[0009] Additionally, traditional gene therapy vectors for delivery of transgenes (e.g. adeno-associated virus (AAV), adenovirus, lentivirus vectors, etc.) are typically limited to a single administration of the vector to patients, due in part to the patient's immune response to viral proteins. Additionally, for sustained long term expression of the transgene, it is typically is required to administer a high titer on the initial administration, which can lead to deleterious side effects. As such, traditional viral vectors for gene therapy lack utility due to lack of sustained, long term transgene expression. In addition, the range of transgene genetic material suitable for delivery in such viral vectors is limited by the viral packaging capacity of the viral capsid proteins (e.g. about 4.5kb for AAV), thereby excluding delivery of larger transgenes for therapy. With respect to conventional adeno-associated virus (AAV) vectors for gene therapy, their use is limited due to the single administration to patients (owing to the patient immune response), the limited range of transgene genetic material suitable for delivery in AAV vectors due to minimal viral packaging capacity (about 4.5kb), and slow AAV-mediated gene expression.

[0010] Additionally, there have been a number of reports raising concern about using too high a dose of a viral vector. Most viral vectors also suffer the immunogenicity concerns raised about AAV.

[0011] Accordingly, there is need in the field for a technology that allows multiple doses of a vector for gene therapy. In addition, there is a need in the art for methods of controlling gene expression from a gene therapy vector with minimal off-target effects, and there remains an important unmet need for controllable recombinant DNA vectors with improved production and/or expression properties. Further, as will be appreciated by a skilled physician, the ability to titrate the expression/dose of a transgene is desired to customize a gene therapy treatment based on a subject's particular set of symptoms and/or severity of disease and further to minimize side effects or toxicity.

SUMMARY

[0012] The disclosure described herein is a capsid-free DNA vector with covalently-closed ends (referred to herein as a "closed-ended DNA vector" or a "ceDNA vector") for controlled expression of a transgene in a cell, e.g., to treat a disease. In particular, the technology described herein relates to capsid-free close-ended DNA (ceDNA) vectors for controlled expression of a transgene, including but not limited to any of, sustained expression of a transgene, long-term controlled expression of a transgene, dose-dependent and/or tritratable expression of a transgene, and repeat dosing of a transgene using the vectors described herein. Accordingly, the methods disclosed herein enable one to personalize gene therapy throughout an individual's lifespan to express a transgene at a level that meets the individual's needs, by sustaining the transgene expression level at a predetermined level for a pre-determined time, or alternatively, increasing the expression of the transgene in a dose-dependent manner, by one or more administration after the initial priming administration, thereby controlling the transgene expression level

to a desired expression level or desired expression level range based on the concentration of ceDNA vector in the re-dose administration, allowing for a controlled and specific increases in the expression of the transgene in the cell or subject.

[0012a] In one aspect, the present disclosure refers to use of a capsid-free close-ended DNA (ceDNA) vector in the manufacture of a medicament to regulate expression of a transgene in a subject, wherein:

a. a first dose of a capsid-free closed-ended DNA (ceDNA) vector comprising a nucleic acid cassette containing at least one transgene operably linked to a promoter between flanking inverted terminal repeats (ITRs) is to be administered, wherein the first dose comprises an amount of the ceDNA vector that is sufficient to express a measurable level of the transgene, wherein the transgene encodes a desired protein; and

b. at least a second dose of the ceDNA vector comprising the at least one transgene between the flanking ITRs is to be administered, wherein the second dose is formulated for administration to (i) continue expression of the desired protein at a predetermined level for a predetermined time or (ii) modulate expression of the desired protein to a predetermined level.

[0013] Accordingly, in some examples a ceDNA vector as disclosed herein can be re-administered (also referred to herein as a "redose" or "booster" administration) to continue transgene expression level at a predetermined level for a predetermined time, or to increase the expression level of the transgene above a prior expression level which was achieved on a first, or prior ceDNA vector administration, where the second administration or booster administration does not generate an immune reaction that prevents expression of the transgene by not generating an immune response to the vector itself that impacts expression of the transgene, or where the immune reaction is less as compared to a re-administration of a viral vector comprising viral proteins, including but not limited to a viral vector comprising a capsid, such as a parvovirus or a lentivirus.

[0014] Without wishing to be limited to theory, research shows that long-term transgene expression using conventional AAV viral vectors wanes over time. One way to ensure long-term transgene expression using conventional AAV vectors has traditionally been to increase the titre and dose of the delivered AAV vector at the initial administration. However, it is known that too high a titre or dose of the AAV vector can result in a variety of side-effects. Furthermore, as discussed above, re-administration of an AAV viral vector is traditionally not possible due to immune responses. Ways to circumvent causing an immune response to re-administration of AAV vectors typically require re-administration of an AAV vector with a different capsid configuration as compared to the AAV vector administered on any previous administrations. Such strategies however, can still pose significant risk to the subject by inducing an immune response to the AAV vector and potentially deleterious effects.

[0015] Herein, the disclosure provides a method for controlled expression of a transgene, including long term expression using a close-ended DNA (ceDNA) vector. It is demonstrated herein that a ceDNA vector can titrated to increase the transgene expression levels by, e.g., repeat or re-administration of the ceDNA vector. Additionally, the level of transgene expression can be maintained over a long term, and if any drop in expression levels is observed, a redose administration of the ceDNA vector can be used to maintain the desired level or even to increase the level of transgene expression if desirable for the subject and/or the disease or disorder to be treated.

[0016] Accordingly, provided herein are methods of administering ceDNA vectors for sustaining and/or increasing the level of expression of a transgene from a ceDNA vector in a cell or a subject, and where the expression level can be sustained or increased with one or more subsequent administrations (e.g., a re-dose or a booster administration). In the event that ceDNA-delivered transgene expression decreases for any reason, re-dosing of the ceDNA vector can re-establish or maintain the desired

expression of the transgene at a desired level. Also provided herein are methods for personalized gene therapy, such that the level of expression of a transgene expressed by the ceDNA vector can be increased incrementally, or in a step-by-step manner, with one or more administrations after an initial priming administration (e.g., at time 0), thereby tailoring (e.g., titration) of the level of expression of the transgene to a desired expression level or within a desired expression level range, as needed by the subject.

[0017] Accordingly, in some examples, the ceDNA vectors and methods disclosed herein can be used to titrate or effectuate an increase in the level of expression of a transgene by a ceDNA vector, and where the expression level of the transgene can be titrated in a dose-dependent manner with one or more subsequent administrations (e.g., a dose-dependent re-dose or booster administration). As such, the methods disclosed herein enable one to personalize gene therapy throughout an individual's lifespan to express a transgene at a level that meets the individual's needs, by sustaining the transgene expression level at a predetermined level, or alternatively, increasing the expression of the transgene in a dose-dependent manner, by one or more administration after the initial priming administration (e.g., at time 0), thereby controlling the transgene expression level to a desired expression level or desired expression level range based on the concentration of ceDNA vector in the re-dose administration, allowing for a controlled and specific increases in the expression of the transgene in the cell or subject.

[0018] Accordingly, in some examples a ceDNA vector as disclosed herein can be re-administered (also referred to herein as a "redose" or "booster" administration) to continue transgene expression level at a predetermined level for a predetermined time, or to increase the expression level of the transgene above a prior expression level which was achieved on a first, or prior ceDNA vector administration.

[0019] Accordingly, one example of the technology described herein relates to the use of ceDNA vector in methods for controlled transgene expression, for example, in a method for modulating expression levels of a transgene, or for a controlled increase in the transgene expression level, or for a dose-dependent expression of a transgene level in a cell or a subject, wherein the ceDNA vector comprises at least one heterologous nucleotide sequence (e.g., a transgene) operatively linked to a promoter and positioned between two inverted terminal repeat sequences, where the ITR sequences can be asymmetric, or symmetric, or substantially symmetrical as these terms are defined herein, wherein at least one of the ITRs comprises a functional terminal resolution site and a Rep binding site, and optionally the heterologous nucleic acid sequence encodes a transgene, and wherein the vector is not in a viral capsid.

[0020] In some examples, a ceDNA vector as described herein are capsid-free, linear duplex DNA molecules formed from a continuous strand of complementary DNA with covalently-closed ends (linear, continuous and non-encapsidated structure), which comprises two inverted terminal repeat (ITR) sequences flanking a transgene which is operatively linked to a promoter or other regulatory switch as

described herein. The 5' ITR and the 3' ITR can have the same symmetrical three-dimensional organization with respect to each other, (i.e., symmetrical or substantially symmetrical), or alternatively, the 5' ITR and the 3' ITR can have different three-dimensional organization with respect to each other (i.e., asymmetrical ITRs), as these terms are defined herein. In addition, the ITRs can be from the same or different serotypes. In some examples, a ceDNA vector can comprise ITR sequences that have a symmetrical three-dimensional spatial organization such that their structure is the same shape in geometrical space, or have the same A, C-C' and B-B' loops in 3D space (i.e., they are the same or are mirror images with respect to each other). In some examples, one ITR can be from one AAV serotype, and the other ITR can be from a different AAV serotype.

[0021] Accordingly, some examples of the technology described herein relate to a ceDNA vector for controlled transgene expression, including but not limited to, sustained or long-term expression of a transgene, dose-dependent or tritratable expression of a transgene, or repeated dosing of a transgene, where the ceDNA vector comprises ITR sequences selected from any of: (i) at least one WT ITR and at least one modified AAV inverted terminal repeat (ITR) (e.g., asymmetric modified ITRs); (ii) two modified ITRs where the mod-ITR pair have a different three-dimensional spatial organization with respect to each other (e.g., asymmetric modified ITRs), or (iii) symmetrical or substantially symmetrical WT-WT ITR pair, where each WT-ITR has the same three-dimensional spatial organization, or (iv) symmetrical or substantially symmetrical modified ITR pair, where each mod-ITR has the same three-dimensional spatial organization. The ceDNA vectors disclosed herein can be produced in eukaryotic cells, thus devoid of prokaryotic DNA modifications and bacterial endotoxin contamination in insect cells.

[0022] In some examples, the methods and ceDNA vectors as described herein allow a personalized genetic medicine approach, i.e., titrating an increase in the level of the transgene expression by re-dose administrations in a concentration-dependent manner. It is envisioned that increases in the transgene expression can be achieved in a dose-dependent, step-by-step manner using re-dose administrations, thus increasing the expression level of the transgene by a defined or certain amount by each re-dose administration. This enables controlled increases in the level of the transgene expression in a dose-dependent manner, and can be done incrementally. Accordingly, 1, 2, 3, 4, 5 or 6 or more than 6 re-doses of a define amount of ceDNA can be administered in order to increase the level of expression the transgene by a defined amount each time, to achieve a desired level, or to a desired expression level range, which is higher than the expression level achieved with the prior administration, or prior to this re-dose administration.

[0023] Accordingly, in some examples, A method of regulating expression of a transgene in a host comprising: (i) administering a sufficient amount of a ceDNA vector as disclosed herein comprising

a nucleic acid cassette containing at least one transgene operably linked to a promoter between flanking inverted terminal repeats (ITRs), to the host to express measurable levels of the transgene, wherein the transgene encodes a desired protein; and (ii) administering at least a second dose of the ceDNA vector comprising the at least one transgene or a modified transgene between flanking ITRs to (i) continue expression of the desired protein at a predetermined level for a predetermined time or (ii) modulate expression of the desired protein to a predetermined level, wherein the second administration of the ceDNA vector does not generate an immune reaction that prevents expression of the desired protein.

[0024] One example of the technology described herein relates to use of a ceDNA vector in a method for sustaining a desired level of expression of a transgene in a cell, method comprising: (a) administering to a cell at a first time point a first dose of a ceDNA vector to achieve expression of a transgene from the ceDNA vector, and (b) administering to the cell at a second time point another dose of the same or a different ceDNA vector to increase the level of expression of the transgene to a desired level, or to compensate for any decrease in expression level of the transgene after the initial ceDNA vector administration. It will be appreciated that such incremental increases in transgene expression permits titration of dosing in a subject to a desired level for such subject. In some examples, use of a ceDNA vector in a method for sustaining the level of expression of a transgene in a cell expresses the transgene at a desired expression level for at least 42 days. In some examples, the ceDNA vector expresses the transgene at a desired expression level for at least 84 days. In some examples, the ceDNA vector expresses the transgene at a desired expression level for at least 132 days.

[0025] In some examples, the ceDNA vector used in the methods described herein, e.g., in a method for sustaining expression of a transgene in a cell and/or for treating a subject with a disease, is administered in combination with a pharmaceutically acceptable carrier and/or excipient. In some examples, a ceDNA vector is administered at a second time point is administered at least 30 days, or at least 60 days or between 60-90 days, or between 90-120 days, or between about 3-6 months, or between 6-12 months, or between 1-2 years, or 2-3 years after the first time point.

[0026] In addition to a re-dose administration of a ceDNA vector to simply increase the level of transgene expression if expression levels have decreased over time (e.g., to continue transgene expression at a desired pre-determined level), in some examples, the methods and compositions of re-administration of a ceDNA vector can increase the level of transgene in a dose-dependent manner - that is, a re-dose administration of a defined amount of a ceDNA vector can effect a defined increase in expression level of a transgene. Stated differently and using arbitrary units for illustrative purposes only, a 1unit dose of the ceDNA in a re-dose administration will achieve a 10% increase in the level of transgene expression from a prior level, and a 2 unit dose of the cDNA vector will achieve a 20% increase in the

level of the transgene from a prior level, and a 0.5 unit dose of the ceDNA will achieve a 5% increase in the level of expression of the transgene from a prior level.

[0027] Accordingly, in one example, a ceDNA vector as disclosed herein for controlled transgene expression can be used for increasing the level of expression of a transgene in a cell or a subject in a controlled manner. For example, the expression level of the transgene can be increased with one or more subsequent administrations (e.g., a re-dose or a booster administration) of the ceDNA vector.

[0028] Another example of the technology herein relates to a method for increasing expression of a transgene in a cell, e.g., to increase the expression level of a transgene above a prior expression level that was achieved with a prior ceDNA administration, the method comprising: (a) administering to a cell at a first time point, a priming dose of a ceDNA vector to achieve expression of a transgene, and (b) administering to the cell at a second time point, a dose of a ceDNA vector to increase the expression level of the transgene as compared to the level of expression of the transgene achieved after administration of the ceDNA vector at the first time point, or to increase the expression level of the transgene to achieve a desired expression level.

[0029] In all examples described herein, a ceDNA vector is administered at any time point (e.g. a first, second, third time point etc.) is administered in combination with a pharmaceutically acceptable carrier, and can be optionally administered with a carrier, for example, a particle, liposome or lipid nanoparticle (LNP). In all examples herein, a ceDNA vector administered at any of: the first, second or any subsequent time point, is administered in combination with a pharmaceutically acceptable carrier.

[0030] In all examples described herein, a ceDNA vector used in the methods for controlled transgene expression as described herein, e.g., in a method for sustaining expression of a transgene, or for a controlled increase in the expression of the transgene, or for a dose-dependent expression of the transgene, and/or for treating a subject with a disease, the ceDNA vector administered at any of: the first, second or any subsequent time point, is administered in combination with a pharmaceutically acceptable carrier and/or excipient.

[0031] In some examples, where more than one administration of the ceDNA is administered (e.g., at a second or any subsequent time point), the second time point, or any subsequent time point is at least 10 days or between 10-30 days, or at least 30 days, or between 30-60 days, at least 60 days, or between 60-90 days, or between 90-120 days, or between about 3-6 months, or between 6-12 months, or at least a year, after the ceDNA vector administration at the first time point, or the previous time point.

[0032] In some examples, a ceDNA vector administered at the first, second or any subsequent time point is the same ceDNA vector comprising the same transgene, or a modified transgene, and in alternative examples, a ceDNA vector administered at the first, second or any subsequent time point

is a different ceDNA vector comprising the same transgene, or a modified transgene, e.g., a different ceDNA vector with a different promoter operatively linked to the same transgene, or a modified transgene. In some examples, the promoter is an inducible or repressible promoter. The transgene can also be part of a regulatory switch, as disclosed herein.

[0033] In some examples, a ceDNA vector used in the methods described herein for controlled transgene expression, the ceDNA vector administered at the first, second or any subsequent time point is the same ceDNA vector comprising the same transgene, or a modified transgene. In alternative examples, the ceDNA vector administered at the first, second or any subsequent time point is a different ceDNA vector comprising the same transgene, or a modified transgene, for example, but not limited to, where the different ceDNA vector has a different promoter operatively linked to the same transgene, or to a modified transgene, or a different transgene. For illustrative purposes only, a ceDNA vector administered at the first timepoint can comprise a transgene and a first promoter or regulatory switch, and a ceDNA administered at a second or subsequent timepoint can comprise the same or a modified transgene and a second promoter or regulatory switch, where the first and second promoter (or regulator switch) are different promoters or different regulatory switches. Exemplary regulatory switches are defined herein.

[0034] In some examples, use of ceDNA vector in the methods for controlled transgene expression as described herein, e.g., in a method for sustaining expression of a transgene, or for a controlled increase in the expression of the transgene, or for a dose-dependent expression of the transgene, and/or for treating a subject with a disease, can optionally comprise a step of administering to the cell, at one or more time points after the second time point, a further dose of the ceDNA vector to increase the expression level of the transgene as compared to the level of expression of the transgene achieved after administration of the ceDNA vector at the second time point or previous time point, or to increase the expression level of the transgene to maintain a desired sustained expression level, wherein the composition administered at the one or more time points after the second time point comprises a ceDNA vector as described herein.

[0035] In some examples, the ceDNA vector useful in the methods disclosed herein for controlled transgene expression allows for expression of the transgene at a therapeutically effective amount.

[0036] In some examples, increasing the predetermined dose of the ceDNA vector administered at a second time point, or any subsequent time point, increases the expression level of the transgene in the cell and/or subject. In some examples, a predetermined dose of a ceDNA vector administered to the cell or subject at second time point, or subsequent time point, is determined using a dose-dependent relationship for the ceDNA vector to achieve the desired level of expression of the transgene in the cell or subject.

[0037] In some examples, a predetermined dose of the ceDNA vector administered at the second or any subsequent time point, is in an amount that is between 2-fold and 10-fold the dose of the ceDNA

vector administered at the first time point. In some examples, a predetermined dose of the ceDNA vector administered at the second or any subsequent time point, is in an amount that increases the expression of the transgene by at least 3-fold, or at least 5-fold, or least 10-fold, or between 2-15 fold or between 2-20 fold, or more than 20-fold as compared the expression of the transgene achieved after administration of the ceDNA at the first time point or previous time point. In some examples, the desired expression level of transgene achieved after the administration of the composition at one or more time points after the second time point is a therapeutically effective amount of the transgene.

[0038] Examples of the disclosure relate to methods to produce a ceDNA vector used in the methods for controlled transgene expression as described herein, e.g., in a method for sustaining expression of a transgene, or for a controlled increase in the expression of the transgene, or for a dose-dependent expression of the transgene, and/or for treating a subject with a disease. In all examples, the capsid free, non-viral DNA vector (ceDNA vector) for controlled transgene expression is obtained from a plasmid (referred to herein as a "ceDNA-plasmid") comprising a polynucleotide expression construct template comprising in this order: a first 5' inverted terminal repeat (e.g. AAV ITR); a heterologous nucleic acid sequence; and a 3' ITR (e.g. AAV ITR), where the 5' ITR and 3'ITR can be asymmetric relative to each other, or symmetric (e.g., WT-ITRs or modified symmetric ITRs) as defined herein.

[0039] A ceDNA vector useful in the methods for controlled transgene expression as described herein, (e.g., in a method for sustaining expression levels of a transgene, and/or for a controlled increase in the transgene expression level, or for a dose-dependent transgene expression level) is obtainable by a number of means that would be known to the ordinarily skilled artisan after reading this disclosure. For example, a polynucleotide expression construct template used for generating the ceDNA vectors of the present disclosure can be a ceDNA-plasmid (e.g. see **FIG. 4B),** a ceDNA-bacmid, and/or a ceDNA-baculovirus. In one example, the ceDNA-plasmid comprises a restriction cloning site (e.g. SEQ ID NO: 123 and/or 124 operably positioned between the ITRs where an expression cassette comprising e.g., a promoter operatively linked to a transgene, e.g., a reporter gene and/or a therapeutic gene) can be inserted. In some examples, ceDNA vectors are produced from a polynucleotide template (e.g., ceDNA-plasmid, ceDNA-bacmid, ceDNA-baculovirus) containing symmetric or asymmetric ITRs (modified or WT ITRs).

[0040] In a permissive host cell, in the presence of e.g., Rep, the polynucleotide template having at least two ITRs replicates to produce ceDNA vectors. ceDNA vector production undergoes two steps: first, excision ("rescue") of template from the template backbone (e.g. ceDNA-plasmid, ceDNA-bacmid, ceDNA-baculovirus genome etc.) via Rep proteins, and second, Rep mediated replication of the excised ceDNA vector. Rep proteins and Rep binding sites of the various AAV serotypes are well known to those of ordinary skill in the art. One of ordinary skill understands to choose a Rep protein from a serotype that

binds to and replicates the nucleic acid sequence based upon at least one functional ITR. For example, if the replication competent ITR is from AAV serotype 2, the corresponding Rep would be from an AAV serotype that works with that serotype such as AAV2 ITR with AAV2 or AAV4 Rep but not AAV5 Rep, which does not. Upon replication, the covalently-closed ended ceDNA vector continues to accumulate in permissive cells and ceDNA vector is preferably sufficiently stable over time in the presence of Rep protein under standard replication conditions, e.g. to accumulate in an amount that is at least 1 pg/cell, preferably at least 2 pg/cell, preferably at least 3 pg/cell, more preferably at least 4 pg/cell, even more preferably at least 5 pg/cell.

[0041] Accordingly, one example of the disclosure relates to a process of producing a ceDNA vector useful in the methods for controlled transgene expression as described herein, comprising the steps of: a) incubating a population of host cells (e.g. insect cells) harboring the polynucleotide expression construct template (e.g., a ceDNA-plasmid, a ceDNA-bacmid, and/or a ceDNA-baculovirus), which is devoid of viral capsid coding sequences, in the presence of a Rep protein under conditions effective and for a time sufficient to induce production of the ceDNA vector within the host cells, and wherein the host cells do not comprise viral capsid coding sequences; and b) harvesting and isolating the ceDNA vector from the host cells. The presence of Rep protein induces replication of the vector polynucleotide with a modified ITR to produce the ceDNA vector in a host cell. However, no viral particles (e.g. AAV virions) are expressed. Thus, there is no virion-enforced size limitation.

[0042] The presence of the ceDNA vector useful for controlled expression of the transgene as described herein is isolated from the host cells can be confirmed by digesting DNA isolated from the host cell with a restriction enzyme having a single recognition site on the ceDNA vector and analyzing the digested DNA material on denaturing and non-denaturing gels to confirm the presence of characteristic bands of linear and continuous DNA as compared to linear and non-continuous DNA.

[0043] In another example of this example and all other examples provided herein, the transgene expressed in a controlled manner from the ceDNA vector is therapeutic transgene, e.g., a protein of interest, including but not limited to, a receptor, a toxin, a hormone, an enzyme, or a cell surface protein. In another example of this example and all other examples provided herein, the protein of interest is a receptor. In another example of this example and all other examples provided herein, the protein of interest is an enzyme. Exemplary genes to be targeted and proteins of interest are described in detail in the methods of use and methods of treatment sections herein.

[0044] In some examples, the present application may be defined in any of the following paragraphs:

[0045] A method of regulating expression of a transgene in a subject comprising: (a) administering to a subject a sufficient amount of a non-viral, capsid-free close-ended DNA (ceDNA) vector comprising

a nucleic acid cassette containing at least one transgene operably linked to a promoter between flanking inverted terminal repeats (ITRs), to express a measurable level of the transgene, wherein the transgene encodes a desired protein to treat a disease; and (b) titrating the ceDNA vector by administering to the subject at least a second dose of the ceDNA vector comprising the at least one transgene between flanking ITRs to obtain the transgene expression of the desired protein at a predetermined level for a predetermined time or to increase the transgene expression of the desired protein to a predetermined level.

[0046] In some examples, the subject is assessed at a predetermined time after step (a), e.g., at least 30 days, or at least 60 days, or between 60-90 days or longer than 90 days after step (a), to determine the titrating dose. For example, in some examples, the subject is assessed to determine the disease state in the subject after step (a) and/or the level of desired protein expressed by the ceDNA vector in the subject. In some examples, assessment of the disease state is an assessment of at least one symptom of the disease in the subject. In some examples, if the disease state of the subject has remained at a steady state, or has not improved, or where the disease state has declined in the subject, for example, as compared to the disease state at the time of the first administration of the ceDNA vector or any time before step (a), the subject is administered a second dose of the ceDNA vector according to step (b). The disease state for any given disease can be determined by a physician or person of skill in the art, and includes assessing one or more clinical symptoms and/or biomarkers of the disease, including protein biomarkers, miRNA and mRNA biomarkers and the like. In some examples, if the level of transgene expression in the subject has declined from a predetermined level or declined from a therapeutically effective amount, the subject is administered a second dose of the ceDNA vector according to step (b). In some examples, the level of the transgene expression is determined by measuring the level of the transgene (e.g., measuring protein level or mRNA levels) expressed from the ceDNA vector in a biological sample obtained from the subject. In some examples, the biological sample is selected from a blood sample, plasma, synovial fluid, CSF, saliva, or tissue biopsy sample. In some examples, where the ceDNA vector expresses a transgene encoding a desired protein or therapeutic gene and a reporter protein, the level of the transgene can be determined by measuring the desired reporter protein expressed from the ceDNA vector *in vivo,* using methods commonly known to persons of ordinary skill in the art. In some examples, the titrating the ceDNA vector is determining the level of transgene expressed from the ceDNA vector and administering a second dose of the ceDNA vector to the subject to adjust or modulate the transgene expression to a predetermined desired level.

[0047] Another example of the technology described herein relates to a method of regulating expression of a transgene in a subject comprising: (a) administering a sufficient amount of a non-viral capsid-free close-ended DNA (ceDNA) vector comprising a nucleic acid cassette containing at least one transgene operably linked to a promoter between flanking inverted terminal repeats (ITRs), to the subject

to express a measurable level of the transgene, wherein the transgene encodes a desired protein; and (b) administering to the subject at least a second dose of the ceDNA vector comprising the at least one transgene, or a modified transgene, between flanking ITRs to (i) continue expression of the desired protein at a predetermined level for a predetermined time or (ii) modulate expression of the desired protein to a predetermined level.

[0048] In all examples herein, the second administration of the ceDNA vector to the subject does not generate an immune reaction sufficient to prevent obtaining the predetermined level of expression of the desired protein.

[0049] In some examples, the ceDNA vector is administered to the subject at first administration, or a second administration or any subsequent administration in combination with a pharmaceutically acceptable carrier.

[0050] In some examples, the second administration of the ceDNA vector is at a time when the level of the expression of the transgene decreases from a desired predetermined level, for example, in some examples, the second administration is at least about 30 days, or at least about 60 days, or at least about 90 days after the first administration. When more than two doses of the ceDNA vector are administered to the subject, each redose (e.g., 3^{rd}, 4^{th}, 5^{th}, 6^{th} and subsequent redoses) are administered at a time when the level of the expression of the transgene decreases or drops from a desired predetermined level achieved from the previous administration, for example, in some examples, each re-administration is at least about 30 days, or at least about 60 days, or at least about 90 days after the previous ceDNA vector administration.

[0051] In some examples, the method comprises administering at least three or more administrations of the ceDNA vector to the subject, and where at least three administrations of the ceDNA vector are administered, none of the administrations generate an immune response to the ceDNA vector that prevents the achieving the predetermined level of expression of the desired protein.

[0052] In some examples, the ceDNA vector is administered to the subject on a periodic schedule, e.g., every 2-months, every 3 months, every 6 months, every 12 months, every 18 months and the like.

[0053] In some examples, the second administration is to increase the level of expression of the desired protein, e.g., to prolong the expression of the desired protein at a predetermined level of expression.

[0054] In all examples herein, the transgene encodes a therapeutic protein and the desired level of expression of the transgene is a therapeutically effective amount of the therapeutic protein. In some examples, the transgene is a genetic medicine selected from any of: a nucleic acid, an inhibitor, peptide or polypeptide, antibody or antibody fragment, fusion protein, antigen, antagonist, agonist, RNAi

molecule, etc. In some examples, the desired protein or therapeutic protein is an inhibitor protein, for example, but not limited to, an antibody or antigen-binding fragment, or a fusion protein. In some examples, the desired protein or therapeutic protein replaces a defective protein or a protein that is not being expressed or being expressed at low levels. In some examples, the transgene is under the control of a regulatory switch, as defined herein.

[0055] In some examples, the ceDNA vector comprises a promoter which is an inducible or repressible promoter.

[0056] In some examples, the ceDNA vector administered at the first, second or any subsequent time point is the same type of ceDNA vector comprising the same transgene, or a modified transgene. For example, stated differently, the same ceDNA vector is administered to the subject multiple times and is comparable to administering the same serotype of viral vector to a subject multiple times.

[0057] In some examples, a ceDNA vector administered to the subject at second administration or any subsequent administration thereafter (e.g., a redose administration) has a different promoter operatively linked to the same transgene, or a modified transgene, as compared to the promoter in the ceDNA vector administered at an earlier timepoint or administration.

[0058] In some examples, a ceDNA vector administered at the first administration, or second administration or any subsequent administration thereafter comprises two inverted terminal repeat sequences (ITRs) that are AAV ITRs, and can be, e.g., AAV-2, or any ITR selected from Table 1, or AAV1, AAV3, AAV4, AAV5, AAV 5, AAV7, AAV8, AAV9, AAV10, AAV 11, AAV12, AAVrh8, AAVrh10, AAV-DJ, and AAV-DJ8. In some examples, at least one ITR comprises a functional terminal resolution site and a Rep binding site. In some examples, the flanking ITRs in a ceDNA vector administered at the first administration, or second administration or any subsequent administration thereafter are symmetric or substantially symmetrical or asymmetric, as defined herein. In some examples, one or both of the ITRs are wild type, or wherein both of the ITRs are wild-type. In some examples, the flanking ITRs are from different viral serotypes. In some examples, where the flanking ITRs are both wild type, they can be selected from any AAV serotype as shown in Table 1.

[0059] In some examples, the flanking ITRs in a ceDNA vector administered at the first administration, or second administration or any subsequent administration thereafter can comprise a sequence selected from the sequences in Tables 2, 4A, 4B or 5 herein.

[0060] In some examples, at least one of the ITRs in a ceDNA vector administered at the first administration, or second administration or any subsequent administration thereafter is altered from a wild-type AAV ITR sequence by a deletion, addition, or substitution that affects the overall three-dimensional conformation of the ITR. In some examples, one or both of the ITRs in a ceDNA vector administered at the first administration, or second administration or any subsequent administration

thereafter is derived from an AAV serotype selected from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, and AAV12.

[0061] In some examples, one or both of the ITRs in a ceDNA vector administered at the first administration, or second administration or any subsequent administration thereafter are synthetic. In some examples, one or both of the ITRs is not a wild type ITR, or wherein both of the ITRs are not wild-type.

[0062] In some examples, one or both of the ITRs in a ceDNA vector administered at the first administration, or second administration or any subsequent administration thereafter is modified by a deletion, insertion, and/or substitution in at least one of the ITR regions selected from A, A', B, B', C, C', D, and D'. In some examples, a deletion, insertion, and/or substitution results in the deletion of all or part of a stem-loop structure normally formed by the A, A', B, B' C, or C' regions. In some examples, one or both of the ITRs are modified by a deletion, insertion, and/or substitution that results in the deletion of all or part of a stem-loop structure normally formed by the B and B' regions. In some examples, one or both of the ITRs are modified by a deletion, insertion, and/or substitution that results in the deletion of all or part of a stem-loop structure normally formed by the C and C' regions. In some examples, one or both of the ITRs are modified by a deletion, insertion, and/or substitution that results in the deletion of part of a stem-loop structure normally formed by the B and B' regions and/or part of a stem-loop structure normally formed by the C and C' regions. In some examples, one or both of the ITRs comprise a single stem-loop structure in the region that normally comprises a first stem-loop structure formed by the B and B' regions and a second stem-loop structure formed by the C and C' regions. In some examples, one or both of the ITRs comprise a single stem and two loops in the region that normally comprises a first stem-loop structure formed by the B and B' regions and a second stem-loop structure formed by the C and C' regions.

[0063] In some examples, both ITRs in a ceDNA vector administered at the first administration, or second administration or any subsequent administration thereafter are altered in a manner that results in an overall three-dimensional symmetry when the ITRs are inverted relative to each other.

[0064] In some examples, a ceDNA vector administered at the first administration, or second administration or any subsequent administration thereafter comprises at least one heterologous nucleotide sequence under the control of at least one regulatory switch, for example, at least one regulatory switch is selected from a binary regulatory switch, a small molecule regulatory switch, a passcode regulatory switch, a nucleic acid-based regulatory switch, a post-transcriptional regulatory switch, a radiation-controlled or ultrasound controlled regulatory switch, a hypoxia-mediated regulatory switch, an inflammatory response regulatory switch, a shear-activated regulatory switch, and a kill switch. Regulatory switches are disclosed herein in more detail below.

[0065] In some examples, a ceDNA vector administered at the first administration, or second administration or any subsequent administration thereafter is administered to a subject that has a disease or disorder selected from, e.g., cancer, autoimmune disease, a neurodegenerative disorder, hypercholesterolemia, acute organ rejection, multiple sclerosis, post-menopausal osteoporosis, skin conditions, asthma, or hemophilia. In some examples, a subject with cancer has a solid tumor, soft tissue sarcoma, lymphoma, and leukemia. In some examples, the subject has an autoimmune disease, e.g., selected from rheumatoid arthritis and Crohn's disease. In some examples, the subject has a skin condition, e.g., is selected from psoriasis and atopic dermatitis. In some examples, the subject has a neurodegenerative disorder, e.g., Alzheimer's disease, ALS, Parkinson's Disease, Huntington's Disease.

[0066] In some examples, the method disclosed herein further comprise administering to the subject, at one or more time points after the second time point, a dose of the ceDNA vector to increase the expression level of the heterologous nucleic acid sequence (e.g., the transgene) as compared to the level of expression of the transgene achieved after administration of the ceDNA vector at the second time point or previous time point, or to increase the expression level of the transgene to achieve a desired expression level.

[0067] In some examples, a predetermined dose of the ceDNA vector administered to the subject at a second or any subsequent time point, is in an amount that is between 2-fold and 10-fold the dose of the ceDNA vector composition administered at the first time point. In some examples, a predetermined dose of the ceDNA vector composition administered at the second or any subsequent time point, is in an amount that increases the expression of the transgene by at least 3-fold, or at least 5-fold, or least 10-fold, or between 2-15 fold or 2-20 fold as compared the level of expression of the transgene after administration of the ceDNA vector at the first time point or previous administration. In some examples, a predetermined dose of the ceDNA vector administered at the second administration, or second time point, is determined using a dose-dependent relationship for the ceDNA vector to achieve the desired level of expression of the transgene in the cell.

[0068] These and other examples of the disclosure are described in further detail below.

DESCRIPTION OF DRAWINGS

[0069] Embodiments of the present disclosure, briefly summarized above and discussed in greater detail below, can be understood by reference to the illustrative embodiments of the disclosure depicted in the appended drawings. However, the appended drawings illustrate only typical embodiments of the disclosure and are therefore not to be considered limiting of scope, for the disclosure may admit to other equally effective embodiments.

CLAIMS

1. Use of a capsid-free close-ended DNA (ceDNA) vector in the manufacture of a medicament to regulate expression of a transgene in a subject, wherein:

   a. a first dose of a capsid-free closed-ended DNA (ceDNA) vector comprising a nucleic acid cassette containing at least one transgene operably linked to a promoter between flanking inverted terminal repeats (ITRs) is to be administered, wherein the first dose comprises an amount of the ceDNA vector that is sufficient to express a measurable level of the transgene, wherein the transgene encodes a desired protein; and

   b. at least a second dose of the ceDNA vector comprising the at least one transgene between the flanking ITRs is to be administered, wherein the second dose is formulated for administration to (i) continue expression of the desired protein at a predetermined level for a predetermined time or (ii) modulate expression of the desired protein to a predetermined level.

2. The use of claim 1, wherein the second dose of the ceDNA vector does not generate an immune reaction sufficient to prevent obtaining the predetermined level of expression of the desired protein.

3. The use of claim 1 or 2, wherein the ceDNA vector is formulated in combination with a pharmaceutically acceptable carrier.

4. The use of any of claims 1-3, wherein the second dose is formulated for administration at a time when the level of the expression of the transgene decreases from a desired level.

5. The use of any of claims 1-4, wherein the second dose is formulated for administration at least 90 days after the first administration; or wherein the administrations are on a periodic schedule.

6. The use of any of claims 1-5, wherein the second dose is formulated for administration to increase the level of expression of the desired protein or the second dose is formulated for administration to prolong the expression of the desired protein at a predetermined level of expression.

7. The use of any of claims 1-6, wherein the desired protein is an inhibitor protein; or the desired protein replaces a defective protein or a protein that is not being expressed.

8. The use of any of claim 1 or 2, wherein the promoter is an inducible or repressible promoter.

9. The use of any of claims 1-8, wherein the transgene is under the control of a regulatory switch.

10. The use of any of claims 1-9, wherein, the two flanking inverted terminal repeat sequences (ITRs) are AAV ITRs.

11. The use of any of claims 1-10, wherein at least one of the flanking ITRs comprises a functional terminal resolution site and a Rep binding site.

12. The use of any of claims 1-11, wherein the flanking ITRs are symmetric or asymmetric;
the flanking ITRs are symmetrical or substantially symmetrical;
the flanking ITRs are asymmetric; or
the flanking ITRs are from different viral serotypes.

13. The use of any of claims 1-12, wherein one or both of the flanking ITRs are synthetic;
one or both of the flanking ITRs is not a wild type ITR;
both of the flanking ITRs are not wild-type;
one or both of the flanking ITRs are wild type; or
both of the flanking ITRs are wild-type.

14. The use of any of claims 1-13, wherein one or both of the flanking ITRs are modified by a deletion, an insertion, and/or a substitution that results in the deletion of all or part of a stem-loop structure normally formed by the B and B' regions;
one or both of the flanking ITRs are modified by a deletion, an insertion, and/or a substitution that results in the deletion of all or part of a stem-loop structure normally formed by the C and C' regions; or
one or both of the flanking ITRs are modified by a deletion, an insertion, and/or a substitution that results in the deletion of part of a stem-loop structure normally formed by the B and B' regions and/or part of a stem-loop structure normally formed by the C and C' regions.

15. The use of any of claims 1-14, wherein one or both of the flanking ITRs comprise a single stem-loop structure in the region that normally comprises a first stem-loop structure formed by the B and B' regions and a second stem-loop structure formed by the C and C' regions, or one or both of the ITRs comprise a single stem and two loops in the region that normally comprises a first stem-loop structure formed by the B and B' regions and a second stem-loop structure formed by the C and C' regions.

16. The use of any of claims 1-15, wherein the subject has a disease or disorder selected from a cancer, an autoimmune disease, a neurodegenerative disorder, hypercholesterolemia, acute organ rejection, multiple sclerosis, post-menopausal osteoporosis, a skin condition, asthma, or hemophilia.

17. The use of claim 16, wherein the cancer is selected from a solid tumor, soft tissue sarcoma, lymphoma, and leukemia;
the autoimmune disease is selected from rheumatoid arthritis and Crohn's disease;
the skin condition is selected from psoriasis and atopic dermatitis; or
the neurodegenerative disorder is Alzheimer's disease.

18. The use of claim 16, wherein a subsequent dose of the ceDNA vector is to be further administered after the second dose, wherein the subsequent dose is formulated to increase the expression level of the heterologous nucleic acid sequence as compared to the level of expression of the heterologous nucleic acid achieved after the second dose, or to increase the expression level of the heterologous nucleic acid sequence to achieve a desired expression level.

19. The use of any of claims 1-18, wherein the second or subsequent dose of the ceDNA vector is selected from: an amount that is between 2-fold and 10-fold the first dose of the ceDNA vector;
an amount that is the same as the first or preceding dose of the ceDNA vector; or
an amount that increases the expression of the transgene by at least 3-fold, or at least 5-fold, or least 10-fold, or between 2-15 fold or 2-20 fold as compared the expression of the transgene after administration of the first or preceding dose of the ceDNA vector.

20. The use of any of claims 1-19, wherein the second or subsequent dose of the ceDNA vector is determined using a dose-dependent relationship for the ceDNA vector and the level of transgene expression from the ceDNA vector to achieve the desired level of expression of the transgene in the cell.