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(54) Title: CELLS DEFICIENT IN HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE

(57) Abstract: Compositions and methods for enhanced protein production provide cells deficient in hypoxanthine-guanine phosphoribosyltransferase. Methods for preparing the deficient cells, and methods for using the deficient cells to produce high levels of recombinant proteins are disclosed.

CELLS DEFICIENT IN HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE

FIELD OF THE INVENTION

[0001] The present invention generally relates to cells that are useful for recombinant protein production. In particular, the invention relates to cells that are deficient in hypoxanthine-guanine phosphoribosyltransferase (HPRT) such that hypoxanthine-guanine phosphoribosyltransferase may be used as an amplifiable metabolic selectable marker.

BACKGROUND OF THE INVENTION

[0002] HPRT is a key enzyme in the purine salvage pathway in eukaryotic cells, and it is widely used as a selectable marker during the production of monoclonal antibodies in hybridoma technology. Although the mechanisms are not well understood, metabolic selection and gene amplification are the cornerstones of pharmaceutical recombinant protein production. These technologies have enabled the generation of highly productive mammalian cells lines, and in particular, Chinese hamster ovary (CHO) cell lines. Currently, there are two amplifiable selectable markers successfully used in biotherapeutic protein production, i.e., dihydrofolate reductase (DHFR) and glutamine synthase (GS).

[0003] Development of HPRT as a third amplifiable selectable marker in addition to DHFR and GS would provide additional tools, and hence versatility, for developing high producing cell lines and/or reducing the time for developing high producing cell lines.

SUMMARY OF THE INVENTION

[0004] Briefly, therefore, one aspect of the present disclosure provides an isolated cell deficient in hypoxanthine-guanine phosphoribosyltransferase (HPRT) and glutamine synthase (GS). In one embodiment, the cell comprises an edited chromosomal sequence encoding HPRT, wherein the chromosomal sequence is edited using targeted endonuclease mediated genome editing. In some embodiments, the

targeting endonuclease is a meganuclease, a transcription activator-like effector (TALE) nuclease, a site-specific nuclease, or a zinc finger nuclease. In one embodiment, the targeting endonuclease is a zinc finger nuclease. In an embodiment, the edited chromosomal sequence comprises a deletion of sequence encoding HPRT. In one embodiment, a zinc finger nuclease is used to delete all or part of the chromosomal sequence encoding HPRT. In another embodiment, all copies of chromosomal sequence encoding HPRT are inactivated, and the cell produces no HPRT. In one embodiment, the cell is a Chinese hamster ovary (CHO) cell line cell or a derivative thereof, and all copies of chromosomal sequence encoding HPRT are inactivated. In an embodiment, all copies of chromosomal sequence encoding HPRT are inactivated by deletion of all or part of the coding sequence. In one embodiment, the cell further comprises an edited chromosomal sequence encoding GS. In an embodiment, all copies of chromosomal sequence encoding GS are inactivated and the cell produces essentially no GS. In a further embodiment, the cell is a Chinese hamster ovary (CHO) cell line cell or a derivative thereof, and all copies of chromosomal sequence encoding HPRT and all copies of chromosomal sequence encoding GS are inactivated such that the cell produces essentially no HPRT and no GS.

[0005] A further aspect of the disclosure encompasses a method for preparing a cell deficient in hypoxanthine-guanine phosphoribosyltransferase (HPRT) by editing a chromosomal sequence encoding HPRT. The method comprises introducing into the cell (i) at least one targeting endonuclease or at least one nucleic acid encoding a targeting endonuclease, each targeting endonuclease being able to introduce a double-stranded break at a targeted cleavage site in the chromosomal sequence encoding HPRT and, optionally, (ii) at least one polynucleotide comprising a sequence having substantial sequence identity to a sequence on at least one side of the targeted cleavage site in the chromosomal sequence encoding HPRT. The method further comprises maintaining the cell under conditions such that the double-stranded break introduced by the targeting endonuclease is repaired by (i) a non-homologous end-joining repair process such that the chromosomal sequence encoding HPRT is edited, or (ii) a homology-directed repair process such that the chromosomal sequence

is exchanged with the sequence of the polynucleotide such that the chromosomal sequence encoding HPRT is edited. In one embodiment, the targeting endonuclease is a zinc finger nuclease. In one embodiment, the optional polynucleotide is a single-stranded nucleic acid. In one embodiment, one targeting endonuclease is introduced into the cell. In another embodiment, one targeting endonuclease is introduced into the cell along with the optional polynucleotide, which comprises a first sequence having substantial sequence identity to a sequence adjacent to the targeted cleavage site and a second sequence having substantial sequence identity to a sequence located distally to the targeted cleavage site. In yet another embodiment, two targeting endonucleases are introduced into the cell. In any of the foregoing embodiments, the cell is a CHO cell line cell or a derivative thereof. In another embodiment, the cell is also deficient in GS. In one embodiment, the cell is a CHO cell and all copies of chromosomal sequence encoding GS are inactivated.

[0006] Still another aspect of the disclosure provides a method for identifying cells that produce high levels of at least one recombinant protein. The method comprises expressing at least one nucleic acid encoding a recombinant protein in a population of cells deficient in hypoxanthine-guanine phosphoribosyltransferase (HPRT), wherein the nucleic acid also comprises sequence encoding HPRT. The method further comprises culturing the population of cells in the presence of hypoxanthine, aminopterin, and thymidine, and selecting for cells that produces high levels of the recombinant protein. In one embodiment, all copies of chromosomal sequence encoding HPRT are inactivated in the population of cell. At the end of the process, the cells that are selected comprise additional copies of the nucleic acid encoding the recombinant protein and HPRT due to amplification of the nucleic acid. In one embodiment, the population of cells is also deficient in GS and the culturing step (b) further comprises methionine sulfoxide. In an embodiment, all copies of chromosomal sequence encoding GS are inactivated. In embodiments in which the population of cells is deficient in both HPRT and GS, the method comprises expressing a first nucleic acid encoding a first recombinant protein and HPRT and a second nucleic acid encoding a second recombinant protein and GS. At the end of such process, the cells that are

selected comprise additional copies of the first and second nucleic acids due to amplification of the nucleic acids. In one embodiment, the first recombinant protein is a therapeutic protein and the second recombinant protein is a therapeutic protein or a protein that provides an improved property to the population of cells. In any of the foregoing embodiments, the cell is a CHO cell line cell or a derivative thereof.

[0007] Other aspects and iterations of the disclosure are described in more detail below.

BRIEF DESCRIPTION OF THE FIGURES

[0008] **FIG. 1** illustrates ZFN-mediated cleavage of the *hprt* locus in CHO-K1 cells. Shown are the results of a Cel-1 nuclease assay performed using mock-transfected cells (i.e., negative control) or cells transfected with DNA encoding a ZFN targeting exon 3 of the *hprt* coding region. Sizes of cleavage products in the ZFN targeted cells are indicated at the right of the image. All intron-exon structure assignment of the CHO *hprt* locus is based on mouse homology.

[0009] **FIG. 2** presents a schematic diagram of a targeted genomic deletion protocol utilizing a ZFN and a single-stranded oligonucleotide.

[0010] **FIG. 3** shows a schematic diagram of a targeted genomic deletion protocol utilizing two ZFNs.

[0011] **FIG. 4** illustrates ZFN-mediated cleavage of the *hprt* locus in CHO-K1 cells. Shown are the results of a Cel-1 nuclease assay using mock-transfected cells (i.e., negative control) or cells transfected with DNA encoding a ZFN targeting intron 2 of the *hprt* coding region. Sizes of cleavage products in the ZFN targeted cells are indicated at the right of the image.

DETAILED DESCRIPTION OF THE INVENTION

[0012] The present disclosure provides compositions and methods for more efficient and/or increased production of recombinant protein pharmaceuticals. In particular, the present disclosure provides cells deficient in HPRT. In some embodiments, the genome of the HPRT deficient cell is edited such that the

chromosomal sequence encoding HPRT is inactivated. In certain embodiments, the inactivated chromosomal sequence comprises a deletion of at least one nucleotide, an insertion of at least one nucleotide, and/or a substitution of at least one nucleotide. In other embodiments, the HPRT deficient cells comprise a deletion of all or most of the chromosomal sequence encoding HPRT. As a consequence, an exogenous sequence encoding HPRT can be used as a metabolic selectable marker and a tool for gene amplification during recombinant protein production. In some embodiments, the HPRT deficient cells are also deficient in glutamine synthase (GS).

[0013] The present disclosure also provides methods for preparing the HPRT deficient cells, wherein the methods comprise using targeting endonucleases to inactivate the chromosomal sequence encoding HPRT. Also provided are methods for using the HPRT deficient cells for the production of high levels of recombinant proteins.

(I) HPRT Deficient Cells

[0014] One aspect of the present disclosure encompasses an isolated cell that is deficient in HPRT. In some embodiments, the HPRT deficient cell produces reduced levels of HPRT. In other embodiments, the HPRT deficient cell produces essentially no HPRT. As used herein, the phrase "essentially no HPRT" means that HPRT mRNA and/or protein cannot be detected in the cell or lysates thereof using procedures well known in the art. Non-limiting examples of suitable procedures for determining the level of mRNA or protein include PCR, qPCR, Western blotting, and ELISA assay.

[0015] In some embodiments, the cell deficient in HPRT comprises an inactivated chromosomal sequence encoding HPRT. For example, the genome of the cell can be edited using targeting endonuclease mediated genome editing to inactivate the chromosomal sequence encoding HPRT. As used herein, the term "inactivated chromosomal sequence" refers to a chromosomal sequence that is unable to generate a protein product. The inactivated chromosomal sequence encoding HPRT is stable and the HPRT deficient cell does not revert to wild type.

[0016] In embodiments in which the cell is euploid, the inactivated

chromosomal sequence encoding HPRT can be monoallelic such that the cell produces reduced levels of HPRT. In other embodiments in which the cell is euploid, the inactivated chromosomal sequence encoding HPRT can be biallelic such that the cell produces no HPRT. In other embodiments in which the cell is aneuploid, one or more copies of the chromosomal sequence(s) encoding HPRT can be inactivated such that the cell produces a reduced amount of HPRT. In still other embodiments in which the cell is aneuploid, all copies of the chromosomal sequence(s) encoding HPRT are inactivated such that the cell line produces no HPRT. In an exemplary embodiment, the cell is haploid for the chromosomal sequence encoding HPRT, and inactivation of the chromosomal sequence encoding HPRT results in a complete loss of HPRT expression.

[0017] The chromosomal sequence encoding HPRT can be inactivated by a deletion of at least one base pair (bp), an insertion of at least one bp, a substitution of at least one bp, or combinations thereof. As a consequence of the deletion(s) insertion(s), and/or substitution(s), the HPRT coding sequence undergoes a shift in the reading frame, thereby preventing production of a protein product. The chromosomal sequence encoding HPRT can be inactivated using targeting endonuclease-mediated genome editing technology as detailed below in section (II). In various embodiments, the chromosomal sequence encoding HPRT can be inactivated by deletion or disruption of all or part of the exonic coding region, deletion or disruption of all or part of a control region, deletion or disruption of all or part of an intron, deletion or disruption of a splice site such that expression of HPRT is abolished, or combinations thereof. In other embodiments, the chromosomal sequence encoding HPRT can be inactivated via deletions, insertions, and/or nucleotide substitutions to introduce a premature stop codon, new splice sites, and/or SNPs into the chromosomal sequence such that the cell is unable to produce HPRT.

[0018] In some embodiments, the HPRT deficient cell comprises a deletion ranging from about 1 bp to the entire coding region of the chromosomal sequence encoding HPRT. The deletion can encompass all or part of an exon and/or an intron in the HPRT chromosomal sequence. For example, the cell can comprise a deletion of all or part of exon 1, intron 1, exon 2, intron 2, exon 3, intron 3, exon 4, intron

4, exon 5, intron 5, exon 6, intron 6, exon 7, intron 7, exon 8, intron 8, and/or exon 9 of the HPRT chromosomal sequence. In exemplary embodiments, the cell can comprise a deletion of exon 3, exon 3-exon 9, exon 3-exon 8, exon 3-exon 7, exon 3-exon 6, exon 3-exon 5, exon 3-exon 4, intron 2-exon 9, intron 2-exon 8, intron 2-exon 7, intron 2-exon 6, intron 2-exon 5, intron 2-exon 4, exon 2-exon 9, exon 2-exon 8, exon 2-exon 7, exon 2-exon 6 exon 2-exon 5, or exon 2-exon 4 of the HPRT chromosomal sequence. In any of these embodiments, the deletion can be a partial or complete deletion of the exon(s) and/or intron(s). In an exemplary embodiment, the cell comprises a deletion of exon 3 of the HPRT coding sequence.

(a) Optional additional deficiency

[0019] In some embodiments, the cell deficient in HPRT is also deficient in GS. In one embodiment, a GS deficient cell produces essentially no GS protein. For example, the HPRT deficient cell also comprises inactivated GS chromosomal sequence(s), in which case the cell is a GS knockout, in addition to a HPRT knockout.

[0020] In some embodiments, the chromosomal sequence encoding GS can be inactivated by targeting endonuclease-mediated genome editing (detailed below in section (II)). That is, the chromosomal sequence encoding GS can be edited to comprise a deletion of at least one nucleotide, an insertion of at least one nucleotide, or a substitution of at least one nucleotide, or a combination thereof such that the chromosomal sequence is inactivated. In other embodiments, the chromosomal sequence encoding GS can be inactivated via random mutagenesis (e.g., exposure to a mutagenic chemical, and/or ionizing radiation). In further embodiments, the chromosomal sequence encoding GS can be inactivated by contact with a transposon-mediated system or a site-specific recombination system. In still other embodiments, the inactivated chromosomal sequence encoding GS can be a naturally occurring mutation.

[0021] In embodiments in which the cell is euploid, the inactivated chromosomal sequence encoding GS can be monoallelic or biallelic. In embodiments in which the cell is aneuploid, at least one or all copies of the chromosomal sequence

encoding GS can be inactivated. In exemplary embodiments, all copies of the chromosomal sequence encoding GS are inactivated such that the cell produced no GS.

(b) Cell types

[0022] The type of cell that is deficient in HPRT can and will vary. The cell can be a human cell, a mammalian cell, a vertebrate cell, or an invertebrate cell. Examples of suitable invertebrate cells include without limit insect cells, such as Sf9 cells from *Spodoptera frugiperda* or S2 cells from *Drosophila melanogaster*. Non-limiting examples of suitable vertebrate cells include those derived from frogs such as *Xenopus*, fish such as zebrafish, and fowl such as chickens. Suitable mammalian cells include without limit those derived from mice, rats, hamsters, rabbits, cats, dogs, horses, pigs, cattle, and non-human primates. In various embodiments, the cells can be primary cells. Examples of suitable primary cells include but are not limited to fibroblasts, myoblasts, T or B cells, macrophages, epithelial cells, hepatocytes, and so forth.

[0023] In other embodiments, the cell is a cell line cell. When mammalian cell lines are used, the cell line can be any mammalian cell line, including, for example, any established cell line or a cell line derived from a primary cell or other cell in culture. Suitable mammalian cell lines include Chinese hamster ovary (CHO) cells, monkey kidney CVI line transformed by SV40 (COS7); human embryonic kidney line 293; baby hamster kidney cells (BHK); monkey kidney cells (CVI-76); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK); buffalo rat liver cells (BRL 3A); human lung cells (W138); human liver cells (Hep G2); rat hepatoma cells (HTC); HIH/3T3 cells, the human U2-OS osteosarcoma cell line, the human A549 cell line, the human A-431 cell line, the human K562 cell line, the human HEK293 cell line, the human HEK293T cell line, and TRI cells. For an extensive list of mammalian non-mouse cell lines, those of ordinary skill in the art may refer to the American Type Culture Collection catalog (ATCC®, Mamassas, VA).

[0024] In still other embodiments, the cell is a stem cell. Suitable stem cells include without limit embryonic stem cells, ES-like stem cells, fetal stem cells, adult stem cells, pluripotent stem cells, induced pluripotent stem cells, multipotent stem cells, oligopotent stem cells, and unipotent stem cells.

[0025] In exemplary embodiments, the cell is of a type that is widely used for the production of recombinant proteins, such as antibodies, glycoproteins, and the like. In an exemplary embodiment, the cell is a CHO cell line cell. Numerous CHO cell lines are available from American Type Culture Collection (ATCC). Suitable CHO cell lines include, but are not limited to, CHO K1 cells and derivatives thereof. In some embodiments, the CHO cells further comprise a deletion of one or both alleles of glutamine synthase (GS). Non-limiting examples of cells deficient in GS include CHO-S cells, CHOK1SV cells, and CHOZN® GS-/- cells.

(c) Optional nucleic acids

[0026] In some embodiments, the cells disclosed herein further comprise at least one nucleic acid sequence encoding a recombinant protein. In general, the recombinant protein is heterologous, meaning that the protein is not native to the cell. In some embodiments, the recombinant protein may be a therapeutic protein, An exemplary recombinant therapeutic protein includes, without limit, an antibody, a fragment of an antibody, a monoclonal antibody, a humanized antibody, a humanized monoclonal antibody, a chimeric antibody, an IgG molecule, an IgG heavy chain, an IgG light chain, an IgA molecule, an IgD molecule, an IgE molecule, an IgM molecule, a vaccine, a growth factor, a cytokine, an interferon, an interleukin, a hormone, a clotting (or coagulation) factor, a blood component, an enzyme, a nutraceutical protein, a functional fragment or functional variant of any of the forgoing, or a fusion protein comprising any of the foregoing proteins and/or functional fragments or variants thereof.

[0027] In other embodiments, the recombinant protein may be a protein that imparts improved properties to the cell or improved properties to a first recombinant protein. Non-limiting examples of improved properties include increased robustness, increased viability, increased survival, increased proliferation, increased cell cycle

progression (i.e., increased progression form G1 to S phase), increased cell growth, increased cell size, increased production of endogenous proteins, increased production of heterologous proteins, increased stability of a recombinant protein, altered post-translational processing of a recombinant protein, and combinations of any of the above. In some embodiments, the protein that improves cell properties can be overexpressed. Non-limiting examples of suitable proteins include serpin proteins (e.g., SerpinB1), cell regulatory proteins, cell cycle control proteins, apoptotic inhibitors, metabolic pathway proteins, post-translation modification proteins, artificial transcription factors, transcriptional activators, transcriptional inhibitors, and enhancer proteins.

[0028] In general, the nucleic acid encoding the recombinant protein is DNA. The DNA can be linear or circular. The nucleic acid encoding the recombinant protein generally is operably linked to at least one expression control sequence. Suitable expression control sequences include promoter sequences, enhancer sequences, polyadenylation signals, termination signals, and translational regulatory sequences (e.g., ribosome binding sites, translation initiation sites, stop codons, etc.). The operably-linked promoter sequence can be constitutive or regulated. Suitable constitutive promoter sequences include the cytomegalovirus immediate early promoter (CMV), the SV40 promoter, the adenovirus major late promoter, Rous sarcoma virus long terminal repeat promoter, ubiquitin promoters, actin promoters, tubulin promoters, immunoglobulin promoters, fragments thereof, or combinations of any of the foregoing. The promoter sequence can be wild type or it can be modified for more efficient or efficacious expression.

[0029] In some embodiments, the nucleic acid encoding the recombinant protein can be linked to a nucleic acid encoding HPRT, such that HPRT may be used as an amplifiable selectable marker. The amplifiable selectable marker not only allows for identification of stably transfected cells but also amplification of the nucleic acid sequence encoding the recombinant protein. The sequence encoding HPRT can be operably linked to the sequence encoding the recombinant protein, such that both sequences are under the control of the same promoter and can be co-expressed. Alternatively, the sequence encoding HPRT can be operably linked to a separate

promoter sequence such that it is expressed separately from the sequence encoding the recombinant protein. Cells comprising a nucleic acid sequence encoding the recombinant protein and HPRT can be grown in the presence of increasing concentrations of hypoxanthine-aminopterin-thymidine (HAT), and cells can be selected for survival due to amplification of the nucleic acid sequence(s) encoding the recombinant protein and/or HPRT.

[0030] In embodiments in which the cell is deficient in both HPRT and GS, the nucleic acid encoding the recombinant protein can be linked to a nucleic acid encoding GS such that GS can also be used as an amplifiable selectable marker. For this, the cells are grown in the presence of methionine sulfoximide (MSX). The sequence encoding GS can be operably linked to the same promoter sequence controlling the expression of the recombinant protein, or the sequence encoding GS can be operably linked to a separate promoter sequence. In embodiments in which the cell comprises a first sequence encoding a first recombinant protein and HPRT and a second sequence encoding a second recombinant protein and GS, the cell can be selected (simultaneously or sequentially) for growth in the presence of HAT and MSX.

[0031] Upon amplification of the nucleic acid sequence encoding the recombinant protein and the amplifiable selectable marker (i.e., HPRT, GS), the cell can contain from 1-10 additional copies, from 10-100 additional copies, from 100-1000, or many thousands of additional copies the nucleic acid sequence encoding the recombinant protein and the amplifiable selectable marker.

[0032] In some embodiments, the one or more copies of the nucleic acid sequence encoding the recombinant protein and the amplifiable selectable marker can be located episomally so as to be stably maintained in the cell or cell line. In other embodiments, the one or more copies of the nucleic acid sequence encoding the recombinant protein and the amplifiable selectable marker can be integrated into the nuclear genome of the cell line.

(d) Exemplary embodiments

[0033] In specific embodiments, the HPRT deficient cell is a CHO cell line cell. CHO cell line cells have a single intact X chromosome (Campbell et al., 1977, Cytogenetics and Cell Genetics 19, 303-319) and only one copy of the X-linked *hprt* gene (Chu et al., 1968, Proc. Natl. Acad. Sci. USA 61, 1306-1312). In an exemplary embodiment, the HPRT deficient CHO cell comprises a deletion of all or part of the single copy of the chromosomal sequence encoding HPRT. The genotype of such cell is $HPRT^{-/0}$, and the cell produces no HPRT protein. In another exemplary embodiment, the HPRT deficient CHO cell is also deficient in GS. That is, the HPRT deficient cell comprises a biallelic inactivation of the chromosomal sequence encoding GS. The genotype of such cell is $HPRT^{-/0}$ and $GS^{-/-}$, and the cell produced no HPRT or GS protein.

(II) Methods for Preparing HPRT Deficient Cells

[0034] Yet another aspect of the present disclosure provides a method for preparing a cell deficient in HPRT by editing a chromosomal sequence encoding HPRT. The method comprises introducing into a cell (i) at least one targeting endonuclease or at least one nucleic acid encoding a targeting endonuclease, wherein each targeting endonuclease is able to introduce a double-stranded break at a targeted cleavage site in the chromosomal sequence encoding HPRT, and optionally, (ii) at least one polynucleotide comprising a sequence having substantial sequence identity to a region on at least one side of the targeted cleavage site in the chromosomal sequence encoding HPRT. The method further comprises maintaining the cell under conditions such that the double-stranded break introduced by the targeting endonuclease(s) is repaired by (i) a non-homologous end-joining repair process such that a mutation is introduced into the chromosomal sequence or, optionally, (ii) a homology-directed repair process such that the chromosomal sequence is exchanged with the sequence of the polynucleotide, thereby editing the chromosomal sequence encoding HPRT such that the cell is deficient in HPRT.

[0035] Suitable cells are detailed above in section (I)(b).

(a) Targeting endonucleases

[0036] The type of targeting endonuclease used in the method disclosed herein can and will vary. The targeting endonuclease can be a naturally-occurring protein or an engineered protein. In one embodiment, the targeting endonuclease is a meganuclease. Meganucleases are endodeoxyribonucleases characterized by a large recognition site, i.e., the recognition site generally ranges from about 12 base pairs to about 40 base pairs. As a consequence of this requirement, the recognition site generally occurs only once in any given genome. Among meganucleases, the family of homing endonucleases named LAGLIDADG has become a valuable tool for the study of genomes and genome engineering. Meganucleases can be targeted to specific chromosomal sequence by modifying their recognition sequence using techniques well known to those skilled in the art.

[0037] In another embodiment, the targeting endonuclease is a transcription activator-like effector (TALE) nuclease. TALEs are transcription factors from the plant pathogen Xanthomonas that can be readily engineered to bind new DNA targets. TALEs or truncated versions thereof may be linked to the catalytic domain of endonucleases such as FokI to create targeting endonuclease called TALE nucleases or TALENs.

[0038] In still another embodiment, the targeting endonuclease is a site-specific nuclease. In particular, the site-specific nuclease can be a "rare-cutter" endonuclease whose recognition sequence occurs rarely in a genome. Preferably, the recognition sequence of the site-specific nuclease occurs only once in a genome. In an alternate further embodiment, the targeting nuclease can be an artificial targeted DNA double strand break inducing agent.

(b) Zinc finger nucleases

[0039] In exemplary embodiments, the targeting endonuclease is a zinc finger nuclease (ZFN). Typically, a zinc finger nuclease comprises a DNA binding domain (i.e., zinc finger) and a cleavage domain (i.e., nuclease), both of which are described below.

(i) Zinc finger binding domain

[0040] .Zinc finger binding domains may be engineered to recognize and bind to any nucleic acid sequence of choice. See, for example, Beerli et al. (2002) Nat. Biotechnol. 20:135-141; Pabo et al. (2001) Ann. Rev. Biochem. 70:313-340; Isalan et al. (2001) Nat. Biotechnol. 19:656-660; Segal et al. (2001) Curr. Opin. Biotechnol. 12:632-637; Choo et al. (2000) Curr. Opin. Struct. Biol. 10:411-416; Zhang et al. (2000) J. Biol. Chem. 275(43):33850-33860; Doyon et al. (2008) Nat. Biotechnol. 26:702-708; and Santiago et al. (2008) Proc. Natl. Acad. Sci. USA 105:5809-5814. An engineered zinc finger binding domain can have a novel binding specificity compared to a naturally-occurring zinc finger protein. Engineering methods include, but are not limited to, rational design and various types of selection. Rational design includes, for example, using databases comprising doublet, triplet, and/or quadruplet nucleotide sequences and individual zinc finger amino acid sequences, in which each doublet, triplet or quadruplet nucleotide sequence is associated with one or more amino acid sequences of zinc fingers which bind the particular triplet or quadruplet sequence. See, for example, U.S. Pat. Nos. 6,453,242 and 6,534,261, the disclosures of which are incorporated by reference herein in their entireties. As an example, the algorithm described in US patent 6,453,242 can be used to design a zinc finger binding domain to target a preselected sequence. Alternative methods, such as rational design using a nondegenerate recognition code table can also be used to design a zinc finger binding domain to target a specific sequence (Sera et al. (2002) Biochemistry 41:7074-7081). Publically available web-based tools for identifying potential target sites in DNA sequences and designing zinc finger binding domains are found at http://www.zincfingertools.org and http://zifit.partners.org/ZiFiT/, respectively (Mandell et al. (2006) Nuc. Acid Res. 34:W516-W523; Sander et al. (2007) Nuc. Acid Res. 35:W599-W605).

[0041] A zinc finger binding domain may be designed to recognize and bind a DNA sequence ranging from about 3 nucleotides to about 21 nucleotides in length, for example, from about 9 to about 18 nucleotides in length. Each zinc finger

recognition region (i.e., zinc finger) recognizes and binds three nucleotides. In general, the zinc finger binding domains of the zinc finger nucleases disclosed herein comprise at least three zinc finger recognition regions (i.e., zinc fingers). In one embodiment, the zinc finger binding domain comprises four zinc finger recognition regions. In another embodiment, the zinc finger binding domain comprises five zinc finger recognition regions. In still another embodiment, the zinc finger binding domain comprises six zinc finger recognition regions. A zinc finger binding domain can be designed to bind to any suitable target DNA sequence. See for example, U.S. Pat. Nos. 6,607,882; 6,534,261 and 6,453,242, the disclosures of which are incorporated by reference herein in their entireties.

[0042] Exemplary methods of selecting a zinc finger recognition region include phage display and two-hybrid systems, and are disclosed in U.S. Pat. Nos. 5,789,538; 5,925,523; 6,007,988; 6,013,453; 6,410,248; 6,140,466; 6,200,759; and 6,242,568; as well as WO 98/37186; WO 98/53057; WO 00/27878; WO 01/88197 and GB 2,338,237, each of which is incorporated by reference herein in its entirety. In addition, enhancement of binding specificity for zinc finger binding domains has been described, for example, in WO 02/077227, the disclosure of which is incorporated herein by reference.

[0043] Zinc finger binding domains and methods for design and construction of fusion proteins (and polynucleotides encoding same) are known to those of skill in the art and are described in detail in U.S. Patent Application Publication Nos. 20050064474 and 20060188987, each incorporated by reference herein in its entirety. Zinc finger recognition regions and/or multi-fingered zinc finger proteins may be linked together using suitable linker sequences, including for example, linkers of five or more amino acids in length. See, U.S. Pat. Nos. 6,479,626; 6,903,185; and 7,153,949, the disclosures of which are incorporated by reference herein in their entireties, for non-limiting examples of linker sequences of six or more amino acids in length. The zinc finger binding domain described herein may include a combination of suitable linkers between the individual zinc fingers (and additional domains) of the protein.

(ii) *Cleavage domain*

[0044] A zinc finger nuclease also includes a cleavage domain. The cleavage domain portion of the zinc finger nuclease can be obtained from any endonuclease or exonuclease. Non-limiting examples of endonucleases from which a cleavage domain can be derived include, but are not limited to, restriction endonucleases and homing endonucleases. See, for example, New England Biolabs catalog (www.neb.com) and Belfort et al. (1997) Nucleic Acids Res. 25:3379-3388. Additional enzymes that cleave DNA are known (e.g., S1 Nuclease; mung bean nuclease; pancreatic DNase I; micrococcal nuclease; yeast HO endonuclease). See also Linn et al. (eds.) Nucleases, Cold Spring Harbor Laboratory Press, 1993. One or more of these enzymes (or functional fragments thereof) can be used as a source of cleavage domains.

[0045] A cleavage domain also can be derived from an enzyme or portion thereof, as described above, that requires dimerization for cleavage activity. Two zinc finger nucleases can be required for cleavage, as each nuclease comprises a monomer of the active enzyme dimer. Alternatively, a single zinc finger nuclease can comprise both monomers to create an active enzyme dimer. As used herein, an "active enzyme dimer" is an enzyme dimer capable of cleaving a nucleic acid molecule. The two cleavage monomers can be derived from the same endonuclease (or functional fragments thereof), or each monomer can be derived from a different endonuclease (or functional fragments thereof).

[0046] When two cleavage monomers are used to form an active enzyme dimer, the recognition sites for the two zinc finger nucleases are preferably disposed such that binding of the two zinc finger nucleases to their respective recognition sites places the cleavage monomers in a spatial orientation to each other that allows the cleavage monomers to form an active enzyme dimer, e.g., by dimerizing. As a result, the near edges of the recognition sites can be separated by about 5 to about 18 nucleotides. For instance, the near edges can be separated by about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18 nucleotides. It will however be understood that any integral number of nucleotides or nucleotide pairs can intervene between two

recognition sites (e.g., from about 2 to about 50 nucleotide pairs or more). The near edges of the recognition sites of the zinc finger nucleases, such as for example those described in detail herein, can be separated by 6 nucleotides. In general, the site of cleavage lies between the recognition sites.

[0047] Restriction endonucleases (restriction enzymes) are present in many species and are capable of sequence-specific binding to DNA (at a recognition site), and cleaving DNA at or near the site of binding. Certain restriction enzymes (e.g., Type IIS) cleave DNA at sites removed from the recognition site and have separable binding and cleavage domains. For example, the Type IIS enzyme FokI catalyzes double-stranded cleavage of DNA, at 9 nucleotides from its recognition site on one strand and 13 nucleotides from its recognition site on the other. See, for example, U.S. Pat. Nos. 5,356,802; 5,436,150 and 5,487,994; as well as Li et al. (1992) Proc. Natl. Acad. Sci. USA 89:4275-4279; Li et al. (1993) Proc. Natl. Acad. Sci. USA 90:2764-2768; Kim et al. (1994a) Proc. Natl. Acad. Sci. USA 91:883-887; Kim et al. (1994b) J. Biol. Chem. 269:31, 978-31, 982. Thus, a zinc finger nuclease can comprise the cleavage domain from at least one Type IIS restriction enzyme and one or more zinc finger binding domains, which may or may not be engineered. Exemplary Type IIS restriction enzymes are described for example in International Publication WO 07/014,275, the disclosure of which is incorporated by reference herein in its entirety. Additional restriction enzymes also contain separable binding and cleavage domains, and these also are contemplated by the present disclosure. See, for example, Roberts et al. (2003) Nucleic Acids Res. 31:418-420.

[0048] An exemplary Type IIS restriction enzyme, whose cleavage domain is separable from the binding domain, is FokI. This particular enzyme is active as a dimer (Bitinaite et al. (1998) Proc. Natl. Acad. Sci. USA 95: 10, 570-10, 575). Accordingly, for the purposes of the present disclosure, the portion of the FokI enzyme used in a zinc finger nuclease is considered a cleavage monomer. Thus, for targeted double-stranded cleavage using a FokI cleavage domain, two zinc finger nucleases, each comprising a FokI cleavage monomer, can be used to reconstitute an active

enzyme dimer. Alternatively, a single polypeptide molecule containing a zinc finger binding domain and two FokI cleavage monomers can also be used.

[0049] In certain embodiments, the cleavage domain comprises one or more engineered cleavage monomers that minimize or prevent homodimerization, as described, for example, in U.S. Patent Publication Nos. 20050064474, 20060188987, and 20080131962, each of which is incorporated by reference herein in its entirety. By way of non-limiting example, amino acid residues at positions 446, 447, 479, 483, 484, 486, 487, 490, 491, 496, 498, 499, 500, 531, 534, 537, and 538 of FokI are all targets for influencing dimerization of the FokI cleavage half-domains. Exemplary engineered cleavage monomers of FokI that form obligate heterodimers include a pair in which a first cleavage monomer includes mutations at amino acid residue positions 490 and 538 of FokI and a second cleavage monomer that includes mutations at amino-acid residue positions 486 and 499 (Miller et al., 2007, Nat. Biotechnol, 25:778-785; Szczpek et al., 2007, Nat. Biotechnol, 25:786-793). For example, the Glu (E) at position 490 can be changed to Lys (K) and the Ile (I) at position 538 can be changed to K in one domain (E490K, I538K), and the Gln (Q) at position 486 can be changed to E and the I at position 499 can be changed to Leu (L) in another cleavage domain (Q486E, I499L). In other embodiments, modified FokI cleavage domains can include three amino acid changes (Doyon et al. 2011, Nat. Methods, 8:74-81). For example, one modified FokI domain (which is termed ELD) can comprise Q486E, I499L, N496D mutations and the other modified FokI domain (which is termed KKR) can comprise E490K, I538K, H537R mutations.

(iii) *Additional domains*

[0050] In some embodiments, the zinc finger nuclease further comprises at least one nuclear localization signal or sequence (NLS). A NLS is an amino acid sequence which facilitates targeting the zinc finger nuclease protein into the nucleus to introduce a double stranded break at the target sequence in the chromosome. Nuclear localization signals are known in the art. See, for example, Makkerh et al. (1996)

Current Biology 6:1025-1027. The NLS can be located at the N-terminus, the C-terminal, or in an internal location of the zinc finger nuclease.

[0051] In additional embodiments, the zinc finger nuclease can also comprise at least one cell-penetrating domain. The cell-penetrating domain can be a cell-penetrating peptide sequence derived from the HIV-1 TAT protein, a cell-penetrating peptide sequence derived from the human hepatitis B virus, a cell penetrating peptide from Herpes simplex virus, MPG peptide, Pep-1 peptide, or a polyarginine peptide sequence. The cell-penetrating domain can be located at the N-terminus, the C-terminal, or in an internal location of the zinc finger nuclease.

(vi) *Exemplary zinc finger nucleases*

[0052] In some embodiments, the targeting endonuclease is a zinc finger nuclease. In one embodiment, the zinc finger nuclease is engineered to recognize and cleave a target sequence in intron 2 of the chromosomal sequence encoding HPRT. In another embodiment, the zinc finger nuclease is engineered to recognize and cleave a target sequence in exon 3 of the chromosomal sequence encoding HPRT.

(c) Optional polynucleotide

[0053] The method for targeted genome editing can further comprise introducing into the cell at least one polynucleotide comprising a sequence having substantial sequence identity to a sequence on at least one side of the targeted cleavage site. For example, the polynucleotide comprises a first sequence having substantial sequence identity to sequence on one side of the targeted cleavage site and a second sequence having substantial sequence identity to sequence on the other side of the targeted cleavage site. Alternatively, the polynucleotide comprises a first sequence having substantial sequence identity to sequence on one side of the targeted cleavage site and a second sequence having substantial sequence identity to a sequence located away from the targeted cleavage site. The sequence located away from the targeted cleavage site may be tens, hundreds, or thousands of nucleotides upstream or downstream of the targeted cleavage site.

[0054] The lengths of the first and second sequences in the polynucleotide that have substantial sequence identity to sequences in the chromosomal sequence encoding HPRT can and will vary. In general, each of the first and second sequences in the polynucleotide is at least about 10 nucleotides in length. In various embodiments, the polynucleotide sequences having substantial sequence identity with chromosomal sequences can be about 15 nucleotides, about 20 nucleotides, about 25 nucleotides, about 30 nucleotides, about 40 nucleotides, about 50 nucleotides, about 100 nucleotides, or more than 100 nucleotides in length.

[0055] The phrase "substantial sequence identity" means that the sequences in the polynucleotide have at least about 75% sequence identity with the chromosomal sequences of interest. In some embodiments, the sequences in the polynucleotide about 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity with the chromosomal sequences of interest.

[0056] The length of the polynucleotide can and will vary. For example, the polynucleotide can range from about 20 nucleotides in length up to about 200,000 nucleotides in length. In various embodiments, the polynucleotide ranges from about 20 nucleotides to about 100 nucleotides in length, from about 100 nucleotides to about 1000 nucleotides in length, from about 1000 nucleotides to about 10,000 nucleotides in length, from about 10,000 nucleotides to about 100,000 nucleotides in length, or from about 100,000 nucleotides to about 200,000 nucleotides in length.

[0057] Typically, the polynucleotide is DNA. The DNA can be single-stranded or double-stranded. The polynucleotide can be a DNA plasmid, a bacterial artificial chromosome (BAC), a yeast artificial chromosome (YAC), a viral vector, a linear piece of DNA, a PCR fragment, a naked nucleic acid, or a nucleic acid complexed with a delivery vehicle such as a liposome or poloxamer. In certain embodiments, the polynucleotide is single-stranded. In exemplary embodiments, the polynucleotide is a single-stranded oligonucleotide comprising less than about 200 nucleotides.

[0058] In some embodiments, the polynucleotide further comprises a marker. Such a marker may enable screening for targeted integrations. In some

embodiments, the marker is a restriction endonuclease site. In other embodiments the marker is a fluorescent protein, a purification tag, or an epitope tag. In some embodiments, the marker can be a fluorescent protein. Non limiting examples of suitable fluorescent proteins include green fluorescent proteins (e.g., GFP, GFP-2, tagGFP, turboGFP, EGFP, Emerald, Azami Green, Monomeric Azami Green, CopGFP, AceGFP, ZsGreen1), yellow fluorescent proteins (e.g. YFP, EYFP, Citrine, Venus, YPet, PhiYFP, ZsYellow1,), blue fluorescent proteins (e.g. EBFP, EBFP2, Azurite, mKalama1, GFPuv, Sapphire, T-sapphire,), cyan fluorescent proteins (e.g. ECFP, Cerulean, CyPet, AmCyan1, Midoriishi-Cyan), red fluorescent proteins (mKate, mKate2, mPlum, DsRed monomer, mCherry, mRFP1, DsRed-Express, DsRed2, DsRed-Monomer, HcRed-Tandem, HcRed1, AsRed2, eqFP611, mRasberry, mStrawberry, Jred), and orange fluorescent proteins (mOrange, mKO, Kusabira-Orange, Monomeric Kusabira-Orange, mTangerine, tdTomato) or any other suitable fluorescent protein. In other embodiments, the marker can be a purification tag and/or an epitope tag. Exemplary tags include, but are not limited to, glutathione-S-transferase (GST), chitin binding protein (CBP), maltose binding protein, thioredoxin (TRX), poly(NANP), tandem affinity purification (TAP) tag, myc, AcV5, AU1, AU5, E, ECS, E2, FLAG, HA, nus, Softag 1, Softag 3, Strep, SBP, Glu-Glu, HSV, KT3, S, S1, T7, V5, VSV-G, 6xHis, biotin carboxyl carrier protein (BCCP), and calmodulin.

(d) Delivery to the cell

[0059] The method further comprises introducing the targeting endonuclease into the cell. The targeting endonuclease can be introduced into the cell as a purified isolated protein or as a nucleic acid encoding the targeting endonuclease. The nucleic acid may be DNA or RNA. In embodiments in which the encoding nucleic acid is mRNA, the mRNA may be 5' capped and/or 3' polyadenylated. In embodiments in which the encoding nucleic acid is DNA, the DNA may be linear or circular. The DNA may be part of a vector, wherein the encoding DNA may be operably linked to a suitable promoter. Those skilled in the art are familiar with appropriate vectors, promoters, other control elements, and means of introducing the vector into the cell of interest.

[0060] The targeting endonuclease molecule(s) and the optional polynucleotide(s) described above can be introduced into the cell by a variety of means. Suitable delivery means include microinjection, electroporation, sonoporation, biolistics, calcium phosphate-mediated transfection, cationic transfection, liposome transfection, dendrimer transfection, heat shock transfection, nucleofection transfection, magnetofection, lipofection, impalefection, optical transfection, proprietary agent-enhanced uptake of nucleic acids, and delivery via liposomes, immunoliposomes, virosomes, or artificial virions. In a specific embodiment, the targeting endonuclease molecule(s) and polynucleotides(s) are introduced into the cell by nucleofection.

[0061] In embodiments in which more than one targeting endonuclease molecule and more than one polynucleotide are introduced into a cell, the molecules can be introduced simultaneously or sequentially. For example, targeting endonuclease molecules, each specific for a targeted cleavage site (and optional polynucleotides) can be introduced at the same time. Alternatively, each targeting endonuclease molecule, as well as the optional polynucleotides(s) can be introduced sequentially.

[0062] The ratio of the targeting endonuclease molecule(s) to the optional polynucleotide(s) can and will vary. In general, the ratio of targeting endonuclease molecule(s) to polynucleotide(s) ranges from about 1:10 to about 10:1. In various embodiments, the ratio of the targeting endonuclease molecule(s) to polynucleotide(s) may be about 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, or 10:1. In one embodiment, the ratio is about 1:1.

(e) Culturing the cell

[0063] The method further comprises maintaining the cell under appropriate conditions such that the double-stranded break introduced by the targeting endonuclease can be repaired by (i) a non-homologous end-joining repair process such that the chromosomal sequence is edited or, optionally, (ii) a homology-directed repair process such that the chromosomal sequence is exchanged with the sequence of the polynucleotide such that the chromosomal sequence is edited. In embodiments in which nucleic acid(s) encoding the targeting endonuclease(s) is introduced into the cell,

the method comprises maintaining the cell under appropriate conditions such that the cell expresses the targeting endonuclease(s).

[0064] In general, the cell is maintained under conditions appropriate for cell growth and/or maintenance. Suitable cell culture conditions are well known in the art and are described, for example, in Santiago et al. (2008) PNAS 105:5809-5814; Moehle et al. (2007) PNAS 104:3055-3060; Urnov et al. (2005) Nature 435:646-651; and Lombardo et al (2007) Nat. Biotechnology 25:1298-1306. Those of skill in the art appreciate that methods for culturing cells are known in the art and can and will vary depending on the cell type. Routine optimization may be used, in all cases, to determine the best techniques for a particular cell type.

[0065] During this step of the process, the targeting endonuclease(s) recognizes, binds, and creates a double-stranded break(s) at the targeted cleavage site(s) in the chromosomal sequence, and during repair of the double-stranded break(s) a deletion, insertion, and/or substitution of at least one nucleotide is introduced into the chromosomal sequence encoding HPRT. In preferred embodiments, the chromosomal sequence encoding HPRT is edited such that the sequence is inactivated.

[0066] The frequency of the targeted editing of the chromosomal sequence can and will vary depending upon a variety of factors. In some embodiments, the frequency of editing is greater than about 0.01%, 0.03%, 0.1%, 0.3%, 1%, 3%, 10%, 20%, or 30%. Single cell clones comprising the edited chromosomal sequence can be isolated using techniques well known in the art.

[0067] In one embodiment, one targeting endonuclease (and no polynucleotide) is introduced into the cell and the double-stranded break introduced by the targeting endonuclease is repaired by a non-homologous end-joining repair process such that the chromosomal sequence encoding HRPT is inactivated. The inactivation can be due to a deletion of at least one nucleotide, an insertion of at least one nucleotide, a substation of at least one nucleotide, or a combination thereof.

[0068] In another embodiment, two targeting endonucleases (are introduced into the cell (with no polynucleotide). As an example, the first endonuclease targets and cleaves a sequence in intron 2 or exon 3 of the HPRT sequence and the

second endonuclease targets and cleaves a sequence in any of exons 4-9 of the HPRT sequence. The two double-stranded breaks are repaired by a non-homologous end-joining repair process such that the sequence between the two breaks is deleted, thereby inactivating the chromosomal sequence encoding HPRT.

[0069] In another embodiment, one targeting endonuclease and one single-stranded polynucleotide are introduced into the cell, wherein the polynucleotide comprises a first sequence having substantial sequence identity to a sequence adjacent to one side of the targeted cleavage site and a second sequence having substantial sequence identity to a sequence located distally to the targeted cleavage site. For example, the endonuclease targets and cleaves intron 2 or exon 3 of the HPRT sequence, and the first sequence of the polynucleotide has substantial sequence identity to a sequence in intron 2 or exon 3 of the HPRT sequence and the second sequence of the polynucleotide has substantial sequence identity to a sequence in any of exons 4-9 of the HPRT sequence. The double-stranded break introduced by the targeting endonuclease is repaired by a homology-directed repair process such that the chromosomal sequence between the two sequences having substantial sequence identity with the first and second sequences in the single-stranded polynucleotide is deleted, thereby inactivating the chromosomal sequence encoding HPRT.

(f) Double knock-out HPRT and GS cells

[0070] In certain embodiments, the HPRT deficient cell is also deficient in GS. In some embodiments, the cell is a double knock-out. That is, all copies of the chromosomal sequences encoding HPRT and GS are inactivated, and the cell produces not HPRT or GS. Such a double knock-out cell can be prepared by using a GS deficient cell as the starting cell in the targeting endonuclease mediated genome editing method detailed above in sections (II)(a)-(e). Non-limiting examples of GS deficient cells are listed above in section (I)(b). In an exemplary embodiment, the starting cell is a CHOZN® GS-/- cell.

[0071] In other embodiments, the targeting endonuclease mediated genome editing method detailed above in sections (II)(a)-(e) can be used to inactivate

the chromosomal sequence encoding GS. The chromosomal sequence encoding GS can be edited before, after, or concurrently with the editing of the chromosomal sequence encoding HPRT. A targeting endonuclease, such as a zinc finger nuclease, can be engineered to target a sequence in exon 1, intron 1, exon 2, intron 2, exon 3, intron 3, exon 4, intron 4, exon 5, intron 6, exon 6, intron 6, or exon 7 of the GS chromosomal sequence. In one embodiment, a zinc finger nuclease is engineered to recognize and cleave a target site in exon 6 of the GS sequence. The double-stranded break in exon 6 is repaired by the error-prone non-homologous end-joining repair process such that a short region is deleted, thereby shifting the reading frame and inactivating the GS gene.

(III) Methods for Identifying Cells that are High Producers

[0072] A further aspect of the present disclosure encompasses a method for identifying cells that produce high levels of at least one recombinant protein. The method comprises (a) expressing at least one nucleic acid encoding a recombinant protein in a population of cells deficient in HPRT, wherein the nucleic acid further comprises sequence encoding HPRT; (b) culturing the population of cells in the presence of hypoxanthine, aminopterin, and thymidine; and (c) selecting for cells that produces high levels of the at least one recombinant protein.

[0073] The recombinant protein can be a therapeutic protein or a protein that imparts improved properties to the cell as detailed above in section (I)(c). Nucleic acids encoding the recombinant protein and the amplifiable selectable marker are also described in section (I)(c). Suitable cells are detailed above in section (I)(b). A protocol for selecting cells that produce high levels of a recombinant protein is detailed below in Example 5. Cells that are selected for producing high levels of the recombinant protein comprise additional copies of the nucleic acid encoding the recombinant protein and HPRT due to amplification of the nucleic acid during the selection procedure (detailed above in section (I)(c)).

[0074] In some embodiments, the population of HPRT deficient cells is also deficient in GS. For example, the cells are double knockouts of HPRT and GS. In

such embodiments, the method comprises expressing in the cells a first nucleic acid encoding a first recombinant protein and HPRT and a second nucleic acid encoding a second recombinant protein and GS, and selecting for cells that produce high levels of both recombinant proteins. In these embodiments, in addition to the methods for culturing and selecting described herein for HPRT deficient cells, the culturing and selecting steps further comprise growth in the presence of MSX (see Example 6). The selection using GS can occur simultaneously with the selection using HPRT. Alternatively, the two selection methods can be performed concurrently (and in either order).

[0075] In embodiments in which the cells are deficient in both HPRT and GS, the first recombinant protein can be a first subunit of a multi-subunit protein (e.g., heavy chain of IgG) and the second recombinant protein can be a second subunit of the multi-subunit protein (e.g., light chain of IgG). In other embodiments, the first recombinant protein can be a therapeutic protein and the second recombinant protein can be a therapeutic protein that is different from the first recombinant protein. In still additional embodiments, the first recombinant protein can be a therapeutic protein and the second recombinant protein can be a protein that provides an improved property to the population of cells (see examples provided above in section (I)(c)).

[0076] In some embodiments, the amount of recombinant protein(s) produced by the selected cells may be increased by at least about 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 12-fold, 15-fold, 20-fold, 25-fold, 30-fold, 40-fold, 50-fold, 60-fold, 80-fold, 100-fold, or more than 100-fold of the amount of recombinant protein(s) produced by a comparable wild-type cell not deficient in HPRT (or deficient in both HPRT and GS) and not subjected to selection process.

DEFINITIONS

[0077] Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton et al., Dictionary of

Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0078] When introducing elements of the present disclosure or the preferred embodiments(s) thereof, the articles "a", "an", "the" and "said" are intended to mean that there are one or more of the elements. The terms "comprising", "including" and "having" are intended to be inclusive and mean that there may be additional elements other than the listed elements.

[0079] As used herein, the term "endogenous sequence" refers to a chromosomal sequence that is native to the cell.

[0080] The term "exogenous sequence" refers to a chromosomal sequence that is not native to the cell, or a chromosomal sequence that is located in a different chromosomal location.

[0081] The terms “editing,” “genome editing,” or "chromosomal editing" refer to a process by which a specific chromosomal sequence is changed. The edited chromosomal sequence may comprise an insertion of at least one nucleotide, a deletion of at least one nucleotide, and/or a substitution of at least one nucleotide.

[0082] A "gene," as used herein, refers to a DNA region (including exons and introns) encoding a gene product, as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites, and locus control regions.

[0083] The term "heterologous" refers to an entity that is not native to the cell or species of interest.

[0084] The terms “nucleic acid” and “polynucleotide” refer to a deoxyribonucleotide or ribonucleotide polymer, in linear or circular conformation. For the purposes of the present disclosure, these terms are not to be construed as limiting with respect to the length of a polymer. The terms can encompass known analogs of natural nucleotides, as well as nucleotides that are modified in the base, sugar and/or phosphate moieties. In general, an analog of a particular nucleotide has the same base-pairing specificity; i.e., an analog of A will base-pair with T. The nucleotides of a nucleic acid or polynucleotide may be linked by phosphodiester, phosphothioate, phosphoramidite, phosphorodiamidate bonds, or combinations thereof. .

[0085] The term "nucleotide" refers to deoxyribonucleotides or ribonucleotides. The nucleotides may be standard nucleotides (i.e., adenosine, guanosine, cytidine, thymidine, and uridine) or nucleotide analogs. A nucleotide analog refers to a nucleotide having a modified purine or pyrimidine base or a modified ribose moiety. A nucleotide analog may be a naturally occurring nucleotide (e.g., inosine) or a non-naturally occurring nucleotide. Non-limiting examples of modifications on the sugar or base moieties of a nucleotide include the addition (or removal) of acetyl groups, amino groups, carboxyl groups, carboxymethyl groups, hydroxyl groups, methyl groups, phosphoryl groups, and thiol groups, as well as the substitution of the carbon and nitrogen atoms of the bases with other atoms (e.g., 7-deaza purines). Nucleotide analogs also include dideoxy nucleotides, 2’-O-methyl nucleotides, locked nucleic acids (LNA), peptide nucleic acids (PNA), and morpholinos.

[0086] The terms “polypeptide” and “protein” are used interchangeably to refer to a polymer of amino acid residues.

[0087] As used herein, the terms "target site" or "target sequence" refer to a nucleic acid sequence that defines a portion of a chromosomal sequence to be edited and to which a targeting endonuclease is engineered to recognize and bind, provided sufficient conditions for binding exist.

[0088] The terms "upstream" and "downstream" refer to locations in a nucleic acid sequence relative to a fixed position. Upstream refers to the region that is

5' (i.e., near the 5' end of the strand) to the position and downstream refers to the region that is 3' (i.e., near the 3' end of the strand) to the position.

[0089] Techniques for determining nucleic acid and amino acid sequence identity are known in the art. Typically, such techniques include determining the nucleotide sequence of the mRNA for a gene and/or determining the amino acid sequence encoded thereby, and comparing these sequences to a second nucleotide or amino acid sequence. Genomic sequences can also be determined and compared in this fashion. In general, identity refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more sequences (polynucleotide or amino acid) can be compared by determining their percent identity. The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov, Nucl. Acids Res. 14(6):6745-6763 (1986). An exemplary implementation of this algorithm to determine percent identity of a sequence is provided by the Genetics Computer Group (Madison, Wis.) in the "BestFit" utility application. Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code=standard; filter=none; strand=both; cutoff=60; expect=10; Matrix=BLOSUM62; Descriptions=50 sequences; sort by=HIGH SCORE; Databases=non-redundant, GenBank+EMBL+DDBJ+PDB+GenBank CDS translations+Swiss protein+Spupdate+PIR. Details of these programs can be found on the GenBank website. With respect to sequences described herein, the range of desired degrees of

sequence identity is approximately 80% to 100% and any integer value therebetween. Typically the percent identities between sequences are at least 70-75%, preferably 80-82%, more preferably 85-90%, even more preferably 92%, still more preferably 95%, and most preferably 98% sequence identity.

[0090] As various changes could be made in the above-described cells and methods without departing from the scope of the invention, it is intended that all matter contained in the above description and in the examples given below, shall be interpreted as illustrative and not in a limiting sense.

EXAMPLES

[0091] The following examples illustrate certain aspects of the invention.

Example 1: Preparation of HPRT Deficient CHO Cells

[0092] A ZFN was designed to target and cleave exon 3 of the *hprt* locus of CHO cells (the exon/intron annotation is based on mousse homology). The ZFN binds to the following sequence in the *hprt* locus: CTTGCCCGAGATGTCatgaaaGAGATGGGAGGC (SEQ ID NO:1), wherein the ZFN cleavage site is designated in lower case. CHO-K1 cells were transfected by electroporation with plasmid DNA encoding the ZFN. After a suitable period of incubation, the cells were harvested and subjected to a Cel-1 assay. The Cel-1 assay detects alleles of the target locus that deviate from wild type as a result of non-homologous end-joining-mediated imperfect repair of ZFN-induced DNA double strand breaks. PCR amplification of the targeted region from a pool of ZFN-treated cells generates a mixture of WT and mutant amplicons. Melting and reannealing of this mixture results in mismatches forming between heteroduplexes of the WT and mutant alleles. A DNA "bubble" formed at the site of mismatch is cleaved by the surveyor nuclease Cel-1, and the cleavage products can be resolved by gel electrophoresis. As shown in FIG. **1**, cleavage was observed in CHO cells transfected with DNA encoding the ZFN (1.4% modified). Cleavage products of 352, 218, and 134 bp were detected. Single cell clones can be isolated using standard procedures.

[0093] Cells with an inactivated *hprt* gene can be selected for by growth in the presence of 6-thioguanine (i.e.., cells deficient in HPRT are resistant to 6-thioguanine). Alternatively, the phenotype of the cells can be confirmed by the requirement for hypoxanthine and thymidine for survival (i.e., the cells die in the absence of hypoxanthine and thymidine).

Example 2: HPRT Deficient CHO Cells

[0094] A ZFN was designed to target and cleave intron 2 of the *hprt* locus of CHO cells. The ZFN binds to the following sequence in the *hprt* locus: AGCATCTGCATTCCAGGAGaccacaAAAGTGTGATGGTGC (SEQ ID NO:2), wherein the ZFN cleavage site is designated in lower case. CHO-K1 cells were transfected by nucleofection with DNA encoding the ZFN. After a suitable period of incubation, the cells were harvested and subjected to a Cel-1 assay to determine whether the ZFN cleaved the target sequence. As shown in FIG. **4**, cleavage products of 401, 246 and 155 bp were detected in CHO cells transfected with DNA encoding the ZFN (10.1% modified).

Example 3: Preparation of HPRT and GS Double Knock-out CHO Cells

[0095] The ZFNs described above in Examples 1 and 2 can be used to target the *hprt* gene in GS-/- knock-out cells. For this, CHOZN® GS-/- cells (Sigma-Aldrich) can be transfected with DNA or mRNA encoding the ZFN. The cells can be screened for ZFN-mediated deletions as detailed above.

Example 4: Alternate Strategies for Targeting the Hprt Locus in CHO cells

[0096] The *hprt* locus in CHO cells can be inactivated via large deletions by using either of the following approaches. In the first approach, CHO cells can be transfected with DNA or mRNA encoding the ZFN targeting intron 2 (described above in Example 2) and a single-stranded oligonucleotide comprising a first sequence having sequence identity with a sequence adjacent to the targeted cleavage site in intron 2 and a second sequence having sequence identity with a sequence located in any of exons

4-9. During repair of the double-stranded break introduced into intron 2, the chromosomal sequence can recombine with the single-stranded oligonucleotide such that sequence between the two targeted sequences is deleted. FIG. **2** presents a schematic diagram of this approach.

[0097] Alternatively, a ZFN targeting exon 3 (detailed in Example 1) and not intron 2 is used. In this method, the single-stranded oligonucleotide comprises a first sequence having sequence identity with a sequence adjacent to the targeted cleavage site in exon 3 and a second sequence having sequence identity with a sequence located in any of exons 4-9.

[0098] In a second approach, CHO cells can be transfected with DNA or mRNA encoding a first ZFN and a second ZFN. In one method, the first ZFN targets intron 2 and the second ZFN targets a sequence in any of exons 4-9. During repair of the two double-stranded DNA breaks introduced by the ZFNs by NHEJ, up to about 33 kb of sequence located between the targeted cleavage sites in intron 2 and exon 9 is deleted. A schematic diagram of this approach is shown in FIG. **3**.

[0099] In an alternate method using the second approach, CHO cells can be transfected with DNA or mRNA encoding a first ZFN targeting exon 3 and a second ZFN targeting a sequence in any of exons 4-9, such that the sequence located between the targeted cleavage sites is deleted during repair of the two double-stranded DNA breaks.

Example 5: Recombinant Protein Production in HPRT deficient cells

[0100] Cells deficient in HPRT can be used to select for cells that produce large amounts of a recombinant protein. The HPRT deficient cells can be transfected using electroporation with DNA encoding the recombinant protein of interest and sequence encoding Chinese hamster HPRT. Twenty-four hours following transfection, the transfected cells are transferred to 6-well plates containing selection media (Ham's F-12 nutrient mix supplemented with 10% fetal bovine serum, 0.1-4 μM aminopterin, 100 μM hypoxanthine, and 16 μM thymidine). Aminopterin inhibits the enzyme dihydrofolate reductase which is responsible for the synthesis of tetrahydrofolate.

Tetrahydrofolate is used to produce nucleotides *de novo*. Only cells that can utilize hypoxanthine and thymidine in the salvage pathways for nucleotide biosynthesis survive in the presence of aminopterin. Cells that are deficient in HPRT cannot use hypoxanthine for salvage nucleotide synthesis. Thus, cells that grow in the presence of aminopterin constitute a selected pool that expresses HPRT and can be screened for the production of the recombinant protein of interest. Specifically, media is changed every 5-7 days during selection until viable cells start to form visible colonies (viewable by microscope). All viable cells are collected when the wells are >80% confluent and the cell cultures are expanded into T-75 and TPP bioreactor tubes. Expression levels of the recombinant protein of interest are evaluated in these stably transfected pools. Individual producing clones can be isolated from these pools for recombinant protein production.

Example 6: Recombinant Protein Production in HPRT and GS deficient cells

[0101] Cells deficient in both HPRT and GS can be used for the amplified production of more than one recombinant protein. In this case, the cells can be transfected with a first DNA encoding a first recombinant protein and sequence encoding Chinese hamster HPRT and a second DNA encoding a second recombinant protein and sequence encoding Chinese hamster GS. In one example, the first recombinant protein can be a therapeutic protein (or other protein of interest) and the second recombinant protein can be a different therapeutic protein (or a second protein of interest). In another example, the first recombinant protein can be a therapeutic protein (or other protein of interest) and the second recombinant protein can be a protein that imparts improved properties to the cell, such as the exemplary recombinant proteins described elsewhere herein. In this example, the second recombinant protein that imparts improved cellular properties may further enhance the production of the first recombinant protein or, alternatively or in addition, may enhance the growth and/or viability of the production cell itself. In another example, the first recombinant protein can be a therapeutic protein (or other protein of interest) and the second recombinant

protein can be a protein that modifies or affects the modification of the first recombinant protein (e.g., sialylation, glycosylation, or other post-translational modification proteins).

[0102] In another example, cells deficient in both HPRT and GS can be used for the production of a multi-subunit therapeutic protein (e.g., IgG protein). Thus, for example, the first recombinant protein can be the heavy chain of a desired IgG protein and the second recombinant protein can be the light chain of the IgG protein.

[0103] In each of these examples, the cells can be transfected with a first DNA encoding a first recombinant protein and sequence encoding Chinese hamster HPRT and a second DNA encoding a second recombinant protein and sequence encoding Chinese hamster GS. The two DNAs can be part of one vector or they can be separate. The cells can be grown as detailed above in Example 5, except the selection media contains 25 μM MSX with or without L-glutamine.

CLAIMS

What is claimed is:

1. An isolated cell deficient in hypoxanthine-guanine phosphoribosyltransferase (HPRT) and glutamine synthase (GS)

2. The isolated cell of claim 1, wherein the cell comprises an edited chromosomal sequence encoding HPRT.

3. The isolated cell of claim 2, wherein the chromosomal sequence is edited via targeting endonuclease mediated genome editing.

4. The isolated cell of claim 3, wherein the targeting endonuclease is a zinc finger nuclease.

5. The isolated cell of any one of claims 2 to 4, wherein the edited chromosomal sequence comprises a deletion of sequence encoding HPRT.

6. The isolated cell of any one of claim 2 to 5, wherein all copies of chromosomal sequence encoding HPRT are inactivated, and the cell produces essentially no HPRT.

7. The isolated cell of any one of claims 1 to 6, wherein the cell further comprises an edited chromosomal sequence encoding GS.

8. The isolated cell of claim 7, wherein all copies of chromosomal sequence encoding GS are inactivated, and the cell produces essentially no GS.

9. The isolated cell of any one of claims 1 to 8, further comprising at least one nucleic acid sequence encoding a recombinant protein.

10. The isolated cell of any one of claims 1 to 9, wherein the cell is a Chinese hamster ovary (CHO) cell line cell or a derivative thereof.

11. A method for preparing a cell deficient in hypoxanthine-guanine phosphoribosyltransferase (HPRT) by editing a chromosomal sequence encoding HPRT, the method comprising:
    a) introducing into the cell (i) at least one targeting endonuclease or at least one nucleic acid encoding a targeting endonuclease, each targeting endonuclease being able to introduce a double-stranded break at a targeted cleavage site in the chromosomal sequence encoding HPRT and, optionally, (ii) at least one polynucleotide comprising a sequence having substantial sequence identity to a sequence on at least one side of the targeted cleavage site in the chromosomal sequence encoding HPRT; and
    b) maintaining the cell under conditions such that the double-stranded break introduced by the targeting endonuclease is repaired by (i) a non-homologous end-joining repair process such that the chromosomal sequence encoding HPRT is edited, or (ii) a homology-directed repair process such that the chromosomal sequence is exchanged with the sequence of the polynucleotide such that the chromosomal sequence encoding HPRT is edited.

12. The method of claim 11, wherein the targeting endonuclease is a zinc finger nuclease.

13. The method of either claim 11 or claim 12, wherein the polynucleotide is a single-stranded nucleic acid.

14. The method of any one of claims 11 to 13, wherein the edited chromosomal sequence comprises a deletion of sequence encoding HPRT.

15. The method of any one of claims 11 to 14, wherein the cell is also deficient in glutamine synthase (GS).

16. The method of any one of claims 11 to 15, wherein the cell is a Chinese hamster ovary (CHO) cell line cell or a derivative thereof.

17. A method for identifying cells that produce high levels of at least one recombinant protein, the method comprising:
    a) expressing at least one nucleic acid encoding a recombinant protein in a population of cells deficient in hypoxanthine-guanine phosphoribosyltransferase (HPRT), wherein the nucleic acid also comprises sequence encoding HPRT;
    b) culturing the population of cells in the presence of hypoxanthine, aminopterin, and thymidine; and
    c) selecting for cells that produces high levels of the recombinant protein.

18. The method of claim 17, wherein all copies of chromosomal sequence encoding HPRT are inactivated in the population of cells.

19. The method of either claim 17 or claim 18, wherein, at step (c), the cells selected comprise additional copies of the nucleic acid encoding the recombinant protein and HPRT due to amplification of the nucleic acid.

20. The method of any one of claims 17 to 19, wherein the population of cells is also deficient in glutamine synthase (GS), and the culturing step (b) further comprises methionine sulfoxide.

21. The method of claim 20, wherein all copies of chromosomal sequence encoding GS are inactivated.

22. The method of either claim 20 or claim 21, wherein the method comprises expressing a first nucleic acid encoding a first recombinant protein and HPRT and a second nucleic acid encoding a second recombinant protein and GS.

23. The method of claim 22, wherein, at step (c), the selected cells comprise additional copies of the first and second nucleic acids due to amplification of the nucleic acids.

24. The method of either claim 22 or claim 23, wherein the first recombinant protein is a therapeutic protein and the second recombinant protein is a therapeutic protein or a protein that provides an improved property to the population of cells.

25. The method of any one of claims 17 to 24, wherein the population of cells is a Chinese hamster ovary (CHO) cell line cell or a derivative thereof.

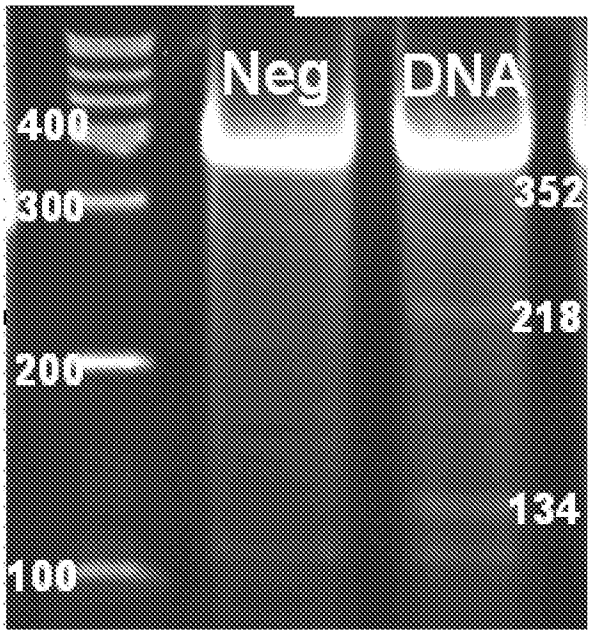
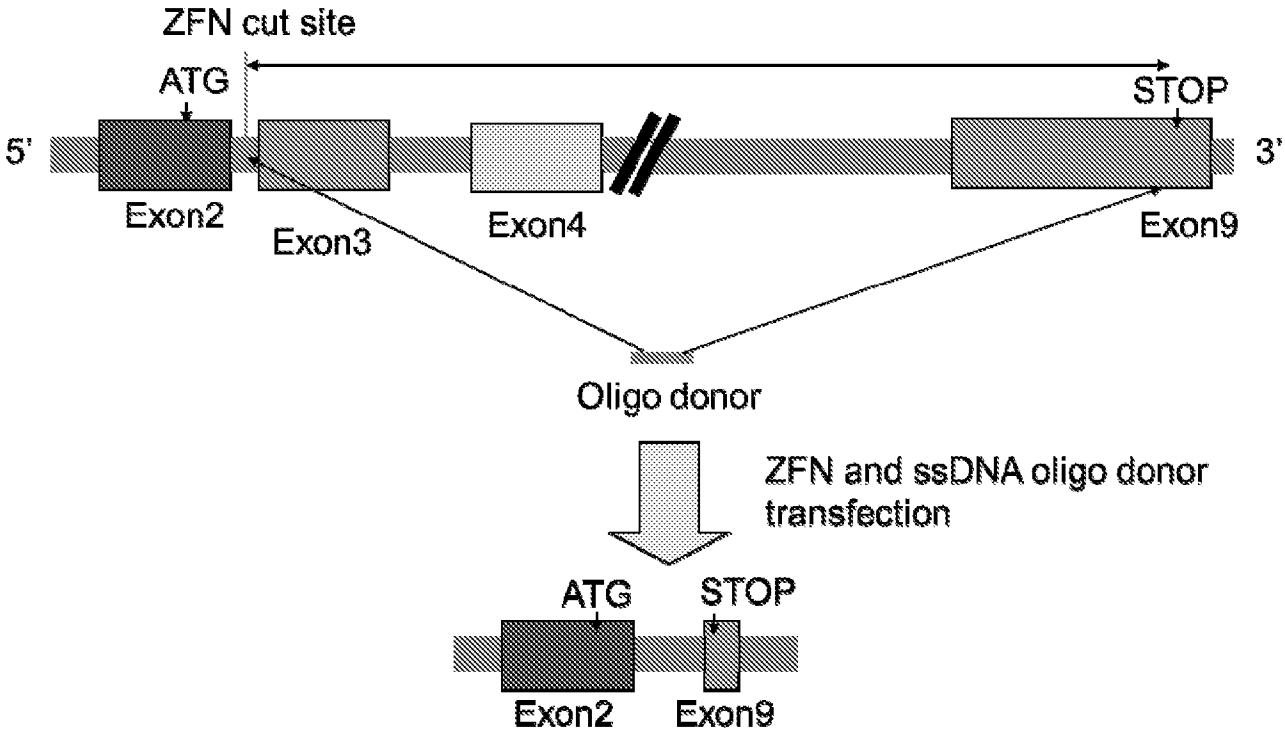

Neg
DNA
400
300
200
100
352
218
134

FIG. 1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ATG
STOP
5'
3'
Exon2
Exon3
Exon4
Exon9
Oligo donor
ZFN and ssDNA oligo donor transfection
ATG
STOP
Exon2
Exon9

FIG. 2

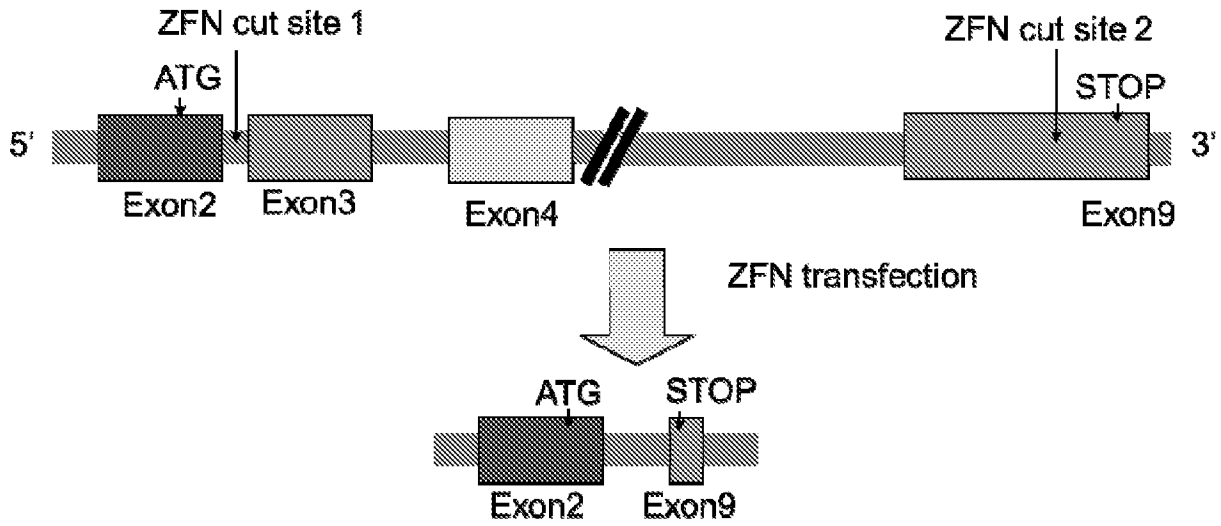
ZFN cut site 1
ATG
ZFN cut site 2
STOP
5'
3'
Exon2
Exon3
Exon4
Exon9
ZFN transfection
ATG
STOP
Exon2
Exon9

FIG. 3

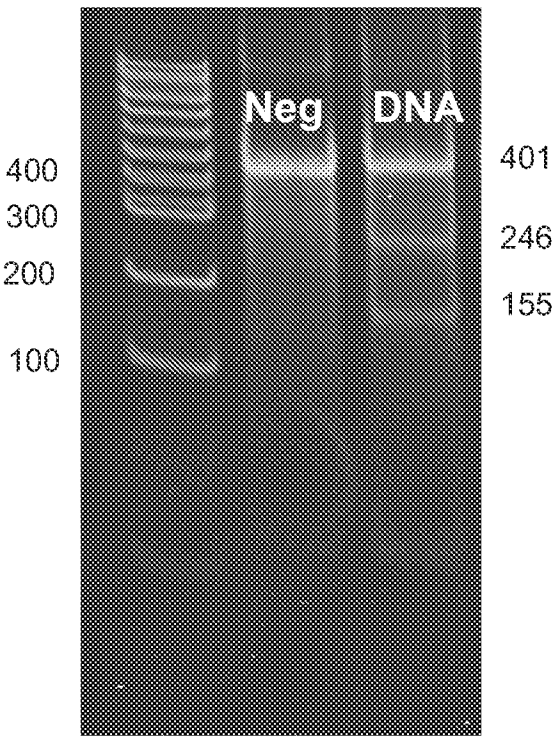
Neg
DNA
400
300
200
100
401
246
155

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/26454

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/01 (2013.01)
USPC - 435/455, 435/440, 435/462, 435/463
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12N 15/01 (2013.01)
USPC: 435/455

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/440, 435/462, 435/463

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, PubWEST, Google, Google Scholar, Google Patents: Deficient, knock out, hypoxanthine-guanine phosphoribosyltransferase, HPRT, glutamine synthase, GS, zinc finger, deletion, mutation, recombinant proteins, cells

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2008/059317 A1 (SMITH) 22 May 2008 (22.05.2008); abstract, pg 12, ln 8; pg 21, ln 4-5	1-5
Y	WO 2010/053518 A2 (LIU et al.) 14 May 2010 (14.05.2010); abstract, para [0009], [0010])	1-5
A	WALLACE et al., Manipulating the Mouse Genome to Engineer Precise Functional Syntenic Replacements with Human Sequence. Cell, 12 January 2007, Vol. 128, No. 1, pg. 197-209. Entire document.	1-5
A	WO 2007/062474 A1 (GODING et al.) 7 June 2007 (07.06.2007) Entire document	1-5
A	US 2004/0053363 A1 (RYLL et al.) 18 March 2004 (18.03.2004) para [0124]; [0125]	1-5

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
04 June 2013 (04.06.2013)	19 JUN 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/26454

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

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

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

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

3. ☒ Claims Nos.: 6-10, 14-16 and 20-25
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: claims 1-5, directed to an isolated cell deficient in hypoxanthine-guanine phosphoribosyltransferase (HPRT) and glutamine synthase (GS).

- Please see extra sheet for continuation -

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/26454

Continuation of Box III: Lack of Unity of Invention

Group II: claims 11-13, directed to a method for preparing a cell deficient in hypoxanthine-guanine phosphoribosyltransferase (HPRT) by editing a chromosomal sequence encoding HPRT, the method comprising:
a) introducing into the cell:
(i) at least one targeting endonuclease or at least one nucleic acid encoding a targeting endonuclease, each targeting endonuclease being able to introduce a double-stranded break at a targeted cleavage site in the chromosomal sequence encoding HPRT; and, optionally
(ii) at least one polynucleotide comprising a sequence having substantial sequence identity to a sequence on at least one side of the targeted cleavage site in the chromosomal sequence encoding HPRT; and
b) maintaining the cell under conditions such that the double-stranded break introduced by the targeting endonuclease is repaired by:
(i) a non-homologous end-joining repair process such that the chromosomal sequence encoding HPRT is edited; or
(ii) a homology-directed repair process such that the chromosomal sequence is exchanged with the sequence of the polynucleotide such that the chromosomal sequence encoding HPRT is edited.

Group III: claims 17-19, directed to a method for identifying cells that produce high levels of at least one recombinant protein, the method comprising:
a) expressing at least one nucleic acid encoding a recombinant protein in a population of cells deficient in hypoxanthine-guanine phosphoribosyltransferase (HPRT), wherein the nucleic acid also comprises sequence encoding HPRT;
b) culturing the population of cells in the presence of hypoxanthine, aminopterin, and thymidine; and
c) selecting for cells that produces high levels of the recombinant protein.

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

Group I contains the special technical element of being related to an isolated cell deficient in both HPRT and GS, a technical element not common to any other group.

Group II contains the special technical element of being related to a method for preparing a cell deficient in hypoxanthine-guanine phosphoribosyltransferase (HPRT) by editing a chromosomal sequence encoding HPRT, a technical element not shared by any other group.

Group III contains the special technical element of being related to a method for identifying cells that produce high levels of at least one recombinant protein, a technical element not shared by any other group.

The only common technical element shared by all of the above groups is that they are related to HPRT deficient cells. Groups II and III share the common technical element of being directed to a nucleic acid comprising a sequence encoding HPRT. These common technical elements do not represent an improvement over the prior art of the article entitled "Manipulating the Mouse Genome to Engineer Precise Functional Syntenic Replacements with Human Sequence" by Wallace et al. (hereinafter "Wallace") in view of WO 2007/062474 A1 to Goding (hereinafter "Goding").

Wallace teaches HPRT deficient cells (pg. 198, col 2, para 5). Although Wallace does not expressly specify wherein the cells may comprise a single construct encoding HPRT, Wallace does teach wherein a supplied heterologous construct encodes portions of the HPRT sequence which, when combined with portions of the HPRT sequence in the genome of the cell, produces a full, recombinant and active HPRT enzyme as a means of selecting cells which were positive for a transfected nucleic acid construct ("Positive genetic selection for loxP 3 loxP recombination involved reconstruction of a functional hypoxanthine phosphoribosyltransferase (Hprt) minigene from defective 5' and 3' components"; pg. 198, col 2, para 4; "reconstructing a functional Hprt minigene"; pg. 199, col 1, para 1). A parallel example by Goding teaches a nucleic acid construct (pg. 6, ln 31) comprising a promoter operably linked to both a first nucleic acid region encoding a protein of interest, and a second nucleic acid region 3' to the first nucleic acid region, the second nucleic acid region comprising an IRES operably linked to a nucleic acid sequence encoding one or more selectable markers (pg. 7, ln 1-9) wherein the selectable marker may be HPRT (pg 10, ln 8-9). Though Goding does not specify wherein the host cells are HPRT negative, as Wallace does. Therefore, it would have been obvious to a person of ordinary skill in the art to have supplied a single nucleic acid construct encoding a fully functional HPRT gene in trans, as a means of establishing positive selection for colonies comprising a transfected plasmid or nucleic acid construct, in a background of HPRT negative cells, based on the combined teachings of Wallace and Goding.

As the common technical element was known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

Therefore, the inventions of Groups I-III lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.