLIQHRXDGSQXFNETWENY
XXGFGRLDGEFWLGLEKIYXIVXQSNYXLRXELXDWXD(X)KXYXEYXFXLGXHETNYTLHX
XXIXGNXXXAXPEXXDLXFSTWXHXAKGXXXCPEXXSGGWWXXXXCGENNLNGKYNKXXXKX
XPERXXGXXWXXQXXXLYXIKXXKMXXXPXX

SEQ ID NO:2
225-455 consensus
TTPXXXXNEXXNXXXXXXPAXCXXXYNXGXHTSGXYXIXPXNSQXFXVYCDXXSGSXWTLIQ
HRXDGSQXFNETWENYXXGFGRLDGEFWLGLEKIYXIVXQSNYXLRXELXDWXD(X)KXYXE
YXFXLGXHETNYTLHXXXIXGNXXXAXPEXXDLXFSTWXHXAKGXXXCPEXXSGGWWXXXXC
GENNLNGKYNKXXXKXXPERXXGXXWXXQXXXLYXIKXXKMXXXPXX

SEQ ID NO:3
207-455 consensus
XIXEXXEXSLSSKXRAPRTTPXXXXNEXXNXXXXXXPAXCXXXYNXGXHTSGXYXIXPXNSQ
XFXVYCDXXSGSXWTLIQHRXDGSQXFNETWENYXXGFGRLDGEFWLGLEKIYXIVXQSNYX
LRXELXDWXD(X)KXYXEYXFXLGXHETNYTLHXXXIXGNXXXAXPEXXDLXFSTWXHXAKG
XXXCPEXXSGGWWXXXXCGENNLNGKYNKXXXKXXPERXXGXXWXXQXXXLYXIKXXKMXXX
PXX

SEQ ID NO:4
Full length consensus
MXTIKLXLXXXPLVIXSXXDXDXXSXDSXXXEPKSRFAMLDDVKILANGLLQLGHGLKDFVH
KTKGQINDIFQKLNIFDQSFYDLSLXTXEIKEEEKELRRXTXXLQVKNEEVKNMSXELXSKX
ESLLEEKXXLQXKVXXLEXQLTXLIXXXXXXXEXXEVTSLKXXVEXQDNSIXXLLQXVEXQY
XQLXQQXXQIKEIEXQLR(X)XXIXEXXEXSLSSKXRAPRTTPXXXXNEXXNXXXXXXPAXC
XXXYNXGXHTSGXYXIXPXNSQXFXVYCDXXSGSXWTLIQHRXDGSQXFNETWENYXXGFGR
LDGEFWLGLEKIYXIVXQSNYXLRXELXDWXD(X)KXYXEYXFXLGXHETNYTLHXXXIXGN
XXXAXPEXXDLXFSTWXHXAKGXXXCPEXXSGGWWXXXXCGENNLNGKYNKXXXKXXPERXX
GXXWXXQXXXLYXIKXXKMXXXPXX(SEXXE)

SEQ ID NO:5
Human ANGPTL3 241-455
GIPAECTTIYNRGEHTSGMYAIRPSNSQVFHVYCDVISGSPWTLIQHRIDGSQNFNETWENY
KYGFGRLDGEFWLGLEKIYSIVKQSNYVLRIELEDWKDNKHYIEYSFYLGNHETNYTLHLVA
ITGNVPNAIPENKDLVFSTWDHKAKGHFNCPEGYSGGWWWHDECGENNLNGKYNKPRAKSKP
ERRRGLSWKSQNGRLYSIKSTKMLIHPTD

SEQ ID NO 6
Human ANGPTL3 225-455
TTPFLQLNEIRNVKHDGIPAECTTIYNRGEHTSGMYAIRPSNSQVFHVYCDVISGSPWTLIQ
HRIDGSQNFNETWENYKYGFGRLDGEFWLGLEKIYSIVKQSNYVLRIELEDWKDNKHYIEYS
FYLGNHETNYTLHLVAITGNVPNAIPENKDLVFSTWDHKAKGHFNCPEGYSGGWWWHDECGE
NNLNGKYNKPRAKSKPERRRGLSWKSQNGRLYSIKSTKMLIHPTD

SEQ ID NO 7

Human ANGPTL3 207-455

IQEPTEISLSSKPRAPRTTPFLQLNEIRNVKHDGIPAECTTIYNRGEHTSGMYAIRPSNSQV
FHVYCDVISGSPWTLIQHRIDGSQNFNETWENYKYGFGRLDGEFWLGLEKIYSIVKQSNYVL
RIELEDWKDNKHYIEYSFYLGNHETNYTLHLVAITGNVPNAIPENKDLVFSTWDHKAKGHFN
CPEGYSGGWWWHDECGENNLNGKYNKPRAKSKPERRRGLSWKSQNGRLYSIKSTKMLIHPTD

SEQ ID NO 8

Full length human ANGPTL3 gi|7656888|ref|NP_055310.1| [Homo sapiens]

MFTIKLLLFIVPLVISSRIDQDNSSFDSLSPEPKSRFAMLDDVKILANGLLQLGHGLKDFVH
KTKGQINDIFQKLNIFDQSFYDLSLQTSEIKEEEKELRRTTYKLQVKNEEVKNMSLELNSKL
ESLLEEKILLQQKVKYLEEQLTNLIQNQPETPEHPEVTSLKTFVEKQDNSIKDLLQTVEDQY
KQLNQQHSQIKEIENQLRRTSIQEPTEISLSSKPRAPRTTPFLQLNEIRNVKHDGIPAECTT
IYNRGEHTSGMYAIRPSNSQVFHVYCDVISGSPWTLIQHRIDGSQNFNETWENYKYGFGRLD
GEFWLGLEKIYSIVKQSNYVLRIELEDWKDNKHYIEYSFYLGNHETNYTLHLVAITGNVPNA
IPENKDLVFSTWDHKAKGHFNCPEGYSGGWWWHDECGENNLNGKYNKPRAKSKPERRRGLSW
KSQNGRLYSIKSTKMLIHPTDSESFE

SEQ ID NO:9

Mouse ANGPTL3 241-455

DLPADCSAVYNRGEHTSGVYTIKPRNSQGFNVYCDTQSGSPWTLIQHRKDGSQDFNETWENY
EKGFGRLDGEFWLGLEKIYAIVQQSNYILRLELQDWKDSKHYVEYSFHLGSHETNYTLHVAE
IAGNIPGALPEHTDLMFSTWNHRAKGQLYCPESYSGGWWWNDICGENNLNGKYNKPRTKSRP
ERRRGIYWRPQSRKLYAIKSSKMMLQPTT

SEQ ID NO 10

Mouse ANGPTL3 225-455

TTPPLQLNETENTEQDDLPADCSAVYNRGEHTSGVYTIKPRNSQGFNVYCDTQSGSPWTLIQ
HRKDGSQDFNETWENYEKGFGRLDGEFWLGLEKIYAIVQQSNYILRLELQDWKDSKHYVEYS
FHLGSHETNYTLHVAEIAGNIPGALPEHTDLMFSTWNHRAKGQLYCPESYSGGWWWNDICGE
NNLNGKYNKPRTKSRPERRRGIYWRPQSRKLYAIKSSKMMLQPTT

SEQ ID NO 11

Mouse ANGPTL3 207-455

IQEPSENSLSSKSRAPRTTPPLQLNETENTEQDDLPADCSAVYNRGEHTSGVYTIKPRNSQG
FNVYCDTQSGSPWTLIQHRKDGSQDFNETWENYEKGFGRLDGEFWLGLEKIYAIVQQSNYIL
RLELQDWKDSKHYVEYSFHLGSHETNYTLHVAEIAGNIPGALPEHTDLMFSTWNHRAKGQLY
CPESYSGGWWWNDICGENNLNGKYNKPRTKSRPERRRGIYWRPQSRKLYAIKSSKMMLQPTT

SEQ ID NO 12

Full length mouse ANGPTL3 gi|33469117|ref|NP_038941.1| [Mus musculus]

MHTIKLFLFVVPLVIASRVDPDLSSFDSAPSEPKSRFAMLDDVKILANGLLQLGHGLKDFVH
KTKGQINDIFQKLNIFDQSFYDLSLRTNEIKEEEKELRRTTSTLQVKNEEVKNMSVELNSKL
ESLLEEKTALQHKVRALEEQLTNLILSPAGAQEHPEVTSLKSFVEQQDNSIRELLQSVEEQY
KQLSQQHMQIKEIEKQLRKTGIQEPSENSLSSKSRAPRTTPPLQLNETENTEQDDLPADCSA
VYNRGEHTSGVYTIKPRNSQGFNVYCDTQSGSPWTLIQHRKDGSQDFNETWENYEKGFGRLD
GEFWLGLEKIYAIVQQSNYILRLELQDWKDSKHYVEYSFHLGSHETNYTLHVAEIAGNIPGA
LPEHTDLMFSTWNHRAKGQLYCPESYSGGWWWNDICGENNLNGKYNKPRTKSRPERRRGIYW
RPQSRKLYAIKSSKMMLQPTT

SEQ ID NO:13

Bovine ANGPTL3 241-454

DIPADCTIIYNQGKHTSGIYSIRPSNSQVFNVYCDVKSGSSWTLIQHRIDGSQNFNETWENY
KYGFGRLDGEFWLGLEKIYSIVMQSNYILRIELEDWKDKYYTEYSFHLGDHETNYTLHLAEI
SGNGPKAFPEHKDLMFSTWDHKAKGHFNCPESNSGGWWYHDVCGENNLNGKYNKPKAKAKPE
RKEGICWKSQDGRLYSIKATKMLIHPSD

SEQ ID NO 14

Bovine ANGPTL3 225-454

TTPSFHSNETKNVEHDDIPADCTIIYNQGKHTSGIYSIRPSNSQVFNVYCDVKSGSSWTLIQ
HRIDGSQNFNETWENYKYGFGRLDGEFWLGLEKIYSIVMQSNYILRIELEDWKDKYYTEYSF
HLGDHETNYTLHLAEISGNGPKAFPEHKDLMFSTWDHKAKGHFNCPESNSGGWWYHDVCGEN
NLNGKYNKPKAKAKPERKEGICWKSQDGRLYSIKATKMLIHPSD

SEQ ID NO 15

Bovine ANGPTL3 207-454

IKESTEISLSSKPRAPRTTPSFHSNETKNVEHDDIPADCTIIYNQGKHTSGIYSIRPSNSQV
FNVYCDVKSGSSWTLIQHRIDGSQNFNETWENYKYGFGRLDGEFWLGLEKIYSIVMQSNYIL
RIELEDWKDKYYTEYSFHLGDHETNYTLHLAEISGNGPKAFPEHKDLMFSTWDHKAKGHFNC
PESNSGGWWYHDVCGENNLNGKYNKPKAKAKPERKEGICWKSQDGRLYSIKATKMLIHPSD

SEQ ID NO 16

Full length bovine ANGPTL3 gi|122692391|ref|NP_001073814.1|
[Bos taurus]

MYTIKLFLIIAPLVISSRTDQDYTSLDSISPEPKSRFAMLDDVKILANGLLQLGHGLKDFVH
KTKGQINDIFQKLNIFDQSFYDLSLQTNEIKEEEKELRRATSKLQVKNEEVKNMSLELDSKL
ESLLEEKILLQQKVRYLEDQLTDLIKNQPQIQEYLEVTSLKTLVEQQDNSIKDLLQIVEEQY
RQLNQQQSQIKEIENQLRRTGIKESTEISLSSKPRAPRTTPSFHSNETKNVEHDDIPADCTI
IYNQGKHTSGIYSIRPSNSQVFNVYCDVKSGSSWTLIQHRIDGSQNFNETWENYKYGFGRLD
GEFWLGLEKIYSIVMQSNYILRIELEDWKDKYYTEYSFHLGDHETNYTLHLAEISGNGPKAF
PEHKDLMFSTWDHKAKGHFNCPESNSGGWWYHDVCGENNLNGKYNKPKAKAKPERKEGICWK
SQDGRLYSIKATKMLIHPSDSENSE

SEQ ID NO:17

Canine ANGPTL3 240-454

DIPANCTTIYNRGEHTSGIYSIRPSNSQVFNVYCDVKSGSSWTLIQHRIDGSQNFNETWENY
RYGFGRLDGEFWLGLEKIYSIVKQSNYILRIELEDWNDNKHYIEYFFHLGNHETNYTLHLVE
ITGNILNALPEHKDLVFSTWDHKAKGHVNCPESYSGGWWWHNVCGENNLNGKYNKQRAKTKP
ERRRGLYWKSQNGRLYSIKSTKMLIHPID

SEQ ID NO:18

Canine ANGPTL3 224-454

TTPFLHLNETKNVEHNDIPANCTTIYNRGEHTSGIYSIRPSNSQVFNVYCDVKSGSSWTLIQ
HRIDGSQNFNETWENYRYGFGRLDGEFWLGLEKIYSIVKQSNYILRIELEDWNDNKHYIEYF
FHLGNHETNYTLHLVEITGNILNALPEHKDLVFSTWDHKAKGHVNCPESYSGGWWWHNVCGE
NNLNGKYNKQRAKTKPERRRGLYWKSQNGRLYSIKSTKMLIHPID

SEQ ID NO:19

Canine ANGPTL3 206-454

IQESTENSLSSKPRAPRTTPFLHLNETKNVEHNDIPANCTTIYNRGEHTSGIYSIRPSNSQV
FNVYCDVKSGSSWTLIQHRIDGSQNFNETWENYRYGFGRLDGEFWLGLEKIYSIVKQSNYIL
RIELEDWNDNKHYIEYFFHLGNHETNYTLHLVEITGNILNALPEHKDLVFSTWDHKAKGHVN
CPESYSGGWWWHNVCGENNLNGKYNKQRAKTKPERRRGLYWKSQNGRLYSIKSTKMLIHPID

SEQ ID NO:20

Full length canine ANGPTL3 gi|57086505|ref|XP_536686.1| [Canis familiaris]

MYTIKLFLFIIPLVISSKIDRDYSSYDSVSPEPKSRFAMLDDVKILANGLLQLGHGLKDFVH
KTKGQINDIFQKLNIFDQSFYDLSLQTNEIKEEEKELRRTTSKLQVKNEEVKNMSLELNSKV
ESLLEEKILLQQKVRYLEKQLTSLIKNQPEIQEHPEVTSLKTFVEQQDNSIKDLLQTVEEQY
RQLNQQHSQIKEIENQLRNVIQESTENSLSSKPRAPRTTPFLHLNETKNVEHNDIPANCTTI
YNRGEHTSGIYSIRPSNSQVFNVYCDVKSGSSWTLIQHRIDGSQNFNETWENYRYGFGRLDG
EFWLGLEKIYSIVKQSNYILRIELEDWNDNKHYIEYFFHLGNHETNYTLHLVEITGNILNAL
PEHKDLVFSTWDHKAKGHVNCPESYSGGWWWHNVCGENNLNGKYNKQRAKTKPERRRGLYWK
SQNGRLYSIKSTKMLIHPIDSESSE

SEQ ID NO:21

Equine ANGPTL3 241-455

DFPADCTTIYNRGEHTSGIYSIKPSNSQVFNVYCDVISGSSWILIQRRIDGSQNFNETWQNY
KYGFGRLDFEFWLGLEKIYSIVKRSNYILRIELEDWKDNKHTIEYSFHLGNHETNYTLHLVE
ITGNVPNALPEHKDLVFSTWDHKAKGQLNCLESYSGGWWWHDVCGGDNPNGKYNKPRSKTKP
ERRRGICWKSQNGRLYTIKSTKMLIHPID

SEQ ID NO:22

Equine ANGPTL3 225-455

TTPSFHLNETKDVEHDDFPADCTTIYNRGEHTSGIYSIKPSNSQVFNVYCDVISGSSWILIQ
RRIDGSQNFNETWQNYKYGFGRLDFEFWLGLEKIYSIVKRSNYILRIELEDWKDNKHTIEYS
FHLGNHETNYTLHLVEITGNVPNALPEHKDLVFSTWDHKAKGQLNCLESYSGGWWWHDVCGG
DNPNGKYNKPRSKTKPERRRGICWKSQNGRLYTIKSTKMLIHPID

SEQ ID NO:23

Equine ANGPTL3 207-455

IQESTENSLSSKPRAPRTTPSFHLNETKDVEHDDFPADCTTIYNRGEHTSGIYSIKPSNSQV
FNVYCDVISGSSWILIQRRIDGSQNFNETWQNYKYGFGRLDFEFWLGLEKIYSIVKRSNYIL
RIELEDWKDNKHTIEYSFHLGNHETNYTLHLVEITGNVPNALPEHKDLVFSTWDHKAKGQLN
CLESYSGGWWWHDVCGGDNPNGKYNKPRSKTKPERRRGICWKSQNGRLYTIKSTKMLIHPID

SEQ ID NO:24

Full length equine ANGPTL3 [equus caballus]

MYTIKLFLVIAPLVISSRIDQDYSSLDSIPPEPKSRFAMLDDVKILANGLLQLGHGLKDFVH
KTKGQINDIFQKLNIFDQSFYALSLQTNEIKEEEKELRRTTSKLQVKNEEVKNMSLELNSKL
ESLLEEKSLLQQKVKYLEEQLTKLIKNQPEIQEHPEVTSLKTFVEQQDNSIKDLLQTMEEQY
RQLNQQHSQIKEIENQLRRTGIQESTENSLSSKPRAPRTTPSFHLNETKDVEHDDFPADCTT
IYNRGEHTSGIYSIKPSNSQVFNVYCDVISGSSWILIQRRIDGSQNFNETWQNYKYGFGRLD
FEFWLGLEKIYSIVKRSNYILRIELEDWKDNKHTIEYSFHLGNHETNYTLHLVEITGNVPNA
LPEHKDLVFSTWDHKAKGQLNCLESYSGGWWWHDVCGGDNPNGKYNKPRSKTKPERRRGICW
KSQNGRLYTIKSTKMLIHPIDSESFELRQIKKPMN

SEQ ID NO:25

241-455 consensus

XXPAXCXXXYNXGXHTSGXYXIXPXNSQXFXVYCDXXSGSXWXLIQXRXDGSQXFNETWXNY
XXGFGRLDXEFWLGLEKIYXIVXXSNYXLEXELXDWXD(X)KXXXEYXFXLGXHETNYTLHX
XXIXGNXXXAXPEXXDLXFSTWXHXAKGXXXCXEXXSGGWWXXXXCGXXNXNGKYNKXXXKX
XPERXXGXXWXXQXXXLYXIKXXKMXXXPXX

SEQ ID NO:26

225-455 consensus

TTPXXXXNEXXXXXXXXXPAXCXXXYNXGXHTSGXYXIXPXNSQXFXVYCDXXSGSXWXLIQ
XRXDGSQXFNETWXNYXXGFGRLDXEFWLGLEKIYXIVXXSNYXLEXELXDWXD(X)KXXXE
YXFXLGXHETNYTLHXXXIXGNXXXAXPEXXDLXFSTWXHXAKGXXXCXEXXSGGWWXXXXC
GXXNXNGKYNKXXXKXXPERXXGXXWXXQXXXLYXIKXXXKMXXXPXX

SEQ ID NO:27

207-455 consensus

XIXEXXEXSLSSKXRAPRTTPXXXXNEXXXXXXXXXPAXCXXXYNXGXHTSGXYXIXPXNSQ
XFXVYCDXXSGSXWXLIQXRXDGSQXFNETWXNYXXGFGRLDXEFWLGLEKIYXIVXXSNYX
LEXELXDWXD(X)KXXXEYXFXLGXHETNYTLHXXXIXGNXXXAXPEXXDLXFSTWXHXAKG
XXXCXEXXSGGWWXXXXCGXXNXNGKYNKXXXKXXPERXXGXXWXXQXXXLYXIKXXKMXXX
PXX

SEQ ID NO:28

Full length consensus

MXTIKLXLXXXPLVIXSXXDXDXXSXDSXXXEPKSRFAMLDDVKILANGLLQLGHGLKDFVH
KTKGQINDIFQKLNIFDQSFYXLSLXTXEIKEEEKELRRXTXXLQVKNEEVKNMSXELXSKX
ESLLEEKXXLQXKVXXLEXQLTXLIXXXXXXXEXXEVTSLKXXVEXQDNSIXXLLQXXEXQY
XQLXQQXXQIKEIEXQLR(X)XXIXEXXEXSLSSKXRAPRTTPXXXXNEXXXXXXXXXPAXC
XXXYNXGXHTSGXYXIXPXNSQXFXVYCDXXSGSXWXLIQXRXDGSQXFNETWXNYXXGFGR
LDXEFWLGLEKIYXIVXXSNYXLEXELXDWXD(X)KXXXEYXFXLGXHETNYTLHXXXIXGN
XXXAXPEXXDLXFSTWXHXAKGXXXCXEXXSGGWWXXXXCGXXNXNGKYNKXXXKXXPERXX
GXXWXXQXXXLYXIKXXKMXXXPXX(SEXXEXXXXXXXXX)

CLAIMS

1. An *in vitro* method of inducing differentiation of mesenchymal stem cells into chondrocytes, the method comprising, contacting mesenchymal stem cells with a sufficient amount of a polypeptide consisting of an ANGPTL3 C-terminal fibrinogen domain peptide comprising an amino acid sequence selected from:

(a) a polypeptide consisting of any one of SEQ ID NO:1, 2, 3, 27, 26, or 25;

(b) a polypeptide consisting of an amino acid sequence having at least 95% identity to SEQ ID NOs: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23;

(c) a polypeptide consisting of an Angptl3 variant amino acid sequence substantially identical to SEQ ID NOs: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23;

(d) a polypeptide consisting of an amino acid sequence of SEQ ID NOs: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23;

(e) a polypeptide consisting of any one of SEQ ID NO:1, 2, 3, 27, 26, or 25 fused to a human serum albumin (HSA), an immunoglobulin heavy chain constant region (Fc), a polyhistidine, a glutathione S transferase (GST), a thioredoxin, a protein A, a protein G, or a maltose binding protein (MBP);

(f) a polypeptide consisting of an amino acid sequence having at least 95% identity to SEQ ID NOs: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23 fused to a human serum albumin (HSA), an immunoglobulin heavy chain constant region (Fc), a polyhistidine, a glutathione S transferase (GST), a thioredoxin, a protein A, a protein G, or a maltose binding protein (MBP);

(g) a polypeptide consisting of an Angptl3 variant amino acid sequence substantially identical to SEQ ID Nos: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23 fused to a human serum albumin (HSA), an immunoglobulin heavy chain constant region (Fc), a polyhistidine, a glutathione S transferase (GST), a thioredoxin, a protein A, a protein G, or a maltose binding protein (MBP); or

(h) a polypeptide consisting of an amino acid sequence of SEQ ID Nos: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23 fused to a human serum albumin (HSA), an immunoglobulin heavy chain constant region (Fc), a polyhistidine, a glutathione S transferase (GST), a thioredoxin, a protein A, a protein G, or a maltose binding protein (MBP);

wherein said polypeptide has chondrogenic activity to induce differentiation of the stem cells into chondrocytes.

2. Use of a polypeptide consisting of an ANGPTL3 C-terminal fibrinogen domain peptide comprising an amino acid sequence selected from:

(a) a polypeptide consisting of an amino acid sequence of any one of SEQ ID Nos: 1, 2, 3, 25, 26, or 27;

(b) a polypeptide consisting of an amino acid sequence having at least 95% identity to SEQ ID NOs: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23;

(c) a polypeptide consisting of an Angptl3 variant amino acid sequence substantially identical to SEQ ID NOs: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23;

(d) a polypeptide consisting of an amino acid sequence of SEQ ID NOs: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23,

(e) a polypeptide consisting of any one of SEQ ID NO:1, 2, 3, 27, 26, or 25 fused to a human serum albumin (HSA), an immunoglobulin heavy chain constant region (Fc), a polyhistidine, a glutathione S transferase (GST), a thioredoxin, a protein A, a protein G, or a maltose binding protein (MBP);

(f) a polypeptide consisting of an amino acid sequence having at least 95% identity to SEQ ID NOs: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23 fused to a human serum albumin (HSA), an immunoglobulin heavy chain constant region (Fc), a polyhistidine, a glutathione S transferase (GST), a thioredoxin, a protein A, a protein G, or a maltose binding protein (MBP);

(g) a polypeptide consisting of an Angptl3 variant amino acid sequence substantially identical to SEQ ID Nos: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23 fused to a human serum albumin (HSA), an immunoglobulin heavy chain constant region (Fc), a polyhistidine, a glutathione S transferase (GST), a thioredoxin, a protein A, a protein G, or a maltose binding protein (MBP); or

(h) a polypeptide consisting of an amino acid sequence of SEQ ID Nos: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23 fused to a human serum albumin (HSA), an immunoglobulin heavy chain constant region (Fc), a polyhistidine, a glutathione S transferase (GST), a thioredoxin, a protein A, a protein G, or a maltose binding protein (MBP);

in the manufacture of a medicament for ameliorating or preventing arthritis or joint injury in a mammal, wherein said polypeptide has chondrogenic activity.

3. A pharmaceutical composition for intra-articular delivery, the composition comprising a pharmaceutically effective amount of a polypeptide consisting of an ANGPTL3 C-terminal fibrinogen domain peptide comprising an amino acid sequence selected from:

(a) a polypeptide consisting of any one of SEQ ID NO:1, 2, 3, 27, 26, or 25;

(b) a polypeptide consisting of an amino acid sequence having at least 95% identity to SEQ ID NOs: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23;

(c) a polypeptide consisting of an Angptl3 variant amino acid sequence substantially identical to SEQ ID NOs: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23;

(d) a polypeptide consisting of an amino acid sequence of SEQ ID NOs: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23;

(e) a polypeptide consisting of any one of SEQ ID NO:1, 2, 3, 27, 26, or 25 fused to a human serum albumin (HSA), an immunoglobulin heavy chain constant region (Fc), a polyhistidine, a glutathione S transferase (GST), a thioredoxin, a protein A, a protein G, or a maltose binding protein (MBP);

(f) a polypeptide consisting of an amino acid sequence having at least 95% identity to SEQ ID NOs: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23 fused to a human serum albumin (HSA), an immunoglobulin heavy chain constant region (Fc), a polyhistidine, a glutathione S transferase (GST), a thioredoxin, a protein A, a protein G, or a maltose binding protein (MBP);

(g) a polypeptide consisting of an Angptl3 variant amino acid sequence substantially identical to SEQ ID Nos: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23 fused to a human serum albumin (HSA), an immunoglobulin heavy chain constant region (Fc), a polyhistidine, a glutathione S transferase (GST), a thioredoxin, a protein A, a protein G, or a maltose binding protein (MBP); or

(h) a polypeptide consisting of an amino acid sequence of SEQ ID Nos: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22 or 23 fused to a human serum albumin (HSA), an immunoglobulin heavy chain constant region (Fc), a polyhistidine, a glutathione S transferase (GST), a thioredoxin, a protein A, a protein G, or a maltose binding protein (MBP);
wherein said polypeptide has chondrogenic activity.

4. The use of claim 2, wherein the arthritis is selected from the group consisting of osteoarthritis, trauma arthritis, and autoimmune arthritis.

5. The composition of claim 3, wherein the composition further comprises hyaluronic acid.

6. The method of claim 1, or the use of claim 2, or the composition of claim 3, wherein the polypeptide is PEGylated.

7. The method of claim 1, or the use of claim 2, or the composition of claim 3, wherein the polypeptide comprises 1, 2, 3, 4, or more unnatural amino acids.

8. The method of claim 1, or the use of claim 2, wherein the mammal is a human, a dog, or a cat.

9. The method of claim 1, or the use of claim 2, or the composition of claim 3, wherein the polypeptide is SEQ ID NO:1 or SEQ ID NO:25.

10. The method of claim 1, or the use of claim 2, or the composition of claim 3, wherein the amino acid sequence is SEQ ID NO: 2 or SEQ ID NO:26.

11. The method of claim 1, or the use of claim 2, or the composition of claim 3, wherein the amino acid sequence is SEQ ID NO: 3 or SEQ ID NO:27.

12. The method of claim 1, or the use of claim 2, or the composition of claim 3, wherein the amino acid sequence is at least 95% identical to SEQ ID NO: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22, or 23.

13. The method of claim 1, or the use of claim 2, or the composition of claim 3, wherein the amino acid sequence is SEQ ID NO: 5, 6, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19, 21, 22, or 23.