[0006] The present disclosure includes, among other things, adenoviral vectors and adenoviral genomes (*e.g.*, "recombinant" or "engineered" adenoviral vectors and adenoviral genomes) for expression of base editors in target cells. The present disclosure includes, among other things, adenoviral vectors and adenoviral genomes for expression of a CRISPR system including CRISPR enzyme that is a CRISPR-associated RNA-guided endonuclease and/or a guide RNA (gRNA) in target cells, optionally wherein expression of at least one component of the CRISPR system is self-inactivating. The present disclosure includes, among other things, adenoviral vectors and adenoviral genomes for expression of a base editing system including base editing enzyme and/or a guide RNA (gRNA) in target cells, optionally wherein expression of at least one component of the base editing system is self-inactivating. The present disclosure includes, among other things, adenoviral vectors and adenoviral genomes that include a regulatory sequence that directs expression of an expression product (*e.g.*, a therapeutic expression product) in target cells, where the regulatory sequence includes an miRNA binding site or where the regulatory sequence includes a β-globin locus control region (LCR), such as a β-globin Long LCR. The present disclosure includes, among other things, combination adenoviral vectors and adenoviral genomes that express a plurality of therapeutic expression products in target cells, *e.g.*, therapeutic expression products that together contribute to treatment of a disease or condition. The present disclosure includes, among other things, adenoviral vectors and adenoviral genomes for integration into a target cell genome of a payload including a β-globin Long LCR. The present disclosure includes, among other things, adenoviral vectors, and adenoviral genomes thereof, that have reduced immunogenicity relative to certain existing vectors (*e.g.*, relative to Ad5 vectors). The present disclosure includes, among other things, Ad35 adenoviral vectors, Ad35 adenoviral genomes, HDAd35 adenoviral vectors, HDAd35 adenoviral genomes, support vectors, support genomes, Ad35 helper vectors, and ad Ad35 helper genomes, where HDAd35 vectors can have reduced immunogenicity relative to certain existing vectors (*e.g.*, relative to Ad5 vectors or Ad5/35 vectors).

[0006a] In one aspect, the present disclosure provides the use of a helper dependent adenoviral donor vector in the manufacture of a medicament for use in treating a subject suffering from cancer, wherein the subject is a human and has received or is receiving an alkylating agent to treat the cancer, wherein the vector comprises: a recombinant adenoviral donor genome comprising: a 5′ Ad5 inverted terminal repeat (ITR); a 3′ Ad5 ITR; an Ad5 packaging sequence; and a nucleic acid sequence encoding $mgmt^{p140K}$.

[0006b] In one aspect, the present disclosure provides a recombinant helper dependent adenoviral donor vector comprising: a recombinant adenoviral donor genome comprising: a 5′ Ad5 inverted terminal repeat (ITR); a 3′ Ad5 ITR; an Ad5 packaging sequence; and a nucleic acid sequence encoding at least one heterologous expression product operably linked with one or more regulatory sequences, wherein the heterologous expression product comprises a β-globin protein or γ-globin protein, and wherein the regulatory sequences comprise a β-globin LCR comprising β-globin LCR DNase I hypersensitive sites (HS) comprising HS1, HS2, HS3, HS4, and HS5.

[0006c] In one aspect, the present disclosure provides a method of modifying a cell, the method comprising contacting the cell with a recombinant helper dependent adenoviral donor vector as disclosed herein.

[0006d] In one aspect, the present disclosure provides the use of a recombinant helper adenoviral donor vector as disclosed herein in the manufacture of a medicament for modifying a cell of a subject, optionally a human subject.

[0006e] In one aspect, the present disclosure provides the use of a recombinant helper adenoviral donor vector as disclosed herein in the manufacture of a medicament for treating a hemoglobinopathy in a subject in need thereof, optionally a human subject.

[0006f] In one aspect, the present disclosure provides a recombinant helper dependent adenoviral donor vector comprising: a recombinant adenoviral donor genome comprising: a 5′ Ad5 inverted terminal repeat (ITR); a 3′ Ad5 ITR; an Ad5 packaging sequence; and a nucleic acid sequence encoding at least one heterologous expression product, wherein the at least one heterologous expression product comprises: (i) a β-globin protein or a γ-globin protein; (ii) a CRISPR-associated RNA-guided endonuclease and/or a base editor, and (iii) one, two, or three gRNAs selected from: a gRNA that binds a target nucleic acid sequence of HBG1; a gRNA that binds a target nucleic acid sequence of HBG2; and a gRNA that binds a target nucleic acid sequence of erythroid enhancer bcl11a, wherein the gRNAs are engineered to increase expression of γ-globin.

[0006g] In one aspect, the present disclosure provides a method of modifying a cell, the method comprising contacting the cell with a recombinant helper dependent adenoviral donor vector as disclosed herein.

[0006h] In one aspect, the present disclosure provides the use of a recombinant helper adenoviral donor vector as disclosed herein in the manufacture of a medicament for modifying a cell of a subject, optionally a human subject.

[0006i] In one aspect, the present disclosure provides the use of a recombinant helper adenoviral donor vector as disclosed herein in the manufacture of a medicament for treating a hemoglobinopathy in a subject in need thereof, optionally a human subject.

[0006j] In one aspect, the present disclosure provides a recombinant helper dependent adenoviral donor vector comprising: a recombinant adenoviral donor genome comprising: a 5′ Ad5 inverted terminal repeat (ITR); a 3′ Ad5 ITR; an Ad5 packaging sequence; and a nucleic acid sequence encoding at least one heterologous expression product, wherein the at least one heterologous expression product comprises a therapeutic expression product operably linked with a regulatory sequence, wherein the therapeutic expression product comprises: (a) a β-globin protein or γ-globin protein; (b) an antibody or an immunoglobulin chain thereof, optionally wherein the antibody is an anti-CD33 antibody; (c) a first antibody or an immunoglobulin chain thereof and a second antibody or an immunoglobulin chain thereof, optionally wherein the first or second antibody is an anti-CD33 antibody; (d) a CRISPR-associated RNA-guided endonuclease and/or a guide RNA (gRNA), optionally wherein the CRISPR-associated RNA-guided endonuclease comprises Cas9 or cpf1; (e) a base editor and/or a gRNA, optionally wherein the base editor is a cytosine base editor (CBE) or adenine base editor (ABE), optionally wherein the base editor comprises a catalytically disabled nuclease selected from a disabled Cas9 and a disabled cpf1; (f) a coagulation factor or a protein that blocks or reduces viral infection, optionally wherein the therapeutic expression produce comprises a Factor VII replacement protein or a Factor VIII replacement protein; (g) a checkpoint inhibitor; (h) chimeric antigen receptor or engineered T cell receptor; or (i) a protein selected from the group consisting of γC, JAK3, IL7RA, RAG1, RAG2, DCLRE1C, PRKDC, LIG4, NHEJ1, CD3D, CD3E, CD3Z, CD3G, PTPRC, ZAP70, LCK, AK2, ADA, PNP, WHN, CHD7, ORAI1, STIM1, CORO1A, CIITA, RFXANK, RFX5, RFXAP, RMRP, DKC1, TERT, TINF2, DCLRE1B, SLC46A1, FancA, FancB, FancC, FancD1, FancD2, FancE, FancF, FancG, FancI, FancJ, FancL, FancM, FancN, FancO, FancP, FancQ, FancR, FancS, FancT, FancU, FancV, FancW, soluble CD40, CTLA, Fas L, an antibody to PD-L1, an antibody to CD4, an antibody to CD5, an antibody to CD7, an antibody to CD52, an antibody to IL-1, an antibody to IL-2, an antibody to IL-4, an antibody to IL-6, an antibody to IL-10, an antibody to TNF, an antibody to a TCR specifically present on autoreactive T cells, a globin family gene, WAS, phox, dystrophin, pyruvate kinase, CLN3,

ABCD1, arylsulfatase A, SFTPB, SFTPC, NLX2.1, ABCA3, GATA1, a ribosomal protein gene, TERT, TERC, DKC1, TINF2, CFTR, LRRK2, PARK2, PARK7, PINK1, SNCA, PSEN1, PSEN2, APP, SOD1, TDP43, FUS, ubiquilin 2, and/or C9ORF72, optionally wherein the protein is a FancA protein.

[0006k] In one aspect, the present disclosure provides a cell including a recombinant helper dependent adenoviral donor genome and a recombinant helper adenoviral vector suitable for producing a recombinant helper dependent adenoviral donor vector as disclosed herein, optionally wherein the cell is a HEK293 cell.

[0006l] In one aspect, the present disclosure provides a recombinant helper dependent adenoviral vector production system comprising a recombinant helper dependent adenoviral donor genome and a recombinant helper adenoviral vector suitable for producing a recombinant helper dependent adenoviral donor vector as disclosed herein.

[0006m] In one aspect, the present disclosure provides a method of producing a recombinant helper dependent adenoviral donor vector as disclosed herein, the method comprising isolating the recombinant helper dependent adenoviral donor vector from a culture of cells, wherein the cells comprise a recombinant helper dependent adenoviral vector production system as disclosed herein, optionally wherein the cells are HEK293 cells.

[0007] The current disclosure describes, among other things, recombinant Ad35 vectors targeting CD46 for *in vivo* gene editing of hematopoietic stem cells and related gene therapy improvements. In particular examples of presently disclosed vector designs, all proteins are derived from serotype 35. In particular examples of Ad35 vectors described herein, no viral genes remain in the vector. In particular examples, the ITR and packaging sequence are derived from Ad35. In particular examples, the Ad35 delivery vector has all viral protein encoding genes removed and replaced with components associated with a therapeutic use.

[0008] In particular examples, the Ad35 vector is helper-dependent, and the current disclosure also provides newly-designed Ad35 helper vectors. Particular examples provide optimized ratios of helper-dependent and transgene plasmid to make Ad35.

[0009] Related gene therapy improvements described within the current disclosure relate to one or more of: (i) novel mutations of the Ad35 knob protein that increase CD46 binding; (ii) vector features allowing for positive selection of *in vivo* modified cells; (iii) microRNA control systems that modulate expression of therapeutic proteins within clinically relevant time windows; (iv) use of homology arms to facilitate targeted genomic insertion at defined sites; (v) use of CRISPR to inactivate genomic suppressor regions, allowing increased expression of endogenous genes; (vi) use of mobilization strategies to increase delivery of Ad35 vectors to targeted CD46-expressing cells; (vii) use of mini- or long-form locus control regions to increase gene expression; (viii) use of recombinase systems to increase the size of transposons that can be inserted with transposase systems; (ix) steroid delivery (*e.g.*, glucocorticoids, dexamethasone) before vector delivery; and (x) erythrocytes to generate and secrete therapeutic proteins. Each of these related gene therapy improvements can be practiced with Ad35 vectors described herein and can also be utilized with other viral vector delivery systems. As one example, mutated Ad35 knob proteins that increase CD46 binding can be utilized with a lentiviral or foamy delivery system.

[00010] Advances described herein also relate to (i) *in vivo* HSC transduction/selection technology for SB100x-mediated transgene addition using HDAd5/35++ vectors; (ii) increased HbF reactivation by simultaneously targeting the erythroid bcl11a-enhancer (*e.g.*, to reduce BCL11A expression) and the HBG1/2 promoter regions (to increase expression of γ-globin); (iii) *in vivo* CRISPR genome engineering; (iv) correction of thalassemia; (v) combination of γ gene addition and reactivation (SB100x system); (vi) self-inactivation of CRISPR/Cas9; (vii) targeted integration using HDAd as donor vectors with self-releasing cassette; (viii) *in vivo* HSC gene therapy using erythroid cells as a factory for high-level production of a secreted therapeutic protein; (ix) therapeutic approaches to treat cancer (prophylactically and therapeutically); and (x) HDAd35++ vectors.

[0011] Certain examples relate to mutated knob proteins that increase targeted binding to CD46, allowing for more targeted and specific delivery of therapeutic genes.

[0012] Certain examples relate to use of homology arms to facilitate targeted genomic insertion, which can be used to provide chromosomal integration into genomic safe harbors, typically open chromatin which allows for higher expression of the transgene levels. As described herein, in particular examples, 1.8 b homology arms work well, with 0.8 as a lower limit. Single nucleotide polymorphisms can begin to impact integration at greater than 1.8 b homology arms.

[0013] Certain examples relate to use of mobilization regimens to alleviate the need for conditioning.

[0014] Particular examples provide an Ad35 *in vivo* gene therapy, with (i) an $MGMT^{P140K}$ system that allows for increasing the therapeutic effect by short-term treatment with low-dose O^6-benzylguanine plus bis-chloroethylnitrosourea, (ii) SB100X transposase-based integration machinery, and (iii) a micro-LCR–driven γ-globin gene.

[0015] Particular examples include an Ad35 adenovirus vector (HDAd-comb) including (i) a CRISPR/Cas9 cassette targeting the BCL11A binding site within the HBG1/2 promoters to reverse suppression of endogenous genes, (ii) a γ-globin gene cassette driven by a 5kb β-globin mini-LCR, and an EF1α- $MGMT^{P140K}$ expression cassette allowing for *in vivo* selection of transduced cells with the latter two cassettes flanked by FRT and transposon sites.

[0016] Particular examples describe CRISPR/Cas9-mediated genome editing approaches in adult CD34+ cells aimed toward the reactivation of fetal γ-globin expression in red blood cells. Because models involving erythroid differentiation of CD34+ cells have limitations in assessing γ-globin reactivation, human β-globin locus-transgenic, a helper-dependent human CD46-targeting adenovirus vector expressing CRISPR/Cas9 (HDAd-HBG-CRISPR) was used to disrupt a repressor binding region within the γ-globin promoter.

[0017] Particular examples provide an integrating CD46 targeted Ad35 vector system: transgene included (i) a β-globin locus control region (LCR) driving expression of a γ globin gene, and (ii) EF1-α (constitutive promoter) driving expression of a $MGMT^{P140K}$ cassette for positive selection of *in vivo* gene-modified HSC.

[0018] Particular examples provide an integrating CD46 targeted Ad35 vector system: transgene included (i) a 21.5 kb (long) human β-globin locus control region (LCR (HS1-HS5)) and a β-globin promoter (1.6 kb), driving expression of a γ globin gene (optionally including its 3′ UTR), and (ii) EF1-α (constitutive promoter) driving expression of a $MGMT^{P140K}$ cassette for positive selection of *in vivo* gene-modified HSC. Some examples can further include a 3′HS1 (human β-globin 3′HS1; 3 kb, *e.g.*, where 3′HS1 has the sequence of positions 5206867-5203839 of chromosome 11). In various examples, a 3′HS1 has the following nucleic acid sequence as shown in SEQ ID NO: 287, or a sequence having at least 80% sequence identity to SEQ ID NO: 287, *e.g.*, a sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 287. These examples can utilize a hyperactive transposase (*e.g.*, SB100X) in combination with a recombinase system (*e.g.*, Flp/Frt; Cre/Lox). Thus, in one particular example, an Ad35 vector system can include, *e.g.*, a transposable transgene insert including a long human β-globin locus control region (21.5 kb), a human β-globin promoter (1.6 kb), a human γ globin gene together with its 3′ UTR (2.7 kb), a human β-globin 3′ UTR, and a 3′HS1 (3 kb). A transposable transgene insert can further include, *e.g.*, EF1- α (constitutive promoter) driving expression of a $MGMT^{P140K}$. In various examples, an Ad35 vector system can include, *e.g.*, a transposable transgene insert of 32.4 kb.

[0019] Particular examples provide miRNA regulation systems that are activated only when HSPCs are recruited to a tumor to control expression of therapeutic transgenes. These features of the disclosure are demonstrated with anti PDL1-γ1 as a transgene. These systems can be used to regulate expression of therapeutic transgene in the context of the tumor microenvironment.

[0020] In various examples, a microRNA control system can refer to a method or composition in which expression of a gene is regulated by the presence of microRNA sites (*e.g.*, nucleic acid sequences with which a microRNA can interact), an example of which has been provided in Example 5. In particular examples, a microRNA control system regulated expression of a gene such that the gene is expressed exclusively in target cells, such as HSPCs *e.g.*, tumor infiltrating HSPCs. In some examples, a nucleic acid (*e.g.*, a therapeutic gene) encoding a protein or nucleic acid of interest (*e.g.*, an anti-cancer agent such as a CAR, TCR, antibody, and/or checkpoint inhibitor, *e.g.*, an αPD-L1 antibody (*e.g.*, an αPD-L1γ1 antibody) that is a checkpoint inhibitor) includes, is associated with, or is operably linked with a microRNA site, a plurality of same microRNA sites, or a plurality of distinct microRNA sites. While those of skill in the art will be familiar with means and techniques of associating a microRNA site with a nucleic acid or portion thereof having a sequence that encodes a gene of interest, certain non-limiting examples are provided herein. For example, a gene of interest (*e.g.*, a sequence encoding an αPD-L1γ1 antibody) can be present in a nucleic acid such that expression of the gene of interest is regulated by the presence of one or more microRNA sites that suppress expression in cells that are not tumor-infiltrating leukocyte cells, but do not suppressed expression in tumor-infiltrating leukocytes. In certain particular examples, a gene of interest (*e.g.*, a sequence encoding an αPD-L1γ1 antibody) can be present in a nucleic acid such that expression of the gene of interest is regulated by the presence of one or more miR423-5p microRNA sites that suppress expression in cells that are not tumor-infiltrating leukocyte cells, but do not suppressed expression in tumor-infiltrating leukocytes. In various examples, a microRNA control system can include a nucleic acid that includes, or in which expression of a protein or nucleic acid of interest is regulated by, one or more microRNA sites, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more microRNA sites. In various examples, a microRNA control system can include a nucleic acid that includes, or in which expression of a protein or nucleic acid of interest is regulated by, one or more miR423-5p microRNA sites, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more miR423-5p microRNA sites. In some particular examples, a microRNA control system can include a nucleic acid that encodes αPD-L1γ1 antibody and includes, or in which expression of αPD-L1γ1 antibody is regulated by, one or more miR423-5p microRNA sites, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more miR423-5p microRNA sites, *e.g.*, miR423-5p microRNA sites.

[0021] The current disclosure describes recombinant Ad35 vectors targeting CD46 for *in vivo* gene editing of hematopoietic stem cells and related gene therapy improvements. In particular examples, the Ad35 delivery vector has all viral protein encoding genes removed and replaced with components associated with a therapeutic use. Removal of all genes encoding viral proteins provides a vector carrying capacity of 30 kb, significantly more space than is available with other viral vector delivery platforms. In particular examples, the Ad35 vector is helper-dependent, and the current disclosure also provides newly-designed Ad35 helper vectors. For the avoidance of doubt, the

term “gene editing” as used herein includes, without limitation, any use of a vector or agent to modify a nucleic acid sequence.

[0022] Further provided herein are vectors that are or include nucleic acids provided herein, including without limitation microRNA control systems and other nucleic acids including microRNA (also referred to herein as miRNA) sites (also referred to herein as target sites) disclosed herein, and/or encode an agent disclosed herein, including without limitation an antibody such as an αPD-L1 antibody (*e.g.*, an αPD-L1γ1 antibody). In any of the various examples of the present disclosure, a vector can be an Ad5/35 vector, optionally wherein the Ad5/35 vector is a helper-dependent Ad5/35 (HDAd5/35). In any of the various examples of the present disclosure, a vector can be an Ad5/35 vector (*e.g.*, HDAd5/35 vector) including variations (*e.g.*, amino acid mutations) provided herein, certain of which such vectors can be designated as Ad5/35++ (*e.g.*, HDAd5/35++). For the avoidance of doubt, it is intended that those of skill in the art appreciate from the present disclosure that any example using any vector, including examples in which a vector other than an Ad5/35 (*e.g.*, other than Ad5/35++ or other than HDAd5/35++) vector is specified, is to be specifically read as disclosing, in addition to such vectors as stated in the relevant text, a vector that is an Ad5/35 vector (including, *e.g.*, any of HDAd5/35, Ad5/35++, and HDAd5/35++ vector).

[0023] In any of the various examples of the present disclosure, a vector can be an Ad35 vector, optionally wherein the Ad35 vector is a HDAd35. In any of the various examples of the present disclosure, a vector can be an Ad35 vector (*e.g.*, HDAd35 vector) including variations (*e.g.*, amino acid mutations) provided herein, certain of which such vectors can be designated as Ad35++ (*e.g.*, HDAd35++). For the avoidance of doubt, it is intended that those of skill in the art appreciate from the present disclosure that any example using any vector, including examples in which a vector other than an Ad35 (*e.g.*, other than Ad35++ or other than HDAd35++) vector is specified, is to be specifically read as disclosing, in addition to such vectors as stated in the relevant text, a vector that is an Ad35 vector (including, *e.g.*, any of HDAd35, Ad35++, and HDAd35++ vector).

[0024] As indicated, the vectors described herein have many uses including in the treatment of sickle cell disease, γ globin gene addition and reactivation, and the targeting of multiple target sites for γ globin reactivation. Further, in addition to factor VIII (FVIII), the application of disclosed approaches can be used for other secreted proteins, including for example: (i) other coagulation factors, specifically FXI, FVII, von Willebrand factor (VWF), and rare clotting factors (*i.e.* factors I, II, V, X, XI, or XIII); (ii) enzymes that are currently used for Enzyme replacement therapies (ERT) for lysosomal storage diseases (taking advantage of the cross-correction mechanism) like Pompe disease (acid alpha (α)-glucosidase), Gaucher disease (glucocerebrosidase), Fabry disease (α-galactosidase A), and Mucopolysaccharidosis type I (α-L-Iduronidase); (iii) immunodeficiencies (*e.g.* SCID-ADA (adenosine deaminase)); (iv) cardiovascular diseases, *e.g.* familial apolipoprotein E deficiency and atherosclerosis (ApoE); (v) viral infections by expression of viral decoy receptors (*e.g.* for HIV- soluble

CD4, or broadly neutralizing antibodies (bNAbs)) for HIV, chronic HCV, or HBV infections; (vi) cancer (*e.g.* controlled expression of monoclonal antibodies (*e.g.* trastuzumab) or checkpoint inhibitors (*e.g.* aPDL1) or protection of HSCs in order to permit therapeutic doses of chemotherapy and (vii) FANCA genes for Fanconi anemia; (viii) a coagulation factor deficiency optionally selected from hemophilia A, hemophilia B, or Von Willebrand Disease, (ix) a platelet disorder, (x) anemia, (xi) alpha-1 antitrypsin deficiency, or (xii) an immune deficiency. Other additional uses are described in more detail elsewhere herein.

[0025] Thus, one example provides a recombinant serotype 35 adenovirus (Ad35) vector targeting CD46 for *in vivo* gene editing of hematopoietic stem cells.

[0026] Another example is an erythrocyte genetically modified to express a therapeutic protein. By way of example, the therapeutic protein in some cases includes a coagulation factor or a protein that blocks or reduces viral infection. Optionally, the erythrocyte secretes the therapeutic protein.

[0027] Also provided are uses of the recombinant Ad35 vectors or erythrocytes described herein. These uses include to increase HbF reactivation by simultaneously targeting the erythroid bcl11a-enhancer and the HBG promoter regions; for a combination of γ-globin gene addition and endogenous γ-globin gene reactivation; for *in vivo* CRISPR genome engineering; to provide a therapeutic gene; to treat a (i) hemoglobinopathy, (ii) Fanconi anemia, (iii) a coagulation factor deficiency optionally selected from hemophilia A, hemophilia B, or Von Willebrand Disease, (iv) a platelet disorder, (v) anemia, (vi) alpha-1 antitrypsin deficiency, or (v) an immune deficiency; to treat thalassemia; to treat cancer, prevent or delay cancer recurrence or prevent or delay cancer onset in carriers of high-risk germ-line mutations, optionally wherein the cancer is breast cancer or ovarian cancer; for self-inactivation of CRISPR/Cas9; and for targeted integration using HDAd as donor vectors with a self-releasing cassette. Any of these uses may optionally include mobilization, for instance wherein the mobilization includes administration of Gro-beta, GM-CSF, S-CSF, and/or AMD3100.

[0028] Yet another use example is use of any of the recombinant Ad35 vectors or erythrocytes described herein which includes administering a steroid (*e.g.*, a glucocorticoid or dexamethasone), an IL-6 receptor antagonist, and/or an IL-1R receptor antagonist to a subject receiving the Ad35 vector and/or erythrocyte.

[0029] Also provided are use examples employing any of the recombinant Ad35 vectors or erythrocytes described herein, which include administering O^6BG and TMZ (temozolomide) or BCNU (Carmustine) to a subject receiving the Ad35 vector and/or erythrocyte. By examples of such uses examples, the subject in is receiving O^6BG and TMZ or BCNU as a treatment for anaplastic astrocytoma, breast cancer, colorectal cancer, diffuse intrinsic brainstem glioma, Ewing sarcoma, glioblastoma multiforme (GBM), malignant glioma, melanoma, metastatic malignant melanoma, nasopharyngeal cancer, or a pediatric cancer.

[0030] Yet another example is a recombinant adenoviral serotype 35 (Ad35) vector production system including: a recombinant Ad35 helper genome including: a nucleic acid sequence encoding an Ad35 fiber shaft; a nucleic acid sequence encoding an Ad35 fiber knob; and recombinase DRs flanking at least a portion of an Ad35 packaging sequence, and a recombinant helper dependent Ad35 donor genome including: a 5′ Ad35 ITR; a 3′ Ad35 ITR; an Ad35 packaging sequence; and a nucleic acid sequence encoding at least one heterologous expression product.

[0031] Also provided are recombinant adenoviral serotype 35 (Ad35) helper vector examples that include: an Ad35 fiber shaft; an Ad35 fiber knob; and an Ad35 genome including recombinase DRs flanking at least a portion of an Ad35 packaging sequence.

[0032] Also provided are recombinant Ad35 helper genome examples that include: a nucleic acid sequence encoding an Ad35 fiber shaft; a nucleic acid sequence encoding an Ad35 fiber knob; and recombinase DRs flanking at least a portion of an Ad35 packaging sequence.

[0033] Also provided are recombinant helper dependent Ad35 donor vector examples that include: a nucleic acid sequence including: a 5′ Ad35 ITR; a 3′ Ad35 ITR; an Ad35 packaging sequence; and a nucleic acid sequence encoding at least one heterologous expression product, wherein the genome does not include a nucleic acid sequence encoding an Ad35 viral structural protein; and an Ad35 fiber shaft and/or an Ad35 fiber knob.

[0034] Also provided are recombinant helper dependent Ad35 donor genome examples that include: a 5′ Ad35 ITR; a 3′ Ad35 ITR; an Ad35 packaging sequence; and a nucleic acid sequence encoding at least one heterologous expression product, wherein the Ad35 donor genome does not include a nucleic acid sequence encoding an expression product encoded by the wild-type Ad35 genome.

[0035] Another example is a method of producing a recombinant helper dependent Ad35 donor vector, the method including isolating the recombinant helper dependent Ad35 donor vector from a culture of cells, wherein the cells include: a recombinant Ad35 helper genome including: a nucleic acid sequence encoding an Ad35 fiber shaft; a nucleic acid sequence encoding an Ad35 fiber knob; and recombinase DRs flanking at least a portion of an Ad35 packaging sequence, and a recombinant helper dependent Ad35 donor genome including: a 5′ Ad35 ITR; a 3′ Ad35 ITR; an Ad35 packaging sequence; and a nucleic acid sequence encoding at least one heterologous expression product.

[0036] Also provided are recombinant Ad35 production system examples including: a recombinant Ad35 helper genome including: a nucleic acid sequence encoding an Ad35 fiber shaft; a nucleic acid sequence encoding an Ad35 fiber knob; and recombinase DRs within 550 nucleotides of the 5’ end of the Ad35 genome that functionally disrupt the Ad35 packaging signal but not the 5’ Ad35 ITR, and a recombinant Ad35 donor genome including: a 5′ Ad35 ITR; a 3′ Ad35 ITR; an Ad35 packaging sequence; and a nucleic acid sequence encoding at least one heterologous expression product.

[0037] Another example is a recombinant Ad35 helper vector including: an Ad35 fiber shaft; an Ad35 fiber knob; and an Ad35 genome including recombinase DRs within 550 nucleotides of the 5' end of the Ad35 genome that functionally disrupt the Ad35 packaging signal but not the 5' Ad35 ITR.

[0038] Another example is a recombinant Ad35 helper genome including: a nucleic acid sequence encoding an Ad35 fiber shaft; a nucleic acid sequence encoding an Ad35 fiber knob; and DRs within 550 nucleotides of the 5' end of the Ad35 genome that functionally disrupt the Ad35 packaging signal but not the 5' Ad35 ITR.

[0039] Another example is a method of producing a recombinant helper dependent Ad35 donor vector, the method including isolating the recombinant helper dependent Ad35 donor vector from a culture of cells, wherein the cells include: a recombinant Ad35 helper genome including: a nucleic acid sequence encoding an Ad35 fiber shaft; a nucleic acid sequence encoding an Ad35 fiber knob; and recombinase DRs within 550 nucleotides of the 5' end of the Ad35 genome that functionally disrupt the Ad35 packaging signal but not the 5' Ad35 ITR, and a recombinant Ad35 donor genome including: a 5′ Ad35 ITR; a 3′ Ad35 ITR; an Ad35 packaging sequence; and a nucleic acid sequence encoding at least one heterologous expression product.

[0040] Yet another example is a cell including a helper vector, a helper genome, a donor vector, or a donor genome as described herein, optionally wherein the cell is a HEK293 cell.

[0041] Another example is a cell including a donor genome of any one of examples described herein, optionally wherein the cell is an erythrocyte, optionally wherein the cell is a hematopoietic stem cell, T-cell, B-cell, or myeloid cell, optionally wherein the cell secretes the expression product.

[0042] Also provided is a method of modifying a cell, the method including contacting the cell with an Ad35 donor vector according to any one of the provided Ad35 donor vector examples.

[0043] Also provided is a method of modifying a cell of a subject, the method including administering to the subject an Ad35 donor vector according to any one of the Ad35 donor vector examples, optionally wherein the method does not include isolation of the cell from the subject.

[0044] Yet another example is a method of treating a disease or condition in a subject in need thereof, the method including administering to the subject an Ad35 donor vector according to any one of the Ad35 donor vector examples provided herein, optionally wherein the administration is intravenous.

DEFINITIONS

[0045] ***A, An, The*****:** As used herein, "a", "an", and "the" refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" discloses embodiments of exactly one element and embodiments including more than one element.

[0046] ***About****:* As used herein, term "about", when used in reference to a value, refers to a value that is similar, in context to the referenced value. In general, those skilled in the art, familiar with the context,

[0189] FIG. 100. Graphical summary describing the combination of γ-globin gene addition and re-activation of endogenous γ-globin.

[0190] FIG. 101. HDAd5/35++ vectors used herein. γ-globin gene addition is achieved through the SB100x transposase system consisting of a transposon vector with IRs and frt sites flanking the expression cassette (see HDAd-combo and HDAd-SB-addition) and a second vector (HDAd-SB) that provides the SB100x and Flpe recombinase *in trans*. The transposon cassette for random integration consists of a mini β-globin LCR/promoter for erythroid specific expression of human γ-globin. The 3'UTR serves for mRNA stabilization in erythroid cells. The γ-globin expression unit is separated by a chicken globin HS4 insulator from a cassette for $mgmt^{P140K}$ expression from a ubiquitously active PGK promoter. The CRISPR/Cas9 cassette in the HDAd-CRISPR and HDAd-combo vectors contains a U6 promote-driven sgRNA specific to the BCL11A binding site within the HBG1/2 promoter, a SpCas9 under EF1α promoter control. Expression of Cas9 in HDAd producer cells is suppressed by a miRNA regulation system (Saydaminova *et al*., *Mol Ther Methods Clin Dev*. 2015, 1: 14057, 2015). In HDAd-combo, the CRISPR/Cas9 cassette is placed outside the transposon so that it will be lost upon Flpe/SB100x-mediated integration (see FIG. 102).

[0191] FIG. 102. Schematic for controlled Cas9 expression. In HDAd-combo, the interaction of Flpe recombinase with the frt sites leads to a circularization of the transposon, leaving linear fragment of the vector containing the CRISPR cassette. Previous studies with the SB100x/Flpe system demonstrated that these vector parts are rapidly lost while the circularized transposon is integrated into the host genome by SB100x (Yant *et al*., *Nat Biotechnol*., 20: 999-1005, 2002).

[0192] FIGs. 103A-103D. *In vitro* studies with HUDEP-2 cells to analyze Cas9 and γ-globin expression. (FIGs. 103A and 103B) Analysis of Cas9 expression by Western blot. HUDEP-2 cells were transduced with HDAd-combo alone and in combination with HDAd-SB (i.e. the vector that provides Flpe and SB100x in trans). *In vitro* erythroid differentiation was started 4 days post transduction and continued for 8 days. (Erythroid differentiation allows for γ-globin expression). Right panel: representative Western blot using Cas9 and β-actin antibodies as probes. Left panel: Summary of the Cas9 signals. The bars compare Cas9 with and without HDAd-SB coinfection, i.e. the reduction of Cas9 by the Flpe/SB100x mechanism. (FIG. 103C) Analysis of γ-globin expression by flow cytometry. HUDEP-2 cells were transduced with HDAd-CRISPR ("cut"), HDAd-SB-add ("add")+HDAd-SB, or HDAd-combo ("combo")+HDAd-SB and analyzed at the indicated time points. (FIG. 103D) γ-globin mRNA levels by qRT-PCR. d.p.t., days post transduction. Diff, differentiation. * $p<0.05$

[0193] FIGs. 104A-104H. γ-globin expression studies after *in vivo* transduction of CD46/β-YAC mice. (FIG. 104A) Schematic of the experiment. HSPCs were mobilized by subcutaneous (s.c.) injections of human recombinant G-CSF for 4 days followed by one s.c. injection of AMD3100. 30 and 60 minutes after AMD3100 injection, animals were intravenously injected with a 1:1 mixture of the following HDAd

vectors (2 injections, each $4x10^{10}$ vp): HDAd-combo+HDAd-SB, HDAd-SB-add+HDAd-SB, and HDAd-cut. Mice were treated with immunosuppressive (IS) drugs for the next 4 weeks to avoid immune responses against the human γ-globin and MGMT. At week 4, O^6-BG/BCNU treatment was started and repeated every 2 weeks for 3 times. With each cycle, the BCNU concentration was increased from 5 mg/kg, to 7.5 mg/kg, to 10 mg/kg. At week 18 animals were sacrificed for tissue sample analysis and harvest of bone marrow Lin^- cells for secondary transplantation into lethally irradiated C57Bl/6 mice, which were then followed for another 16 weeks. (FIG. 104B) Detection of γ-globin expression in peripheral red blood cells by flow cytometry for the "combo" and "cut" groups. (FIG. 104C) γ-globin protein levels measured by HPLC. Right panel: Chromatogram of RBC lysates (week 18) with human β-globin, reactivated human Aγ, and added γ-globin chains marked. Left panel: Summary of HPLC data. Shown is the percentage of total γ-globin relative to human β -globin for CD46/β-YAC mice treated with the "cut", "add", and "combo" vector. *: $p<0.05$, n.s.. (FIG. 104D) γ-globin mRNA expression relative to mouse β-major mRNA expression (measured by qRT-PCR). (FIG. 104E) Percent target site cleavage by CRISPR/Cas9. Genomic DNA from PBMCs and bone marrow MNCs harvested at week 18 from *in vivo* "cut" and "combo" transduced mice were subjected to T7EI assay. Shown is the summary of data from FIG. 105. * $p<0.05$). (FIG. 104F) Integrated vector copy numbers measured in bone marrow HSPCs at week 18 after transduction with the "add" and "combo" vectors. The difference between the groups is not significant. (FIG. 104G) Spectrum of VCNs in individual CFU's from "combo" vector treated mice. Bone marrow Lin^- cells were plated for progenitor assays and VCN was measured in individual colonies by qPCR. Shown are data from four different mice. (FIG. 104H) Human γ/human β globin protein by HPLC.

[0194] FIG. 105. Chromatograms of RBC lysates with marked human β- and γ-globin peaks. Upper panel shows β-YAC mice before treatment. Middle panels show week 18 after HDAd-CRISPR ("cut") transduction. The left panel shows the reactivation of both Gγ and Aγ. Lower panels show week 18 after HDAd-CRISPR ("cut") transduction. The peaks are labeled in the last bottom panel. Each chromatogram is an individual animal. Note that human β-globin decreases with increased and γ-globin (reverse globin switch).

[0195] FIG. 106. T7EI assay data from MNCs from blood, spleen, and bone marrow at week 16 after transduction with "cut" and "combo" vectors. The specific CRISPR/Cas9 cleavage fragments (255 and 110 bp) are marked by arrows. The percentage of cleavage based on band signal quantification is shown below each lane.

[0196] FIGs. 107A-107F. Analysis of secondary recipients of Lin^- cells from CD46/β-YAC transduced mice. (FIG. 107A) Percentage of human γ-globin expressing peripheral blood RBCs at the indicated time points. All mice received immunosuppression starting from week 4 post-transplantation. (FIG. 107B) Level of γ-globin protein relative to human β-globin at week 16 after transplantation. (FIGs.

globin coding sequence is operably linked with a 3′UTR (*e.g.*, a γ-globin 3′UTR), where the β-globin mini-LCR is also operably linked with the γ-globin coding sequence (c) a cHS4 insulator sequence, and (d) a gene including a PGK promoter operably linked with an $MGMT^{P140K}$ coding sequence, where the $MGMT^{P140K}$ coding sequence is operably linked with a polyadenylation signal, optionally where any of (a)-(d) can be encoded in a 5′ to 3′ orientation on either of the two strands of an Ad35 payload.

[0519] In various embodiments, an Ad35 payload further includes, outside of the integration element and outside of the recombinase sites, a nucleic acid sequence encoding a CRISPR system. In certain particular embodiments, the nucleic acid sequence encoding a CRISPR system includes, optionally from 5′ to 3′, (a) a gRNA gene including a U6 promoter operably linked with a gRNA-encoding sequence, where the gRNA targets an HBG promoter, and (b) a CRISPR enzyme gene including an EF1α promoter operably linked with a CRISPR/Cas9 coding sequence, wherein the CRISPR/Cas9 coding sequence is operably linked with a 3′UTR/miR sequence and a polyadenylation signal. In various embodiments, the CRISPR system targets the BCL11A binding site of the HBG promoter, which can result in γ-globin activation or re-activation. As disclosed herein, the CRISPR system can be self-inactivating, in that cleavage of donor vector by transposition results in degradation of non-integrated donor vector nucleic acids. In various embodiments, a miR sequence can be a sequence that suppresses Cas9 expression in a producer cell during HDAd35 donor vector production (see, *e.g.*, Saydaminova *et al.*, *Mol. Ther. Meth. Clin. Dev.* 1: 14057, 2015; Li *et al.*, *Mol. Ther. Meth. Clin. Dev.* 9: 390-401, 2018).

[0520] In various embodiments, an Ad35 system of the present disclosure further includes an Ad35 support vector, where the support vector includes, optionally from 5′ to 3′, (a) a recombines gene including an EF1α promoter operably linked with a FLPe recombinase coding sequence, and (b) a transposase gene including a PGK promoter operably linked with an SB100x transposase coding sequence.

[0521] In various embodiments an Ad35 payload is present in an Ad35 donor vector genome. In various embodiments an Ad35 payload present in an Ad35 donor vector genome is flanked by Ad35 ITRs. In various embodiments, an Ad35 donor vector genome is present in an Ad35 donor vector. In various embodiments, the donor vector is an Ad35 ++ vector.

[0522] In various embodiments a support genome includes Ad35 ITRs. In various embodiments, a support genome is present in an Ad35 vector. In various embodiments, the support vector is an Ad35 ++ vector.

[0523] In various embodiments, an Ad35 donor vector is a helper dependent donor vector (HDAd35). In certain such embodiments, systems of the present disclosure can include an HDAd35 donor vector or genome, and Ad35 helper vector or genome, and in various embodiments can further include an Ad35 support vector.

[0524] Certain exemplary embodiments are illustrated in FIG. 166.

expression in tumor-infiltrating leukocytes. In various embodiments, a microRNA control system can include a nucleic acid that includes, or in which expression of a protein or nucleic acid of interest is regulated by, one or more microRNA sites, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more microRNA sites. In various embodiments, a microRNA control system can include a nucleic acid that includes, or in which expression of a protein or nucleic acid of interest is regulated by, one or more miR423-5p microRNA sites, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more miR423-5p microRNA sites. In some particular embodiments, a microRNA control system can include a nucleic acid that encodes αPD-L1γ1 antibody and includes, or in which expression of αPD-L1γ1 antibody is regulated by, one or more miR423-5p microRNA sites, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more miR423-5p microRNA sites, *e.g.*, miR423-5p microRNA sites.

[0557] In various embodiments, a microRNA site can be a sequence that suppresses expression of an operably linked coding sequence in a producer cell during HDAd35 donor vector production, *e.g.*, a coding sequence encoding a CRISPR enzyme, base editing enzyme, or gRNA (see, *e.g.*, Saydaminova *et al*., *Mol. Ther. Meth. Clin. Dev.* 1: 14057, 2015; Li *et al*., *Mol. Ther. Meth. Clin. Dev.* 9: 390-401, 2018).

I(C)(iii). Selection Sequences

[0558] In particular embodiments vectors include a selection element including a selection cassette. In particular embodiments, a selection cassette includes a promoter, a cDNA that adds or confers resistance to a selection agent, and a poly A sequence enabling stopping the transcription of this independent transcriptional element.

[0559] A selection cassette can encode one or more proteins that (a) confer resistance to antibiotics or other toxins, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for Bacilli. Any number of selection systems may be used to recover transformed cell lines. In particular embodiments, a positive selection cassette includes resistance genes to neomycin, hygromycin, ampicillin, puromycin, phleomycin, zeomycin, blasticidin, viomycin. In particular embodiments, a positive selection cassette includes the DHFR (dihydrofolate reductase) gene providing resistance to methotrexate, the $MGMT^{P140K}$ gene responsible for the resistance to O^6BG/BCNU, the HPRT (Hypoxanthine phosphoribosyl transferase) gene responsible for the transformation of specific bases present in the HAT selection medium (aminopterin, hypoxanthine, thymidine) and other genes for detoxification with respect to some drugs. In particular embodiments, the selection agent includes neomycin, hygromycin, puromycin, phleomycin, zeomycin, blasticidin, viomycin, ampicillin, O^6BG/BCNU, methotrexate, tetracycline, aminopterin, hypoxanthine, thymidine kinase, DHFR, Gln synthetase, or ADA.

[0560] In particular embodiments, negative selection cassettes include a gene for transformation of a substrate present in the culture medium into a toxic substance for the cell that expresses the gene. These molecules include detoxification genes of diphtheria toxin (DTA) (Yagi *et al., Anal Biochem*.

lo implies within the left lower quadrant or in the middle between the right and left quadrant (but shifted relative to the negative population). In particular embodiments, “hi” refers to greater than 20-fold of +, greater than 30-fold of +, greater than 40-fold of +, greater than 50-fold of +, greater than 60-fold of +, greater than 70-fold of +, greater than 80-fold of +, greater than 90-fold of +, greater than 100-fold of +, or more of an increase in detectable fluorescence relative to + cells. Conversely, “lo” can refer to a reciprocal population of those defined as “hi”.

II(E). Other Targets

[0632] In addition to HSCs, T Cells, B Cells, and tumors (or cancer cells), vectors can target other antigens for bacteria and fungi.

[0633] Antigens targeting bacteria can be derived from, for example, anthrax, gram-negative bacilli, chlamydia, diphtheria, Helicobacter pylori, Mycobacterium tuberculosis, pertussis toxin, pneumococcus, rickettsiae, staphylococcus, streptococcus and tetanus.

[0634] As particular examples of bacterial antigen markers, anthrax antigens include anthrax protective antigen; gram-negative bacilli antigens include lipopolysaccharides; diphtheria antigens include diphtheria toxin; Mycobacterium tuberculosis antigens include mycolic acid, heat shock protein 65 (HSP65), the 30 kDa major secreted protein and antigen 85A; pertussis toxin antigens include hemagglutinin, pertactin, FIM2, FIM3 and adenylate cyclase; pneumococcal antigens include pneumolysin and pneumococcal capsular polysaccharides; rickettsiae antigens include rompA; streptococcal antigens include M proteins; and tetanus antigens include tetanus toxin.

[0635] Antigens targeting fungi can be derived from, for example, candida, coccidiodes, cryptococcus, histoplasma, leishmania, plasmodium, protozoa, parasites, schistosomae, tinea, toxoplasma, and *Trypanosoma cruzi*.

[0636] As particular examples of fungal antigens, coccidiodes antigens include spherule antigens; cryptococcal antigens include capsular polysaccharides; histoplasma antigens include heat shock protein 60 (HSP60); leishmania antigens include gp63 and lipophosphoglycan; plasmodium falciparum antigens include merozoite surface antigens, sporozoite surface antigens, circumsporozoite antigens, gametocyte/gamete surface antigens, protozoal and other parasitic antigens including the blood-stage antigen pf 155/RESA; schistosomae antigens include glutathione-S-transferase and paramyosin; tinea fungal antigens include trichophyton; toxoplasma antigens include SAG-1 and p30; and *Trypanosoma cruzi* antigens include the 75-77 kDa antigen and the 56 kDa antigen.

III. Dosages, Formulations, and Administration

[0637] A vector can be formulated such that it is pharmaceutically acceptable for administration to cells or animals, *e.g.*, to humans. A vector may be administered *in vitro*, *ex vivo*, or *in vivo*. The Ad35 viral vector vectors described herein can be formulated for administration to a subject. Formulations

[0653] Exemplary buffering agents include citrate buffers, succinate buffers, tartrate buffers, fumarate buffers, gluconate buffers, oxalate buffers, lactate buffers, acetate buffers, phosphate buffers, histidine buffers, and/or trimethylamine salts.

[0654] An exemplary chelating agent is EDTA.

[0655] Exemplary isotonic agents include polyhydric sugar alcohols including trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol, or mannitol.

[0656] Exemplary preservatives include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides, hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, and 3-pentanol.

[0657] Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the active ingredients or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can include polyhydric sugar alcohols; amino acids, such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, and threonine; organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinositol, galactitol, glycerol, and cyclitols, such as inositol; PEG; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol, and sodium thiosulfate; low molecular weight polypeptides (*i.e.*, <10 residues); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on therapeutic weight.

[0658] The formulations disclosed herein can be formulated for administration by, for example, injection. For injection, formulation can be formulated as aqueous solutions, such as in buffers including Hanks' solution, Ringer's solution, or physiological saline, or in culture media, such as Iscove's Modified Dulbecco's Medium (IMDM). The aqueous solutions can include formulatory agents such as suspending, stabilizing, and/or dispersing agents. Alternatively, the formulation can be in lyophilized and/or powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

[0659] Any formulation disclosed herein can advantageously include any other pharmaceutically acceptable carriers which include those that do not produce significantly adverse, allergic, or other untoward reactions that outweigh the benefit of administration. Exemplary pharmaceutically acceptable carriers and formulations are disclosed in Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990. Moreover, formulations can be prepared to meet sterility, pyrogenicity,

CLAIMS

What is claimed is:

1. Use of a helper dependent adenoviral donor vector in the manufacture of a medicament for use in treating a subject suffering from cancer, wherein the subject is a human and has received or is receiving an alkylating agent to treat the cancer, wherein the vector comprises:

a recombinant adenoviral donor genome comprising:

a 5′ Ad5 inverted terminal repeat (ITR);

a 3′ Ad5 ITR;

an Ad5 packaging sequence; and

a nucleic acid sequence encoding $mgmt^{p140K}$.

2. The use according to claim 1, wherein the alkylating agent comprises a nitrosourea, optionally bischloroethyl nitrosourea (BCNU) or temozolomide (TMZ).

3. The use according to claim 1 or 2, wherein the subject has also received or is also receiving O^6-benzyl guanine (O^6BG).

4. The use according to claim 3, wherein the alkylating agent comprises bischloroethyl nitrosourea (BCNU).

5. The use according to any one of claims 1-4, wherein the cancer is anaplastic astrocytoma, breast cancer, ovarian cancer, colorectal cancer, diffuse intrinsic brainstem glioma, Ewing sarcoma, glioblastoma multiforme, malignant glioma, melanoma, metastatic malignant melanoma, nasopharyngeal cancer, or a pediatric cancer.

6. The use according to claim 5, wherein the cancer is glioblastoma multiforme.

7. The use according to any one of claims 1-6, wherein the vector further comprises: an Ad35 fiber knob and an Ad5 fiber shaft.

8. The use according to claim 7, wherein:

the Ad35 fiber knob is a wild-type Ad35 fiber knob, or

the Ad35 fiber knob is an engineered Ad35 fiber knob, wherein the engineered fiber knob comprises a mutation that increases affinity of the fiber knob with CD46.

9. The use according to claim 8, wherein the mutation comprises a mutation selected from Ile192Val, Asp207Gly (or Glu207Gly), Asn217Asp, Thr226Ala, Thr245Ala, Thr254Pro, Ile256Leu, Ile256Val, Arg259Cys, and Arg279His; or comprises each of mutations Ile192Val, Asp207Gly (or Glu207Gly), Asn217Asp, Thr226Ala, Thr245Ala, Thr254Pro, Ile256Leu, Ile256Val, Arg259Cys, and Arg279His, optionally where the mutation comprises Asp207Gly and Thr245Ala mutations.

10. A recombinant helper dependent adenoviral donor vector comprising:

a recombinant adenoviral donor genome comprising:

a 5′ Ad5 inverted terminal repeat (ITR);

a 3′ Ad5 ITR;

an Ad5 packaging sequence; and

a nucleic acid sequence encoding at least one heterologous expression product operably linked with one or more regulatory sequences,

wherein the heterologous expression product comprises a β-globin protein or γ-globin protein, and

wherein the regulatory sequences comprise a β-globin LCR comprising β-globin LCR DNase I hypersensitive sites (HS) comprising HS1, HS2, HS3, HS4, and HS5.

11. The recombinant helper dependent adenoviral donor vector of claim 10, further comprising: an Ad35 fiber knob and an Ad5 fiber shaft.

12. The recombinant helper dependent adenoviral donor vector of claim 11, wherein:

the Ad35 fiber knob is a wild-type Ad35 fiber knob, or

the Ad35 fiber knob is an engineered Ad35 fiber knob, wherein the engineered fiber knob comprises a mutation that increases affinity of the fiber knob with CD46.

13. The recombinant helper dependent adenoviral donor vector of claim 12, wherein the mutation:

comprises a mutation selected from Ile192Val, Asp207Gly (or Glu207Gly), Asn217Asp, Thr226Ala, Thr245Ala, Thr254Pro, Ile256Leu, Ile256Val, Arg259Cys, and Arg279His; or

comprises each of mutations Ile192Val, Asp207Gly (or Glu207Gly), Asn217Asp, Thr226Ala, Thr245Ala, Thr254Pro, Ile256Leu, Ile256Val, Arg259Cys, and Arg279His, optionally where the mutation comprises Asp207Gly and Thr245Ala mutations.

14. The recombinant helper dependent adenoviral donor vector of any one of claims 10-13, wherein the β-globin LCR:

is from a Homo sapiens β-globin region on chromosome 11;

has a length of about 21.5 kb; or

comprises a sequence according to positions 5292319-5270789 of chromosome 11.

15. The recombinant helper dependent adenoviral donor vector of any one of claims 10-14, wherein the nucleic acid encoding the heterologous expression product is part of an integration element that is engineered for integration into a target genome by homologous recombination, wherein the integration element is flanked by homology arms that correspond to contiguously linked sequences of the target genome, optionally wherein:

the homology arms are between 0.8 and 1.8 kb; and/or

the homology arms are homologous to nucleic acid sequences of the target genome that flank a chromosomal safe harbor locus, optionally wherein the safe harbor loci is selected from AAVS1, CCR5, HPRT, or Rosa.

16. The recombinant helper dependent adenoviral donor vector of any one of claims 10-14, wherein the nucleic acid encoding the heterologous expression product is part of an integration element that is engineered for integration into a target genome by transposition, wherein the integration element is flanked by transposon inverted repeats (IRs), optionally wherein the transposon IRs are flanked by recombinase direct repeats (DRs).

17. The recombinant helper dependent adenoviral donor vector of claim 16, wherein:

the transposon IRs are Sleeping Beauty (SB) IRs, optionally wherein the SB IRs are pT4 IRs; or

the transposon IRs are piggyback, Mariner, frog prince, Tol2, TcBuster, or spinON IRs.

18. The recombinant helper dependent adenoviral donor vector of claim 16 or 17, wherein the recombinase DRs that flank the transposon IRs are FRT, loxP, rox, vox, AttB, or AttP sites.

19. The recombinant helper dependent adenoviral donor vector of any one of claims 16-18, wherein the integration element comprises a selection cassette.

20. The recombinant helper dependent adenoviral donor vector of claim 19, wherein the selection cassette comprises a nucleic acid sequence encoding $mgmt^{P140K}$ or wherein the selection cassette comprises a nucleic acid sequence encoding an anti-CD33 shRNA.

21. A method of modifying a cell, the method comprising contacting the cell with a recombinant helper dependent adenoviral donor vector of any one of claims 10-20.

22. Use of a recombinant helper adenoviral donor vector of any one of claims 10-20 in the manufacture of a medicament for modifying a cell of a subject, optionally a human subject.

23. Use of a recombinant helper adenoviral donor vector of any one of claims 10-20 in the manufacture of a medicament for treating a hemoglobinopathy in a subject in need thereof, optionally a human subject.

24. The use according to claim 23, wherein the hemoglobinopathy is thalassemia.

25. The use according to claim 23, wherein the hemoglobinopathy is sickle cell disease.

26. The use according to any one of claims 23-25, wherein the nucleic acid encoding the heterologous expression product is part of an integration element that is engineered for integration into a target genome, and wherein the use causes integration of the integration element and/or expression of the heterologous expression product in at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of cells expressing CD46, in at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of hematopoietic stem cells, and/or in at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of erythroid Ter119+ cells.

27. The use according to claim 26, wherein the use causes integration of an average of at least 2 copies or at least 2.5 copies of the integration element in target cell genomes comprising at least 1 copy of the integration element.

28. The use according to any one of claims 23-27, wherein the use causes expression of the heterologous expression product at a level that is at least about 20% of the level of a reference or at least about 25% of the level of a reference, optionally wherein the reference is expression of an endogenous reference protein in the subject or in a reference population.

29. The use according to any one of claims 23-28, wherein the use increases HbF.

30. A recombinant helper dependent adenoviral donor vector comprising:
a recombinant adenoviral donor genome comprising:

a 5′ Ad5 inverted terminal repeat (ITR);

a 3′ Ad5 ITR;

an Ad5 packaging sequence; and

a nucleic acid sequence encoding at least one heterologous expression product,

wherein the at least one heterologous expression product comprises:

(i) a β-globin protein or a γ-globin protein;

(ii) a CRISPR-associated RNA-guided endonuclease and/or a base editor, and

(iii) one, two, or three gRNAs selected from:

a gRNA that binds a target nucleic acid sequence of HBG1;

a gRNA that binds a target nucleic acid sequence of HBG2; and

a gRNA that binds a target nucleic acid sequence of erythroid enhancer bcl11a,

wherein the gRNAs are engineered to increase expression of γ-globin.

31. The recombinant helper dependent adenoviral donor vector of claim 30, further comprising: an Ad35 fiber knob and an Ad5 fiber shaft.

32. The recombinant helper dependent adenoviral donor vector of claim 31, wherein:

the Ad35 fiber knob is a wild-type Ad35 fiber knob, or

the Ad35 fiber knob is an engineered Ad35 fiber knob, wherein the engineered fiber knob comprises a mutation that increases affinity of the fiber knob with CD46.

33. The recombinant helper dependent adenoviral donor vector of claim 30 or 31, wherein the mutation:

comprises a mutation selected from Ile192Val, Asp207Gly (or Glu207Gly), Asn217Asp, Thr226Ala, Thr245Ala, Thr254Pro, Ile256Leu, Ile256Val, Arg259Cys, and Arg279His; or

comprises each of mutations Ile192Val, Asp207Gly (or Glu207Gly), Asn217Asp, Thr226Ala, Thr245Ala, Thr254Pro, Ile256Leu, Ile256Val, Arg259Cys, and Arg279His, optionally where the mutation comprises Asp207Gly and Thr245Ala mutations.

34. The recombinant helper dependent adenoviral donor vector of any one of claims 30-33, wherein the nucleic acid sequence comprises:

(i) an integration element encoding the β-globin protein or γ-globin protein, wherein the integration element is engineered for integration into a target genome by homologous recombination, wherein the integration element is flanked by homology arms that correspond to contiguously linked sequences of the target genome, optionally wherein:

the homology arms are between 0.8 and 1.8 kb; and/or

the homology arms are homologous to nucleic acid sequences of the target genome that flank a chromosomal safe harbor locus, optionally wherein the safe harbor loci is selected from AAVS1, CCR5, HPRT, or Rosa; and

(ii) a non-integrating nucleic acid sequence that:

(a) encodes the CRISPR-associated RNA-guided endonuclease and/or base editor and the one, two, or three gRNAs, and

(b) is not comprised by the integration element, in that it is not flanked by the homology arms.

35. The recombinant helper dependent adenoviral donor vector of any one of claims 30-33, wherein the nucleic acid sequence comprises:

(i) an integration element encoding the β-globin protein or γ-globin protein, wherein the integration element is engineered for integration into a target genome by transposition, wherein the integration element is flanked by transposon inverted repeats (IRs), optionally wherein the transposon IRs are flanked by recombinase direct repeats (DRs); and

(ii) a non-integrating nucleic acid sequence that:

(a) encodes the CRISPR-associated RNA-guided endonuclease and/or base editor and the one, two, or three gRNAs, and

(b) is not comprised by the integration element, in that it is not flanked by the transposon inverted repeats (IRs).

36. The recombinant helper dependent adenoviral donor vector of claim 35, wherein:

the transposon IRs are Sleeping Beauty (SB) IRs, optionally wherein the SB IRs are pT4 IRs; or

the transposon IRs are piggyback, Mariner, frog prince, Tol2, TcBuster, or spinON IRs.

37. The recombinant helper dependent adenoviral donor vector of claim 35 or 26, wherein the recombinase DRs that flank the transposon IRs are FRT, loxP, rox, vox, AttB, or AttP sites.

38. The recombinant helper dependent adenoviral donor vector of any one of claims 34-37, wherein the integration element comprises a selection cassette.

39. The recombinant helper dependent adenoviral donor vector of claim 38, wherein the selection cassette comprises a nucleic acid sequence encoding $mgmt^{P140K}$ or wherein the selection cassette comprises a nucleic acid sequence encoding an anti-CD33 shRNA.

40. A method of modifying a cell, the method comprising contacting the cell with a recombinant helper dependent adenoviral donor vector of any one of claims 30-39.

41. Use of a recombinant helper adenoviral donor vector of any one of claims 30-39 in the manufacture of a medicament for modifying a cell of a subject, optionally a human subject.

42. Use of a recombinant helper adenoviral donor vector of any one of claims 30-39 in the manufacture of a medicament for treating a hemoglobinopathy in a subject in need thereof, optionally a human subject.

43. The use according to claim 42, wherein the hemoglobinopathy is thalassemia.

44. The use according to claim 42, wherein the hemoglobinopathy is sickle cell disease.

45. The use according to any one of claims 42-44, wherein the nucleic acid sequence comprises an integration element encoding the β-globin protein or γ-globin protein and the integration element is engineered for integration into a target genome, and wherein the use causes integration of the integration element and/or expression of the β-globin protein or γ-globin protein in at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of cells expressing CD46, in at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of hematopoietic stem cells, and/or in at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of erythroid Ter119+ cells.

46. The use according to claim 45, wherein the use causes integration of an average of at least 2 copies or at least 2.5 copies of the integration element in target cell genomes comprising at least 1 copy of the integration element.

47. The use according to any one of claims 42-46, wherein the use causes expression of the heterologous expression product at a level that is at least about 20% of the level of a reference or at least about 25% of the level of a reference, optionally wherein the reference is expression of an endogenous reference protein in the subject or in a reference population.

48. The use according to any one of claims 42-47, wherein the use increases HbF.

49. A recombinant helper dependent adenoviral donor vector comprising:

a recombinant adenoviral donor genome comprising:

a 5′ Ad5 inverted terminal repeat (ITR);

a 3′ Ad5 ITR;

an Ad5 packaging sequence; and

a nucleic acid sequence encoding at least one heterologous expression product,

wherein the at least one heterologous expression product comprises a therapeutic expression product operably linked with a regulatory sequence, wherein the therapeutic expression product comprises:

(a) a β-globin protein or γ-globin protein;

(b) an antibody or an immunoglobulin chain thereof, optionally wherein the antibody is an anti-CD33 antibody;

(c) a first antibody or an immunoglobulin chain thereof and a second antibody or an immunoglobulin chain thereof, optionally wherein the first or second antibody is an anti-CD33 antibody;

(d) a CRISPR-associated RNA-guided endonuclease and/or a guide RNA (gRNA), optionally wherein the CRISPR-associated RNA-guided endonuclease comprises Cas9 or cpf1;

(e) a base editor and/or a gRNA, optionally wherein the base editor is a cytosine base editor (CBE) or adenine base editor (ABE), optionally wherein the base editor comprises a catalytically disabled nuclease selected from a disabled Cas9 and a disabled cpf1;

(f) a coagulation factor or a protein that blocks or reduces viral infection, optionally wherein the therapeutic expression produce comprises a Factor VII replacement protein or a Factor VIII replacement protein;

(g) a checkpoint inhibitor;

(h) chimeric antigen receptor or engineered T cell receptor; or

(i) a protein selected from the group consisting of γC, JAK3, IL7RA, RAG1, RAG2, DCLRE1C, PRKDC, LIG4, NHEJ1, CD3D, CD3E, CD3Z, CD3G, PTPRC, ZAP70, LCK, AK2, ADA, PNP, WHN, CHD7, ORAI1, STIM1, CORO1A, CIITA, RFXANK, RFX5, RFXAP, RMRP, DKC1, TERT, TINF2, DCLRE1B, SLC46A1, FancA, FancB, FancC, FancD1, FancD2, FancE, FancF, FancG, FancI, FancJ, FancL, FancM, FancN, FancO, FancP, FancQ, FancR, FancS, FancT, FancU, FancV, FancW, soluble CD40, CTLA, Fas L, an antibody to PD-L1, an antibody to CD4, an antibody to CD5, an antibody to CD7, an antibody to CD52, an antibody to IL-1, an antibody to IL-2, an antibody to IL-4, an antibody to IL-6, an antibody to IL-10, an antibody to TNF, an antibody to a TCR specifically present on autoreactive T cells, a globin family gene, WAS, phox, dystrophin, pyruvate kinase, CLN3, ABCD1, arylsulfatase A, SFTPB, SFTPC, NLX2.1, ABCA3, GATA1, a ribosomal protein gene, TERT, TERC, DKC1, TINF2, CFTR, LRRK2, PARK2, PARK7, PINK1, SNCA, PSEN1, PSEN2, APP, SOD1, TDP43, FUS, ubiquilin 2, and/or C9ORF72, optionally wherein the protein is a FancA protein.

50. The recombinant helper dependent adenoviral donor vector of claim 49, further comprising: an Ad35 fiber knob and an Ad5 fiber shaft.

51. The recombinant helper dependent adenoviral donor vector of claim 49 or 50, wherein the therapeutic expression product comprises a coagulation factor, optionally wherein the therapeutic expression product comprises a Factor VII replacement protein or a Factor VIII replacement protein.

52. The recombinant helper dependent adenoviral donor vector of any one of claims 49-51, wherein the therapeutic expression product comprises a checkpoint inhibitor, optionally wherein the checkpoint inhibitor is an aPD-L1 antibody.

53. A cell including a recombinant helper dependent adenoviral donor genome and a recombinant helper adenoviral vector suitable for producing a recombinant helper dependent adenoviral donor vector of any one of claims 10-20, 30-39, and 49-52, optionally wherein the cell is a HEK293 cell.

54. A recombinant helper dependent adenoviral vector production system comprising a recombinant helper dependent adenoviral donor genome and a recombinant helper adenoviral vector suitable for producing a recombinant helper dependent adenoviral donor vector of any one of claims 10-20, 30-39, and 49-52.

55. The cell of claim 53 or the recombinant helper dependent adenoviral vector production system of claim 54, wherein the recombinant helper adenoviral vector comprises:

a nucleic acid sequence encoding an Ad5 fiber shaft;

a nucleic acid sequence encoding an Ad35 fiber knob; and

recombinase direct repeats (DRs) flanking at least a portion of an Ad5 packaging sequence.

56. The cell of claim 55 or the recombinant helper dependent adenoviral vector production system of claim 55, wherein the recombinant helper dependent adenoviral donor genome comprises:

a 5′ Ad5 inverted terminal repeat (ITR);

a 3′ Ad5 ITR;

an Ad5 packaging sequence; and

a nucleic acid sequence encoding at least one heterologous expression product.

57. A method of producing a recombinant helper dependent adenoviral donor vector of any one of claims 10-20, 30-39, and 49-52, the method comprising isolating the recombinant helper dependent

adenoviral donor vector from a culture of cells, wherein the cells comprise a recombinant helper dependent adenoviral vector production system of any one of claims 54-56, optionally wherein the cells are HEK293 cells.