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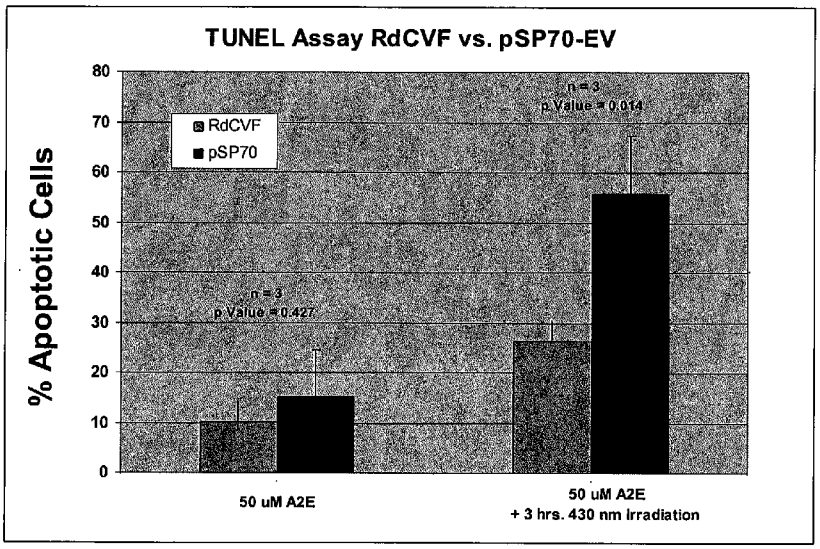
(54) **Title**: METHODS TO PRODUCE ROD-DERIVED CONE VIABILITY FACTOR (RDCVF)

FIGURE 18



(57) **Abstract**: The invention is related to methods of producing rod-derived cone viability factor (RdCVF). This invention also relates to the treatment of an ocular disease in a mammal using RdCVF. Also provided are expression vectors for high secreted expression of RdCVF of using nucleotide sequences encoding heterologous signal proteins and optionally markers for furin cleavage.

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METHODS TO PRODUCE ROD-DERIVED CONE VIABILITY FACTOR (RDCVF)

Technical Field

[0001] This invention relates to methods of producing rod-derived cone viability factor (RdCVF) using engineered cells. This invention also relates to the treatment of an ocular disease in a mammal using RdCVF. The invention further concerns the cells and the expression vectors that are used to secrete RdCVF.

Background

[0002] Retinitis pigmentosa is an untreatable, inherited retinal disease that leads to blindness. The disease initiates with the loss of night vision due to rod photoreceptor degeneration, followed by irreversible progressive loss of cone photoreceptors. Although loss of rods has substantial functional consequences such as night blindness and some constriction of visual fields, rod death by itself does not cause severe vision loss. Rather, it is the delayed wave of non-autonomous cone photoreceptor degeneration that results in blindness.

[0003] It has previously been shown that factors secreted from rods are essential for cone viability. One such trophic factor, RdCVF, has been identified (see U.S. Pub. No. 2004/0204350, Chalmel et al., BMC Molecular Biology 2007, 8:74; Leveillard et al., 2004, Nature Genetics, 36: 755-759; and Leveillard et al. at http://www.hal.inserm.fr/docs/00/31/23/78/DOC/Manuscript_on_April_15.doc). The feasibility of delivering RdCVF to the retina of a mouse model of retinitis pigmentosa (rd1) using recombinant AAV vectors has been evaluated. *In vivo* results suggests that efficient delivery of vector derived RdCVF protein to target photoreceptor cells requires novel secreted versions of the protein by the addition of specific signal peptides. Therapeutic applications of RdCVF have been explored, e.g., see WO 03/066810A2, EP 2027889A1, WO 2005/113586A2, Purification of secreted RdCVF and subsequent biochemical analysis confirms that RdCVF(LF) has anti-oxidative properties.

[0004] Recombinant expression of RdCVF at a large scale has been challenging. Effective expression of RdCVF from a gene therapy vector has also been challenging. Without being limited as to theory, it is theorized that post-translational events are largely responsible for the less than optimal levels of protein expression in either recombinant protein expression systems or in expression from a gene therapy vector. Following translation, proteins are routed specifically to secretory or endocytic pathways in the endoplasmic reticulum and traverse the Golgi network. Secreted proteins comprise a signal peptide, which is recognized during translation by the signal recognition pathway. Subsequently, the signal peptide is cleaved by a membrane – bound signal peptidase and the mature secreted protein enters a secretory vesicle. Thus secreted proteins are destined for the secretory pathway by recognition of their N-terminal signal peptides. The presently disclosed invention seeks to improve RdCVF expression profiles, including improving RdCVF secretion from cells both *in vitro* and *in vivo*.

Summary of the Invention

[0005] In one aspect, the invention relates to an expression vector for expression of rod-derived cone viability factor (RdCVF) comprising a) a RdCVF-encoding nucleotide sequence lacking the initiating methionine coding sequence; b) an exogenous signal peptide-encoding nucleotide sequence positioned upstream of the RdCVF-encoding nucleotide sequence, wherein the exogenous signal peptide-encoding sequence is selected from the group consisting of: human growth hormone (hGH), brain-derived neurotrophic factor (BDNF), insulin growth factor-1 (IGF-1), or β-glucoronidase (GUSB).

[0006] In one aspect, the invention relates to an expression vector for expression of rod-derived cone viability factor (RdCVF) comprising a) a RdCVF-encoding nucleotide sequence; b) a signal peptide-encoding nucleotide sequence positioned upstream of the RdCVF-encoding nucleotide sequence, wherein the signal peptide-encoding sequence encodes an exogenous signal peptide; and c) a nucleotide sequence encoding a furin cleavage marker located downstream of the signal peptide-encoding nucleotide sequence and upstream of the RdCVF-encoding nucleotide sequence.

[0007] The signal peptide-encoding sequence and the furin cleavage marker sequence according to some embodiments may be separated by a sequence encoding an amino acid linker.

[0008] In one embodiment, the RdCVF-encoding nucleotide sequence lacks the initiating methionine coding sequence.

[0009] The signal peptide–encoding sequence may be that of human growth hormone (hGH), brain-derived neurotrophioc factor (BDNF), insulin growth factor-1 (IGF-1), or β-glucoronidase (GUSB).

[0010] In one aspect, the invention relates to an expression vector for high secreted expression of rod-derived cone viability factor (RdCVF) in a cell, said vector comprising a) a signal peptide-encoding nucleotide sequence of a heterologous secreted protein upstream of the N-terminus of a RdCVF-encoding nucleotide sequence, wherein the heterologous secreted protein is human growth hormone (hGH), brain-derived neurotrophic factor (BDNF), Insulin growth factor-1 (IGF-1), or β-glucoronidase (GUSB); b) a RdCVF-encoding nucleotide sequence; and c) optionally a nucleotide sequence encoding for a subtilisin-like serine endoprotease furin cleavage marker located downstream of the signal peptide-encoding nucleotide sequence of (a) and immediately upstream of the sequence encoding the initiating methionine of RdCVF, wherein the signal peptide-encoding sequence (a) and sequence encoding the for a subtilisin-like serine endoprotease furin cleavage marker (c) are separated by a sequence encoding an amino acid linker.

[0011] Another aspect of the invention is a method of treating an ocular disease in a mammal using RdCVF. The method comprises administering to a diseased eye of the mammal the chimeric RdCVF fusion protein of the invention at a therapeutically effective amount.

[0012] In another aspect, is a method of treating an ocular disease in a mammal using RdCVF wherein the method comprises administering to a diseased eye of the mammal a nucleic acid encoding the RdCVF fusion protein of the invention, where the RdCVF is expressed at a therapeutically effective amount. In certain aspects, the nucleic acid is delivered via a recombinant viral vector, such as an adeno-associated virus (AAV) or an adenovirus (Ad). The viral vector comprises the nucleic acid encoding for rod-derived cone viability factor (RdCVF) wherein the nucleic acid comprises a) a signal peptide-encoding nucleotide sequence of a heterologous secreted protein upstream of the N-terminus of a RdCVF-encoding nucleotide sequence, wherein the heterologous secreted protein is human growth hormone (hGH), brain-derived neurotrophic factor (BDNF), Insulin growth factor-1 (IGF-1), or β-glucoronidase (GUSB); b) a RdCVF-encoding nucleotide sequence; and c) optionally a nucleotide sequence encoding for a subtilisin-like serine endoprotease furin cleavage marker located downstream of

the signal peptide-encoding nucleotide sequence of (a) and immediately upstream of the sequence encoding the initiating methionine of RdCVF, wherein the signal peptide-encoding sequence (a) and sequence encoding the for a subtilisin-like serine endoprotease furin cleavage marker (c) are separated by a sequence encoding an amino acid linker. The viral vector may be injected into the eye to produce RdCVF protein intra-ocularly.

[0013] In another aspect, the invention relates to a method for producing a recombinant rod-derived cone viability factor (RdCVF) in the eye, said method comprising injecting a recombinant viral vector, e.g AAV or Ad, into the eye, wherein ocular cells are transduced and produce RdCVF. The produced RdCVF may be secreted into the vitreal humor and provide therapeutic effects. The virus may be injected into a subject with retinitis pigmentosa, wherein the secreted RdCVF provides therapeutic effects.

[0014] In some embodiments, the cell is a mammalian cell and said mammalian cell is a CHO cell or a COS cell. In some embodiments, the amino acid linker is between 10 and 25 amino acids long. In some embodiments, the amino acid linker is MPLESGLSSEDSASSESFA (SEQ ID NO. 1). In some embodiments, the RdCVF-encoding sequence encodes the long or short form of RdCVF. In some embodiments the heterologous protein is HGH, BDNF, IGF-1 or GUSB. In some embodiments, the nucleotide sequence encoding for a subtilisin-like serine endoprotease furin cleavage marker is a sequence encoding the amino acid sequence KRIKR (SEQ ID NO. 2).

[0015] In another aspect, the invention relates to a mammalian cell producing recombinant RdCVF, comprising a vector disclosed herein.

[0016] In a another aspect, the invention relates to a method for high secreted expression of rod-derived cone viability factor (RdCVF) in a mammalian cell, said method comprises culturing a cell comprising a chimeric protein expression vector disclosed herein.

[0017] In one embodiment, the mammalian cell is a CHO cell or a COS cell.

[0018] In a another aspect, the invention relates to a method for producing a recombinant rod-derived cone viability factor (RdCVF), said method comprising growing a cell disclosed herein and isolating the protein produced.

Brief Description of the Drawings

[0019] Figure 1 show Western blots of cells transfected with plasmid encoding for RdCVF and signal peptides according to one embodiment. Immunoblot of cell lysates (Sup) and cell pellet (Pellet) following transfection with plasmid encoding cDNA for RdCVF (LF) with signal peptides from BDNF, GUSB, IGF1 or HGH are indicated in the figure. The immunoblot is probed with an antibody generated against RdCVF (LF). This demonstrates inclusion of a signal peptide in the RdCVF sequence results in successful secretion of RdCVF from cells, as evidenced by the presence of RdCVF in cell supernatants "sup". The pellet fraction represents the fraction of RdCVF that remains intracellular.

[0020] Figure 2 shows SDS-PAGE analysis of purified RdCVF protein (with affinity tag) produced either in CHO or Drosophila cells according to one embodiment. The immunoblot is probed with an antibody against the affinity tag.

[0021] Figure 3 shows absorbance measurements at 595nm as a function of time indicating oxidoreductase activity of RdCVF according to one embodiment. In this assay, insulin polypeptide chains that are normally held together by a disulfide bond are reduced in the presence of either RdCVF(LF) or Thioredoxin protein. Reduced insulin forms a precipitate which is measured as an increase in absorbance at 595 nm RdCVF(LF) has measurable oxidoreductase activity as shown here.

[0022] Figure 4 shows expressed protein samples of pGZ6 RdCVF SF and pGZ6 RdCVF LF by HPC4 Western analysis according to one embodiment.

[0023] Figure 5 shows the nucleic acid sequence (A) and the amino acid sequence (B) of the GUSB signal peptide-furin- hRdCVF Long Form (LF) according to one embodiment.

[0024] Figure 6 shows the nucleic acid sequence (A) and the amino acid sequence (B) of the BDNF signal peptide-furin- hRdCVF Long Form (LF) according to one embodiment.

[0025] Figure 7 shows the nucleic acid sequence (A) and the amino acid sequence (B) of the IGF-1 signal peptide-furin- hRdCVF Long Form (LF) according to one embodiment.

[0026] Figure 8 shows the nucleic acid sequence (A) and the amino acid sequence (B) of the GUSB signal peptide-furin- hRdCVF Short Form (SF) according to one embodiment.

[0027] Figure 9 shows the nucleic acid sequence (A) and the amino acid sequence (B) of the BDNF signal peptide-furin- hRdCVF Short Form (SF) according to one embodiment.

[0028] Figure 10 shows the nucleic acid sequence (A) and the amino acid sequence (B) of the IGF1 signal peptide-furin- hRdCVF Short Form (SF) according to one embodiment.

[0029] Figure 11 shows the nucleic acid sequence (A) and the amino acid sequence (B) of the hGH signal peptide hRdCVF Long Form (LF) according to one embodiment. The predicted amino acid sequence of secreted hRdCVF (LF) is shown, following signal peptidase cleavage of HGH signal peptide is depicted below.

[0030] Figure 12 shows the nucleic acid sequence (A) and the amino acid sequence (B) of the hGH signal peptide hRdCVF Short Form (SF) according to one embodiment. The predicted amino acid sequence of secreted hRdCVF (SF) is shown, following signal peptidase cleavage of HGH signal peptide is depicted below.

[0031] Figure 13 shows the A. nucleic acid sequence and the B. amino acid sequence of the hGH signal peptide hRdCVF (LF) with HPC4 tag according to one embodiment. The predicted amino acid sequence of secreted hRdCVF (Long Form) HPC4 tag is shown, following signal peptidase cleavage of HGH signal peptide is depicted below. The twelve amino acid HPC4 tag is depicted by underline.

[0032] Figure 14 shows the nucleic acid sequence (A) and the amino acid sequence (B) of the hGH signal peptide hRdCVF (SF) with HPC4 tag according to one embodiment. The predicted amino acid sequence of secreted hRdCVF (Short Form) HPC4 tag is shown, following signal peptidase cleavage of HGH signal peptide is depicted below. The sequence for the HPC4 tag is underlined.

[0033] Figure 15 shows an example of RdCVF protein sequence according to one embodiment.

[0034] Figure 16 shows an example of RdCVF nucleotide sequence according to one embodiment.

[0035] Figure 17 shows example signal peptides according to some embodiments.

[0036] Figure 18 shows a TUNEL assay result according to one embodiment.

Detailed Description of the Invention

[0037] The instant invention provides an expression system that provides improved secretion of recombinantly expressed RdCVF, which is poorly secreted in its native form. The invention provides a chimeric protein that can be generated by adding the signal peptide from efficiently secreted proteins to the N terminus of the poorly secreted protein. The resulting chimeric protein will be secreted via the classical secretory pathway. Preferably the chimeric protein further comprises a cleavable linker for separating the signal peptide from the recombinant RdCVF.

[0038] The RdCVF gene encodes two products through alternative splicing. The short form RdCVF is a truncated thioredoxin (txn)-like protein specifically expressed by photoreceptors which has been shown to promote cone survival. An alternatively spliced long form of RdCVF is encoding a longer protein with a C-terminal extension and could have oxidoreductase activity. Similar to Txn, RdCVF might be a new example of a bifunctional protein, with the long form having a putative thiol-oxydoreductase activity and the short form having trophic activity for cones but no redox activity. Polypeptide sequences of human RdCVF and nucleic acid sequences encoding such human RdCVF have been reported. Examples of such polypeptide

sequences include, but are not limited to, those disclosed in Figure 15, or homologue or a portion thereof. Examples of such nucleic acid sequences include, but are not limited to, those disclosed in Figure 16, or homologue or a portion thereof.

[0039] Insertion of a signal peptide upstream of the initiating methionine in the chimeric protein often results in inefficient recognition of the signal sequence by its cognate signal peptidase. To overcome this limitation, the codon for the initiating methionine from the cDNA sequence of the chimeric protein may be removed; this generates a final secreted protein product that lacks an initiating methionine. Alternatively, the signal sequence for the subtilisin-like serine endoprotease furin can be inserted downstream of the signal peptide but immediately upstream of the initiating methionine of the chimeric protein. Furin which resides in the trans Golgi network cleaves specifically at the C terminal side of the consensus sequence Arg-X-Lys/Arg-Arg ↓ - (R X (K/R)R) (SEQ ID NO:3) in the trans Golgi network (TGN). Using this approach the secreted chimeric protein still retains its initiating methionine following processing by both the relevant signal peptidase in the ER and the furin endoprotease in the TGN.

[0040] As used herein, the singular form "a", "an", and "the" includes plural references unless indicated otherwise. For example, "a" target cell includes one or more target cells.

[0041] The convention for writing peptide sequences is to put the C-terminal end on the right and write the sequence from N- to C-terminus. Reference to the C terminal is defined as the end of the amino acid chain terminated typically, but not necessarily, by a carboxyl group. Reference to the N terminal is defined as the end of the amino acid chain terminated typically, but not necessarily, by an amino group.

[0042] "Amino acid" or "amino acid sequence" as used herein, include references to oligopeptide, peptide, polypeptide, or protein sequence, or to a fragment, portion, or subunit of any of these, and to naturally occurring or synthetic molecules. The terms "polypeptide" and "protein" include amino acids joined to each other by peptide bonds or modified peptide bonds, *i.e.*, peptide isosteres, and may contain modified amino acids other than the 20 gene-encoded amino acids. The term "polypeptide" also includes peptides and polypeptide fragments, motifs and the like.

[0043] The terms "polypeptide," "peptide," and "protein" are used interchangeably to refer to polymers of amino acids of any length. Peptides and polypeptides 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.

[0044] The terms "transformation" and "transfection" or "transduction" refer to intracellular introduction of a nucleic acid. A nucleic acid can be introduced into a plant or an animal cell or a prokaryotic or eukaryotic cell by a number of methods known in the art or described herein.

[0045] The term "isolated" refers to a deoxyribonucleic acid, a ribonucleic acid, or a nucleic acid analog having a polynucleotide sequence that is separated from other nucleic acid sequences in such a way that does not naturally occur. An isolated nucleic acid encompasses nucleic acids that may be partially or wholly chemically or recombinantly synthesized and/or purified by standard techniques known in the art.

[0046] The term "variant" refers to a nucleotide sequence that is substantially similar, over the entire length, to another nucleotide sequence. The term "substantially similar" when used herein with respect to a nucleotide sequence means a nucleotide sequence corresponding to a reference nucleotide sequence, wherein the corresponding sequence encodes a polypeptide having substantially the same structure and function as the polypeptide encoded by the reference nucleotide sequence, e.g. where only changes in amino acids not affecting the polypeptide function occur. In some embodiments, the expression vector comprises variants of the nucleotide sequences disclosed herein.

[0047] "Heterologous protein" as used herein, is a protein that is not normally produced by or required for viability of the host organism. This term contemplates the functional insertion of DNA encoding such protein, via recombinant DNA technology, into an expression vehicle. Non-limiting examples of heterologous proteins of this invention include hGH, BDNF, IGF-1, GUSB.

[0048] The term "vector" refers to viral or non-viral, prokaryotic or eukaryotic, deoxyribonucleic acid, ribonucleic acid or a nucleic acid analog, that is capable of carrying

another nucleic acid. A vector may either carry a nucleic acid into a cell, referred to as "host cell", so that all or a part of the nucleic acid is transcribed or expressed. Alternatively, a vector may be used in an *in vitro* transcription assay. Vectors are frequently assembled as composites of elements derived from different viral, bacterial, or mammalian genes. Vectors contain various coding and non-coding sequences including sequences coding for selectable markers (e. g., an antibiotic resistance gene), sequences that facilitate their propagation in bacteria, or one or more transcription units that are expressed only in certain cell types. For example, mammalian expression vectors often contain both prokaryotic sequences that facilitate the propagation of the vector in bacteria and one or more eukaryotic transcription units that are expressed only in eukaryotic cells. It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc.

[0049] Vectors include, for example, plasmids, phagemids, and viral vectors. Vectors that have an existing promoter can be modified by standard recombinant DNA techniques known in the art to replace the promoter with any promoter sequence variation. In general, suitable vectors can either be chosen from those that are commercially available or they can be constructed using standard recombinant DNA techniques known in the art. (See, e. g., Molecular Cloning : A Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press.) The vector comprises DNA that encodes for the chimeric RdCVF protein construct. The chimeric RdCVF protein may or may not contain the initiating methionine of the RdCVF protein. A gene delivery vehicle is any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are liposomes, biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; metal particles; and bacteria, viruses, such as baculovirus, adeno-associated virus, adenovirus and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

[0050] Gene delivery, gene transfer, and the like as used herein, are terms referring to the introduction of an exogenous polynucleotide (sometimes referred to as a "transgene") into a host

cell, irrespective of the method used for the introduction. Such methods include a variety of well-known techniques such as vector-mediated gene transfer (by, *e.g.*, viral infection/transfection, or various other protein-based or lipid-based gene delivery complexes) as well as techniques facilitating the delivery of "naked" polynucleotides (such as electroporation, "gene gun" delivery and various other techniques used for the introduction of polynucleotides). The introduced polynucleotide may be stably or transiently maintained in the host cell. Stable maintenance typically requires that the introduced polynucleotide either contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosomal replicon (*e.g.*, a plasmid) or a nuclear or mitochondrial chromosome. A number of vectors are known to be capable of mediating transfer of genes to mammalian cells, as is known in the art and described herein.

[0051] The exogenous polynucleotide is inserted into a vector such as adenovirus, adeno-associated virus (AAV), retrovirus, lentivirus, naked plasmid, plasmid/liposome complex, etc. for delivery to the host via intravenous, intramuscular, intraportal or other route of administration. Expression vectors which can be used in the methods and compositions of the present invention include, for example, viral vectors. One of the most frequently used methods of administration of gene therapy, both *in vivo* and *ex vivo*, is the use of viral vectors for delivery of the gene. Many species of virus are known, and many have been studied for gene therapy purposes. The most commonly used viral vectors include those derived from adenoviruses, adeno-associated viruses (AAV) and retroviruses, including lentiviruses, such as human immunodeficiency virus (HIV). In aspects where the vector is a DNA viral vector, such as an adenovirus (Ad) or adeno-associated virus (AAV), a vector construct refers to the polynucleotide comprising the viral genome or part thereof, and a transgene comprising DNA encoding for the chimeric RdCVF protein construct of the instant invention. Adenoviruses (Ads) are a relatively well characterized, homogenous group of viruses, including over 50 serotypes. *See*, e.g., International PCT Application No. WO 95/27071. Ads are easy to grow and do not require integration into the host cell genome. Recombinant Ad derived vectors, particularly those that reduce the potential for recombination and generation of wild-type virus, have also been constructed. *See*, International PCT Application Nos. WO 95/00655 and WO 95/11984.

[0052] Adenovirus is a non-enveloped, nuclear DNA virus with a genome of about 36 kb, which has been well-characterized through studies in classical genetics and molecular biology (Hurwitz, M.S., Adenoviruses *Virology*, 3rd edition, Fields *et al.*, eds., Raven Press, New York, 1996; Hitt, M.M. *et al.*, Adenovirus Vectors, *The Development of Human Gene Therapy*, Friedman, T. ed., Cold Spring Harbor Laboratory Press, New York 1999). The viral genes are classified into early (designated E1-E4) and late (designated L1-L5) transcriptional units, referring to the generation of two temporal classes of viral proteins. The demarcation of these events is viral DNA replication. The human adenoviruses are divided into numerous serotypes (approximately 47, numbered accordingly and classified into 6 groups: A, B, C, D, E and F), based upon properties including hemaglutination of red blood cells, oncogenicity, DNA and protein amino acid compositions and homologies, and antigenic relationships.

[0053] Recombinant adenoviral vectors have several advantages for use as gene delivery vehicles, including tropism for both dividing and non-dividing cells, minimal pathogenic potential, ability to replicate to high titer for preparation of vector stocks, and the potential to carry large inserts (Berkner, K.L., *Curr. Top. Micro. Immunol.* 158:39-66, 1992; Jolly, D., *Cancer Gene Therapy* 1:51-64 1994). Adenoviral vectors with deletions of various adenoviral gene sequences, such as pseudoadenoviral vectors (PAVs) and partially-deleted adenoviral (termed "DeAd"), have been designed to take advantage of the desirable features of adenovirus which render it a suitable vehicle for delivery of nucleic acids to recipient cells.

[0054] AAV vectors are derived from single-stranded (ss) DNA parvoviruses that are nonpathogenic for mammals (reviewed in Muzyscka (1992) Curr. Top. Microb. Immunol., 158:97-129). Briefly, AAV-based vectors have the rep and cap viral genes that account for 96% of the viral genome removed, leaving the two flanking 145-basepair (bp) inverted terminal repeats (ITRs), which are used to initiate viral DNA replication, packaging and integration. In the absence of helper virus, wild-type AAV integrates into the human host-cell genome with preferential site-specificity at chromosome 19q 13.3 or it may remain expressed episomally. A single AAV particle can accommodate up to 5 kb of ssDNA, therefore leaving about 4.5 kb for a transgene and regulatory elements, which is typically sufficient. However, trans-splicing systems as described, for example, in United States Patent No. 6,544,785, may nearly double this limit.

[0055] In the methods of the invention, AAV of any serotype can be used. The serotype of the viral vector used in certain embodiments of the invention is selected from the group consisting from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, and AAV8 (see, e.g., Gao et al. (2002) PNAS, 99:11854-11859; and Viral Vectors for Gene Therapy: Methods and Protocols, ed. Machida, Humana Press, 2003). Other serotype besides those listed herein can be used. Furthermore, pseudotyped AAV vectors may also be utilized in the methods described herein. Pseudotyped AAV vectors are those which contain the genome of one AAV serotype in the capsid of a second AAV serotype; for example, an AAV vector that contains the AAV2 capsid and the AAV1 genome or an AAV vector that contains the AAV5 capsid and the AAV 2 genome (Auricchio et al., (2001) Hum. Mol. Genet., 10(26):3075-81).

[0056] In an illustrative embodiment, AAV is AAV2 or AAV1. Adeno-associated virus of many serotypes, especially AAV2, have been extensively studied and characterized as gene therapy vectors. Those skilled in the art will be familiar with the preparation of functional AAV-based gene therapy vectors. Numerous references to various methods of AAV production, purification and preparation for administration to human subjects can be found in the extensive body of published literature (see, e.g., Viral Vectors for Gene Therapy: Methods and Protocols, ed. Machida, Humana Press, 2003). The terms "genome particles (gp)," or "genome equivalents," or "genome copies" (gc) as used in reference to a viral titer, refer to the number of virions containing the recombinant AAV DNA genome, regardless of infectivity or functionality. The number of genome particles in a particular vector preparation can be measured by procedures such as described in the Examples herein, or for example, in Clark et al. (1999) Hum. Gene Ther., 10:1031-1039; Veldwijk et al. (2002) Mol. Ther., 6:272-278. The terms "infection unit (iu)," "infectious particle," or "replication unit," as used in reference to a viral titer, refer to the number of infectious and replication-competent recombinant AAV vector particles as measured by the infectious center assay, also known as replication center assay, as described, for example, in McLaughlin et al. (1988) J. Virol., 62:1963-1973. The term "transducing unit (tu)" as used in reference to a viral titer, refers to the number of infectious recombinant AAV vector particles that result in the production of a functional transgene product as measured in functional assays such as described in Examples herein, or for example, in Xiao et al. (1997) Exp. Neurobiol., 144:113-124; or in Fisher et al. (1996) J. Virol., 70:520-532 (LFU

assay). The term "dnase resistant particles (drp)" as used in reference to a viral titer, refers to the number of recombinant AAV vector particles that are resistant to DNase treatment. It is typically measured using DNA dot-blot hybridization as known in the art.

[0057] Retrovirus vectors are a common tool for gene delivery (Miller, *Nature* (1992) 357:455-460). The ability of retrovirus vectors to deliver an unrearranged, single copy gene into a broad range of rodent, primate and human somatic cells makes retroviral vectors well suited for transferring genes to a cell. Retroviruses are RNA viruses wherein the viral genome is RNA. When a host cell is infected with a retrovirus, the genomic RNA is reverse transcribed into a DNA intermediate which is integrated very efficiently into the chromosomal DNA of infected cells. This integrated DNA intermediate is referred to as a provirus. Transcription of the provirus and assembly into infectious virus occurs in the presence of an appropriate helper virus or in a cell line containing appropriate sequences enabling encapsidation without coincident production of a contaminating helper virus. A helper virus is not required for the production of the recombinant retrovirus if the sequences for encapsidation are provided by co-transfection with appropriate vectors.

[0058] The retroviral genome and the proviral DNA have three genes: the gag, the pol, and the env, which are flanked by two long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (matrix, capsid, and nucleocapsid) proteins; the pol gene encodes the RNA-directed DNA polymerase (reverse transcriptase) and the env gene encodes viral envelope glycoproteins. The 5' and 3' LTRs serve to promote transcription and polyadenylation of the virion RNAs. The LTR contains all other cis-acting sequences necessary for viral replication. Lentiviruses have additional genes including vit vpr, tat, rev, vpu, nef, and vpx (in HIV-1, HIV-2 and/or SIV). Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the Psi site). If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the result is a cis defect which prevents encapsidation of genomic RNA. However, the resulting mutant is still capable of directing the synthesis of all varion proteins.

[0059] Lentiviruses are complex retroviruses which, in addition to the common retroviral genes gag, pol and env, contain other genes with regulatory or structural function. The higher complexity enables the lentivirus to modulate the life cycle thereof, as in the course of latent infection. A typical lentivirus is the human immunodeficiency virus (HIV), the etiologic agent of AIDS. *In vivo*, HIV can infect terminally differentiated cells that rarely divide, such as lymphocytes and macrophages. *In vitro*, HIV can infect primary cultures of monocyte-derived macrophages (MDM) as well as HeLa-Cd4 or T lymphoid cells arrested in the cell cycle by treatment with aphidicolin or gamma irradiation. Infection of cells is dependent on the active nuclear import of HIV preintegration complexes through the nuclear pores of the target cells. That occurs by the interaction of multiple, partly redundant, molecular determinants in the complex with the nuclear import machinery of the target cell. Identified determinants include a functional nuclear localization signal (NLS) in the gag matrix (MA) protein, the karyophilic virion-associated protein, vpr, and a C-terminal phosphotyrosine residue in the gag MA protein. The use of retroviruses for gene therapy is described, for example, in United States Patent 6,013,516; and U.S. Patent 5,994,136, the disclosures of which are hereby incorporated herein by reference.

[0060] Other methods for delivery of DNA to cells do not use viruses for delivery. For example, cationic amphiphilic compounds can be used to deliver the nucleic acid of the present invention. Because compounds designed to facilitate intracellular delivery of biologically active molecules must interact with both non-polar and polar environments (in or on, for example, the plasma membrane, tissue fluids, compartments within the cell, and the biologically active molecular itself), such compounds are designed typically to contain both polar and non-polar domains. Compounds having both such domains may be termed amphiphiles, and many lipids and synthetic lipids that have been disclosed for use in facilitating such intracellular delivery (whether for *in vitro* or *in vivo* application) meet this definition. One particularly important class of such amphiphiles is the cationic amphiphiles. In general, cationic amphiphiles have polar groups that are capable of being positively charged at or around physiological pH, and this property is understood in the art to be important in defining how the amphiphiles interact with the many types of biologically active (therapeutic) molecules including, for example, negatively charged polynucleotides such as DNA.

[0061] The use of compositions comprising cationic amphiphilic compounds for gene delivery is described, for example, in United States Patent 5,049,386; US 5,279,833; US 5,650,096; US 5,747,471; US 5,767,099; US 5,910,487; US 5,719,131; US 5,840,710; US 5,783,565; US 5,925,628; US 5,912,239; US 5,942,634; US 5,948,925; US 6,022,874; U.S. 5,994,317; U.S. 5,861,397; U.S. 5,952,916; U.S. 5,948,767; U.S. 5,939,401; and U.S. 5,935,936, the disclosures of which are hereby incorporated herein by reference.

[0062] In addition, nucleic acid of the present invention can be delivered using "naked DNA." Methods for delivering a non-infectious, non-integrating DNA sequence encoding a desired polypeptide or peptide operably linked to a promoter, free from association with transfection-facilitating proteins, viral particles, liposomal formulations, charged lipids and calcium phosphate precipitating agents are described in U.S. Patent 5,580,859; U.S. 5,963,622; U.S. 5,910,488; the disclosures of which are hereby incorporated herein by reference.

[0063] Gene transfer systems that combine viral and nonviral components have also been reported. Cristiano *et al.,* (1993) *Proc. Natl. Acad. Sci. USA* 90:11548; Wu *et al.* (1994) *J. Biol. Chem.* 269:11542; Wagner *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6099; Yoshimura *et al.* (1993) *J. Biol. Chem.* 268:2300; Curiel *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8850; Kupfer *et al.* (1994) *Human Gene Ther.* 5:1437; and Gottschalk *et al.*(1994) *Gene Ther.* 1:185. In most cases, adenovirus has been incorporated into the gene delivery systems to take advantage of its endosomolytic properties. The reported combinations of viral and nonviral components generally involve either covalent attachment of the adenovirus to a gene delivery complex or co-internalization of unbound adenovirus with cationic lipid: DNA complexes.

[0064] Signal peptides, as used herein, are peptide chains that direct post-translational transport of a protein. Signal peptides may also be referred to as targeting signals, signal sequences, transit peptides or localizing signals. Signal peptides are involved in directing proteins to particular organelles such as the nucleus, mitochondrial matrix, endoplasmic reticulum, chloroplast, apoplast and peroxixome.

[0065] The structure of a typical Signal peptide includes three distinct regions: (i) an N-terminal region (n-region) that contains a number of positively charged amino acids (lysines and

arginines); (ii) a central hydrophobic core region (h-region); (iii) a hydrophilic cleavage region (c-region) that contains the sequence motif recognized by the signal peptidase. (von Heijne,G. (1983) Eur. J. Biochem., 133, 17–21; von Heijne,G. (1985) J. Mol. Biol., 184, 99–105; von Heijne,G. (1997) Protein Engineering (10), pp1-6). The (-3, -1) rule states that residues at -3 and -1 must be small and neutral for cleavage to occur efficiently (vonHeijne, 1997). Figure 17 demonstrate the structures of various of types of signal peptides. Common examples of proteins with signal peptides include BMP7,BMP2,CNTF, BDNF, GDNF, HGF, VEGF, LIF.

[0066] Some signal peptides are cleaved from the protein by signal peptidase after the proteins are transported. Non-limiting examples of signal peptides include those present in secreted proteins bovine growth hormone, bovine proalbumin, human proinsulin, human interferon-γ, human α-fibrinogen, human IgG heavy chain, rat amylase, murine α-fetoprotein, chicken lysozyme, *Zea mays* rein protein 22.1. In some embodiments, preferred non-limiting examples of signal peptides include those present in secreted proteins human growth hormone (HGH), brain derived neurotrophic factor (BDNF), insulin growth factor 1 (IGF1), and β-glucoronidase (GUSB).

[0067] In some embodiments of the invention, the signal peptide nucleotide sequence exists at or near the N terminal or the C terminal of a protein. In preferred embodiments, the signal peptide exists at or near the N terminal of the protein. In preferred embodiments, the signal peptide directs transit of the protein to secretory pathways and, in particular, to the endoplasmic reticulum (ER). In some embodiments, the signal peptide associates or binds to ER bound receptors, or signal recognition particles in the cytosol. In some embodiments, the signal peptide facilitates protein transport from the cytoplasm to destinations outside the cell. In some embodiments, the signal peptide sequence is selected from a signal peptide sequence from efficiently secreted proteins. Signal peptide sequences may be selected from naturally occurring, signal peptide sequences, derivatives thereof, or a synthetic designed sequence. In some embodiments, non-limiting parameters for a designed signal peptide sequences include a sequence of preferably 3-40 residues, comprising a 3- to 20- residue hydrophobic core flanked by several relatively hydrophilic residues.

[0068] In some embodiments, the signal peptide sequence lacks a hydrophobic core. Non-limiting examples of mammalian secretory proteins that lack a typical hydrophobic signal sequence include human IL-1α, IL1β, bFGF, aFGF, PDEGF, anticoagulant protein, lectin L-14, ATL-derived factor, Factor XIIIa, Anchorin CII, lipocortin I, parathymosin, α-prothymosin, and rodent transglutaminase, parathymosin and MDGI.

[0069] In some embodiments, the signal peptide-encoding nucleotide sequence encodes a signal peptide disclosed herein.

[0070] Furin catalyses a biochemical reaction, the proteolytic maturation of proprotein substrates in the secretory pathway. Furin is subtilisin-like serine endoprotease and known as a proprotein convertase. It is expressed in cells and tissues, mainly localized in the trans-Golgi network. A typical consensus site that furin cleaves is positioned after the carboxy-terminal arginine (Arg) residue in the sequence –Arg–Xaa–(Lys/Arg)–Arg$^{\downarrow}$– (where Lys is lysine, Xaa is any amino acid and $^{\downarrow}$ identifies the cleavage site) (SEQ ID NO:3). In some embodiments, the nucleotide sequence encoding for the furin cleavage marker is a sequence encoding the amino acid sequence of –Xaa–Arg–Xaa–(Lys/Arg)–Arg$^{\downarrow}$– (where Xaa is any amino acid and $^{\downarrow}$ identifies the cleavage site) (SEQ ID NO:4). In some embodiments, the nucleotide sequence encoding for the furin cleavage marker is a sequence encoding the amino acid sequence of –Arg–Xaa–(Lys/Arg)–Arg$^{\downarrow}$– (where X is any amino acid and $^{\downarrow}$ identifies the cleavage site) (SEQ ID NO:3). In some preferred embodiments, the nucleotide sequence encoding for the furin cleavage marker is a sequence encoding the amino acid sequence of KRIKR (SEQ ID NO:2).

[0071] In some embodiments, the expression vector optionally comprises a sequence encoding for an amino acid linker. In some embodiments the expression vector encodes for an amino acid linker wherein the amino acid linker separates the signal peptide sequence from the furin cleavage marker. In some preferred embodiments, the signal peptide-encoding sequence and the sequence encoding the furin cleavage marker are separated by a sequence encoding an amino acid linker. In some embodiments the expression vector may encode an amino acid linker comprised of 2-50 amino acids. In some embodiments the amino acid linker is comprised of 10-25 acids. In preferred embodiments the amino acid linker is comprised of 15-20 amino acids. In even more preferred embodiments, the amino acid linker is comprised of the sequence

MPLESGLSSEDSASSESFA (SEQ ID NO:1), although variations of the length and amino acid choice may also be included.

[0072] In some embodiments, the expression vector disclosed herein, contains, in addition, a nucleotide sequence encoding an amino acid tag that enables the purification of the RdCVF protein using affinity chromatography. In some embodiments, the location of the nucleotide sequence encoding the amino acid tag is located at the C terminus. For example, the amino acid tag is EDQVDPRLIDGK (SEQ ID NO:5), a sequence from protein C that encodes the epitope for a monoclonal antibody HPC4. Inclusion of this peptide epitope enables the purification of the RdCVF protein using HPC4 affinity chromatography.

[0073] "Promoter" refers to a regulatory element that directs the transcription of a nucleic acid to which it is operably linked. A promoter used to control the transcription of the recombinant proteins herein may be chosen from any of those which are functional in the host cell. Non-limiting examples of promoters include SV40 early promoter, adenovirus major late promoter, herpes simplex (HSV) thymidine kinase promoter, rous sarcoma (RSV) LTR promoter, human cytomegalovirus (CMV) immediate early promoter, mouse mammary tumor virus (MMTV) LTR promoter, interferon-β promoter, heat shock protein 70 (hsp 70) promoter, as well as many others well known in the art. In preferred embodiments, the promoter is SV40 early promoter or the 3 kb hamster β-actin promoter.

[0074] "Host cells" as used herein, refers to a cell that contains heterologous DNA that has been introduced into the cell by any means. Non-limiting examples of means for introduction of DNA into the cell include electroporation, calcium phosphate precipitation, microinjection, transformation, viral infection, DEAE-Dextran transfection, liposome-mediated transfection, transduction using viral vectors and the like.

[0075] The invention further provides host cells that have been transfected with a vector of the invention disclosed herein. Such a host cell can be a prokaryotic cell or a eukaryotic cell. Host cells can either be cells in culture or be present in an animal.

[0076] Non-limiting example of non-mammalian cell line includes insect and Drosophila cells. Non-limiting examples of mammalian cell lines include 3T3 cells, COS cells, human osteosarcoma cells, MRC-5 cells, BHK cells, VERO cells, CHO cells, rCHO-tPA cells, rCHO-Hep B Surface Antigen cells, HEK 293 cells, rHEK 293 cells, rC127-Hep B Surface Antigen cells, Normal Human fibroblast cells, Stroma cells, Hepatocyte cells, PER.C6 cells and human permanent amniocytic cells. Examples of host cells in culture include, but are not limited to, HeLa cells, CHO cells, NSO, HEK cells, BHK cells, NIH-3T3, MDCK cells, and COS cells. Host cells in culture can be grown either in suspension or on microcarriers. In some embodiments, host cells are chosen from cells that are capable of secreting the RdCVF protein, or derivative thereof, into the surrounding medium, so that the expressed protein according to the invention can be directly purified from the media. In a preferred embodiment, the host cells are mammalian cells COS or CHO cells.

[0077] Many suitable expression systems can be employed for the production of proteins using vectors of the invention. The expression of the protein may be stable or transient expression. The introduced polynucleotide may be stably or transiently maintained in the host cell. Stable maintenance typically requires that the introduced polynucleotide either contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosomal replicon (*e.g.*, a plasmid) or a nuclear or mitochondrial chromosome. Transient expression comprises introduction of the DNA encoding for the chimeric RdCVF protein into the cell wherein the DNA does not contains an origin of replication compatible with the host cell or does not integrate into a replicon or chromosome of the host cell but rather is maintained episomally. One particular expression system employs a dihydrofolate reductase (DHFR) gene which is introduced into a vector of the invention or a variant thereof. Alternatively, an expression vector expressing DHFR can be co-transfected into the host cell, if a DHFR-deficient cell is used for expression. When increasing concentrations of methotrexate (MTX), a competitive inhibitor of the essential enzyme DHFR, are applied to the transfected cells, only cells with higher expression levels of DHFR survive. As MTX levels are increased further, only cells which amplify the copy number of the DHFR gene survive. In this way, by increasing the copy number of the vector, increased expression of the heterologous nucleic acid can be achieved, thereby leading to increased protein production. A second expression system employs a glutamin synthetase (GS) gene that is introduced into a vector of

the invention or a variant thereof. Addition of a competitive inhibitor of GS, e. g., methionine sulphoximine (MSX), is used for increasing the copy number of the vector leading to increased protein production. Any suitable prokaryotic or eukaryotic expression system can be used for expression of proteins using vectors of the invention.

[0078] "Secretion" as used herein means exportation of product through the plasma membrane and into the medium supporting the cell culture. In some embodiments, preferred products of this invention are any proteins expressed by the vectors disclosed herein. In some preferred embodiments, non-limiting examples of products are rod-derived cone viability factor (RdCVF) or derivatives thereof.

[0079] The protein of the present invention can be produced by cultivating transformants, especially mammalian cells, as mentioned above and allowing them to secrete the protein into the culture supernatant.

[0080] A culture filtrate (supernatant) may be obtained by a method such as filtration or centrifugation of the obtained culture, and the protein of the present invention is purified and isolated from the culture filtrate by methods commonly used in order to purify and isolate a natural or synthetic protein. Non-limiting examples of the isolation and purification method are a method utilizing solubility, such as salting out and solvent precipitation method; a method utilizing the difference in molecular weight, such as dialysis, ultrafiltration, gel filtration, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis; a method utilizing charge, such as ion exchange chromatography and hydroxylapatite chromatography; a method utilizing specific affinity, such as affinity column chromatography; a method utilizing the difference in hydrophobicity, such as reverse phase high performance liquid chromatography; and a method utilizing the difference in isoelectric point, such as isoelectric focusing. An example of RdCVF purification using HPC4 affinity chromatography is described in Example 4.

[0081] Ocular diseases that may be treated according to the present invention include, but are not limited to, Stargardt's disease, Retinitis Pigmentosa, Age-related Macular Degeneration (AMD, Dry & Wet forms), Glaucoma/Ocular Hypertension, Diabetic Retinopathy, Thyroid

related eye disease – Grave's disease, Diseases associated with Retinal Pigmented Epithelial Cells, Anterior segment disease, Lens disease / Cataracts, Eye cup disorders, Uveitis.

[0082] For delivery of the RdCVF protein of the instant invention to the eye (whether via gene therapy or protein therapy), administration will typically be local. This has the advantage of limiting the amount of material (protein or DNA) that needs to be administered and limiting systemic side-effects. Many possible modes of delivery can be used, including, but not limited to: topical administration on the cornea by a gene gun; subconjunctival injection, intracameral injection, via eye drops to the cornea, injection into the anterior chamber via the termporal limbus, intrastromal injection, corneal application combined with electrical pulses, intracorneal injection, subretinal injection, intravitreal injection (e.g., front, mid or back vitreal injection), and intraocular injection. Alternatively cells can be transfected or transduced *ex vivo* and delivered by intraocular implantation. See, Auricchio, *Mol. Ther. 6:* 490-494, 2002; Bennett, *Nature Med. 2:* 649-654, 1996; Borrás, *Experimental Eye Research 76:* 643-652, 2003; Chaum, *Survey of Ophthalmology 47:* 449-469, 2002; Campochiaro, *Expert Opinions in Biological Therapy 2:* 537-544 (2002); Lai, *Gene Therapy 9:* 804 813, 2002; Pleyer, *Progress in Retinal and Eye Research, 22:* 277-293, 2003.

[0083] The effects of various proposed therapeutic agents and administrations can be tested in suitable animal models for particular diseases. For example, the effect of delivering RdCVF to the retina can be tested in a mouse model of retinitis pigmentosa (rd1).

[0084] For the prevention or treatment of disease, the appropriate dosage of the RdCVF protein of the present invention (protein or gene therapy) will depend on the type of disease to be treated, the severity and course of the disease, whether the RdCVF is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the RdCVF, and the discretion of the attending physician. The RdCVF is suitably administered to the patient at one time or over a series of treatments. In a combination therapy regimen, the compositions of the present invention are administered in a therapeutically effective or synergistic amount. As used herein, a therapeutically effective amount is such that co-administration of RdCVF and one or more other therapeutic agents, or administration of a composition of the present invention, results in reduction or inhibition of the targeting disease or

condition. A therapeutically synergistic amount is that amount of RdCVF and one or more other therapeutic agents necessary to synergistically or significantly reduce or eliminate conditions or symptoms associated with a particular disease.

[0085] Depending on the type and severity of the disease, the RdCVF of the instant invention may be administered so that the local concentration provided is about 100 pg/ml to about 100 μg/ml, 1 ng/ml to about 95 μg/ml, 10 ng/ml to about 85 μg/ml, 100 ng/ml to about 75 μg/ml, from about 100 ng/ml to about 50 μg/ml, from about 1 μg/ml to about 25 μg/ml, from about 1 μg/ml to about 15 μg/ml, from about 1 μg/ml to about 10 μg/ml, or from about 1 μg/ml to about 4 μg/ml. When the RdCVF is delivered by gene therapy through viral virions, the dose may be from about $2x10^6$ to about $2x10^{12}$, from about $2x10^7$ to about $2x10^{11}$ drp, or from about $2x10^8$ to about $2x10^{10}$ drp per unit dose. When the RdCVF is administered by protein therapy, the dose may be from about 0.1 mg to about 50 mg, from about 0.1 mg to about 25 mg, from about 0.1 mg to about 10 mg, from about 0.5 mg to about 5 mg, or from about 0.5 mg to about 2.5 mg per unit dose.

[0086] The RdCVF of the present invention may be administered as a single dose or repeatedly whether as the protein or as a gene therapy vector). For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The progress of the therapy of the invention is monitored by conventional techniques and assays.

[0087] Within this application, unless otherwise stated, definitions of the terms and illustration of the techniques of this application may be found in any of several well-known references such as: Sambrook, J., et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989); Goeddel, D., ed., Gene Expression Technology, Methods in Enzymology, 185, Academic Press, San Diego, CA (1991); "Guide to Protein Purification" in Deutshcer, M.P., ed., Methods in Enzymology, Academic Press, San Diego, CA (1989); Innis, et al., PCR Protocols: A Guide to Methods and Applications, Academic Press, San Diego, CA (1990); Freshney, R.I., Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed., Alan Liss, Inc. New York, NY (1987); Murray, E.J., ed., Gene Transfer and Expression Protocols, pp. 109-128, The Humana Press Inc., Clifton, NJ and Lewin, B., Genes VI, Oxford University Press,

New York (1997). All methods and techniques described herein are known to those skilled in the art.

[0088] The following examples are offered to illustrate but not to limit the invention. All references disclosed herein are expressly incorporated by reference.

Example 1

Generation of cDNAs encoding RdCVF with different signal peptides

[0089] Expression vectors were constructed to evaluate the efficiency of RdCVF expression using a number of signal peptides. Signal peptides from brain derived neurotrophic factor (BDNF), insulin growth factor 1 (IGF1), and beta-glucoronidase signal peptide (GUSB) were evaluated.

[0090] In the case of these signal peptides, the relevant sequences were cloned immediately before the initiating methionine for RdCVF. In Figures 5-10, the signal peptide sequence (depicted in **bold** in the following sequences) is followed by an amino acid linker sequence (depicted by underline) followed by a furin cleavage site depicted in *italics*. The remainder of the sequence indicates the amino acid sequence for RdCVF (Short Form or Long Form). The different signal peptides are cleaved by their respective signal peptidases in the ER (specific site depicted by arrow). Inclusion of the furin cleavage site KRIKR, results in removal of the remaining amino acids before the initiating methionine for RdCVF. Furin is an enzyme that resides in the Golgi, and cleaves directly after the terminal Arginine in the KRIKR sequence. The Furin cleavage site is depicted by arrow.

Example 2

Human Growth Hormone Signal Peptide LONG FORM human RdCVF

[0091] Expression vectors were constructed to evaluate the efficiency of RdCVF expression using the signal peptide from HGH signal peptide (Human growth hormone signal peptide). For the HGH signal peptide, the cDNA construct is designed such that the initiating methionine for RdCVF is removed resulting in a RdCVF protein that initiates with an Alanine.

[0092] In Figures 11 and 12, the signal peptide sequence is depicted in bold in the following sequences. The remainder of the sequence indicates the amino acid sequence for RdCVF (Short Form or Long Form). The signal peptide is cleaved by the respective signal peptidase in the ER (specific site depicted by arrow).

Example 3

Human Growth Hormone Signal Peptide LONG FORM human RdCVF HPC4 Tag

[0093] As in Example 2, expression vectors were constructed to evaluate the efficiency of RdCVF expression using the signal peptide from HGH signal peptide (Human growth hormone signal peptide) that contain a purification tag. For the HGH signal peptide, the cDNA construct is designed such that the initiating methionine for RdCVF is removed resulting in a RdCVF protein that initiates with an Alanine. In Figures 13 and 14, the signal peptide sequence is depicted in bold. The remainder of the sequence indicates the amino acid sequence for RdCVF (Short Form or Long Form). The signal peptide is cleaved by the respective signal peptidase in the ER (specific site depicted by arrow). The twelve amino acid HPC4 tag is depicted by underline.

Example 4

Method to produce purified RdCVF protein (LF) and (SF) from mammalian cells

Vector	Insert	Length	Signal sequence	Cell line
pGZ6	rhRdCVF	short form	hGH	CHODXB11
pGZ6	rhRdCVF	long form	hGH	CHODXB11

CHO DXB11 transient expression

[0094] PGZ6 vectors expressing RdCVF(LF) and RdCVF(SF) were generated. The signal peptide from human growth hormone was included at the N terminus of RdCVF sequence to increase secretion of the protein. In addition the epitope for antibody HPC4 was included at the C terminus. The twelve amino acid tag (EDQVDPRLIDGK (SEQ ID NO:5)), is a sequence from protein C and encodes the epitope for a monoclonal antibody HPC4. Inclusion of this peptide epitope enables the purification of the RdCVF protein using HPC4 affinity chromatography in the presence of calcium ions. Release of bound RdCVF from the HPC4 affinity resin is achieved by elution with EGTA containing buffers. Transient transfection in

CHO DXB11 cells confirmed expression of RdCVF-HGH in the mammalian expression system. Transfections were done in duplicate with a change of media after 4 hour incubation to either DMEM + 1% FBS or ExCell 302. Samples were harvested after 72h and examined by HPC4 western. All transient transfections showed expression of the recombinant proteins in the conditioned medium.

Example 5

Method to produce purified RdCVF protein (LF) and (SF) from mammalian cells

CHO DXB11 stable expression

[0095] Expression was observed in the transient transfection with CHODXB11 mammalian system and stable cell line developments were initiated. An expression system utilizing dihydrofolate reductase (DHFR) selection and methotrexate (MTX) amplification was used. The vector pGZ6 was derived from the pCLHAXSV2DHFR plasmid, so as to contain the 3 kb hamster β-actin promoter in addition to a DHFR gene under the control of the SV40 early promoter. The pCLHAXSV2DHFR plasmid has been previously described by Cole et al. (1993) Biotechnology, 11:1014-1024. Briefly, the metallothionine (MT) promoter in the pCLHAXSV2DHFR vector was replaced with the β-actin promoter to create the pGZ6 vector. The DHFR-deficient CHO-K1 cell line DXB11 was transfected in triplicate with the RdCVF expression vectors. Typically, for industrial production of proteins, high expression is achieved by selecting cells with a higher gene copy number through a process that involves increasing the concentration of MTX. The RdCVF pools initially selected at 100 nM MTX were amplified by selection at five-fold higher levels of MTX (100 nM). Cells stably expressing RdCVF were selected by growth in 100nM MTX. Cells recovered from the 100nM MTX selection were cryopreserved and a small scale expression experiment was performed. A T150 cell culture flask of each of the four stables above was seeded. Cells were confluent 2 days later and medium was changed from FBS containing medium to Serum-free Excel 302. Samples were removed after 72hrs and analyzed by HPC4 Western analysis, see Figure 4. pGZ6 (hGH) RdCVF SF and pGZ6 (hGH) RdCVF LF expressed protein at the appropriate size in reduced SDS PAGE gels HPC4 Western analysis. Selection of single clones of CHO-DXB11 expressing hRdCVF long and short forms (with growth hormone signal sequence) was achieved by limiting dilution. Single clones were isolated and used for purification studies.

Example 6

Reductase activity of RdCVF(LF)

[0096] The insulin disulfide reductase activity of purified RdCVF(LF) protein was measured by monitoring the increase in the turbidity of reaction mixtures due to the formation of fine precipitates of the dissociated B chain of insulin (Figure 3). In a negative control with dithiothreitol alone, no precipitation was observed during incubation for up to 40 minutes. The addition of RdCVF (LF) or Trx1 resulted in an increase in optical density at A595 nm.

Example 7

TUNEL assay

[0097] A2E is a bis retinoid pyridinium that accumulates as a lipofuscin pigment in RPE cells in association with aging and in some inherited forms of retinal degeneration eg dry AMD and Stargardt;s disease. Upon photooxidation the A2E forms oxiranes (these are toxic). RdCVF(LF) protein protects A2E / Blue light irradiated cells from photooxidation and decreases apoptosis. The number of apoptotic cells is reduced by ~30% compared to control cells, following treatment with RdCVF(LF) protein (Figure 19). Figure 18 is the average of 3 separate experiments of RdCVF (LF) treated cells compared to control pSP70-Ev empty vector.

Example 8

In Vivo Evaluation of RdCVF (LF)

[0098] Secretable forms of RdCVF(LF) are evaluated in a model of retinal degeneration, the rd10 mouse. In this model there is a missense mutation in the rod phosphodiesterase which results in rod degeneration that starts at P18. Later, cones are also lost. AAV2/5 RdCVF (LF) are delivered to P0 rd10 pups by sub retinal injection (1e 9 DRPs, 1 ul); control eyes are injected with titer matched AAV2/8 EV. Mice are sacrificed at various times post injection, P18, P25, P32 & P46. At each time point retinal thickness is measured following retinal sectioning, in an additional cohort cone survival is assessed by PNA lectin labeling of cones followed by cone counting using morphometric analysis. Rd10 mice treated with AAV2/5 RdCVF(LF) demonstrates a greater number of surviving cones resulting in a thicker outer nuclear layer (ONL) following RdCVF(LF) treatment.

Claims

1. An expression vector for expression of rod-derived cone viability factor (RdCVF) comprising

a) a RdCVF-encoding nucleotide sequence;

b) a signal peptide-encoding nucleotide sequence positioned upstream of the RdCVF-encoding nucleotide sequence, wherein the signal peptide-encoding sequence encodes an exogenous signal peptide; and

c) a nucleotide sequence encoding a furin cleavage marker located downstream of the signal peptide-encoding nucleotide sequence and upstream of the RdCVF-encoding nucleotide sequence.

2. The expression vector of claim 1, wherein the signal peptide-encoding sequence and the furin cleavage marker sequence are separated by a sequence encoding an amino acid linker.

3. The expression vector of claim 1, wherein the RdCVF-encoding nucleotide sequence lacks the initiating methionine coding sequence.

4. The expression vector of claim 1, wherein the signal peptide–encoding sequence is that of human growth hormone (hGH), brain-derived neurotrophioc factor (BDNF), insulin growth factor-1 (IGF-1), or β-glucoronidase (GUSB).

5. The expression vector of claim 2, wherein the amino acid linker is MPLESGLSSEDSASSESFA (SEQ ID NO:4).

6. The expression vector of claim 1, wherein the RdCVF-encoding sequence encodes the long form of RdCVF (SEQ ID NO:26).

7. The expression vector of claim 1, wherein the RdCVR-encoding sequence encodes the short form of RdCVF (SEQ ID NO:27).

8. The expression vector of claim 4, wherein the signal peptide is the hGH signal peptide (SEQ ID NO:28).

9. The expression vector of claim 4, wherein the signal peptide is the BDNF signal peptide (SEQ ID NO:29).

10. The expression vector of claim 4, wherein the signal peptide is the IGF-1 signal peptide (SEQ ID NO:30).

11. The expression vector of claim 4, wherein the signal peptide is the GUSB signal peptide (SEQ ID NO:31).

12. The expression vector of claim 1, wherein the nucleotide sequence encoding the furin cleavage marker encodes the amino acid sequence KRIKR (SEQ ID NO:3).

13. The expression vector of claim 1, wherein the vector comprises a nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:6..

14. The expression vector of claim 1, wherein the vector comprises SEQ ID NO:7.

15. The expression vector of claim 1, wherein said vector comprises a nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:8..

16. The expression vector of claim 1, wherein said vector comprises SEQ ID NO:9.

17. The expression vector of claim 1, wherein said vector comprises a nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:10.

18. The expression vector of claim 1, wherein said vector comprises SEQ ID NO:11.

19. The expression vector of claim 1, wherein said vector comprises SEQ ID NO:13.

20. The expression vector of claim 1, wherein said vector comprises a nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:12.

21. The expression vector of claim 1, wherein said vector comprises SEQ ID NO:15.

22. The expression vector of claim 1, wherein said vector comprises a nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:14.

23. The expression vector of claim 1, wherein said vector comprises SEQ ID NO:17.

24. The expression vector of claim 1, wherein said vector comprises a nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:16.

25. The expression vector of claim 1, wherein said vector comprises SEQ ID NO:18.

26. The expression vector of claim 1, wherein said vector comprises a nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:19.

27. The expression vector of claim 1, wherein said vector comprises SEQ ID NO:20.

28. The expression vector of claim 1, wherein said vector comprises a nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:21.

29. The expression vector of claim 1, wherein said vector comprises SEQ ID NO:22.

30. The expression vector of claim 1, wherein said vector comprises a nucleic acid sequence that codes for the amino acid sequence of SEQ ID NO:23.

31. The expression vector of claim 1, wherein said vector comprises SEQ ID NO:24.

32. The expression vector of claim 1, wherein said vector comprises a nucleic acid sequence that encodes the amino acid sequence of SEQ ID NO:25.

33. An expression vector for expression of rod-derived cone viability factor (RdCVF) comprising

a) a RdCVF-encoding nucleotide sequence lacking the initiating methionine coding sequence; and

b) an exogenous signal peptide-encoding nucleotide sequence positioned upstream of the RdCVF-encoding nucleotide sequence, wherein the exogenous signal peptide-encoding sequence is selected from the group consisting of: human growth hormone (hGH), brain-derived neurotrophic factor (BDNF), insulin growth factor-1 (IGF-1), or β-glucoronidase (GUSB).

34. A mammalian cell comprising an expression vector for expression of rod-derived cone viability factor (RdCVF) in a cell, said vector comprising:

a) a RdCVF-encoding nucleotide sequence;

b) a signal peptide-encoding nucleotide sequence positioned upstream of the RdCVF-encoding nucleotide sequence, wherein the signal peptide-encoding sequence encodes an exogenous signal peptide; and

c) a nucleotide sequence encoding a furin cleavage marker located downstream of the signal peptide-encoding nucleotide sequence and upstream of the RdCVF-encoding nucleotide sequence.

35. The cell of claim 34, wherein the signal peptide-encoding sequence and the furin cleavage marker sequence are separated by a sequence encoding an amino acid linker.

36. The cell of claim 34, wherein the RdCVF-encoding nucleotide sequence lacks the initiating methionine coding sequence.

37. The cell of claim 34, wherein the signal peptide–encoding sequence is that of human growth hormone (hGH), brain-derived neurotrophioc factor (BDNF), insulin growth factor-1 (IGF-1), or β-glucoronidase (GUSB).

38. A method for expressing of rod-derived cone viability factor (RdCVF), in a host cell, said method comprises:

providing a mammalian cell comprising a RdCVF expression vector, wherein the vector comprises:

a) a RdCVF-encoding nucleotide sequence;

b) a signal peptide-encoding nucleotide sequence positioned upstream of the RdCVF-encoding nucleotide sequence, wherein the signal peptide-encoding sequence encodes an exogenous signal peptide; and

c) a nucleotide sequence encoding a furin cleavage marker located downstream of the signal peptide-encoding nucleotide sequence and immediately upstream of the RdCVF-encoding nucleotide sequence; and

culturing the mammalian cell under conditions such that RdCVF is expressed.

39. The cell of claim 38, wherein the signal peptide-encoding sequence and the furin cleavage marker sequence are separated by a sequence encoding an amino acid linker.

40. The cell of claim 38, wherein the RdCVF-encoding nucleotide sequence lacks the initiating methionine coding sequence.

41. The cell of claim 38, wherein the signal peptide–encoding sequence is that of human growth hormone (hGH), brain-derived neurotrophioc factor (BDNF), insulin growth factor-1 (IGF-1), or β-glucoronidase (GUSB).

42. The method of claim 38, wherein the mammalian cell is a CHO cell or a COS cell.

43. The method of claim 38, wherein the RdCVF-encoding nucleotide sequence encodes the long form of RdCVF.

44. The method of claim 38, wherein the RdCVF-encoding nucleotide sequence encodes the short form of RdCVF.

45. The method of claim 38, wherein the nucleotide sequence the furin cleavage marker is a sequence encoding the amino acid sequence KRIKR.

46. The method of claim 38, further comprising the step of isolating the expressed RdCVF protein.

47. A method of treating an ocular disease in a mammal, the method comprising:

(1) administering to a diseased eye of the mammal a nucleic acid encoding a RdCVF fusion protein, where the nucleic acid encoding the RdCVF fusion protein comprises:

a) a RdCVF-encoding nucleotide sequence;

b) a signal peptide-encoding nucleotide sequence positioned upstream of the RdCVF-encoding nucleotide sequence, wherein the signal peptide-encoding sequence encodes an exogenous signal peptide; and

c) a nucleotide sequence encoding a furin cleavage marker located downstream of the signal peptide-encoding nucleotide sequence and upstream of the RdCVF-encoding nucleotide sequence; and

(2) expressing the RdCVF fusion protein at a therapeutically effective amount in the diseased eye to treat the ocular disease.

48. The method of claim 47, wherein the nucleic acid encoding the RdCVF fusion protein is an expression vector that is a recombinantn adeno-associated virus (AAV) or adenovirus (Ad).

49. The method of claim 47, wherein the ocular diseases is Stargardt's disease, Retinitis Pigmentosa, Dry Age-related Macular Degeneration (Dry AMD), Wet Age-related Macular Degeneration (Wet AMD), Glaucoma/Ocular Hypertension, Diabetic Retinopathy, Thyroid related eye disease, Grave's disease, a diseases associated with Retinal Pigmented Epithelial Cells, Anterior segment disease, Lens disease / Cataracts, an Eye cup disorder, or Uveitis.

50. The method of claim 47, wherein the signal peptide-encoding sequence and the furin cleavage marker sequence are separated by a sequence encoding an amino acid linker.

51. The method of claim 47, wherein the RdCVF-encoding nucleotide sequence lacks the initiating methionine coding sequence.

52. The method of claim 47, wherein the signal peptide–encoding sequence is that of human growth hormone (hGH), brain-derived neurotrophioc factor (BDNF), insulin growth factor-1 (IGF-1), or β-glucoronidase (GUSB).

53. A method of treating an ocular disease in a mammal, the method comprising:

(1) administering to a diseased eye of the mammal a nucleic acid encoding a RdCVF fusion protein, where the nucleic acid encoding the RdCVF fusion protein comprises:

a) a RdCVF-encoding nucleotide sequence lacking the initiating methionine coding sequence;

b) an exogenous signal peptide-encoding nucleotide sequence positioned upstream of the RdCVF-encoding nucleotide sequence, wherein the exogenous signal peptide-encoding sequence is selected from the group consisting of: human growth hormone (hGH), brain-derived neurotrophic factor (BDNF), insulin growth factor-1 (IGF-1), or β-glucoronidase (GUSB); and

(2) expressing the RdCVF fusion protein at a therapeutically effective amount in the diseased eye to treat the ocular disease.

54. The method of claim 53, wherein the nucleic acid encoding the RdCVF fusion protein is an expression vector that is a recombinantn adeno-associated virus (AAV) or adenovirus (Ad).

55. The method of claim 53, wherein the ocular diseases is Stargardt's disease, Retinitis Pigmentosa, Dry Age-related Macular Degeneration (Dry AMD), Wet Age-related Macular Degeneration (Wet AMD), Glaucoma/Ocular Hypertension, Diabetic Retinopathy, Thyroid related eye disease, Grave's disease, a diseases associated with Retinal Pigmented Epithelial Cells, Anterior segment disease, Lens disease / Cataracts, an Eye cup disorder, or Uveitis.

Figure 1

Western Blots of CHODXB11 Cells Transiently Transfected with pcDNA-hRdCVF-FL ±Signal Peptides Using anti-hRdCVF-FL

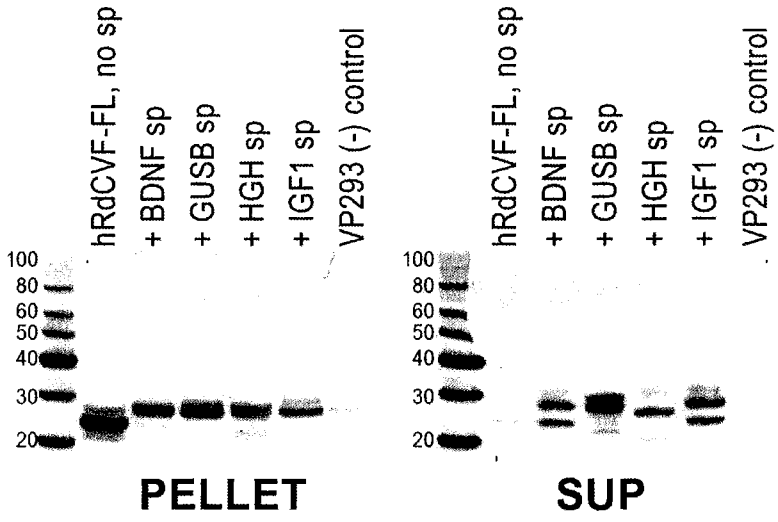


Figure 2

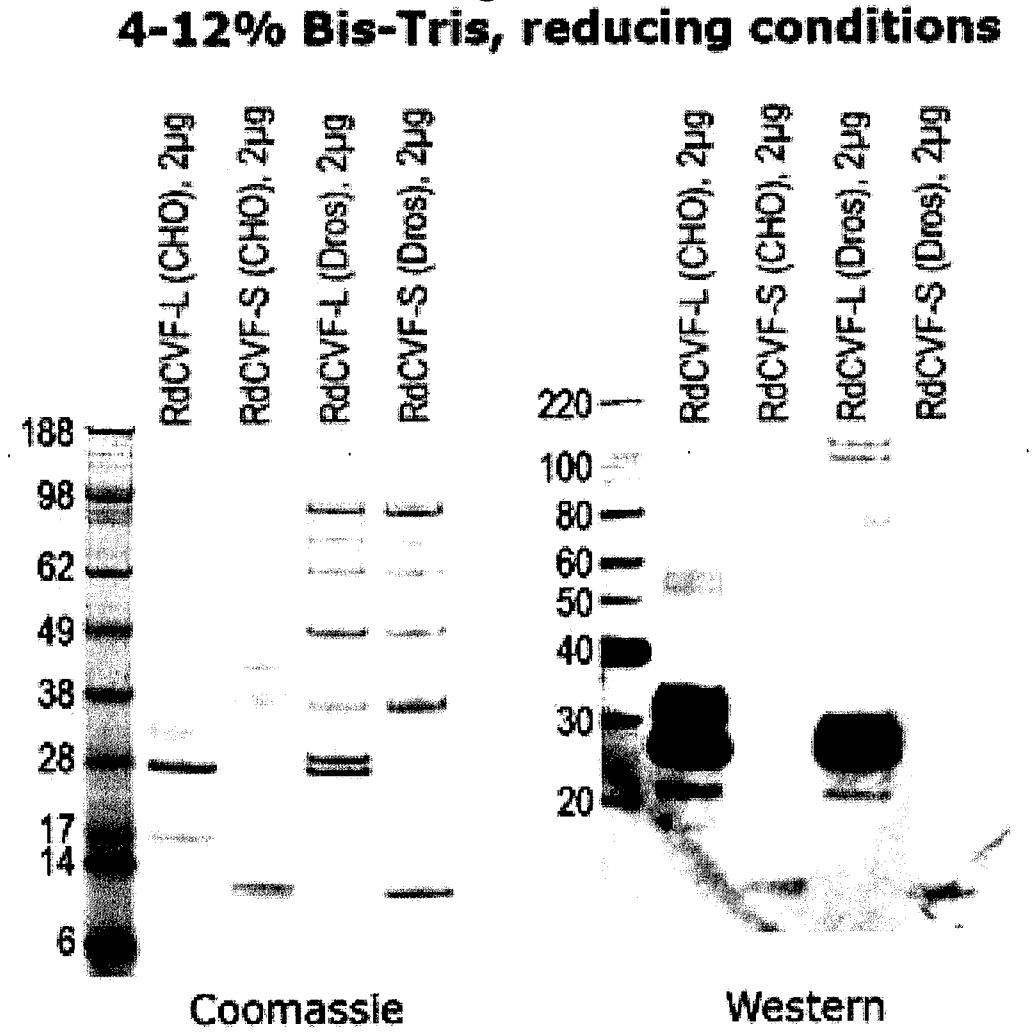

4-12% Bis-Tris, reducing conditions
RdCVF-L (CHO), 2µg
RdCVF-S (CHO), 2µg
RdCVF-L (Dros), 2µg
RdCVF-S (Dros), 2µg
188
98
62
49
38
28
17
14
6
Coomassie
220
100
80
60
50
40
30
20
Western

Figure 3

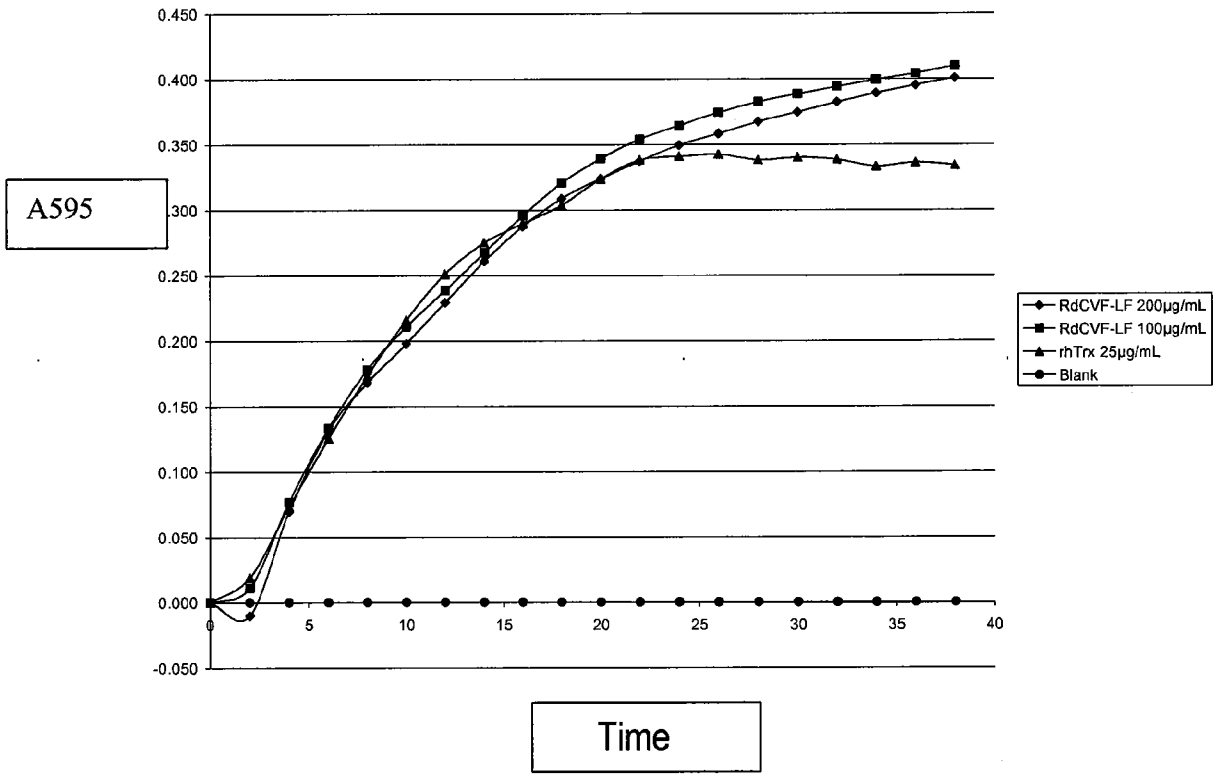
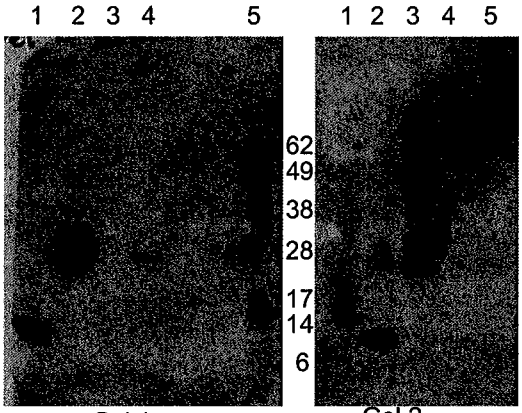

A595
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
-0.050
0
5
10
15
20
25
30
35
40
Time
RdCVF-LF 200µg/mL
RdCVF-LF 100µg/mL
rhTrx 25µg/mL
Blank

Figure 4



Gel 1
Lane 1: pGZ6 RdCVF SF
Lane 2: pGZ6 RdCVF LF
Lane 5: SeeBlue Plus2 Marker

Gel 2
Lane 1: SeeBlue Plus2 Marker
Lane 2: pGZ6 RdCVF SF
Lane 3: pGZ6 RdCVF LF

Figure 5
GUSB signal peptide-FURIN-hRdCVF (Long Form)

A.

MARGSAVAWAALGPLLWGCALG↓LQMPLESGLSSEDSASSESFA*KRIKR*↓MASLFSGR
ILIRNNSDQDELDTEAEVSRRLENRLVLLFFGAGACPQCQAFVPILKDFFVRLTDEFYVL
RAAQLALVYVSQDSTEEQQDLFLKDMPKKWLFLPFEDDLRRDLGRQFSVERLPAVVVL
KPDGDVLTRDGADEIQRLGTACFANWQEAAEVLDRNFQLPEDLEDQEPRSLTECLRRH
KYRVEKAARGGRDPGGGGGEEGGAGGLF (SEQ ID NO:6)

B.

gaattcctccggggaccacacgccgcgctgtccccagcacttaccatggcccgggggtcggcggttgcctgggcggcgctcgggccgtt
gttgtggggctgcgcgctggggctgcagatgcccctcgagtccggcttgctgtcctccgaggactccgccagctccgagagcttcgccaa
gcgcatcaagcgcatggcctccctgttctctggccgcatcctgatccgcaacaatagcgaccaggacgagctggatacggaggctgaggt
cagtcgcaggctggagaaccggctggtgctgctgttctttggtgctggggcttgtccacagtgccaggccttcgtgcccatcctcaaggact
tcttcgtgcggctcacagatgagttctatgtactgcgggcggctcagctggccctggtgtacgtgtcccaggactccacggaggagcagca
ggacctgttcctcaaggacatgccaaagaaatggcttttcctgccctttgaggatgatctgaggagggacctcgggcgccagttctcagtgg
agcgcctgccggcggtcgtggtgctcaagccggacggggacgtgctcactcgcgacggcgccgacgagatccagcgcctgggcacc
gcctgcttcgccaactggcaggaggcggccgaggtgctggaccgcaacttccagctgccagaggacctggaggaccaggagccacgg
agcctcaccgagtgcctgcgccgccacaagtaccgcgtggaaaaggcggcgcgaggcgggcgcgaccccgggggaggggggtggg
gaggagggcggggccgggggctgttctgacccgctagggtggaggagaggagtggggtttgttgatgaacctccacccccacccccac
ccccgcacgcctgtaatcccagcacttggggaggccaaggcgggaggatcgcttgagcccagaggttcgagatcaacctgggcaagag
agtgagaccctgactctacgaaaattaaaagttagcccggtgtggtggcgcgcacctgtggcttagctaccctgaaaaaaaaaaaaaaaaa
aaaaaaaaaaaaaaaaaaaaaaaaaaaagcttcc (SEQ ID NO:7)

Figure 6
BDNF signal peptide-FURIN-hRdCVF (Long Form)

A.

MTILFLTMVISYFGCMKA↓APMPLESGLSSEDSASSESFA*KRIKR*↓MASLFSGRILIRNNS
DQDELDTEAEVSRRLENRLVLLFFGAGACPQCQAFVPILKDFFVRLTDEFYVLRAAQLA
LVYVSQDSTEEQQDLFLKDMPKKWLFLPFEDDLRRDLGRQFSVERLPAVVVLKPDGDV
LTRDGADEIQRLTACFANWQEAAEVLDRNFQLPEDLEDQEPRSLTECLRRHKYRVEKA
ARGGRDPGGGGGEEGGAGGLF (SEQ ID NO:8)

B.

gaattcctccggggaccacacgccgcgctgtccccagcacttaccatgaccatcctttccttactatggttatttcatactttggttgcatgaag
gctgcccccatgcccctcgagtccggcctgtcctccgaggactccgccagctccgagagcttcgccaagcgcatcaagcgcatggcctc
cctgttctctggccgcatcctgatccgcaacaatagcgaccaggacgagctggatacggaggctgaggtcagtcgcaggctggagaacc
ggctggtgctgctgttctttggtgctggggcttgtccacagtgccaggccttcgtgcccatcctcaaggacttcttcgtgcggctcacagatg
agttctatgtactgcgggcggctcagctggccctggtgtacgtgtcccaggactccacggaggagcagcaggacctgttcctcaaggaca
tgccaaagaaatggcttttcctgccctttgaggatgatctgaggagggacctcgggcgccagttctcagtggagcgcctgccggcggtcgt
ggtgctcaagccggacggggacgtgctcactcgcgacggcgccgacgagatccagcgcctgggcaccgcctgcttcgccaactggca
ggaggcggccgaggtgctggaccgcaacttccagctgccagaggacctggaggaccaggagccacggagcctcaccgagtgcctgc
gccgccacaagtaccgcgtggaaaaggcggcgcgaggcgggcgcgaccccgggggaggggggtggggaggagggcggggccgg
ggggctgttctgacccgctagggtggaggagaggagtggggtttgttgatgaacctccacccccacccccacccccgcacgcctgtaatcc
cagcacttggggaggccaaggcgggaggatcgcttgagcccagaggttcgagatcaacctgggcaagagagtgagaccctgactctac
gaaaattaaaagttagcccggtgtggtggcgcgcacctgtggcttagctaccctgaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
aaaaaaaaagcttg (SEQ ID NO:9)

Figure 7
IGF-1 signal peptide-FURIN-hRdCVF (Long Form)

A.

MGKISSLPTQLFKCCFCDFLKVKMHTMSSSHLFYLALCLLTFTSSATA↓GPMPLESG LSSEDSASSESFA*KRIKR*↓MASLFSGRILIRNNSDQDELDTEAEVSRRLENRLVLLFFGAGA CPQCQAFVPILKDFFVRLTDEFYVLRAAQLALVYVSQDSTEEQQDLFLKDMPKKWLFL PFEDDLRRDLGRQFSVERLPAVVVLKPDGDVLTRDGADEIQRLGTACFANWQEAAEVL DRNFQLPEDLEDQEPRSLTECLRRHKYRVEKAARGGRDPGGGGGEEGGAGGLF (SEQ ID NO:10)

B.

gaattcctccggggaccacacgccgcgctgtccccagcacttaccatgggaaaaatcagcagtcttccaacccaattatttaagtgctgcttt tgtgatttcttgaaggtgaagatgcacaccatgtcctcctcgcatctcttctacctggcgctgtgcctgctcaccttcaccagctctgccacggc tggaccggagatgcccctcgagtccggcctgtcctccgaggactccgccagctccgagagcttcgccaagcgcatcaagcgcatggcct ccctgttctctggccgcatcctgatccgcaacaatagcgaccaggacgagctggatacggaggctgaggtcagtcgcaggctggagaac cggctggtgctgctgttctttggtgctggggcttgtccacagtgccaggccttcgtgcccatcctcaaggacttcttcgtgcggctcacagat gagttctatgtactgcgggcggctcagctggccctggtgtacgtgtcccaggactccacggaggagcagcaggacctgttcctcaaggac atgccaaagaaatggcttttcctgccctttgaggatgatctgaggagggacctcgggcgccagttctcagtggagcgcctgccggcggtcg tggtgctcaagccggacggggacgtgctcactcgcgacggcgccgacgagatccagcgcctgggcaccgcctgcttcgccaactggca ggaggcggccgaggtgctggaccgcaacttccagctgccagaggacctggaggaccaggagccacggagcctcaccgagtgcctgc gccgccacaagtaccgcgtggaaaaggcggcgcgaggcgggcgcgaccccgggggaggggtggggaggagggcggggccgg ggggctgttctgacccgctagggtggaggagaggagtggggtttgttgatgaacctccacccccacccccacccccgcacgcctgtaatcc cagcacttggggaggccaaggcgggaggatcgcttgagcccagaggttcgagatcaacctgggcaagagagtgagaccctgactctac gaaaattaaaagttagcccggtgtggtggcgcgcacctgtggcttagctaccctgaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa aaaaaaaaagcttc (SEQ ID NO:11)

Figure 8
GUSB signal peptide – Furin - hRdCVF (Short Form)

A.

MARGSAVAWAALGPLLWGCALG↓LQMPLESGLSSEDSASSESFA*KRIKR*↓MASLFSGR
ILIRNNSDQDELDTEAEVSRRLENRLVLLFFGAGACPQCQAFVPILKDFFVRLTDEFYVL
RAAQLALVYVSQDSTEEQQDLFLKDMPKKWLFLPFEDDLRR (SEQ ID NO:12)

B.

gaattcctccggggaccacacgccgcgctgtccccagcacttaccatggcccgggggtcggcggttgcctgggcggcgctcgggccgtt
gttgtggggctgcgcgctggggctgcagatgcccctcgagtccggcttgctgtcctccgaggactccgccagctccgagagcttcgccaa
gcgcatcaagcgcatggcctccctgttctctggccgcatcctgatccgcaacaatagcgaccaggacgagctggatacggaggctgaggt
cagtcgcaggctggagaaccggctggtgctgctgttctttggtgctggggcttgtccacagtgccaggccttcgtgcccatcctcaaggact
tcttcgtgcggctcacagatgagttctatgtactgcgggcggctcagctggccctggtgtacgtgtcccaggactccacggaggagcagca
ggacctgttcctcaaggacatgccaaagaaatggcttttcctgccctttgaggatgatctgaggaggtgaggccccagggaagaccaggg
agggcttcctggagaaggcatttccctggaggtttactgtcctggtactacttgtgcataaagaggtaaagctt (SEQ ID NO:13)

Figure 9
BDNF signal peptide -Furin-hRdCVF (Short Form)

A.

MTILFLTMVISYFGCMKA↓**AP**MPLESGLSSEDSASSESFA*KRIKR*↓MASLFSGRILIRNNS
DQDELDTEAEVSRRLENRLVLLFFGAGACPQCQAFVPILKDFFVRLTDEFYVLRAAQLA
LVYVSQDSTEEQQDLFLKDMPKKWLFLPFEDDLRR (SEQ ID NO:14)

B.

gaattcctccggggaccacacgccgcgctgtccccagcacttaccatgaccatcctttccttactatggttatttcatactttggttgcatgaag
gctgccccccatgcccctcgagtccggcctgtcctccgaggactccgccagctccgagagcttcgccaagcgcatcaagcgcatggcctc
cctgttctctggccgcatcctgatccgcaacaatagcgaccaggacgagctggatacggaggctgaggtcagtcgcaggctggagaacc
ggctggtgctgctgttctttggtgctggggcttgtccacagtgccaggccttcgtgcccatcctcaaggacttcttcgtgcggctcacagatg
agttctatgtactgcgggcggctcagctggccctggtgtacgtgtcccaggactccacggaggagcagcaggacctgttcctcaaggaca
tgccaaagaaatggcttttcctgccctttgaggatgatctgaggaggtgaggccccagggaagaccagggagggcttcctggagaaggc
atttccctggaggtttactgtcctggtactacttgtgcataaagaggtaaagctt (SEQ ID NO:15)

Figure 10
IGF1signal peptide- Furin- hRdCVF (Short Form)

A.

MGKISSLPTQLFKCCFCDFLKVKMHTMSSSHLFYLALCLLTFTSSATA↓GPMPLESGLSS
EDSASSESFA*KRIKR*↓MASLFSGRILIRNNSDQDELDTEAEVSRRLENRLVLLFFGAGACP
QCQAFVPILKDFFVRLTDEFYVLRAAQLALVYVSQDSTEEQQDLFLKDMPKKWLFLPFE
DDLRR (SEQ ID NO:16)

B.

gaattcctccggggaccacacgccgcgctgtccccagcacttaccatgggaaaaatcagcagtcttccaacccaattatttaagtgctgctttgtgatttcttgaaggtgaagatgcacaccatgtcctcctcgcatctcttctacctggcgctgtgcctgctcaccttcaccagctctgccacggctggaccggagatgcccctcgagtccggcctgtcctccgaggactccgccagctccgagagcttcgccaagcgcatcaagcgcatggcctccctgttctctggccgcatcctgatccgcaacaatagcgaccaggacgagctggatacggaggctgaggtcagtcgcaggctggagaaccggctggtgctgctgttctttggtgctggggcttgtccacagtgccaggccttcgtgcccatcctcaaggacttcttcgtgcggctcacagatgagttctatgtactgcgggcggctcagctggccctggtgtacgtgtcccaggactccacggaggagcagcaggacctgttcctcaaggacatgccaaagaaatggcttttcctgccctttgaggatgatctgaggaggtgaggccccagggaagaccagggagggcttcctggagaaggcatttccctggaggtttactgtcctggtactacttgtgcataaagaggtaaagctt. (SEQ ID NO:17)

Figure 11
HGH Signal Peptide Long Form hRdCVF

A.

ATGGCTACAGGCTCCCGGACGTCCCTGCTCCTGGCTTTTGGCCTGCTCTGCCT
CCCCTGGCTTCAAGAGGGATCCGCC↓GCCTCCCTGTTCTCTGGCCGCATCCTGATC
CGCAACAATAGCGACCAGGACGAGCTGGATACGGAGGCTGAGGTCAGTCGCAGGC
TGGAGAACCGGCTGGTGCTGCTGTTCTTTGGTGCTGGGGCTTGTCCACAGTGCCAGG
CCTTCGTGCCCATCCTCAAGGACTTCTTCGTGCGGCTCACAGATGAGTTCTATGTAC
TGCGGGCGGCTCAGCTGGCCCTGGTGTACGTGTCCCAGGACTCCACGGAGGAGCAG
CAGGACCTGTTCCTCAAGGACATGCCAAAGAAATGGCTTTTCCTGCCCTTTGAGGAT
GATCTGAGGAGGGACCTCGGGCGCCAGTTCTCAGTGGAGCGCCTGCCGGCGGTCGT
GGTGCTCAAGCCGGACGGGGACGTGCTCACTCGCGACGGCGCCGACGAGATCCAGC
GCCTGGGCACCGCCTGCTTCGCCAACTGGCAGGAGGCGGCCGAGGTGCTGGACCGC
AACTTCCAGCTGCCAGAGGACCTGGAGGACCAGGAGCCACGGAGCCTCACCGAGTG
CCTGCGCCGCCACAAGTACCGCGTGGAAAAGGCGGCGCGAGGCGGGCGCGACCCC
GGGGGAGGGGGTGGGGAGGAGGGCGGGGCCGGGGGGCTGTTCTGA (SEQ ID NO:18)

B.

ASLFSGRILIRNNSDQDELDTEAEVSRRLENRLVLLFFGAGACPQCQAFVPILKDFFVRLT
DEYVLRAAQLALVYVSQDSTEEQQDLFLKDMPKKWLFLPFEDDLRRDLGRQFSVERLP
AVVVLKPDGDVLTRDGADEIQRLGTACFANWQEAAEVLDRNFQLPEDLEDQEPRSLTE
CLRRHKYRVEKAARGGRDPGGGGGEEGGAGGLF (SEQ ID NO:19)

Figure 12
HGH Signal Peptide Short Form hRdCVF

A.

ATGGCTACAGGCTCCCGGACGTCCCTGCTCCTGGCTTTTGGCCTGCTCTGCCT
CCCCTGGCTTCAAGAGGGATCCGCC↓GCCTCCCTGTTCTCTGGCCGCATCCTGATC
CGCAACAATAGCGACCAGGACGAGCTGGATACGGAGGCTGAGGTCAGTCGCAGGC
TGGAGAACCGGCTGGTGCTGCTGTTCTTTGGTGCTGGGGCTTGTCCACAGTGCCAGG
CCTTCGTGCCCATCCTCAAGGACTTCTTCGTGCGGCTCACAGATGAGTTCTATGTAC
TGCGGGCGGCTCAGCTGGCCCTGGTGTACGTGTCCCAGGACTCCACGGAGGAGCAG
CAGGACCTGTTCCTCAAGGACATGCCAAAGAAATGGCTTTTCCTGCCCTTTGAGGAT
GATCTGAGGAGGTGA (SEQ ID NO:20)

B.

ASLFSGRILIRNNSDQDELDTEAEVSRRLENRLVLLFFGAGACPQCQAFVPILKDFFVRLT
DEFYLRAAQLALVYVSQDSTEEQQDLFLKDMPKKWLFLPFEDDLRR (SEQ ID NO:21)

Figure 13
HGH Signal Peptide Long Form hRdCVF HPC4 Tag

A.

ATGGCTACAGGCTCCCGGACGTCCCTGCTCCTGGCTTTTGGCCTGCTCTGCCT
CCCCTGGCTTCAAGAGGGATCCGCC↓GCCTCCCTGTTCTCTGGCCGCATCCTGATC
CGCAACAATAGCGACCAGGACGAGCTGGATACGGAGGCTGAGGTCAGTCGCAGGC
TGGAGAACCGGCTGGTGCTGCTGTTCTTTGGTGCTGGGGCTTGTCCACAGTGCCAGG
CCTTCGTGCCCATCCTCAAGGACTTCTTCGTGCGGCTCACAGATGAGTTCTATGTAC
TGCGGGCGGCTCAGCTGGCCCTGGTGTACGTGTCCCAGGACTCCACGGAGGAGCAG
CAGGACCTGTTCCTCAAGGACATGCCAAAGAAATGGCTTTTCCTGCCCTTTGAGGAT
GATCTGAGGAGGGACCTCGGGCGCCAGTTCTCAGTGGAGCGCCTGCCGGCGGTCGT
GGTGCTCAAGCCGGACGGGGACGTGCTCACTCGCGACGGCGCCGACGAGATCCAGC
GCCTGGGCACCGCCTGCTTCGCCAACTGGCAGGAGGCGGCCGAGGTGCTGGACCGC
AACTTCCAGCTGCCAGAGGACCTGGAGGACCAGGAGCCACGGAGCCTCACCGAGTG
CCTGCGCCGCCACAAGTACCGCGTGGAAAAGGCGGCGCGAGGCGGGCGCGACCCC
GGGGGAGGGGGTGGGGAGGAGGGCGGGGCCGGGGGGCTGTTCGAAGATCAAGTCG
ATCCACGCCTCATCGATGGCAAATGA (SEQ ID NO:22)

B.

ASLFSGRILIRNNSDQDELDTEAEVSRRLENRLVLLFFGAGACPQCQAFVPILKDFFVRLT
DEYVLRAAQLALVYVSQDSTEEQQDLFLKDMPKKWLFLPFEDDLRRDLGRQFSVERLP
AVVVLKPDGDVLTRDGADEIQRLGTACFANWQEAAEVLDRNFQLPEDLEDQEPRSLTE
CLRRHKYRVEKAARGGRDPGGGGGEEGGAGGLFEDQVDPRLIDGK. (SEQ ID NO:23)

Figure 14

HGH Signal Peptide SHORT FORM human RdCVF HPC4

A.

ATGGCTACAGGCTCCCGGACGTCCCTGCTCCTGGCTTTTGGCCTGCTCTGCCTCCCCTGGCTTCAAGAGGGA
TCCGCC↓GCCTCCCTGTTCTCTGGCCGCATCCTGATCCGCAACAATAGCGACCAGGACGAGCTGGATACGGAG
GCTGAGGTCAGTCGCAGGCTGGAGAACCGGCTGGTGCTGCTGTTCTTTGGTGCTGGGGCTTGTCCACAGTGC
CAGGCCTTCGTGCCCATCCTCAAGGACTTCTTCGTGCGGCTCACAGATGAGTTCTATGTACTGCGGGCGGCTC
AGCTGGCCCTGGTGTACGTGTCCCAGGACTCCACGGAGGAGCAGCAGGACCTGTTCCTCAAGGACATGCCAA
AGAAATGGCTTTTCCTGCCCTTTGAGGATGATCTGAGGAGGGAAGATCAAGTCGATCCACGCCTCATCGATGGC
AAATGA (SEQ ID NO:24)

B.

ASLFSGRILIRNNSDQDELDTEAEVSRRLENRLVLLFFGAGACPQCQAFVPILKDFFVRLT
DEFYLRAAQLALVYVSQDSTEEQQDLFLKDMPKKWLFLPFEDDLRR
EDQVDPRLIDGK. (SEQ ID NO:25)

Figure 15

nucleoredoxin-like 1 [Homo sapiens]

```
LOCUS       NP_612463                212 aa            linear   PRI 14-SEP-2008
DEFINITION  nucleoredoxin-like 1 [Homo sapiens].
ACCESSION   NP_612463
VERSION     NP_612463.1  GI:19923987
DBSOURCE    REFSEQ: accession NM_138454.1
FEATURES             Location/Qualifiers
     source          1..212
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
                     /chromosome="19"
                     /map="19p13.11"
     Protein         1..212
                     /product="nucleoredoxin-like 1"
                     /note="rod-derived cone viability factor; thioredoxin-like
                     6"
                     /calculated_mol_wt=23812
     Region          8..153
                     /region_name="TryX_like_RdCVF"
                     /note="Tryparedoxin (TryX)-like family, Rod-derived cone
                     viability factor (RdCVF) subfamily; RdCVF is a thioredoxin
                     (TRX)-like protein specifically expressed in
                     photoreceptors. RdCVF was isolated and identified as a
                     factor that supports cone survival in...; cd03008"
                     /db_xref="CDD:48557"
     Site            order(44,47)
                     /site_type="other"
                     /note="putative catalytic residues"
                     /db_xref="CDD:48557"
     CDS             1..212
                     /gene="NXNL1"
                     /gene_synonym="RDCVF; TXNL6"
                     /coded_by="NM_138454.1:48..686"
                     /db_xref="CCDS:CCDS12360.1"
                     /db_xref="GeneID:115861"
                     /db_xref="HGNC:25179"
                     /db_xref="HPRD:12300"
                     /db_xref="MIM:608791"
ORIGIN
        1 maslfsgril irnnsdqdel dteaevsrrl enrlvllffg agacpqcqaf vpilkdffvr
       61 ltdefyvlra aqlalvyvsq dsteeqqdlf lkdmpkkwlf lpfeddlrrd lgrqfsverl
      121 pavvvlkpdg dvltrdgade iqrlgtacfa nwqeaaevld rnfqlpedle dqeprsltec
      181 lrrhkyrvek aarggrdpgg gggeeggagg lf  (SEQ ID NO. 32)
```

FIGURE 16

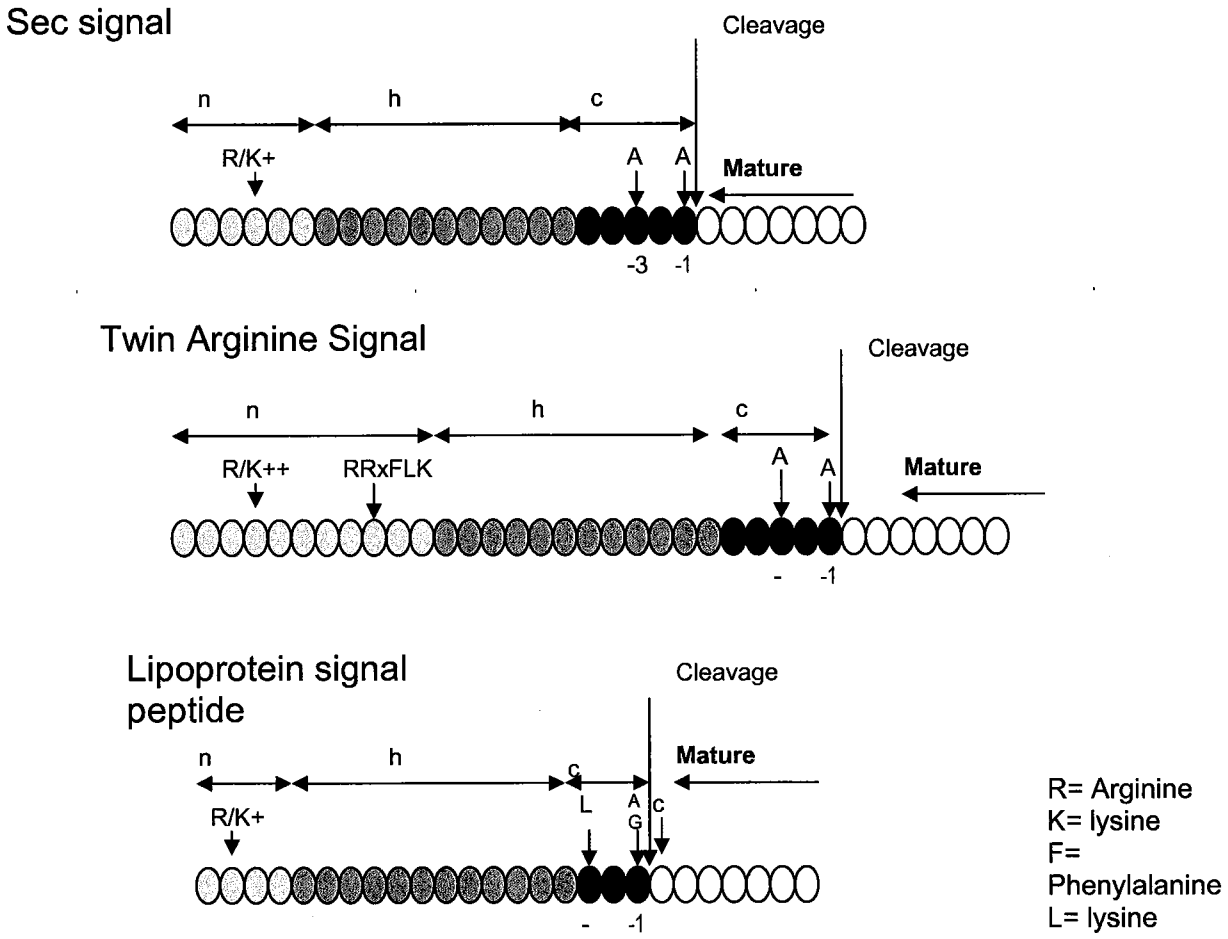
Homo sapiens nucleoredoxin-like 1 (NXNL1), mRNA

```
LOCUS       NM_138454               948 bp    mRNA    linear   PRI 14-SEP-2008
DEFINITION  Homo sapiens nucleoredoxin-like 1 (NXNL1), mRNA.
ACCESSION   NM_138454
VERSION     NM_138454.1  GI:19923986
FEATURES             Location/Qualifiers
     source          1..948
                     /organism="Homo sapiens"
                     /mol_type="mRNA"
                     /db_xref="taxon:9606"
                     /chromosome="19"
                     /map="19p13.11"
     gene            1..948
                     /gene="NXNL1"
                     /gene_synonym="RDCVF; TXNL6"
                     /note="nucleoredoxin-like 1"
                     /db_xref="GeneID:115861"
                     /db_xref="HGNC:25179"
                     /db_xref="HPRD:12300"
                     /db_xref="MIM:608791"
     exon            1..373
                     /gene="NXNL1"
                     /gene_synonym="RDCVF; TXNL6"
                     /inference="alignment:Splign"
                     /number=1
     CDS             48..686
                     /gene="NXNL1"
                     /gene_synonym="RDCVF; TXNL6"
                     /note="rod-derived cone viability factor; thioredoxin-like
                     6"
                     /codon_start=1
                     /product="nucleoredoxin-like 1"
                     /protein_id="NP_612463.1"
                     /db_xref="GI:19923987"
                     /db_xref="CCDS:CCDS12360.1"
                     /db_xref="GeneID:115861"
                     /db_xref="HGNC:25179"
                     /db_xref="HPRD:12300"
                     /db_xref="MIM:608791"
                     /translation="MASLFSGRILIRNNSDQDELDTEAEVSRRLENRLVLLFFGAGAC
                     PQCQAFVPILKDFFVRLTDEFYVLRAAQLALVYVSQDSTEEQQDLFLKDMPKKWLFLP
                     FEDDLRRDLGRQFSVERLPAVVVLKPDGDVLTRDGADEIQRLGTACFANWQEAAEVLD
                     RNFQLPEDLEDQEPRSLTECLRRHKYRVEKAARGGRDPGGGGGEEGGAGGLF" (SEQ
ID NO. 32)
     exon            374..908
                     /gene="NXNL1"
                     /gene_synonym="RDCVF; TXNL6"
                     /inference="alignment:Splign"
                     /number=2
ORIGIN
        1 ccggggacca cacgccgcgc tgtccccagc acccaaccca ggttaccatg gcctccctgt
       61 tctctggccg catcctgatc cgcaacaata gcgaccagga cgagctggat acggaggctg
      121 aggtcagtcg caggctggag aaccggctgg tgctgctgtt ctttggtgct ggggcttgtc
      181 cacagtgcca ggccttcgtg cccatcctca aggacttctt cgtgcggctc acagatgagt
      241 tctatgtact gcgggcggct cagctggccc tggtgtacgt gtcccaggac tccacggagg
      301 agcagcagga cctgttcctc aaggacatgc caaagaaatg gcttttcctg ccctttgagg
      361 atgatctgag gagggacctc gggcgccagt tctcagtgga gcgcctgccg gcggtcgtgg
```

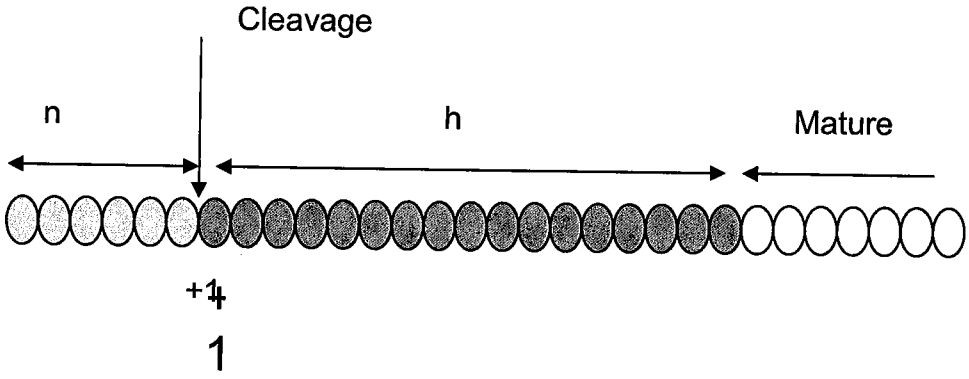
421 tgctcaagcc ggacggggac gtgctcactc gcgacggcgc cgacgagatc cagcgcctgg
481 gcaccgcctg cttcgccaac tggcaggagg cggccgaggt gctggaccgc aacttccagc
541 tgccagagga cctggaggac caggagccac ggagcctcac cgagtgcctg cgccgccaca
601 agtaccgcgt ggaaaaggcg gcgcgaggcg ggcgcgaccc cgggggaggg ggtggggagg
661 agggcggggc cgggggctg ttctgacccg ctagggtgga ggagaggagt ggggtttgtt
721 gatgaacctc cacccccacc ccacccccgc acgcctgtaa tcccagcact tggggaggcc
781 aaggcgggag gatcgcttga gcccagaggt tcgagatcaa cctgggcaag agagtgagac
841 cctgactcta cgaaaattaa aagttagccc ggtgtggtgg cgcgcacctg tggcttagct
901 accctgaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaa (SEQ ID NO. 33)

FIGURE 17

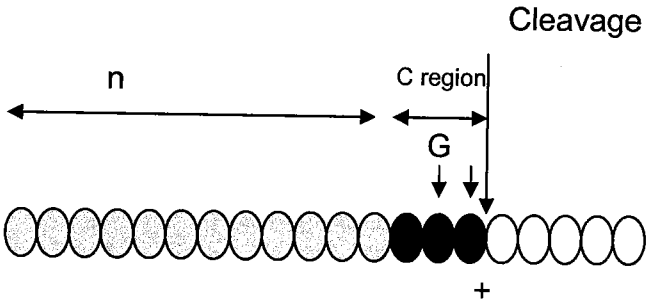
SIGNAL PEPTIDES



Prepillin like signal

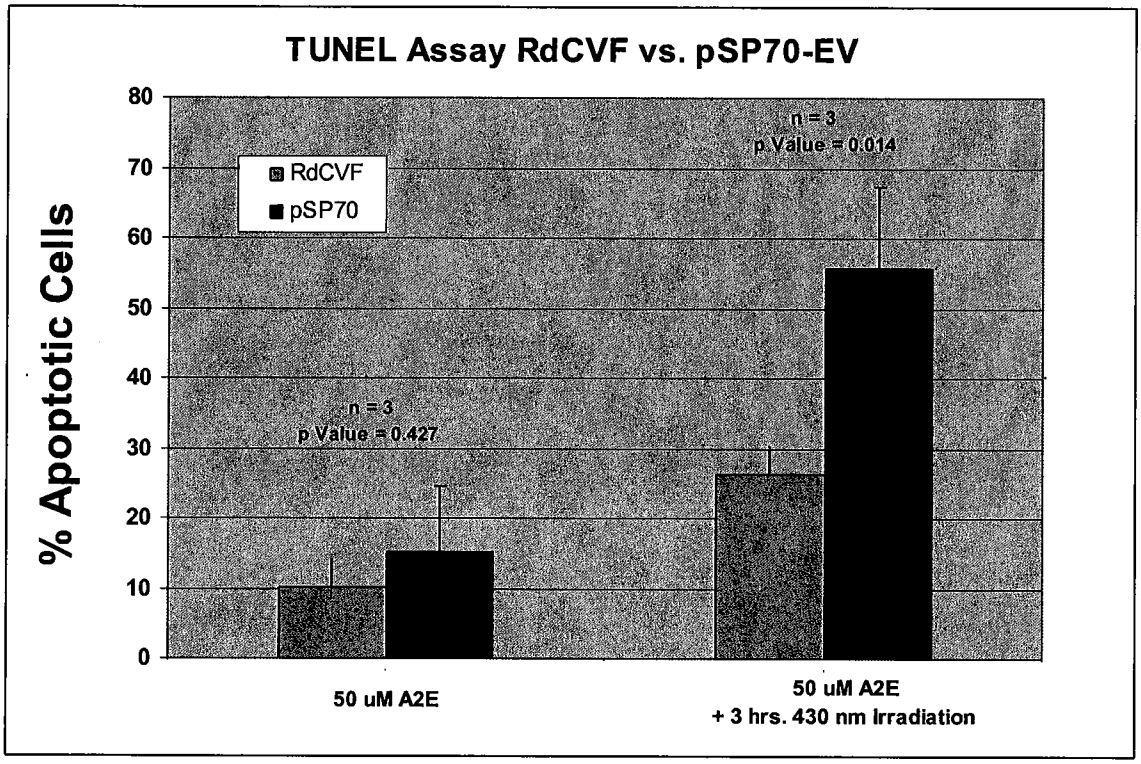


Bacterioicin Signal



All white circles are part of the mature protein
+1 indicates the first position of the mature protein

FIGURE 18


TUNEL Assay RdCVF vs. pSP70-EV
% Apoptotic Cells
80
70
60
50
40
30
20
10
0
RdCVF
pSP70
n = 3
p Value = 0.427
n = 3
p Value = 0.014
50 uM A2E
50 uM A2E
+ 3 hrs. 430 nm Irradiation

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/40645

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/64; C12N 15/09; A61K 31/713 (2009.01)
USPC - 435/91.4; 435/320.1; 435/455; 514/44
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) - C12N 15/64; C12N 15/09; A61K 31/713 (2009.01)
USPC - 435/91.4; 435/320.1; 435/455; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/6, 91.4, 320.1, 455; 514/44; class 800 as a whole
(Text Search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB, USPT, USOC, EPAB, JPAB); DialogPRO (Engineering); PubMed and Google Scholar.
Search Terms: rod-derived cone viability factor, signal peptide, furin, RdCVF, linker, human growth hormone, hGH, BDNF, IGF-1, GUSB, secret, secret$, cleavage, initiat$, methionine, Met, lack, lack$, missing, delet$, absent, absence

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2004/0204350 A1 (LEVEILLARD et al.) 14 October 2004 (14.10.2004) SEQ ID NO: 2, 6, 8, 10, 14; claim 28; para [0009], [0012], [0014], [0016], [0017], [0030], [0074], [0083], [0090], [0094]-[0097], [0130], [0150].	1-6, 8, 12 and 33-55
Y	US 2007/0166788 A1 (JIN et al.) 19 July 2007 (19.07.2007) SEQ ID NO: 91; table 5; para [0256], [0260], [0291], [0293], [0304], [0308], [0315], [0319], [0377], [0390], [0404].	1-6, 8, 12 and 33-55
Y	US 2002/0194643 A1 (MEROT et al.) 19 December 2002 (19.12.2002) para [0189], [0191].	3, 33, 36, 40, 51 and 53-55
Y	CHEN, T. et al. Isolation and Cloning of Exendin Precursor cDNAs from Single Samples of Venom from the Mexican Beaded Lizard (Heloderma rorridum) and the Gila Monster (Heloderma suspectum). Toxicon. March 2006, Vol 47(3), pages 288-295: fig 4.	5
Y	US 2005/0107318 A1 (WADSWORTH et al.) 19 May 2005 (19.05.2005) SEQ ID NO: 36; para [0021].	12 and 45

☐ 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
12 August 2009 (12.08.2009)	31 AUG 2009
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/40645

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.:
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. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: claims 1-55, directed to an expression vector for expression of rod-derived cone viability factor (RdCVF) and its use, restricted to the amino acid linker SEQ ID NO:4, the RdCVF-encoding sequence SEQ ID NO: 26, the signal peptide SEQ ID NO:28, and the furin cleavage marker SEQ ID NO: 3. In order for more inventions to be searched, an additional fee must be paid for each sequence and/or nucleotide/polypeptide coding pair to be searched.

Groups II - XI: claims 1-55 limited to a single nucleic acid/polypeptide sequence pair (SEQ ID NOs: 6 and 7; 8 and 9; 10 and 11; 12 and 13; 14 and 15; 16 and 17; 18 and 19, 20 and 21; 22 and 23; and 24 and 25 are the coding pairs).

Groups XII - XV: claims 1-55 limited to one of SEQ ID NOs 27, 29, 30 and 31, respectively.

**********Continued in Supplemental Box**********

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.:

Group I: Claims 1-55 restricted to SEQ ID NOs: 3, 4, 26 and 28 (namely, claims 1-6, 8, 12 and 33-55)

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.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US 09/40645

Continuation of :

Box No. III - Observations where unity of invention is lacking:

The groups of inventions listed above 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:

The Groups represented by the unique nucleic acid/amino acid sequences as designated do not relate to a single general inventive concept because, under PCT Rule 13.2, the different polynucleotides/polypeptides represented by the sequences are not common to one another but are different because they are composed of unique nucleic acid/amino acid sequences. No shared significant structural elements can be ascertained. Therefore, the groups do not have a shared special technical feature.

A separate fee must be paid for each sequence to be searched. In the case of a nucleotide sequence and its translated amino acid sequence cognate (e.g. SEQ ID NO:11 IGF-1 signal peptide-Furin-humanRdCVF(Long Form) Nucleotide Sequence and (SEQ ID NO: 10) IGF-I signal peptide-Furin-humanRdCVF (Long Form) Amino Acid Sequence), only a single fee will be required to search the nucleic acid/polypeptide sequence pair (SEQ ID NOs: 6 and 7; 8 and 9; 10 and 11; 12 and 13; 14 and 15; 16 and 17; 18 and 19, 20 and 21; 22 and 23; and 24 and 25 are the coding pairs). Apart from the first named invention comprising SEQ ID NO: 4, SEQ ID. NO: 26, SEQ ID NO: 28 and SEQ ID NO:3, a serparate fee will be necessary to search each of the sequences represented by each of the ten cognate pairs, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:30, or SEQ ID NO:31.