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(54) **Title**: AAV-EPO FOR TREATING COMPANION ANIMALS

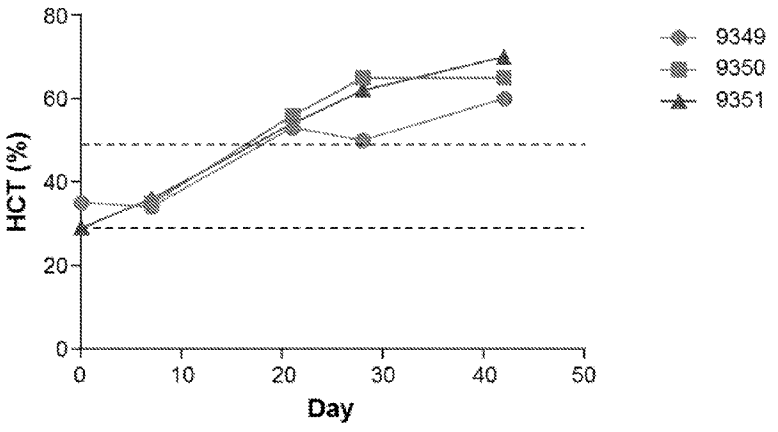

FIG. 1.

(57) **Abstract**: Compositions and methods are provided for treating companion animals are provided. An adeno-associated viral vector is provided which includes a nucleic acid molecule comprising a sequence encoding erythropoietin (EPO). In desired embodiments, the subject is a cat or dog.

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AAV-EPO FOR TREATING COMPANION ANIMALS

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED IN ELECTRONIC FORM

[0001] Applicant hereby incorporates by reference the Sequence Listing material filed in electronic form herewith. This file is labeled "15-7472PCT_Seq_Listing.txt".

BACKGROUND OF THE INVENTION

[0001] Erythropoietin (EPO) is a hormone made predominantly within the peritubular cells of the kidney. It acts on the bone marrow, stimulating erythropoiesis. Erythropoietin also controls apoptosis (programmed cell death) of mature red blood cells. Renal disease reduces erythropoietin production. In humans, the management of anemia in chronic kidney disease has been revolutionized by the development of recombinant human erythropoietin (epoetin). Many of the symptoms that had been ascribed to chronic kidney disease such as fatigue, lethargy, somnolence and shortness of breath, which all impact unfavorably on quality of life, were resolved or markedly improved when anemia was corrected.

[0002] There are over 2 million cats and 350,000 dogs that suffer from chronic kidney disease (CKD). Companion animals with CKD- related renal failure suffer in similar ways. They do not have sufficient EPO and subsequently become very anemic. In the past veterinarians have given human recombinant EPO until the animals would develop an immune response to the infused EPO. Effectively, this leaves no long term treatment in the market for a very well understood physiological process that has a clear need in the clinic.

[0003] Therefore, compositions useful for expressing EPO in subjects, particularly companion animals, are needed.

SUMMARY OF THE INVENTION

[0004] Novel engineered erythropoietin (EPO) constructs are provided herein. These constructs can be delivered to subjects in need thereof via a number of routes, and particularly by

expression *in vivo* mediated by a recombinant vector such as a recombinant adeno-associated virus (rAAV) vector.

[0005] In some embodiments, the EPO is encoded by an endogenous sequence. That is, the EPO sequence is derived from the same subject species for which administration is ultimately intended.

[0006] In some embodiments, a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a recombinant vector as described herein is provided. Also provided are methods for treating chronic kidney disease by administering to a subject in need thereof a recombinant vector described herein that has an expression cassette, wherein said expression cassette further comprises regulatory control sequences which direct expression of the EPO construct in the subject. In some embodiments, the subject being treated is a companion animal. In one embodiment, the subject is a feline. In another embodiment, the subject is a canine. As used herein, the terms "patient" and "subject" are used interchangeably, and can refer to a human or veterinary subject.

[0007] In yet another embodiment, methods for increasing the amount of circulating EPO in a subject comprising providing a recombinant vector described herein that has an expression cassette encoding EPO.

[0008] The recombinant vectors described above can be used in a regimen for treating chronic kidney disease and other conditions characterized by a decrease in the amount of circulating red blood cells.

[0009] Other aspects and advantages of the invention will be readily apparent from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[00010] FIG. 1 is a graph showing hematocrits of cats treated with AAV8 expressing feline erythropoietin. Dashed lines indicate the normal range.

[00011] FIG. 2 is a graph showing hematocrits of dogs treated with AAV8 expressing canine erythropoietin. Dashed lines indicate the normal range.

[00012] FIG. 3A shows the canine EPO propeptide sequence, with the leader sequence underlined. FIG. 3B shows the feline EPO propeptide sequence, with the leader sequence underlined.

[00013] FIG. 4 is a graph showing hematocrits of cats treated with AAV8 expressing feline erythropoietin. Cats were treated with 3.0 $x10^7$ GC, 3.0 $x10^8$ GC, 3.0 $x10^9$ GC, or 3.0 $x10^{10}$ GC AAV8f.EPO.

DETAILED DESCRIPTION OF THE INVENTION

[00014] Adeno-associated viral vectors carrying EPO expression constructs have been developed for use in subjects including companion animals (e.g., feline and canine). Though likely effective, a recombinant canine or feline specific EPO protein therapeutic would cost much more to develop and manufacture than a viral vector mediated system for delivery of EPO to the affected animal. With a viral vector therapeutic, there is also the convenience of being able to treat the animal once, as opposed to frequent injections of recombinant EPO. Stable expression of EPO would correct anemia and give the animal an improved quality of life. The EPO constructs described herein are also characterized in that they provide an EPO sequence which is endogenous to the subject, which reduces the risk of the subject developing an immune response to a non-native protein.

[00015] Also provided are uses for the constructs described herein. Delivery of these constructs to subjects in need thereof via a number of routes, and particularly for expression *in vivo* which is mediated by a recombinant vector such as a rAAV vector, is described. In one embodiment, methods of using the constructs in regimens for treating chronic kidney disease in a subject in need thereof and increasing the EPO in a subject are also provided. In one embodiment, methods of using these constructs in regimens for treating anemia in a subject in need thereof are provided. In another embodiment, the subject's anemia is related to the use of other medications. Possible medications which contribute to anemia include, but are not limited to, HIV/AIDS treatments (including AZT) and cancer therapeutics, including chemotherapy. In another embodiment, the subject's anemia is related to a medical condition. Possible medical conditions that contribute to anemia include, but are not limited to, cancer, HIV/AIDS,

rheumatoid arthritis, Crohn's disease and other chronic inflammatory diseases and dysfunctional bone marrow (e.g., aplastic anemia, leukemia, myelodysplasia or myelofibrosis), multiple myeloma, myeloproliferative disorders and lymphoma, hemolytic anemia, sickle cell anemia and thalassemia. In addition, methods are provided for enhancing the activity of EPO in a subject.

[00016] EPO is expressed in vivo as a propeptide, with the leader sequences sharing some homology across species. SEQ ID NO: 3 shows the sequence of the canine EPO propeptide, with the mature protein beginning at amino acid 41. The leader sequence is underlined in Figure 3a. SEQ ID NO: 4 shows the sequence of the feline EPO propeptide, with the mature protein beginning at amino acid 27. The leader sequence is underlined in Figure 3B.

[00017] In one embodiment, functional variants of EPO include variants which may include up to about 10% variation from an EPO nucleic acid or amino acid sequence described herein or known in the art, which retain the function of the wild type sequence. The sequence on which the EPO variant is based may, in some embodiments, include the propeptide leader sequence (e.g., as shown in SEQ ID NO: 3 and SEQ ID NO: 4). In another embodiment, the EPO variant described herein refers only to the mature peptide (e.g., amino acids 41-206 of SEQ ID NO: 3 or amino acids 27-192 of SEQ ID NO: 4). As used herein, by "retain function" it is meant that the nucleic acid or amino acid functions in the same way as the wild type sequence, although not necessarily at the same level of expression or activity. For example, in one embodiment, a functional variant has increased expression or activity as compared to the wild type sequence. In another embodiment, the functional variant has decreased expression or activity as compared to the wild type sequence. In one embodiment, the functional variant has 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater increase or decrease in expression or activity as compared to the wild type sequence. The amino acid sequence of canine EPO is provided herein as SEQ ID NO: 3. The amino acid sequence of feline EPO is provided herein as SEQ ID NO: 4.

[00018] In another embodiment, functional variants of EPO include variants which may include up to about 20% variation from an EPO nucleic acid or amino acid sequence described herein or known in the art, which retain the function of the wild type sequence. In one embodiment, functional variants of EPO include variants which may include up to about 30%

variation from an EPO nucleic acid or amino acid sequence described herein or known in the art, which retain the function of the wild type sequence. The following alignment shows the canine sequence on top, the feline sequence on the bottom, with the consensus sequence in the middle.

Canine (query; part of SEQ ID NO: 3) v. Feline (subject, part of SEQ ID NO: 4)

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Query  19   ECPALLLLLSLLLLPLGLPVLGAPPRLICDSRVLERYILEAREAENVTMGCAQGCSFSEN  78
            ECPALLLLLSLLLLPLGLPVLGAPPRLICDSRVLERYILEAREAENVTMGCA+GCSFSEN
Sbjct  5    ECPALLLLLSLLLLPLGLPVLGAPPRLICDSRVLERYILEAREAENVTMGCAEGCSFSEN  64

Query  79   ITVPDTKVNFYTWKRMDVGQQALEVWQGLALLSEAILRGQALLANASQPSETPQLHVDKA  138
            ITVPDTKVNFYTWKRMDVGQQA+EVWQGLALLSEAILRGQALLAN+SQPSET QLHVDKA
Sbjct  65   ITVPDTKVNFYTWKRMDVGQQAVEVWQGLALLSEAILRGQALLANSSQPSETLQLHVDKA  124

Query  139  VSSLRSLTSLLRALGAQKEAMSLPEEASPAPLRTFTVDTLCKLFRIYSNFLRGKLTLYTG  198
            VSSLRSLTSLLRALGAQKEA SLPE  S APLRTFTVDTLCKLFRIYSNFLRGKLTLYTG
Sbjct  125  VSSLRSLTSLLRALGAQKEATSLPEATSAAPLRTFTVDTLCKLFRIYSNFLRGKLTLYTG  184

Query  199  EACRRGDR  206
            EACRRGDR
Sbjct  185  EACRRGDR  192
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[00019] In one embodiment, the term EPO refers to active EPO in which one or more amino acid substitutions have been made, as compared to the wild type sequence (SEQ ID NO: 3 or SEQ ID NO: 4, either sequence with or without the leader peptide). In one embodiment, one or more amino acid substitutions are made in a residue in which variation is shown across species as in the alignment above. In another embodiment, one or more amino acid substitutions are made in a residue in which conservation is shown across species. Although EPO shares a high degree of identity across species, in one embodiment, it is desirable to select the EPO sequence based on the species of the subject for which administration of the vector is ultimately

intended. In one example, the subject is a mammal. For example, in one embodiment, if the subject is a feline, the EPO sequence is derived from a feline protein. In another embodiment, the EPO sequence is derived from a canine protein. In another embodiment, the EPO sequence is derived from a non-human primate protein. In another embodiment, the EPO is derived from bovine, ovine, or porcine protein. In another embodiment, the EPO sequence is SEQ ID NO: 3. In another embodiment, the EPO sequence is SEQ ID NO: 4.

[00020] The EPO peptide or nucleic acid coding sequence may include a heterologous leader sequence in conjunction with the EPO mature protein sequence. The term "heterologous" when used with reference to a protein or a nucleic acid indicates that the protein or the nucleic acid comprises two or more sequences or subsequences which are not found in the same relationship to each other in nature. For instance, the expression cassette is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid. For example, in one embodiment, the leader sequence may be from a different gene than EPO. Thus, with reference to the EPO coding sequences, the leader is heterologous. In one embodiment, the leader sequence is derived from a different species than the EPO sequence.

[00021] In one embodiment, the sequence encodes an IL-2 leader peptide fused upstream of the EPO mature polypeptide. In one embodiment, the leader sequence is SEQ ID NO: 9: M Y R M Q L L S C I A L S L A L V T N S. However, another heterologous leader sequence may be substituted for the IL-2 signal/leader peptide. The leader may be a signal sequence which is natively found in a cytokine (*e.g.,* IL-2, IL12, IL18, or the like), immunoglobulin, insulin, albumin, β-glucuronidase, alkaline protease or the fibronectin secretory signal peptides, or sequences from tissue specific secreted proteins, amongst others. In one embodiment, the leader sequence is the endogenous leader sequence from the EPO propeptide.

[00022] As used herein, the terms "derived" or "derived from" mean the sequence or protein is sourced from a specific subject species or shares the same sequence as a protein or sequence sourced from a specific subject species. For example, a propeptide sequence which is "derived from" a canine, shares the same sequence (or a variant thereof, as defined herein) as the same propeptide sequence as expressed in a canine. However, the specified nucleic acid or

amino acid need not actually be sourced from a canine. Various techniques are known in the art which are able to produce a desired sequence, including mutagenesis of a similar protein (e.g., a homolog) or artificial production of a nucleic acid or amino acid sequence. The "derived" nucleic acid or amino acid retains the function of the same nucleic acid or amino acid in the species from which it is "derived", regardless of actual source of the derived sequence.

[00023] As used herein the terms "EPO construct", "EPO expression construct" and synonyms include the EPO sequence as described herein. The terms "EPO construct", "EPO expression construct" and synonyms can be used to refer to the nucleic acid sequences encoding the EPO (including the EPO mature protein or propeptide with endogenous or heterologous leader) or the expression products thereof.

[00024] The term "amino acid substitution" and its synonyms are intended to encompass modification of an amino acid sequence by replacement of an amino acid with another, substituting, amino acid. The substitution may be a conservative substitution. It may also be a non-conservative substitution. The term conservative, in referring to two amino acids, is intended to mean that the amino acids share a common property recognized by one of skill in the art. For example, amino acids having hydrophobic nonacidic side chains, amino acids having hydrophobic acidic side chains, amino acids having hydrophilic nonacidic side chains, amino acids having hydrophilic acidic side chains, and amino acids having hydrophilic basic side chains. Common properties may also be amino acids having hydrophobic side chains, amino acids having aliphatic hydrophobic side chains, amino acids having aromatic hydrophobic side chains, amino acids with polar neutral side chains, amino acids with electrically charged side chains, amino acids with electrically charged acidic side chains, and amino acids with electrically charged basic side chains. Both naturally occurring and non-naturally occurring amino acids are known in the art and may be used as substituting amino acids in embodiments. Methods for replacing an amino acid are well known to the skilled in the art and include, but are not limited to, mutations of the nucleotide sequence encoding the amino acid sequence. Reference to "one or more" herein is intended to encompass the individual embodiments of, for example, 1, 2, 3, 4, 5, 6, or more.

[00025] Also provided are the assembled EPO proteins described herein. In one embodiment, the EPO protein is produced by a described AAV construct. In one embodiment, the EPO protein includes a heterologous leader combined with the mature EPO protein. In one embodiment, the heterologous leader is from IL-2. The assembled EPO proteins have many uses including diagnostic assays. Thus, in one embodiment, the EPO protein is labeled. As used herein, "labels" are chemical or biochemical moieties useful for labeling the EPO protein. "Labels" include fluorescent agents, chemiluminescent agents, chromogenic agents, quenching agents, radionucleotides, enzymes, substrates, cofactors, inhibitors, radioactive isotopes, magnetic particles, and other moieties known in the art. "Labels" or "reporter molecules" are capable of generating a measurable signal and may be covalently or noncovalently joined to an oligonucleotide or nucleotide (e.g., a non-natural nucleotide) or ligand. Most desirably, the label is detectable visually, e.g. colorimetrically. Many such labels are known in the art and include, without limitation, fluorescent detectable fluorochromes, e.g., fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), coriphosphine-O (CPO) or tandem dyes, PE-cyanin-5 (PC5), and PE-Texas Red (ECD). Commonly used fluorochromes include fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), and also include the tandem dyes, PE-cyanin-5 (PC5), PE-cyanin-7 (PC7), PE-cyanin-5.5, PE-Texas Red (ECD), rhodamine, PerCP, fluorescein isothiocyanate (FITC) and Alexa dyes. Combinations of such labels, such as Texas Red and rhodamine, FITC +PE, FITC + PECy5 and PE + PECy7, among others may be used depending upon assay method. Other desirable labels or tags include those which allow physical separation or immobilization on a substrate of the protein. Such labels include biotin. Other suitable labels or tags are described, e.g., in US 2011-0177967 A1, which is incorporated herein by reference.

[00026] In another embodiment, the EPO peptide includes variants which may include up to about 10% variation from the EPO sequence. That is, the EPO peptide shares about 90% identity to about 99.9 % identity, about 95% to about 99% identity or about 97% to about 98% identity to the EPO sequences provided herein and/or known in the art.

[00027] In addition to the EPO peptides provided herein, nucleic acid sequences encoding these peptides are provided. In one embodiment, a nucleic acid sequence is provided which

encodes for the EPO peptides described herein. In another embodiment, this includes any nucleic acid sequence which encodes the canine EPO protein of SEQ ID NO: 3 or a sequence sharing at least 90% identity with SEQ ID NO: 3. In another embodiment, this includes any nucleic acid sequence which encodes the feline EPO protein of SEQ ID NO: 4 or a sequence sharing at least 90% identity with SEQ ID NO: 4.

[00028] In one embodiment, the nucleic acid sequence encoding canine EPO is SEQ ID NO: 5. In one embodiment, the nucleic acid sequence encoding feline EPO is SEQ ID NO: 6. In yet another embodiment, the EPO nucleic acid includes variants which may include up to about 10% variation from an EPO sequence disclosed herein or known in the art. In yet another embodiment, the EPO nucleic acid includes variants which may include up to about 20% variation from an EPO sequence disclosed herein or known in the art. In yet another embodiment, the EPO nucleic acid includes variants which may include up to about 30% variation from an EPO sequence disclosed herein or known in the art. In another embodiment, the EPO nucleic acid includes variants which may include up to about 40% variation from an EPO sequence disclosed herein or known in the art.

[00029] In one embodiment, the nucleic acid sequence encoding EPO is a codon optimized sequence encoding any of the EPO peptides described herein, including sequences sharing at least 90% identity with the described sequence. In one embodiment, the nucleic acid sequence is codon optimized for expression in the subject for which administration is desired. In one embodiment, the nucleic acid sequence encoding canine EPO is SEQ ID NO: 7. In one embodiment, the nucleic acid sequence encoding feline EPO is SEQ ID NO: 8.

[00030] When a variant of the EPO peptide is desired, the coding sequences for these peptides may be generated using site-directed mutagenesis of the wild-type nucleic acid sequence. Web-based or commercially available computer programs, as well as service based companies may be used to back translate the amino acids sequences to nucleic acid coding sequences, including both RNA and/or cDNA. *See, e.g.,* backtranseq by EMBOSS, http://www.ebi.ac.uk/Tools/st/ ; Gene Infinity (http://www.geneinfinity.org/sms-/sms_backtranslation.html); ExPasy (http://www.expasy.org/tools/). In one embodiment, the RNA and/or cDNA coding sequences are designed for optimal expression in the subject species

for which administration is ultimately intended, as discussed herein. Thus, in one embodiment, the coding sequences are designed for optimal expression in a feline. In another embodiment, the coding sequences are designed for optimal expression in a canine. In yet another embodiment, the coding sequences are designed for optimal expression in a primate.

[00031] The coding sequences may be designed for optimal expression using codon optimization. Codon-optimized coding regions can be designed by various different methods. This optimization may be performed using methods which are available on-line, published methods, or a company which provides codon optimizing services. One codon optimizing method is described, *e.g.,* in International Patent Publication No. WO 2015/012924, which is incorporated by reference herein. Briefly, the nucleic acid sequence encoding the product is modified with synonymous codon sequences. Suitably, the entire length of the open reading frame (ORF) for the product is modified. However, in some embodiments, only a fragment of the ORF may be altered. By using one of these methods, one can apply the frequencies to any given polypeptide sequence, and produce a nucleic acid fragment of a codon-optimized coding region which encodes the polypeptide.

[00032] The terms "percent (%) identity", "sequence identity", "percent sequence identity", or "percent identical" in the context of nucleic acid sequences refers to the bases in the two sequences which are the same when aligned for correspondence. The length of sequence identity comparison may be over the full-length of the genome, the full-length of a gene coding sequence, or a fragment of at least about 100 to 150 nucleotides, or as desired. However, identity among smaller fragments, *e.g.* of at least about nine nucleotides, usually at least about 20 to 24 nucleotides, at least about 28 to 32 nucleotides, at least about 36 or more nucleotides, may also be desired. Multiple sequence alignment programs are also available for nucleic acid sequences. Examples of such programs include, "Clustal W", "CAP Sequence Assembly", "BLAST", "MAP", and "MEME", which are accessible through Web Servers on the internet. Other sources for such programs are known to those of skill in the art. Alternatively, Vector NTI utilities are also used. There are also a number of algorithms known in the art that can be used to measure nucleotide sequence identity, including those contained in the programs described above. As another example, polynucleotide sequences can be compared using Fasta™, a

program in GCG Version 6.1. Fasta™ provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences. For instance, percent sequence identity between nucleic acid sequences can be determined using Fasta™ with its default parameters (a word size of 6 and the NOPAM factor for the scoring matrix) as provided in GCG Version 6.1, herein incorporated by reference.

[00033] The terms "percent (%) identity", "sequence identity", "percent sequence identity", or "percent identical" in the context of amino acid sequences refers to the residues in the two sequences which are the same when aligned for correspondence. Percent identity may be readily determined for amino acid sequences over the full-length of a protein, polypeptide, about 70 amino acids to about 100 amino acids, or a peptide fragment thereof or the corresponding nucleic acid sequence coding sequencers. A suitable amino acid fragment may be at least about 8 amino acids in length, and may be up to about 150 amino acids. Generally, when referring to "identity", "homology", or "similarity" between two different sequences, "identity", "homology" or "similarity" is determined in reference to "aligned" sequences. "Aligned" sequences or "alignments" refer to multiple nucleic acid sequences or protein (amino acids) sequences, often containing corrections for missing or additional bases or amino acids as compared to a reference sequence. Alignments are performed using any of a variety of publicly or commercially available Multiple Sequence Alignment Programs. Sequence alignment programs are available for amino acid sequences, *e.g.,* the "Clustal X", "MAP", "PIMA", "MSA", "BLOCKMAKER", "MEME", and "Match-Box" programs. Generally, any of these programs are used at default settings, although one of skill in the art can alter these settings as needed. Alternatively, one of skill in the art can utilize another algorithm or computer program which provides at least the level of identity or alignment as that provided by the referenced algorithms and programs. See, *e.g.,* J. D. Thomson et al, Nucl. Acids. Res., "A comprehensive comparison of multiple sequence alignments", 27(13):2682-2690 (1999).

[00034] In one embodiment, the nucleic acid sequences encoding the EPO constructs described herein are engineered into any suitable genetic element, *e.g.,* naked DNA, phage, transposon, cosmid, RNA molecule (*e.g.,* mRNA), episome, *etc.,* which transfers the EPO sequences carried thereon to a host cell, *e.g.,* for generating nanoparticles carrying DNA or

RNA, viral vectors in a packaging host cell and/or for delivery to a host cells in subject. In one embodiment, the genetic element is a plasmid. The selected genetic element may be delivered by any suitable method, including transfection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion. The methods used to make such constructs are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. *See, e.g.,* Green and Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (2012).

[00035] As used herein, an "expression cassette" refers to a nucleic acid molecule which comprises coding sequences for the EPO peptide, promoter, and may include other regulatory sequences therefor, which cassette may be engineered into a genetic element and/or packaged into the capsid of a viral vector (*e.g.,* a viral particle). Typically, such an expression cassette for generating a viral vector contains the EPO construct sequences described herein flanked by packaging signals of the viral genome and other expression control sequences such as those described herein. Any of the expression control sequences can be optimized for a specific species using techniques known in the art including, e.g, codon optimization, as described herein.

[00036] The expression cassette typically contains a promoter sequence as part of the expression control sequences. In one embodiment, a CB7 promoter is used. CB7 is a chicken β-actin promoter with cytomegalovirus enhancer elements. Alternatively, other liver-specific promoters may be used [*see, e.g.,* The Liver Specific Gene Promoter Database, Cold Spring Harbor, http://rulai.schl.edu/LSPD, alpha 1 anti-trypsin (A1AT); human albumin Miyatake et al., J. Virol., 71:5124 32 (1997), humAlb; and hepatitis B virus core promoter, Sandig *et al.,* Gene Ther., 3:1002 9 (1996)]. TTR minimal enhancer/promoter, alpha-antitrypsin promoter, LSP (845 nt)25(requires intron-less scAAV). In one embodiment, the liver-specific promoter thyroxin binding globulin (TBG) is used.Other promoters, such as viral promoters, constitutive promoters, regulatable promoters [*see, e.g.,* WO 2011/126808 and WO 2013/04943], or a promoter responsive to physiologic cues may be used may be utilized in the vectors described herein.

[00037] In addition to a promoter, an expression cassette and/or a vector may contain other appropriate transcription initiation, termination, enhancer sequences, efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; TATA sequences; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.*, Kozak consensus sequence); introns; sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. The expression cassette or vector may contain none, one or more of any of the elements described herein. Examples of suitable polyA sequences include, *e.g.*, SV40, bovine growth hormone (bGH), and TK polyA. Examples of suitable enhancers include, *e.g.*, the CMV enhancer, the RSV enhancer, the alpha fetoprotein enhancer, the TTR minimal promoter/enhancer, LSP (TH-binding globulin promoter/alpha1-microglobulin/bikunin enhancer), amongst others.

[00038] In one embodiment, the viral vector includes a nucleic acid expression cassette comprising: a 5' AAV inverted terminal repeat sequence (ITR), a promoter with optional enhancer, an EPO sequence, a poly A sequence, and a 3' AAV ITR, wherein said expression cassette expresses a functional EPO in a host cell.

[00039] These control sequences are "operably linked" to the EPO construct sequences. As used herein, the term "operably linked" refers to both expression control sequences that are contiguous with the gene of interest and expression control sequences that act *in trans* or at a distance to control the gene of interest.

[00040] The expression cassette may be engineered onto a plasmid which is used for production of a viral vector. The minimal sequences required to package the expression cassette into an AAV viral particle are the AAV 5' and 3' ITRs, which may be of the same AAV origin as the capsid, or of a different AAV origin (to produce an AAV pseudotype). In one embodiment, the ITR sequences from AAV2, or the deleted version thereof (ΔITR), are used for convenience and to accelerate regulatory approval. However, ITRs from other AAV sources may be selected. Where the source of the ITRs is from AAV2 and the AAV capsid is from another AAV source, the resulting vector may be termed pseudotyped. Typically, an expression cassette for an AAV vector comprises an AAV 5' ITR, the propeptide-EPO active peptide coding sequences and any regulatory sequences, and an AAV 3' ITR. However, other

configurations of these elements may be suitable. A shortened version of the 5' ITR, termed ΔITR, has been described in which the D-sequence and terminal resolution site (trs) are deleted. In other embodiments, the full-length AAV 5' and 3' ITRs are used.

[00041] Exemplary plasmids are provided in the sequence listing. SEQ ID NO: 1 provides the sequence of a plasmid encoding a canine EPO construct, entitled pn1044.CB7.caEPO. In one embodiment, the expression cassette is engineered into the plasmid of SEQ ID NO: 1. SEQ ID NO: 2 provides the sequence of a plasmid encoding a feline EPO construct, entitled pn1044.CB7.feEPO. In one embodiment, the expression cassette is engineered into the plasmid of SEQ ID NO: 2. Plasmids, such as those, shown in SEQ ID NO: 1 and SEQ ID NO: 2 may be modified to include one or more additional components as described herein, or to remove or replace components as necessary. In one embodiment, the plasmid has the sequence of SEQ ID NO: 1 or a sequence sharing at least 80% identity therewith. In another embodiment, the plasmid has the sequence of SEQ ID NO: 2 or a sequence sharing at least 80% identity therewith.

[00042] The abbreviation "sc" refers to self-complementary. "Self-complementary AAV" refers a plasmid or vector having an expression cassette in which a coding region carried by a recombinant AAV nucleic acid sequence has been designed to form an intra-molecular double-stranded DNA template. Upon infection, rather than waiting for cell mediated synthesis of the second strand, the two complementary halves of scAAV will associate to form one double stranded DNA (dsDNA) unit that is ready for immediate replication and transcription. *See, e.g.,* D M McCarty *et al*, "Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis", Gene Therapy, (August 2001), Vol 8, Number 16, Pages 1248-1254. Self-complementary AAVs are described in, *e.g.,* U.S. Patent Nos. 6,596,535; 7,125,717; and 7,456,683, each of which is incorporated herein by reference in its entirety.

[00043] An adeno-associated virus (AAV) viral vector is an AAV DNase-resistant particle having an AAV protein capsid into which is packaged nucleic acid sequences for delivery to target cells. An AAV capsid is composed of 60 capsid (cap) protein subunits, VP1, VP2, and VP3, that are arranged in an icosahedral symmetry in a ratio of approximately 1:1:10 to 1:1:20, depending upon the selected AAV. AAV serotypes may be selected as sources for capsids of

AAV viral vectors (DNase resistant viral particles) including, e.g., AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, rh10, AAVrh64R1, AAVrh64R2, rh8, rh.10, variants of any of the known or mentioned AAVs or AAVs yet to be discovered. In one embodiment, the AAV is an AAV8 capsid, or a variant thereof. *See, e.g.,* US Published Patent Application No. 2007-0036760-A1; US Published Patent Application No. 2009-0197338-A1; EP 1310571. *See also*, WO 2003/042397 (AAV7 and other simian AAV), US Patent 7790449 and US Patent 7282199 (AAV8), WO 2005/033321 and US 7,906,111 (AAV9), and WO 2006/110689, and WO 2003/042397 (rh.10). Alternatively, a recombinant AAV based upon any of the recited AAVs, may be used as a source for the AAV capsid. These documents also describe other AAV which may be selected for generating AAV and are incorporated by reference. In some embodiments, an AAV cap for use in the viral vector can be generated by mutagenesis (i.e., by insertions, deletions, or substitutions) of one of the aforementioned AAV Caps or its encoding nucleic acid. In some embodiments, the AAV capsid is chimeric, comprising domains from two or three or four or more of the aforementioned AAV capsid proteins. In some embodiments, the AAV capsid is a mosaic of Vpl, Vp2, and Vp3 monomers from two or three different AAVs or recombinant AAVs. In some embodiments, an rAAV composition comprises more than one of the aforementioned Caps. In another embodiment, the AAV capsid includes variants which may include up to about 10% variation from any described or known AAV capsid sequence. That is, the AAV capsid shares about 90% identity to about 99.9 % identity, about 95% to about 99% identity or about 97% to about 98% identity to an AAV capsid provided herein and/or known in the art. In one embodiment, the AAV capsid shares at least 95% identity with an AAV capsid. When determining the percent identity of an AAV capsid, the comparison may be made over any of the variable proteins (e.g., vp1, vp2, or vp3). In one embodiment, the AAV capsid shares at least 95% identity with the AAV8 vp3. In another embodiment, a self-complementary AAV is used.

[00044] For packaging an expression cassette into virions, the ITRs are the only AAV components required in cis in the same construct as the gene. In one embodiment, the coding sequences for the replication (rep) and/or capsid (cap) are removed from the AAV genome and supplied *in trans* or by a packaging cell line in order to generate the AAV vector. For example,

as described above, a pseudotyped AAV may contain ITRs from a source which differs from the source of the AAV capsid. Additionally or alternatively, a chimeric AAV capsid may be utilized. Still other AAV components may be selected. Sources of such AAV sequences are described herein and may also be isolated or obtained from academic, commercial, or public sources (e.g., the American Type Culture Collection, Manassas, VA). Alternatively, the AAV sequences may be obtained through synthetic or other suitable means by reference to published sequences such as are available in the literature or in databases such as, e.g., GenBank®, PubMed®, or the like.

[00045] Methods for generating and isolating AAV viral vectors suitable for delivery to a subject are known in the art. *See, e.g.,* US Patent 7790449; US Patent 7282199; WO 2003/042397; WO 2005/033321, WO 2006/110689; and US 7588772 B2]. In a one system, a producer cell line is transiently transfected with a construct that encodes the transgene flanked by ITRs and a construct(s) that encodes rep and cap. In a second system, a packaging cell line that stably supplies rep and cap is transiently transfected with a construct encoding the transgene flanked by ITRs. In each of these systems, AAV virions are produced in response to infection with helper adenovirus or herpesvirus, requiring the separation of the rAAVs from contaminating virus. More recently, systems have been developed that do not require infection with helper virus to recover the AAV - the required helper functions (*i.e.,* adenovirus E1, E2a, VA, and E4 or herpesvirus UL5, UL8, UL52, and UL29, and herpesvirus polymerase) are also supplied, *in trans*, by the system. In these newer systems, the helper functions can be supplied by transient transfection of the cells with constructs that encode the required helper functions, or the cells can be engineered to stably contain genes encoding the helper functions, the expression of which can be controlled at the transcriptional or posttranscriptional level. In yet another system, the transgene flanked by ITRs and rep/cap genes are introduced into insect cells by infection with baculovirus-based vectors. For reviews on these production systems, see generally, *e.g.*, Zhang et al., 2009, "Adenovirus-adeno-associated virus hybrid for large-scale recombinant adeno-associated virus production," Human Gene Therapy 20:922-929, the contents of each of which is incorporated herein by reference in its entirety. Methods of making and using these and other AAV production systems are also described in the following U.S. patents, the contents of each of

which is incorporated herein by reference in its entirety: 5,139,941; 5,741,683; 6,057,152; 6,204,059; 6,268,213; 6,491,907; 6,660,514; 6,951,753; 7,094,604; 7,172,893; 7,201,898; 7,229,823; and 7,439,065. *See generally, e.g.*, Grieger & Samulski, 2005, "Adeno-associated virus as a gene therapy vector: Vector development, production and clinical applications," Adv. Biochem. Engin/Biotechnol. 99: 119-145; Buning et al., 2008, "Recent developments in adeno-associated virus vector technology," J. Gene Med. 10:717-733; and the references cited below, each of which is incorporated herein by reference in its entirety. The methods used to construct any embodiment of this invention are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. *See, e.g.,* Green and Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (2012). Similarly, methods of generating rAAV virions are well known and the selection of a suitable method is not a limitation on the present invention. See, e.g., K. Fisher et al, (1993) J. Virol., 70:520-532 and US Patent No. 5,478,745.

[00046] Also provided are compositions which include the viral vector constructs described herein. The pharmaceutical compositions described herein are designed for delivery to subjects in need thereof by any suitable route or a combination of different routes. Direct delivery to the liver (optionally via intravenous, via the hepatic artery, or by transplant), oral, inhalation, intranasal, intratracheal, intraarterial, intraocular, intravenous, intramuscular, subcutaneous, intradermal, and other parental routes of administration. The viral vectors described herein may be delivered in a single composition or multiple compositions. Optionally, two or more different AAV may be delivered, or multiple viruses [*see, e.g.*, WO 2011/126808 and WO 2013/049493]. In another embodiment, multiple viruses may contain different replication-defective viruses (*e.g.,* AAV and adenovirus).

[00047] The replication-defective viruses can be formulated with a physiologically acceptable carrier for use in gene transfer and gene therapy applications. In the case of AAV viral vectors, quantification of the genome copies ("GC") may be used as the measure of the dose contained in the formulation or suspension. Any method known in the art can be used to determine the genome copy (GC) number of the replication-defective virus compositions of the invention. One method for performing AAV GC number titration is as follows: Purified AAV

vector samples are first treated with DNase to eliminate un-encapsidated AAV genome DNA or contaminating plasmid DNA from the production process. The DNase resistant particles are then subjected to heat treatment to release the genome from the capsid. The released genomes are then quantitated by real-time PCR using primer/probe sets targeting specific region of the viral genome (usually poly A signal).

[00048] Also, the replication-defective virus compositions can be formulated in dosage units to contain an amount of replication-defective virus that is in the range of about 1.0×10^9 GC to about 1.0×10^{15} GC. In another embodiment, this amount of viral genome may be delivered in split doses. In one embodiment, the dosage is about 1.0×10^{10} GC to about 1.0×10^{12} GC for an average feline or small canine subject of about 5 kg. In one embodiment, the dosage is about 1.0×10^{11} GC to about 1.0×10^{13} GC for an average medium canine subject of about 20 kg. The average canine ranges from about 5 to about 50 kg in body weight. In one embodiment, the dosage is about 1.0×10^{11} GC to 1.0×10^{13} GC for a subject. In another embodiment, the dose about 3×10^{12} GC. For example, the dose of AAV virus may be about 1×10^{11} GC, about 5×10^{11} GC, about 1×10^{12} GC, about 5×10^{12} GC, or about 1×10^{13} GC. In one embodiment, the dosage is about 3×10^{10} GC/kg. In another example, the constructs may be delivered in an amount of about 0.001 mg to about 10 mg per mL. In one embodiment, the constructs may be delivered in volumes from 1μL to about 100 mL for a veterinary subject. See, e.g., Diehl et al, J. Applied Toxicology, 21:15-23 (2001) for a discussion of good practices for administration of substances to various veterinary animals. This document is incorporated herein by reference. As used herein, the term "dosage" can refer to the total dosage delivered to the subject in the course of treatment, or the amount delivered in a single (of multiple) administration.

[00049] The above-described recombinant vectors may be delivered to host cells according to published methods. The rAAV, preferably suspended in a physiologically compatible carrier, diluent, excipient and/or adjuvant, may be administered to a desired subject including without limitation, a cat, dog, or other non-human mammalian subject. Suitable carriers may be readily selected by one of skill in the art in view of the indication for which the transfer virus is directed. For example, one suitable carrier includes saline, which may be

formulated with a variety of buffering solutions (*e.g.*, phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water. The selection of the carrier is not a limitation of the present invention.

[00050] Optionally, the compositions of the invention may contain, in addition to the rAAV and/or variants and carrier(s), other conventional pharmaceutical ingredients, such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

[00051] The viral vectors and other constructs described herein may be used in preparing a medicament for delivering an EPO construct to a subject in need thereof and/or for treating chronic kidney disease in a subject. Thus, in another aspect a method of treating chronic kidney disease is provided. The method includes administering a composition as described herein to a subject in need thereof. In one embodiment, the composition includes a viral vector containing an EPO expression cassette, as described herein. In one embodiment, the subject is a mammal. In another embodiment, the subject is a feline or canine. In yet another aspect a method of treating anemia is provided. The method includes administering a composition as described herein to a subject in need thereof. In one embodiment, the composition includes a viral vector containing an EPO expression cassette, as described herein. In one embodiment, the subject is a mammal. In another embodiment, the subject is a feline or canine.

[00052] In another embodiment, a method for treating chronic kidney disease in a feline is provided. The method includes administering an AAV viral vector comprising a nucleic acid molecule comprising a sequence encoding feline EPO. In another embodiment, a method for treating chronic kidney disease in a canine is provided. The method includes administering an AAV viral vector comprising a nucleic acid molecule comprising a sequence encoding canine EPO.

[00053] A course of treatment may optionally involve repeat administration of the same viral vector (*e.g.,* an AAV8 vector) or a different viral vector (*e.g.,* an AAV8 and an AAVrh10).

Still other combinations may be selected using the viral vectors described herein. Optionally, the composition described herein may be combined in a regimen involving other drugs or protein-based therapies, including *e.g.,* recombinant EPO. Optionally, the composition described herein may be combined in a regimen involving lifestyle changes including dietary and exercise regimens.

[00054] It is to be noted that the term "a" or "an" refers to one or more. As such, the terms "a" (or "an"), "one or more," and "at least one" are used interchangeably herein.

[00055] The words "comprise", "comprises", and "comprising" are to be interpreted inclusively rather than exclusively. The words "consist", "consisting", and its variants, are to be interpreted exclusively, rather than inclusively. While various embodiments in the specification are presented using "comprising" language, under other circumstances, a related embodiment is also intended to be interpreted and described using "consisting of" or "consisting essentially of" language.

[00056] As used herein, the term "about" means a variability of 10% from the reference given, unless otherwise specified.

[00057] The term "regulation" or variations thereof as used herein refers to the ability of a composition to inhibit one or more components of a biological pathway.

[00058] A "subject" is a mammal, *e.g.*, a human, mouse, rat, guinea pig, dog, cat, horse, cow, pig, or non-human primate, such as a monkey, chimpanzee, baboon or gorilla. As used herein, the term "subject" is used interchangeably with "patient".

[00059] As used herein, "disease", "disorder" and "condition" are used interchangeably, to indicate an abnormal state in a subject.

[00060] Unless defined otherwise in this specification, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art and by reference to published texts, which provide one skilled in the art with a general guide to many of the terms used in the present application.

[00061] The following examples are illustrative only and are not intended to limit the present invention.

Example 1 - Construction of EPO vectors

[00062] The amino acid sequences of canine and feline erythropoietin were obtained from Genbank. The amino acid sequences were backtranslated and codon optimized, followed by addition of a kozac consensus sequence, stop codon, and cloning sites. The sequences were produced by GeneArt, and cloned into an expression vector containing a chicken-beta actin promoter with CMV enhancer (p1044). The expression construct is flanked by AAV2 ITRs. The canine and feline constructs were packaged in an AAV serotype 8 capsid by triple transfection and iodixanol gradient purification and titered by Taqman quantitative PCR.

Example 2 - AAV-mediated expression of feline erythropoietin in cats

[00063] Three cats were treated with a single intramuscular injection of 3×10^{10} genome copies per kilogram body weight (GC/kg) AAV8 expressing feline erythropoietin (Figure 1). Blood samples were collected at the time of injection and periodically thereafter for measurement of hematocrit. Therapeutic phlebotomy was initiated on day 42 after vector injection. To date, the results have shown seen sustained expression of EPO for greater than 100 days.

Example 3 - AAV-mediated expression of canine erythropoietin in dogs

[00064] Three dogs were treated with a single intramuscular injection of 3×10^{10} GC/kg AAV8 expressing canine erythropoietin. Blood samples were collected at the time of injection and periodically thereafter for measurement of hematocrit (Figure 2). Blood samples from untreated littermates were included as controls. Therapeutic phlebotomy was initiated on day 60 after vector injection.

Example 4 - Dosage study of AAV-mediated expression of feline erythropoietin in cats

[00065] Cats were treated with a single intramuscular injection of up to 3×10^{7}, 3×10^{8}, 3×10^{9}, or 3×10^{10} genome copies per kilogram body weight (GC/kg) of AAV8 expressing feline

erythropoietin. These three cohorts were of 4 cats per dosage. All cats were normal/wildtype and selected randomly. The purpose of this study was show long term safety and efficacy to highlight a possible clinical candidate for a client owned animal study. FIG. 4.

Example 5 - AAV-mediated expression of feline erythropoietin in cats

[00066] Cats are treated with a single intramuscular injection of up to 3 x 10^9 genome copies per kilogram body weight (GC/kg) AAV8 expressing feline erythropoietin in the left or right quadriceps in a total volume of up to 400μL. Blood samples are collected at the time of injection and periodically thereafter for measurement of hematocrit. Vector may be readministered 28 days or more post the initial vector administration using the same criteria.

[00067] This study will include up to nine cats with anemia related to stage III chronic kidney disease. CKD related anemia will be defined as a hematocrit less than 29% on two occasions at least one month apart and without another apparent cause for the anemia. Enrolled subjects will receive a single intramuscular injection of an adenoassociated virus vector carrying a feline erythropoietin transgene (AAV8.fEpo). Subjects will be evaluated at the study site at the time of vector administration and at 2, 4, 6, and 8 weeks after administration. At each visit blood will be collected for evaluation of erythropoietin concentration, hematocrit, reticulocyte count, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration. Patients will return to the study site or follow up with their primary veterinarian at 3 months, 6 months and 12 months after study drug administration for measurement of hematocrit. The study drug has previously been found to be safe in 4 normal cats at doses up to 3E8 genome copies/kg. The first three subjects enrolled in this trial will receive a dose of 1E8 genome copies/kg AAV8.fEpo. An initial evaluation of safety and vector activity will take place after the first three subjects have reached 8 weeks post vector administration. Depending on the results at the 8 week analysis in this initial cohort of 3 animals, dosing of the second cohort of three animals will proceed using the following scheme:

1. If any severe adverse events occur in the initial cohort or if any animal reaches a hematocrit 55%, the dose will be reduced threefold and three additional animals will be enrolled at this reduced dose. (total study enrollment of 6 subjects)

2. If there are no adverse events and all cats demonstrate an increase in hematocrit of at least 5% or reach the normal hematocrit range, 3 additional cats will be treated at the starting dose. (total study enrollment of 6 subjects)
3. If there are no adverse events and all cats do not achieve at least a 5% increase in hematocrit or reach the normal range, 3 additional cats will be treated at a 3fold higher dose of 3E8 genome copies/kg. There will again be an interim evaluation of safety and activity after all three animals in this cohort have reached 6 weeks post vector administration. If there are no adverse events and all cats demonstrate an increase in hematocrit of at least 5% or reach the normal hematocrit range, 3 additional cats will be treated at this dose. If there are no adverse events and all cats do not achieve at least a 5% increase in hematocrit or reach the normal range, 3 additional cats will be treated at a dose of 6E8 genome copies/kg. (total study enrollment of 9 subjects) This study will therefore enroll at least 6 and as many as 9 subjects depending on the results of interim analyses of safety and change in hematocrit. Primary endpoints include safety and evaluation of vector expression
Secondary endpoints include quality of life for animals and long-term sustained expression of vector
Inclusion criteria:
Cats with stage III renal failure (serum creatinine 2.95 mg/dL)
Hematocrit 29% on two occasions at least one month apart
Owner willing to return to study site for visits at 2 weeks, 4 weeks, 6 weeks, and 8 weeks after study drug administration, and to study site or primary veterinary clinic at 3 months, 6 months and 12 months after study drug administration.
Exclusion criteria:
Anticipated life expectancy less than 3 months
Kidney transplant
Past treatment with recombinant erythropoietin (epoetin, darbepoietin)
Any other condition that in the opinion of the principle investigator would preclude evaluation of the safety and activity of the study drug

Preexisting neutralizing antibodies to AAV8

Eligible cats will be screened during first visit. This will include a full history and clinical exam, CBC/chemistry, and preexisting antibodies to AAV8, and full release on consent form.

All eligible cats that agree to terms of research protocol will receive a single intramuscular injection of AAV8.fEPO of up to 3E9 genome copies/kg at least one week after being accepted into study.

Following vector administration, cats will be evaluated every other week for a duration of 8 weeks. These clinical check ups will include CBC retic/chem, full clinical evaluation, and serum collection. After 8 weeks, these clinical evaluations will move to every 3 months, starting on day 90 after vector administration. The 3 month, 6 month, 9 month, and 1 year evaluations will be done.

Possible Complications

Any animal that displays polycythemia of 65% Hematocrit will be given a therapeutic phlebotomy of up to 10% blood volume per every 3 weeks.

[00068] All publications cited in this specification, as well as provisional patent application nos. 62/212,144 and 62/336,211 are incorporated herein by reference. Similarly, the SEQ ID NOs which are referenced herein and which appear in the appended Sequence Listing are incorporated by reference. While the invention has been described with reference to particular embodiments, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A recombinant adeno-associated virus (rAAV) comprising an AAV capsid having packaged therein a vector genome, wherein said vector genome comprises nucleic acid sequences encoding feline erythropoietin (EPO), inverted terminal repeat sequences and expression control sequences that direct expression of the EPO in a host cell.

2. A recombinant adeno-associated virus (rAAV) comprising an AAV capsid having packaged therein a vector genome, wherein said vector genome comprises nucleic acid sequences encoding canine erythropoietin (EPO), inverted terminal repeat sequences and expression control sequences that direct expression of the EPO in a host cell.

3. The rAAV of claim 1 or 2, wherein the EPO sequence encodes a full length EPO protein.

4. The rAAV of claim 1 or 2, wherein the EPO sequence encodes the mature EPO protein in combination with a heterologous leader sequence.

5. The rAAV of claim 1, wherein the EPO sequence encodes the protein sequence of SEQ ID NO: 4.

6. The rAAV of claim 2, wherein the EPO sequence encodes the protein sequence of SEQ ID NO: 3.

7. The rAAV of claim 1, wherein the EPO sequence comprises the nucleic acid sequence of SEQ ID NO: 6 or a variant thereof.

8. The rAAV of claim 7, wherein the variant is a codon optimized variant of SEQ ID NO: 6.

9. The rAAV of claim 8, wherein codon optimized variant is SEQ ID NO: 8.

10. The rAAV of claim 2, wherein the EPO sequence comprises the nucleic acid sequence of SEQ ID NO: 5 or a variant thereof.

11. The rAAV of claim 10, wherein the variant is a codon optimized variant of SEQ ID NO: 5.

12. The rAAV of claim 11, wherein codon optimized variant is SEQ ID NO: 7.

13. The rAAV of claim 1 or 2, wherein the expression control sequences comprise a promoter.

14. The rAAV of claim 13, wherein the promoter is a CB7 promoter.

15. The rAAV of claim 13, wherein the promoter is a TBG promoter.

16. The rAAV of claim 13, wherein the expression control sequences comprise a tissue-specific promoter.

17. The rAAV of claim 16, wherein the tissue-specific promoter is a kidney-specific promoter.

18. The rAAV of claim 16, wherein the tissue-specific promoter is selected from a Nkcc2 promoter, uromodulin promoter, Ksp-cadherin promoter and THP gene promoter.

19. The rAAV of claim 1 or claim 2, further comprising one or more of an intron, a Kozak sequence, a polyA, and post-transcriptional regulatory elements.

21. The recombinant vector according to claim 1 or claim 2, wherein the AAV capsid is selected from AAV8, rh64R1, AAV9, AAVhu.37, or rh10 and variants thereof.

22. The viral vector of claim 1 or claim 2, wherein the capsid is an AAV8 capsid.

23. The viral vector of claim 1 or claim 2, further comprising AAV inverted terminal repeat sequences.

24. A recombinant adeno-associated virus (rAAV) comprising an AAV8 capsid having packaged therein a vector genome, wherein said vector genome comprises nucleic acid sequences encoding feline erythropoietin (EPO), inverted terminal repeat sequences and expression control sequences that direct expression of EPO in a host cell.

25. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a viral vector according to any one of claims 1 to 24.

26. A method for treating chronic kidney disease, said method comprising administering a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a viral vector according to claim 1 or claim 2.

27. The method according to claim 26, wherein said composition is administered intravenously.

28. The method according to claim 26, wherein said subject is feline or canine.

29. The method according to claim 26, wherein said subject is feline and the EPO is a feline sequence.

30. The method according to claim 26, wherein said composition is administered in combination with another therapy.

31. The method according to claim 27, wherein said composition is administered at a dosage of about $3x10^{10}$ GC/kg.

32. The method according to claim 28, wherein said composition is administered more than once.

33. Use of a recombinant AAV according to any one of claims 1 to 24 in the use of treating chronic kidney disease.

34. An rAAV according to any one of claims 1 to 24 suitable for use in treating chronic kidney disease.

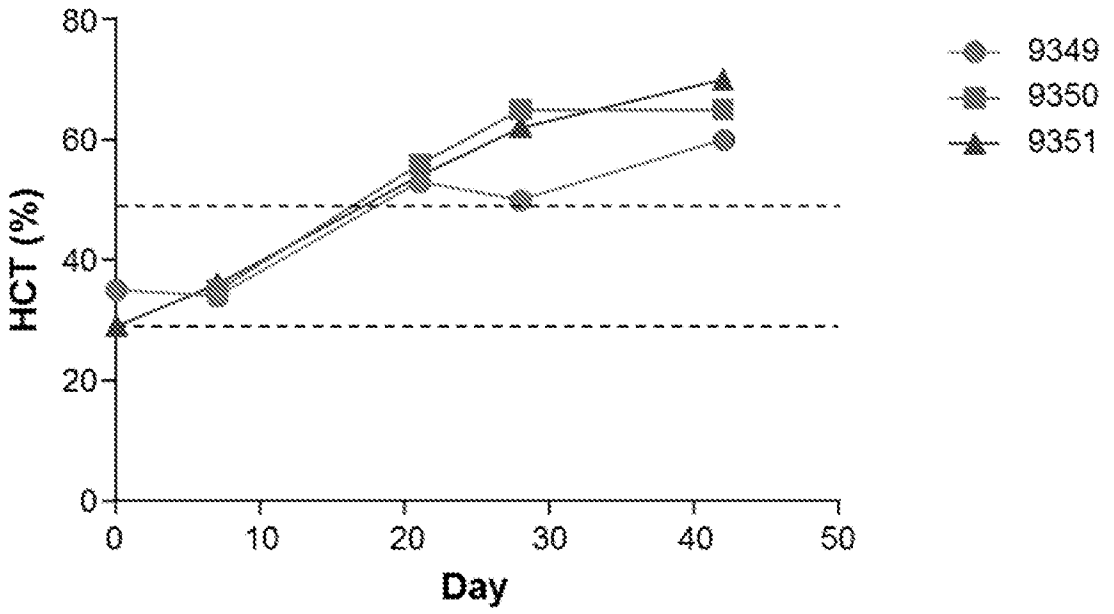

80
60
40
20
0
HCT (%)
0
10
20
30
40
50
Day
9349
9350
9351

FIG. 1.

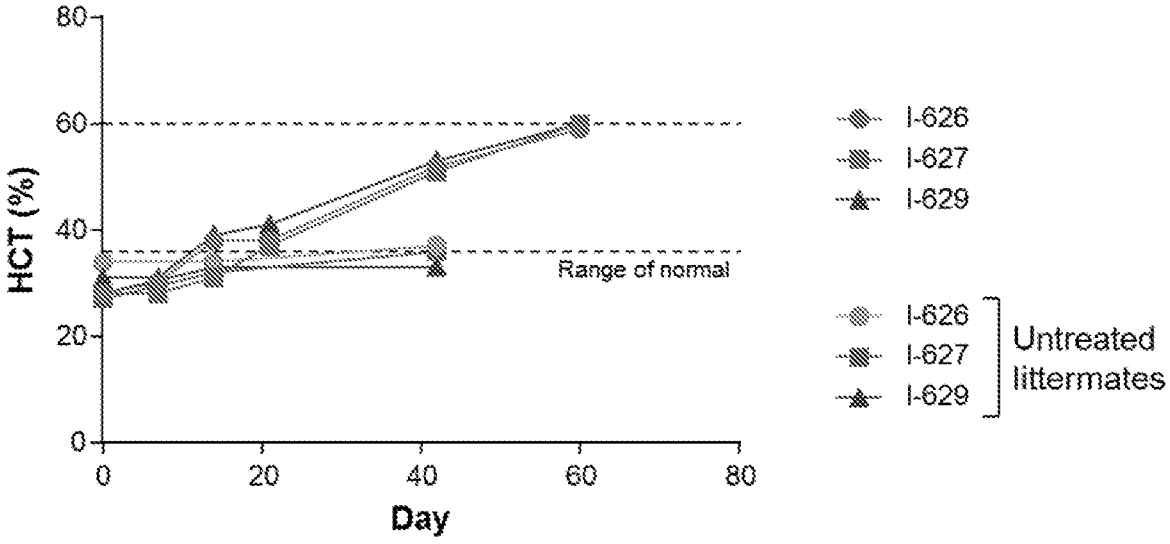

80
60
40
20
0
HCT (%)
0
20
40
60
80
Day
Range of normal
I-626
I-627
I-629
I-626
I-627
I-629
Untreated littermates

FIG. 2.

FIG. 3A

SEQ ID NO: 3 CANINE EPO

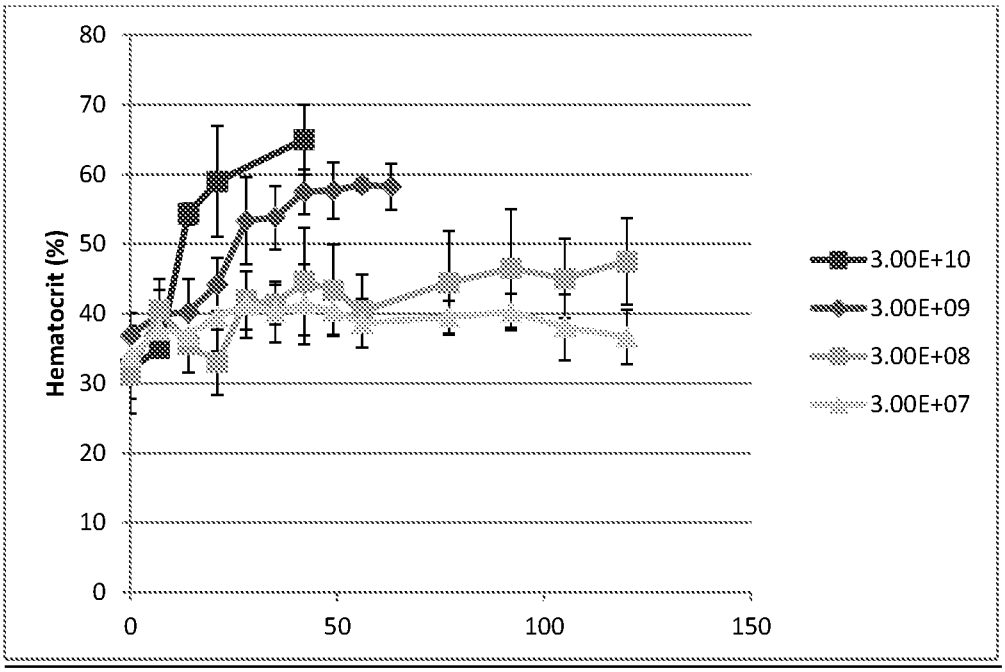
MCEPAPPKPTQSAWHSFPECPALLLLLSLLLLPLGLPVLGAPPRLICDSRVLERYILE
AREAENVTMGCAQGCSFSENITVPDTKVNFYTWKRMDVGQQALEVWQGLALLSE
AILRGQALLANASQPSETPQLHVDKAVSSLRSLTSLLRALGAQKEAMSLPEEASPA
PLRTFTVDTLCKLFRIYSNFLRGKLTLYTGEACRRGDR

FIG. 3B

SEQ ID NO: 4 FELINE EPO

MGSCECPALLLLLSLLLLPLGLPVLGAPPRLICDSRVLERYILEAREAENVTMGCAE
GCSFSENITVPDTKVNFYTWKRMDVGQQAVEVWQGLALLSEAILRGQALLANSSQ
PSETLQLHVDKAVSSLRSLTSLLRALGAQKEATSLPEATSAAPLRTFTVDTLCKLFRI
YSNFLRGKLTLYTGEACRRGDR

FIG 4.


80
70
60
50
40
30
20
10
0
Hematocrit (%)
0
50
100
150
3.00E+10
3.00E+09
3.00E+08
3.00E+07

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/49487

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 14/505, C12N 15/10, A61K 38/00, A01K 21/00, A01K 29/00, A61K 9/19 (2016.01)
CPC - C07K 14/505, C12N 15/102, A61K 38/00, A61K 38/1816, A61K 47/48215, A01K 21/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C07K 14/505, C12N 15/10, A61K 38/00, A01K 21/00, A01K 29/00, A61K 9/19 (2016.01)
CPC - C07K 14/505, C12N 15/102, A61K 38/00, A61K 38/1816, A61K 47/48215, A01K 21/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC(8) - A61K 31/713, C07K 16/26 (2016.01); CPC - A61K 31/713, A01K 29/00, A61K 9/19, C07K 16/26
USPC- 514/44R, 426/2, 435/320.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); PatBase, Google/Scholar: Feline erythropoietin, cat EPO, fEPO, feEpo, promoter, CB7, CB-7; AAV8, AAV-8; tissue specific
GenCore 6.4.1: SEQ ID NO: 3, 4, 6, 8

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	Beall, et al. Transfer of the feline erythropoietin gene to cats using a recombinant adeno-associated virus vector. Gene Ther. 2000, 7(6):534-9; Abstract, pg 534, col 2; pg 535, Figure 1 and its legend; pg 537, Table 2; pg 538, col 2	1, (3,4,13,19,23)/1 ---------- 5, 7, 8, (14,16)/(13/1), (21,22)/1, 24
Y	GenBank Submission U00685.1. Felis domesticus erythropoietin mRNA, complete cds (1994) [Retrieved from the Internet 08 January 2017: <https://www.ncbi.nlm.nih.gov/nuccore/392793>]; pg 1, nucleotide sequence, 100% identity to SEQ ID NO: 6	7, 8
Y	US 2015/0230430 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 20 August 2015 (20.08.2015) Abstract, para [0019], [0070], [0087]	16/(13/1), (21,22)/1, 24
Y	US 2012/0058102 A1 (Wilson, et al.) 08 March 2012 (08.03.2012) Abstract, para [0384]	14/(13/1)
Y	WO 2010/036964 A2 AMBRX INC. 01 April 2010 (01.04.2010) pg 161, Example 2; Table 2, SEQ ID NO 1, 100% identity to SEQ ID NO: 4	5
A	WO 2014/182684 A2 (CELL MACHINES INC.) 13 November 2014 (13.11.2014) SEQ ID NO: 19, nucleotides 4-579, 73.6% identity to SEQ ID NO:8	9

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 January 2017	03 FEB 2017

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 25, 33, 34
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

********** See Supplemental Sheet to continue **********

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, (3-4)/1, 5, 7-9, 13/1, (14,16)/(13/1), (19, 21-23)/1, 24, restricted to feline EPO, SEQ ID NO:4, 6, 8 and CB7 promoter

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

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********** Supplemental Sheet **********

In Continuation of Box III. Observations where unity of invention is lacking:

Group I+: claims 1-19, 21-24, directed to a rAAV comprising an AAV capsid having packaged therein a vector genome. The rAAV will be searched to the extent that the EPO encompasses feline EPO of SEQ ID NO:4, and is encoded by SEQ ID NO:6 or 8 and encompasses CB7 promoter. It is believed that claims 1, (3-4)/1, 5, 7-9, 13/1, (14,16)/(13/1), (19, 21-23)/1, 24 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass a rAAV comprising an AAV capsid having packaged therein a vector genome comprising a nucleic acid sequences SEQ ID NO:6 or 8, said nucleic acid sequences encoding feline EPO of SEQ ID NO:4 and comprising CB7 promoter, and a method of using said rAAV. Additional kind of EPO encoded by another nucleic acid and/or additional promoter(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected kind of EPO encoded by another nucleic acid and/or additional promoter(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a rAAV comprising a nucleic acid of SEQ ID NO: 5 or 7, said nucleic acid encoding canine EPO of SEQ ID NO:3, and comprises a Nkcc2 promoter, and a method of using said rAAV, i.e, claims 2, (3-4)/2, 6, 10-12, 13/2, 16/13/2, (17-18)/16/13/2, (19, 21-23)/2.

Group II+: claims 26-32, directed to a method of treating chronic kidney disease. Group II+ will be searched upon payment of additional fees. The method may be searched, for example, to the extent that the EPO encompasses feline EPO for an additional fee and election as such, i.e., claims 26/1, (27-32)/(26/1). Additional method encompassing use of an additional EPO will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected EPO. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. Another exemplary election would be a method comprising the use of canine EPO, i.e., claims 26/2, (27,28, 30-32)/(26/2).

The inventions listed as Group I+ and II+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features
The special technical feature of each invention of Groups I+ and II+ is a specific kind of EPO recited therein.

The special technical feature of some inventions of Group I+ is a specific amino acid sequence and/or specific nucleic acid sequence encoding thereof.

The special technical feature of some inventions of Group I+ is a specific kind of a tissue specific promoter recited therein.

Common Technical Features
The inventions of Groups I+ and II+ share the technical feature of a rAAV comprising an AAV capsid having packaged therein a vector genome, wherein said vector genome comprises nucleic acid sequences encoding EPO, inverted terminal repeat sequences and expression control sequences that direct expression of the EPO in a host cell. However, this shared technical feature does not represent a contribution over prior art as being anticipated by a paper titled "Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein" by Kessler, et al. (Proc Natl Acad Sci USA 1996, 93(24):14082-7) (hereinafter "Kessler"). Kessler discloses a rAAV comprising an AAV capsid having packaged therein a vector genome (pg 14083, col 1, Vector Encapsidation, pg 14086, Table 2. Epo expression and hematocrit: AAV-Epo dose-response, "Values representing means +/- SD; EPO,... .v. injection was 3 x 10.exp.11 of encapsidated vector"), wherein said vector genome comprises nucleic acid sequences encoding EPO, inverted terminal repeat sequences and expression control sequences that direct expression of the EPO in a host cell (pg 14083, FIG. 1 and its legend, pAAV-Epo comprising ITR and CMV promoter, "(B) pAAV-Epo. Epo, human Epo coding sequence; An, SV40 polyadenylylation signal; noncoding, 2.2-kb noncoding fragment from the lacZ gene"). As said technical feature was known in the art at the time of the invention, this cannot be considered special technical feature that would otherwise unify the inventions.

Some inventions of Groups I+ and II+ share the technical feature of a rAAV of claim 1. The inventions of Group II+ share the technical feature of a method for treating chronic kidney disease by administering said rAAV. However, these shared technical features do not represent a contribution over prior art as being anticipated by a paper titled "Transfer of the feline erythropoietin gene to cats using a recombinant adeno-associated virus vector" by Beall, et al. (Gene Ther. 2000, 7(6):534-9) (hereinafter "Beall").
Beall discloses a rAA V (Abstract, "we have developed a recombinant an rAAV vector containing the feline Epo gene (rAAV/feEpo) for the express purpose of treating the anemia of chronic renal failure in cats") comprising an AA V capsid having packaged therein a vector genome (pg 538, col 2, "The pTP-feEpo plasmid was transfected into HeLa cells... 51 cell lines were screened for rAAV synthesis by induction and measurement of packaged virus in crude lysates. Titers of rAAV particles were determined..."), wherein said vector genome comprises nucleic acid sequences encoding feline EPO, inverted terminal repeat sequences and expression control sequences that direct expression of the EPO in a host cell (pg 535, Figure 1 and its legend, "Map of the tripartite cat Epo rAAV production plasmid. The various sequence elements are referenced as follows: rep/cap, AAV-2-derived rep and cap genes; ITR, inverted terminal repeat sequences; CMVIE, human cytomegalovirus immediate early promoter; C.I., chimeric intron sequence from SV40; Epo, feline Epo coding sequence; SV40 pA, SV40 polyadenylation sequence; neoR, neomycin resistance gene").

Beall also discloses a method for treating chronic kidney disease (Abstract, "the rAAV/feEpo vector holds promise as a simple, safe and effective therapy for the anemia of chronic renal failure in domestic cats"), said method comprising administering a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a viral vector according to claim 1 (pg 538, col 2, last para to pg 539, col 1, "Injection of rAAV-feEpo into cat skeletal muscle Specific pathogen-free (SPF) cats were purchased f... Six females were anesthetized with 15-25 mg/kg ketamine HCl intramuscularly (Fort Dodge, Overland Park, KS, USA). Each animal was injected in the right semitendenosus/semi-mebranosus muscle with varying doses of rAAV/feEpo contained in 0.6 ml of sterile normal saline"). As said technical features were known in the art at the time of the invention, these cannot be considered special technical feature that would otherwise unify the inventions.

********** See the Following Supplemental Sheet to continue **********

INTERNATIONAL SEARCH REPORT

International application No.
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In Continuation of Box III. Observations where unity of invention is lacking and the Preceding Supplemental Sheet:

Some inventions of Group I+ and II+ share the technical feature of a rAAV of claim 2. The inventions of Group II+ share the technical feature of a method for treating chronic kidney disease by administering said rAAV. However, these shared technical features do not represent a contribution over prior art as being obvious over a paper titled "Clinical efficacy and safety of recombinant canine erythropoietin in dogs with anemia of chronic renal failure and dogs with recombinant human erythropoietin-induced red cell aplasia" by Randolph, et al. (J Vet Intern Med. 2004, 18(1):81-91) (hereinafter "Randolph") in view of a paper titled "Human erythropoietin gene therapy for patients with chronic renal failure" by Lippin, et al. (Blood 2005, 106(7):2280-6) (hereinafter "Lippin").

Randolph discloses recombinant canine erythropoietin (Abstract, "recombinant canine erythropoietin (rcEPO) therapy was evaluated in 19 dogs with anemia of chronic renal failure (group 1) and 6 dogs with chronic renal failure and recombinant human erythropoietin (rhEPO)-induced red cell aplasia (group 2)") and its use for treating chronic kidney disease (Abstract, "For group 1 dogs, median Hct and ARC increased significantly during the 1st week of rcEPO treatment, and median Hct was sustained at .35% after week 5... Appetite and energy level improved in most group 1 dogs with increases in Hct. Recombinant cEPO stimulated erythrocyte production in dogs with nonregenerative anemia secondary to chronic renal failure without causing the profound erythroid hypoplasia that can occur in rhEPO-treated dogs").
Randolph does not disclose a recombinant adeno-associated virus (rAAV) comprising an AAV capsid having packaged therein a vector genome.
Lippin discloses a recombinant adeno-associated virus (rAAV) comprising an AAV capsid having packaged therein a vector genome, wherein said vector genome comprises nucleic acid sequences encoding EPO and expression control sequences that direct expression of the EPO in a host cell (pg 2283, col 1, "Clinical-grade adenovector. To construct and produce the clinical-grade Ad-MG/EPO-1 (Ad5 E1/E3 deleted), the human EPO cDNA was inserted into the pAd-lox shuttle vector9 containing the cytomegalovirus (CMV) promoter and simian virus-40 (SV40) polyA site ... The Ad-MG/EPO-1 used to manufacture the clinical-grade material was... the infection was initiated with a predetermined multiplicity of infection, infection, and harvest times using the Ad-MG/EPO-1 Master Viral Bank... The particle count of the production lot, by OD260 was 1.45 x 10.exp.12 particles/mL, the viral titer was 1.6 x 10.exp.9 plaque-forming units (pfu)/mL").
It would have been obvious to one of ordinary skill in the art to combine, in the course of routine experimentation and with a reasonable expectation of success, Randolph and Lippin by substituting nucleic acid encoding human EPO in the rAAV disclosed by Lippin for canince EPO disclosed by Randolph, to be used for the treatment of chronic kidney disease as in Randolph (Abstract). The motivation to do so is provided by Lippin (pg 2280, col 2, "The treatment of the anemia of kidney disease with recombinant human EPO (hEPO) has been successfully carried out for more than a decade. However, the short half-life of the currently available recombinant products, their potential to cause antibodies against hEPO, and their high cost provide an incentive to significantly improve care in this area with the use of a genetic therapy approach"). As said technical feature s would have been obvious to one of ordinary skill in the art at the time of the invention, these cannot be considered special technical feature that would otherwise unify the inventions.

Finally, some inventions of Group I+ share the technical feature of a tissue specific promoter. However, this shared technical feature does not represent a contribution over prior art because tissue specific promoters were known in the art at the time of the invention (please see US 2012/0058102 A1 to Wilson, et al. (08 March 2012), para [0072], "Examples of tissue-specific promoters suitable for use in the present invention include, but are not limited to those listed in Table 1 and other tissue-specific promoters known in the art").

The inventions of Groups I+ and II+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

摘要

本公开提供了用于治疗伴侣动物的组合物和方法。提供了腺相关病毒载体，其包括含有编码促红细胞生成素(EPO)的序列的核酸分子。在所需的实施方案中，受试者是猫或狗。