for improved methods and optimized nucleic acid expression cassettes and vectors for expressing genes in mammalian cells.

[0005] The present invention meets this need.

SUMMARY ~~OF THE INVENTION~~

[0006] The ~~invention~~disclosure relates generally to the fields of molecular biology and virology, and in particular, to gene expression cassettes and vectors comprising them for the delivery of nucleic acid segments encoding selected therapeutic constructs (for example, peptides and polypeptides) to selected cells and tissues of vertebrate animals. These genetic constructs are useful in the development of gene delivery vectors, including for example, herpes simplex virus (HSV), adenovirus (AV), and AAV vectors, for the treatment of mammalian, and in particular, human diseases, disorders, and dysfunctions.

[0007] More specifically, the ~~invention~~disclosure is directed to gene expression cassettes for enhanced expression of a secreted protein by a eukaryotic or mammalian cell, and to methods of making and using such cassettes for use in both research and therapeutic applications, including but not limited to a method of treating a disease or disorder caused by or associated with the deficiency, absence, or loss of function of one or more proteins in a subject in need thereof.

[0008] The present cassettes generally include a nucleic acid sequence, or transgene, encoding a polypeptide effective in the treatment of a medical condition in a human or non-human animal subject. In some ~~embodiment~~examples, the polypeptide is a secretory polypeptide. The cassette can be incorporated into a viral vector, such as an adeno-associated virus (AAV) vector, which can then be administered to eukaryotic or mammalian cells in vitro (e.g., in cell culture) or to a mammalian subject suffering from or at risk for developing a medical condition.

[0009] The disclosed compositions may be utilized in a variety of investigative, diagnostic and therapeutic regimens, including the prevention and treatment of human diseases. For example, the present compositions may find use in the treatment of an ocular disease, angiogenesis-dependent disease, disease that responds to treatment with a vascular endothelial growth factor (VEGF) inhibitor, or in enzyme replacement therapy, wherein the disease is caused by or associated with a deficiency in or loss of function of an enzyme.

[0010] Methods and compositions are provided for preparing polynucleotide expression cassettes and gene delivery vector compositions, e.g., viral vectors, comprising these expression cassettes for use in the preparation of medicaments useful in central and targeted gene therapy of diseases, disorders, and dysfunctions in an animal, and in humans in particular.

[0011] ~~Embodiment~~Examples of the present ~~invention~~disclosure comprise a non-naturally occurring polynucleotide cassette for enhanced expression of a transgene in a mammalian cell, comprising in 5' to 3' order: (a) a first enhancer region; (b) a promoter region; (c) a coding sequence encoding a polypeptide gene product; (d) a second enhancer region; and (e) a polyadenylation site. In some ~~embodiment~~examples, the polynucleotide cassette further comprises a ribonucleic acid (RNA) export signal located downstream of the second enhancer and upstream of the polyadenylation site. In one ~~embodiment~~example the polynucleotide cassette does not contain an RNA export signal. In a more specific form, the cassette does not contain an RNA export signal downstream of the second enhancer and upstream of the polyadenylation site.

[0012] In yet other ~~embodiment~~examples, the polynucleotide cassette further comprises an intron located downstream of the promoter and upstream of the coding sequence.

[0013] In still other ~~embodiment~~examples, the polynucleotide cassette further comprises a 5' untranslated region (5'UTR) that is located upstream of the coding sequence and downstream of the promoter.

[0014] In preferred ~~embodiment~~examples, the polypeptide gene product is a secretory protein.

[0015] Polynucleotide cassettes of the present ~~invention~~disclosure comprise a first enhancer region located upstream of a promoter region. In preferred ~~embodiment~~examples, the first enhancer region comprises a cytomegalovirus (CMV) sequence. In other ~~embodiment~~examples, the first enhancer region comprises an elongation factor 1 alpha (EF1α) sequence. In certain ~~embodiment~~examples the first enhancer region comprises a sequence with at least 85% identity SEQ ID NO: 1. In certain ~~embodiment~~examples, the identity is at least 90%, at least 95% or at least 99%.

[0016] Polynucleotide cassettes of the present ~~invention~~disclosure comprise a promoter region comprising a promoter sequence, or a functional fragment thereof. In some ~~embodiment~~examples, the promoter region is specific for eukaryotic cells, or more specifically mammalian cells. In some ~~embodiment~~examples, the promoter region comprises a promoter sequence selected from the

group consisting of an actin promoter, a cytomegalovirus (CMV) promoter, an elongation factor 1 alpha (EF1a) promoter, and a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter. In preferred ~~embodiment~~examples, the promoter region comprises a CMV promoter sequence or an EF1a promoter sequence. In certain ~~embodiment~~examples the promoter region comprises a sequence with at least 85% identity to one of the following SEQ ID NO: 3, 4, or 96. In certain ~~embodiment~~examples, the identity is at least 90%, at least 95% or at least 99%.

[0017] In some ~~embodiment~~examples, polynucleotide cassettes of the present ~~invention~~disclosure further comprise an intron region downstream of the promoter region. In some ~~embodiment~~examples, the intron sequence is located downstream of the promoter and upstream of the 5'UTR. In some ~~embodiment~~examples, the intron region comprises an elongation factor 1 alpha (EF1a), actin, or CMVc sequence. In certain ~~embodiment~~examples the intron comprises a sequence with at least 85% identity to one of the following SEQ ID NO: 5, 18, or 97. In certain ~~embodiment~~examples, the identity is at least 90%, at least 95% or at least 99%.

[0018] Polynucleotide cassettes of the present ~~invention~~disclosure comprise an untranslated region 5' of the coding sequence, referred to herein as a 5'UTR. In some ~~embodiment~~examples, the 5'UTR sequence is heterologous to the promoter sequence. The 5'UTR is located downstream of the promoter and upstream of the coding sequence. In some such ~~embodiment~~examples, the 5'UTR comprises a sequence selected from the group consisting of UTR1, UTR2, an enhancer element from the adenovirus major late promoter (eMLP), and the tripartite leader sequence from adenovirus (TPL sequence). In some ~~embodiment~~examples, the 5'UTR comprises a UTR1 sequence. In one ~~embodiment~~example, the 5'UTR comprises a UTR2 sequence. In preferred ~~embodiment~~examples, the 5'UTR comprises, in 5' to 3' order, a TPL sequence and an eMLP sequence. In some ~~embodiment~~examples, the 5'UTR does not comprise a polynucleotide ATG. In certain ~~embodiment~~examples the 5' UTR comprises a sequence with at least 85% identity to one of the following SEQ ID NO: 6, 11, 12, or 19. In certain ~~embodiment~~examples, the identity is at least 90%, at least 95% or at least 99%.

[0019] ~~Embodiment~~Examples of the present ~~invention~~disclosure comprise polynucleotide cassettes for the enhanced expression of transgenes. Accordingly, polynucleotide cassettes of the present ~~invention~~disclosure comprise a coding sequence, also referred to as a transgene. In one specific ~~embodiment~~example, the polynucleotide cassette comprises one coding sequence, or transgene, and does not comprise two or more transgenes. The transgene may encode a therapeutic agent such as for example a peptide or a polypeptide. In preferred ~~embodiment~~examples, the transgene

encodes a secretory polypeptide (also referred to herein as a secreted protein or secretory protein). Examples of secretory polypeptides that can be encoded by the coding sequence include, but are not limited to, soluble fms-like tyrosine kinase-1 (also known as sFLT-1), a VEGF-binding fragment of sFLT-1, aflibercept, and alpha-1 antitrypsin or α_1-antitrypsin (A1AT). In some ~~embodiment~~examples, the secretory polypeptide is an anti-angiogenic or anti-vascular endothelial growth factor (anti-VEGF) polypeptide.

[0020] The subject expression cassettes provide for enhanced expression of the transgene product (e.g., polypeptide) in a mammalian cell relative to the expression of the transgene product in the mammalian cell from a reference cassette. In preferred ~~embodiment~~examples a subject expression cassette provides for enhanced expression of a secretory protein in a eukaryotic cell in vivo or in vitro (e.g., in a cell culture or tissue explant) relative to the expression of the secretory protein in the eukaryotic cell in vivo or in vitro from a reference cassette. In some ~~aspect~~examples the eukaryotic cell is a mammalian cell. In still further ~~aspect~~examples the mammalian cell is a human cell.

[0021] In some ~~embodiment~~examples, the expression of a secretory polypeptide from the polynucleotide cassette in mammalian cells is at least about 2X, 3X, 5X, 9X, 10X, 20X, or 50X higher than the expression of the secretory polypeptide from a reference cassette in the mammalian cells in vitro or in vivo.

[0022] In some ~~embodiment~~examples, the expression level of a non-secreted polypeptide obtained from the polynucleotide cassette in mammalian cells is approximately the same as, less than about 1.5X greater than, or less than about 2X greater than the expression level of the non-secreted polypeptide obtained from a reference cassette in the mammalian cells in vitro or in vivo. One non-limiting example of a non-secreted polypeptide is green fluorescent protein (GFP).

[0023] According to some ~~embodiment~~examples, the reference cassette (also referred to herein as the CMV reference control cassette), referred to herein above and below, comprises, in 5' to 3' order, a CMV enhancer sequence (SEQ ID NO:2), a CMV promoter (SEQ ID NO:21), a chimeric intron (SEQ ID NO:22), a 5'UTR (SEQ ID NO:23), a coding sequence encoding the peptide or polypeptide gene product, a 3'UTR (SEQ ID NO:25), and an SV40 polyA sequence (SEQ ID NO:26).

[0024] According to specific ~~embodiment~~examples, enhanced expression of the peptide or polypeptide is observed in vitro in mammalian cells selected from the group consisting of HeLa cells, HEK-293 cells, and ARPE-19 cells. In another ~~embodiment~~example, the mammalian cells are contained in a retinal tissue explant.

[0025] In some forms, the expression of the secretory protein from the polynucleotide cassette in mammalian cells in vitro or in vivo (e.g., in a subject) is at least 2X, at least 5X, at least 10X, or from about 5X to about 10X greater than the expression of the secretory protein from the CMV reference control cassette in the mammalian cells under the same conditions, wherein the CMV reference control cassette comprises, in 5' to 3' order, the CMV enhancer sequence set forth in SEQ ID NO:2, the CMV promoter sequence set forth in SEQ ID NO:21, the chimeric intron sequence set forth in SEQ ID NO:22, the 5'UTR sequence set forth in SEQ ID NO:23, a coding sequence encoding the secretory polypeptide, the 3'UTR sequence set forth in SEQ ID NO:25, and the SV40 polyA sequence set forth in SEQ ID NO:26. In some ~~embodiment~~examples, the polynucleotide cassette increases the expression of the secretory protein in mammalian cells by at least 2-fold (2X), 5-fold (5X), 10-fold (10X), or from about 5- to about 10-fold as compared to the expression of the protein from the CMV reference control cassette in the mammalian cells. In one ~~embodiment~~example, the polynucleotide cassette enhances or increases the expression of a secreted protein in a transduced mammalian cell relative to the expression of the protein from the CMV reference control cassette in a transduced mammalian cell.

[0026] The polynucleotide cassette comprises a second enhancer region located downstream of the coding sequence and upstream of the polyadenylation signal sequence. When an RNA export signal sequence is present, the second enhancer sequence is located upstream of the RNA export signal (i.e., between the coding sequence and the RNA export signal), and the RNA export signal is located upstream of the polyadenylation signal. In preferred ~~embodiment~~examples, the second enhancer region comprises an expression enhancer sequence (EES) selected from the group consisting of the full EES, 410-564 EES, and 511-810 EES.

[0027] In some ~~embodiment~~examples, the polynucleotide cassette comprises an RNA export signal downstream of the second enhancer and upstream of the polyadenylation sequence (also referred to herein as a polyA sequence, polyA site, or polyadenylation region). The RNA export signal may comprise a sequence selected from the group consisting of human hepatitis

B virus post-transcriptional element (HPRE) sequence and woodchuck hepatitis virus post-transcriptional element (WPRE) sequence. In certain ~~embodiment~~examples the RNA export signal comprises a sequence with at least 85% identity to one of the following SEQ ID NO: 8, or 17. In certain ~~embodiment~~examples, the identity is at least 90%, at least 95% or at least 99%.

[0028] A polynucleotide cassette according to the present ~~invention~~disclosure comprises a polyadenylation site. The polyadenylation site may be located downstream of the second enhancer region or downstream of the RNA export region. In some ~~embodiment~~examples, the polyadenylation region comprises a human growth hormone (HGH or hGH), bovine growth hormone (BGH or bGH), or beta-globin (βglobin) polyA sequence. In certain ~~embodiment~~examples, the polyadenylation region comprises a sequence with at least 85% identity to one of the following SEQ ID NO: 9, 14, or 20. In certain ~~embodiment~~examples, the identity is at least 90%, at least 95% or at least 99%.

[0029] Some ~~embodiment~~examples of the present ~~invention~~disclosure are directed to a polynucleotide cassette for enhanced or increased expression of a secretory protein by mammalian cells, as measured by the amount (e.g., concentration or quantity) of protein in the extracellular environment, relative to the expression of the protein by a reference cassette in the mammalian cells. The amount of protein in a sample can be measured by, for example, immunoassay.

[0030] In some ~~embodiment~~examples, the polynucleotide cassette for enhanced expression of a transgene in a mammalian cell, comprises in 5' to 3' order: (a) a first enhancer region comprising a CMV sequence (SEQ ID NO:1); (b) a promoter region, comprising an EF1α sequence (SEQ ID NO:3); (c) an intron region comprising an EF1α sequence (SEQ ID NO:5); (d) a 5'UTR region comprising an UTR2 sequence (SEQ ID NO:6); (e) a coding sequence encoding a peptide or polypeptide; (f) a second enhancer region comprising a 511-810 EES sequence (SEQ ID NO:7); (g) an WPRE RNA export sequence (SEQ ID NO:8); and (h) a BGH polyadenylation site (SEQ ID NO:9). In certain of these ~~embodiment~~examples, the polynucleotide cassette comprises one or more sequences selected from SEQ ID NO: 70–75 or a sequence with at least 85% identity thereto. In certain of these ~~embodiment~~examples the 5' arm of the polynucleotide cassette comprises or consists of SEQ ID NO: 45 or a sequence with at least 85% identity thereto. In certain of these ~~embodiment~~examples the 3' arm of the polynucleotide cassette comprises or consists of SEQ ID NO: 46 or a sequence with at least 85% identity thereto.

[0031] In other ~~embodiment~~examples, the polynucleotide cassette for enhanced expression of a transgene in a mammalian cell, comprises in 5' to 3' order: (a) a first enhancer region comprising a CMV sequence (SEQ ID NO:1); (b) a promoter region, comprising a CMV sequence (SEQ ID NO:4); (c) a 5'UTR region comprising, in 5' to 3' order, TPL and eMLP sequences (SEQ ID NO:11 and SEQ ID NO:12, respectively); (d) a coding sequence encoding a peptide or polypeptide; (e) a second enhancer region comprising a full EES sequence (SEQ ID NO:13); and (f) a HGH polyadenylation site (SEQ ID NO:14). In one aspect, the present disclosure refers to a non-naturally occurring polynucleotide cassette comprising in 5' to 3' order: (a) a first enhancer region comprising a CMV sequence consisting of SEQ ID NO:1 or a sequence having at least 99% identity to SEQ ID NO:1; (b) a promoter region comprising a CMV sequence consisting of SEQ ID NO:4 or a sequence having at least 99% identity to SEQ ID NO:4; (c) a 5'UTR region comprising, in 5' to 3' order, a tripartite leader (TPL) sequence consisting of SEQ ID NO:11 or a sequence having at least 99% identity to SEQ ID NO:11, an enhancer element from the adenovirus major late promoter (eMLP) sequence consisting of SEQ ID NO:12 or a sequence having at least 99% identity thereto, a mammalian intron acceptor, and a Kozak sequence; (d) a coding sequence encoding a secretory polypeptide, wherein the coding sequence is codon optimized for translation in primates and operably linked to the promoter region; (e) an enhancer region comprising a full expression enhancer sequence (EES) sequence consisting of SEQ ID NO:13 or a sequence having at least 99% identity to SEQ ID NO:13; and (f) a human growth hormone (HGH) polyadenylation site consisting of SEQ ID NO:14 or a sequence having at least 99% identity to SEQ ID NO:14; and wherein: the first enhancer region (a), the promoter region (b), and the 5' UTR region (c) define a 5' arm; the second enhancer region (e), and the HGH polyadenylation site (f) define a 3' arm. In certain of these ~~embodiment~~examples, the polynucleotide cassette comprises one or more sequences selected from SEQ ID NO: 76–80 or a sequence with at least 85% identity thereto. In certain of these ~~embodiment~~examples the 5' arm of the polynucleotide cassette comprises or consists of SEQ ID NO: 47 or a sequence with at least 85% identity thereto. In certain of these ~~embodiment~~examples the 3' arm of the polynucleotide cassette comprises or consists of SEQ ID NO: 48 or a sequence with at least 85% identity thereto.

[0032] In other ~~embodiment~~examples, the polynucleotide cassette for enhanced expression of a transgene in a mammalian cell, comprises in 5' to 3' order: (a) a first enhancer region comprising a CMV sequence (SEQ ID NO:1); (b) a promoter region, comprising a CMV sequence (SEQ ID NO:4); (c) a 5'UTR region comprising, in 5' to 3' order, TPL and eMLP sequences (SEQ ID NO:11 and SEQ ID NO:12, respectively); (e) a coding sequence encoding a peptide or polypeptide; (f) a second enhancer region comprising a 410-564 EES sequence (SEQ ID NO:16); (g) an HPRE RNA export sequence (SEQ ID NO:17); and (h) a BGH polyadenylation site (SEQ ID NO:9). In certain of these ~~embodiment~~examples, the polynucleotide cassette comprises one or more sequences selected from SEQ ID NO: 81–86 or a sequence with at least 85% identity thereto. In certain of these ~~embodiment~~examples the 5' arm of the polynucleotide cassette comprises or consists of SEQ ID NO: 49 or a sequence with at least 85% identity thereto. In certain of these ~~embodiment~~examples the 3' arm of the polynucleotide cassette comprises or consists of SEQ ID NO: 50 or a sequence with at least 85% identity thereto.

[0033] In other ~~embodiment~~examples, the polynucleotide cassette for enhanced expression of a transgene in a mammalian cell, comprises in 5' to 3' order: (a) a first enhancer region comprising a CMV sequence (SEQ ID NO:1); (b) a promoter region, comprising an actin sequence (SEQ ID NO:96); (c) a 5' UTR comprising an eMLP sequences (SEQ ID NO:12); (e) a coding sequence encoding a peptide or polypeptide; (f) a second enhancer region

comprising a 511-810 EES sequence (SEQ ID NO:7); (g) an HPRE RNA export sequence (SEQ ID NO:17); and (h) a rabbit Beta Globin polyadenylation site (SEQ ID NO:20). In certain of these ~~embodiment~~examples, the polynucleotide cassette comprises one or more sequences selected from SEQ ID NO: 87–91 or a sequence with at least 85% identity thereto. In certain of these ~~embodiment~~examples the 5' arm of the polynucleotide cassette comprises or consists of SEQ ID NO: 51 or a sequence with at least 85% identity thereto. In certain of these ~~embodiment~~examples the 3' arm of the polynucleotide cassette comprises or consists of SEQ ID NO: 52 or a sequence with at least 85% identity thereto.

[0034] In other ~~embodiment~~examples, the polynucleotide cassette for enhanced expression of a transgene in a mammalian cell, comprises in 5' to 3' order: (a) a first enhancer region comprising a CMV sequence (SEQ ID NO:1); (b) a promoter region, comprising a CMV sequence (SEQ ID NO:4); (c) an intron region comprising an CMVc sequence (SEQ ID NO:18); (d) a 5'UTR region comprising a UTR1 sequence (SEQ ID NO:19); (e) a coding sequence encoding a peptide or a polypeptide; (f) a second enhancer region comprising a full EES sequence (SEQ ID NO:13); (g) an WPRE RNA export sequence (SEQ ID NO:8); and (h) a rabbit Beta Globin polyadenylation site (SEQ ID NO:20). In certain of these ~~embodiment~~examples, the polynucleotide cassette comprises one or more sequences selected from SEQ ID NO: 92–95 or a sequence with at least 85% identity thereto. In certain of these ~~embodiment~~examples the 5' arm of the polynucleotide cassette comprises or consists of SEQ ID NO: 53 or a sequence with at least 85% identity thereto. In certain of these ~~embodiment~~examples the 3' arm of the polynucleotide cassette comprises or consists of SEQ ID NO: 54 or a sequence with at least 85% identity thereto.

[0035] In other ~~embodiment~~examples, the polynucleotide cassette for enhanced expression of a transgene in a mammalian cell, comprises in 5' to 3' order: (a) a first enhancer region comprising a CMV sequence (SEQ ID NO:1); (b) a promoter region, comprising an actin sequence (SEQ ID NO:96); (c) an intron region comprising a chicken beta-actin sequence (SEQ ID NO:97); (d) a 5'UTR region comprising a UTR1 sequence (SEQ ID NO:19); (e) a coding sequence encoding a peptide or a polypeptide; (f) a second enhancer region comprising a 410-564 EES sequence (SEQ ID NO:16); (g) an HPRE RNA export sequence (SEQ ID NO:17); and (h) a BGH polyadenylation site (SEQ ID NO:9). In certain of these ~~embodiment~~examples, the polynucleotide cassette comprises one or more sequences selected from SEQ ID NO: 92–95 or a sequence with at least 85% identity thereto. In certain of these

~~embodiment~~examples the 5’ arm of the polynucleotide cassette comprises or consists of SEQ ID NO: 39 or a sequence with at least 85% identity thereto. In certain of these ~~embodiment~~examples the 3’ arm of the polynucleotide cassette comprises or consists of SEQ ID NO: 40 or a sequence with at least 85% identity thereto.

[0036] In certain ~~embodiment~~examples, the polynucleotide cassette comprises or consists essentially of in 5’ to 3’ order: (a) a 5’ arm; (b) a coding sequence encoding a peptide or a polypeptide; and (c) a 3’ arm. In certain of these ~~embodiment~~examples, the 5’ arm comprises a sequence selected from the group consisting of SEQ ID NO: 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, and 61 or a sequence with at least 85% identity thereto, and the 3’ arm comprises a sequence selected from the group consisting of SEQ ID NO: 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, and 62 or a sequence with at least 85% identity thereto. In particular ~~embodiment~~examples, the 5’ arm and 3’ arm are, respectively, SEQ ID NO: 27 and 28, or SEQ ID NO: 29 and 30, or SEQ ID NO: 31 and 32, or SEQ ID NO: 33 and 34, or SEQ ID NO: 35 and 36, or SEQ ID NO: 37 and 38, or SEQ ID NO: 39 and 40, or SEQ ID NO: 41 and 42, or SEQ ID NO: 43 and 44, or SEQ ID NO: 45 and 46, or SEQ ID NO: 47 and 48, or SEQ ID NO: 49 and 50, or SEQ ID NO: 51 and 52, or SEQ ID NO: 53 and 54 or SEQ ID NO: 55 and 56, or SEQ ID NO: 57 and 58, or SEQ ID NO: 59 and 60, or SEQ ID NO: 61 and 62.

[0037] In preferred forms of the ~~invention~~disclosure, the peptide or polypeptide encoded by the coding sequence in the subject polynucleotide cassette is one that is secreted or exported from the cell following its expression in the cell.

[0038] In some ~~aspect~~examples of the ~~invention~~disclosure, gene delivery vectors are provided comprising a polynucleotide cassette of the present ~~invention~~disclosure. In some ~~embodiment~~examples, the gene delivery vector is a recombinant virus comprising (a) a capsid protein, and (b) a polynucleotide cassette of the present ~~invention~~disclosure. In more specific ~~embodiment~~examples, the recombinant virus is a recombinant adeno-associated virus (AAV), wherein the recombinant adeno-associated virus comprises an AAV capsid protein and a polynucleotide cassette of the present ~~invention~~disclosure. In some ~~embodiment~~examples, the AAV capsid protein is a wild type AAV capsid protein. In other ~~embodiment~~examples, the AAV capsid protein is a variant AAV capsid protein, wherein the variant capsid protein contains a substitution, deletion, or an insertion of one or more amino acids relative to the parental capsid protein or the capsid protein from which it is derived. For example, in one ~~embodiment~~example from about 5 to about 11 amino acids are inserted in an

insertion site in the GH loop or loop IV of the VP1 capsid protein relative to a corresponding parental AAV capsid protein. Suitable examples include AAV variant capsids having the 7m8 variant capsid protein or a capsid protein that is derived from the AAV variant 7m8 capsid protein. In one particular example, the variant AAV comprises the AAV2.7m8 capsid protein. In one aspect, the present disclosure refers to a recombinant adeno-associated virus comprising: a) a capsid protein, wherein the capsid protein is an AAV variant 7m8 capsid protein or is derived from the AAV variant 7m8 capsid protein, and b) an adeno-associated virus vector genome comprising the polynucleotide cassette as disclosed herein.

[0039] In some ~~aspect~~examples of the ~~invention~~disclosure, pharmaceutical compositions are provided comprising a polynucleotide cassette of the ~~invention~~disclosure and a pharmaceutically acceptable excipient. In some ~~embodiment~~examples, the pharmaceutical composition comprises a gene delivery vector of the ~~invention~~disclosure and a pharmaceutical excipient. In one aspect, the present disclosure refers to a pharmaceutical composition comprising the recombinant adeno-associated virus as disclosed herein and a pharmaceutically acceptable excipient.

[0040] One ~~embodiment~~example is an isolated host cell transfected or transduced with a polynucleotide cassette of the present ~~invention~~disclosure.

[0041] In some ~~aspect~~examples of the ~~invention~~disclosure, methods are provided for expressing a transgene in mammalian cells in vitro or in vivo, the method comprising contacting one or more mammalian cells, in vivo or in vitro, with an amount of a polynucleotide cassette, gene delivery vector, or pharmaceutical composition of the ~~invention~~disclosure, wherein the transgene is expressed at detectable levels in the one or more mammalian cells, or, more preferably, is expressed at a level that is about 2-fold, 5-fold, or 10-fold higher than that obtained from (i.e., as compared to the expression of the transgene from) a reference CMV cassette comprising, in 5' to 3' order, a CMV enhancer sequence (SEQ ID NO:2), a CMV promoter (SEQ ID NO:21), a chimeric intron (SEQ ID NO:22), a 5'UTR (SEQ ID NO:23), the transgene or coding sequence of interest, a 3'UTR (SEQ ID NO:25), and an SV40 polyA sequence (SEQ ID NO:26), in the cells or cell culture, measured under the same conditions (i.e., other variables remaining constant). Some ~~embodiment~~examples provide a method for expressing a transgene in mammalian cells in vitro or in vivo, comprising contacting one or more mammalian cells in vitro or in vivo with an amount of recombinant virus of the present ~~invention~~disclosure, wherein the recombinant virus comprises a polynucleotide cassette according to the present disclosure. In preferred ~~embodiment~~examples the cassette or transgene encodes a secretory protein (also referred to herein as a secretory polypeptide) and the secretory protein is expressed in the one or more mammalian cells at a level that is at least 2X, 5X, 10X, from about 5X to about 10X, or greater than 20X higher than that obtained by contacting the cells with a recombinant virus comprising the CMV reference control cassette encoding the same protein. In some ~~aspect~~examples, the mammalian cells are contacted with an amount of polynucleotide cassette, pharmaceutical composition, or vector effective for altering one or

more characteristics of the cell or for reducing one or more signs or symptoms of a disease in an individual.

[0042] In some ~~aspect~~examples of the ~~invention~~disclosure, methods are provided for the treatment or prophylaxis of a disease or disorder in a mammal in need of treatment or prophylaxis for a disease or disorder. In some ~~embodiment~~examples, the method comprises administering to the mammal an effective amount of a pharmaceutical composition of the ~~invention~~disclosure, wherein the coding sequence encodes a therapeutic gene product. In one ~~embodiment~~example the disease is an ocular disease and/or is a disease associated with a loss of function of or deficiency in a cellular protein product. In one ~~embodiment~~example, the ocular disease is associated with or caused by ocular neovascularization. One ~~embodiment~~example is a method for treating an ocular disease in a mammal in need thereof, comprising administering to the eye of a mammal in need of treatment a therapeutically effective amount of a pharmaceutical composition of the ~~invention~~disclosure. In a more specific ~~embodiment~~example, a therapeutically effective amount of a pharmaceutical composition of the ~~invention~~disclosure is administered to an eye of the individual in need of treatment by way of intraocular injection or by way of intravitreal injection.

[0043] Ocular diseases for which the subject cassettes, compositions, and methods may find use include acute macular neuroretinopathy; Behcet's disease; choroidal neovascularization; diabetic uveitis; histoplasmosis; macular degeneration, such as acute macular degeneration, non-exudative age related macular degeneration, and exudative age related macular degeneration; edema, such as macular edema, cystoid macular edema and diabetic macular edema; multifocal choroiditis; ocular trauma which affects a posterior ocular site or location; ocular tumors; retinal disorders, such as retinal vein occlusion, central retinal vein occlusion, diabetic retinopathy (including proliferative diabetic retinopathy), proliferative vitreoretinopathy (PVR), retinal arterial occlusive disease, retinal detachment, uveitic retinal disease; sympathetic ophthalmia; Vogt Koyanagi-Harada (VKH) syndrome; uveal diffusion; a posterior ocular condition caused by or influenced by an ocular laser treatment; posterior ocular conditions caused by or influenced by a photodynamic therapy; photocoagulation, radiation retinopathy; epiretinal membrane disorders; branch retinal vein occlusion; anterior ischemic optic neuropathy; non-retinopathy diabetic retinal dysfunction; retinoschisis; retinitis pigmentosa; glaucoma; Usher syndrome, cone-rod dystrophy; Stargardt disease (fundus flavimaculatus); inherited macular degeneration; chorioretinal degeneration; Leber congenital amaurosis; congenital stationary night blindness; choroideremia; Bardet-Biedl

CLAIMS

What is claimed is:

1. A non-naturally occurring polynucleotide cassette ~~for enhanced expression of a transgene in a mammalian cell,~~ comprising in 5' to 3' order:

(a) a first enhancer region comprising a CMV sequence consisting of SEQ ID NO:1 or a sequence having at least 99% identity to SEQ ID NO:1;

(b) a promoter region comprising a CMV sequence consisting of SEQ ID NO:4 or a sequence having at least 99% identity to SEQ ID NO:4;

(c) a 5'UTR region comprising, in 5' to 3' order, a tripartite leader (TPL) sequence consisting of SEQ ID NO:11 or a sequence having at least 99% identity to SEQ ID NO:11, an enhancer element from the adenovirus major late promoter (eMLP) sequence consisting of SEQ ID NO:12 or a sequence having at least 99% identity thereto, a mammalian intron acceptor, and a Kozak sequence;

(d~~c~~) a coding sequence encoding a secretory polypeptide, wherein the coding sequence is codon optimized for translation in primates and operably linked to the promoter region;

(e~~d~~) an ~~second~~ enhancer region comprising a full expression enhancer sequence (EES) sequence consisting of SEQ ID NO:13 or a sequence having at least 99% identity to SEQ ID NO:13; and

(f~~e~~) a human growth hormone (HGH) polyadenylation site consisting of SEQ ID NO:14 or a sequence having at least 99% identity to SEQ ID NO:14;~~,~~

and wherein:

the first enhancer region (a), the promoter region (b), and the 5' UTR region (c) define a 5' arm;

the second enhancer region (e), and the HGH polyadenylation site (f) define a 3' arm.

~~wherein the coding sequence is operably linked to the promoter region,~~

~~wherein the expression of the secretory polypeptide from the polynucleotide cassette in mammalian cells is at least 5X higher than the expression of the secretory polypeptide from a reference cassette in the mammalian cells, and~~

~~wherein the reference cassette comprises, in 5' to 3' order, a CMV enhancer sequence (SEQ ID NO:2), a CMV promoter (SEQ ID NO:21), a chimeric intron (SEQ ID NO:22), a 5'UTR (SEQ ID NO:23), a coding sequence encoding the secretory polypeptide, a 3'UTR (SEQ ID NO:25), and an SV40 polyA sequence (SEQ ID NO:26).~~

2. The polynucleotide cassette according toof claim 1, wherein the cassette does not comprisecontain an RNA export signal.

~~3. The polynucleotide cassette of any of the preceding claims, wherein the first enhancer region comprises the cytomegalovirus (CMV) sequence set forth in SEQ ID NO:1 or a sequence with at least 85% identity thereto.~~

~~4. The polynucleotide cassette of any of the preceding claims, wherein the promoter region comprises the CMV promoter sequence set forth in SEQ ID NO:4 or a sequence with at least 85% identity thereto.~~

~~5. The polynucleotide cassette of any of the preceding claims, further comprising an untranslated region (5'UTR) downstream of the promoter region and upstream of the coding sequence, wherein the 5'UTR comprises, in 5' to 3' order, a TPL sequence according to SEQ ID NO:11 or a sequence with at least 85% identity thereto, and an eMLP sequence according to SEQ ID NO:12 or a sequence with at least 85% identity thereto.~~

~~6. The polynucleotide cassette of any of the preceding claims, wherein the second enhancer comprises the full EES sequence set forth in SEQ ID NO:13 or a sequence with at least 85% identity thereto.~~

~~7. The polynucleotide cassette of any of the preceding claims, wherein the polyadenylation site comprises the human growth hormone (HGH) polyadenylation site set forth in SEQ ID NO:14 or a sequence with at least 85% identity thereto.~~

~~8~~3. The polynucleotide cassette of ~~any of the preceding~~ claim~~s~~ 1, further ~~comprising one or more regions~~ comprising each of~~a sequence selected from~~ SEQ ID NO~~s~~: 76–80 ~~or a sequence with at least 85% identity thereto~~.

4. The polynucleotide cassette according to claim 1, wherein the 5' arm comprises a sequence with at least 99% identity to SEQ ID NO: 47.

5. The polynucleotide cassette according to claim 1, wherein the 5' arm consists of SEQ ID NO: 47.

6. The polynucleotide cassette according to claim 1, wherein the 3' arm comprises a sequence with at least 99% identity to SEQ ID NO: 48.

7. The polynucleotide cassette according to claim 1, wherein the 3' arm consists of SEQ ID NO: 48.

8. The polynucleotide cassette according to claim 4, wherein the 3' arm comprises a sequence with at least 99% identity to SEQ ID NO: 48.

9. The polynucleotide cassette according to claim 5, wherein the 3' arm consists of SEQ ID NO: 48.

~~9. The polynucleotide cassette of any of the preceding claims, wherein the secretory polypeptide is an anti-angiogenic polypeptide.~~

10. The polynucleotide cassette of claim 1~~any of the preceding claims~~, wherein the secretory polypeptide is selected from the group consisting of~~comprises~~ soluble fms-like tyrosine kinase-1 ~~(sFLT-1),~~ ~~or~~ a VEGF-binding fragment of sFLT-1, and aflibercept.

11. The polynucleotide cassette of claim 1, wherein the secretory polypeptide is aflibercept.

~~11. A polynucleotide cassette according to claim 1, comprising in 5' to 3':~~

~~(a) a first enhancer region comprising a CMV sequence consisting of SEQ ID NO:1 or a sequence having at least 85% identity thereto;~~

~~(b) a promoter region comprising a CMV sequence consisting of SEQ ID NO:4 or a sequence having at least 85% identity thereto;~~

~~(c) a 5'UTR region comprising, in 5' to 3' order, a TPL sequence consisting of SEQ ID NO:11 or a sequence having at least 85% identity thereto, and an eMLP sequence consisting of SEQ ID NO:12 or a sequence having at least 85% identity thereto;~~

~~(d) a coding sequence encoding said secretory polypeptide, wherein the coding sequence is operably linked to the promoter region;~~

~~(e) a second enhancer region comprising a full EES sequence consisting of SEQ ID~~

~~NO:13 or a sequence having at least 85% identity thereto; and~~

~~(f) a HGH polyadenylation site consisting of SEQ ID NO:14 or a sequence having at least 85% identity thereto, and~~

~~optionally wherein the cassette does not comprise an RNA export signal.~~

~~12. The polynucleotide cassette of claim 11, further comprising each of SEQ ID NO: 76–80.~~

~~13. A polynucleotide cassette according to claim 1, comprising in 5' to 3':~~

~~(a) a first enhancer region comprising a CMV sequence consisting of SEQ ID NO:1 or a sequence having at least 85% identity thereto;~~

~~(b) a promoter region, comprising an EF1α sequence consisting of SEQ ID NO:3 or a sequence having at least 85% identity thereto;~~

~~(c) an intron region comprising an EF1α sequence consisting of SEQ ID NO:5 or a sequence having at least 85% identity thereto;~~

~~(d) a 5'UTR region comprising an UTR2 sequence consisting of SEQ ID NO:6 or a sequence having at least 85% identity thereto;~~

~~(e) a coding sequence encoding said secretory polypeptide, wherein the coding sequence is operably linked to the promoter region;~~

~~(f) a second enhancer region comprising a 511-810 EES sequence consisting of SEQ ID NO:7 or a sequence having at least 85% identity thereto;~~

~~(g) an WPRE RNA export sequence consisting of SEQ ID NO:8 or a sequence having at least 85% identity thereto; and~~

~~(h) a BGH polyadenylation site consisting of SEQ ID NO:9 or a sequence having at least 85% identity thereto.~~

~~14. The polynucleotide cassette of claim 13, further comprising each of SEQ ID NO: 70–75.~~

~~15. A polynucleotide cassette according to claim 1, comprising in 5' to 3':~~

~~(a) a first enhancer region comprising a CMV sequence consisting of SEQ ID NO:1 or a sequence having at least 85% identity thereto;~~

~~(b) a promoter region, comprising a CMV sequence consisting of SEQ ID NO:4 or a~~

~~sequence having at least 85% identity thereto;~~

~~(c) a 5'UTR region comprising, in 5' to 3' order, TPL and eMLP sequences consisting of SEQ ID NO:11 and SEQ ID NO:12, respectively or a sequence having at least 85% identity thereto;~~

~~(e) a coding sequence encoding a peptide or polypeptide;~~

~~(f) a second enhancer region comprising a 410-564 EES sequence consisting of SEQ ID NO:16 or a sequence having at least 85% identity thereto;~~

~~(g) an HPRE RNA export sequence consisting of SEQ ID NO:17 or a sequence having at least 85% identity thereto; and~~

~~(h) a BGH polyadenylation site consisting of SEQ ID NO:9 or a sequence having at least 85% identity thereto.~~

~~16. The polynucleotide cassette of claim 15, further comprising each of SEQ ID NO: 81–86.~~

~~17. A polynucleotide cassette according to claim 1, comprising in 5' to 3':~~

~~(a) a first enhancer region comprising a CMV sequence consisting of SEQ ID NO:1 or a sequence having at least 85% identity thereto;~~

~~(b) a promoter region, comprising an actin sequence consisting of SEQ ID NO:96 or a sequence having at least 85% identity thereto;~~

~~(c) a 5' UTR comprising an eMLP sequences consisting of SEQ ID NO:12 or a sequence having at least 85% identity thereto;~~

~~(e) a coding sequence encoding a peptide or polypeptide;~~

~~(f) a second enhancer region comprising a 511-810 EES sequence consisting of SEQ ID NO:7 or a sequence having at least 85% identity thereto;~~

~~(g) an HPRE RNA export sequence consisting of SEQ ID NO:17 or a sequence having at least 85% identity thereto; and~~

~~(h) a rabbit Beta Globin polyadenylation site consisting of SEQ ID NO:20 or a sequence having at least 85% identity thereto.~~

~~18. The polynucleotide cassette of claim 17, further comprising each of SEQ ID NO: 87–91.~~

~~19. A polynucleotide cassette according to claim 1, comprising in 5' to 3':~~

~~(a) a first enhancer region comprising a CMV sequence consisting of SEQ ID NO:1 or a sequence having at least 85% identity thereto;~~

~~(b) a promoter region, comprising a CMV sequence consisting of SEQ ID NO:4 or a sequence having at least 85% identity thereto;~~

~~(c) an intron region comprising an CMVc sequence consisting of SEQ ID NO:18 or a sequence having at least 85% identity thereto;~~

~~(d) a 5'UTR region comprising a UTR1 sequence consisting of SEQ ID NO:19 or a sequence having at least 85% identity thereto;~~

~~(e) a coding sequence encoding a peptide or a polypeptide;~~

~~(f) a second enhancer region comprising a full EES sequence consisting of SEQ ID NO:13 or a sequence having at least 85% identity thereto;~~

~~(g) an WPRE RNA export sequence consisting of SEQ ID NO:8 or a sequence having at least 85% identity thereto; and~~

~~(h) a rabbit Beta Globin polyadenylation site consisting of SEQ ID NO:20 or a sequence having at least 85% identity thereto.~~

~~20. The polynucleotide cassette of claim 19, further comprising each of SEQ ID NO: 92–95.~~

~~21~~12. A recombinant adeno-associated virus comprising:

a) a capsid protein, wherein the capsid protein is an AAV variant 7m8 capsid protein or is derived from the AAV variant 7m8 capsid protein, and

b) an adeno-associated virus vector genome comprising the polynucleotide cassette according to claim 1~~any of the preceding claims~~.

~~22. The recombinant virus of claim 21 wherein the recombinant virus is a recombinant adeno-associated virus.~~

~~23. The recombinant virus of claim 22 wherein the capsid protein is an AAV variant 7m8 capsid protein or is derived from the AAV variant 7m8 capsid protein.~~

13. The recombinant adeno-associated virus of claim 12, wherein the secretory polypeptide is aflibercept.

~~24~~14. A pharmaceutical composition comprising the recombinant adeno-associated virus of claim 12~~any one of claims 21–23~~ and a pharmaceutically acceptable excipient.

15. The pharmaceutical composition of claim 14, wherein the secretory polypeptide is aflibercept.

16. The polynucleotide cassette according to claim 1, wherein the 5' arm comprises a sequence with at least 99% identity to SEQ ID NO: 47, and wherein the 3' arm comprises a sequence with at least 99% identity to SEQ ID NO: 48.

17. The polynucleotide cassette according to claim 1, wherein the 5' arm comprises SEQ ID NO: 47, and wherein the 3' arm comprises SEQ ID NO: 48.

~~25. An isolated host cell transfected or transduced with the polynucleotide cassette of claim 1.~~

~~26. A method for expressing a transgene in mammalian cells, comprising contacting one or more mammalian cells with an amount of the recombinant virus of any one of claims 21–23, wherein the secretory polypeptide is expressed in the one or more mammalian cells at a level that is at least 5X higher than that obtained by contacting the cells with a recombinant virus comprising a reference cassette encoding the secretory polypeptide, wherein the reference cassette comprises, in 5' to 3' order, a CMV enhancer sequence (SEQ ID NO:2), a CMV promoter (SEQ ID NO:21), a chimeric intron (SEQ ID NO:22), a 5'UTR (SEQ ID NO:23), a coding sequence encoding the secretory polypeptide, a 3'UTR (SEQ ID NO:25), and an SV40 polyA sequence (SEQ ID NO:26).~~

~~27. A method for the treatment or prophylaxis of a disease in a mammal in need of treatment or prophylaxis for a disease, comprising administering to the mammal an effective amount of the pharmaceutical composition of claim 24.~~

~~28. The method of claim 27, wherein the disease is an ocular disease and the pharmaceutical composition is administered to the eye of the mammal.~~

~~29. The method of claim 28, wherein the pharmaceutical composition is administered to an eye of the mammal via intraocular injection or intravitreal injection.~~

~~30. The method of claim 29, wherein the ocular disease is selected from the group consisting of choroidal neovascularization and macular degeneration.~~