GENE THERAPY VECTORS AND CYTOSINE DEAMINASES

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 61/100,666, filed September 26, 2008, U.S. Provisional Application Serial No. 61/120,618, filed December 8, 2008 and U.S. Provisional Application Serial No. 61/186,823, filed June 13, 2009 the disclosures of which are incorporated herein by reference.

TECHNICAL FIELD

[0002] This disclosure relates to modified cytosine deaminases (CDs). The disclosure further relates to cells expressing such modified CDs and methods of using such modified CDs in the treatment of disease and disorders.

BACKGROUND

[0003] The yeast, or bacterial, cytosine deaminase converts the innocuous antibiotic pro-drug 5-FC into the cytotoxic chemotherapeutic agent 5-fluorouracil (5-FU). Humans (and mammals in general) are not known to posess a naturally occurring gene encoding an enzyme with significant cytosine deaminase activity. Yeast and bacterial cytosine deaminase have gained recognition in the treatment of cancers using gene delivery and viral vectors for the delivery of the enzyme followed by treatment with 5-FC, which is then converted by the enzyme to a cytotoxic drug (Miller *et al.*, Can Res 62:773-780 2002; Kievit *et al.*, Can Res 59:1417-1421 1999).

SUMMARY

[0004] ~~Provided~~Described herein are polypeptides that convert 5-FC to 5-FU. Also ~~provided~~described are nucleic acid molecules that encode such polypeptides, cells expressing such polypeptides, vectors containing such polynucleotide and polypeptides and methods of synthesizing 5-FU or derivatives thereof from a suitable using such polypeptides. In a first aspect, the present invention refers to an isolated polynucleotide comprising:

a human codon optimized polynucleotide comprising SEQ ID NO:19 from base pairs 8877 to 9353 which encodes a polypeptide of SEQ ID NO:4, wherein the polypeptide comprises cytosine deaminase (CD) activity.

Also~~Accordingly~~, in various ~~embodiments~~examples, isolated or recombinant polypeptides comprising a cytosine deaminase having a sequence as set forth in SEQ ID NO:2 ~~is~~ are ~~provided~~described. In other ~~embodiments~~examples, the polypeptide comprise SEQ ID NO:2 with a mutation selected from the

group consisting of A23L, V108I, I140L and any combination thereof. In yet another ~~embodiment~~example, the the polypeptide comprises the sequence as set forth in SEQ ID NO:4 and includes up to 50, 25, 10, or 5 conservative amino acid substitutions excluding residues: (a) 23, 108 and 140. In general, polypeptides ~~provided~~described herein display cytosine deaminase activity useful for converting 5-FC to 5-FU.

[0005] The disclosure also ~~provides~~describes polypeptide that comprise a sequence that is at least 80%, 90%, 95%, 98% or 99% identical to SEQ ID NO:4, wherein the polypeptide has a leucine at position 23, an isoleucine at position 108 and a leucine at position 140, and wherein the polypeptide has cytosine deaminase activity. In yet another ~~embodiment~~example, the polypeptide comprises the sequence as set forth in SEQ ID NO:4.

[0006] The disclosure further ~~provides~~describes fusion constructs comprising any one of the foregoing polypeptides operably linked to a uracil phosphoribosyltransferase (UPRT) or an orotate phosphoribosyltransferase (OPRT). In one ~~embodiment~~example, the fusion construct comprises a first polypeptide comprising SEQ ID NO:2 with a mutation selected from the group consisting of A23L, V108I, I140L and any combination thereof linked to a second polypeptide having UPRT or OPRT activity. In yet another ~~embodiment~~example, the fusion construct comprises a first polypeptide having the sequence as set forth in SEQ ID NO:4 and includes up to 50, 25, 10, or 5 conservative amino acid substitutions excluding residues: 23, 108 and 140 wherein the first polypeptide is linked to a second polypeptide having UPRT or OPRT activity. The disclosure also ~~provides~~describes a fusion construct comprising a first polypeptide having cytosine deaminase activity and that comprise a sequence that is at least 80%, 90%, 95%, 98% or 99% identical to SEQ ID NO:4, wherein the polypeptide has a leucine at position 23, an isoleucine at position 108 and a leucine at position 140, and wherein the first polypeptide is linked to a second polypeptide comprising UPRT or OPRT activity. In one ~~embodiment~~example, the polypeptides having UPRT or OPRT activity comprises a sequence as set forth in SEQ ID NO:8 and 10, respectively, or variants thereof. In yet another ~~embodiment~~example, a first polypeptide comprising cytosine deaminase activity is linked to a polypeptide having UPRT or OPRT activity, wherein the

polypeptides are separated by a peptide linker. In another ~~embodiment~~example, the fusion construct comprises a sequence selected from the group consisting of SEQ ID NO:12, 14, 16 or 18.

[0007] The disclosure further ~~provides~~describes polynucleotides encoding any of the foregoing polypeptides. For example, the disclosure ~~provides~~describes a polynucleotide encoding a polypeptide comprising SEQ ID NO:2 with a mutation selected from the group consisting of A23L, V108I, I140L and any combination thereof. In yet another ~~embodiment~~example, the polynucleotide encodes a polypeptide comprising a sequence as set forth in SEQ ID NO:4 and includes up to 50, 25, 10, or 5 conservative amino acid substitutions excluding residues: (a) 23, 108 and 140. In a further ~~embodiment~~example, the disclosure ~~provides~~ describes a polynucleotide encoding a polypeptide comprising a sequence that is at least 80%, 90%, 95%, 98% or 99% identical to SEQ ID NO:4, wherein the polypeptide has a leucine at position 23, an isoleucine at position 108 and a leucine at position 140, and wherein the polypeptide has cytosine deaminase activity. In yet another ~~embodiment~~example, the polynucleotide encodes a polypeptide comprising SEQ ID NO: 4. In a further ~~embodiment~~example, the polynucleotide encodes a polypeptide comprising a sequence as set forth in SEQ ID NO:4, 6, 8, 10, 11, 12, or 13.

[0008] The disclosure ~~provides~~describes a human codon optimized polynucleotide encoding a cytosine deaminase. In one ~~embodiment~~example, the human codon optimized polynucleotide comprises a sequence as set forth in SEQ ID NO:3. In yet another ~~embodiment~~example, the polynucleotide comprises a sequence as set forth in SEQ ID NO:5, 7, or 9.

[0009] In other ~~embodiments~~examples, a cytosine deaminase or fusion construct of the disclosure is delivered using a gene delivery system (GDS). Thus, in another aspect, the present invention refers to a vector comprising a polynucleotide of the present invention. Also~~In another aspect~~, the polynucleotide encoding a cytosine deaminase is delivered with a GDS that is a viral or viral derived vector. The viral vector can be replicating or non-replicating, and can be an adenoviral vector, a measles vector, a herpes vector, a retroviral vector (including a lentiviral vector),

a rhabdoviral vector such as a Vesicular Stomatitis viral vector, a reovirus vector, a Seneca Valley Virus vector, a poxvirus vector (including animal pox or vaccinia derived vectors), a parvovirus vector (including an AAV vector), an alphavirus vector or other

viral vector known to one skilled in the art. In one ~~embodiment~~example, the viral vector can be a replication competent retroviral vector capable of infecting replicating mammalian cells. The replication competent retroviral vector can comprise an Orthoretrovirus or more typically a gamma retrovirus vector. In one ~~aspect~~example, a replication competent retroviral vector comprises an internal ribosomal entry site (IRES) 5′ to the polynucleotide encoding a cytosine deaminase of the disclosure. In one ~~embodiment~~example, the polynucleotide encoding a cytosine deaminase is 3′ to a ENV polynucleotide of a retroviral vector. In another aspect, the present invention refers to a vector of the present invention, wherein the vector is a plasmid and the vector is an expression vector.

[0010] In other ~~embodiments~~examples, host cells transfected with a cytosine deaminase or fusion construct of the disclosure, or a vector that includes a polynucleotide or fusion construct of the disclosure, are ~~provided~~described. Host cells include eukaryotic cells such as yeast cells, insect cells, or animal cells. Host cells also include procaryotic cells such as bacterial cells. In another aspect, the present invention refers to the use of a polynucleotide of the present invention and 5-fluorocytosine in the manufacture of a medicament for treating a subject with a cell proliferative disorder wherein the use comprises to contact the subject with said polynucleotide under conditions such that the polynucleotide is expressed and to contact the subject with the 5-fluorocytosine. In another aspect, the present invention refers to the use of a retrovirus of the present invention and 5-fluorocytosine in the manufacture of a medicament for treating a cell proliferative disorder in a subject wherein the use comprises to contact the subject with said retrovirus and to contact the subject with the 5-fluorocytosine.

[0011] The disclosure also ~~provides~~describes methods of treating a cell proliferative disorder including a cancer comprising administering a polynucleotide or polypeptide of the disclosure to a subject and contacting the subject with a cytotoxic drug comprising 5-fluorocytosine (5-FC).

[0012] In another aspect, the present invention refers to a recombinant replication competent retrovirus (RCR) comprising:

a retroviral GAG protein;

a retroviral POL protein;

a retroviral envelope;

a retroviral polynucleotide comprising Long-Terminal Repeat (LTR) sequences at the 3' end of the retroviral polynucleotide sequence, a promoter sequence at the 5' end of the retroviral polynucleotide, said promoter being suitable for expression in a mammalian cell, a gag nucleic acid domain, a pol nucleic acid domain and an env nucleic acid domain;

a cassette comprising an internal ribosome entry site (IRES) operably linked to a heterologous nucleic acid comprising a polynucleotide of the present invention, wherein the cassette is positioned 5' to the 3' LTR and 3' to the env nucleic acid domain encoding the retroviral envelope; and

cis-acting sequences necessary for reverse transcription, packaging and integration in a target cell.

The disclosure ~~provides~~describes a recombinant replication competent retrovirus (RCR) comprising: a retroviral GAG protein; a retroviral POL protein; a retroviral envelope; a retroviral polynucleotide comprising Long-Terminal Repeat (LTR) sequences at the 3' end of the retroviral polynucleotide sequence, a promoter sequence at the 5' end of the retroviral polynucleotide, said promoter being suitable for expression in a mammalian cell, a gag nucleic acid domain, a pol nucleic acid domain and an env nucleic acid domain; a cassette comprising an internal ribosome entry site (IRES) operably linked to a heterologous polynucleotide, wherein the cassette is positioned 5' to the 3' LTR and 3' to the env nucleic acid domain encoding the retroviral envelope; and cis-acting sequences necessary for reverse transcription, packaging and integration in a target cell,wherein the RCR maintains higher replication competency after 6 passages compared to a pACE vector (SEQ ID NO:21). In one ~~embodiment~~example, the ~~the~~ retroviral

polynucleotide sequence is derived from murine leukemia virus (MLV), Moloney murine leukemia virus (MoMLV),Feline leukemia virus or Gibbon ape leukemia virus (GALV). In another ~~embodiment~~example, the MLV is an amphotropic MLV. In yet another ~~embodiment~~example, the retrovirus is an oncoretrovirus. In yet another ~~embodiment~~example, the target cell is a cell having a cell proliferative disorder. The cell proliferative disorder can be selectedfrom the group consisting of, but is not limited to, lung cancer, colon-rectum cancer, breast cancer, prostate cancer, urinary tract cancer, uterine cancer, brain cancer, head and neck cancer, pancreatic cancer, melanoma, stomach cancer and ovarian cancer, rheumatoid arthritis and other auto-immune diseases. In one ~~embodiment~~example, the promoter comprises a CMV promoter having a sequence as set forth in SEQ ID NO:19, 20, or 22 from nucleotide 1 to about nucleotide 582 and may include modification to one or more nucleic acid bases and which is capable of directing and initiating transcription. In yet a further ~~embodiment~~example, the promoter comprises a sequence as set forth in SEQ ID NO:19, 20, or 22 from nucleotide 1 to about nucleotide 582. In a further ~~embodiment~~example, the promoter comprises a CMV-R-U5 domain polynucleotide. In one ~~embodiment~~example, the CMV-R-U5 domain comprise the immediately early promoter from human cytomegalovirus linked to an MLV R-U5 region. In yet another ~~embodiment~~example, the CMV-R-U5 domain polynucleotide comprises a sequence as set forth in SEQ ID NO:19, 20, or 22 from about nucleotide 1 to about nucleotide 1202 or sequences that are at least 95% identical to a sequence as set forth in SEQ ID NO:19, 20, or 22, wherein the polynucleotide promotes transcription of a nucleic acid molecule operably linked thereto. In another ~~embodiment~~example, the gag and pol of the polynucleotide are derived from an oncoretrovirus. The gag nucleic acid domain can comprise a sequence from about nucleotide number 1203 to about nucleotide 2819 of SEQ ID NO: 19 or a sequence having at least 95%, 98%, 99% or 99.8% identity thereto. The pol domain can comprise a sequence from about nucleotide number 2820 to about nucleotide 6358 of SEQ ID NO:19 or a sequence having at least 95%, 98%, 99% or 99.9% identity thereto. In one ~~embodiment~~example, the env domain encodes an amphoteric env protein. The env domain can comprise a sequence from about nucleotide number 6359 to about nucleotide 8323 of SEQ ID NO:19 or a

sequence having at least 95%, 98%, 99% or 99.8% identity thereto. The IRES domain of the vector can be any IRES, however, in one ~~embodiment~~example the IRES is derived from an encephalomyocarditis virus. In a further ~~embodiment~~example, the IRES comprises a sequence from about nucleotide number 8327 to about nucleotide 8876 of SEQ ID NO:19 or a sequence having at least 95%, 98%, or 99% identity thereto. In yet a further ~~embodiment~~example, the heterologous polynucleotide comprises a polynucleotide having a sequence as set forth in SEQ ID NO:3, 5, 11, 13, 15 or 17. In a further ~~embodiment~~example, the heterologous sequence encodes a polypeptide comprising a sequence as set forth in SEQ ID NO:4. The heterologous nucleic acid is human codon optimized and encodes a polypeptide as set forth in SEQ ID NO:4. In a further ~~embodiment~~example, the heterologous nucleic acid comprises a sequence as set forth in SEQ ID NO: 19 from about nucleotide number 8877 to about 9353. In one ~~embodiment~~example, the 3′ LTR is derived from an oncoretrovirus. In a further ~~embodiment~~example, the 3′ LTR comprises a U3-R-U5 domain. In yet a further ~~embodiment~~example, the 3′ LTR comprises a sequence as set forth in SEQ ID NO:19 from about nucleotide 9405 to about 9998 or a sequence that is at least 95%, 98% or 99.5% identical thereto.

[0013] The disclosure ~~provides~~describes a polynucleotide comprising a sequence as set forth in SEQ ID NO:19, 20, or 22. In another aspect, the present invention refers to an isolated polynucleotide comprising from 5′ to 3′:

a CMV-R-U5 fusion of the immediate early promoter from human cytomegalovirus to an MLV R-U5 region;

a PBS, primer binding site for reverse transcriptase;

a 5′ splice site;

ψ packaging signal;

a gag coding sequence for MLV group specific antigen;

a pol coding sequence for MLV polymerase polyprotein;

a 3′ splice site;

a 4070A env coding sequence for envelope protein of MLV strain 4070A;

an internal ribosome entry site (IRES) from encephalomyocarditis virus;

a modified cytosine deaminase coding sequence according to the present invention;

a polypurine tract; and

a U3-R-U5 MLV long terminal repeat.

[0014] The disclosure ~~provides~~describes an isolated polynucleotide comprising from 5′ to 3′: a CMV-R-U5 fusion of the immediate early promoter from human cytomegalovirus to an MLV R-U5 region; a PBS, primer binding site for reverse transcriptase; a 5′ splice site; ψ packaging signal; a gag coding sequence for MLV group specific antigen; a pol coding sequence for MLV polymerase polyprotein; a 3′ splice site; a 4070A env coding sequence for envelope protein of MLV strain 4070A; an internal ribosome entry site (IRES) from encephalomyocarditis virus; a modified cytosine deaminase coding sequence; a polypurine tract; and a U3-R-U5 MLV long terminal repeat.

[0015] The disclosure ~~provides~~describes a method of treating a subject with a cell proliferative disorder comprising contacting the subject with a polynucleotide a polypeptide of the disclosure having cytosine deaminase activity under conditions such that the

polynucleotide is expressed and contacting the subject with 5-fluorocytosine.

[0016] The disclosure also ~~provides~~describes a method of treating a cell proliferative disorder in a subject comprising contacting the subject with a retrovirus of the disclosure, wherein the heterologous nucleic acid sequence encodes a therapeutic protein that inhibits proliferation of a neoplastic cell. In one ~~embodiment~~example, the retrovirus comprises a polynucleotide encoding a polypeptide having a sequence as set forth in SEQ ID NO:4, 12, 14, 16, or 18.

[0017] The details of one or more ~~embodiments~~examples of the disclosure are set forth in the accompanying drawings and the description below. Other features, objects, and advantages will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF DRAWINGS

[0018] **Figure 1A-D** shows (a) a schematic of a recombinant retroviral vector of the disclosure; (b) a plasmid map of a polynucleotide of the disclosure; (c and d) a sequence of a polynucleotide of the disclosure (SEQ ID NO:19).

[0019] **Figure 2A-D** shows schemes for the generation of various embodiments of the disclosure comprising polypeptides with CD, OPRT and UPRT activity.

[0020] **Figure 3** shows that higher levels of yCD2 protein are observed compared to wild type yCD protein in infected U-87 cells.

[0021] **Figure 4** shows the stability of a vector comprising a CD polypeptide of the disclosure and the comparison to other vectors of the disclosure.

[0022] **Figure 5A-B** show that a cytosine deaminase activity and vector of the disclosure provide comparable or better expression, and hence killing, of infected rat RG2(5A) or U87 cells (5B) compared to wild type yCD activity (T5.0007),when infected cells are exposed to increasing levels of 5-FC.

[0023] **Figure 6** shows that the specific activity in infected U87 cells of T5.0002 (yCD2) is greater than T5.0001 (partially optimized yCD) which is greater than T5.0007 (wt yCD).