ENGINEERED CELLS EXPRESSING MULTIPLE IMMUNOMODULATORS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is claims priority benefit of U.S. Provisional Application No. 61/103,810, filed October 8, 2008, which is hereby incorporated by reference in its entirety.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY VIA EFS-WEB

[0002] The content of the electronically submitted sequence listing (Name: sequence listing.ST25.txt; Size: 213,102 bytes; Date Of Creation: October 8, 2009) filed with this application is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

[0003] This invention relates to the field of gene therapy for the treatment of diseases and disorders, such as cancer. In one embodiment, the invention provides the engineering of immune cells or therapy support cells (TSC) to express one or more immunomodulators and use of the cells as therapeutics.

Background

[0004] Interleukin-12 (IL-12) is a member of the type I cytokine family involved in contributing to a number of biological processes including, but not limited to, protective immune response and suppression of tumorigenesis (Abdi *et al.*, 2006; Adorini, 1999; Adorini, 2001; Adorini *et al.*, 2002; Adorini *et al.*, 1996; Akhtar *et al.*, 2004; Akiyama *et al.*, 2000; Al-Mohanna *et al.*, 2002; Aliberti *et al.*, 1996; Allavena *et al.*, 1994; Alli and Khar, 2004; Alzona *et al.*, 1996; Amemiya *et al.*, 2006; Araujo *et al.*, 2001; Arulanandam *et al.*, 1999; Athie *et al.*, 2000; Athie-Morales *et al.*, 2004; Bertagnolli *et al.*, 1992; Bhardwaj *et al.*, 1996; Biedermann *et al.*, 2006; Brunda and Gately, 1994; Buchanan *et al.*, 1995; Romani *et al.*, 1997; Rothe *et al.*, 1996; Satoskar *et al.*, 2000; Schopf *et al.*,

1999; Thomas *et al.*, 2000; Tsung *et al.*, 1997; Wolf *et al.*, 1994; Yuminamochi *et al.*, 2007). A growing body of evidence suggests that IL-12 may be a promising target to control human diseases (*e.g.*, cancer).

[0005] Despite the fact that IL-12 remains promising as a cancer therapeutic agent based on its potent supportive activity on Type-1 anti-tumor NK cells, $CD4^+$ T cells and $CD8^+$ T cells (Trinchieri, 2003), the reported toxicity of recombinant human IL-12 (rhIL-12) in patients (Atkins *et al.*, 1997), together with limited sources of GMP-grade rhIL-12 for clinical application, have prevented successful IL-12-based therapeutic approaches. Thus it seems reasonable that gene therapy approaches may represent safer, more tenable treatment options. Indeed, phase I clinical trials implementing intra- or peri-tumoral delivery of recombinant viral- (Sangro *et al.*, 2004; Triozzi *et al.*, 2005) or plasmid-based IL-12 cDNA (Heinzerling *et al.*, 2005), or IL-12 gene modified autologous fibroblasts (Kang *et al.*, 2001) have been found safe and well-tolerated.

[0006] However, objective clinical responses in patients with melanoma or a diverse range of carcinomas receiving these gene therapies have been rare, variable, transient and largely focused at the site of treatment (Heinzerling *et al.*, 2005; Kang *et al.*, 2001; Sangro *et al.*, 2004; Triozzi *et al.*, 2005). In cases where disease resolution was partial or complete, increased frequencies of tumor-infiltrating lymphocytes (Heinzerling *et al.*, 2005; Sangro *et al.*, 2004) and elevated levels of circulating tumor-specific $CD8^+$ T cells (Heinzerling *et al.*, 2005) have been noted, consistent with the improved cross-priming of antigen-specific T cells in these patients.

[0007] Since the cross-priming of specific T cells is best accomplished by dendritic cells (DC) that serve as a natural but regulated source of IL-12 (Berard *et al.*, 2000), recent reports of the superior pre-clinical efficacy of DC-based IL-12 gene therapy have been of great interest (Satoh *et al.*, 2002; Tatsumi *et al.*, 2003; Yamanaka *et al.*, 2002). For example, it was shown that intratumoral (i.t.) injection of DC engineered to produce IL-12p70 (via recombinant adenovirus infection) results in the dramatically improved cross-priming of a broadly-reactive, tumor-specific $CD8^+$ T cell repertoire in concert with tumor rejection in murine models (Tatsumi *et al.*, 2003). Given the previous use of a recombinant adenovirus encoding mIL-12 under a CMV-based promoter (rAd.cIL12, (Tatsumi *et al.*, 2003)), engineered DC production of IL-12 was constitutive, hence the immunologic impact of this cytokine early within the tumor lesion and later within tumor-

draining lymph nodes could not be resolved with regards to therapeutic outcome. Thus, a need exists for DC engineered for conditional expression of IL-12 for the purpose of regulating both the level of transgene expression and the timing of the transgene activation. The invention provides a promising therapeutic outcome for the use of such cells.

SUMMARY OF THE INVENTION

[0008] The invention provides a recombinant vector encoding protein(s) having the function(s) of one or more immunomodulators, under the control of one or more promoters. In one embodiment, the one or more promoters are conditional. In another embodiment, the one or more promoters are constitutive. In another embodiment, the vector is an adenovirus vector encoding the protein(s) driven off a promoter that can be conditionally activated by provision of a soluble small molecule ligand such as diacylhydrazines (e.g., RG-115819, RG-115830 or RG-115932). This vector allows for the control of expression of the protein(s) from immune cells and TSC.

[0009] In one embodiment, the invention provides a vector for conditionally expressing protein(s) having the function(s) of one or more immunomodulators comprising a polynucleotide encoding a gene switch, wherein said polynucleotide encoding a gene switch comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, and (2) a polynucleotide encoding one or more proteins having the function of an immunomodulator linked to a promoter which is activated by said ligand-dependent transcription factor. In one embodiment, the immunomodulator is selected from IL-1, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10R DN or a subunit thereof, IL-15, IL-18, IL-21, IL-23, IL-24, IL-27, GM-CSF, IFN-alpha, IFN-gamma, CCL3 (MIP-1a), CCL5 (RANTES), CCL7 (MCP3), XCL1(lymphotactin), CXCL1 (MGSA-alpha), CCR7, CCL19 (MIP-3b), CXCL9 (MIG), CXCL10 (IP-10), CXCL12 (SDF-1), CCL21 (6Ckine), OX40L, 4-1BBL, CD40, CD70, GITRL, LIGHT, b-Defensin, HMGB1, Flt3L, IFN-beta, TNF-alpha, dnFADD, TGF-alpha, PD-L1RNAi, a PD-L1 antisense oligonucleotide, TGFbRII DN, ICOS-L and S100.

[0010] In another embodiment, the invention provides a vector for expressing protein(s) having the function(s) of one or more immunomodulators and a protein having the

function of IL-12, comprising a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, (2) a polynucleotide encoding said protein(s) having the function(s) of the one or more immunomodulators, and (3) a polynucleotide encoding a protein having the function of the IL-12; wherein at least one polynucleotide of (2) and (3) are linked to the promoter which is activated by the ligand-dependent transcription factor.

[0011] The invention further provides a method of producing a population of cells, *e.g.*, immune cells or TSC, expressing protein(s) having the function of one or more immunomodulators, by modifying (*e.g.*, transfecting, electroporating, *etc.*) the cells with a recombinant vector conditionally expressing protein(s) having the function(s) of the one or more immunomodulators, wherein the vector comprises a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, and (2) a polynucleotide encoding one or more proteins having the function of an immunomodulator linked to a promoter which is activated by said ligand-dependent transcription factor.

[0012] In another embodiment, the invention provides a method of producing a population of cells, *e.g.*, immune cells or TSC, expressing proteins having the function(s) of one or more immunomodulators and a protein having the function of IL-12, by modifying the cells with a recombinant vector comprising a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, (2) a polynucleotide encoding said protein(s) having the function(s) of the one or more immunomodulators, and (3) a polynucleotide encoding a protein having the function of the IL-12; wherein at least one polynucleotide of (2) and (3) are linked to the promoter which is activated by said ligand-dependent transcription factor.

[0013] The invention further provides a population of cells, *e.g.*, immune cells or TSC, expressing protein(s) having the function of one or more immunomodulators, which has been modified (*e.g.*, transfected, electroporated, *etc.*) with a recombinant vector

conditionally the expressing protein(s) having the function(s) of the one or more immunomodulators, wherein the vector comprises a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, and (2) a polynucleotide encoding one or more proteins having the function of an immunomodulator linked to the promoter which is activated by said ligand-dependent transcription factor.

[0014] In another embodiment, the invention provides a population of cells, *e.g.*, immune cells or TSC, expressing proteins having the function(s) of one or more immunomodulators and a protein having the function of IL-12, which has been modified with a recombinant vector comprising a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, (2) a polynucleotide encoding said protein(s) having the function(s) of the one or more immunomodulators and (3) a polynucleotide encoding a protein having the function of the IL-12; wherein at least one polynucleotide of (2) and (3) are linked to a promoter which is activated by said ligand-dependent transcription factor.

[0015] In another embodiment, the invention provides a composition comprising two or more populations of cells of the present invention, *e.g.*, immune cells or TSC, wherein each population of cells in the composition expresses one or more immunomodulators that are different from the one or more immunomodulators expressed in the other population(s) of cells in the composition. In one embodiment, the composition contains two populations of cells. In another embodiment, the composition contains more than two populations of cells. In another embodiment, the composition contains three populations of cells. In another embodiment, the composition contains four populations of cells.

[0016] In another embodiment, the invention provides an *in vitro* engineered cell, *e.g.*, immune cell or TSC, comprising a vector comprising a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, and (2) a polynucleotide encoding a protein having the function of an immunomodulator linked to a promoter

which is activated by said ligand-dependent transcription factor. In another embodiment, the invention provides an *in vitro* engineered cell, *e.g.*, immune cell or TSC, comprising a vector comprising a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, (2) a polynucleotide encoding a protein having the function of an immunomodulator, and (3) a polynucleotide encoding a protein having the function of IL-12; wherein at least one polynucleotide of (2) and (3) are linked to a promoter which is activated by said ligand-dependent transcription factor.

[0017] In another embodiment, the invention provides a composition comprising two or more populations of *in vitro* engineered cells, *e.g.*, immune cells or TSCs, of the present invention, wherein each of the populations of *in vitro* engineered cells in the composition comprises a vector comprising a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, and (2) a polynucleotide encoding a protein having the function of an immunomodulator linked to a promoter which is activated by said ligand-dependent transcription factor, and wherein each population of *in vitro* engineered cells in the composition expresses one or more immunomodulators that are different from the one or more immunomodulators expressed in the other population(s) of *in vitro* engineered cell in the composition. In one embodiment, the invention provides a composition comprising two or more populations of *in vitro* engineered cells, *e.g.*, immune cell or TSC, each of said populations of cells comprising a vector comprising a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, (2) a polynucleotide encoding a protein having the function of an immunomodulator, and (3) a polynucleotide encoding a protein having the function of IL-12; wherein at least one polynucleotide of (2) and (3) are linked to a promoter which is activated by said ligand-dependent transcription factor. In one embodiment, the composition contains two populations of *in vitro* engineered cells. In another embodiment, the composition contains more than two populations of *in vitro* engineered cells. In another embodiment,

the composition contains three populations of *in vitro* engineered cells. In another embodiment, the composition contains four populations of *in vitro* engineered cells.

[0018] The invention also provides a pharmaceutical composition comprising a population of cells, *e.g.*, immune cells or TSC, as described herein.

[0019] In one embodiment, the polynucleotide coding for the one or more proteins having the functions of the immunomodulator is under control of the promoter of the gene switch and the polynucleotide coding for a protein having the function of IL-12 is under control of a constitutive promoter. In another embodiment, both the polynucleotide coding for protein(s) having the functions of the immunomodulator(s) and the polynucleotide coding for a protein having the function of IL-12 are both under control of a multicistronic promoter of the gene switch. In another embodiment, the polynucleotide coding for a protein(s) having the function of the immunomodulator(s) is under control of the promoter of the gene switch and the polynucleotide coding for a protein having the function of IL-12 is under control of a conditional promoter which is different than the gene switch promoter. In a further embodiment, the gene regulation system for the polynucleotide coding for the protein(s) having the function of the immunomodulator(s) and the gene regulation system for the polynucleotide having the function of IL-12 are orthogonal. In a further embodiment, the gene regulation system for each polynucleotide coding for each protein is orthogonal.

[0020] In one embodiment, the invention also provides a treatment of cancer, such as, but not limited to, melanoma tumors, glioma tumors, renal cancer, and prostate cancers, as well as the cancers listed herein in Table 1. IL-12 gene therapy has demonstrated anti-tumor efficacy in animal model studies when applied as a recombinant cDNA vector (Faure *et al.*, 1998; Sangro *et al.*, 2005), but even more so, when applied in the context of gene-modified DC (Satoh *et al.*, 2002; Svane *et al.*, 1999; Tatsumi *et al.*, 2003; Yamanaka *et al.*, 2002). To date, however, human phase I trials of IL-12 gene therapy implementing plasmids or viral vectors have failed to achieve durable, objective clinical responses in the cancer setting (Heinzerling *et al.*, 2005; Kang *et al.*, 2001; Sangro *et al.*, 2004; Triozzi *et al.*, 2005). gene therapy as described herein provides a promising therapeutic modality.

[0021] In one embodiment, the invention provides a method for treating a tumor in a mammal, comprising the steps of:

(a) administering intratumorally to tumor microenvironments a population of immune cells or TSC, which are *in vitro* engineered to conditionally express one or more proteins having the function of an immunomodulator; and

(b) administering to said mammal a therapeutically effective amount of an activating ligand;

thereby inducing expression of a protein having the function of the immunomodulator and treating said tumor.

[0022] In another embodiment, the invention provides a method for treating a tumor in a mammal, comprising the steps of:

(a) administering intratumorally to tumor microenvironments two or more populations of immune cells or TSCs, which are *in vitro* engineered to conditionally express one or more proteins having the function of an immunomodulator, wherein each population of immune cells or TSCs expresses a different set of one or more immunomodulators; and

(b) administering to said mammal a therapeutically effective amount of one or more activating ligands;

thereby inducing expression of proteins having the function of the immunomodulators and treating said tumor.

[0023] In another embodiment, the invention provides a method for treating a tumor in a mammal, comprising the steps of:

(a) administering intratumorally to tumor microenvironments a population of an immune cells or TSC, which are *in vitro* engineered to conditionally express one or more proteins having the function of an immunomodulator and a protein having the function of IL-12, wherein at least one of the proteins having the function of the immunomodulator or IL-12 is under control of a conditional promoter that is activated by a ligand; and

(b) administering to said mammal a therapeutically effective amount of the activating ligand;

thereby inducing expression of a protein having the function of the immunomodulator and/or the protein having the function of IL-12 and treating said tumor.

[0024] In another embodiment, the invention provides a method for treating a tumor in a mammal, comprising the steps of:

(a) administering intratumorally to tumor microenvironments two or more populations of an immune cells or TSCs, which are *in vitro* engineered to conditionally express one or more proteins having the function of an immunomodulator and a protein having the function of IL-12, wherein each population of immune cells or TSCs expresses a different set of one or more proteins having the function of an immunomodulator, wherein at least one of the proteins having the function of the immunomodulator or IL-12 is under control of a conditional promoter that is activated by a ligand; and

(b) administering to said mammal a therapeutically effective amount of one or more activating ligands;

thereby inducing expression of a protein having the function of the immunomodulators and/or the protein having the function of IL-12 and treating said tumor.

[0025] In another embodiment, the invention provides a method for treating a disease or disorder in a mammal, comprising the steps of:

(a) administering to said mammal a population of modified cells, which are modified to conditionally express one or more proteins having the function of an immunomodulator; and

(b) administering to said mammal a therapeutically effective amount of an activating ligand;

thereby inducing expression of a protein having the function of the immunomodulator and treating said disease or disorder.

[0026] In another embodiment, the invention provides a method for treating a disease or disorder in a mammal, comprising the steps of:

(a) administering to said mammal two or more populations of modified cells, which are modified to conditionally express one or more proteins having the function of an immunomodulator, wherein each population of modified cells expresses a different set of one or more immunomodulators; and

(b) administering to said mammal a therapeutically effective amount of one or more activating ligands;

thereby inducing expression of proteins having the function of the immunomodulators and treating said disease or disorder.

[0027] In another embodiment, the invention provides a method for treating a disease or disorder in a mammal, comprising the steps of:

(a) administering to said mammal a population of a modified cells, which are modified to conditionally express one or more proteins having the function of an immunomodulator and a protein having the function of IL-12, wherein at least one of the proteins having the function of the immunomodulator or IL-12 is under control of a conditional promoter that is activated by a ligand; and

(b) administering to said mammal a therapeutically effective amount of the activating ligand;

thereby inducing expression of a protein having the function of the immunomodulator and/or the protein having the function of IL-12 and treating said disease or disorder.

[0028] In another embodiment, the invention provides a method for treating a disease or disorder in a mammal, comprising the steps of:

(a) administering to said mammal two or more populations of modified cells, which are modified to conditionally express one or more proteins having the function of an immunomodulator and a protein having the function of IL-12, wherein each population of modified cells expresses a different set of one or more proteins having the function of an immunomodulator, wherein at least one of the proteins having the function of the immunomodulator or IL-12 is under control of a conditional promoter that is activated by a ligand; and

(b) administering to said mammal a therapeutically effective amount of one or more activating ligands;

thereby inducing expression of a protein having the function of the immunomodulators and/or the protein having the function of IL-12 and treating said disease or disorder.

[0029] The invention also provides a method for determining the efficacy of engineered cell-, *e.g.*, immune cell- or TSC-, based therapy by measuring the level of expression or activity of IFN- gamma in a patient before the start of therapy, thereby generating a control level, followed by the administration of cells engineered to express one or more

proteins having the functions of an immunomodulator and optionally a protein having the function of IL-12, administering an effective amount of an activating ligand, and then measuring the level of expression of IFN- gamma to generate a test level, and comparing the control level to the test level to determine if the therapeutic regime is effective.

[0030] In one embodiment, the invention provides a method for determining the efficacy of an *in vitro* engineered cell-, *e.g.*, immune cell- or TSC-, based therapeutic regime in a patient comprising:

(a) measuring the level of expression or the level of activity or both of interferon-gamma (IFN- gamma) in a first biological sample obtained from said patient in need thereof before administration of the *in vitro* engineered cells, thereby generating a control level;

(b) administering to a patient in need thereof the *in vitro* engineered cells engineered to conditionally express one or more proteins having the functions of an immunomodulator and optionally a protein having the function of IL-12;

(c) administering to said patient in need thereof an effective amount of an activating ligand;

(d) measuring the level of expression or the level of activity or both of IFN-gamma in a second biological sample obtained from said patient in need thereof following administration of *in vitro* engineered immune cells and activating ligand, thereby generating a test level; and

(e) comparing the control level to the test level of IFN-gamma, wherein an increase in the test level of expression, activity or both of IFN-gamma relative to the control level indicates that the therapeutic regime is effective in said patient in need thereof.

DETAILED DESCRIPTION OF DRAWINGS

[0031] FIG. 1 shows a plasmid map for a regulated promoter expression system for a bicistronic transcript encoding hIL-12.

[0032] FIG. 2 shows a plasmid map for a regulated promoter expression system for a bicistronic transcript encoding hIL-21 and hIL-15.

[0033] FIG. 3 shows a plasmid map for a regulated promoter expression system for a bicistronic transcript encoding mIL-12.

[0034] FIG. 4 shows a plasmid map for a regulated promoter expression system for a bicistronic transcript encoding mIL-21 and mIL-15.

[0035] FIG. 5 shows a plasmid map for a regulated promoter expression system for hIL-21.

[0036] FIG. 6 shows a plasmid map for a regulated promoter expression system for mIL-21.

[0037] FIG. 7 shows a plasmid map for a regulated promoter expression system for a tricistronic transcript encoding hIL-12 and hIL-21.

[0038] FIG. 8 shows a plasmid map for a regulated promoter expression system for a tricistronic transcript encoding mIL-12 and mIL-21.

[0039] FIG. 9 shows the structure of the vector rAd.RheoIL12 in which the E1 and E3 regions have been deleted and the RheoSwitch® Therapeutic System (RTS)-IL-12 components replace the E1 region. The box labeled "IL12" represents the IL-12p40 and IL-12p35 coding sequences separated by IRES.

DETAILED DESCRIPTION OF SEQUENCES

Immunomodulators

Cytokines

[0040] The polynucleotide sequences of interleukin 1 (IL-1), which are cytokines important for inflammatory response against infection, are available from public databases as accession numbers M28983 (human IL-1α); M15330 (human IL-1β); AF201830 (human IL-1δ); AF201831 (human IL-1ε); AF201832 (human 1L-1ζ); AF201833 (human IL-1η); NM_010554 (mouse IL-1α); NM_008361 (mouse IL-1β); NM_019451 (mouse L-1δ); NM_019450 (mouse IL-1f6); NM_027163 (mouse IL-1f8); NM_153511 (mouse IL-1f9); NM_204524 (chicken IL-1β); NM_017019 (rat IL-1α); and NM_031512 (rat IL-1β), sequences of which are incorporated by reference herein.

[0041] The amino acid sequences of interleukin 1 (IL-1) are available from public databases as accession numbers AAA59134 (human IL-1α); AAA59135 (human IL-1β); AAF25210 (human IL-1δ); AAF25211 (human IL-1ε); AAF25212 (human 1L-1ζ); AAF25213 (human IL-1η); NP_034684 (mouse IL-1α); NP_032387 (mouse IL-1β); NP_062324 (mouse L-1δ); NP_062323 (mouse IL-1f6); NP_081439 (mouse IL-1f8);

NP_705731 (mouse IL-1f9); NP_989855 (chicken IL-1β); NP_058715 (rat IL-1α); and NP_113700 (rat IL-1β), sequences of which are incorporated by reference herein. Laurent *et al., Psychiatr. Genet. 7*: 103 (1997) identified polymorphic mutations in human interleukin-1 beta gene.

[0042] The polynucleotide sequences of interleukin 2 (IL-2), which belongs to a family of cytokines, including IL-4, IL-7, IL-9, IL-15, and IL-21, are available from public databases as accession numbers U25676 (human); NM_008366 (mouse); NM_204153 (chicken); and NM_053836 (rat), sequences of which are incorporated by reference herein.

[0043] The amino acid sequences of interleukin 2 (IL-2) are available from public databases as accession numbers AAA70092 (human); NP_032392 (mouse); NP_989484 (chicken); and NP_446288 (rat), sequences of which are incorporated by reference herein.

[0044] Liu *et al., Appl. Biochem. Biotechnol. 133*: 77 (2006) generated mutant human IL-2, and Lorberboum *et al., J. Biol. Chem. 265*: 16311 (1990) describes generation of chimeric IL-2.

[0045] The polynucleotide sequences of interleukin 4 (IL-4), which is a cytokine that induces differentiation of naïve helper T cells to Th2 cells, are available from public databases as accession numbers M23442 (human); NM_021283 (mouse); NM_001007079 (chicken); and NM_201270 (rat), sequences of which are incorporated by reference herein.

[0046] The amino acid sequences of interleukin 4 (IL-4) are available from public databases as accession numbers AAA59150 (human); NP_067258 (mouse); NP_001007080 (chicken); and NP_958427 (rat), sequences of which are incorporated by reference herein.

[0047] Kawashima *et al., J. Med. Genet. 35*: 502 (1998) describes polymorphisms in IL-4 gene, that are associated with atopic dermatitis.

[0048] Interleukin 7 (IL-7) is a cytokine important for B and T cell development. The polynucleotide sequences of IL-7 are available from public databases as accession numbers J04156 (human); NM_008371 (mouse); NM_001037833 (chicken); and NM_013110 (rat), sequences of which are incorporated by reference herein.

[0049] The amino acid sequences of interleukin 7 (IL-7) are available from public databases as accession numbers AAA59156 (human); NP_032397 (mouse);

NP_001032922 (chicken); and NP_037242 (rat), sequences of which are incorporated by reference herein.

[0050] Feng *et al., Genetics 175*:545 (2007) have identified point mutations in IL-7 that results in functional deficiency.

[0051] Interleukin 9 (IL-9) is a cytokine produced by T-cells and is a regulator of hematopoietic cells. The polynucleotide sequences of IL-9 are available from public databases as accession numbers NM_000590 (human); NM_008373 (mouse); NM_001037825 (chicken); and NM_001105747 (rat), sequences of which are incorporated by reference herein.

[0052] The amino acid sequences of interleukin 9 (IL-9) are available from public databases as accession numbers NP_000581 (human); NP_032399 (mouse); NP_001032914 (chicken); and NP_001099217 (rat), sequences of which are incorporated by reference herein.

[0053] IL-12 is a cytokine that can act as a growth factor for activated T and NK cells, enhance the lytic activity of NK/lymphokine-activated Killer cells, and stimulate the production of IFN-gamma by resting peripheral blood mononuclear cells (PBMC). The polynucleotide sequences of IL-12 are available from public databases as accession numbers NM_000882 (human IL12A); NM_002187 (human IL12B); NM_008351 (mouse IL12a); NM_008352 (mouse IL12b); NM_213588 (chicken IL12A); NM_213571 (chicken IL12B); NM_053390 (rat IL12a); and NM_022611 (rat IL12b), sequences of which are incorporated by reference herein.

[0054] The amino acid sequences of interleukin 12 (IL-12) are available from public databases as accession numbers NP_000873 (human IL12A); NP_002178 (human IL12B); NP_032377 (mouse IL12a); NP_032378 (mouse IL12b); NP_998753 (chicken IL12A); NP_998736 (chicken IL12B); NP_445842 (rat IL12a); and NP_072133 (rat IL12b), sequences of which are incorporated by reference herein.

[0055] Interleukin 15 (IL-15) is a cytokine that regulates T and natural killer cell activation and proliferation. The polynucleotide sequences of IL-15 are available from public databases as accession numbers U14407 (human); NM_008357 (mouse); EU334509 (chicken); and AF015719 (rat), sequences of which are incorporated by reference herein.

[0056] The amino acid sequences of interleukin 15 (IL-15) are available from public databases as accession numbers AAA21551 (human); NP_032383 (mouse); ABY55312 (chicken); and AAB94536 (rat), sequences of which are incorporated by reference herein.

[0057] Interleukin 18 (IL-18), a cytokine produced by macrophage that together with interleukin 12 induces cell-mediated immunity following infection with microbial products. The polynucleotide sequences of IL-18 are available from public databases as accession numbers U90434 (human); NM_008360 (mouse); EU747333 (chicken); and AY258448 (rat), sequences of which are incorporated by reference herein.

[0058] The amino acid sequences of interleukin 18 (IL-18) are available from public databases as accession numbers AAB50010 (human); NP_032386 (mouse); ACE79188 (chicken); and AAP14669 (rat), sequences of which are incorporated by reference herein.

[0059] The polynucleotide sequences of interleukin 21 (IL-21), which is a cytokine that has a potent regulatory effects on cells of the immune system, including natural killer cells and cytotoxic T cells by inducing cell proliferation, are available from public databases as accession numbers AF254069 (human); NM_021782 (mouse); NM_001024835 (chicken); and NM_001108943 (rat), sequences of which are incorporated by reference herein.

[0060] The amino acid sequences of interleukin 21 (IL-21) are available from public databases as accession numbers, AAG29348 (human); NP_068554 (mouse); NP_001020006 (chicken); and NP_001102413 (rat), sequences of which are incorporated by reference herein.

[0061] Interleukin 27 (IL-27) is a cytokine that plays important function in regulating the activity of B and T lymphocytes. The polynucleotide sequences of IL-27 are available from public databases as accession numbers AY099296 (human); NM_145636 (mouse); and XM_344962 (rat), sequences of which are incorporated by reference herein.

[0062] The amino acid sequences of interleukin 27 (IL-27) are available from public databases as accession numbers AAM34498 (human); NP_663611 (mouse); and XP_344963 (rat), sequences of which are incorporated by reference herein.

[0063] The polynucleotide sequences of interferon beta 1 (IFNB1), which is a member of group of interferon proteins that bind to specific cell surface receptors (IFNAR), and stimulates both macrophages and natural killer (NK) cells to elicit an anti-viral response, are available from public databases as accession numbers NM_002176 (human);

NM_010510 (mouse); NM_001024836 (chicken); and NM_019127 (rat), sequences of which are incorporated by reference herein.

[0064] The amino acid sequences of interferon beta 1 (IFNB1) are available from public databases as accession numbers NP_002167 (human); NP_034640 (mouse); NP_001020007 (chicken); and NP_062000 (rat), sequences of which are incorporated by reference herein.

[0065] Interferon gamma (IFN- gamma) is a soluble cytokine that is the only Type II interferon and has antiviral, immunoregulatory, and anti-tumor activity. The polynucleotide sequences of IFN- gamma are available from public databases as accession numbers NM_000619 (human); NM_008337 (mouse); and NM_138880 (rat), sequences of which are incorporated by reference herein.

[0066] The amino acid sequences of interferon gamma (IFN- gamma) are available from public databases as accession numbers NP_000610 (human); NP_032363 (mouse); and NP_620235 (rat) sequences of which are incorporated by reference herein.

[0067] The polynucleotide sequences of tumor necrosis factor (TNF-alpha), which is a multifunctional proinflammatory cytokine secreted predominantly by monocytes/macrophages that has effects on lipid metabolism, coagulation, insulin resistance, and endothelial function, are available from public databases as accession numbers X02910 (human); NM_013693 (mouse); and BC107671 (rat), sequences of which are incorporated by reference herein.

[0068] The amino acid sequences of TNF-alpha are available from public databases as accession numbers CAA26669 (human); NP_038721 (mouse); and AAI07672 (rat), sequences of which are incorporated by reference herein.

Chemokines

[0069] Chemokine (C motif) ligand 1 (XCL1, also known as Lymphotactin) is chemotactic for CD4+ and CD8+ T cells but not for monocytes, and induces a rise in intracellular calcium in peripheral blood lymphocytes. The polynucleotide sequences of XCL1 are available from public databases as accession numbers NM_002995 (human); NM_008510 (mouse); and NM_134361 (rat), sequences of which are incorporated by reference herein.

[0070] The amino acid sequences of XCL1 are available from public databases as accession numbers NP_002986 (human); NP_032536 (mouse); and NP_599188 (rat),

sequences of which are incorporated by reference herein. U.S. Patent No. 6,022,534 discloses lymphotactin and use to either attract cytotoxic T cells and/or NK cells, and/or to induce proliferation or resident cells. Methods for isolation and usage of an anti-lymphotactin antibody, and XCL1 fusion protein are also disclosed.

[0071] The polynucleotide sequences of CC chemokine ligand 3 (CCL3), also known as macrophage inflammatory protein-1 (MIP-1), which is a so-called monokine (a type of cytokine produced primarily by monocytes and macrophages) that is involved in the acute inflammatory state in the recruitment and activation of polymorphonuclear leukocytes, are available from public databases as accession numbers NM_002983 (human); NM_011337 (mouse); and NM_013025 (rat), sequences of which are incorporated by reference herein.

[0072] The amino acid sequences of CCL3 are available from public databases as accession numbers NP_002974 (human); NP_035467 (mouse); and NP_037157 (rat), sequences of which are incorporated by reference herein.

[0073] The polynucleotide sequences of CCL5 (RANTES), which is a proinflammatory cytokine involved in inflammation and asthma, are available from public databases as accession numbers AF043341 (human); NM_013653 (mouse); and NM_031116 (rat), sequences of which are incorporated by reference herein.

[0074] The amino acid sequences of CCL5 are available from public databases as accession numbers AAC03541 (human); NP_038681 (mouse); and NP_112378 (rat), sequences of which are incorporated by reference herein.

[0075] The polynucleotide sequences of CC chemokine ligand 7 (CCL7), which is a chemokine involved in macrophage recruitment during inflammation and cancer invasion, are available from public databases as accession numbers NM_006273 (human); NM_013654 (mouse); and NM_001007612 (rat), sequences of which are incorporated by reference herein.

[0076] The amino acid sequences of CCL7 are available from public databases as accession numbers NP_006264 (human); NP_038682 (mouse); and NP_001007613 (rat), sequences of which are incorporated by reference herein.

[0077] Chemokine (CXC motif) ligand 9 (CXCL9, also known as MIG) is a T-cell chemoattractant inducible by gamma interferon. The polynucleotide sequences of CXCL9 are available from public databases as accession numbers NM_002416 (human);

NM_0108599 (mouse); and NM_145672 (rat), sequences of which are incorporated by reference herein.

[0078] The amino acid sequences of CXCL9 are available from public databases as accession numbers NP_002407 (human); NP_032625 (mouse); and NP_663705 (rat), sequences of which are incorporated by reference herein.

[0079] Chemokine (C-X-C motif) ligand 10 (CXCL10) is a small cytokine with roles in chemoattraction for cells in the immune system, adhesion of T cells to endothelial cells, anti-tumor activity and angiogenesis. The polynucleotide sequences of CXCL10 are available from public databases as accession numbers X02530 (human); NM_021274 (mouse); and BC058444 (rat), sequences of which are incorporated by reference herein.

[0080] The amino acid sequences of chemokine (C-X-C motif) ligand 10 (CXCL10) are available from public databases as accession numbers CAA26370 (human); NP_067249 (mouse); and AAH58444 (rat), sequences of which are incorporated by reference herein.

[0081] Chemokine (C-X-C motif) ligand 12 (CXCL12), also known as stromal cell-derived factor 1 (SDF-1), is a small cytokine that belong to the intercrine family, members of which activate leukocytes and are often induced by proinflammatory stimuli such as LPS, TNF or IL1. The polynucleotide sequences of CXCL12 are available from public databases as accession numbers NM_000609 (human); NM_001012477 (mouse); NM_204510 (chicken); and NM_001033883 (rat), sequences of which are incorporated by reference herein.

[0082] The amino acid sequences of CXCL12 are available from public databases as accession numbers NP_000600 (human); NP_001012495 (mouse); NP_989841 (chicken); and NP_001029055 (rat), sequences of which are incorporated by reference herein.

[0083] Hansson *et al.*, *Microbes and Infection* *8*:841 (2006) discusses that interaction between chemokine (C-C motif) receptor 7 (CCR7) and chemokine (C-C motif) ligand 19 (CCL19, also known as MIP-3β) is crucial for the generation of primary immune responses. The polynucleotide sequences of CCR7 are available from public databases as accession numbers NM_001838 (human); and NM_007719 (mouse), sequences of which are incorporated by reference herein.

[0084] The amino acid sequences of CCR7 are available from public databases as accession numbers NP_001829 (human); and NP_031745 (mouse), sequences of which are incorporated by reference herein.

[0085] The polynucleotide sequences of CCL19 are available from public databases as accession numbers NM_006274 (human); and NM_011888 (mouse), sequences of which are incorporated by reference herein.

[0086] The amino acid sequences of CCL19 are available from public databases as accession numbers NP_006265 (human); and NP_036018 (mouse), sequences of which are incorporated by reference herein.

[0087] The polynucleotide sequences of CC chemokine ligand 21 (CCL21), a well established ligand for CCR7 which is necessary for CD4+ but not CD8+ T cells to reach their steady state 'set point', and perturbations in the expression of CCL21 may alter susceptibility to autoimmunity, are available from public databases as accession numbers AB002409 (human); NM_011335 (mouse CCL21a); NM_011124 (mouse CCL21b); and NM_023052 (mouse CCL21c); sequences of which are incorporated by reference herein.

[0088] The amino acid sequences of CCL21 are available from public databases as accession numbers BAA21817 (human); NP_035465 (mouse CCL21a); NP_035254 (mouse CCL21b); and NP_075539 (mouse CCL21c), sequences of which are incorporated by reference herein.

[0089] Interleukin-8 (IL-8), is a chemokine, also called neutrophil-activating peptide-1 or SCYB8, is a tissue-derived peptide secreted by several types of cells in response to inflammatory stimuli. U.S. Patent Nos. 6,133,426 and 6,177,980 disclose amino acid and polynucleotide sequences of humanized anti-IL-8 antibodies. The polynucleotide sequence of human IL-8 is available from public database as accession number NM_000584, sequence of which is incorporated by reference herein.

[0090] The amino acid sequence of human IL-8 is available from public database as accession number NP_000575, sequence of which is incorporated by reference herein.

Growth Factors

[0091] Granulocyte/macrophage colony-stimulating factor (GM-CSF) is a cytokine that functions as a white blood cell growth factor, stimulates stems cells to produce granulocytes (neutrophils, eosinophils, and basophils) and monocytes. The polynucleotide sequences of GM-CSF are available from public databases as accession

numbers M11734 (human); NM_009969 (mouse); EU520303 (chicken); NM_001037660 (rat Csf2ra); and NM_133555 (rat Csf2rb), sequences of which are incorporated by reference herein.

[0092] The amino acid sequences of granulocyte/macrophage colony-stimulating factor (GM-CSF) are available from public databases as accession numbers AAA52122 (human); NP_034099 (mouse); ACB11534 (chicken); NP_001032749 (rat Csf2ra); and NP_598239 (Csf2rb), sequences of which are incorporated by reference herein.

[0093] The polynucleotide sequences of FMS-related tyrosine kinase ligand (FLT3/FLK2 ligand, Flt3L), which may function as a growth factor receptor on hematopoietic stem cells or progenitor cells or both, are available from public databases as accession numbers U04806 (human); and NM_013520 (mouse), sequences of which are incorporated by reference herein.

[0094] The amino acid sequences of FLT3/FLK2 ligand (Flt3L) are available from public databases as accession numbers AAA17999 (human); and NP_038548 (mouse), sequences of which are incorporated by reference herein.

[0095] The polynucleotide sequence of transforming growth factor, alpha (TGF-alpha), which is upregulated in some human cancers can reversibly confer the transformed phenotype on cultured cells, is available from public databases as accession numbers NM_001099691 (human); NM_031199 (mouse); NM_001001614 (chicken); and NM_012671 (rat), sequences of which are incorporated by reference herein.

[0096] The amino acid sequences of TGF-alpha is available from public databases as accession numbers NP_001093161 (human); NP_112476 (mouse); NP_001001614 (chicken); and NP_036803 (rat), sequences of which are incorporated by reference herein.

Adjuvants

[0097] Beta-defensins are antimicrobial peptides implicated in innate immune response against many Gram-negative and Gram-positive bacteria, fungi and viruses. The polynucleotide sequences of beta-defensins are available from public databases as accession numbers X92744 (human hBD-1); AJ000152 (human hBD-2); AF217245 (human beta defensin-3); AJ314835 (human beta defensin-4); AB089180 (human hBD-5); AY122466 (human defensin beta 106, DEFB106); AF540979 (human beta defensin 107, DEFB107); AF529416 (human beta defensin, DEFB108); DQ012014 (human beta defensin 110, DEFB110); DQ012015 (human beta defensin 111, DEFB111); DQ012016

(human beta defensin 112, DEFB112); DQ012017 (human beta defensin 113, DEFB113); DQ012018 (human beta defensin 114, DEFB114); DQ012019 (human beta defensin 115, DEFB115); DQ012020 (human beta defensin 116, DEFB116); DQ012021 (human beta defensin 117, DEFB117); NM_007843 (mouse defensin beta 1); NM_010030 (mouse defensin beta 2, Defb2); NM_013756 (mouse defensin beta 3, Defb3); NM_019728 (mouse defensin beta 4, Defb4); NM_030734 (mouse defensin beta 5, Defb5); NM_054074 (mouse defensin beta 6, Defb6); NM_139220 (mouse defensin beta 7); NM_153108 (mouse defensin beta 8, Defb8); NM_139219 (mouse defensin beta 9, Defb9); and NM_139225 (mouse defensin beta 10, Defb10); sequences of which are incorporated by reference herein.

[0098] The amino acid sequences of beta-defensins are available from public databases as accession numbers CAA63405 (human hBD-1); CAB65126 (human hBD-2); AAF73853 (human beta defensin-3); CAC85520 (human beta defensin-4); BAC10630 (human hBD-5); AAM93908 (human defensin beta 106, DEFB106); AAN33115 (human beta defensin 107, DEFB107); AAQ09525 (human beta defensin, DEFB108); AAY59750 (human beta defensin 110, DEFB110); AAY59751 (human beta defensin 111, DEFB111); AAY59752 (human beta defensin 112, DEFB112); AAY59753 (human beta defensin 113, DEFB113); AAY59754 (human beta defensin 114, DEFB114); AAY59755 (human beta defensin 115, DEFB115); AAY59756 (human beta defensin 116, DEFB116); AAY59757 (human beta defensin 117, DEFB117); NP_031869 (mouse defenin beta 1); NP_034160 (mouse defensin beta 2, Defb2); NP_038784 (mouse defensin beta 3, Defb3); NP_062702 (mouse defensin beta 4, Defb4); NP_109659 (mouse defensin beta 5, Defb5); NP_473415 (mouse defensin beta 6, Defb6); NP_631966 (mouse defensin beta 7, Defb7); NP_694748 (mouse defensin beta 8, Defb8); NP_631965 (mouse defensin beta 9, Defb9); and NP_631971 (mouse defensin beta 10, Defb10), sequences of which are incorporated by reference herein. See also U.S. Patent No. 5,242,902 for additional human and rat defensin peptide sequences.

[0099] High-mobility group box-1 (HMGB1) proteins are nonhistone chromosomal proteins that function as cytokines, mediating local and systemic responses to necrotic cell death and cancer, invasion by pathogens, trauma, and sepsis. The polynucleotide sequences of HMGB1 proteins are available from public databases as accession numbers

NM_002128 (human); NM_010439 (mouse); NM_204902 (chicken); and NM_012963 (rat), sequences of which are incorporated by reference herein.

[00100] The amino acid sequences of high-mobility group box-1 (HMGB1) are available from public databases as accession numbers NP_002119 (human); NP_034569 (mouse); NP_990233 (chicken); and NP_037095 (rat), sequences of which are incorporated by reference herein.

[00101] Phagocytic S100 proteins mediate inflammatory responses and recruit inflammatory cells to sites of tissue damage, and are members of Damage-associated molecular pattern (DAMP) molecules that are important for innate immunity. See Foell *et al.*, *J. Leukocyte Biol. 81*:1 (2006). The polynucleotide sequences of S100 proteins are available from public databases as accession numbers BC014392 (human S100 A1); BC002829 (human S100 A2); BC012893 (human S100 A3); BC016300 (human S100 A4); Z18954 (human S100D); BC001431 (human S100 A6); BC 034687 (human S100 A7); BC005928 (human S100 A8); BC047681 (human S100 A9); BC015973 (human S100 A10); D38583 (human clagizzarin); NM_011309 (mouse S100a1); NM_009115 (mouse S100b); NM_013650 (mouse S100a8); NM_009114 (mouse S100a9); NM_011310 (mouse S100a3); NM_011311 (mouse S100a4); and NM_011312 (mouse S100a5), sequences of which are incorporated by reference herein.

[00102] The amino acid sequences of S100 proteins are available from public databases as accession numbers AAH14392 (human S100 A1); AAH02829 (human S100 A2); AAH12893 (human S100 A3); AAH16300 (human S100 A4); CAA79479 (human S100D); AAH01431 (human S100 A6); AAH34687 (human S100 A7); AAH05928 (human S100 A8); AAH47681 (human S100 A9); AAH15973 (human S100 A10); BAA07597 (human clagizzarin); NP_035439 (mouse S100a1); NP_033141 (mouse S100b); NP_038678 (mouse S100a8); NP_033140 (mouse S100a9); NP_035440 (mouse S100a3); NP_035441 (mouse S100a4); and NP_035442 (mouse S100a5), sequences of which are incorporated by reference herein.

[00103] Mannan, a plant polysaccharide, that is a polymer of the sugar mannose, is useful for generation of an immune response. U.S. Patent No. 5,807,559, discloses immunogenic conjugates of Mannan that may be useful for generating T cell immunity against tumor-associated carbohydrate structures or against carbohydrate structures expressed on infectious agents and/or infected host cells. U.S. Patent No. 5,773,425

discloses use of mannan to relieve symptoms and/or cure viral diseases and to enhance immune response.

[00104] Bacille Calmette-Guerin (BCG), live attenuated Mycobacterium species, are used as vaccine against to prevent severe and fatal tuberculosis. U.S. Patent No. 7,393,541 discloses generation of an adjuvant vaccine for producing an *in vivo* T-cell mediated immune response to a mycobacterium in a mammalian subject. See also Hubbard and Collins, *Infect. Immun. 59(2)*: 570. U.S. Patent No. 5,292,513 discloses a method for priming macrophages *in vivo* in patients in need of enhanced bactericidal and anti-viral activity with heat killed BCG. The complete genome sequence of BCG is available from public databases as accession number NC_008769 (*M. bovis* BCG str. Pasteur 1173P2, complete genome).

[00105] Bacterial lipopolysaccharides (LPS) are endotoxins that induces a strong immune response upon infection with Gram-negative bacteria. U.S. Patent No. 4,148,877 discloses fractionation of LPS from bacterial culture and use the fraction as a drug to induce resistance to bacterial infection. U.S. Patent No. 5,292,513 discloses a method for priming macrophages *in vivo* in patients in need of enhanced bactericidal and anti-viral activity with LPS.

Co-stimulatory Molecules (Positive)

[00106] OX40 ligand (OX40L) belongs to tumor necrosis factor (ligand) superfamily member 4 (Tnfsf4), is expressed on dendritic cells and promotes Th2 cell differentiation. The polynucleotide sequences of OX40 ligand are available from public databases as accession numbers X79929 (human); U12763 (mouse); and AF037067 (rat), sequences of which are incorporated by reference herein.

[00107] The amino acid sequences of OX40 ligand (OX40L) are available from public databases as accession numbers CAA56284 (human); AAA21871 (mouse); and AAC67236 (rat), sequences of which are incorporated by reference herein.

[00108] The 4-1BB ligand (4-1BBL) belongs to tumor necrosis factor (ligand) superfamily member 9 (Tnfsf9), which is a type 2 transmembrane glycoprotein and is expressed on activated T lymphocytes. The polynucleotide sequences of 4-1BBL are available from public databases as accession numbers NM_003811 (human); NM_009404 (mouse); and AY332409 (rat), sequences of which are incorporated by reference herein.

[0100] The amino acid sequences of 4-1BB ligand (4-1BBL) are available from public databases as accession numbers NP_003802 (human); NP_033430 (mouse); and AAQ01228 (rat), sequences of which are incorporated by reference herein.

[0101] The CD40 protein belongs to the tumor necrosis factor receptor superfamily member 5, is essential in mediating a broad variety of immune and inflammatory responses including T cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation. The polynucleotide sequences of CD40 proteins are available from public databases as accession numbers X60592 (human); NM_170701 (mouse); NM_204665 (chicken); and NM_134360 (rat), sequences of which are incorporated by reference herein.

[0102] The amino acid sequences of CD40 proteins are available from public databases as accession numbers CAA43045 (human); NP_733802 (mouse); NP_989996 (chicken); and NP_599187 (rat), sequences of which are incorporated by reference herein.

[0103] The glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) can evoke effective tumor immunity via T cell stimulation. Administration of anti-GITR monoclonal antibody (mAb) can provoke potent tumor-specific immunity and eradicated established tumors without eliciting overt autoimmune disease. See Ko *et al.*, *J. Exp. Med.* *7*: 885 (2005). U.S. Patent No. 6,503,184 B1 discloses an Anti-GITR antibody.

[0104] The polynucleotide sequences of GITR ligand (GITRL) are available from public databases as accession numbers AY358868 (human); and AY359852 (mouse), sequences of which are incorporated by reference herein.

[0105] The amino acid sequences of GITR ligand (GITRL) are available from public databases as accession numbers AAQ89227 (human); and AAQ55265 (mouse), sequences of which are incorporated by reference herein.

[0106] Herpes virus entry mediator (HVEM) binding ligand (HSVgD), also referred to as p30, or LIGHT is a TNF family member involved in co-stimulation of T cells. LIGHT has two receptors, herpes virus entry mediator (HVEM) and lymphotoxin-β receptor (LT-βR). Being a ligand for HVEM, HSVgD activates T cells by acting as a costimulatory factor to T cells that results in T cell proliferation and cytokine secretion. See U.S. Patent No. 7,118,742 for polynucleotide and amino acid sequences of LIGHT. U.S. Patent 5,654,174 describes a variant gD protein with deletion of carboxy terminal residues.

[0107] CD70 is a cytokine that binds to CD27. It plays a role in T-cell activation. Induces the proliferation of costimulated T-cells and enhances the generation of cytolytic T-cells. The polynucleotide sequences of CD70 are available from public databases as accession numbers NM_001252 (human); NM_011617 (mouse); and NM_001106878 (rat), sequences of which are incorporated by reference herein.

[0108] The amino acid sequences of CD70 are available from public databases as accession numbers NP_001243 (human); NP_035747 (mouse); and NP_001100348 (rat), sequences of which are incorporated by reference herein.

[0109] ICOS-L is a ligand for the T-cell-specific cell surface receptor ICOS and acts as a costimulatory signal for T-cell proliferation and cytokine secretion. ICOS-L also induces B-cell proliferation and differentiation into plasma cells. ICOS-L could play an important role in mediating local tissue responses to inflammatory conditions, as well as in modulating the secondary immune response by co-stimulating memory T-cell function. The polynucleotide sequences of ICOS-L are available from public databases as accession numbers NM_015259 (human); and NM_015790 (mouse), sequences of which are incorporated by reference herein.

[0110] The amino acid sequences of ICOS-L are available from public databases as accession numbers NP_056074 (human); and NP_056605 (mouse), sequences of which are incorporated by reference herein.

[0111] PD-L1 (also known as CD274) protein is expressed in activated monocytes, T and B cells. PD-L1 is upregulated in monocytes upon treatment with IFN-gamma, and in dendritic cells and keratinocytes upon treatment with IFN-gamma, together with other activators. The polynucleotide sequences of PD-L1 proteins are available from public databases as accession numbers NM_014143 (human); and NM_021893 (mouse), sequences of which are incorporated by reference herein.

[0112] The amino acid sequences of PD-L1 proteins are available from public databases as accession numbers NP_054862 (human); and NP_068693 (mouse), sequences of which are incorporated by reference herein.

Co-stimulatory Molecule (negative)

[0113] Cytotoxic T lymphocyte-associated 4 (CTLA4) is a member of the immunoglobulin superfamily and is a costimulatory molecule expressed in activated T cells. U.S. Patent Nos. 7,034,121 and 6,984,720 disclose methods of preparation and

usage of antibodies against CTLA4. U.S. Patent 6,984,720 also discloses amino acid sequences of heavy and light chain of anti-CTLA4 antibody.

[0114] PD-1 molecules are members of the immunoglobulin gene superfamily, which binds to PD-1 ligand (PD-L1). Binding of a PD-1 receptor on a T-cell by PD-L1 transmits a costimulatory signal to the cell, which prevents the cells from progressing through the cell cycle, and increases T cell proliferation. Inhibition of an interaction between PD-L1 and receptor on the T cell with an anti-PD-L1 antibody results in the down regulation of the immune response termed as immune cell energy. U.S. Patent No. 7,029,674 discloses methods of preparation and sequence of anti-PD-L1 antibody.

[0115] PD-L2 is primarily known as a ligand for PD-1 (or the human homologue PDCD1). However, PD-12 has been reported to be involved in the costimulatory signal, essential for T lymphocyte proliferation and IFN-gamma production in a PDCD1-independent manner. Interaction with PDCD1 inhibit T-cell proliferation by blocking cell cycle progression, and cytokine production. Yamazaki *et al., J. of Immunol. 169*: 5538 (2002) and Ansari *et al., J. Exp. Med.* 198: 63 (2003) describe preparation of anti-PD-L2 monoclonal antibodies.

Counter Immune Suppressants (Tolerance Inhibitors)

[0116] Transforming growth factor-beta (TGF-β) is a multifunctional protein that regulates cell proliferation and differentiation, by interacting with one of the two transmembrane serine/threonine kinase receptors, type I and type II. See Chen *et al., Science 28*: 1335 (1993). TGF receptor type II (TGFR2) phosphorylate and activate type I receptors which autophosphorylate, then bind and activate SMAD transcriptional regulators. Lynch MA *et al., Cancer Res. 58*: 4227 (1998) describes mutations in the transforming growth factor β receptor type II gene (TGFBR2) that are associated with human ovarian carcinomas. Brand *et al., J. Biol. Chem. 268*:11500-11503 (1993) describes that deletion of predicted serine/theronine kinase cytoplasmic domain (nucleotides 1172-2036 of TGFβR2 cDNA H2-3FF, available from public databases as accession number M85079 and amino acid sequence available as accession number AAA61164) impairs the all three TGF-β (1,2 and 3) dependent gene expressions. TGF-β is produced in most human tumors and inhibits tumor antigen-specific cellular immunity. Foster *et al., J. Immunother. 31*:500 (2008) describes that expression of dominant

negative TGFβR2 in cytotoxic T lymphocytes can lead to resistance to the inhibitory effects of TGF-β.

[0117] TGFβ acts synergistically with TGFα in inducing transformation. It also acts as a negative autocrine growth factor. Dysregulation of TGFβ activation and signaling may result in apoptosis. Ziyadeh *et al.*, *Proc. Natl. Acad. Sci. 97*: 8015 (2000) describes that administration of anti-TGFβ antibody can prevent renal insufficiency and glomerulosclerosis in the db/db mouse, a model of type II diabetes that develops overt nephropathy. Methods of generation and use of TGFβ monoclonal antibodies are described in U.S. Patent No. 6,419,928. Barcellos-Hoff *et al.*, *Am J. Pathol. 147*:5 (1995) also describes a method for generation of TGFβ antibody. Amino acid and nucleotide sequences for TGFβ fusion protein constructs are described in US Patent No. 6,756,215.

[0118] IL-10 is a cytokine produced by activated Th2 cells, B cells, keratinocytes, monocytes, and macrophages. IL-10 inhibits the synthesis of a number of cytokines, including IFN-gamma, IL-2, IL-3, TNF and GM-CSF produced by activated macrophages and by helper T-cells. IL-10 is useful in promoting growth and differentiation of activated human B cells, inhibiting Th1 responses to prevent transplant rejection and T cell-mediated autoimmune diseases. O'Farrell *et al., EMBO J.* 17:1006 (1998); Kanbayashi *et al., Cell Immunol.* 171:153 (1996); Fukushima *et al., Br. J. Ophthalmol.* 90:1535 (2006); and van Lent *et al., Ann. Rheum. Dis.* 66:334 (2007) describe the preparation of anti-IL10 antibodies. U.S. Patent No. 7,326,567 discloses polynucleotide sequence of IL-10 antibody. U.S. Patent No. 5,837,232 discloses a method to treat a B-cell mediated autoimmune disorder with anti-IL-10 antibodies.

[0119] Suppressor of cytokine signaling (SOCS) family proteins form part of a classical negative feedback system that regulates cytokine signal transduction. Alexander *et al. Cell 98*: 597 (1999) describes that suppressor of cytokine signaling 1 (SOCS1) is a critical inhibitor of interferon-gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. Hilton *et al., Proc. Natl. Acad. Sci. USA 95*:114 (1999) discusses that SOCS1 is involved in negative regulation of cytokines that signal through the JAK/STAT3 pathway. Ohya *et al. J. Biol. Chem.* 272: 27178 (1997) describes that SOCS proteins appear to be a major regulator of signaling by interleukin 6 (IL-6) and leukemia inhibitory factor (LIF). U.S. Patent No. 6,534,277 discloses a method for the preparation and use of anti-SOCS1 antibody, where a nucleic acid sequence encoding SOCS1

antibody is introduced into cells such that the antibody is expressed by the cells or their progeny, and the recombinant cells are then administered in vivo for therapeutic effect. U.S. Patent Nos. 6,323,317 and 7,049,418 also disclose anti-SOCS1 antibodies.

[0120] TGF-α is a mitogenic polypeptide that is able to bind to the EGF receptor and to act synergistically with TGF-β to promote anchorage-independent cell proliferation in soft agar. Ellis *et al., N. Engl. J. Med. 317*:158 (1987) describes that TGF-α plays a role in certain paraneoplastic manifestations of melanoma. U.S. Patent No. 4,742,003 and Xian *et al., The J. of Histochem. & Cytochem.* 47:949 (1999) describe methods of preparation of Anti-TGF-α antibodies.

[0121] Both tumor necrosis factor receptor (TNFR1) and Fas contain cytoplasmic Fas-associated protein with death domain (FADD), which is essential for Fas and TNF-induced signaling for programmed cell death (apoptosis) and receptor oligomerization. A mammalian protein designated FADD having the ability to bind the cytoplasmic region or domain of the Fas receptor and inhibits FAS mediated apoptosis has been identified. The polynucleotide sequence of FADD is available from public database as accession number U24231, and the amino acid sequence as accession number AAA86517, which are incorporated by reference herein. A FADD fragment or nucleic acid encoding it which is a dominant negative inhibitor of functionally intact native FADD is described in U.S. Patent No. 6,562,797 B1.

DESCRIPTION OF SEQUENCE LISTING

[0122] SEQ ID NO: 1 is a polynucleotide sequence of a construct coding for mIL-12 and m-IL21.

[0123] SEQ ID NO: 2 is a polynucleotide sequence of a construct coding for hIL-12 and hIL-21.

[0124] SEQ ID NO: 3 is a polynucleotide sequence of a construct coding for mIL-21 and mIL-15.

[0125] SEQ ID NO: 4 is a polynucleotide sequence of a construct coding for mIL-12.

[0126] SEQ ID NO: 5 is a polynucleotide sequence of a construct coding for hIL-21 and hIL-15.

[0127] SEQ ID NO: 6 is a polynucleotide sequence of a construct coding for hIL-21.

[0128] SEQ ID NO: 7 is a polynucleotide sequence of a construct coding for mIL-21.

[0129] SEQ ID NO: 8 is a polynucleotide sequence of a construct coding for hIL-21.

[0130] SEQ ID NO: 9 is a polynucleotide sequence coding for mIL-21.

[0131] SEQ ID NO: 10 is an amino acid sequence of mIL-21.

[0132] SEQ ID NO: 11 is a polynucleotide sequence coding for mIL-15.

[0133] SEQ ID NO: 12 is an amino acid sequence of mIL-15.

[0134] SEQ ID NO: 13 is a polynucleotide sequence coding for mp40 of mIL-12.

[0135] SEQ ID NO: 14 is the amino acid sequence of mp40 of mIL-12.

[0136] SEQ ID NO: 15 is a polynucleotide sequence coding for mp35 of mIL-12.

[0137] SEQ ID NO: 16 is the amino acid sequence of mp35 of mIL-12.

[0138] SEQ ID NO: 17 is a polynucleotide sequence coding for hIL-21.

[0139] SEQ ID NO: 18 is the amino acid sequence of hIL-21.

[0140] SEQ ID NO: 19 is a polynucleotide sequence coding for hIL-15.

[0141] SEQ ID NO: 20 is the amino acid sequence of hIL-15.

[0142] SEQ ID NO: 21 is a polynucleotide sequence coding for p40 of hIL-12.

[0143] SEQ ID NO: 22 is the amino acid sequence of p40 of hIL-12.

[0144] SEQ ID NO: 23 is a polynucleotide sequence coding for p35 of hIL-12.

[0145] SEQ ID NO: 24 is the amino acid sequence of p35 of hIL-12.

[0146] SEQ ID NO: 25 is a nucleic acid sequence of an ecdysone response element found in *Drosophila.*

[0147] SEQ ID NO: 26 is a nucleic acid sequence of an ecdysone response element found in *Drosophila melanogaster.*

[0148] SEQ ID NO: 27 is a nucleic acid sequence of an ecdysone response element found in *Drosophila melanogaster.*

[0149] SEQ ID NO: 28 is a restriction site of a homing endonuclease (HE) enzyme (I-SceI)

[0150] SEQ ID NO: 29 is a DNA sequence of adenovirus vector comprising human IL-12 coding sequence: Ad-RTS-hIL-12 (SP1-RheoIL-12).

DETAILED DESCRIPTION OF INVENTION

DEFINITIONS

[0151] Unless otherwise defined, all terms of art, notations and other scientific terms or terminology used herein are intended to have the meanings commonly understood by

those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference and understanding, and the inclusion of such definitions herein should not necessarily be construed to mean a substantial difference over what is generally understood in the art. Commonly understood definitions of molecular biology terms and/or methods and/or protocols can be found in Rieger *et al.*, Glossary of Genetics: Classical and Molecular, 5th edition, Springer-Verlag: New York, 1991; Lewin, Genes V, Oxford University Press: New York, 1994; Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (3d ed. 2001) and Ausubel *et al.*, Current Protocols in Molecular Biology (1994). As appropriate, procedures involving the use of commercially available kits and/or reagents are generally carried out in accordance with manufacturer's guidance and/or protocols and/or parameters unless otherwise noted.

[0152] The term "isolated" for the purposes of the invention designates a biological material (cell, nucleic acid or protein) that has been removed from its original environment (the environment in which it is naturally present). For example, a polynucleotide present in the natural state in a plant or an animal is not isolated, however the same polynucleotide separated from the adjacent nucleic acids in which it is naturally present, is considered "isolated."

[0153] The term "purified," as applied to biological materials does not require the material to be present in a form exhibiting absolute purity, exclusive of the presence of other compounds. It is rather a relative definition.

[0154] "Nucleic acid," "nucleic acid molecule," "oligonucleotide," "nucleotide," and "polynucleotide" are used interchangeably and refer to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, supercoiled

DNA and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the non-transcribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation. DNA includes, but is not limited to, cDNA, genomic DNA, plasmid DNA, synthetic DNA, and semi-synthetic DNA.

[0155] The term "fragment," as applied to polynucleotide sequences, refers to a nucleotide sequence of reduced length relative to the reference nucleic acid and comprising, over the common portion, a nucleotide sequence identical to the reference nucleic acid. Such a nucleic acid fragment according to the invention may be, where appropriate, included in a larger polynucleotide of which it is a constituent. Such fragments comprise, or alternatively consist of, oligonucleotides ranging in length from at least 6, 8, 9, 10, 12, 15, 18, 20, 21, 22, 23, 24, 25, 30, 39, 40, 42, 45, 48, 50, 51, 54, 57, 60, 63, 66, 70, 75, 78, 80, 90, 100, 105, 120, 135, 150, 200, 300, 500, 720, 900, 1000, 1500, 2000, 3000, 4000, 5000, or more consecutive nucleotides of a nucleic acid according to the invention.

[0156] As used herein, an "isolated nucleic acid fragment" refers to a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

[0157] A "gene" refers to a polynucleotide comprising nucleotides that encode a functional molecule, including functional molecules produced by transcription only (*e.g.*, a bioactive RNA species) or by transcription and translation (*e.g.*, a polypeptide). The term "gene" encompasses cDNA and genomic DNA nucleic acids. "Gene" also refers to a nucleic acid fragment that expresses a specific RNA, protein or polypeptide, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and/or coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding

sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. A chimeric gene may comprise coding sequences derived from different sources and/or regulatory sequences derived from different sources. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene or "heterologous" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure. For example, the interleukin-12 (IL-12) gene encodes the IL-12 protein. IL-12 is a heterodimer of a 35-kD subunit (p35) and a 40-kD subunit (p40) linked through a disulfide linkage to make fully functional IL-12p70. The IL-12 gene encodes both the p35 and p40 subunits.

[0158] "Heterologous DNA" refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. The heterologous DNA may include a gene foreign to the cell.

[0159] The term "genome" includes chromosomal as well as mitochondrial, chloroplast and viral DNA or RNA.

[0160] A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known and exemplified in Sambrook *et al.* in *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989), particularly Chapter 11 and Table 11.1 therein). The conditions of temperature and ionic strength determine the "stringency" of the hybridization.

[0161] Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m of 55°, can be used, *e.g.*, 5X SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5X SSC, 0.5% SDS. Moderate

stringency hybridization conditions correspond to a higher T_m, *e.g.*, 40% formamide, with 5X or 6X SSC. High stringency hybridization conditions correspond to the highest T_m, *e.g.*, 50% formamide, 5X or 6X SSC.

[0162] Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The term "complementary" is used to describe the relationship between nucleotide bases that are capable of hybridizing to one another. For example, with respect to DNA, adenosine is complementary to thymine and cytosine is complementary to guanine. Accordingly, the invention also includes isolated nucleic acid fragments that are complementary to the complete sequences as disclosed or used herein as well as those substantially similar nucleic acid sequences.

[0163] In one embodiment of the invention, polynucleotides are detected by employing hybridization conditions comprising a hybridization step at T_m of 55°C, and utilizing conditions as set forth above. In other embodiments, the T_m is 60°C, 63°C, or 65°C.

[0164] Post-hybridization washes also determine stringency conditions. One set of conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 minutes (min), then repeated with 2X SSC, 0.5% SDS at 45°C for 30 min, and then repeated twice with 0.2X SSC, 0.5% SDS at 50°C for 30 min. A preferred set of stringent conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS is increased to 60°C. Another preferred set of highly stringent conditions uses two final washes in 0.1X SSC, 0.1% SDS at 65°C.

[0165] The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (see Sambrook *et al.*, *supra*, 9.50-0.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook *et al.*, *supra*, 11.7-11.8).

[0166] In one embodiment of the invention, polynucleotides are detected by employing hybridization conditions comprising a hybridization step in less than 500 mM salt and at least 37°C, and a washing step in 2X SSPE at a temperature of at least 63°C. In another embodiment, the hybridization conditions comprise less than 200 mM salt and at least 37°C for the hybridization step. In a further embodiment, the hybridization conditions comprise 2X SSPE and 63°C for both the hybridization and washing steps.

[0167] In another embodiment, the length for a hybridizable nucleic acid is at least about 10 nucleotides. Preferably a minimum length for a hybridizable nucleic acid is at least about 15 nucleotides; *e.g.*, at least about 20 nucleotides; *e.g.*, at least 30 nucleotides. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as length of the probe.

[0168] The term "probe" refers to a single-stranded nucleic acid molecule that can base pair with a complementary single stranded target nucleic acid to form a double-stranded molecule.

[0169] As used herein, the term "oligonucleotide" refers to a short nucleic acid that is hybridizable to a genomic DNA molecule, a cDNA molecule, a plasmid DNA or an mRNA molecule. Oligonucleotides can be labeled, *e.g.*, with ^{32}P-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. A labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. Oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of a nucleic acid, for DNA sequencing, or to detect the presence of a nucleic acid. An oligonucleotide can also be used to form a triple helix with a DNA molecule. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer. Accordingly, oligonucleotides can be prepared with non-naturally occurring phosphoester analog bonds, such as thioester bonds, *etc.*

[0170] A "primer" refers to an oligonucleotide that hybridizes to a target nucleic acid sequence to create a double stranded nucleic acid region that can serve as an initiation point for DNA synthesis under suitable conditions. Such primers may be used in a polymerase chain reaction or for DNA sequencing.

[0171] "Polymerase chain reaction" is abbreviated PCR and refers to an *in vitro* method for enzymatically amplifying specific nucleic acid sequences. PCR involves a repetitive

series of temperature cycles with each cycle comprising three stages: denaturation of the template nucleic acid to separate the strands of the target molecule, annealing a single stranded PCR oligonucleotide primer to the template nucleic acid, and extension of the annealed primer(s) by DNA polymerase. PCR provides a means to detect the presence of the target molecule and, under quantitative or semi-quantitative conditions, to determine the relative amount of that target molecule within the starting pool of nucleic acids.

[0172] "Reverse transcription-polymerase chain reaction" is abbreviated RT-PCR and refers to an *in vitro* method for enzymatically producing a target cDNA molecule or molecules from an RNA molecule or molecules, followed by enzymatic amplification of a specific nucleic acid sequence or sequences within the target cDNA molecule or molecules as described above. RT-PCR also provides a means to detect the presence of the target molecule and, under quantitative or semi-quantitative conditions, to determine the relative amount of that target molecule within the starting pool of nucleic acids.

[0173] A DNA "coding sequence" refers to a double-stranded DNA sequence that encodes a polypeptide and can be transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of suitable regulatory sequences. "Suitable regulatory sequences" refers to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from mRNA, genomic DNA sequences, and even synthetic DNA sequences. If the coding sequence is intended for expression in an eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

[0174] "Open reading frame" is abbreviated ORF and refers to a length of nucleic acid sequence, either DNA, cDNA or RNA, that comprises a translation start signal or initiation codon, such as an ATG or AUG, and a termination codon and can be potentially translated into a polypeptide sequence.

[0175] The term "head-to-head" is used herein to describe the orientation of two polynucleotide sequences in relation to each other. Two polynucleotides are positioned in a head-to-head orientation when the 5' end of the coding strand of one polynucleotide is adjacent to the 5' end of the coding strand of the other polynucleotide, whereby the direction of transcription of each polynucleotide proceeds away from the 5' end of the other polynucleotide. The term "head-to-head" may be abbreviated (5')-to-(5') and may also be indicated by the symbols (← →) or (3'←5'5'→3').

[0176] The term "tail-to-tail" is used herein to describe the orientation of two polynucleotide sequences in relation to each other. Two polynucleotides are positioned in a tail-to-tail orientation when the 3' end of the coding strand of one polynucleotide is adjacent to the 3' end of the coding strand of the other polynucleotide, whereby the direction of transcription of each polynucleotide proceeds toward the other polynucleotide. The term "tail-to-tail" may be abbreviated (3')-to-(3') and may also be indicated by the symbols (→ ←) or (5'→3'3'←5').

[0177] The term "head-to-tail" is used herein to describe the orientation of two polynucleotide sequences in relation to each other. Two polynucleotides are positioned in a head-to-tail orientation when the 5' end of the coding strand of one polynucleotide is adjacent to the 3' end of the coding strand of the other polynucleotide, whereby the direction of transcription of each polynucleotide proceeds in the same direction as that of the other polynucleotide. The term "head-to-tail" may be abbreviated (5')-to-(3') and may also be indicated by the symbols (→ →) or (5'→3'5'→3').

[0178] The term "downstream" refers to a nucleotide sequence that is located 3' to a reference nucleotide sequence. In particular, downstream nucleotide sequences generally relate to sequences that follow the starting point of transcription. For example, the translation initiation codon of a gene is located downstream of the start site of transcription.

[0179] The term "upstream" refers to a nucleotide sequence that is located 5' to a reference nucleotide sequence. In particular, upstream nucleotide sequences generally relate to sequences that are located on the 5' side of a coding sequence or starting point of transcription. For example, most promoters are located upstream of the start site of transcription.

[0180] The terms "restriction endonuclease" and "restriction enzyme" are used interchangeably and refer to an enzyme that binds and cuts within a specific nucleotide sequence within double stranded DNA.

[0181] "Homologous recombination" refers to the insertion of a foreign DNA sequence into another DNA molecule, *e.g.*, insertion of a vector in a chromosome. Preferably, the vector targets a specific chromosomal site for homologous recombination. For specific homologous recombination, the vector will contain sufficiently long regions of homology to sequences of the chromosome to allow complementary binding and incorporation of the vector into the chromosome. Longer regions of homology, and greater degrees of sequence similarity, may increase the efficiency of homologous recombination.

[0182] Several methods known in the art may be used to propagate a polynucleotide according to the invention. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As described herein, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

[0183] A "vector" refers to any vehicle for the cloning of and/or transfer of a nucleic acid into a host cell. A vector may be a replicon to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" refers to any genetic element (*e.g.*, plasmid, phage, cosmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, *i.e.*, capable of replication under its own control. The term "vector" includes both viral and nonviral vehicles for introducing the nucleic acid into a cell *in vitro*, *ex vivo* or *in vivo*. A large number of vectors known in the art may be used to manipulate nucleic acids, incorporate response elements and promoters into genes, *etc*. Possible vectors include, for example, plasmids or modified viruses including, for example bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives, or the Bluescript vector. Another example of vectors that are useful in the invention is the UltraVector™ Production System (Intrexon Corp., Blacksburg, VA) as described in WO 2007/038276. For example, the insertion of the DNA fragments corresponding to response elements and promoters into a suitable vector can be accomplished by ligating the appropriate DNA fragments into a

chosen vector that has complementary cohesive termini. Alternatively, the ends of the DNA molecules may be enzymatically modified or any site may be produced by ligating nucleotide sequences (linkers) into the DNA termini. Such vectors may be engineered to contain selectable marker genes that provide for the selection of cells that have incorporated the marker into the cellular genome. Such markers allow identification and/or selection of host cells that incorporate and express the proteins encoded by the marker.

[0184] Viral vectors, and particularly retroviral vectors, have been used in a wide variety of gene delivery applications in cells, as well as living animal subjects. Viral vectors that can be used include, but are not limited to, retrovirus, adeno-associated virus, pox, baculovirus, vaccinia, herpes simplex, Epstein-Barr, adenovirus, geminivirus, and caulimovirus vectors. Non-viral vectors include plasmids, liposomes, electrically charged lipids (cytofectins), DNA-protein complexes, and biopolymers. In addition to a nucleic acid, a vector may also comprise one or more regulatory regions, and/or selectable markers useful in selecting, measuring, and monitoring nucleic acid transfer results (transfer to which tissues, duration of expression, *etc.*).

[0185] The term "plasmid" refers to an extra-chromosomal element often carrying a gene that is not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear, circular, or supercoiled, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.

[0186] A "cloning vector" refers to a "replicon," which is a unit length of a nucleic acid, preferably DNA, that replicates sequentially and which comprises an origin of replication, such as a plasmid, phage or cosmid, to which another nucleic acid segment may be attached so as to bring about the replication of the attached segment. Cloning vectors may be capable of replication in one cell type and expression in another ("shuttle vector"). Cloning vectors may comprise one or more sequences that can be used for selection of cells comprising the vector and/or one or more multiple cloning sites for insertion of sequences of interest.

[0187] The term "expression vector" refers to a vector, plasmid or vehicle designed to enable the expression of an inserted nucleic acid sequence following transformation into the host. The cloned gene, *i.e.*, the inserted nucleic acid sequence, is usually placed under the control of control elements such as a promoter, a minimal promoter, an enhancer, or the like. Initiation control regions or promoters, which are useful to drive expression of a nucleic acid in the desired host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving expression of these genes can be used in an expression vector, including but not limited to, viral promoters, bacterial promoters, animal promoters, mammalian promoters, synthetic promoters, constitutive promoters, tissue specific promoters, pathogenesis or disease related promoters, developmental specific promoters, inducible promoters, light regulated promoters; *CYC1, HIS3, GAL1, GAL4, GAL10, ADH1, PGK, PHO5, GAPDH, ADC1, TRP1, URA3, LEU2, ENO, TPI*, alkaline phosphatase promoters (useful for expression in *Saccharomyces*); *AOX1* promoter (useful for expression in *Pichia*); β-lactamase, *lac, ara, tet, trp*, lP_L, lP_R, *T7, tac, and trc* promoters (useful for expression in *Escherichia coli*); light regulated-, seed specific-, pollen specific-, ovary specific-, cauliflower mosaic virus 35S, CMV 35S minimal, cassava vein mosaic virus (CsVMV), chlorophyll a/b binding protein, ribulose 1,5-bisphosphate carboxylase, shoot-specific, root specific, chitinase, stress inducible, rice tungro bacilliform virus, plant super-promoter, potato leucine aminopeptidase, nitrate reductase, mannopine synthase, nopaline synthase, ubiquitin, zein protein, and anthocyanin promoters (useful for expression in plant cells); animal and mammalian promoters known in the art including, but are not limited to, the SV40 early (SV40e) promoter region, the promoter contained in the 3' long terminal repeat (LTR) of Rous sarcoma virus (RSV), the promoters of the E1A or major late promoter (MLP) genes of adenoviruses (Ad), the cytomegalovirus (CMV) early promoter, the herpes simplex virus (HSV) thymidine kinase (TK) promoter, a baculovirus IE1 promoter, an elongation factor 1 alpha (EF1) promoter, a phosphoglycerate kinase (PGK) promoter, a ubiquitin (Ubc) promoter, an albumin promoter, the regulatory sequences of the mouse metallothionein-L promoter and transcriptional control regions, the ubiquitous promoters (HPRT, vimentin, α-actin, tubulin and the like), the promoters of the intermediate filaments (desmin, neurofilaments, keratin, GFAP, and the like), the promoters of therapeutic genes (of the MDR, CFTR or factor VIII type, and the like), pathogenesis or disease related-promoters,

and promoters that exhibit tissue specificity and have been utilized in transgenic animals, such as the elastase I gene control region which is active in pancreatic acinar cells; insulin gene control region active in pancreatic beta cells, immunoglobulin gene control region active in lymphoid cells, mouse mammary tumor virus control region active in testicular, breast, lymphoid and mast cells; albumin gene, Apo AI and Apo AII control regions active in liver, alpha-fetoprotein gene control region active in liver, alpha 1-antitrypsin gene control region active in the liver, beta-globin gene control region active in myeloid cells, myelin basic protein gene control region active in oligodendrocyte cells in the brain, myosin light chain-2 gene control region active in skeletal muscle, and gonadotropic releasing hormone gene control region active in the hypothalamus, pyruvate kinase promoter, villin promoter, promoter of the fatty acid binding intestinal protein, promoter of the smooth muscle cell α-actin, and the like. In addition, these expression sequences may be modified by addition of enhancer or regulatory sequences and the like.

[0188] Vectors may be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (see, *e.g.*, Wu *et al.*, *J. Biol. Chem. 267*:963 (1992); Wu *et al.*, *J. Biol. Chem. 263*:14621 (1988); and Hartmut *et al.*, Canadian Patent Application No. 2,012,311).

[0189] A polynucleotide according to the invention can also be introduced *in vivo* by lipofection. For the past decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids *in vitro*. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome-mediated transfection can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner *et al.*, *Proc. Natl. Acad. Sci. USA. 84*:7413 (1987); Mackey *et al.*, *Proc. Natl. Acad. Sci. USA 85*:8027 (1988); and Ulmer *et al.*, *Science 259*:1745 (1993)). The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes (Felgner *et al.*, *Science* 337:387 (1989)). Particularly useful lipid compounds and compositions for transfer of nucleic acids are described in WO95/18863, WO96/17823 and U.S. 5,459,127. The use of lipofection to introduce exogenous genes into the specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one

area of benefit. It is clear that directing transfection to particular cell types would be particularly preferred in a tissue with cellular heterogeneity, such as pancreas, liver, kidney, and the brain. Lipids may be chemically coupled to other molecules for the purpose of targeting (Mackey *et al.* 1988, *supra*). Targeted peptides, *e.g.*, hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

[0190] Other molecules are also useful for facilitating transfection of a nucleic acid *in vivo*, such as a cationic oligopeptide (*e.g.*, WO95/21931), peptides derived from DNA binding proteins (*e.g.*, WO96/25508), or a cationic polymer (*e.g.*, WO95/21931).

[0191] It is also possible to introduce a vector *in vivo* as a naked DNA plasmid (see U.S. Patent Nos. 5,693,622, 5,589,466 and 5,580,859). Receptor-mediated DNA delivery approaches can also be used (Curiel *et al., Hum. Gene Ther. 3*:147 (1992); and Wu *et al., J. Biol. Chem.* 262:4429 (1987)).

[0192] The term "transfection" refers to the uptake of exogenous or heterologous RNA or DNA by a cell. A cell has been "transfected" by exogenous or heterologous RNA or DNA when such RNA or DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous RNA or DNA when the transfected RNA or DNA effects a phenotypic change. The transforming RNA or DNA can be integrated (covalently linked) into chromosomal DNA making up the genome of the cell.

[0193] "Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

[0194] In addition, the recombinant vector comprising a polynucleotide according to the invention may include one or more origins for replication in the cellular hosts in which their amplification or their expression is sought, markers or selectable markers.

[0195] The term "selectable marker" refers to an identifying factor, usually an antibiotic or chemical resistance gene, that is able to be selected for based upon the marker gene's effect, *i.e.*, resistance to an antibiotic, resistance to a herbicide, colorimetric markers, enzymes, fluorescent markers, and the like, wherein the effect is used to track the inheritance of a nucleic acid of interest and/or to identify a cell or organism that has inherited the nucleic acid of interest. Examples of selectable marker genes known and

used in the art include: genes providing resistance to ampicillin, streptomycin, gentamycin, kanamycin, hygromycin, bialaphos herbicide, sulfonamide, and the like; and genes that are used as phenotypic markers, *i.e.*, anthocyanin regulatory genes, isopentanyl transferase gene, and the like.

[0196] The term "reporter gene" refers to a nucleic acid encoding an identifying factor that is able to be identified based upon the reporter gene's effect, wherein the effect is used to track the inheritance of a nucleic acid of interest, to identify a cell or organism that has inherited the nucleic acid of interest, and/or to measure gene expression induction or transcription. Examples of reporter genes known and used in the art include: luciferase (Luc), green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT), β-galactosidase (LacZ), β-glucuronidase (Gus), and the like. Selectable marker genes may also be considered reporter genes.

[0197] "Promoter" and "promoter sequence" are used interchangeably and refer to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters that cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters." Promoters that cause a gene to be expressed in a specific cell type are commonly referred to as "cell-specific promoters" or "tissue-specific promoters." Promoters that cause a gene to be expressed at a specific stage of development or cell differentiation are commonly referred to as "developmentally-specific promoters" or "cell differentiation-specific promoters." Promoters that are induced and cause a gene to be expressed following exposure or treatment of the cell with an agent, biological molecule, chemical, ligand, light, or the like that induces the promoter are commonly referred to as "inducible promoters" or "regulatable promoters." It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

[0198] In any of the vectors of the present invention, the vector optionally comprises a promoter disclosed herein. In one embodiment, the promoter is a promoter listed in Table 1 herein.

[0199] In any of the vectors of the present invention, the vector optionally comprises a tissue-specific promoter. In one embodiment, the tissue-specific promoter is a tissue specific promoter disclosed herein. In another embodiment, the tissue-specific promoter is a tissue specific promoter listed in Table 2 herein.

[0200] The promoter sequence is typically bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

[0201] "Therapeutic switch promoter" ("TSP") refers to a promoter that controls expression of a gene switch component. Gene switches and their various components are described in detail elsewhere herein. In certain embodiments a TSP is constitutive, *i.e.*, continuously active. A consitutive TSP may be either constitutive-ubiquitous (*i.e.*, generally functions, without the need for additional factors or regulators, in any tissue or cell) or constitutive-tissue or cell specific (*i.e.*, generally functions, without the need for additional factors or regulators, in a specific tissue type or cell type). In certain embodiments a TSP of the invention is activated under conditions associated with a disease, disorder, or condition. In certain embodiments of the invention where two or more TSPs are involved the promoters may be a combination of constitutive and activatable promoters. As used herein, a "promoter activated under conditions associated with a disease, disorder, or condition" includes, without limitation, disease-specific promoters, promoters responsive to particular physiological, developmental, differentiation, or pathological conditions, promoters responsive to specific biological molecules, and promoters specific for a particular tissue or cell type associated with the disease, disorder, or condition, *e.g.* tumor tissue or malignant cells. TSPs can comprise the sequence of naturally occurring promoters, modified sequences derived from naturally occurring promoters, or synthetic sequences (*e.g.*, insertion of a response element into a minimal promoter sequence to alter the responsiveness of the promoter).

[0202] A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced (if the coding sequence contains introns) and translated into the protein encoded by the coding sequence.

[0203] "Transcriptional and translational control sequences" refer to DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

[0204] The term "response element" refers to one or more cis-acting DNA elements which confer responsiveness on a promoter mediated through interaction with the DNA-binding domains of a transcription factor. This DNA element may be either palindromic (perfect or imperfect) in its sequence or composed of sequence motifs or half sites separated by a variable number of nucleotides. The half sites can be similar or identical and arranged as either direct or inverted repeats or as a single half site or multimers of adjacent half sites in tandem. The response element may comprise a minimal promoter isolated from different organisms depending upon the nature of the cell or organism into which the response element is incorporated. The DNA binding domain of the transcription factor binds, in the presence or absence of a ligand, to the DNA sequence of a response element to initiate or suppress transcription of downstream gene(s) under the regulation of this response element. Examples of DNA sequences for response elements of the natural ecdysone receptor include: RRGG/TTCANTGAC/ACYY (SEQ ID NO: 25) (see Cherbas *et. al.*, *Genes Dev. 5*:120 (1991)); AGGTCA$N_{(n)}$AGGTCA, where $N_{(n)}$ can be one or more spacer nucleotides (SEQ ID NO: 26) (see D'Avino *et al.*, *Mol. Cell. Endocrinol. 113*:1 (1995)); and GGGTTGAATGAATTT (SEQ ID NO: 27) (see Antoniewski *et al.*, *Mol. Cell Biol. 14*:4465 (1994)).

[0205] The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (*i.e.*, that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

[0206] The term "expression" as used herein refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from a nucleic acid or polynucleotide. Expression may also refer to translation of mRNA into a protein or polypeptide.

[0207] The terms "cassette," "expression cassette" and "gene expression cassette" refer to a segment of DNA that can be inserted into a nucleic acid or polynucleotide at specific restriction sites or by homologous recombination. The segment of DNA comprises a polynucleotide that encodes a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation. "Transformation cassette" refers to a specific vector comprising a polynucleotide that encodes a polypeptide of interest and having elements in addition to the polynucleotide that facilitate transformation of a particular host cell. Cassettes, expression cassettes, gene expression cassettes and transformation cassettes of the invention may also comprise elements that allow for enhanced expression of a polynucleotide encoding a polypeptide of interest in a host cell. These elements may include, but are not limited to: a promoter, a minimal promoter, an enhancer, a response element, a terminator sequence, a polyadenylation sequence, and the like.

[0208] For purposes of this invention, the term "gene switch" refers to the combination of a response element associated with a promoter, and a ligand-dependent transcription factor-based system which, in the presence of one or more ligands, modulates the expression of a gene into which the response element and promoter are incorporated. The term "a polynucleotide encoding a gene switch" refers to the combination of a response element associated with a promoter, and a polynucleotide encoding a ligand-dependent transcription factor-based system which, in the presence of one or more ligands, modulates the expression of a gene into which the response element and promoter are incorporated.

[0209] The therapeutic switch promoters of the invention may be any promoter that is useful for treating, ameliorating, or preventing a specific disease, disorder, or condition. Examples include, without limitation, promoters of genes that exhibit increased expression only during a specific disease, disorder, or condition and promoters of genes that exhibit increased expression under specific cell conditions (*e.g.*, proliferation, apoptosis, change in pH, oxidation state, oxygen level). In some embodiments where the

gene switch comprises more than one transcription factor sequence, the specificity of the therapeutic methods can be increased by combining a disease- or condition- specific promoter with a tissue- or cell type-specific promoter to limit the tissues in which the therapeutic product is expressed. Thus, tissue- or cell type-specific promoters are encompassed within the definition of therapeutic switch promoter.

[0210] As an example of disease-specific promoters, useful promoters for treating cancer include the promoters of oncogenes. Examples of classes of oncogenes include, but are not limited to, growth factors, growth factor receptors, protein kinases, programmed cell death regulators and transcription factors. Specific examples of oncogenes include, but are not limited to, sis, erb B, erb B-2, ras, abl, myc and bcl-2 and TERT. Examples of other cancer-related genes include tumor associated antigen genes and other genes that are overexpressed in neoplastic cells (*e.g.*, MAGE-1, carcinoembryonic antigen, tyrosinase, prostate specific antigen, prostate specific membrane antigen, p53, MUC-1, MUC-2, MUC-4, HER-2/neu, T/Tn, MART-1, gp100, GM2, Tn, sTn, and Thompson-Friedenreich antigen (TF)).

[0211] Examples of promoter sequences and other regulatory elements (*e.g.*, enhancers) that are known in the art and are useful as therapeutic switch promoters in the present invention are disclosed in the references listed in Tables 1 and 2, along with the disease/disorder (Table 1) or tissue specificity (Table 2) associated with each promoter. The promoter sequences disclosed in these references are herein incorporated by reference in their entirety.

Table 1

Promoter Sequence	**Disease/Disorder**	**Patent/Published Application No.**
Her-2/neu (ERBB2/c-erbB-2)	cancer	5,518,885
osteocalcin	calcified tumors	5,772,993
stromelysin-1	cancer	5,824,794
prostate specific antigen	prostate cancer	5,919,652
human sodium-iodide symporter	thyroid carcinoma	6,015,376
H19, IF-1, IGF-2	cancer	6,306,833
thymosin β15	breast, pancreatic, prostate cancer	6,489,463

Promoter Sequence	Disease/Disorder	Patent/Published Application No.
T cell factor	cancer	6,608,037
cartilage-derived retinoic acid-sensitive protein	chondrosarcoma, mammary tumor	6,610,509
insulin	pancreatic cancer	6,716,824
PEG-3	cancer	6,737,523
telomerase reverse transcriptase	cancer	6,777,203
melanoma differentiation associated gene-7	cancer	6,841,362
prostasin	cancer	6,864,093
telomerase catalytic subunit; cyclin-A	cancer	6,936,595
midkine; c-erbB-2	cancer	7,030,099
prostate-specific membrane antigen	prostate cancer	7,037,647
p51	cancer	7,038,028
telomerase RNA	cancer	7,084,267
prostatic acid phosphatase	prostate cancer	7,094,533
$PCA3_{dd3}$	prostate cancer	7,138,235
DF3/MUC1	cancer	7,247,297
hex II	cancer	2001/0011128
cyclooxygenase-2	cancer	2002/0107219
super PSA	prostate cancer	2003/0078224
skp2	cancer	2003/0109481
PRL-3	metastatic colon cancer	2004/0126785
CA125/M17S2	ovarian cancer	2004/0126824
IAI.3B	ovarian cancer	2005/0031591
CRG-L2	liver cancer	2005/0124068
TRPM4	prostate cancer	2006/0188990
RTVP	glioma	2006/0216731
TARP	prostate cancer, breast cancer	2007/0032439
telomere reverse transcriptase	cancer	2007/0059287
A4 amyloid protein	Alzheimer's disease	5,151,508

Promoter Sequence	Disease/Disorder	Patent/Published Application No.
amyloid β-protein precursor	Alzheimer's disease	5,643,726
precursor of the Alzheimer's Disease A4 amyloid protein	Alzheimer's disease	5,853,985
neuropeptide FF	CNS disorders	6,320,038
endoplasmic reticulum stress elements	stress	7,049,132
urocortin II	psychopathologies	7,087,385
tyrosine hydroxylase	neurological disorders	7,195,910
complement factor 3; serum amyloid A3	inflammation	5,851,822
tissue inhibitor of metalloproteinase-3 (TIMP-3)	rheumatism, cancer, autoimmune disease, inflammation	5,854,019
p75 tumor necrosis factor receptor	autoimmune disease	5,959,094
tumor necrosis factor-α	inflammation	6,537,784
peroxisome proliferator activated receptor/IIA-1 nonpancreatic secreted phospholipase A2	inflammation	6,870,044
SOCS-3	growth disorders, autoimmune disease, inflammation	2002/0174448
SR-BI	lipid disorders	5,965,790
Ob	obesity	5,698,389
site-1 protease	obesity, diabetes	7,045,294
TIGR	glaucoma	7,138,511
VL30	anoxia	5,681,706
excitatory amino acid transporter-2	nervous system ischemia	2004/0171108
MDTS9	renal failure	2006/0014931
LIM, pyrroline 5-carboxylate reductase, SIM2	prostate disorders	2006/0134688
Bax	apoptosis	5,744,310
fas	apoptosis	5,888,764
bbc3	apoptosis	7,202,024
PINK-1	PI-3 kinase/Akt pathway disorders	2006/0228776

Table 2

Promoter Sequence	Tissue Specificity	Patent/Published Application No.
troponin T	skeletal muscle	5,266,488
myoD	muscle	5,352,595
actin	muscle	5,374,544
smooth muscle 22α	arterial smooth muscle	5,837,534
utrophin	muscle	5,972,609
myostatin	muscle	6,284,882
smooth muscle myosin heavy chain	smooth muscle	6,780,610
cardiac ankyrin repeat protein	cardiac muscle	7,193,075
MLP	muscle	2002/0042057
smoothelin	smooth muscle	2003/0157494
MYBPC3	cardiomyocytes	2004/0175699
Tα1 α-tubulin	neurons	5,661,032
intercellular adhesion molecule-4 (ICAM-4)	neurons	5,753,502
γ-aminobutyric acid type A receptor β1 subunit	hippocampus	6,066,726
neuronal nicotinic acetylcholine receptor β2-subunit	neurons	6,177,242
presenilin-1	neurons	6,255,473
calcium-calmodulin-dependent kinase IIα	forebrain	6,509,190
$CRF_{2\alpha}$ receptor	brain	7,071,323
nerve growth factor	neurons	2003/ 159159
GLP-2 receptor	gut, brain	2002/0045173
type I transglutaminase	keratinocytes	5,643,746
K14	keratinocytes	6,596,515
stearoyl-CoA desaturase	skin	2002/0151018
megsin	renal cells	6,790,617
prolactin	pituitary	5,082,779

Promoter Sequence	Tissue Specificity	Patent/Published Application No.
GDF-9	ovary, testes, hypothalamus, pituitary, placenta	7,227,013
PSP94	prostate	2003/0110522
NRL; NGAL	mammary gland	5,773,290
long whey acidic protein	mammary gland	5,831,141
mammary associated amyloid A	mammary ductal epithelial cells	2005/0107315
endothelin-1	endothelial cells	5,288,846
serglycin	hematopoietic cells	5,340,739
platelet-endothelial cell adhesion molecule-1 (PECAM-1)	platelets, leukocytes, endothelial cells	5,668,012
Tie receptor tyrosine kinase	endothelial cells, bone marrow	5,877,020
KDR/flk-1	endothelial cells	5,888,765
endoglin	endothelial cells	6,103,527
CCR5	myeloid and lymphoid cells	6,383,746
CD11d	myeloid cells	6,881,834
platelet glycoprotein IIb	hematopoietic cells	6,884,616
preproendothelin-1	endothelial cells	7,067,649
interleukin-18 binding protein	mononuclear cells	2006/0239984
CD34	hematopoietic stem cells	5,556,954
Tec tyrosine kinase	hematopoietic stem cells, liver	6,225,459

[0212] Other genes that exhibit changes in expression levels during specific diseases or disorders and therefore may provide promoters that are useful in the present invention include, without limitation, the genes (along with the associated disease/disorder) listed in Table 3.

Table 3

Gene	Disease/Disorder	Patent/Published Application No.
MLH1, MSH2, MSH6, PMS1, APC	Colorectal cancer	7,148,016
LEF-1	Colon cancer	2002/0169300
F_2 receptor	Colon cancer	2002/0187502
TGF-β type II receptor	Colon cancer	2004/0038284
EYA4	Colon cancer	2005/0003463
PCA3	Prostate cancer	7,138,235
K2	Prostate cancer	6,303,361
PROST 03	Prostate cancer metastases	2002/0009455
PCAM-1	Prostate cancer	2002/0042062
PCADM-1	Prostate cancer	2003/0100033
$PCA3_{dd3}$	Prostate cancer	2003/0165850
PCAV	Prostate cancer	2006/0275747
PAcP	Androgen-insensitive prostate cancer	2006/0294615
SEQ ID NO: 1 of the patent 5,866,329, incorporated by reference herein	Liver cancer	5,866,329
SEQ ID NOS: 1, 3 of the U.S. patent application publication 2002/0115094, incorporated by reference herein	Hepatocellular cancer	2002/0115094
SEQ ID NO: 1 of the patent U.S. application publication 2005/0037372, incorporated by reference herein	Hepatocellular carcinoma	2005/0037372
ATB_0	Hepatocellular carcinoma	2006/0280725
SEQ ID NOS: 1, 3 of the U.S. patent application publication 2007/0042420	Liver cancer	2007/0042420
CSA-1	Chondrosarcoma	2001/0016649
SEQ ID NOS: 1-15 of the U.S. patent application publication 2001/0016651, incorporated by reference herein	Pancreatic cancer	2001/0016651

Gene	Disease/Disorder	Patent/Published Application No.
SEQ ID NOS: 1-15 of the U.S. patent application publication 2003/0212264, incorporated by reference herein	Pancreatic cancer	2003/0212264
SYG972	Breast cancer	2002/0055107
Urb-ctf	Breast cancer	2003/0143546
BCU399	Breast cancer	2003/0180728
TBX2	Breast cancer	2004/0029185
Cyr61	Breast cancer	2004/0086504
DIAPH3	Breast cancer	2005/0054826
SEQ ID NOS: 1-24 of the U.S. patent application publication 2007/0134669, incorporated by reference herein	Breast cancer	2007/0134669
Human aspartyl (asparaginyl) beta-hydroxylase	CNS cancer	2002/0102263
BEHAB	CNS cancer	2003/0068661
IL-8	Kaposi's Sarcoma	2003/0096781
SEQ ID NOS: 1-278 of the U.S. patent application publication 2002/0198362, incorporated by reference herein	Hematological cancers	2002/0198362
BLSA	B-cell cancer	2003/0147887
BP1	Leukemia	2003/0171273
DAP-kinase, HOXA9	Non-small cell lung cancer	2003/0224509
ARP	Clear cell renal carcinoma, inflammatory disorders	2004/0010119
Nbk	Renal cancer	2005/0053931
CD43	Ovarian cancer	2006/0216231
SEQ ID NOS: 1-84 of the U.S. patent application publication 2007/0054268, incorporated by reference herein	Ovarian cancer	2007/0054268
β7-hcG, β6-hCG, β6e-hCG, β5-hCG, β8-hcG, β3-hCG	Uterine tumors	2006/0292567

Gene	Disease/Disorder	Patent/Published Application No.
MTA1s	Hormone insensitive cancer	2006/0204957
Old-35, Old-64	Tumor proliferation	2003/0099660
LAGE-1	Cancer	6,794,131
CIF150/hTAF$_{II}$150	Cancer	6,174,679
P65 oncofetal protein	Cancer	5,773,215
Telomerase	Cancer	2002/0025518
CYP1B1	Cancer	2002/0052013
14-3-3σ	Cancer	2002/0102245
NES1	Cancer	2002/0106367
CAR-1	Cancer	2002/0119541
HMGI, MAG	Cancer	2002/0120120
ELL2	Cancer	2002/0132329
Ephrin B2	Cancer	2002/0136726
WAF1	Cancer	2002/0142442
CIF130	Cancer	2002/0143154
C35	Cancer	2002/0155447
BMP2	Cancer	2002/0159986
BUB3	Cancer	2002/0160403
Polymerase kappa	Cancer	2003/0017573
EAG1, EAG2	Cancer	2003/0040476
SEQ ID NOS: 18, 20, 22 of the U.S. patent application publication 2003/0044813, incorporated by reference herein	Cancer	2003/0044813
HMG I	Cancer	2003/0051260
HLTF	Cancer	2003/0082526
Barx2	Cancer	2003/0087243
SEQ ID NOS: 18, 20, 22, 32, 34, 36of the U.S. patent application publication 2003/0108920, incorporated by reference herein	Cancer	2003/0108920
Cables	Cancer	2003/0109443

Gene	Disease/Disorder	Patent/Published Application No.
Pp 32r1	Cancer	2003/0129631
BMP4	Cancer	2003/0134790
TS10q23.3	Cancer	2003/0139324
Nuclear spindle-associating protein	Cancer	2003/0157072
PFTAIRE	Cancer	2003/0166217
SEMA3B	Cancer	2003/0166557
MOGp	Cancer, multiple sclerosis, inflammatory disease	2003/0166898
Fortilin	Cancer	2003/0172388
SEQ ID NO: 1 of the U.S. patent application publication 2003/0215833, incorporated by reference herein	Cancer	2003/0215833
IGFBP-3	Cancer	2004/0005294
Polyhomeotic 2	Cancer	2004/0006210
PNQALRE	Cancer	2004/0077009
SEQ ID NOS: 1, 3 of the U.S. patent application publication 2004/0086916, incorporated by reference herein	Cancer	2004/0086916
SCN5A	Cancer	2004/0146877
miR15, miR16	Cancer	2004/0152112
Headpin	Cancer	2004/0180371
PAOh1/SMO	Cancer	2004/0229241
Hippo, Mst2	Cancer	2005/0053592
PSMA-like	Cancer, neurological disorders	2005/0064504
JAB1	Cancer	2005/0069918
NF-AT	Cancer	2005/0079496
P28ING5	Cancer	2005/0097626
MTG16	Cancer	2005/0107313
ErbB-2	Cancer	2005/0123538
HDAC9	Cancer	2005/0130146
GPBP	Cancer	2005/0130227

Gene	Disease/Disorder	Patent/Published Application No.
MG20	Cancer	2005/0153352
KLF6	Cancer	2005/0181374
ARTS1	Cancer	2005/0266443
Dock 3	Cancer	2006/0041111
Annexin 8	Cancer	2006/0052320
MH15	Cancer	2006/0068411
DELTA-N p73	Cancer	2006/0088825
RapR6	Cancer	2006/099676
StarD10	Cancer	2006/0148032
Ciz1	Cancer	2006/0155113
HLJ1	Cancer	2006/0194235
RapR7	Cancer	2006/0240021
A34	Cancer	2006/0292154
Sef	Cancer	2006/0293240
Killin	Cancer	2007/0072218
SGA-1M	Cancer	2007/0128593
TGFβ Type II receptor	Cancer	2002/0064786
GCA-associated genes	Giant cell arteritis	6,743,903
PRV-1	Polycythemia vera	6,686,153
SEQ ID NOS: 2, 4 of the U.S. patent 5,948,637, incorporated by reference herein	Ischemia	5,948,637
Vezf1	Vascular disorders	2002/0023277
MLP	Dilatative cardiomyopathy	2002/0042057
VEGI	Pathological angiogenesis	2002/0111325
PRO256	Cardiovascular disorders	2002/0123091
AOP2	Atherosclerosis	2002/0142417
Remodelin	Arterial restenosis, fibrosis	2002/0161211
Phosphodiesterase 4D	Stroke	2003/0054531
Prostaglandin receptor subtype EP3	Peripheral arterial occlusive disease	2003/0157599
CARP	Heart disorders	2004/0014706

Gene	Disease/Disorder	Patent/Published Application No.
HOP	Congenital heart disease	2004/0029158
SEQ ID NOS: 1-4 of the U.S. patent application publication 2004/0087784, incorporated by reference herein	Apoplexy	2004/0087784
PLTP	Atherosclerosis, vascular disease, hypercholesterolemia, Tangier's disease, familial HDL deficiency disease	2006/0252787
SEQ ID NOS: 1, 3-8, 15, 16 of the U.S. patent application publication 2007/0160996, incorporated by reference herein	Thrombosis	2007/0160996
UCP-2	Stroke	2002/0172958
FLJ11011	Fanconi's Anemia	2006/0070134
Codanin-1	Anemia	2006/0154331
SEQ ID NOS: 1, 6, 8 of the U.S. patent 5,763,591, incorporated by reference herein	Insulin-dependent diabetes mellitus	5,763,591
Resistin	Type II diabetes	2002/0161210
Archipelin	Diabetes	2003/0202976
SEQ ID NOS: 2, 7, 16, 27 of the U.S. patent application publication 2004/0053397, incorporated by reference herein	Diabetes, hyperlipidemia	2004/0053397
Neuronatin	Metabolic disorders	2004/0259777
Ncb5or	Diabetes	2005/0031605
7B2	Endocrine disorders	2005/0086709
PTHrP, PEX	Metabolic bone diseases	2005/0113303
KChIPl	Type II diabetes	2005/0196784
SLIT-3	Type II diabetes	2006/0141462
CX3CR1	Type II diabetes	2006/0160076
SMAP-2	Diabetes	2006/0210974

Gene	Disease/Disorder	Patent/Published Application No.
SEQ ID NOS: 2, 8, 12, 16, 22, 26, 28, 32 of the U.S. patent application publication 2006/0228706, incorporated by reference herein	Type II diabetes	2006/0228706
IC-RFX	Diabetes	2006/0264611
E2IG4	Diabetes, insulin resistance, obesity	2007/0036787
SEQ ID NOS: 2, 8, 10, 14, 18, 24, 26, 30, 34, 38, 44, 50, 54, 60, 62, 68, 74, 80, 86, 92, 98, 104, 110 of the U.S. patent application publication 2007/0122802, incorporated by reference herein	Diabetes	2007/0122802
UCP2	Body weight disorders	2002/0127600
Ob receptor	Body weight disorders	2002/0182676
Ob	Bodyweight disorders	2004/0214214
Dp1	Neurodegenerative disorders	2001/0021771
NRG-1	Schizophrenia	2002/0045577
Synapsin III	Schizophrenia	2002/0064811
NRG1AG1	Schizophrenia	2002/0094954
AL-2	Neuronal disorders	2002/0142444
Proline dehydrogenase	Bipolar disorder, major depressive disorder, schizophrenia, obsessive compulsive disorder	2002/0193581
MNR2	Chronic neurodegenerative disease	2002/0197678
ATM	Ataxia-telangiectasia	2004/0029198
Ho-1	Dementing diseases	2004/0033563
CON202	Schizophrenia	2004/0091928
Ataxin-1	Neurodegenerative disorders	2004/0177388
NR3B	Motor neuron disorders	2005/0153287
NIPA-1	Hereditary spastic paraplegia	2005/0164228

Gene	Disease/Disorder	Patent/Published Application No.
DEPP, adrenomedullin, csdA	Schizophrenia	2005/0227233
Inf-20	Neurodegenerative diseases	2006/0079675
EOPA	Brain development and degeneration disorders	2007/0031830
SERT	Autism	2007/0037194
FRP-1	Glaucoma	2002/0049177
Serum amyloid A	Glaucoma	2005/0153927
BMP2	Osteoporosis	2002/0072066
BMPR1A	Juvenile polyposis	2003/0072758
ACLP	Gastroschisis	2003/0084464
Resistin-like molecule β	Familial adenomatous polyposis, diabetes, insulin resistance, colon cancer, inflammatory bowel disorder	2003/0138826
Dlg5	Inflammatory bowel disease	2006/0100132
SEQ ID NOS: 1-82 of the U.S. patent application publication 2002/0119452, incorporated by reference herein	Osteoarthritis	2002/0119452
TRANCE	Immune system disorders	2003/0185820
Matrilin-3	Osteoarthritis	2003/0203380
Synoviolin	Rheumatoid arthritis	2004/0152871
SEQ ID NOS: 9, 35 of the U.S. patent application publication 2007/0028314, incorporated by reference herein	Osteoarthritis	2007/0028314
HIV LTR	HIV infection	5,627,023
SHIVA	HIV infection	2004/0197770
EBI 1, EBI 2, EBI 3	Epstein Barr virus infection	2002/0040133
NM23 family	Skin/intestinal disorders	2002/0034741

Gene	Disease/Disorder	Patent/Published Application No.
SEQ ID NO: 1 of the U.S. patent application publication 2002/0169127, incorporated by reference herein	Psoriasis	2002/0169127
Eps8	Skin disorders, wound healing	2003/0180302
Beta-10	Thyroid gland pathology	2002/0015981
SEQ ID NO: 2 of the U.S. patent application publication 2003/0207403, incorporated by reference herein	Thyroid conditions	2003/0207403
SEQ ID NO: 3 of the U.S. patent application publication 2007/0020275, incorporated by reference herein	Thyroid disorders	2007/0020275
Hair follicle growth factor	Alopecia	2003/0036174
Corneodesmosin	Alopecia	2003/0211065
GCR9	Asthma, lymphoma, leukemia	2003/0166150
SEQ ID NO: 1-71 of the U.S. patent application publication 2004/0002084, incorporated by reference herein	Asthma	2004/0002084
Bg	Chediak-Higashi syndrome	2002/0115144
SEQ ID NOS: 1-16 of the U.S. patent application publication 2002/0127555, incorporated by reference herein	Endometriosis	2002/0127555
FGF23	Hypophosphatemic disorders	2005/0156014
BBSR	Bardet-Biedl syndrome	2003/0152963
MIC-1	Fetal abnormalities, cancer, inflammatory disorders, miscarriage, premature birth	2004/0053325
MIA-2	Liver damage	2004/0076965
IL-17B	Cartilage degenerative disorders	2004/0171109

Gene	Disease/Disorder	Patent/Published Application No.
Formylglycine generating enzyme	Multiple sulfatase deficiency	2004/0229250
LPLA2	Pulmonary alveolar proteinosis	2006/0008455
CXCL1O	Respiratory illnesses	2006/0040329
SEQ ID NOS: 1, 2 of the U.S. patent application publication 2006/0140945, incorporated by reference herein	Nephropathy	2006/0140945
HFE2A	Iron metabolism disease	2007/0166711

[0213] Once a gene with an expression pattern that is modulated during a disease, disorder, or condition is identified, the promoter of the gene may be used in the gene switch of the invention. The sequence of many genes, including the promoter region, is known in the art and available in public databases, *e.g.*, GenBank. Thus, once an appropriate gene is identified, the promoter sequence can be readily identified and obtained. Another aspect of the present invention is directed towards identifying suitable genes whose promoter can be isolated and placed into a gene switch. The identity of the gene, therefore, may not be critical to specific embodiments of the present invention, provided the promoter can be isolated and used in subsequent settings or environments. The current invention thus includes the use of promoters from genes that are yet to be identified. Once suitable genes are identified, it is a matter of routine skill or experimentation to determine the genetic sequences needed for promoter function. Indeed, several commercial protocols exist to aid in the determination of the promoter region of genes of interest. By way of example, Ding *et al.* recently elucidated the promoter sequence of the novel *Sprouty4* gene (*Am. J. Physiol. Lung Cell. Mol. Physiol. 287*: L52 (2004), which is incorporated by reference) by progressively deleting the 5′-flanking sequence of the human *Sprouty4* gene. Briefly, once the transcription initiation site was determined, PCR fragments were generated using common PCR primers to clone segments of the 5′-flanking segment in a unidirectional manner. The generated segments were cloned into a luciferase reporter vector and luciferase activity was measured to determine the promoter region of the human *Sprouty4* gene.

[0214] Another example of a protocol for acquiring and validating gene promoters includes the following steps: (1) acquire diseased and non-diseased cell/tissue samples of similar/same tissue type; (2) isolate total RNA or mRNA from the samples; (3) perform differential microarray analysis of diseased and non-diseased RNA; (4) identify candidate disease-specific transcripts; (5) identify genomic sequences associated with the disease-specific transcripts; (6) acquire or synthesize DNA sequence upstream and downstream of the predicted transcription start site of the disease-specific transcript; (7) design and produce promoter reporter vectors using different lengths of DNA from step 6; and (8) test promoter reporter vectors in diseased and non-diseased cells/tissues, as well as in unrelated cells/tissues.

[0215] The source of the promoter that is inserted into the gene switch can be natural or synthetic, and the source of the promoter should not limit the scope of the invention described herein. In other words, the promoter may be directly cloned from cells, or the promoter may have been previously cloned from a different source, or the promoter may have been synthesized.

Gene Switch Systems

[0216] The gene switch may be any gene switch that regulates gene expression by addition or removal of a specific ligand. In one embodiment, the gene switch is one in which the level of gene expression is dependent on the level of ligand that is present. Examples of ligand-dependent transcription factor complexes that may be used in the gene switches of the invention include, without limitation, members of the nuclear receptor superfamily activated by their respective ligands (*e.g.*, glucocorticoid, estrogen, progestin, retinoid, ecdysone, and analogs and mimetics thereof) and rTTA activated by tetracycline. In one aspect of the invention, the gene switch is an EcR-based gene switch. Examples of such systems include, without limitation, the systems described in U.S. Patent Nos. 6,258,603, 7,045,315, U.S. Published Patent Application Nos. 2006/0014711, 2007/0161086, and International Published Application No. WO 01/70816. Examples of chimeric ecdysone receptor systems are described in U.S. Patent No. 7,091,038, U.S. Published Patent Application Nos. 2002/0110861, 2004/0033600, 2004/0096942, 2005/0266457, and 2006/0100416, and International Published Application Nos. WO 01/70816, WO 02/066612, WO 02/066613, WO 02/066614, WO 02/066615, WO 02/29075, and WO 2005/108617, each of which is incorporated by reference in its

entirety. An example of a non-steroidal ecdysone agonist-regulated system is the RheoSwitch® Mammalian Inducible Expression System (New England Biolabs, Ipswich, MA). In another aspect of the invention, the gene switch is based on heterodimerization of FK506 binding protein (FKBP) with FKBP rapamycin associated protein (FRAP) and is regulated through rapamycin or its non-immunosuppressive analogs. Examples of such systems, include, without limitation, the ARGENT™ Transcriptional Technology (ARIAD Pharmaceuticals, Cambridge, MA) and the systems described in U.S. Patent Nos. 6,015,709, 6,117,680, 6,479,653, 6,187,757, and 6,649,595.

[0217] In one embodiment, the gene switch comprises a single transcription factor sequence encoding a ligand-dependent transcription factor complex under the control of a therapeutic switch promoter. The transcription factor sequence may encode a ligand-dependent transcription factor complex that is a naturally occurring or an artificial ligand-dependent transcription factor complex. An artificial transcription factor is one in which the natural sequence of the transcription factor has been altered, *e.g.*, by mutation of the sequence or by the combining of domains from different transcription factors. In one embodiment, the transcription factor comprises a Group H nuclear receptor ligand binding domain. In one embodiment, the Group H nuclear receptor ligand binding domain is from an ecdysone receptor, a ubiquitous receptor (UR), an orphan receptor 1 (OR-1), a steroid hormone nuclear receptor 1 (NER-1), a retinoid X receptor interacting protein–15 (RIP-15), a liver X receptor β (LXRβ), a steroid hormone receptor like protein (RLD-1), a liver X receptor (LXR), a liver X receptor α (LXRα), a farnesoid X receptor (FXR), a receptor interacting protein 14 (RIP-14), or a farnesol receptor (HRR-1). In another embodiment, the Group H nuclear receptor LBD is from an ecdysone receptor.

A. Ecdysone -based Gene Switch

[0218] The EcR and the other Group H nuclear receptors are members of the nuclear receptor superfamily wherein all members are generally characterized by the presence of an amino-terminal transactivation domain (AD, also referred to interchangeably as "TA" or "TD"), optionally fused to a heterodimerization partner (HP) to form a coactivation protein (CAP), a DNA binding domain (DBD), and a LBD fused to the DBD via a hinge region to form a ligand-dependent transcription factor (LTF). As used herein, the term "DNA binding domain" comprises a minimal polypeptide sequence of a DNA binding protein, up to the entire length of a DNA binding protein, so long as the DNA binding

domain functions to associate with a particular response element. Members of the nuclear receptor superfamily are also characterized by the presence of four or five domains: A/B, C, D, E, and in some members F (see US 4,981,784 and Evans, *Science 240*:889 (1988)). The "A/B" domain corresponds to the transactivation domain, "C" corresponds to the DNA binding domain, "D" corresponds to the hinge region, and "E" corresponds to the ligand binding domain. Some members of the family may also have another transactivation domain on the carboxy-terminal side of the LBD corresponding to "F".

[0219] The following polypeptide sequence was reported as a polypeptide sequence of Ecdysone receptor (Ecdysteroid receptor) (20-hydroxy-ecdysone receptor) (20E receptor) (EcRH) (Nuclear receptor subfamily 1 group H member 1) and has the accession number P34021 in Genbank.

[0220] Ecdysone receptor (878aa) from Drosophila melanogaster (Fruit fly) (SEQ ID NO:5)

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  1 mkrrwsnngg fmrlpeesss evtsssnglv lpsgvnmsps sldshdycdq dlwlcgnesg
 61 sfggsnghgl sqqqqsvitl amhgcsstlp aqttiiping nangnggstn gqyvpgatnl
121 galangmlng gfngmqqqiq nghglinstt pstpttplhl qqnlggaggg giggmgilhh
181 angtpnglig vvgggggvgl gvggggvggl gmqhtprsds vnsissgrdd lspssslngy
241 sanescdakk skkgpaprvq eelclvcgdr asgyhynalt cegckgffrr svtksavycc
301 kfgracemdm ymrrkcqecr lkkclavgmr pecvvpenqc amkrrekkaq kekdkmttsp
361 ssqhggngsl asgggqdfvk keildlmtce ppqhatipll pdeilakcqa rnipsltynq
421 laviykliwy qdgyeqpsee dlrrimsqpd enesqtdvsf rhiteitilt vqlivefakg
481 lpaftkipqe dqitllkacs sevmmlrmar rydhssdsif fannrsytrd sykmagmadn
541 iedllhfcrq mfsmkvdnve yalltaivif sdrpglekaq lveaiqsyyi dtlriyilnr
601 hcgdsmslvf yakllsilte lrtlgnqnae mcfslklknr klpkfleeiw dvhaippsvq
661 shlqitqeen erleraermr asvggaitag idcdsastsa aaaaaqhqpq pqpqpqpssl
721 tqndsqhqtq pqlqpqlppq lqgqlqpqlq pqlqtqlqpq iqpqpqllpv sapvpasvta
781 pgslsavsts seymggsaai gpitpattss itaavtasst tsavpmgngv gvgvgvggnv
841 smyanaqtam almgvalhsh qeqliggvav ksehstta
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[0221] The DBD is characterized by the presence of two cysteine zinc fingers between which are two amino acid motifs, the P-box and the D-box, which confer specificity for response elements. These domains may be either native, modified, or chimeras of different domains of heterologous receptor proteins. The EcR, like a subset of the nuclear receptor family, also possesses less well-defined regions responsible for heterodimerization properties. Because the domains of nuclear receptors are modular in nature, the LBD, DBD, and AD may be interchanged.

[0222] In another embodiment, the transcription factor comprises a AD, a DBD that recognizes a response element associated with the therapeutic protein or therapeutic polynucleotide whose expression is to be modulated; and a Group H nuclear receptor

LBD. In certain embodiments, the Group H nuclear receptor LBD comprises a substitution mutation.

[0223] In another embodiment, the gene switch comprises a first transcription factor sequence, *e.g.*, a CAP, under the control of a first therapeutic switch promoter (TSP-1) and a second transcription factor sequence, *e.g.*, a LTF, under the control of a second therapeutic switch promoter (TSP-2), wherein the proteins encoded by said first transcription factor sequence and said second transcription factor sequence interact to form a protein complex (LDTFC), *i.e.*, a "dual switch"- or "two-hybrid"-based gene switch. The first and second TSPs may be the same or different. In this embodiment, the presence of two different TSPs in the gene switch that are required for therapeutic molecule expression enhances the specificity of the therapeutic method (see Figure 2). Figure 2 also demonstrates the ability to modify the therapeutic gene switch to treat any disease, disorder, or condition simply by inserting the appropriate TSPs.

[0224] In a further embodiment, both the first and the second transcription factor sequence, *e.g.*, a CAP or a LTF, are under the control of a single therapeutic switch promoter (*e.g.* TSP-1 in Figure 1). Activation of this promoter will generate both CAP and LTF with a single open reading frame. This can be achieved with the use of a transcriptional linker such as an IRES (internal ribosomal entry site). In this embodiment, both portions of the ligand-dependent transcription factor complex are synthesized upon activation of TSP-1. TSP-1 can be a constitutive promoter or only activated under conditions associated with the disease, disorder, or condition.

[0225] In a further embodiment, one transcription factor sequence, *e.g.* a LTF, is under the control of a therapeutic switch promoter only activated under conditions associated with the disease, disorder, or condition (*e.g.*, TSP-2 or TSP-3 in Figure 4) and the other transcription factor sequence, *e.g.*, CAP, is under the control of a constitutive therapeutic switch promoter (*e.g.*, TSP-1 in Figure 4). In this embodiment, one portion of the ligand-dependent transcription factor complex is constitutively present while the second portion will only be synthesized under conditions associated with the disease, disorder, or condition.

[0226] In another embodiment, one transcription factor sequence, *e.g.*, CAP, is under the control of a first TSP (*e.g.*, TSP-1 in Figure 3) and two or more different second transcription factor sequences, *e.g.*, LTF-1 and LTF-2 are under the control of different

TSPs (*e.g.*, TSP-2 and TSP-3 in Figure 3). In this embodiment, each of the LTFs may have a different DBD that recognizes a different factor-regulated promoter sequence (*e.g.*, DBD-A binds to a response element associated with factor-regulated promoter-1 (FRP-1) and DBD-B binds to a response element associated with factor-regulated promoter-2 (FRP-2). Each of the factor-regulated promoters may be operably linked to a different therapeutic gene. In this manner, multiple treatments may be provided simultaneously.

[0227] In one embodiment, the first transcription factor sequence encodes a polypeptide comprising a AD, a DBD that recognizes a response element associated with the therapeutic product sequence whose expression is to be modulated; and a Group H nuclear receptor LBD, and the second transcription factor sequence encodes a transcription factor comprising a nuclear receptor LBD selected from a vertebrate retinoid X receptor (RXR), an invertebrate RXR, an ultraspiracle protein (USP), or a chimeric nuclear receptor comprising at least two different nuclear receptor ligand binding domain polypeptide fragments selected from a vertebrate RXR, an invertebrate RXR, and a USP (see WO 01/70816 A2 and US 2004/0096942 A1). The "partner" nuclear receptor ligand binding domain may further comprise a truncation mutation, a deletion mutation, a substitution mutation, or another modification.

[0228] In another embodiment, the gene switch comprises a first transcription factor sequence encoding a first polypeptide comprising a nuclear receptor LBD and a DBD that recognizes a response element associated with the therapeutic product sequence whose expression is to be modulated, and a second transcription factor sequence encoding a second polypeptide comprising an AD and a nuclear receptor LBD, wherein one of the nuclear receptor LBDs is a Group H nuclear receptor LBD. In a preferred embodiment, the first polypeptide is substantially free of an AD and the second polypeptide is substantially free of a DBD. For purposes of the invention, "substantially free" means that the protein in question does not contain a sufficient sequence of the domain in question to provide activation or binding activity.

[0229] In another aspect of the invention, the first transcription factor sequence encodes a protein comprising a heterodimerization partner and an AD (a "CAP") and the second transcription factor sequence encodes a protein comprising a DBD and a LBD (a "LTF").

[0230] When only one nuclear receptor LBD is a Group H LBD, the other nuclear receptor LBD may be from any other nuclear receptor that forms a dimer with the Group

H LBD. For example, when the Group H nuclear receptor LBD is an EcR LBD, the other nuclear receptor LBD "partner" may be from an EcR, a vertebrate RXR, an invertebrate RXR, an ultraspiracle protein (USP), or a chimeric nuclear receptor comprising at least two different nuclear receptor LBD polypeptide fragments selected from a vertebrate RXR, an invertebrate RXR, or a USP (see WO 01/70816 A2, International Patent Application No. PCT/US02/05235 and US 2004/0096942 A1, incorporated herein by reference in their entirety). The "partner" nuclear receptor ligand binding domain may further comprise a truncation mutation, a deletion mutation, a substitution mutation, or another modification.

[0231] In one embodiment, the vertebrate RXR LBD is from a human *Homo sapiens*, mouse *Mus musculus*, rat *Rattus norvegicus*, chicken *Gallus gallus*, pig *Sus scrofa domestica*, frog *Xenopus laevis*, zebrafish *Danio rerio*, tunicate *Polyandrocarpa misakiensis*, or jellyfish *Tripedalia cysophora* RXR.

[0232] In one embodiment, the invertebrate RXR ligand binding domain is from a locust *Locusta migratoria* ultraspiracle polypeptide ("LmUSP"), an ixodid tick *Amblyomma americanum* RXR homolog 1 ("AmaRXR1"), an ixodid tick *Amblyomma americanum* RXR homolog 2 ("AmaRXR2"), a fiddler crab *Celuca pugilator* RXR homolog ("CpRXR"), a beetle *Tenebrio molitor* RXR homolog ("TmRXR"), a honeybee *Apis mellifera* RXR homolog ("AmRXR"), an aphid *Myzus persicae* RXR homolog ("MpRXR"), or a non-Dipteran/non-Lepidopteran RXR homolog.

[0233] In one embodiment, the chimeric RXR LBD comprises at least two polypeptide fragments selected from a vertebrate species RXR polypeptide fragment, an invertebrate species RXR polypeptide fragment, or a non-Dipteran/non-Lepidopteran invertebrate species RXR homolog polypeptide fragment. A chimeric RXR ligand binding domain for use in the present invention may comprise at least two different species RXR polypeptide fragments, or when the species is the same, the two or more polypeptide fragments may be from two or more different isoforms of the species RXR polypeptide fragment.

[0234] In one embodiment, the chimeric RXR ligand binding domain comprises at least one vertebrate species RXR polypeptide fragment and one invertebrate species RXR polypeptide fragment.

[0235] In another embodiment, the chimeric RXR ligand binding domain comprises at least one vertebrate species RXR polypeptide fragment and one non-Dipteran/non-Lepidopteran invertebrate species RXR homolog polypeptide fragment.

[0236] The ligand, when combined with the LBD of the nuclear receptor(s), which in turn are bound to the response element of a FRP associated with a therapeutic product sequence, provides external temporal regulation of expression of the therapeutic product sequence. The binding mechanism or the order in which the various components of this invention bind to each other, that is, for example, ligand to LBD, DBD to response element, AD to promoter, *etc.*, is not critical.

[0237] In a specific example, binding of the ligand to the LBD of a Group H nuclear receptor and its nuclear receptor LBD partner enables expression of the therapeutic product sequence. This mechanism does not exclude the potential for ligand binding to the Group H nuclear receptor (GHNR) or its partner, and the resulting formation of active homodimer complexes (*e.g.* GHNR + GHNR or partner + partner). Preferably, one or more of the receptor domains is varied producing a hybrid gene switch. Typically, one or more of the three domains, DBD, LBD, and AD, may be chosen from a source different than the source of the other domains so that the hybrid genes and the resulting hybrid proteins are optimized in the chosen host cell or organism for transactivating activity, complementary binding of the ligand, and recognition of a specific response element. In addition, the response element itself can be modified or substituted with response elements for other DNA binding protein domains such as the GAL-4 protein from yeast (see Sadowski *et al.*, *Nature 335*:563 (1988)) or LexA protein from *Escherichia coli* (see Brent *et al.*, *Cell 43*:729 (1985)), or synthetic response elements specific for targeted interactions with proteins designed, modified, and selected for such specific interactions (see, for example, Kim *et al.*, *Proc. Natl. Acad. Sci. USA*, *94*:3616 (1997)) to accommodate hybrid receptors. Another advantage of two-hybrid systems is that they allow choice of a promoter used to drive the gene expression according to a desired end result. Such double control may be particularly important in areas of gene therapy, especially when cytotoxic proteins are produced, because both the timing of expression as well as the cells wherein expression occurs may be controlled. When genes, operably linked to a suitable promoter, are introduced into the cells of the subject, expression of the exogenous genes is controlled by the presence of the system of this invention. Promoters

may be constitutively or inducibly regulated or may be tissue-specific (that is, expressed only in a particular type of cells) or specific to certain developmental stages of the organism.

[0238] The DNA binding domain of the first hybrid protein binds, in the presence or absence of a ligand, to the DNA sequence of a response element to initiate or suppress transcription of downstream gene(s) under the regulation of this response element.

[0239] The functional LDTFC, *e.g.*, an EcR complex, may also include additional protein(s) such as immunophilins. Additional members of the nuclear receptor family of proteins, known as transcriptional factors (such as DHR38 or *betaFTZ-1*), may also be ligand dependent or independent partners for EcR, USP, and/or RXR. Additionally, other cofactors may be required such as proteins generally known as coactivators (also termed adapters or mediators). These proteins do not bind sequence-specifically to DNA and are not involved in basal transcription. They may exert their effect on transcription activation through various mechanisms, including stimulation of DNA-binding of activators, by affecting chromatin structure, or by mediating activator-initiation complex interactions. Examples of such coactivators include RIP140, TIF1, RAP46/Bag-1, ARA70, SRC-1/NCoA-1, TIF2/GRIP/NCoA-2, ACTR/AIB1/RAC3/pCIP as well as the promiscuous coactivator C response element B binding protein, CBP/p300 (for review see Glass *et al.*, *Curr. Opin. Cell Biol.* 9:222 (1997)). Also, protein cofactors generally known as corepressors (also known as repressors, silencers, or silencing mediators) may be required to effectively inhibit transcriptional activation in the absence of ligand. These corepressors may interact with the unliganded EcR to silence the activity at the response element. Current evidence suggests that the binding of ligand changes the conformation of the receptor, which results in release of the corepressor and recruitment of the above described coactivators, thereby abolishing their silencing activity. Examples of corepressors include N-CoR and SMRT (for review, see Horwitz *et al.*, *Mol Endocrinol. 10*:1167 (1996)). These cofactors may either be endogenous within the cell or organism, or may be added exogenously as transgenes to be expressed in either a regulated or unregulated fashion.

B. Rapamycin based Gene Switch

[0240] The present invention further provides a gene switch system which utilizes FK506 binding protein as the ligand-dependent transcription factor complex and rapamycin as the ligand. In one embodiment, the construct encoding the gene switch comprises

(a) a first polynucleotide encoding a first chimeric protein which binds to rapamycin or an analog thereof and which comprises at least one *FK506*-binding protein (FKBP) domain and at least one protein domain heterologous thereto, wherein the FKBP domain comprises a peptide sequence selected from:

(1) a naturally occurring FKBP

(2) a variant of a naturally occurring FKBP in which up to 10 amino acid residues have been deleted, inserted, or replaced with substitute amino acids, and

(3) an FKBP encoded by a DNA sequence which selectively hybridizes to a DNA sequence encoding an FKBP of (1) or (2);

(b) a second polynucleotide encoding a second chimeric protein which forms a complex with both (a) rapamycin or a rapamycin analog and (b) the first chimeric protein, and which comprises at least one FKBP:rapamycin binding (FRB) domain and at least one protein domain heterologous thereto, wherein the FRB domain comprises a peptide sequence selected from:

(4) a naturally occurring FRB domain,

(5) a variant of a naturally occuring FRB domain in which up to 10 amino acid residues have been deleted, inserted, or replaced with substitute amino acids, and

(6) an FRB domain encoded by a DNA sequence which selectively hybridizes to a DNA sequence encoding an FRB of (4) or (5).

[0241] In this gene switch system, each of the first polynucleotide and the second polynucleotide are under the control of one or more therapeutic switch promoters as

described elsewhere herein. Furthermore, in certain embodiments, at least one protein domain heterologous to the FKBP and/or FRB domains in the first and second chimeric protein may be one or more "action" or "effector" domains. Effector domains may be selected from a wide variety of protein domains including DNA binding domains, transcription activation domains, cellular localization domains and signaling domains (*i.e.*, domains which are capable upon clustering or multimerization, of triggering cell growth, proliferation, differentiation, apoptosis, gene transcription, etc.).

[0242] In certain embodiments, one fusion protein contains at least one DNA binding domain (*e.g.*, a GAL4 or ZFHD1 DNA-binding domain) and another fusion protein contains at least one transcription activation domain (*e.g.*, a VP16 or p65 transcription activation domain). Ligand-mediated association of the fusion proteins represents the formation of a transcription factor complex and leads to initiation of transcription of a target gene linked to a DNA sequence recognized by (*i.e.*, capable of binding with) the DNA-binding domain on one of the fusion proteins. Information regarding the gene expression system as well as the ligand is disclosed in U.S. Patent Nos. 6,187,757 B1, 6,649,595 B1, 6,509,152 B1, 6,479,653 B1, and 6,117,680 B1.

[0243] In other embodiments, the present invention provides a gene switch system which comprises polynucleotides encoding two fusion proteins which self-aggregate in the absence of a ligand, wherein (a) the first fusion protein comprises a conditional aggregation domain which binds to a selected ligand and a transcription activation domain, and (b) the second fusion protein comprising a conditional aggregation domain which binds to a selected ligand and a DNA binding domain, and (c) in the absence of ligand, the cells express a gene operably linked to regulatory DNA to which said DNA binding domain binds. Modified cells comprising the gene switch system are expanded in the presence of the ligand in an amount sufficient for repression of the gene. Ligand removal induces expression of the encoded protein that causes cell death. The nucleic acids encoding the two fusion proteins are under the control of at least one conditional promoter. The gene expression system utilizing conditional aggregation domains is disclosed in U.S. Publication No. 2002/0048792.

C. Procaryotic Repressor/ Operator based Gene Switch System

[0244] In one embodiment, the present invention provides gene switch system comprising (a) a first polynucleotide coding for a transactivator fusion protein comprising

a prokaryotic tetracycline ("tet") repressor and a eucaryotic transcriptional activator protein domain; and (b) a second polynucleotide coding for a therapeutic protein or therapeutic polypeptide, wherein said second polynucleotide is operably linked to a minimal promoter and at least one tet operator sequence. The first polynucleotide coding for a transactivator fusion protein may comprise therapeutic switch promoter as described elsewhere herein. The expression of the lethal protein is up-regulated in the absence of tetracycline. (*see, e.g.*, Gossen *et al.* (1992) *Proc. Natl. Acad. Sci.* 89: 5547-5551; Gossen *et al.* (1993) *TIBS* 18 : 471-475; Furth *et al.* (1994) *Proc. Natl. Acad. Sci.* 91: 9302-9306; and Shockett *et al.* (1995) *Proc. Natl. Acad. Sci.* 92: 6522-6526). The TetO expression system is disclosed in U.S. Patent No. 5,464,758 B1.

[0245] In another embodiment, the gene switch system comprises the lactose ("Lac") repressor-operator systems from the bacterium *Escherichia coli*. The gene switch system of the present invention may also comprise (a) a first polynucleotide coding for a transactivator fusion protein comprising a prokaryotic lac I repressor and a eucaryotic transcriptional activator protein domain; and (b) a second polynucleotide coding for a therapeutic protein or therapeutic polypeptide, wherein said second polynucleotide is operably linked to a therapeutic switch promoter. In the Lac system, a lac operon is inactivated in the absence of lactose, or synthetic analogs such as isopropyl-b-D-thiogalactoside.

[0246] Additional gene switch systems include those described in the following: US 7,091,038; WO2004078924; EP1266015; US20010044151; US20020110861; US20020119521; US20040033600; US20040197861; US20040235097; US20060020146; US20040049437; US20040096942; US20050228016; US20050266457; US20060100416; WO2001/70816; WO2002/29075; WO2002/066612; WO2002/066613; WO2002/066614; WO2002/066615; WO2005/108617; US 6,258,603; US20050209283; US20050228016; US20060020146; EP0965644; US 7,304,162; US 7,304,161; MX234742; KR10-0563143; AU765306; AU2002-248500; and AU2002-306550.

D. Combination of the Gene Switch Systems

[0247] The present invention provides nucleic acid compositions, modified cells, and bioreactors comprising two or more gene switch systems comprising different ligand-dependent transcription factor complexes which are activated by an effective amount of

one or more ligands, wherein the two or more gene switch systems comprise a first gene switch and a second gene switch, both of which selectively induce expression of one or more therapeutic polypeptides or therapeutic polynucleotides, upon binding to one or more ligands. Within the scope of the present invention are any numbers of and/or combinations of gene switch systems.

[0248] In one embodiment, the present invention provides a nucleic acid composition comprising:

a. a first gene switch system which comprises:

i. a first gene expression cassette comprising a polynucleotide encoding a first hybrid polypeptide which comprises:

1. a transactivation domain, which activates a factor-regulated promoter operably associated with a polynucleotide encoding a therapeutic polypeptide or therapeutic polynucleotide; and

2. a heterodimer partner domain,

ii. a second gene expression cassette comprising a polynucleotide encoding a second hybrid polypeptide which comprises:

1. a DNA-binding domain, which recognizes a factor-regulated promoter operably associated with a polynucleotide encoding a therapeutic polypeptide or therapeutic polynucleotide; and

2. a ligand binding domain; and

iii. a third gene expression cassette comprising a polynucleotide encoding a therapeutic polypeptide or therapeutic polynucleotide comprising:

1. a factor-regulated promoter, which is activated by the transactivation domain of the second hybrid polypeptide; and,

2. a polynucleotide encoding a therapeutic polypeptide or therapeutic polynucleotide, and

b. a second gene expression system which comprises:

i. a first gene expression cassette comprising a polynucleotide encoding a first hybrid polypeptide which comprises:

1. a transactivation domain, which activates a factor-regulated promoter operably associated with a polynucleotide encoding a therapeutic polypeptide or therapeutic polynucleotide; and

2. a heterodimer partner domain,

ii. a second gene expression cassette comprising a polynucleotide encoding a second hybrid polypeptide which comprises:

1. a DNA-binding domain, which recognizes a factor-regulated promoter operably associated with a polynucleotide encoding a therapeutic polypeptide or therapeutic polynucleotide; and

2. a ligand binding domain; and

iii. a third gene expression cassette comprising a polynucleotide encoding a therapeutic polypeptide or therapeutic polynucleotide comprising:

1. a factor-regulated promoter, which is activated by the transactivation domain of the second hybrid polypeptide; and,

2. a polynucleotide encoding a therapeutic polypeptide or therapeutic polynucleotide.

[0249] The multiple inducible gene expression systems provide for expression of a given therapeutic polynucleotide or therapeutic polypeptide under conditions associated with different diseases, disorders or conditions, or expression of multiple therapeutic polypeptides or therapeutic polynucleotides either under the same conditions associated with the same disease disorder or condition, or under different conditions associated with different diseases, disorders, or conditions.

[0250] In certain embodiments, the combination of two or more gene switch systems may be (1) a dual-switch ecdysone receptor based gene expression system and (2) a single-switch ecdysone receptor based gene switch. In other embodiments, the combination may

be (1) an single- or dual-switch ecdysone receptor based gene switch and (2) a rapamycin based gene switch. Alternatively, the combination of gene switch systems may be two identical rapamycin based gene switch systems disclosed above. Any possible combinations of the gene switch systems are within the scope of the invention.

Ligands

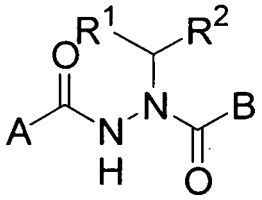
[0251] As used herein, the term "ligand," as applied to LDTFC-based gene switches *e.g.*, EcD complex based gene switches, describes small and soluble molecules having the capability of activating a gene switch to stimulate expression of a polypeptide encoded therein. The ligand for a ligand-dependent transcription factor complex of the invention binds to the protein complex comprising one or more of the ligand binding domain, the heterodimer partner domain, the DNA binding domain, and the transactivation domain. The choice of ligand to activate the ligand-dependent transcription factor complex depends on the type of the gene switch utilized.

[0252] Examples of ligands include, without limitation, an ecdysteroid, such as ecdysone, 20-hydroxyecdysone, ponasterone A, muristerone A, and the like, 9-cis-retinoic acid, synthetic analogs of retinoic acid, N,N'-diacylhydrazines such as those disclosed in U.S. Patent Nos. 6,013,836; 5,117,057; 5,530,028; and 5,378,726 and U.S. Published Application Nos. 2005/0209283 and 2006/0020146; oxadiazolines as described in U.S. Published Application No. 2004/0171651; dibenzoylalkyl cyanohydrazines such as those disclosed in European Application No. 461,809; N-alkyl-N,N'-diaroylhydrazines such as those disclosed in U.S. Patent No. 5,225,443; N-acyl-N-alkylcarbonylhydrazines such as those disclosed in European Application No. 234,994; N-aroyl-N-alkyl-N'-aroylhydrazines such as those described in U.S. Patent No. 4,985,461; amidoketones such as those described in U.S. Published Application No. 2004/0049037; each of which is incorporated herein by reference and other similar materials including 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide, 8-O-acetylharpagide, oxysterols, 22(R) hydroxycholesterol, 24(S) hydroxycholesterol, 25-epoxycholesterol, T0901317, 5-alpha-6-alpha-epoxycholesterol-3-sulfate (ECHS), 7-ketocholesterol-3-sulfate, famesol, bile acids, 1,1-biphosphonate esters, juvenile hormone III, and the like. Examples of diacylhydrazine ligands useful in the present invention include RG-115819 (3,5-Dimethyl-benzoic acid N-(1-ethyl-2,2-dimethyl-propyl)-N'-(2-methyl-3-methoxy-benzoyl)-hydrazide), RG-115932 ((*R*)-3,5-Dimethyl-benzoic acid N-(1-*tert*-butyl-butyl)-

N'-(2-ethyl-3-methoxy-benzoyl)-hydrazide), and RG-115830 (3,5-Dimethyl-benzoic acid N-(1-*tert*-butyl-butyl)-N'-(2-ethyl-3-methoxy-benzoyl)-hydrazide). See, *e.g.*, U.S. Patent Appl. Serial No. 12/155,111, and PCT Appl. No. PCT/US2008/006757, both of which are incorporated herein by reference in their entireties.

[0253] For example, a ligand for the edysone receptor based gene switch may be selected from any suitable ligands. Both naturally occurring ecdysone or ecdyson analogs (*e.g.*, 20-hydroxyecdysone, muristerone A, ponasterone A, ponasterone B, ponasterone C, 26-iodoponasterone A, inokosterone or 26-mesylinokosterone) and non-steroid inducers may be used as a ligand for gene switch of the present invention. U.S. Patent No. 6,379,945 B1, describes an insect steroid receptor isolated from *Heliothis virescens* ("HEcR") which is capable of acting as a gene switch responsive to both steroid and certain non-steroidal inducers. Non-steroidal inducers have a distinct advantage over steroids, in this and many other systems which are responsive to both steroids and non-steroid inducers, for a number of reasons including, for example: lower manufacturing cost, metabolic stability, absence from insects, plants, or mammals, and environmental acceptability. U.S. Patent No. 6,379,945 B1 describes the utility of two dibenzoylhydrazines, 1,2-dibenzoyl-1-tert-butyl-hydrazine and tebufenozide (N-(4-ethylbenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butyl-hydrazine) as ligands for an ecdysone-based gene switch. Also included in the present invention as a ligand are other dibenzoylhydrazines, such as those disclosed in U.S. Pat. No. 5,117,057 B1. Use of tebufenozide as a chemical ligand for the ecdysone receptor from *Drosophila melanogaster* is also disclosed in U.S. Patent No. 6,147,282. Additional, non-limiting examples of ecdysone ligands are 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide, 8-O-acetylharpagide, a 1,2-diacyl hydrazine, an N'-substituted-N,N'-disubstituted hydrazine, a dibenzoylalkyl cyanohydrazine, an N-substituted-N-alkyl-N,N-diaroyl hydrazine, an N-substituted-N-acyl-N-alkyl, carbonyl hydrazine or an N-aroyl-N'-alkyl-N'-aroyl hydrazine. (*See* U.S. Patent No. 6,723,531).

[0254] In one embodiment, the ligand for an ecdysone based gene switch system is a diacylhydrazine ligand or chiral diacylhydrazine ligand. The ligand used in the gene switch system may be compounds of Formula I



Formula I

wherein

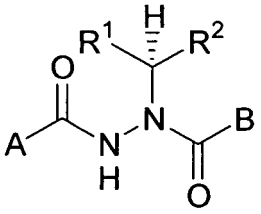
A is alkoxy, arylalkyloxy or aryloxy;

B is optionally substituted aryl or optionally substituted heteroaryl; and

R^1 and R^2 are independently optionally substituted alkyl, arylalkyl, hydroxyalkyl, haloalkyl, optionally substituted cycloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted heterocyclo, optionally substituted aryl or optionally substituted heteroaryl;

or pharmaceutically acceptable salts, hydrates, crystalline forms or amorphous forms thereof.

[0255] In another embodiment, the ligand may be enantiomerically enriched compounds of Formula II



Formula II

wherein

A is alkoxy, arylalkyloxy, aryloxy, arylalkyl, optionally substituted aryl or optionally substituted heteroaryl;

B is optionally substituted aryl or optionally substituted heteroaryl; and

R^1 and R^2 are independently optionally substituted alkyl, arylalkyl, hydroxyalkyl,

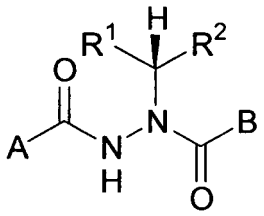
haloalkyl, optionally substituted cycloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted heterocyclo, optionally substituted aryl or optionally substituted heteroaryl;

with the proviso that R^1 does not equal R^2;

wherein the absolute configuration at the asymmetric carbon atom bearing R^1 and R^2 is predominantly *S*;

or pharmaceutically acceptable salts, hydrates, crystalline forms or amorphous forms thereof.

[0256] In certain embodiments, the ligand may be enantiomerically enriched compounds of Formula III



Formula III

wherein

A is alkoxy, arylalkyloxy, aryloxy, arylalkyl, optionally substituted aryl or optionally substituted heteroaryl;

B is optionally substituted aryl or optionally substituted heteroaryl; and

R^1 and R^2 are independently optionally substituted alkyl, arylalkyl, hydroxyalkyl, haloalkyl, optionally substituted cycloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted heterocyclo, optionally substituted aryl or optionally substituted heteroaryl;

with the proviso that R^1 does not equal R^2;

wherein the absolute configuration at the asymmetric carbon atom bearing R^1 and R^2 is predominantly *R*;

or pharmaceutically acceptable salts, hydrates, crystalline forms or amorphous forms thereof.

[0257] In one embodiment, a ligand may be *(R)*-3,5-dimethyl-benzoic acid N-(1-*tert*-butyl-butyl)-N'-(2-ethyl-3-methoxy-benzoyl)-hydrazide having an enantiomeric excess of at least 95% or a pharmaceutically acceptable salt, hydrate, crystalline form or amorphous form thereof.

[0258] The diacylhydrazine ligands of Formula I and chiral diacylhydrazine ligands of Formula II or III, when used with an ecdysone-based gene switch system, provide the means for external temporal regulation of expression of a therapeutic polypeptide or therapeutic polynucleotide of the present invention. See U.S. Appl. No. 12/155,111, filed May 29, 2008, which is fully incorporated by reference herein.

[0259] The ligands used in the present invention may form salts. The term "salt(s)" as used herein denotes acidic and/or basic salts formed with inorganic and/or organic acids and bases. In addition, when a compound of Formula I, II or III contains both a basic moiety and an acidic moiety, zwitterions ("inner salts") may be formed and are included within the term "salt(s)" as used herein. Pharmaceutically acceptable (*i.e.*, non-toxic, physiologically acceptable) salts are used, although other salts are also useful, *e.g.*, in isolation or purification steps which may be employed during preparation. Salts of the compounds of Formula I, II or III may be formed, for example, by reacting a compound with an amount of acid or base, such as an equivalent amount, in a medium such as one in which the salt precipitates or in an aqueous medium followed by lyophilization.

[0260] The ligands which contain a basic moiety may form salts with a variety of organic and inorganic acids. Exemplary acid addition salts include acetates (such as those formed with acetic acid or trihaloacetic acid, for example, trifluoroacetic acid), adipates, alginates, ascorbates, aspartates, benzoates, benzenesulfonates, bisulfates, borates, butyrates, citrates, camphorates, camphorsulfonates, cyclopentanepropionates, digluconates, dodecylsulfates, ethanesulfonates, fumarates, glucoheptanoates, glycerophosphates, hemisulfates, heptanoates, hexanoates, hydrochlorides (formed with hydrochloric acid), hydrobromides (formed with hydrogen bromide), hydroiodides, 2-hydroxyethanesulfonates, lactates, maleates (formed with maleic acid), methanesulfonates (formed with methanesulfonic acid), 2-naphthalenesulfonates, nicotinates, nitrates, oxalates, pectinates, persulfates, 3-phenylpropionates, phosphates, picrates, pivalates,

propionates, salicylates, succinates, sulfates (such as those formed with sulfuric acid), sulfonates (such as those mentioned herein), tartrates, thiocyanates, toluenesulfonates such as tosylates, undecanoates, and the like.

[0261] The ligands which contain an acidic moiety may form salts with a variety of organic and inorganic bases. Exemplary basic salts include ammonium salts, alkali metal salts such as sodium, lithium, and potassium salts, alkaline earth metal salts such as calcium and magnesium salts, salts with organic bases (for example, organic amines) such as benzathines, dicyclohexylamines, hydrabamines (formed with N,N-bis(dehydroabietyl)ethylenediamine), N-methyl-D-glucamines, N-methyl-D-glucamides, t-butyl amines, and salts with amino acids such as arginine, lysine and the like.

[0262] Non-limiting examples of the ligands for the inducible gene expression system utilizing the FK506 binding domain are FK506, Cyclosporin A, or Rapamycin. FK506, rapamycin, and their analogs are disclosed in U.S. Patent Nos. 6,649,595 B2 and 6,187,757. *See also* U.S. Patent Nos. 7,276,498 and 7,273,874.

[0263] The ligands described herein may be administered alone or as part of a pharmaceutical composition comprising a pharmaceutically acceptable carrier. In one embodiment, the pharmacetical compoistion are in the form of solutions, suspensions, tablets, capsules, ointments, elixirs, or injectable compositions.

[0264] The term "ecdysone receptor-based," with respect to a gene switch, refers to a gene switch comprising at least a functional part of a naturally occurring or synthetic ecdysone receptor ligand binding domain and which regulates gene expression in response to a ligand that binds to the ecdysone receptor ligand binding domain. Examples of ecdysone-responsive systems are described in U.S. Patent Nos. 7,091,038 and 6,258,603. In one embodiment, the system is the RheoSwitch® Therapeutic System (RTS), which contains two fusion proteins, the DEF domains of a mutagenized ecdysone receptor (EcR) fused with a Gal4 DNA binding domain and the EF domains of a chimeric RXR fused with a VP16 transcription activation domain, expressed under a constitutive promoter as illustrated in FIG. 1.

[0265] The terms “modulate” and “modulates” mean to induce, reduce or inhibit nucleic acid or gene expression, resulting in the respective induction, reduction or inhibition of protein or polypeptide production.

[0266] The polynucleotides or vectors according to the invention may further comprise at least one promoter suitable for driving expression of a gene in a host cell.

[0267] Enhancers that may be used in embodiments of the invention include but are not limited to: an SV40 enhancer, a cytomegalovirus (CMV) enhancer, an elongation factor 1 (EF1) enhancer, yeast enhancers, viral gene enhancers, and the like.

[0268] Termination control regions, *i.e.*, terminator or polyadenylation sequences, may also be derived from various genes native to the preferred hosts. Optionally, a termination site may be unnecessary, however, it is most preferred if included. In one embodiment of the invention, the termination control region may be comprised or be derived from a synthetic sequence, synthetic polyadenylation signal, an SV40 late polyadenylation signal, an SV40 polyadenylation signal, a bovine growth hormone (BGH) polyadenylation signal, viral terminator sequences, or the like.

[0269] The terms "3' non-coding sequences" or "3' untranslated region (UTR)" refer to DNA sequences located downstream (3') of a coding sequence and may comprise polyadenylation [poly(A)] recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

[0270] "Regulatory region" refers to a nucleic acid sequence that regulates the expression of a second nucleic acid sequence. A regulatory region may include sequences which are naturally responsible for expressing a particular nucleic acid (a homologous region) or may include sequences of a different origin that are responsible for expressing different proteins or even synthetic proteins (a heterologous region). In particular, the sequences can be sequences of prokaryotic, eukaryotic, or viral genes or derived sequences that stimulate or repress transcription of a gene in a specific or non-specific manner and in an inducible or non-inducible manner. Regulatory regions include origins of replication, RNA splice sites, promoters, enhancers, transcriptional termination sequences, and signal sequences which direct the polypeptide into the secretory pathways of the target cell.

[0271] A regulatory region from a "heterologous source" refers to a regulatory region that is not naturally associated with the expressed nucleic acid. Included among the heterologous regulatory regions are regulatory regions from a different species, regulatory regions from a different gene, hybrid regulatory sequences, and regulatory sequences

which do not occur in nature, but which are designed by one having ordinary skill in the art.

[0272] "RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from post-transcriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA (mRNA)" refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a double-stranded DNA that is complementary to and derived from mRNA. "Sense" RNA refers to RNA transcript that includes the mRNA and so can be translated into protein by the cell. "Antisense RNA" refers to a RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene. The complementarity of an antisense RNA may be with any part of the specific gene transcript, *i.e.*, at the 5' non-coding sequence, 3' non-coding sequence, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that is not translated yet has an effect on cellular processes.

[0273] "Polypeptide," "peptide" and "protein" are used interchangeably and refer to a polymeric compound comprised of covalently linked amino acid residues.

[0274] An "isolated polypeptide," "isolated peptide" or "isolated protein" refer to a polypeptide or protein that is substantially free of those compounds that are normally associated therewith in its natural state (*e.g.*, other proteins or polypeptides, nucleic acids, carbohydrates, lipids). "Isolated" is not meant to exclude artificial or synthetic mixtures with other compounds, or the presence of impurities which do not interfere with biological activity, and which may be , for example, due to incomplete purification, addition of stabilizers, or compounding into a pharmaceutically acceptable preparation.

[0275] A "substitution mutant polypeptide" or a "substitution mutant" will be understood to mean a mutant polypeptide comprising a substitution of at least one wild-type or naturally occurring amino acid with a different amino acid relative to the wild-type or naturally occurring polypeptide. A substitution mutant polypeptide may comprise only one wild-type or naturally occurring amino acid substitution and may be referred to as a "point mutant" or a "single point mutant" polypeptide. Alternatively, a substitution mutant polypeptide may comprise a substitution of two or more wild-type or naturally

occurring amino acids with two or more amino acids relative to the wild-type or naturally occurring polypeptide. According to the invention, a Group H nuclear receptor ligand binding domain polypeptide comprising a substitution mutation comprises a substitution of at least one wild-type or naturally occurring amino acid with a different amino acid relative to the wild-type or naturally occurring Group H nuclear receptor ligand binding domain polypeptide.

[0276] When the substitution mutant polypeptide comprises a substitution of two or more wild-type or naturally occurring amino acids, this substitution may comprise either an equivalent number of wild-type or naturally occurring amino acids deleted for the substitution, *i.e.*, 2 wild-type or naturally occurring amino acids replaced with 2 non-wild-type or non-naturally occurring amino acids, or a non-equivalent number of wild-type amino acids deleted for the substitution, *i.e.*, 2 wild-type amino acids replaced with 1 non-wild-type amino acid (a substitution+deletion mutation), or 2 wild-type amino acids replaced with 3 non-wild-type amino acids (a substitution+insertion mutation).

[0277] Substitution mutants may be described using an abbreviated nomenclature system to indicate the amino acid residue and number replaced within the reference polypeptide sequence and the new substituted amino acid residue. For example, a substitution mutant in which the twentieth (20th) amino acid residue of a polypeptide is substituted may be abbreviated as "x20z", wherein "x" is the amino acid to be replaced, "20" is the amino acid residue position or number within the polypeptide, and "z" is the new substituted amino acid. Therefore, a substitution mutant abbreviated interchangeably as "E20A" or "Glu20Ala" indicates that the mutant comprises an alanine residue (commonly abbreviated in the art as "A" or "Ala") in place of the glutamic acid (commonly abbreviated in the art as "E" or "Glu") at position 20 of the polypeptide.

[0278] A substitution mutation may be made by any technique for mutagenesis known in the art, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson *et al.*, *J. Biol. Chem. 253*:6551 (1978); Zoller *et al.*, *DNA 3*:479 (1984); Oliphant *et al.*, *Gene 44*:177 (1986); Hutchinson *et al.*, *Proc. Natl. Acad. Sci. USA 83*:710 (1986)), use of TAB® linkers (Pharmacia), restriction endonuclease digestion/fragment deletion and substitution, PCR-mediated/oligonucleotide-directed mutagenesis, and the like. PCR-based techniques are preferred for site-directed mutagenesis (see Higuchi, 1989, "Using

PCR to Engineer DNA", in *PCR Technology: Principles and Applications for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61-70).

[0279] The term "fragment," as applied to a polypeptide, refers to a polypeptide whose amino acid sequence is shorter than that of the reference polypeptide and which comprises, over the entire portion with these reference polypeptides, an identical amino acid sequence. Such fragments may, where appropriate, be included in a larger polypeptide of which they are a part. Such fragments of a polypeptide according to the invention may have a length of at least 2, 3, 4, 5, 6, 8, 10, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 25, 26, 30, 35, 40, 45, 50, 100, 200, 240, or 300 or more amino acids.

[0280] A "variant" of a polypeptide or protein refers to any analogue, fragment, derivative, or mutant which is derived from a polypeptide or protein and which retains at least one biological property of the polypeptide or protein. Different variants of the polypeptide or protein may exist in nature. These variants may be allelic variations characterized by differences in the nucleotide sequences of the structural gene coding for the protein, or may involve differential splicing or post-translational modification. The skilled artisan can produce variants having single or multiple amino acid substitutions, deletions, additions, or replacements. These variants may include, *inter alia*: (a) variants in which one or more amino acid residues are substituted with conservative or non-conservative amino acids, (b) variants in which one or more amino acids are added to the polypeptide or protein, (c) variants in which one or more of the amino acids includes a substituent group, and (d) variants in which the polypeptide or protein is fused with another polypeptide such as serum albumin. The techniques for obtaining these variants, including genetic (suppressions, deletions, mutations, *etc.*), chemical, and enzymatic techniques, are known to persons having ordinary skill in the art. In one embodiment, a variant polypeptide comprises at least about 14 amino acids.

[0281] The term "homology" refers to the percent of identity between two polynucleotide or two polypeptide moieties. The correspondence between the sequence from one moiety to another can be determined by techniques known to the art. For example, homology can be determined by a direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs. Alternatively, homology can be determined by hybridization of polynucleotides under conditions that form stable duplexes between homologous regions,

followed by digestion with single-stranded-specific nuclease(s) and size determination of the digested fragments.

[0282] As used herein, the term "homologous" in all its grammatical forms and spelling variations refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) and homologous proteins from different species (*e.g.*, myosin light chain, *etc.*) (Reeck *et al., Cell 50*:667 (1987)). Such proteins (and their encoding genes) have sequence homology, as reflected by their high degree of sequence similarity. However, in common usage and in the application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and not a common evolutionary origin.

[0283] Accordingly, the term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that may or may not share a common evolutionary origin (see Reeck *et al., Cell 50*:667 (1987)). In one embodiment, two DNA sequences are "substantially homologous" or "substantially similar" when at least about 50% (*e.g.*, at least about 75%, 90%, or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art (see *e.g.*, Sambrook *et al.*, 1989, *supra*).

[0284] As used herein, "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. "Substantially similar" also refers to nucleic acid fragments wherein changes in one or more nucleotide bases do not affect the ability of the nucleic acid fragment to mediate alteration of gene expression by antisense or co-suppression technology. "Substantially similar" also refers to modifications of the nucleic acid fragments of the invention such as deletion or insertion of one or more nucleotide bases that do not substantially affect the functional properties of the resulting transcript. It is therefore understood that the invention encompasses more than the specific exemplary sequences.

Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products.

[0285] Moreover, the skilled artisan recognizes that substantially similar sequences encompassed by this invention are also defined by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS), with the sequences exemplified herein. Substantially similar nucleic acid fragments of the invention are those nucleic acid fragments whose DNA sequences are at least about 70%, 80%, 90% or 95% identical to the DNA sequence of the nucleic acid fragments reported herein.

[0286] Two amino acid sequences are "substantially homologous" or "substantially similar" when greater than about 40% of the amino acids are identical, or greater than 60% are similar (functionally identical). Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, *Version 7*, Madison, Wisconsin) pileup program.

[0287] The term "corresponding to" is used herein to refer to similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. A nucleic acid or amino acid sequence alignment may include spaces. Thus, the term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

[0288] A “substantial portion” of an amino acid or nucleotide sequence comprises enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to putatively identify that polypeptide or gene, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul *et al., J. Mol. Biol. 215*:403 (1993)); available at ncbi.nlm.nih.gov/BLAST/). In general, a sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene specific oligonucleotide probes comprising 20-30 contiguous nucleotides may be used in sequence-dependent methods of gene identification (*e.g.*, Southern hybridization) and isolation (*e.g.*, *in situ* hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12-15 bases may be used as amplification primers in

PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises enough of the sequence to specifically identify and/or isolate a nucleic acid fragment comprising the sequence.

[0289] The term "percent identity," as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in: *Computational Molecular Biology* (Lesk, A. M., ed.) Oxford University Press, New York (1988); *Biocomputing: Informatics and Genome Projects* (Smith, D. W., ed.) Academic Press, New York (1993); *Computer Analysis of Sequence Data, Part I* (Griffin, A. M., and Griffin, H. G., eds.) Humana Press, New Jersey (1994); *Sequence Analysis in Molecular Biology* (von Heinje, G., ed.) Academic Press (1987); and *Sequence Analysis Primer* (Gribskov, M. and Devereux, J., eds.) Stockton Press, New York (1991). Preferred methods to determine identity are designed to give the best match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations may be performed using sequence analysis software such as the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences may be performed using the Clustal method of alignment (Higgins *et al.*, *CABIOS. 5*:151 (1989)) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method may be selected: KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

[0290] The term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. "Sequence analysis software" may be commercially available or independently developed. Typical sequence analysis software includes, but is not limited to, the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI), BLASTP, BLASTN, BLASTX (Altschul *et al.*, *J. Mol. Biol. 215*:403

(1990)), and DNASTAR (DNASTAR, Inc. 1228 S. Park St. Madison, WI 53715 USA). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters which originally load with the software when first initialized.

[0291] "Chemically synthesized," as related to a sequence of DNA, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of DNA may be accomplished using well-established procedures, or automated chemical synthesis can be performed using one of a number of commercially available machines. Accordingly, the genes can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell where sequence information is available.

[0292] As used herein, two or more individually operable gene regulation systems are said to be "orthogonal" when; a) modulation of each of the given systems by its respective ligand, at a chosen concentration, results in a measurable change in the magnitude of expression of the gene of that system, and b) the change is statistically significantly different than the change in expression of all other systems simultaneously operable in the cell, tissue, or organism, regardless of the simultaneity or sequentiality of the actual modulation. Preferably, modulation of each individually operable gene regulation system effects a change in gene expression at least 2-fold greater than all other operable systems in the cell, tissue, or organism, *e.g.*, at least 5-fold, 10-fold, 100-fold, or 500-fold greater. Ideally, modulation of each of the given systems by its respective ligand at a chosen concentration results in a measurable change in the magnitude of expression of the gene of that system and no measurable change in expression of all other systems operable in the cell, tissue, or organism. In such cases the multiple inducible gene regulation system is said to be "fully orthogonal." Useful orthogonal ligands and orthogonal receptor-based gene expression systems are described in US 2002/0110861 A1.

[0293] The term "exogenous gene" means a gene foreign to the subject, that is, a gene which is introduced into the subject through a transformation process, an unmutated version of an endogenous mutated gene or a mutated version of an endogenous unmutated gene. The method of transformation is not critical to this invention and may be any method suitable for the subject known to those in the art. Exogenous genes can be either natural or synthetic genes which are introduced into the subject in the form of DNA or RNA which may function through a DNA intermediate such as by reverse transcriptase. Such genes can be introduced into target cells, directly introduced into the subject, or indirectly introduced by the transfer of transformed cells into the subject.

[0294] The term "therapeutic product" refers to a therapeutic polypeptide or therapeutic polynucleotide which imparts a beneficial function to the host cell in which such product is expressed. Therapeutic polypeptides may include, without limitation, peptides as small as three amino acids in length, single- or multiple-chain proteins, and fusion proteins. Therapeutic polynucleotides may include, without limitation, antisense oligonucleotides, small interfering RNAs, ribozymes, and RNA external guide sequences. The therapeutic product may comprise a naturally occurring sequence, a synthetic sequence or a combination of natural and synthetic sequences.

[0295] The term "ligand-dependent transcription factor complex" or "LDTFC" refers to a transcription factor comprising one or more protein subunits, which complex can regulate gene expression driven by a "factor-regulated promoter" as defined herein. A model LDTFC is an "ecdysone receptor complex" generally refers to a heterodimeric protein complex having at least two members of the nuclear receptor family, ecdysone receptor ("EcR") and ultraspiracle ("USP") proteins (see Yao *et al.*, *Nature 366*:476 (1993)); Yao *et al., Cell 71*:63 (1992)). A functional LDTFC such as an EcR complex may also include additional protein(s) such as immunophilins. Additional members of the nuclear receptor family of proteins, known as transcriptional factors (such as DHR38, *betaFTZ-1* or other insect homologs), may also be ligand dependent or independent partners for EcR and/or USP. A LDTFC such as an EcR complex can also be a heterodimer of EcR protein and the vertebrate homolog of ultraspiracle protein, retinoic acid-X-receptor ("RXR") protein or a chimera of USP and RXR. The terms "LDTFC" and "EcR complex" also encompass homodimer complexes of the EcR protein or USP, as well as single polypeptides or trimers, tetramer, and other multimers serving the same function.

[0296] A LDTFC such as an EcR complex can be activated by an active ecdysteroid or non-steroidal ligand bound to one of the proteins of the complex, inclusive of EcR, but not excluding other proteins of the complex. A LDTFC such as an EcR complex includes proteins which are members of the nuclear receptor superfamily wherein all members are characterized by the presence of one or more polypeptide subunits comprising an amino-terminal transactivation domain ("AD," "TD," or "TA," used interchangeably herein), a DNA binding domain ("DBD"), and a ligand binding domain ("LBD"). The AD may be present as a fusion with a "heterodimerization partner" or "HP." A fusion protein comprising an AD and HP of the invention is referred to herein as a "coactivation protein" or "CAP." The DBD and LBD may be expressed as a fusion protein, referred to herein as a "ligand-inducible transcription factor ("LTF"). The fusion partners may be separated by a linker, *e.g.*, a hinge region. Some members of the LTF family may also have another transactivation domain on the carboxy-terminal side of the LBD. The DBD is characterized by the presence of two cysteine zinc fingers between which are two amino acid motifs, the P-box and the D-box, which confer specificity for ecdysone response elements. These domains may be either native, modified, or chimeras of different domains of heterologous receptor proteins.

[0297] The DNA sequences making up the exogenous gene, the response element, and the LDTFC, *e.g.*, EcR complex, may be incorporated into archaebacteria, procaryotic cells such as *Escherichia coli*, *Bacillus subtilis*, or other enterobacteria, or eucaryotic cells such as plant or animal cells. However, because many of the proteins expressed by the gene are processed incorrectly in bacteria, eucaryotic cells are preferred. The cells may be in the form of single cells or multicellular organisms. The nucleotide sequences for the exogenous gene, the response element, and the receptor complex can also be incorporated as RNA molecules, preferably in the form of functional viral RNAs such as tobacco mosaic virus. Of the eucaryotic cells, vertebrate cells are preferred because they naturally lack the molecules which confer responses to the ligands of this invention for the EcR. As a result, they are "substantially insensitive" to the ligands of this invention. Thus, the ligands useful in this invention will have negligible physiological or other effects on transformed cells, or the whole organism. Therefore, cells can grow and express the desired product, substantially unaffected by the presence of the ligand itself.

[0298] The term "ecdysone receptor complex" generally refers to a heterodimeric protein complex having at least two members of the nuclear receptor family, ecdysone receptor ("EcR") and ultraspiracle ("USP") proteins (see Yao *et al.*, *Nature 366*:476 (1993)); Yao *et al., Cell 71*:63 (1992)). The functional EcR complex may also include additional protein(s) such as immunophilins. Additional members of the nuclear receptor family of proteins, known as transcriptional factors (such as DHR38, *betaFTZ-1* or other insect homologs), may also be ligand dependent or independent partners for EcR and/or USP. The EcR complex can also be a heterodimer of EcR protein and the vertebrate homolog of ultraspiracle protein, retinoic acid-X-receptor ("RXR") protein or a chimera of USP and RXR. The term EcR complex also encompasses homodimer complexes of the EcR protein or USP.

[0299] An EcR complex can be activated by an active ecdysteroid or non-steroidal ligand bound to one of the proteins of the complex, inclusive of EcR, but not excluding other proteins of the complex. As used herein, the term "ligand," as applied to EcR-based gene switches, describes small and soluble molecules having the capability of activating a gene switch to stimulate expression of a polypeptide encoded therein. Examples of ligands include, without limitation, an ecdysteroid, such as ecdysone, 20-hydroxyecdysone, ponasterone A, muristerone A, and the like, 9-cis-retinoic acid, synthetic analogs of retinoic acid, N,N'-diacylhydrazines such as those disclosed in U.S. Patent Nos. 6,013,836; 5,117,057; 5,530,028; and 5,378,726 and U.S. Published Application Nos. 2005/0209283 and 2006/0020146; oxadiazolines as described in U.S. Published Application No. 2004/0171651; dibenzoylalkyl cyanohydrazines such as those disclosed in European Application No. 461,809; N-alkyl-N,N'-diaroylhydrazines such as those disclosed in U.S. Patent No. 5,225,443; N-acyl-N-alkylcarbonylhydrazines such as those disclosed in European Application No. 234,994; N-aroyl-N-alkyl-N'-aroylhydrazines such as those described in U.S. Patent No. 4,985,461; amidoketones such as those described in U.S. Published Application No. 2004/0049037; and other similar materials including 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide, 8-O-acetylharpagide, oxysterols, 22(R) hydroxycholesterol, 24(S) hydroxycholesterol, 25-epoxycholesterol, T0901317, 5-alpha-6-alpha-epoxycholesterol-3-sulfate (ECHS), 7-ketocholesterol-3-sulfate, famesol, bile acids, 1,1-biphosphonate esters, juvenile hormone III, and the like. Examples of diacylhydrazine ligands useful in the invention include RG-115819 (3,5-Dimethyl-

benzoic acid N-(1-ethyl-2,2-dimethyl-propyl)-N'-(2-methyl-3-methoxy-benzoyl)-hydrazide), RG-115932 ((*R*)-3,5-Dimethyl-benzoic acid N-(1-*tert*-butyl-butyl)-N'-(2-ethyl-3-methoxy-benzoyl)-hydrazide), and RG-115830 (3,5-Dimethyl-benzoic acid N-(1-*tert*-butyl-butyl)-N'-(2-ethyl-3-methoxy-benzoyl)-hydrazide). See U.S. Appln. 12/155,111, filed May 29, 2008, and PCT/US2008/006757 filed May 29, 2008, for additional diacylhydrazines that are useful in the practice of the invention.

[0300] The EcR complex includes proteins which are members of the nuclear receptor superfamily wherein all members are characterized by the presence of an amino-terminal transactivation domain ("TA"), a DNA binding domain ("DBD"), and a ligand binding domain ("LBD") separated by a hinge region. Some members of the family may also have another transactivation domain on the carboxy-terminal side of the LBD. The DBD is characterized by the presence of two cysteine zinc fingers between which are two amino acid motifs, the P-box and the D-box, which confer specificity for ecdysone response elements. These domains may be either native, modified, or chimeras of different domains of heterologous receptor proteins.

[0301] The DNA sequences making up the exogenous gene, the response element, and the EcR complex may be incorporated into archaebacteria, procaryotic cells such as *Escherichia coli*, *Bacillus subtilis*, or other enterobacteria, or eucaryotic cells such as plant or animal cells. However, because many of the proteins expressed by the gene are processed incorrectly in bacteria, eucaryotic cells are preferred. The cells may be in the form of single cells or multicellular organisms. The nucleotide sequences for the exogenous gene, the response element, and the receptor complex can also be incorporated as RNA molecules, preferably in the form of functional viral RNAs such as tobacco mosaic virus. Of the eucaryotic cells, vertebrate cells are preferred because they naturally lack the molecules which confer responses to the ligands of this invention for the EcR. As a result, they are "substantially insensitive" to the ligands of this invention. Thus, the ligands useful in this invention will have negligible physiological or other effects on transformed cells, or the whole organism. Therefore, cells can grow and express the desired product, substantially unaffected by the presence of the ligand itself.

[0302] EcR ligands, when used with the EcR complex which in turn is bound to the response element linked to an exogenous gene (*e.g.*, IL-12), provide the means for external temporal regulation of expression of the exogenous gene. The order in which the

various components bind to each other, that is, ligand to receptor complex and receptor complex to response element, is not critical. Typically, modulation of expression of the exogenous gene is in response to the binding of the EcR complex to a specific control, or regulatory, DNA element. The EcR protein, like other members of the nuclear receptor family, possesses at least three domains, a transactivation domain, a DNA binding domain, and a ligand binding domain. This receptor, like a subset of the nuclear receptor family, also possesses less well-defined regions responsible for heterodimerization properties. Binding of the ligand to the ligand binding domain of EcR protein, after heterodimerization with USP or RXR protein, enables the DNA binding domains of the heterodimeric proteins to bind to the response element in an activated form, thus resulting in expression or suppression of the exogenous gene. This mechanism does not exclude the potential for ligand binding to either EcR or USP, and the resulting formation of active homodimer complexes (*e.g.,* EcR+EcR or USP+USP). In one embodiment, one or more of the receptor domains can be varied producing a chimeric gene switch. Typically, one or more of the three domains may be chosen from a source different than the source of the other domains so that the chimeric receptor is optimized in the chosen host cell or organism for transactivating activity, complementary binding of the ligand, and recognition of a specific response element. In addition, the response element itself can be modified or substituted with response elements for other DNA binding protein domains such as the GAL-4 protein from yeast (see Sadowski *et al.*, *Nature 335*:563 (1988) or LexA protein from *E. coli* (see Brent *et al.*, *Cell 43*:729 (1985)) to accommodate chimeric EcR complexes. Another advantage of chimeric systems is that they allow choice of a promoter used to drive the exogenous gene according to a desired end result. Such double control can be particularly important in areas of gene therapy, especially when cytotoxic proteins are produced, because both the timing of expression as well as the cells wherein expression occurs can be controlled. When exogenous genes, operatively linked to a suitable promoter, are introduced into the cells of the subject, expression of the exogenous genes is controlled by the presence of the ligand of this invention. Promoters may be constitutively or inducibly regulated or may be tissue-specific (that is, expressed only in a particular type of cell) or specific to certain developmental stages of the organism.

[0303] In certain embodiments, the therapeutic switch promoter described in the methods is consititutive. In certain embodiments, the therapeutic switch promoter is activated under conditions associated with a disease, disorder, or condition, *e.g.*, the promoter is activated in response to a disease, in response to a particular physiological, developmental, differentiation, or pathological condition, and/or in response to one or more specific biological molecules; and/or the promoter is activated in particular tissue or cell types. In certain embodiments, the disease, disorder, or condition is responsive to the therapeutic polypeptide or polynucleotide. For example in certain non-limiting embodiments the therapeutic polynucleotide or polypeptide is useful to treat, prevent, ameliorate, reduce symptoms, prevent progression, or cure the disease, disorder or condition, but need not accomplish any one or all of these things. In certain embodiments, the first and second polynucleotides are introduced so as to permit expression of the ligand-dependent transcription factor complex under consitions associated with a disease, disorder or condition. In one embodiment, the therapeutic methods are carried out such that the therapeutic polypeptide or therapeutic polynucleotide is expressed and disseminated through the subject at a level sufficient to treat, ameliorate, or prevent said disease, disorder, or condition. As used herein, "disseminated" means that the polypeptide is expressed and released from the modified cell sufficiently to have an effect or activity in the subject. Dissemination may be systemic, local or anything in between. For example, the therapeutic polypeptide or therapeutic polynucleotide might be systemically disseminated through the bloodstream or lymph system. Alternatively, the therapeutic polypeptide or therapeutic polynucleotide might be disseminated locally in a tissue or organ to be treated.

[0304] Numerous genomic and cDNA nucleic acid sequences coding for a variety of polypeptides, such as transcription factors and reporter proteins, are well known in the art. Those skilled in the art have access to nucleic acid sequence information for virtually all known genes and can either obtain the nucleic acid molecule directly from a public depository, the institution that published the sequence, or employ routine methods to prepare the molecule. See for example the description of the sequence accession numbers, *infra*.

[0305] The gene switch may be any gene switch system that regulates gene expression by addition or removal of a specific ligand. In one embodiment, the gene switch is one in

which the level of gene expression is dependent on the level of ligand that is present. Examples of ligand-dependent transcription factors that may be used in the gene switches of the invention include, without limitation, members of the nuclear receptor superfamily activated by their respective ligands (*e.g.*, glucocorticoid, estrogen, progestin, retinoid, ecdysone, and analogs and mimetics thereof) and rTTA activated by tetracycline. In one aspect of the invention, the gene switch is an EcR-based gene switch. Examples of such systems include, without limitation, the systems described in U.S. Patent Nos. 6,258,603, 7,045,315, U.S. Published Patent Application Nos. 2006/0014711, 2007/0161086, and International Published Application No. WO 01/70816. Examples of chimeric ecdysone receptor systems are described in U.S. Patent No. 7,091,038, U.S. Published Patent Application Nos. 2002/0110861, 2004/0033600, 2004/0096942, 2005/0266457, and 2006/0100416, and International Published Application Nos. WO 01/70816, WO 02/066612, WO 02/066613, WO 02/066614, WO 02/066615, WO 02/29075, and WO 2005/108617. An example of a non-steroidal ecdysone agonist-regulated system is the RheoSwitch® Mammalian Inducible Expression System (New England Biolabs, Ipswich, MA).

[0306] In one embodiment, a polynucleotide encoding the gene switch comprises a single transcription factor sequence encoding a ligand-dependent transcription factor under the control of a promoter. The transcription factor sequence may encode a ligand-dependent transcription factor that is a naturally occurring or an artificial transcription factor. An artificial transcription factor is one in which the natural sequence of the transcription factor has been altered, *e.g.*, by mutation of the sequence or by the combining of domains from different transcription factors. In one embodiment, the transcription factor comprises a Group H nuclear receptor ligand binding domain (LBD). In one embodiment, the Group H nuclear receptor LBD is from an EcR, a ubiquitous receptor, an orphan receptor 1, a NER-1, a steroid hormone nuclear receptor 1, a retinoid X receptor interacting protein–15, a liver X receptor β, a steroid hormone receptor like protein, a liver X receptor, a liver X receptor α, a farnesoid X receptor, a receptor interacting protein 14, or a farnesol receptor. In another embodiment, the Group H nuclear receptor LBD is from an ecdysone receptor.

[0307] The EcR and the other Group H nuclear receptors are members of the nuclear receptor superfamily wherein all members are generally characterized by the presence of

an amino-terminal transactivation domain (TD), a DNA binding domain (DBD), and a LBD separated from the DBD by a hinge region. As used herein, the term "DNA binding domain" comprises a minimal polypeptide sequence of a DNA binding protein, up to the entire length of a DNA binding protein, so long as the DNA binding domain functions to associate with a particular response element. Members of the nuclear receptor superfamily are also characterized by the presence of four or five domains: A/B, C, D, E, and in some members F (see US 4,981,784 and Evans, *Science 240*:889 (1988)). The "A/B" domain corresponds to the transactivation domain, "C" corresponds to the DNA binding domain, "D" corresponds to the hinge region, and "E" corresponds to the ligand binding domain. Some members of the family may also have another transactivation domain on the carboxy-terminal side of the LBD corresponding to "F".

[0308] The DBD is characterized by the presence of two cysteine zinc fingers between which are two amino acid motifs, the P-box and the D-box, which confer specificity for response elements. These domains may be either native, modified, or chimeras of different domains of heterologous receptor proteins. The EcR, like a subset of the nuclear receptor family, also possesses less well-defined regions responsible for heterodimerization properties. Because the domains of nuclear receptors are modular in nature, the LBD, DBD, and TD may be interchanged.

[0309] In another embodiment, the transcription factor comprises a TD, a DBD that recognizes a response element associated with the exogenous gene whose expression is to be modulated; and a Group H nuclear receptor LBD. In certain embodiments, the Group H nuclear receptor LBD comprises a substitution mutation.

[0310] In another embodiment, a polynucleotide encoding the gene switch comprises a first transcription factor sequence under the control of a first promoter and a second transcription factor sequence under the control of a second promoter, wherein the proteins encoded by said first transcription factor sequence and said second transcription factor sequence interact to form a protein complex which functions as a ligand-dependent transcription factor, *i.e.*, a "dual switch"- or "two-hybrid"-based gene switch. The first and second promoters may be the same or different.

[0311] In certain embodiments, the polynucleotide encoding a gene switch comprises a first transcription factor sequence and a second transcription factor sequence under the control of a promoter, wherein the proteins encoded by said first transcription factor

sequence and said second transcription factor sequence interact to form a protein complex which functions as a ligand-dependent transcription factor, *i.e.*, a "single gene switch". The first transcription factor sequence and a second transcription factor sequence may be connected by an internal ribosomal entry site (IRES). The IRES may be an EMCV IRES.

[0312] In one embodiment, the first transcription factor sequence encodes a polypeptide comprising a TD, a DBD that recognizes a response element associated with the exogenous gene whose expression is to be modulated; and a Group H nuclear receptor LBD, and the second transcription factor sequence encodes a transcription factor comprising a nuclear receptor LBD selected from a vertebrate RXR LBD, an invertebrate RXR LBD, an ultraspiracle protein LBD, and a chimeric LBD comprising two polypeptide fragments, wherein the first polypeptide fragment is from a vertebrate RXR LBD, an invertebrate RXR LBD, or an ultraspiracle protein LBD, and the second polypeptide fragment is from a different vertebrate RXR LBD, invertebrate RXR LBD, or ultraspiracle protein LBD.

[0313] In another embodiment, the gene switch comprises a first transcription factor sequence encoding a first polypeptide comprising a nuclear receptor LBD and a DBD that recognizes a response element associated with the exogenous gene whose expression is to be modulated, and a second transcription factor sequence encoding a second polypeptide comprising a TD and a nuclear receptor LBD, wherein one of the nuclear receptor LBDs is a Group H nuclear receptor LBD. In a preferred embodiment, the first polypeptide is substantially free of a TD and the second polypeptide is substantially free of a DBD. For purposes of the invention, "substantially free" means that the protein in question does not contain a sufficient sequence of the domain in question to provide activation or binding activity.

[0314] In another aspect of the invention, the first transcription factor sequence encodes a protein comprising a heterodimer partner and a TD and the second transcription factor sequence encodes a protein comprising a DBD and a LBD.

[0315] When only one nuclear receptor LBD is a Group H LBD, the other nuclear receptor LBD may be from any other nuclear receptor that forms a dimer with the Group H LBD. For example, when the Group H nuclear receptor LBD is an EcR LBD, the other nuclear receptor LBD "partner" may be from an EcR, a vertebrate RXR, an invertebrate RXR, an ultraspiracle protein (USP), or a chimeric nuclear receptor comprising at least

two different nuclear receptor LBD polypeptide fragments selected from a vertebrate RXR, an invertebrate RXR, and a USP (see WO 01/70816 A2, International Patent Application No. PCT/US02/05235 and US 2004/0096942 A1). The "partner" nuclear receptor ligand binding domain may further comprise a truncation mutation, a deletion mutation, a substitution mutation, or another modification.

[0316] In one embodiment, the vertebrate RXR LBD is from a human *Homo sapiens*, mouse *Mus musculus*, rat *Rattus norvegicus*, chicken *Gallus gallus*, pig *Sus scrofa domestica*, frog *Xenopus laevis*, zebrafish *Danio rerio*, tunicate *Polyandrocarpa misakiensis*, or jellyfish *Tripedalia cysophora* RXR.

[0317] In one embodiment, the invertebrate RXR ligand binding domain is from a locust *Locusta migratoria* ultraspiracle polypeptide ("LmUSP"), an ixodid tick *Amblyomma americanum* RXR homolog 1 ("AmaRXR1"), an ixodid tick *Amblyomma americanum* RXR homolog 2 ("AmaRXR2"), a fiddler crab *Celuca pugilator* RXR homolog ("CpRXR"), a beetle *Tenebrio molitor* RXR homolog ("TmRXR"), a honeybee *Apis mellifera* RXR homolog ("AmRXR"), an aphid *Myzus persicae* RXR homolog ("MpRXR"), or a non-Dipteran/non-Lepidopteran RXR homolog.

[0318] In one embodiment, the chimeric RXR LBD comprises at least two polypeptide fragments selected from a vertebrate species RXR polypeptide fragment, an invertebrate species RXR polypeptide fragment, and a non-Dipteran/non-Lepidopteran invertebrate species RXR homolog polypeptide fragment. A chimeric RXR ligand binding domain for use in the invention may comprise at least two different species RXR polypeptide fragments, or when the species is the same, the two or more polypeptide fragments may be from two or more different isoforms of the species RXR polypeptide fragment.

[0319] In one embodiment, the chimeric RXR ligand binding domain comprises at least one vertebrate species RXR polypeptide fragment and one invertebrate species RXR polypeptide fragment.

[0320] In another embodiment, the chimeric RXR ligand binding domain comprises at least one vertebrate species RXR polypeptide fragment and one non-Dipteran/non-Lepidopteran invertebrate species RXR homolog polypeptide fragment.

[0321] The ligand, when combined with the LBD of the nuclear receptor(s), which in turn are bound to the response element linked to the exogenous gene, provides external temporal regulation of expression of the exogenous gene. The binding mechanism or the

order in which the various components of this invention bind to each other, that is, for example, ligand to LBD, DBD to response element, TD to promoter, *etc.*, is not critical.

[0322] In a specific example, binding of the ligand to the LBD of a Group H nuclear receptor and its nuclear receptor LBD partner enables expression of the exogenous gene. This mechanism does not exclude the potential for ligand binding to the Group H nuclear receptor (GHNR) or its partner, and the resulting formation of active homodimer complexes (*e.g.,* GHNR + GHNR or partner + partner). Preferably, one or more of the receptor domains is varied producing a hybrid gene switch. Typically, one or more of the three domains, DBD, LBD, and TD, may be chosen from a source different than the source of the other domains so that the hybrid genes and the resulting hybrid proteins are optimized in the chosen host cell or organism for transactivating activity, complementary binding of the ligand, and recognition of a specific response element. In addition, the response element itself can be modified or substituted with response elements for other DNA binding protein domains such as the GAL-4 protein from yeast (see Sadowski *et al.*, *Nature 335*:563 (1988)) or LexA protein from *Escherichia coli* (see Brent *et al.*, *Cell 43*:729 (1985)), or synthetic response elements specific for targeted interactions with proteins designed, modified, and selected for such specific interactions (see, for example, Kim *et al.*, *Proc. Natl. Acad. Sci. USA*, *94*:3616 (1997)) to accommodate hybrid receptors.

[0323] The functional EcR complex may also include additional protein(s) such as immunophilins. Additional members of the nuclear receptor family of proteins, known as transcriptional factors (such as DHR38 or *betaFTZ-1*), may also be ligand dependent or independent partners for EcR, USP, and/or RXR. Additionally, other cofactors may be required such as proteins generally known as coactivators (also termed adapters or mediators). These proteins do not bind sequence-specifically to DNA and are not involved in basal transcription. They may exert their effect on transcription activation through various mechanisms, including stimulation of DNA-binding of activators, by affecting chromatin structure, or by mediating activator-initiation complex interactions. Examples of such coactivators include RIP140, TIF1, RAP46/Bag-1, ARA70, SRC-1/NCoA-1, TIF2/GRIP/NCoA-2, ACTR/AIB1/RAC3/pCIP as well as the promiscuous coactivator C response element B binding protein, CBP/p300 (for review see Glass *et al.*, *Curr. Opin. Cell Biol.* 9:222 (1997)). Also, protein cofactors generally known as

corepressors (also known as repressors, silencers, or silencing mediators) may be required to effectively inhibit transcriptional activation in the absence of ligand. These corepressors may interact with the unliganded EcR to silence the activity at the response element. Current evidence suggests that the binding of ligand changes the conformation of the receptor, which results in release of the corepressor and recruitment of the above described coactivators, thereby abolishing their silencing activity. Examples of corepressors include N-CoR and SMRT (for review, see Horwitz *et al.*, *Mol Endocrinol. 10*:1167 (1996)). These cofactors may either be endogenous within the cell or organism, or may be added exogenously as transgenes to be expressed in either a regulated or unregulated fashion.

[0324] The exogenous gene is operably linked to a promoter comprising at least one response element that is recognized by the DBD of the ligand-dependent transcription factor encoded by the gene switch. In one embodiment, the promoter comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more copies of the response element. Promoters comprising the desired response elements may be naturally occurring promoters or artificial promoters created using techniques that are well known in the art, *e.g.*, one or more response elements operably linked to a minimal promoter.

[0325] To introduce the polynucleotides into the cells, a vector can be used. The vector may be, for example, a plasmid vector or a single-or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells by well-known techniques for introducing DNA and RNA into cells. Viral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells. As used herein, the term "host cell" or "host" is used to mean a cell of the invention that is harboring one or more polynucleotides of the invention.

[0326] Thus, at a minimum, the vectors must include the polynucleotides of the invention. Other components of the vector may include, but are not limited to, selectable markers, chromatin modification domains, additional promoters driving expression of other polypeptides that may also be present on the vector (*e.g.*, a lethal polypeptide), genomic integration sites, recombination sites, and molecular insertion pivots. The vectors may comprise any number of these additional elements, either within or not within the polynucleotides, such that the vector can be tailored to the specific goals of the therapeutic methods desired.

[0327] In one embodiment of the invention, the vectors that are introduced into the cells further comprise a "selectable marker gene" which, when expressed, indicates that the gene switch construct of the invention has been integrated into the genome of the host cell. In this manner, the selector gene can be a positive marker for the genome integration. While not critical to the methods of the invention, the presence of a selectable marker gene allows the practitioner to select for a population of live cells where the vector construct has been integrated into the genome of the cells. Thus, certain embodiments of the invention comprise selecting cells where the vector has successfully been integrated. As used herein, the term "select" or variations thereof, when used in conjunction with cells, is intended to mean standard, well-known methods for choosing cells with a specific genetic make-up or phenotype. Typical methods include, but are not limited to, culturing cells in the presence of antibiotics, such as G418, neomycin and ampicillin. Other examples of selectable marker genes include, but are not limited to, genes that confer resistance to dihydrofolate reductase, hygromycin, or mycophenolic acid. Other methods of selection include, but are not limited to, a selectable marker gene that allows for the use of thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase or adenine phosphoribosyltransferase as selection agents. Cells comprising a vector construct comprising an antibiotic resistance gene or genes would then be capable of tolerating the antibiotic in culture. Likewise, cells not comprising a vector construct comprising an antibiotic resistance gene or genes would not be capable of tolerating the antibiotic in culture.

[0328] As used herein, a "chromatin modification domain" (CMD) refers to nucleotide sequences that interact with a variety of proteins associated with maintaining and/or altering chromatin structure, such as, but not limited to, DNA insulators. *See* Ciavatta *et al., Proc. Nat'l Acad. Sci. U.S.A.,* 103:9958 (2006). Examples of CMDs include, but are not limited to, the chicken β-globulin insulator and the chicken hypersensitive site 4 (cHS4). The use of different CMD sequences between one or more gene programs (*i.e.*, a promoter, coding sequence, and 3' regulatory region), for example, can facilitate the use of the differential CMD DNA sequences as "mini homology arms" in combination with various microorganism or *in vitro* recombineering technologies to "swap" gene programs between existing multigenic and monogenic shuttle vectors. Other examples of chromatin modification domains are known in the art or can be readily identified.

[0329] Particular vectors for use with the invention are expression vectors that code for proteins or polynucleotides. Generally, such vectors comprise *cis*-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate *trans*-acting factors are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

[0330] A great variety of expression vectors can be used to express proteins or polynucleotides. Such vectors include chromosomal, episomal and virus-derived vectors, *e.g.,* vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, from viruses such as adeno-associated viruses, lentiviruses, baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. All may be used for expression in accordance with this aspect of the invention. Generally, any vector suitable to maintain, propagate or express polynucleotides or proteins in a host may be used for expression in this regard.

[0331] The polynucleotide sequence in the expression vector is operatively linked to appropriate expression control sequence(s) including, for instance, a promoter to direct mRNA transcription. Representatives of additional promoters include, but are not limited to, constitutive promoters and tissue specific or inducible promoters. Examples of constitutive eukaryotic promoters include, but are not limited to, the promoter of the mouse metallothionein I gene (Hamer *et al., J. Mol. Appl. Gen. 1*:273 (1982)); the TK promoter of Herpes virus (McKnight, *Cell 31*:355 (1982)); the SV40 early promoter (Benoist *et al.*, *Nature 290*:304 (1981)); and the vaccinia virus promoter. Additional examples of the promoters that could be used to drive expression of a protein or polynucleotide include, but are not limited to, tissue-specific promoters and other endogenous promoters for specific proteins, such as the albumin promoter (hepatocytes), a proinsulin promoter (pancreatic beta cells) and the like. In general, expression constructs will contain sites for transcription, initiation and termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs may include a translation initiating AUG at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

[0332] In addition, the constructs may contain control regions that regulate, as well as engender expression. Generally, such regions will operate by controlling transcription, such as repressor binding sites and enhancers, among others.

[0333] Examples of eukaryotic vectors include, but are not limited to, pW-LNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; pSVK3, pBPV, pMSG and pSVL available from Amersham Pharmacia Biotech; and pCMVDsRed2-express, pIRES2-DsRed2, pDsRed2-Mito, and pCMV-EGFP available from Clontech. Many other vectors are well-known and commercially available.

[0334] Particularly useful vectors, which comprise molecular insertion pivots for rapid insertion and removal of elements of gene programs, are described in United States Published Patent Application No. 2004/0185556, United States Patent Application No. 11/233,246 and International Published Application Nos. WO 2005/040336 and WO 2005/116231. An example of such vectors is the UltraVector™ Production System (Intrexon Corp., Blacksburg, VA), as described in WO 2007/038276. As used herein, a "gene program" is a combination of genetic elements comprising a promoter (P), an expression sequence (E) and a 3′ regulatory sequence (3), such that "PE3" is a gene program. The elements within the gene program can be easily swapped between molecular pivots that flank each of the elements of the gene program. A molecular pivot, as used herein, is defined as a polynucleotide comprising at least two non-variable rare or uncommon restriction sites arranged in a linear fashion. In one embodiment, the molecular pivot comprises at least three non-variable rare or uncommon restriction sites arranged in a linear fashion. Typically any one molecular pivot would not include a rare or uncommon restriction site of any other molecular pivot within the same gene program. Cognate sequences of greater than 6 nucleotides upon which a given restriction enzyme acts are referred to as "rare" restriction sites. There are, however, restriction sites of 6 bp that occur more infrequently than would be statistically predicted, and these sites and the endonucleases that cleave them are referred to as "uncommon" restriction sites. Examples of either rare or uncommon restriction enzymes include, but are not limited to, AsiS I, Pac I, Sbf I, Fse I, Asc I, Mlu I, SnaB I, Not I, Sal I, Swa I, Rsr II, BSiW I, Sfo I, Sgr AI, AflIII, Pvu I, Ngo MIV, Ase I, Flp I, Pme I, Sda I, Sgf I, Srf I, Nru I, Acl I, Cla I, Csp45 I, Age I, Bst1107 I, BstB I, Hpa I, Aat II, EcoR V, Nhe I, Spe I, Avi II, Avr II, Mfe I, Afe I, Fsp I, Kpn I, Sca I, BspE I, Nde I, Bfr I, Xho I, Pml I, ApaL I, Kas I, Xma I,

BsrB I, Nsi I, Sac II, Sac I, Blp I, PspoM I, Pci I, Stu I, Sph I, BamH I, Bsu36 I, Xba I, BbvC I, Bgl II, Nco I, Hind III, EcoR I, BsrG I and Sse8781 I.

[0335] The vector may also comprise restriction sites for a second class of restriction enzymes called homing endonuclease (HE) enzymes. HE enzymes have large, asymmetric restriction sites (12-40 base pairs), and their restriction sites are infrequent in nature. For example, the HE known as I-SceI has an 18 bp restriction site (5'TAGGGATAACAGGGTAAT3' (SEQ ID NO: 28)), predicted to occur only once in every $7x10^{10}$ base pairs of random sequence. This rate of occurrence is equivalent to only one site in a genome that is 20 times the size of a mammalian genome. The rare nature of HE sites greatly increases the likelihood that a genetic engineer can cut a gene program without disrupting the integrity of the gene program if HE sites are included in appropriate locations in a cloning vector plasmid.

[0336] Selection of appropriate vectors and promoters for expression in a host cell is a well-known procedure, and the requisite techniques for vector construction and introduction into the host, as well as its expression in the host are routine skills in the art.

[0337] The introduction of the polynucleotides into the cells can be a transient transfection, stable transfection, or can be a locus-specific insertion of the vector. Transient and stable transfection of the vectors into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, Basic Methods in Molecular Biology (1986); Keown *et al.*, 1990, Methods Enzymol. 185: 527-37; Sambrook *et al.*, 2001, Molecular Cloning, A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, N.Y. These stable transfection methods result in random insertion of the vector into the genome of the cell. Further, the copy number and orientation of the vectors are also, generally speaking, random.

[0338] In one embodiment of the invention, the vector is inserted into a bio-neutral site in the genome. A bio-neutral site is a site in the genome where insertion of the polynucleotides interferes very little, if any, with the normal function of the cell. Bio-neutral sites may be analyzed using available bioinformatics. Many bio-neutral sites are known in the art, *e.g.*, the ROSA-equivalent locus. Other bio-neutral sites may be identified using routine techniques well known in the art. Characterization of the

genomic insertion site(s) is performed using methods known in the art. To control the location, copy number and/or orientation of the polynucleotides when introducing the vector into the cells, methods of locus-specific insertion may be used. Methods of locus-specific insertion are well-known in the art and include, but are not limited to, homologous recombination and recombinase-mediated genome insertion. Of course, if locus-specific insertion methods are to be used in the methods of the invention, the vectors may comprise elements that aid in this locus-specific insertion, such as, but not limited to, homologous recombination. For example, the vectors may comprise one, two, three, four or more genomic integration sites (GISs). As used herein, a "genomic integration site" is defined as a portion of the vector sequence which nucleotide sequence is identical or nearly identical to portions of the genome within the cells that allows for insertion of the vector in the genome. In particular, the vector may comprise two genomic insertion sites that flank at least the polynucleotides. Of course, the GISs may flank additional elements, or even all elements present on the vector.

[0339] In another embodiment, locus-specific insertion may be carried out by recombinase-site specific gene insertion. Briefly, bacterial recombinase enzymes, such as, but not limited to, PhiC31 integrase can act on "pseudo" recombination sites within the human genome. These pseudo recombination sites can be targets for locus-specific insertion using the recombinases. Recombinase-site specific gene insertion is described in Thyagarajan *et al., Mol. Cell Biol.* 21:3926 (2001). Other examples of recombinases and their respective sites that may be used for recombinase-site specific gene insertion include, but are not limited to, serine recombinases such as R4 and TP901-1 and recombinases described in WO 2006/083253.

[0340] In a further embodiment, the vector may comprise a chemo-resistance gene, *e.g.*, the multidrug resistance gene mdr1, dihydrofolate reductase, or O^6-alkylguanine-DNA alkyltransferase. The chemo-resistance gene may be under the control of a constitutive (*e.g.*, CMV) or inducible (*e.g.*, RheoSwitch®) promoter. In this embodiment, if it is desired to treat a disease in a subject while maintaining the modified cells within the subject, a clinician may apply a chemotherapeutic agent to destroy diseased cells while the modified cells would be protected from the agent due to expression of a suitable chemo-resistance gene and may continue to be used for treatment, amelioration, or prevention of a disease or disorder. By placing the chemo-resistance gene under an

inducible promoter, the unnecessary expression of the chemo-resistance gene can be avoided, yet it will still be available in case continued treatment is needed. If the modified cells themselves become diseased, they could still be destroyed by inducing expression of a lethal polypeptide as described below.

[0341] The methods of the invention are carried out by introducing the polynucleotides encoding the gene switch and the exogenous gene into cells of a subject. Any method known for introducing a polynucleotide into a cell known in the art, such as those described above, can be used.

[0342] When the polynucleotides are to be introduced into cells *ex vivo*, the cells may be obtained from a subject by any technique known in the art, including, but not limited to, biopsies, scrapings, and surgical tissue removal. The isolated cells may be cultured for a sufficient amount of time to allow the polynucleotides to be introduced into the cells, *e.g.*, 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, hours or more. Methods for culturing primary cells for short periods of time are well known in the art. For example, cells may be cultured in plates (*e.g.*, in microwell plates) either attached or in suspension.

[0343] For *ex vivo* therapeutic methods, cells are isolated from a subject and cultured under conditions suitable for introducing the polynucleotides into the cells. Once the polynucleotides have been introduced into the cells, the cells are incubated for a sufficient period of time to allow the ligand-dependent transcription factor to be expressed, *e.g.*, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 18, or 24 hours or more. At some point after the introduction of the polynucleotides into the cells (either before or after significant levels of the ligand-dependent transcription factor is expressed), the cells are introduced back into the subject. Reintroduction may be carried out by any method known in the art, *e.g.*, intravenous infusion or direct injection into a tissue or cavity. In one embodiment, the presence of the polynucleotides in the cells is determined prior to introducing the cells back into the subject. In another embodiment, cells containing the polynucleotides are selected (*e.g.*, based on the presence of a selectable marker in the polynucleotides) and only those cells containing the polynucleotides are reintroduced into the subject. After the cells are reintroduced to the subject, ligand is administered to the subject to induce expression of the therapeutic polypeptide or therapeutic polynucleotide. In an alternative embodiment, the ligand may be added to the cells even before the cells are reintroduced to the subject such that the therapeutic polypeptide or therapeutic polynucleotide is

expressed prior to reintroduction of the cells. The ligand may be administered by any suitable method, either systemically (*e.g.*, orally, intravenously) or locally (*e.g.*, intraperitoneally, intrathecally, intraventricularly, direct injection into the tissue or organ where the cells are reintroduced). The optimal timing of ligand administration can be determined for each type of cell and disease or disorder using only routine techniques.

[0344] The *in vivo* therapeutic methods of the invention involve direct *in vivo* introduction of the polynucleotides into the cells of the subject. The polynucleotides may be introduced into the subject systemically or locally (*e.g.*, at the site of the disease or disorder). Once the polynucleotides have been introduced to the subject, the ligand may be administered to induce expression of the therapeutic polypeptide or therapeutic polynucleotide. The ligand may be administered by any suitable method, either systemically (*e.g.*, orally, intravenously) or locally (*e.g.*, intraperitoneally, intrathecally, intraventricularly, direct injection into the tissue or organ where the disease or disorder is occurring). The optimal timing of ligand administration can be determined for each type of cell and disease or disorder using only routine techniques.

[0345] For *in vivo* use, the ligands described herein may be taken up in pharmaceutically acceptable carriers, such as, for example, solutions, suspensions, tablets, capsules, ointments, elixirs, and injectable compositions. Pharmaceutical compositions may contain from 0.01 % to 99% by weight of the ligand. Compositions may be either in single or multiple dose forms. The amount of ligand in any particular pharmaceutical composition will depend upon the effective dose, that is, the dose required to elicit the desired gene expression or suppression.

[0346] Suitable routes of administering the pharmaceutical preparations include oral, rectal, topical (including dermal, buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous, intratumoral, intradermal, intrathecal and epidural) and by naso-gastric tube. It will be understood by those skilled in the art that the preferred route of administration will depend upon the condition being treated and may vary with factors such as the condition of the recipient.

[0347] As used herein, the term "rAD.RheoIL12" refers to an adenoviral polynucleotide vector harboring the IL-12 gene under the control of a gene switch of the RheoSwitch® Therapeutic System (RTS), which is capable of producing IL-12 protein in the presence of activating ligand. As used herein, the term "rAd.cIL12" refers to an adenoviral

polynucleotide control vector containing the IL-12 gene under the control of a constitutive promoter.

[0348] As used herein, the term "IL-12p70" refers to IL-12 protein, which naturally has two subunits commonly referred to as p40 and p35. The term IL-12p70 encompasses fusion proteins comprising the two subunits of IL-12 (p40 and p35), wherein the fusion protein may include linker amino acids between subunits.

[0349] As used herein, the term "a protein having the function of an immunomodulator" refers to a protein that has at least 20% (*e.g.*, at least 30%, 40%, 50%, 60%, 70%, 80% or 90%) of any bioactivity of an immunomodulator selected from IL-1, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10R or a subunit thereof DN, IL-15, IL-18, IL-21, IL-23, IL-24, IL-27, GM-CSF, IFN-alpha, IFN-gamma, CCL3 (MIP-1a), CCL5 (RANTES), CCL7 (MCP3), XCL1(lymphotactin), CXCL1 (MGSA-alpha), CCR7, CCL19 (MIP-3b), CXCL9 (MIG), CXCL10 (IP-10), CXCL12 (SDF-1), CCL21 (6Ckine), OX40L, 4-1BBL, CD40, CD70, GITRL, LIGHT, b-Defensin, HMGB1, Flt3L, IFN-beta, TNF-alpha, dnFADD, BCG, TGF-alpha, PD-L1, TGFbRII DN, ICOS-L and S100. Likewise, the term "a protein having the function of IL-12" refers to a protein that has at least 20% (*e.g.*, at least 30%, 40%, 50%, 60%, 70%, 80% or 90%) of any bioactivity of human IL-12. The bioactivities of such immunomodulators are well known. See the following Table.

Table 4. Immunomodulators and their functions

Immunomodulator	**Function**
Cytokines	
Interleukin-1 (IL-1)	IL-1 is a cytokine produced by activated macrophages. IL-1 stimulates thymocyte proliferation by inducing IL-2 release, B-cell maturation and proliferation, and fibroblast growth factor activity. IL-1 proteins are involved in the inflammatory response.
Interleukin-2 (IL-2)	IL-2 is a family of cytokines, that is produced by T-cells in response to antigenic or mitogenic stimulation, this protein is required for T-cell proliferation and other activities crucial to regulation of the immune response. IL-2 can stimulate B-cells, monocytes, lymphokine-activated killer cells, natural killer cells, and glioma cells.
Interleukin-3 (IL-3)	IL-3 stimulates the proliferation of hematopoietic pluripotent progenitor cells. It is secreted by activated T cells to support growth and differentiation of T cells from the bone marrow in an immune response. The combined intratumoral Ad-mIL-3 gene therapy in combination with radiation therapy was shown to

Immunomodulator	Function
	significantly suppress tumor growth (Oh 2004).
Interleukin-4 (IL-4)	IL-4 is a cytokine that participates in at least several B-cell activation processes as well as of other cell types. It is a costimulator of DNA-synthesis. It induces the expression of class II MHC molecules on resting B-cells. It enhances both secretion and cell surface expression of IgE and IgG1. It also regulates the expression of the low affinity Fc receptor for IgE (CD23) on both lymphocytes and monocytes.
Interleukin-5 (IL-5)	IL-5 stimulates B cell growth and increase immunoglobulin secretion and induce tumor suppression (Nakashima 1993, Wu 1992).
Interleukin-7 (IL-7)	IL-7 is a cytokine that is a hematopoietic growth factor capable of stimulating the proliferation of lymphoid progenitors. It is important for proliferation during certain stages of B-cell maturation.
Interleukin-9 (IL-9)	IL-9 supports IL-2 independent and IL-4 independent growth of helper T-cells.
Interleukin-15 (IL-15)	IL-15 is a cytokine that stimulates the proliferation of T-lymphocytes. Stimulation by IL-15 requires interaction of IL-15 with components of IL-2R, including IL-2R beta and probably IL-2R gamma but not IL-2R alpha.
Interleukin-18 (IL-18)	IL-18 augments natural killer cell activity in spleen cells and stimulates interferon gamma production in T-helper type I cells.
Interleukin-21 (IL-21)	IL-21 is a cytokine with immunoregulatory activity. IL-21 may promote the transition between innate and adaptive immunity.
Interleukin-23 (IL-23)	IL-23 acts directly on DC to promote immunogenic presentation of tumor peptide and can I resulted in robust intratumoral CD8(+) and CD4(+) T-cell infiltration and induced a specific TH1-type response to the tumor in regional lymph nodes and spleen. (Hu 2006).
Interleukin-27 (IL-27)	IL-27 is a cytokine with pro- and anti-inflammatory properties, that can regulate T helper cell development, suppress T-cell proliferation, stimulate cytotoxic T cell activity, induce isotype switching in B-cells, and that has diverse effects on innate immune cells.
Intereukin-24 (IL-23)	IL-24 has been shown to suppress tumor growth (Susan 2004, Fisher 2003).
INF-alpha (IFNα)	IFN-alpha has anti-tumor function (Taqliaferri 2005).
Interferon beta 1 (IFNB1)	IFNB1 is a member of group of interferon proteins that bind to specific cell surface receptors (IFNAR), and stimulates both macrophages and natural killer (NK) cells to elicit an antiviral, antibacterial and anticancer activities.
Interferon gamma (IFN-gamma)	IFN-gamma is produced by lymphocytes activated by specific antigens or mitogens. IFN-gamma, in addition to having antiviral activity, has important immunoregulatory functions. It is a potent activator of macrophages, it has antiproliferative effects on transformed cells and it can potentiate the antiviral and antitumor effects of the type I interferons.
Tumor necrosis factor (TNF-alpha)	TNF-α is mainly secreted by macrophages and can induce cell death of certain tumor cell lines. It is a potent pyrogen, causing

Immunomodulator	Function
	fever by direct action or by stimulation of interleukin-1 secretion.
Chemokines	
Chemokine (C motif) ligand 1 (XCL1)	Chemokine (C motif) ligand 1 (XCL1, also known as Lymphotactin) is chemotactic for CD4+ and CD8+ T cells but not for monocytes, and induces a rise in intracellular calcium in peripheral blood lymphocytes. The combination of XCL1 with IL-2 and IL-12 can enhance immunotherapy and augment the antitumor response (Emtage 1999, Wang 2002).
CC chemokine ligand 3 (CCL3)	CC chemokine ligand 3 (CCL3), also known as macrophage inflammatory protein-1 (MIP-1), which is a so-called monokine (a type of cytokine produced primarily by monocytes and macrophages) that is involved in the acute inflammatory state in the recruitment and activation of polymorphonuclear leukocytes.
CCL5 (RANTES)	CCL5 (RANTES), is a chemoattractant for blood monocytes, memory T-helper cells and eosinophils. Causes the release of histamine from basophils and activates eosinophils. Binds to CCR1, CCR3, CCR4 and CCR5. One of the major HIV-suppressive factors produced by CD8+ T-cells.
CC chemokine ligand 7 (CCL7)	CCL7 is a chemotactic factor that attracts monocytes and eosinophils, but not neutrophils. CCL7 also augments monocyte anti-tumor activity. Also induces the release of gelatinase B.
Chemokine (CXC motif) ligand 9 (CXCL9)	CXCL9 is a cytokine that affects the growth, movement, or activation state of cells that participate in immune and inflammatory response. Chemotactic for activated T-cells.
Chemokine (C-X-C motif) ligand 10 (CXCL10)	Chemokine (C-X-C motif) ligand 10 (CXCL10) is a small cytokine with roles in chemoattraction for cells in the immune system, adhesion of T cells to endothelial cells, anti-tumor activity and angiogenesis.
Chemokine (C-X-C motif) ligand 12 (CXCL12)	Chemokine (C-X-C motif) ligand 12 (CXCL12), also known as stormal cell-derived factor 1 (SDF-1), is a small cytokine that belong to the intercrine family, members of which activate leukocytes and are often induced by proinflammatory stimuli such as LPS, TNF or IL1.
Chemokine (C-C motif) receptor 7 (CCR7)	CCR7 is the receptor for the MIP-3-beta chemokine. Probable mediator of EBV effects on B-lymphocytes or of normal lymphocyte functions.
Chemokine (C-C motif) ligand 19 (CCL19, also known as MIP-3β)	CCL19 plays a role not only in inflammatory and immunological responses but also in normal lymphocyte recirculation and homing. CCL19 has an important role in trafficking of T-cells in thymus, and T-cell and B-cell migration to secondary lymphoid organs. It specifically binds to chemokine receptor CCR7.
CC chemokine ligand 21 (CCL21)	CCL21 inhibits hemopoiesis and stimulates chemotaxis. CCL21 is chemotactic in vitro for thymocytes and activated T-cells, but not for B-cells, macrophages, or neutrophils.
Interleukin-8 (IL-8)	IL-8 is a chemotactic factor that attracts neutrophils, basophils, and T-cells, but not monocytes. It is also involved in neutrophil activation. It is released from several cell types in response to an inflammatory stimulus.
Growth Factors	
Granulocyte/macrophage	GM-CSF is a cytokine that stimulates the growth and

Immunomodulator	Function
colony-stimulating factor (GM-CSF)	differentiation of hematopoietic precursor cells from various lineages, including granulocytes, macrophages, eosinophils and erythrocytes.
FMS-related tyrosine kinase ligand (FLT3/FLK2 ligand, Flt3L)	FMS-related tyrosine kinase ligand (FLT3/FLK2 ligand, Flt3L), which may function as a growth factor receptor on hematopoietic stem cells or progenitor cells or both.
TGFA	TGF alpha is a mitogenic polypeptide that is able to bind to the EGF receptor and to act synergistically with TGF beta to promote anchorage-independent cell proliferation in soft agar.
Adjuvants	
Beta-defensin	Beta-defensins are antimicrobial peptides implicated in innate immune response against many Gram-negative and Gram-positive bacteria, fungi and viruses.
High-mobility group box-1 (HMGB1)	High-mobility group box-1 (HMGB1) proteins are nonhistone chromosomal proteins that function as cytokines, mediating local and systemic responses to necrotic cell death and cancer, invasion by pathogens, trauma, and sepsis.
S100	Phagocytic S100 proteins mediate inflammatory responses and recruit inflammatory cells to sites of tissue damage, and are members of Damage-associated molecular pattern (DAMP) molecules that are important for innate immunity.
Mannan	Mannan, a plant polysaccharide, that is a polymer of the sugar mannose, is useful for generation of a immune response.
Bacille Calmette-Guerin (BCG)	Bacille Calmette-Guerin (BCG), live attenuated Mycobacterium species, are used as vaccine against to prevent severe and fatal tuberculosis.
Bacterial lipopolysaccharides (LPS)	Bacterial lipopolysaccharides (LPS) are endotoxins that induces a strong immune response upon infection with Gram-negative bacteria.
Co-stimulatory Molecule (Positive)	
OX40 ligand	OX40 ligand (OX40L) belongs to tumor necrosis factor (ligand) superfamily member 4 (Tnfsf4), is expressed on dendritic cells and promotes Th2 cell differentiation.
4-1BB ligand (4-1BBL)	4-1BB ligand (4-1BBL) belongs to tumor necrosis factor (ligand) superfamily member 9 (Tnfsf9), which is a type 2 transmembrane glycoprotein and is expressed on activated T lymphocytes. 4-1BBL induces the proliferation of activated peripheral blood T-cells, and has a role in activation-induced cell death (AICD).
CD40	The CD40 protein belongs to the tumor necrosis factor receptor superfamily member 5, is essential in mediating a broad variety of immune and inflammatory responses including T cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation.
Glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR)	GITR can evoke effective tumor immunity via T cell stimulation. Administration of anti-GITR monoclonal antibody (mAb) can provoke potent tumor-specific immunity and eradicated established tumors without eliciting overt autoimmune disease.
GITR Ligand (GITRL)	GITRL is the ligand for GITR.
CD70	CD70 is a cytokine that binds to CD27. It plays a role in T-cell

Immunomodulator	Function
	activation. Induces the proliferation of costimulated T-cells and enhances the generation of cytolytic T-cells.
LIGHT (HSVgD)	Herpes virus entry mediator (HVEM) binding ligand (HSVgD), also referred to as p30, or LIGHT is a TNF family member involved in co-stimulation of T cells.
PD-L1 (also known as CD274)	PD-L1 (also known as CD274) protein is expressed in activated monocytes, T and B cells. PD-L1 is upregulated in monocytes upon treatment with IFN-gamma, and in dendritic cells and keratinocytes upon treatment with IFN-gamma, together with other activators.
ICOS-L	ICOS-L is a ligand for the T-cell-specific cell surface receptor ICOS and acts as a costimulatory signal for T-cell proliferation and cytokine secretion; induces also B-cell proliferation and differentiation into plasma cells.
Co-stimulatory Molecule (Negative)	
Anti-CTLA4	Cytotoxic T lymphocyte-associated 4 (CTLA4) is a member of the immunoglobulin superfamily and is a costimulatory molecule expressed in activated T cells.
Anti-PD-L1	Binding of a PD-1 receptor on a T-cell by PD-L1 transmits a negative costimulatory signal to the cell, which prevents the cells to progress through the cell cycle, and increases T cell proliferation. Inhibition of an interaction between PD-L1 and receptor on the T cell with an anti-PD-L1 antibody results in the downregulation of the immune response termed as immune cell anergy.
Anti-PD-L2	PD-L2 is involved in the costimulatory signal, essential for T lymphocyte proliferation and IFN-gamma production in a PDCD1-independent manner, but the ligand is known to primarily act through PD-1 resulting in anergic responses.
Counter Immune Suppressant (Tolerance Inhibitors)	
TGFR2DN	On ligand binding, TGFR2 forms a receptor complex consisting of two type II and two type I transmembrane serine/threonine kinases. Type II receptors phosphorylate and activate type I receptors which autophosphorylate, then bind and activate SMAD transcriptional regulators. Receptor for TGF-beta. Deletion of predicted serine/theronine kinase cytoplasmic domain (nucleotides 1172-2036 of TGFβR2 cDNA H2-3FF, available from public databases as accession number M85079 and amino acid sequence available as accession number AAA61164) impairs the all three TGF-β (1,2 and 3) dependent gene expressions.
Anti-TGFβ	TGFβ is a multifunctional peptide that controls proliferation, differentiation, and other functions in many cell types. TGFβ acts synergistically with TGFα in inducing transformation. It also acts as a negative autocrine growth factor. Dysregulation of TGFβ activation and signaling may result in apoptosis. administration of anti-TGFβ antibody can prevent renal insufficiency and glomerulosclerosis in the db/db mouse, a model of type II diabetes that develops overt nephropathy.
Anti-IL10	IL-10 is a cytokine produced by activated Th2 cells, B cells,

Immunomodulator	Function
	keratinocytes, monocytes, and macrophages. IL-10 is useful in promoting growth and differentiation of activated human B cells, inhibiting Th1 responses to prevent transplant rejection and T cell-mediated autoimmune diseases.
Anti-Suppressor of cytokine signaling1 (SOCS1)	Suppressor of cytokine signaling1 (SOCS1) is a critical inhibitor of interferon-gamma signaling and prevents the potentially fatal neonatal actions of this cytokine.
Anti-TGF-α	TGF-α is a mitogenic polypeptide that is able to bind to the EGF receptor and to act synergistically with TGF-β to promote anchorage-independent cell proliferation in soft agar.
Fas contain cytoplasmic Fas-associated protein with death domain (FADD)	FADD is essential for Fas and TNF-induced signaling for programmed cell death (apoptosis) and receptor oligomerization.

[0350] The bioactivities of IL-12 are also well known and include, without limitation, differentiation of naive T cells into Th1 cells, stimulation of the growth and function of T cells, production of interferon-gamma (IFN-gamma) and tumor necrosis factor-alpha (TNF-α) from T and natural killer (NK) cells, reduction of IL-4 mediated suppression of IFN-gamma, enhancement of the cytotoxic activity of NK cells and $CD8^+$ cytotoxic T lymphocytes, stimulation of the expression of IL-12R-β1 and IL-12R-β2, facilitation of the presentation of tumor antigens through the upregulation of MHC I and II molecules, and anti-angiogenic activity. The term "a protein having the function of IL-12" encompasses mutants of a wild type IL-12 sequence, wherein the wild type sequence has been altering by one or more of addition, deletion, or substitution of amino acids, as well as non-IL-12 proteins that mimic one or more of the bioactivities of IL-12.

[0351] As used herein, the terms "activating" or "activate" refer to any measurable increase in cellular activity of a gene switch, resulting in expression of a gene of interest (*e.g.*, selected from IL-1, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10R or a subunit thereof DN, IL-15, IL-18, IL-21, IL-23, IL-24, IL-27, GM-CSF, IFN-alpha, IFN-gamma, CCL3 (MIP-1a), CCL5 (RANTES), CCL7 (MCP3), XCL1(lymphotactin), CXCL1 (MGSA-alpha), CCR7, CCL19 (MIP-3b), CXCL9 (MIG), CXCL10 (IP-10), CXCL12 (SDF-1), CCL21 (6Ckine), OX40L, 4-1BBL, CD40, CD70, GITRL, LIGHT, b-Defensin, HMGB1, Flt3L, IFN-beta, TNF-alpha, dnFADD, TGF-alpha, PD-L1 RNAi, a PD-L1 antisense oligonucleotide, TGFbRII DN, ICOS-L and S100.

[0352] As used herein, the terms "treating" or "treatment" of a disease refer to executing a protocol, which may include administering one or more drugs or *in vitro* engineered cells

to a mammal (human or non-human), in an effort to alleviate signs or symptoms of the disease. Thus, "treating" or "treatment" should not necessarily be construed to require complete alleviation of signs or symptoms, does not require a cure, and specifically includes protocols that have only marginal effect on the subject.

[0353] As used herein, "immune cells" include dendritic cells, macrophages, neurophils, mast cells, eosinophils, basophils, natural killer cells and lymphocytes (*e.g.*, B and T cells).

[0354] As used herein, the terms "dendritic cells" and "DC" are interchangeably used.

[0355] As used herein, the term "therapy support cells" (TSC) are cells that can be modified (*e.g.*, transfected, electroporated, *etc.*) with the vector of the invention to deliver the one or more proteins having the function of an immunomodulator and, optionally, a protein having the function of IL-12, to tumor microenvironments. Such TSC include, but are not limited to, stem cells, fibroblasts, endothelial cells and keratinocytes.

[0356] As used herein, the terms "*in vitro* engineered immune cells" or "*in vitro* engineered population of immune cells" or "a population of engineered immune cells" or "immune cells expressing an immunomodulator" or "immune cells expressing IL-12" refer to immune cells, *e.g.*, dendritic cells, conditionally expressing an immunomodulator and/or IL-12 as the case may be under the control of a gene switch, which can be activated by an activating ligand.

[0357] As used herein, the terms "*in vitro* engineered TSC" or "*in vitro* engineered population of TSC" or "a population of engineered TSC" or "TSC expressing an immunomodulator" or "TSC expressing IL-12" refer to therapy support cells, *e.g.*, stem cells, fibroblasts, endothelial cells and keratinocytes, conditionally expressing an immunomodulator and/or IL-12 as the case may be under the control of a gene switch, which can be activated by activating ligand.

[0358] As used herein, the term "modified cell" refers to cells which have been altered by a process including, but not limited to, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation and lipofection (lysosome fusion).

[0359] As used herein, the terms "MOI" or "Multiplicity of Infection" refer to the average number of adenovirus particles that infect a single cell in a specific experiment (*e.g.*, recombinant adenovirus or control adenovirus)

[0360] As used herein, the term "tumor" refers to all benign or malignant cell growth and proliferation either *in vivo* or *in vitro*, whether precancerous or cancerous cells and/or tissues.

[0361] Examples of cancers that can be treated according to the invention include breast cancer, prostate cancer, lymphoma, skin cancer, pancreatic cancer, colon cancer, melanoma, malignant melanoma, ovarian cancer, brain cancer, primary brain carcinoma, head–neck cancer, glioma, glioblastoma, liver cancer, bladder cancer, non-small cell lung cancer, head or neck carcinoma, breast carcinoma, ovarian carcinoma, lung carcinoma, small-cell lung carcinoma, Wilms' tumor, cervical carcinoma, testicular carcinoma, bladder carcinoma, pancreatic carcinoma, stomach carcinoma, colon carcinoma, prostatic carcinoma, genitourinary carcinoma, thyroid carcinoma, esophageal carcinoma, myeloma, multiple myeloma, adrenal carcinoma, renal cell carcinoma, endometrial carcinoma, adrenal cortex carcinoma, malignant pancreatic insulinoma, malignant carcinoid carcinoma, choriocarcinoma, mycosis fungoides, malignant hypercalcemia, cervical hyperplasia, leukemia, acute lymphocytic leukemia, chronic lymphocytic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, chronic granulocytic leukemia, acute granulocytic leukemia, hairy cell leukemia, neuroblastoma, rhabdomyosarcoma, Kaposi's sarcoma, polycythemia vera, essential thrombocytosis, Hodgkin's disease, non-Hodgkin's lymphoma, soft-tissue sarcoma, mesothelioma, osteogenic sarcoma, primary macroglobulinemia, and retinoblastoma, and the like.

[0362] The invention provides engineering of cells, *e.g.*, immune cells and TSC, to conditionally express a protein having the function of an immunomodulator and, optionally, IL-12 and therapeutic uses and/or applications for the treatment of cancer or tumors or both. *In vitro* engineered immune cells and TSC that conditionally express a protein having the function of an immunomodulator and optionally IL-12 are a safe improvement over constitutive production of the protein(s). Additionally, the ability to control the timing and level of immunomodulator and optionally IL-12 expression provides improved control of the efficacy of the treatment. Therefore, *in vitro* engineered immune cells and TSC may be formulated into pharmaceutical compositions as therapeutics for the treatment of a cancer or a tumor in a human or a non-human organism. Alternatively, *in vitro* engineered populations of immune cells, TSC or subsets thereof may be used as vehicles to conditionally deliver an immunomodulator and

optionally IL-12 protein production to a specific area (normal tissue, cancer, or tumor) in the body of a human or non-human organism. The immune cells may be autologous or non-autologous dendritic cells. The dendritic cells may be isolated from bone marrow or from peripheral blood circulation. In human patients, dendritic cell populations may be isolated via a leukophoresis procedure, where a white blood cell fraction is isolated and removed and other blood components are re-infused to the patient.

[0363] In another embodiment, the dendritic cells may be prepared by transfecting human hematopoietic stem cells with a vector of the invention expressing a protein having the function of an immunomodulator and optionally a protein having the function of IL-12, and differentiating the transfected stem cell to give a dendritic cell. See U.S. Pat. 6,734,014.

[0364] In one embodiment, a nucleic acid adenoviral vector is provided containing a gene switch, wherein the coding sequences for VP16-RXR and Gal4-EcR are separated by the EMCV internal ribosome entry site (IRES) sequence are inserted into the adenoviral shuttle vector under the control of the human ubiquitin C promoter. The coding sequences for the p40 and p35 subunits of IL12 separated by an IRES sequence, and placed under the control of a synthetic inducible promoter, are inserted upstream of the ubiquitin C promoter.

[0365] In another embodiment, the invention provides a shuttle vector carrying transcription units (VP16-RXR and Gal4-EcR) for the two fusion proteins and inducible IL-12 subunits recombined with the adenoviral backbone (AdEasy1) in *E. coli* BJ5183 cells. After verifying the recombinant clone, the plasmid carrying the rAd.RheoIL12 genome is grown in and purified from XL10-Gold cells, digested off the plasmid backbone and packaged by transfection into HEK 293 cells.

[0366] In a particular embodiment, the resulting primary viral stock is amplified by re-infection of HEK 293 cells and is purified by CsCl density-gradient centrifugation.

[0367] In one embodiment the immunomodulator and/or IL-12 gene is a wild-type gene sequence. In another embodiment, the immunomodulator and/or IL-12 gene is a modified gene sequence, *e.g.*, a chimeric sequence or a sequence that has been modified to use preferred codons.

[0368] In one embodiment, the immunomodulator and/or IL-12 gene is the human wild type sequence. In another embodiment, the sequence is at least 85% identical to wild

type human sequence, *e.g.*, at least 90%, 95%, or 99% identical to wild type human sequence. In a further embodiment, the gene sequence encodes the human polypeptide. In another embodiment, the gene encodes a polypeptide that is at least 85% identical to wild type human polypeptide *e.g.*, at least 90%, 95%, or 99% identical to wild type human polypeptide.

[0369] In one embodiment, the IL-12 gene is the wild type mouse IL-12 sequence. In another embodiment, the sequence is at least 85% identical to wild type mouse IL-12, *e.g.*, at least 90%, 95%, or 99% identical to wild type mouse IL-12. In a further embodiment, the IL-12 gene sequence encodes the mouse IL-12 polypeptide. In another embodiment, the gene encodes a polypeptide that is at least 85% identical to wild type mouse IL-12, *e.g.*, at least 90%, 95%, or 99% identical to wild type mouse IL-12.

[0370] DC may be isolated from bone marrow from humans, mice, or other mammals. The dendritic cells may be isolated from the blood of humans, mice or other mammals. In human patients, dendritic cell populations may be isolated via a leukophoresis procedure as is known in the art, where a white blood cell fraction is isolated and removed and other blood components are re-infused to the patient. In one embodiment, DC are derived from murine bone marrow as previously described (Tatsumi *et al.*, 2003). Briefly, wild-type or EGFP Tg mouse bone marrow (BM) is cultured in conditioned medium (CM) supplemented with 1000 units/ml recombinant murine granulocyte/macrophage colony-stimulating factor and recombinant mIL-4 (Peprotech, Rocky Hill, NJ) at 37°C in a humidified, 5% CO_2 incubator for 7 days. $CD11c^+$ DC are then isolated, *e.g.*, using specific MACSTM beads, per the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). $CD11c^+$ DC produced in this manner are >95% pure based on morphology and co-expression of the CD11b, CD40, CD80, and class I and class II MHC antigens.

[0371] One embodiment of the invention provides engineered immune cells and TSC conditionally expressing a protein having the function of an immunomodulator and optionally IL-12 suitable for therapeutic applications for the treatment of cancer, or tumors or both as gene therapy in human or non-human organism. In an embodiment, the invention provides engineered immune cells and TSC containing the gene switch.

[0372] In another embodiment, the invention provides engineered immune cells and TSC containing at least a portion of an ecdysone receptor. In another embodiment, the invention provides engineered immune cells and TSC containing an ecdysone receptor-

based gene switch. In another embodiment, the invention provides engineered immune cells and TSC containing RheoSwitch. In another embodiment, the invention provides a kit comprising engineered immune cells and TSC containing a gene switch and a ligand that modulates the gene switch. In another embodiment, the kits further comprise a diacylhydrazine ligand. In another embodiment, the kit further comprises RG-115830 or RG-115932.

[0373] In one embodiment, the invention provides an engineered population of immune cells and TSC. In one embodiment, day 7 cultured DC are treated with recombinant adenovirus encoding an immunomodulator and/or IL-12 driven off a constitutive or inducible promoter, or are infected with mock, control adenovirus vector (rAdψ5), over a range of multiplicity of infection (MOIs). After 48 h, infected DC are harvested and analyzed for phenotype and for production of an immunomodulator and/or IL-12 using a specific ELISA kit (BD-PharMingen, San Diego, CA), with a lower level of detection of 62.5 pg/ml.

[0374] In another embodiment, the invention provides *in vitro* engineered population of immune cells and TSC comprising a vector, *e.g.*, a DNA vector, having a gene switch capable of conditionally expressing a protein having the function of an immunomodulator and/or IL-12, and further comprising activating ligand.

[0375] In a further embodiment, the invention provides a method of treating cancer, *e.g.*, melanoma or glioma, by administering engineered DC to a patient and then administering an activating ligand, such as RG-115819, RG-115830 or RG-115932, to said patient. The patient may be a human or an animal with cancer. The treatment methods and products, engineered cells, kits, and ligands have application in human therapy and in veterinary animal therapy. Therefore, the products and methods are contemplated to be used for human and veterinary animal purposes.

[0376] In one aspect, the invention provides a pharmaceutical composition suitable for administration to a human or a non-human comprising a population of *in vitro* engineered immune cells or TSC expressing a protein having the function of an immunomodulator and/or IL-12, wherein the formulation is suitable for administration by intratumoral administration. The invention further provides a pharmaceutical composition comprising an activating ligand, such as RG-115819, RG-115830 or RG-115932, wherein the

composition is suitable for administration by intraperitoneal, oral, or subcutaneous administration.

[0377] In the particular embodiment described herein, the invention provides a method for treating a tumor, comprising the steps in order of:

a. administering intratumorally in a mammal a population of an in vitro engineered immune cells or TSC; and

b. administering to said mammal a therapeutically effective amount of an activating ligand.

[0378] In one embodiment, the activating ligand is administered at substantially the same time as the *in vitro* engineered immune cells or TSC, *e.g.*, within one hour before or after administration of the cells. In another embodiment, the activating ligand is administered at or less than about 24 hours after administration of the *in vitro* engineered immune cells or TSC. In still another embodiment, the activating ligand is administered at or less than about 48 hours after the *in vitro* engineered immune cells or TSC. In another embodiment, the ligand is RG-115932. In another embodiment, the ligand is administered at a dose of about 1 to 50 mg/kg/day. In another embodiment, the ligand is administered at a dose of about 30 mg/kg/day. In another embodiment, the ligand is administered daily for a period of 7 to 28 days. In another embodiment, the ligand is administered daily for a period of 14 days. In another embodiment, about 1×10^6 to 1×10^8 cells are administered. In another embodiment, about 1×10^7 cells are administered.

[0379] In one embodiment, dendritic cells are engineered to conditionally express IL-2 and IL-12. IL-2 exerts potent immunoregulatory effects on effector and regulatory T, NK and NK-T cells. It is expected that expressing IL-2 and IL-12 in cells will result in reciprocal upregulation of each others receptor and induce different by complementary biological effects by virtue of separate signaling pathways. It is also expected that the combination of IL-2 and IL-12 will lengthen the duration of immune stimulation and reduce the effective dose of cells that may be more tolerated by the animal. See Dietrich 2002, Wigginton 2002, 2001, 1996 and Koyama, 1997, McDermott and Atkins 2008; Berntsen et al 2008; Tarhini et al 2008; Heemskerk et al 2008; Horton et al 2008. The polynucleotide sequences of IL-2 are available under accession numbers U25676 (human); NM_008366 (mouse); NM_204153 (chicken); and NM_053836 (rat). The polynucleotide sequences of IL-12 are available under accession numbers NM_000882

(human IL12A); NM_002187 (human IL12B); NM_008351 (mouse IL12a); NM_008352 (mouse IL12b); NM_213588 (chicken IL12A); NM_213571 (chicken IL12B); NM_053390 (rat IL12a); and NM_022611 (rat IL12b). SEQ ID NOS: 13, 15, 21 and 23 code for human and mouse IL-12 and subunits thereof.

[0380] In another embodiment, dendritic cells are engineered to conditionally express IL-18 and IL-12. IL-18 induces IFN-gamma production and promotes T helper cell development and NK activation. In addition, IL-18 can augment GM-CSF production and decrease IL-10 production. It is expected that expressing IL-18 and IL-12 will overcome the limitations observed when either cytokine is administered alone. It is expected that expression of IL-12 and IL-18 in dendritic cells will stimulate more vigorous tumor antigen-specific Th1 responses than when dendritic cells are transduced with either cytokine alone.

[0381] The intratumoral injection of DCs engineered to secrete both IL-12 and IL-18 mediated the highest levels of INF-γ production and complete tumor rejection (Tatsumi 2003). See, Vujanovic, 2006. See also Coughlin, 1998, Subleski, 206, Tatsumi, 2003, and Sabel, 2007; Shiratori et al 2007; Lian et al 2007; Iinuma et al 2006. See above for IL-12 polynucleotide sequences. The polynucleotide sequences of IL-18 are available under accession numbers U90434 (human); NM_008360 (mouse); EU747333 (chicken); and AY258448 (rat).

[0382] In another embodiment, dendritic cells are engineered to conditionally express IL-15 and IL-12. IL-15 shares some biologic activities with IL-2 that also makes it potentially useful for therapies against cancer. IL-15 stimulates the proliferation of NK cells and activated T cells, and supports the expansion of effector T cells. It has been reported that IL-15 presentation synergized with IL-12 for enhanced IFN-gamma production by NK cells. Koka, 2004; Basak 2008; Lasek et al 2004. Intratumoral delivery of IL-15 and IL-12 induced significant tumor regression in a melanoma model (Lasek 1999). See above for the IL-12 polynucleotide sequences. SEQ ID NOS: 11 and 19 code for the human and mouse IL-15. FIGs. 2 and 4 are plasmid maps for expression systems which may be used for the human and mouse IL-12 and IL-15.

[0383] In another embodiment, dendritic cells are engineered to conditionally express IL-21 and IL-12. IL-21 and its receptor shares sequence homology with IL-2 and IL-15. IL-21 promotes the expansion and maturation of NK cells. The biologic effects of IL-21

potentially synergize with IL-12 as treatment of NK cells with IL-21 results in a significant upregulation of IL-12 receptor. In addition, IL-21 can enhance IL-12 signal transduction and cooperated for increased IFN-gamma production. See above for IL-12 polynucleotide sequences. The polynucleotide sequences of IL-21 are available under accession numbers AF254069 (human); NM_021782 (mouse); NM_001024835 (chicken); and NM_001108943 (rat). SEQ ID NOS: 6, 7, 8, 9, and 17 code for human and mouse IL-21. SEQ ID NOS: 1 and 2 are polynucleotide constructs that code for mouse and human IL-12 and IL-21. FIGs. 7 and 8 are plasmid maps for expression systems which may be used to express human and mouse IL-12 and IL-21, respectively.

[0384] In another embodiment, dendritic cells are engineered to conditionally express TNF-alpha and IL-12. TNF-alpha is a potent activator of immune cells and mediates antitumor properties. In addition, TNF-alpha can synergize with IL-12 for enhanced expression of IFN-gamma and IL-12 receptor on T cells. In an animal study, application of both IL-12 and TNF-alpha resulted in tumor infiltration by DN8+ T cells, significant IFN-gamma production, and subsequent tumor regression. See Sabel, 2003, 2004, 2007, Taniguchi, 1998, Lasek, 2000; and Xia et al 2008. See above for IL-12 polynucleotide sequences. The polynucleotide sequences coding for TNF-alpha are available from under accession numbers X02910 (human); NM_013693 (mouse); and BC107671 (rat).

[0385] In another embodiment, dendritic cells are engineered to conditionally express IL-7 and IL-12. IL-7 is a member of the IL-2 family and is important for T cell and B cell lympophoiesis. IL-7 regulates the homeostasis of survival and proliferation of naïve and memory CD8+ T cells. IL-7 has been proved to enhance CTL generation against tumors. In addition, IL-12 acts directed on CD8+ T cells to enhance IL-7 mediated proliferation. Further, it has been reported that IL-7 and IL-12 synergistically enhance CD8+ T cell cytotoxicity. Mehrotra, 1995; Sharma et al 2003; Tirapu et al 2002. Thus, it is expected that IL-7 and IL-12 coexpression will provide more optimal antitumor responses. See above for polynucleotide sequences coding for IL-12. The polynucleotide sequences coding for IL-7 are available under accession numbers J04156 (human); NM_008371 (mouse); NM_001037833 (chicken); and NM_013110 (rat).

[0386] In another embodiment, dendritic cells are engineered to conditionally express GM-CSF and IL-12. GM-CSF regulates hematopoietic progenitor cell differentiation and proliferation, and plays a particularly important role in the maturation of professional

antigen presenting cells (APC) such as dendritic cells. GM-CSF also enhances the capacity of dendritic cells to process and present antigens. GM-CSF functions differently than IL-12 and both elicit significant antitumor responses in animal studies. The combination of IL-12 (T cell activation) and GM-CSF (dendritic cell activation) is expected to result in more potent antitumor immunity. In animal studies, GM-CSF in combination with IL-12 treatment significantly suppressed tumor growth in multiple cancer models. Wang, 2001; Chang, 2007; Jean, 2004; Nair, 2006; Hill 2002; Small et al 2007. In human trials, GM-CSF + IL-12 were used successfully for treating myeloma patients, where the combined actions of both cytokines led to a reduction in circulating B cells. Rasmussen, 2003; Hansson, 2007; Abdalla, 2007. It is expected that coexpression of GM-CSF and IL-12 in a single cell will avoid unwanted systemic effects such as reductions in circulating B cells. See above for polynucleotide sequences coding for IL-12. The polynucleotide sequences of GM-CSF are available under accession numbers M11734 (human); NM_009969 (mouse); EU520303 (chicken); NM_001037660 (rat Csf2ra); and NM_133555 (rat Csf2rb).

[0387] In another embodiment, dendritic cells are engineered to conditionally express a chemokine (*e.g.*, CCL3 (MIP-1a), CCL5 (RANTES), CCL7 (MCP3), XCL1 (lymphotactin), CCL19 (MIP-3b), CXCL9 (MIG), CXCL10 (IP-10), CXCL12 (SDF-1), or CCL21 (6Ckine)) and IL-12. Chemokines are chemoattractant cytokines that regulate the trafficking and activation of leukocytes and other cell types under a variety of inflammatory and noninflammatory conditions. Inflammatory cytokines control the recruitment of leukocytes in inflammation and tissue injury. Homeostatic chemokines fulfill housekeeping functions such as navigating leukocytes (*e.g.*, dendritic cells) to and within secondary lymphoid organs as well as in bone marrow and the thymus during hematopoiesis. In animal studies, intratumoral co-injection of two separate adenoviruses expressing IL-12 and CXCL10 led to 100% regression of tumor nodules derived from the CT26 murine colorectal adenocarcinoma cell line. Narvaiza *et al.*, 2000. Emtage *et al.*, 1999, describe two double recombinant adenovirus vectors expressing either IL2 and XCL1 (lymphotactin) or IL-12 and XCL1. Intratumoral injection of the vectors breast adenocarcinoma tumors in mice elicited potent antitumor responses and gave rise to protective immunity. In other animal studies, co transduction of adenoviral vectors expressing IL-12 and CCL27 resulted in tumor regression and long term specific

immunity. Gao *et al.,* 2007. Thus, it is expected that the coexpression of a chemokine and IL-12 according to the invention will result in synergistic antitumor activity.

[0388] In another embodiment, dendritic cells are engineered to conditionally express an antiangiogenic cytokine (*e.g.*, IP-10 and Mig) and IL-12. IP-10 and Mig are chemoattractants for T cells and NK cells and their ability to inhibit angiogenesis is dependent on NK cells. Animal studies have shown that combination therapy with two adenoviruses, one expressing IP10 and another expressing IL-12, resulted in marked antitumoral synergy. Narvaiza *et al.,* 2000. In other studies, adenovirus vectors expressing IP10 or MIG and/or IL-12 were administered intratumorally in a murine model of mammary adenocarcinoma and fibrosarcoma. It was found that administration of IP-10 or MIG in combination with IL-12 resulted in considerable tumor regression and increased survival time of tumor-bearing animals as compared to IP10, MIG, IL-12 alone or control treated animals, with the IP-10, IL12 combination being most effective. Palmer, 2001. See also Mazzolini, 2003; and Huang 2004. Thus, it is expected that the coexpression of an antiangiogenic cytokine and IL-12 will result in synergistic antitumor activity.

[0389] To demonstrate an effective IL-12-mediated gene therapy, a conditional cDNA expression system is used that allows one to turn on an immunomodulator and/or IL-12 production by immune cells or TSC at various time points post-intratumoral injection. Based on the results in the aggressive B16 melanoma model in C57BL/6 mice, the following conclusions are made: 1) elevated levels of IL-12 are secreted from DC.RheoIL12 in the presence of the activating ligand RG-115830 but not in the absence of the ligand; 2) intratumoral DC.RheoIL12-based therapy is as effective as intratumoral DC.cIL12-based therapy as long as RG-115830 is administered to treated animals within 24 h of DC injection (and at later time points of ligand provision, RG-115830 therapy fails); 3) IL-12 expression in DC appears to prolong the survival of these cells in the tumor microenvironment and is associated with higher numbers of intratumorally-injected DC that migrate to tumor-draining lymph nodes; and 4) the strongest immune correlate to therapy outcome is the level of tumor-specific $CD8^+$ T cells cross-primed by the therapy and not the number of injected DC sustained in the tumor microenvironment. Overall, these data suggest that DC.IL12-based therapies likely succeed based on their positive influence on the afferent (cross-priming) of Type-1 $CD8^+$ T cell effectors and not on later

efferent events, such as injected DC-mediated recruitment of anti-tumor T cells into the tumor microenvironment, *etc.*

[0390] Prior to intratumoral injection, the cells (immune cells or TSC) may be treated with a factor to stimulate the activity of the cells. For example, the cells may be treated with a co-stimulatory molecule such as positive co-stimulatory molecule including OX40L, 4-1BBL, CD40, CD40L, GITRL, CD70, LIGHT or ICOS-L or a negative co-stimulatory molecule such as anti-CTLA4, anti-PD-L1 or anti-PD-L2 antibodies. For example, the cells (*e.g.*, immune cells or TSC) may be incubated with a cell expressing one or more co-stimulatory molecule, *e.g.*, J588 lymphoma cells expressing CD40 ligand molecule. In another embodiment, the cells (immune cells or TSC) may be treated with a counter immune suppressant molecule (tolerance inhibitor) such as anti-TGF-beta antibodies (for inhibiting TGF signaling within the microenvironment), anti-IL10 antibodies, TGFbRII DN (to inhibit TGF signaling within gene modified cells), IL-10R DN, dnFADD (to inhibit cell death pathways within the cells), anti-SOCS1 antibodies, siRNA or decoy (to inhibit suppressive cytokine signaling within the cells), or anti-TGFa antibodies.

[0391] The recombinant adenoviruses carrying the polynucleotide sequences shown in Figures 1-8 are produced. For example, hIL-21 is produced by cotransfection of the hIL-21 expression vector, linearized by restriction digestion at a site upstream of the left ITR, and the appropriate (example E3 deleted) adenoviral backbone in a permissive cell line such as HEK293 cells. The adenoviral vector carrying the murine immunomodulatory genes is used for transduction of murine dendritic cells or TSC for use in murine therapeutic models. For human therapeutic application, a polynucleotide encoding the human homologue of the immunomodulatory gene is inserted in the appropriate vector. The adenoviral vector for human therapeutic application is produced under GMP conditions. Example of a treatment outline (clinical trial) for stage III/IV melanoma patients is as follows: The treatment in this case involves an intratumoral injection of the adenoviral transduced dendritic cells and 14 daily oral administration of the activator drug (ligand). Subjects are screened 30 days to one week prior to the clinical trial. Each subject is asked to sign an informed consent before any procedures are initiated. The investigator will inform all subjects of the nature, aims, duration, potential hazards, and procedures to be performed during the trial and the possibility that their medical records may be

reviewed by FDA. Subjects (a total of 16 to 20) are randomly grouped into 4 cohorts. All cohorts will receive an intratumoral injection of up to $5x10^7$ transduced dendritic cells approximately 3 hours after the first dose of oral administration of the ligand. The 4 cohorts differ in the daily oral dose of ligand received: example cohort 1= 0.01mg/kg; cohort 2= 0.3 mg/kg; cohort 3= 1 mg/kg; cohort 4=3 mg/kg. During the course of the treatment, blood is drawn at specified time intervals for evaluation of single dose and steady state pharmacokinetics of the Activator Drug and its major metabolites. Also, blood is drawn at specified time points for the evaluation of humoral and cellular immune responses against the viral vector, RTS components and the tumor. Urine is collected and blood drawn at specific time points for serum chemistry, urinalysis, and hematology (safety profile). Tumor and/or draining lymph node biopsies are taken at specified time points to assess the transgene expression and the immune response to the tumor as a result of the therapy. Criteria for early termination are established for patients in case of adverse events, and the adverse events are recorded. The patients are followed up at 1, 2, 3 and 4 months for adverse events and therapeutic outcome.

[0392] In another embodiment, a subject in need of treatment of a tumor is (a) administered dendritic cells engineered to express an immunomodulator, for example, an immunomodulator disclosed herein, either consitutively or conditionally, and (b) a vector expressing an immunomodulator, for example, an immunomodulator disclosed herein, either constitutively or conditionally, is injected intratumorally to the subject. In a preferred embodiment, the dentritic cells are engineered to express an Ad-immunomodulator vector, and particularly the Ad-RTS-immunomodulator vector. In another preferred embodiment, the vector that is injected intratumorally to the subject is an Ad-immunomodulator vector, and particularly the Ad-RTS-immunomodulator vector.

[0393] In another embodiment, a subject in need of treatment of a tumor is (a) administered dendritic cells engineered to express IL-12, either consitutively or conditionally, and (b) a vector expressing IL-12, either constitutively or conditionally, is injected intratumorally to the subject. In a preferred embodiment, the dentritic cells are engineered to express an Ad-IL-12 vector, and particularly the Ad-RTS-IL-12 vector. In another preferred embodiment, the vector that is injected intratumorally to the subject is an Ad-IL-12 vector, and particularly the Ad-RTS-IL-12 vector.

[0394] In another embodiment, a subject in need of treatment of a tumor is (a) administered dendritic cells engineered to express IL-12, either consitutively or conditionally, and (b) the subject is administered one or more anticancer chemotherapeutic agents. In a preferred embodiment, the engineered dentritic cells are engineered to express an Ad-IL-12 vector, and particularly the Ad-RTS-IL-12 vector. The one or more anticancer chemotherapeutic agents can be administered prior to the engineered dendritic cells are administered, after the engineered dendritic cells are administered, or concurrently with the administration of the engineered dendritic cells. In a preferred embodiment, the anticancer chemotherapeutic is paclitaxel, a paclitaxel derivative or analog, temozolomide, a temozolomide derivative or analog, sunitinib, a sunitinib derivative or analog, gemcitabine, or a gemcitabine derivative or analog.

[0395] In another embodiment, a subject in need of treatment of a tumor is (a) administered dendritic cells engineered to express IL-12, either consitutively or conditionally, (b) a vector expressing IL-12, either constitutively or conditionally, is injected intratumorally to the subject, and (c) the subject is administered one or more anticancer chemotherapeutic agents. In a preferred embodiment, the dentritic cells are engineered to express an Ad-IL-12 vector, and particularly the Ad-RTS-IL-12 vector. In another preferred embodiment, the vector that is injected intratumorally to the subject is an Ad-IL-12 vector, and particularly the Ad-RTS-IL-12 vector. The one or more anticancer chemotherapeutic agents can be administered prior to the engineered dendritic cells and the vector expressing IL-12 are administered, after the engineered dendritic cells and vector expressing IL-12 are administered, or concurrently with the administration of the engineered dendritic cells and the vector expressing IL-12. In a preferred embodiment, the anticancer chemotherapeutic is paclitaxel, a paclitaxel derivative or analog, temozolomide, a temozolomide derivative or analog, sunitinib, a sunitinib derivative or analog, gemcitabine, or a gemcitabine derivative or analog.

[0396] In another embodiment, a subject in need of treatment of a tumor is (a) administered dendritic cells engineered to express an immunomodulator, for example, an immunomodulator disclosed herein, either consitutively or conditionally, and (b) a vector expressing an immunomodulator, for example, an immunomodulator disclosed herein, either constitutively or conditionally, is injected intratumorally to the subject. In a preferred embodiment, the dentritic cells are engineered to express an Ad-

immunomodulator vector, and particularly the Ad-RTS-immunomodulator vector. In another preferred embodiment, the vector that is injected intratumorally to the subject is an Ad-IL-immunomodulator vector, and particularly the Ad-RTS-immunomodulator vector.

[0397] In another embodiment, a subject in need of treatment of a tumor is (a) administered dendritic cells engineered to express an immunomodulator, for example, an immunomodulator disclosed herein, either consitutively or conditionally, and (b) the subject is administered one or more anticancer chemotherapeutic agents. In a preferred embodiment, the engineered dentritic cells are engineered to express an Ad-immunomodulator vector, and particularly the Ad-RTS-immunomodulator vector. The one or more anticancer chemotherapeutic agents can be administered prior to the engineered dendritic cells are administered, after the engineered dendritic cells are administered, or concurrently with the administration of the engineered dendritic cells. In a preferred embodiment, the anticancer chemotherapeutic is paclitaxel, a paclitaxel derivative or analog, temozolomide, a temozolomide derivative or analog, sunitinib, a sunitinib derivative or analog, gemcitabine, or a gemcitabine derivative or analog.

[0398] In another embodiment, a subject in need of treatment of a tumor is (a) administered dendritic cells engineered to express an immunomodulator, for example, an immunomodulator disclosed herein, either consitutively or conditionally, (b) a vector expressing an immunomodulator, for example, an immunomodulator disclosed herein, either constitutively or conditionally, is injected intratumorally to the subject, and (c) the subject is administered one or more anticancer chemotherapeutic agents. In a preferred embodiment, the dentritic cells are engineered to express an Ad-immunomodulator vector, and particularly the Ad-RTS-immunomodulator vector. In another preferred embodiment, the vector that is injected intratumorally to the subject is an Ad-immunomodulator vector, and particularly the Ad-RTS-immunomodulator vector. The one or more anticancer chemotherapeutic agents can be administered prior to the engineered dendritic cells and the vector expressing the immunomodulator are administered, after the engineered dendritic cells and vector expressing the immunomodulator are administered, or concurrently with the administration of the engineered dendritic cells and the vector expressing the immunomodulator. In a preferred embodiment, the anticancer chemotherapeutic is paclitaxel, a paclitaxel derivative or

analog, temozolomide, a temozolomide derivative or analog, sunitinib, a sunitinib derivative or analog, gemcitabine, or a gemcitabine derivative or analog.

[0399] In any of the methods of the present invention, the disease or disorder may be a disease or disorder disclosed in the present application. In one embodiment, the disease or disorder is a disease or disorder listed in Table 1 herein. In another embodiment, the disease or disorder is a disease or disorder listed in Table 3 herein.

[0400] In any of the methods of the present invention, the cancer or tumor may be a disease or disorder disclosed in the present application. In one embodiment, the cancer or tumor is a cancer or tumor listed in Table 1 herein. In another embodiment, the cancer or tumor is a cancer or tumor listed in Table 3 herein.

[0401] It is possible to measure the effect of an immunomodulator and/or IL-12 expression on a population of cells by measuring the level of expression or activity of the Th1/Tc1 type cytokine, IFN-gamma in a biological sample from a patient.

[0402] For the purposes of the invention, the invention provides a method for determining the efficacy of an *in vitro* engineered immune- or TSC-based therapeutic regimen in a cancer patient, comprising:

a. measuring the level of expression or the level of activity or both of interferon-gamma (IFN-gamma) in a first biological sample obtained from a human patient before administration of in vitro engineered cells, e.g., immune cells or TSC, thereby generating a control level;

b. administering intratumorally to said patient the in vitro engineered cells;

c. administering to said patient an effective amount of activating ligand;

d. measuring the level of expression or the level of activity or both of IFN-gamma in a second biological sample obtained from said patient at a time following administration of said activating ligand, thereby generating data for a test level; and

e. comparing the control level to the test level of IFN- gamma, wherein data showing an increase in the level of expression, activity, or both of IFN- gamma in the test level relative to the control level indicates that the therapeutic treatment regimen is effective in said patient. The invention may also optionally comprise the additional steps of

f. taking biopsy and counting tumor infiltrating lymphocytes (TIL) and/or

g. observing tumor regression in response to the treatment.

[0403] The term "subject" means an intact insect, plant or animal. It is also anticipated that the ligands will work equally well when the subject is a fungus or yeast. Animals for use with the invention include, but are not limited to, vertebrates, *e.g.*, mammals such as humans, rodents, monkeys, and other animals, with humans or mice being more preferred. Other animals include veterinary animals such as dogs, cats, horses, cattle, sheep, goats, pigs and the like.

[0404] Without wishing to be bound by theory, it is expected that the invention will support the use of intratumorally administered *in vitro* engineered immune- and TSC based gene therapy in the clinical setting, focusing on the objective clinical response as a primary study endpoint, and cross-primed anti-tumor $CD8^+$ T cells (producing IFN-gamma) as a secondary study endpoint. The ability to turn the immunomodulator and/or IL-12 expression on and off *in vivo* adds an element of safety and therapeutic control to the treatment in that both the timing and level of protein expression may be controlled by the administration of ligand, and further that the timing of immunomodulator and/or IL-12 expression is expected to be critical to the therapeutic effectiveness of the method.

[0405] The invention further supports the therapeutic applications of *in vitro* engineered cells with conditionally expressed genes of interest as innovative approaches for the effective and efficient treatment of human diseases.

[0406] In the event of conflict between any teaching or suggestion of any reference cited herein and the specification, the latter shall prevail, for purposes of the invention.

[0407] All patents, patent applications and publications cited herein are fully incorporated by reference in their entireties.

[0408] It is to be understood that the foregoing described embodiments and exemplifications are not intended to be limiting in any respect to the scope of the invention, and that the claims presented herein are intended to encompass all embodiments and exemplifications whether or not explicitly presented herein.

[0409] U.S. Application No. 12/247,738, entitled "Engineered Dendritic Cells And Uses For Treatment Of Cancer," filed October 8, 2008, is hereby incorporated by reference in its entirety. U.S. application no. 12/241,018, entitled "Therapeutic Gene-Switch Constructs And Bioreactors For The Expression Of Biotherapeutic Molecules, And Uses

Thereof," filed September 29, 2008, is also hereby incorporated by reference in its entirety.

EXAMPLE 1

[0410] A study is undertaken to determine the dose of dendritic cells and the most effective cytokine that is able to induce tumor-specific immune responses and antitumor activty in a Renca renal cell cancer tumor model

[0411] Two tumor cell lines are used in this study: Renca and Renca-HA. The latter cell line is made by transfection of Renca cells with influenza virus hemagglutinin (HA). The advantage of Renca-HA model is the ability to trace antigen-specific T cells, since both CD8 and CD4 specific HA-derived epitopes are known and have been used.

[0412] Specific Aim - determine the induction of HA-specific immune responses after intratumoral administration of dendritic cells.

[0413] The Renca-HA tumor is established subcutaneously in BALB/c mice. When the tumor becoms palpable, dendritic cells are injected intratumorally. Dendritic cell administration is be repeated twice at 7-day intervals, for a total of 3 administrations.

[0414] The following groups of mice are used (each group includes 3 mice):

1. Untreated mice;
2. Mice treated with $5x10^5$ dendritic cells transduced with control plasmid;
3. Mice treated with 10^6 dendritic cells transuced with control plasmid;
4. Mice treated with $5x10^6$ dendritic cells transduced with control plasmid;
5. The same as groups 2-4 using dendritic cells transduced with IL-12;
6. The same as groups 2-4 using dendritic cells transduced with IL-15; and
7. The same as groups 2-4 using dendritic cells transduced with IL-21.

To test the effect of combination of different cytokines, mice are treated simultaneously with:

8. $5x10^5$ dendritic cells transduced with IL-12, and $5x10^5$ dendritic cells transduced with IL-15,

9. $5x10^5$ dendritic cells transduced with IL-12 and $5x10^5$ dendritic cells transduced with IL-21, and

10. $5x10^5$ dendritic cells transduced with IL-15 and $5x10^5$ dendritic cells transduced with IL-21.

[0415] Four days after the last administration, lymph nodes of tumor-bearing mice are collected, and cells are stimulated with either MHC class I matched peptide (to detect $CD8^+$ T cell responses) or MHC class II matched peptide (to detect $CD4^+$ T cell responses).

[0416] The following assays are used:

[0417] 1. ELISPOT IFN-γ and IL-2;

2. T-cell proliferation;

3. Detection of TNFα, IL-10, IL-4, and GM-CSF release by lymph node cells.

[0418] In addition, NK activity of lymph node cells is evaluated using YAC cells as targets.

[0419] In parallel, cells are stimulated with anti-CD3/CD28 antibodies to evaluate non-specific response of T cells.

[0420] The most effective dose of dendritic cells capable of inducing antigen-specific immune responses are determined.

[0421] Specific Aim 2 - evaluate antitumor activity of dendritic cells transduced with cytokine genes.

[0422] Only those cytokine transduced dendritic cells that demonstrated statistically significant induction of immune responses are used in further experiments.

[0423] Treatment of Renca-HA tumor-bearing mice is performed as described in specific aim 1. One dose of DCs transduced with cytokines that shows specific activity in previous experiments is used. As a control, dendritic cells transduced with control adenovirus are used. To achieve statistical significance, each group includes 10 mice.

[0424] Tumor growth is evaluated. Renca-HA tumor contains an immunogeneic epitope that is useful for immunological monitoring and intitial testing of antitumor effect. However, to verify potential antitumor activity of the treatment non-transfected tumor cells needs to be used. Therefore, the experiments described above are repeated using the Renca tumor model.

EXAMPLE 2

[0425] The safety, tolerance, transgene function, and immunological effects of intratumoral injection(s) of adenoviral transduced autologous dendritic cells engineered to express hIL-12 and one or more other immunodulators under control of the RTS, in

subjects with stage III and IV melanoma will be evaluated through procedures such as those described below.

[0426] A study involving study subjects with stage III and IV melanoma will be conducted in 4 cohorts (groups) of subjects each subject receiving a single intratumoral injection (into a melanoma tumor) of adenoviral transduced autologous (reinserted into the same subject that they came from) dendritic cells (DCs) engineered to express human interleukin-12 (hIL-12), and one or more other immunodulators, at a dose of 5 x 10^7 in combination with daily oral doses of activator drug (activating ligand). The study will use injections of dendritic cells transduced *ex vivo* (after the cells are removed from the subjects) with adenoviral vector for inducible expression of human IL-12 and one or more other immunodulators. The production off IL-12 and the one or more or other immunomodulators is "turned on" (induced) from the injected DCs through the activation of the RTS by the oral administration of the activator drug (RG-115932). Safety and tolerance will be assessed through physical examinations (including ECOG performance status), vital signs measurements, serum chemistry, urinalysis, hematology, adverse events "side-effects", and antibodies and cellular immune response to the adenovirus, components of RTS, and the Activator Drug. To evaluate progress, single dose and steady-state pharmacokinetics/ADME of oral Activator Drug and its major metabolites, analysis of hIL-12 levels, other immunomodulator levels, and cellular immune response (T cells) in biopsies of the target tumors, draining lymph nodes, and peripheral circulation, as well as a serum cytokine profile will be measured.

[0427] For instance, 16 subjects with stage III and IV melanoma are divided into four cohorts with cohorts 1 and 2 containing three subjects and cohorts 3 and 4 containing 5 subjects. All subjects will receive a single intratumoral injection of $5x10^7$ autologous DC transduced with adenoviral vector encoding human IL-12 and one or more other immunodulators under the RTS control. For example, the subjects are administered an intratumoral injection of autologous DC transduced with adenoviral vector encoding human IL-12 under the RTS control and an immunomodulator such as IL-15 or IL-21.

[0428] The subjects will receive a single daily oral dose of activator drug (cohort 1: 0.01 mg/kg, cohort 2: 0.1 mg/kg, cohort 3: 1.0 mg/kg or cohort 4: 3 mg/kg) the first dose starting approximately 3 hours prior to the DC injection on day 1 and continuing for 13 more consecutive days. Additional injection(s) of adenovirally transduced autologous

dendritic cells in combination with 14 single (once) daily oral doses of activator drug may be administered to eligible subjects who meet the criteria for retreatment. Safety, tolerance, and dendritic cell function are assessed for all subjects in each group of cohort 1 for up to one month after injection of the *in vitro* engineered dendritic cells before enrolling subjects to receive the next highest dose of the activator drug. The safety assessment will continue in all subjects for 3 months after the initial injection of the engineered dendritic cells with the possibility of extending the follow-up period to a total of six months to monitor subject safety if toxicity is observed or the subject receives additional injection(s) of the dendritic cells.

[0429] Such a study demonstrates the safety and tolerance of a single or multiple intratumoral injection(s) of adenoviral transduced autologous dendritic cells in combination with an oral activator drug in subjects with melanoma. The study provides steady-state pharmacokinetics/ADME of the oral activator drug. The study demonstrates functionality of the RTS in subjects by measuring hIL-12 expression and the expression of the one or more other immunomodulators of adenovirus transduced autologous dendritic cells in target tumor and/or draining lymph nodes in response to the activation of the RTS by the oral administration of the activator drug. Furthermore, the study demonstrates the immunological effects of the adenoviral transduced autologous dendritic cells in terms of the cellular immune response in the target tumor, draining lymph nodes, and peripheral circulation following oral administration of the activator drug.

[0430] Melanoma is selected as an exemplary cancer. Melanoma in particular among solid tumors has been shown to respond to immunotherapy approaches, and melanoma tumors are readily accessible for intratumoral injection and biopsy. The subjects included in the study have unresectable stage III or IV melanoma, which has at least 0.5 cm in diameter, any tumor thickness, any number of lymph node involvement, in-transit metastases, or distant metastases.

Preparation of Adenovirus Harboring the RheoSwitch Therapeutic System, hIL-12 and One or More Other Immunomodulatiors

[0431] The recombinant DNA is transferred to dendritic cells (DC) by *ex vivo* adenoviral vector transduction. The recombinant DNA is used to express human IL-12(p70) and one or more other immunodulators from intratumorally injected immature dendritic cells

which confers survival and stimulates maturation of DC in the tumor environment resulting in their subsequent migration to the draining lymph nodes. This leads to a bias toward the differentiation of T helper cells to Th1 type and also activation of tumor-specific cytotoxic T cells by cross priming with the tumor antigens.

[0432] The recombinant DNA used as the recombinant adenoviral vector allows the expression of human IL-12 and one or more other immunodulators under the control of the RheoSwitch® Therapeutic System (RTS). The RTS comprises a bicistronic message expressed from the human Ubiquitin C promoter and codes for two fusion proteins: Gal4-EcR and VP16-RXR. Gal4-EcR is a fusion between the DNA binding domain (amino acids 1-147) of yeast Gal4 and the DEF domains of the ecdysone receptor from the insect *Choristoneura fumiferana*. In another embodiment, the RTS consists of a bicistronic message expressed from the human Ubiquitin C promoter and codes for two fusion proteins: Gal4-EcR and VP16-RXR. Gal4-EcR is a fusion between the DNA binding domain (amino acids 1-147) of yeast Gal4 and the DEF domains of the ecdysone receptor from the insect *Choristoneura fumiferana*. VP16-RXR is a fusion between the transcription activation domain of HSV-VP16 and the EF domains of a chimeric RXR derived from human and locust sequences. These Gal4-EcR and VP16-RXR sequences are separated by an internal ribosome entry site (IRES) from EMCV. These two fusion proteins dimerize when Gal4-EcR binds to a small molecule drug (RG-115932) and activate transcription of hIL-12 and one or more other immunodulators from a Gal4-responsive promoter that contains six Gal4-binding sites and a synthetic minimal promoter. The RTS transcription unit described above is placed downstream of the hIL-12 and one or more other immunodulators transcription units. This whole RTS-hIL12-immunomodualtor cassette is incorporated into the adenovirus 5 genome at the site where the E1 region has been deleted. The adenoviral backbone also lacks the E3 gene. A map for the adenoviral vector Ad-RTS-hIL-12 is shown in FIG. 8 of US 2009/0123441 A1.

[0433] The recombinant adenoviral vector used in this study contains the following exemplary regulatory elements in addition to the viral vector sequences: Human Ubiquitin C promoter, Internal ribosome entry site derived from EMCV, an inducible promoter containing 6 copies of Gal4-binding site, 3 copies of SP-1 binding sites, and a synthetic minimal promoter sequence, SV40 polyadenylation sites, and a transcription

termination sequence derived from human alpha-globin gene. It should be understood that other regulatory elements could be utilized as alternatives.

[0434] An exemplary recombinant adenoviral vector Ad-RTS-hIL-12-immunomodulator(s) is produced in the following manner. The coding sequences for the receptor fusion proteins, VP16-RXR and Gal4-EcR separated by the EMCV-IRES (internal ribosome entry site), are inserted into the adenoviral shuttle vector under the control of the human ubiquitin C promoter (constitutive promoter). Subsequently, the coding sequences for the p40 and p35 subunits of hIL-12 separated by IRES, and one or more other immunomodulators, is placed under the control of a synthetic inducible promoter containing 6 copies of Gal4-binding site are inserted upstream of the ubiquitin C promoter and the receptor sequences. The shuttle vector contains the adenovirus serotype 5 sequences from the left end to map unit 16 (mu16), from which the E1 sequences are deleted and replaced by the RTS, IL-12 and one or more other immunomodulator sequences (RTS- hIL-12). The shuttle vector carrying the RTS-hIL-12-immunodulator(s) is tested by transient transfection in HT-1080 cells for Activator Drug-dependent IL-12 and other immunomodulator(s) expression. The shuttle vector is then recombined with the adenoviral backbone by cotransfection into HEK 293 cells to obtain recombinant adenovirus Ad-RTS-hIL-12-immunomodulator(s). The adenoviral backbone contains sequence deletions of mu 0 to 9.2 at the left end of the genome and the E3 gene. The shuttle vector and the adenoviral backbone contain the overlapping sequence from mu 9.2 to mu 16 that allows the recombination between them and production of the recombinant adenoviral vector. Since the recombinant adenoviral vector is deficient in the E1 and E3 regions, the virus is replication-deficient in normal mammalian cells. However, the virus can replicate in HEK 293 cells that harbor the adenovirus-5 E1 region and hence provide the E1 function in trans.

[0435] An exemplary recombinant adenoviral vector is produced in the following manner: The linearized shuttle vector carrying the DNA elements for inducible expression of human IL12 and one or more other immunomodulators, and the adenoviral backbone are co-transfected into HEK293 cells. Recombination between the overlapping sequences on the shuttle vector and the viral backbone results in the production of recombinant adenovirus and is packaged into viral particles in the HEK293 cells. The HEK293 cells are grown in DMEM containing fetal bovine serum.

[0436] The virus used for the proposed study was purified by CsCl density gradient centrifugation. The recombinant adenovirus undergoes two rounds of plaque purification and the resulting seed stock is used to produce a master viral bank (MVB) by amplification in HEK293 cells from a fully characterized master cell bank. The MVB undergoes extensive cGMP/GLP release tests including replication competent adenovirus (RCA), sterility, mycoplasma, adventitious viruses, retrovirus, human viruses HIV1/2, HTLV1/2, HAV, HBV, HCV, EBV, B19, CMV, HHV-6, 7 and 8, bovine and porcine virus, complete vector sequencing and functional testing by AD-induced expression of IL-12 and one or more other immunomodulators in human cell lines.

[0437] The virus from MVB may be used for production of the purified virus in a cGMP facility and may again undergo release tests including identity, RCA, sterility, mycoplasma, adventitious viruses, viral particle-to-infectious units ratio, contamination of host cell DNA, endotoxin and proteins and functional testing by AD-induced expression of IL-12 and one or more other immunomodulators in human cell lines.

Transduction of Autologous Dendritic Cells by Adenovirus Containing hIL-12 Transgene and One or More Other Immunodulators and RheoSwitch® Therapeutic System (RTS)

[0438] Dendritic cells derived from the human subjects are transduced *ex vivo* and injected into the tumor. The DC will be characterized before viral transduction for viability, purity (typically >80% cells showing DC phenotype), sterility, mycoplasma and endotoxin. After viral transduction, the cells are washed repeatedly to remove any unabsorbed virus. Supernatant from the last wash will be tested for the content of residual virus by PCR. Since the DCs are transduced *ex vivo* by adenoviral vector (non-integrating virus) and the life span of DCs after intratumoral injection and the subsequent migration to draining lymph nodes is short, it is not expected that the viral DNA will be incorporated into any non-target cells. The protocol used for adenoviral transduction of DCs is expected to yield 80-90% transduction and is considered very efficient.

[0439] Harvesting of PBMC by leukapheresis: Subjects undergo a standard 90 to 120 minutes leukapheresis at the Apheresis Unit of the UPCI Outpatient. The leukapheresis procedure involves the removal of blood from a vein in one arm; the passage of blood through a centrifuge (cell separator), where its components are separated and one or more components are removed; and the return of the remaining components to the subject's

vein in the same or other arm. No more than 15% of the subject's total blood volume is withdrawn at any one time as blood is processed through the cell separator device. In the cell separator, blood is separated into plasma, platelets, white cells and red blood cells. White blood cells (WBC) are removed and all the other components are returned into the subject's circulation. Every attempt is made to use two peripheral IV lines for this procedure. If that is not possible, a central line may be necessary. The subject has to be cleared by physician to undergo leukapheresis, and is routinely screened for vital signs (including blood pressure) prior to the procedure.

[0440] Processing: After collection, the leukapack is delivered by hand to the CPL, and is immediately processed by centrifugal elutriation in ELUTRA™. This is a closed system validated for clinical use. The monocyte fraction is recovered, and after the recovery and viability of cells are established, they are transferred to an Aastrom cartridge for 6-day culture in the presence of IL-4 and GM-CSF. All processing and washing procedures are performed under sterile conditions.

[0441] Initial plating: Monocytes recovered from a single leukapack are counted in the presence of a trypan blue dye to determine the number of viable cells. Monocytes are evaluated for purity by flow cytometry. Monocytes are resuspended at 5 to 10 x 10^6 cells/mL in serum-free and antibiotic-free CellGenix medium, containing 1,000 IU/mL of IL-4 and 1,000 IU/mL of GM-CSF per SOP-CPL-0166, and placed in an Aastrom cartridge. A minimum loading volume of 50 ml and a minimum cell number are required for cassette inoculation.

[0442] Culture: The Aastrom cartridge is placed in the incubator in the Replicell System, a fully closed, cGMP-compatible automated culture device for immature DC generation.

[0443] Immature DC harvest: On day 6, the Aastrom cartridge is removed from the incubator and immature DCs are harvested. The cells are recovered by centrifugation at 1,500 rpm, washed in CellGenix medium, counted in the presence of a trypan blue dye and checked for morphologic and phenotypic characteristics.

[0444] Viability: This is determined by performing hemocytometer cell counts in the presence of trypan blue. Generally, >95% of harvested cells are viable, *i.e.*, exclude a trypan blue dye. If viability is less than 70% the immature DCs will be discarded.

[0445] Phenotyping: The cells generated in culture are counted by microscopic observation on a hemocytometer, and a preliminary differential count (DC vs.

lymphocytes) is obtained using a trypan blue dye. Confirmation of the differential count is made by flow cytometry, gating on DC vs. lymphocytes and using high forward and side scatter properties of immature DC as the criterion for their identification. Immature DCs routinely contain >80% of cells with dendritic cell morphology and have DC phenotype.

[0446] IL-12p70 potency assay: It has been established that mature DCs (mDCs) have the ability to produce IL-12p70 spontaneously or upon activation with CD40L with or without addition of innate immunity signals (*e.g.*, LPS). A standardized IL-12p70 production assay was recently established and is applicable to small samples or large lots of DC vaccines generated under a variety of conditions. The current potency assay consists of two distinct steps, the first involving co-incubation of responder DCs with J588 lymphoma cells stably transfected with the human CD40 ligand gene as stimulators. The second step involves testing of supernatants from these co-cultures for levels of IL-12p70 secreted by DCs stimulated with J558/CD40L +/- LPS in the Luminex system. This potency assay has an inter-assay CV of 18.5% (n=30) and a broad dynamic range, which facilitates evaluation of various DC products characterized by vastly different levels of IL-12p70 production. The normal range for the assay established using DC products generated from monocytes of 13 normal donors was 8-999 pg/mL, with a mean of 270 pg/mL

Production and Release Criteria for Dendritic Cells

[0447] Each lot of the *in vitro* generated dendritic cells is tested for the presence of microbial contaminants (aerobic and anaerobic bacteria, fungi and mycoplasma), as well as endotoxin and are phenotypically and functionally characterized. All dendritic cells to be injected into subjects will be fresh and will not undergo croypreservation.

[0448] Quality assurance testing of DC: DC generated as described above are evaluated for sterility, viability, purity, potency and stability. Criteria for release of the cellular product are established and rigorously followed.

[0449] Viability: The cells generated in culture are counted by microscopic observation on a hemacytometer, and a differential count (DC vs. lymphocytes) is obtained using a trypan blue dye. This count provides the percentage of viable cells in the tested culture. More than 70% cell viability by trypan blue exclusion and minimum 70% cells expressing HLA-DR and CD86 as the monocyte-derived DC markers are required for

passing the release criteria. Additional markers may be included for exploratory analysis such as CD83 and CCR7 for assessing the DC maturation status, and CD3 and CD19 to assess the lymphocytes contamination.

[0450] Purity: Two-color flow cytometry analysis of cells stained with FITC- and PE-conjugated mAbs is used to determine that the DC population identified morphologicallly expresses the surface antigens defined for DC and lack the monocyte and T and B cell lineage antigens. For vaccine preparation, the DC generated must express HLA-DR and CD86 and must not express CD3, CD19, or CD14. To be considered as mDC, the cells must express CD83+ and CCR7+.

[0451] Potency: To define a measure of potency for the DC, we determine their ability to produce IL-12p70 as described above.

[0452] Sterility: DC are tested by bacterial (Aerobic and anaerobic) and fungal cultures using the BD Bactec system (Becton Dickinson Co., Sparks, MD) at the University of Pittsburgh Medical Center Microbiology Laboratory. Final results of the microbial cultures are available in 14 days. Prior to release of the DC for vaccine use, a gram stain is performed and must be negative for the presence of microorganisms.

[0453] The IMCPL tests for mycoplasma by the use of the Gen-Probe Mycoplasma Tissue Culture Rapid Detection System (Gen-Probe, Inc. San Diego, CA), which is based on nucleic acid hybridization technology. Endotoxin testing is performed using the Limulus Amoebocyte Lysate Pyrogen Plus assay (Bio Whittaker, Inc., Walkerville, MD). Endotoxin testing is performed on the cell culture at the time of harvest and prior to release of the final product. The acceptable endotoxin level is <5EU/kg body weight. Untransduced and transduced dendritic cells will be cryopreserved for future analysis.

[0454] It is expected that all the transduced cells will express the transgene. More than 80 % of the DCs are expected to be transduced. The product will be biologically active since the native coding sequence is maintained in the transgene. The viral-transduced DCs injected into the tumor are of immature DC phenotype and do not express IL-12 and one or more other immunomodulators until they undergo maturation, and hence at this stage, the expression of IL-12 and one or more other immunomodulators is mostly from the transgene. Since the expression of the IL-12 and one or more other immunomodulators transgene is induced by the small molecule activator drug RG-115932 in a dose dependent way, one can control the level of transgene expression in the transduced DCs

to the desired levels. A small portion of the transduced DCs prepared for administration to the human subjects may be tested *in vitro* for the activator drug-dependent induction of expression of IL12 and one or more other immunomodulators. Expression of IL-12 and one or more other immunomodulators may be assayed by ELISA with a sensitivity of 4 ng/ml.

[0455] It is expected that *in vitro* induction of IL-12 and one or more other immunomodulators from cells transduced by the vector used in the proposed study yields about 500 ng IL-12 and one or more other immunomodulators per 10^6 cells in 24 hours, determined by ELISA. In preclinical studies using mouse model of melanoma, intratumoral injection of 10^6 or more transduced DCs show efficacy. However, it is expected that the required intratumoral injection may show efficacy at levels below this amount and therefore injections of $5x10^7$ transduced DCs may be utilized as a starting point to determine if less or greater amounts are required.

[0456] For instance, *in vitro*, human and mouse cell lines and primary dendritic cells transduced with recombinant adenoviral vector carrying the genes for IL12 and one or more other immunomodulators show induction of IL12 expression in response to the activator drug in a dose dependent way.

6.3. Formulation of Activator Drug

[0457] The activator drug used herein is formulated in any one of the following formulations:

(1) 100% Labrasol;

(2) Listerine flavored Labrasol (Latitude Pharmaceuticals Inc., USA) comprising (a) menthol, (b) thymol, (c) eucalyptol, (d) aspartame, (e) sodium saccharine, (f) citric acid, (g) peppermint flavor, (h) cream flavor, (i) labrasol;

(3) Miglyol 812 and phospholipon 90G (Latitude Pharmaceuticals Inc., USA); or

(4) Miglyol 812, phospholipon 90G and Vitamin E tocopheryl polyethylene glycol succinate (Latitude Pharmaceuticals Inc., USA).

Delivery

[0458] While a variety of concentrations and specific protocols may be imagined, one example for treating patients would include patients receiving intratumoral injection(s) of transduced autologous dendritic cells (AdDCs) at a concentration of 5×10^7 suspended in

sterile saline engineered to express hIL-12 (human interleukin 12) and one or more other immunodulators under control of the RTS, in combination with the oral activator drug (RG-115932).

[0459] Initial Treatment

[0460] Day 1 Inpatient Visit: On day 1, a baseline physical examination (including vital signs, weight, and ECOG status) is performed. Urine is collected and blood drawn for baseline serum chemistry, urinanalysis, and hematology (safety profile). Approximately 3 hours before the intratumoral injection of the *in vitro* engineered dendritic cells, each subject is dosed with an activator drug (cohort 1 - 0.01 mg/kg, 0.3 mg/kg, 1.0 mg/kg, and 3 mg/kg) immediately after a meal. Blood is drawn at specified time intervals (predose, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 16, and 24 hours after the AD dose) on day 1 for evaluation of single dose pharmacokinetics of the activator drug and its major metabolites. Each subject receives a single intratumoral injection of adenoviral transduced autologous dendritic cells at a concentration of 5×10^7 cells, engineered to express hIL-12 and one or more other immunomodulators under the control of the RTS. The subjects are carefully monitored for local injection site reactions and/or hypersensitivity reactions. Day 2 through 14 Inpatient Visit: On days 2 through 14, each subject is dosed with the activator drug immediately after a meal. Vital signs and adverse events are collected daily on days 2 through 14. On day 4 ± 24 hours, biopsies of the tumor and/or draining lymph nodes are removed from approximately 50% of the subjects for measurement of hIL-12 and cellular immune response. On day 8, weight is measured. On day 8 ± 24 hours, biopsies of the tumor and/or draining lymph nodes are removed from subjects who did not have a biopsy performed on day 4 for measurement of hIL-12 and one or more other immunomodulators and cellular immune response. Blood is drawn on day 4 ± 24 hours and day 8 ± 24 hours for assay of potential antibodies and cellular immune response against the adenovirus and/or the RTS components. A serum cytokine profile is also obtained to determine if the expression of other cytokines is affected by treatment with the hIL-12 and one or more other immunomodulators transgene. On day 8, urine is collected and blood is drawn for baseline serum chemistry, urine analysis, and hematology (safety profile). On Day 8, blood is drawn at specified time intervals (predose, 0.5, 1, 2, 4, 6, 8, 12, 16, and 24 hours after the AD dose) for evaluation of steady-state pharmacokinetics/ADME of the activator drug and its major metabolites.

[0461] Day 14 Inpatient Visit: On day 14, each subject is dosed with the Activator Drug immediately after a meal. Each subject receives a physical examination (including vital signs, height, weight and ECOG status). Urine is collected and blood is drawn for serum chemistry, urinalysis, and hematology (safety profile). Blood is drawn on day 14 ± 24 hours for assay of potential antibodies and cellular immune response against the adenovirus and/or the RTS components. A serum cytokine profile is also obtained to determine if the expression of other cytokines is affected.

[0462] Blood is collected from the subjects at specified inpatient and outpatient visits to measure potential antibodies and cellular immune response to the adenovirus and components of the RTS. Blood is obtained for a baseline serum cytokine profile. The AdVeGFP infectivity blocking type assay is used to detect an antibody response to the adenoviral vector (Gambotto, Robins *et al.* 2004). Antibody response to the RTS components will be assessed by western blot and/or ELISA using serum from the patient and the RTS proteins produced from an expression vector. In addition, multiplex cytokine testing will be done in the serum by Luminex for IL-12, IFN-gamma, IP-10, and other Th1/Th2 cytokines such as IL-2, TNF-alpha, IL-4, IL-5, and IL-10. These antibody and cytokine assays will need about 10 ml of blood.

[0463] Potential Antibody and Cellular Immune Response to Adenovirus and/or Components of the RTS: Blood will be collected from the subjects at specified inpatient and outpatient visits to evaluate the potential antibody and cellular immune response to the adenovirus and components of the RTS and tumor antigens. The AdVeGFP infectivity blocking type assay will be used to detect an antibody response to the adenoviral vector (Nwanegbo, *et al.* 2004). Antibody response to the RTS components will be assessed by western blot and/or ELISA using serum from the subjects and the RTS proteins produced from an expression vector. In addition, multiplex cytokine testing will be done in the serum by Luminex for IL-12, IFN-gamma, IP-10, and other Thl/Th2 cytokines such as IL-2, TNFa, IL-4, IL-5 and IL-10. These antibody and cytokine assays will need about 10 ml of blood.

[0464] The cellular immune response assays use about 50-60 ml blood and CD4 and CD8 T cell subsets will be separated from it. The separated T cells will be mixed with autologous DCs transduced with empty AdV vector, AdV-RTS, or AdV-RTS-hIL12-immunomodulator(s) vectors in an ELISPOT assay for IFN-gamma production by the T

cells activated by the AdV- and RTS-derived antigens, if any. Similar assays will be performed using the tumor cells as such and/or DCs expressing shared melanoma antigens to assess the early immune response to the tumor. Additional assays may also be performed as necessary.

[0465] PREGNANCY TESTING: Females of childbearing potential is administered a urine pregnancy test at the screening visit and before the first inpatient visit of the retreatment phase. The testing is performed at least 72, 48, 24, or 12 hours prior to the administration of Activator Drug during both the initial treatment and all retreatment periods. If the urine pregnancy test is positive, then confirmation will be obtained with a serum pregnancy test. If pregnancy is confirmed, the subject will not be allowed to enter the trial or continue into the retreatment phase. The pregnancy testing may be reperformed as many times as necessary.

[0466] CONCOMITANT MEDICATION INQUIRY: At screening, and before the first inpatient visit of the retreatment phase, each subject will be asked to provide a list of concurrent medications to determine any possible relationship to adverse events that occur during the trial and follow-up phase.

[0467] RETREATMENT CRITERIA: If a subject has tolerated prior AdDC inoculation without adverse reactions that are limiting, and has shown no progression of disease or symptomatic decline at the time of potential retreatment, they will be considered for retreatment. If, in the opinion of the principal investigator, and treating physician there is a potential clinical benefit for additional intratumoral injection(s) of AdDCs in combination with Activator Drug (maximum tolerated dose from cohort 1) for 14 consecutive days, retreatment will be offered to the subject, provided the following criteria are met:

1. There have been no limiting toxicities,
2. The subject's disease is stable or showing clinical or subjective signs of improvement, and
3. There is no evidence of antibody or cellular immune response to adenovirus components of RheoSwitch® Therapeutic System.

[0468] ASSESSMENT OF TRANSGENE FUNCTION AND IMMUNOLOGICAL EFFECTS: Punch or excisional biopsies of the tumor and associated draining lymph nodes will be collected during screening (day -12 to day -7), day 4, day 8 and day 14 of

the trial and at month 1 of the follow-up (see Tables 3-5) for *in vivo* assessment of transgene expression of hIL-12 and one or more other immunomodulators, and cellular immune response. Fine needle aspiration biopsies of the tumor and associated draining lymph nodes will be collected on day -12 to -7 and day 14 of the retreatment period for *in vivo* assessment of transgene expression of hIL-12 and one or more other immunomodulators, and cellular immune response. Biopsies will be evaluated by standard light microscopy and immunohistochemistry to assess cellular infiltration of T cells into the tumor and draining lymph nodes. Biopsy sections will be read by a pathologist unaware of study subject background. To distinguish between endogenous and induced IL-12 expression by DCs in the tumor and draining lymph nodes, RT-PCR on RNA will be used with appropriately designed primers. Blood will be drawn for a serum cytokine profile at screening, day 4, day 8 and day 14 of the trial, at month 1 of the follow-up and on day -12 to -7, day 8 and day 14 of the retreatment period (see Tables 3-5). A serum cytokine profile will be obtained to determine if the expression of other cytokines is affected by treatment with the hIL-12 transgene. Multiplex cytokine testing will be done in the serum by Luminex for IL-12, IFN-gamma, IP-10, and other Thl/Th2 cytokines such as IL-2, TNFa, IL-4, IL-5 and IL-10. These antibody and cytokine assays will need about 10 ml of blood.

[0469] SINGLE DOSE AND STEADY-STATE PHARMACOKINETICS OF ACTIVATOR DRUG: Blood will be drawn at specified time intervals (predose, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 16, and 24 hours after the morning dose) on day 1 of the trial for evaluation of single dose pharmacokinetics and on day 8 of the trial for measurement of steady state pharmacokinetics/ADME of the Activator Drug and its major metabolites. Plasma will be evaluated by HPLC to obtain the following steady-state pharmacokinetic endpoints of the Activator Drug and major metabolites: Cmax (maximum observed plasma concentration), Tmax (time to maximum observed plasma concentration), Ctrough (minimum observed plasma concentration computed as the average of the concentrations at 0 and 24 hours), C24h (plasma concentration at 24 hours), AUC24h (area under plasma concentration-time curve from time 0 to 24 hours), Ke (apparent elimination rate), and T112 (apparent half-life).

[0470] It is to be understood that the foregoing described embodiments and exemplifications are not intended to be limiting in any respect to the scope of the

invention, and that the claims presented herein are intended to encompass all embodiments and exemplifications whether or not explicitly presented herein.

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WHAT IS CLAIMED IS:

1. A vector for conditionally expressing protein(s) having the function(s) of one or more immunomodulators comprising a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence which is operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, and (2) a polynucleotide encoding one or more proteins having the function of an immunomodulator operably linked to a promoter which is activated by said ligand-dependent transcription factor, wherein the one or more immunomodulators are selected from IL-1, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10R DN or a subunit thereof, IL-15, IL-18, IL-21, IL-23, IL-24, IL-27, GM-CSF, IFN-alpha, IFN-gamma, IFN-alpha 1, IFN alpha 2, IL-15-R-alpha, CCL3 (MIP-1a), CCL5 (RANTES), CCL7 (MCP3), XCL1(lymphotactin), CXCL1 (MGSA-alpha), CCR7, CCL19 (MIP-3b), CXCL9 (MIG), CXCL10 (IP-10), CXCL12 (SDF-1), CCL21 (6Ckine), OX40L, 4-1BBL, CD40, CD70, GITRL, LIGHT, b-Defensin, HMGB1, Flt3L, IFN-beta, TNF-alpha, dnFADD, BCG, TGF-alpha, PD-L1 RNAi, a PD-L1 antisense oligonucleotide, TGFbRII DN, ICOS-L and S100.

2. The vector of claim 1, which is an adenoviral vector.

3. The vector of claim 2, further comprising a polynucleotide encoding a protein having the function of IL-12.

4. The vector of claim 1, wherein said polynucleotide encoding said one or more proteins having the functions of the immunomodulator and said polynucleotide encoding said protein(s) having the function of IL-12 are under control of a regulated promoter of said gene switch.

5. The vector of claim 1, wherein said gene switch is an ecdysone receptor (EcR)-based gene switch.

6. The vector of claim 1, wherein said polynucleotide encoding a gene switch comprises a first transcription factor sequence under the control of a first promoter and a second transcription factor sequence under the control of a second promoter, wherein the proteins encoded by said first transcription factor sequence and said second transcription factor

sequence interact to form a protein complex which functions as a ligand-dependent transcription factor.

7. The vector of claim 1, wherein said polynucleotide encoding the one or more proteins having the functions of the immunomodulator encodes the human immunomodulator(s).

8. The vector of claim 3, wherein said polynucleotide encoding the protein having the function of IL-12 encodes human IL-12.

9. A method of producing a population of immune cells or therapy support cells (TSC) expressing protein(s) having the function of one or more immunomodulators, comprising modifying the immune cells with a recombinant vector conditionally expressing protein(s) having the function(s) of the one or more immunomodulators, wherein the vector comprises a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, and (2) a polynucleotide encoding one or more proteins having the functions of the immunomodulator linked to a promoter which is activated by said ligand-dependent transcription factor, wherein the one or more immunomodulators are selected from IL-1, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10R DN or a subunit thereof, IL-15, IL-18, IL-21, IL-23, IL-24, IL-27, GM-CSF, IFN-alpha, IFN-gamma, IFN-alpha 1, IFN-alpha 2, IL-15-R-alpha, CCL3 (MIP-1a), CCL5 (RANTES), CCL7 (MCP3), XCL1(lymphotactin), CXCL1 (MGSA-alpha), CCR7, CCL19 (MIP-3b), CXCL9 (MIG), CXCL10 (IP-10), CXCL12 (SDF-1), CCL21 (6Ckine), OX40L, 4-1BBL, CD40, CD70, GITRL, LIGHT, b-Defensin, HMGB1, Flt3L, IFN-beta, TNF-alpha, dnFADD, TGF-alpha, PD-L1 RNAi, a PD-L1 antisense oligonucleotide, TGFbRII DN, ICOS-L and S100.

10. The method of claim 9, wherein said vector is an adenoviral vector.

11. The method of claim 9, wherein said vector further comprises a polynucleotide encoding a protein having the function of IL-12.

12. The method of claim 11, wherein said polynucleotide encoding said protein having the function of the immunomodulator and the polynucleotide encoding said protein having the function of IL-12 are under control of said gene switch.

13. The method of claim 9, wherein said gene switch is an EcR-based gene switch.

14. The method of claim 9, wherein said polynucleotide encoding a gene switch comprises a first transcription factor sequence under the control of a first promoter and a second transcription factor sequence under the control of a second promoter, wherein the proteins encoded by said first transcription factor sequence and said second transcription factor sequence interact to form a protein complex which functions as a ligand-dependent transcription factor.

15. The method of claim 9, wherein said polynucleotide encoding the protein having the function of the immunomodulator encodes the human immunomodulator.

16. The method of claim 11, wherein said polynucleotide encoding a protein having the function of IL-12 encodes human IL-12.

17. The method of claim 9, wherein said cells are human dendritic cells.

18. The method of claim 17, wherein said dendritic cells are bone marrow dendritic cells.

19. A population of immune cells or TSC expressing protein(s) having the function of one or more immunomodulators, which have been modified with a recombinant vector conditionally expressing protein(s) having the function(s) of the one or more immunomodulators, wherein the vector comprises a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, and (2) a polynucleotide encoding one or more proteins having the function of an immunomodulator linked to a promoter which is activated by said ligand-dependent transcription factor, wherein the immunomodulator is selected from IL-1, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10R DN or a subunit thereof, IL-15, IL-18, IL-21, IL-23, IL-24, IL-27, GM-CSF, IFN-alpha, IFN-alpha 1, INF-alpha 2, IL-15R alpha, IFN-gamma, CCL3 (MIP-1a), CCL5

(RANTES), CCL7 (MCP3), XCL1(lymphotactin), CXCL1 (MGSA-alpha), CCR7, CCL19 (MIP-3b), CXCL9 (MIG), CXCL10 (IP-10), CXCL12 (SDF-1), CCL21 (6Ckine), OX40L, 4-1BBL, CD40, CD70, GITRL, LIGHT, b-Defensin, HMGB1, Flt3L, IFN-beta, TNF-alpha, dnFADD, TGF-alpha, PD-L1 RNAi, a PD-L1 antisense olignucleotide, TGFbRII DN, ICOS-L and S100.

20. The population of claim 19, wherein said vector is an adenoviral vector.

21. The population of claim 19, wherein said vector further comprises a polynucleotide encoding a protein having the function of IL-12.

22. The population of claim 19, wherein said polynucleotide encoding said protein having the function of the immunomodulator and the polynucleotide encoding said protein having the function of IL-12 are under control of said gene switch.

23. The population of claim 19, wherein said gene switch is an EcR-based gene switch.

24. The population of claim 19, wherein said polynucleotide encoding a gene switch comprises a first transcription factor sequence under the control of a first promoter and a second transcription factor sequence under the control of a second promoter, wherein the proteins encoded by said first transcription factor sequence and said second transcription factor sequence interact to form a protein complex which functions as a ligand-dependent transcription factor.

25. The population of claim 19, wherein said polynucleotide encoding the protein having the function of the immunomodulator encodes the human immunomodulator.

26. The population of claim 21, wherein said polynucleotide encoding a protein having the function of IL-12 encodes human IL-12.

27. The population of claim 19, wherein said cells are human dendritic cells.

28. The population of claim 27, wherein said dendritic cells are bone marrow dendritic cells.

29. An *in vitro* engineered immune cell or a TSC comprising a vector comprising a polynucleotide encoding a gene switch, wherein said polynucleotide encoding a gene switch comprises (1) at least one transcription factor sequence, wherein said at least one

transcription factor sequence encodes a ligand-dependent transcription factor, operably linked to a promoter, and (2) a polynucleotide encoding a protein having the function of an immunomodulator linked to a promoter which is activated by said ligand-dependent transcription factor, wherein the immunomodulator is selected from IL-1, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10R DN or a subunit thereof, IL-15, IL-18, IL-21, IL-23, IL-24, IL-27, GM-CSF, IFN-alpha, IFN-gamma, IFN-alpha 1, INF-alpha 2, IL-15R alpha, CCL3 (MIP-1a), CCL5 (RANTES), CCL7 (MCP3), XCL1(lymphotactin), CXCL1 (MGSA-alpha), CCR7, CCL19 (MIP-3b), CXCL9 (MIG), CXCL10 (IP-10), CXCL12 (SDF-1), CCL21 (6Ckine), OX40L, 4-1BBL, CD40, CD70, GITRL, LIGHT, b-Defensin, HMGB1, Flt3L, IFN-beta, TNF-alpha, dnFADD, TGF-alpha, PD-L1 RNAi, a PD-L1 antisense oligonucleotide, TGFbRII DN, ICOS-L and S100.

30. The *in vitro* engineered immune cell of claim 29, wherein said vector is an adenoviral vector.

31. The *in vitro* engineered immune cell of claim 29, wherein said vector further comprises a polynucleotide encoding a protein having the function of IL-12.

32. The *in vitro* engineered immune cell of claim 29, wherein said polynucleotide encoding said protein having the function of the immunomodulator and the polynucleotide encoding said protein having the function of IL-12 are under control of said gene switch.

33. The *in vitro* engineered immune cell of claim 29, wherein said gene switch is an EcR-based gene switch.

34. The *in vitro* engineered immune cell of claim 29, wherein said polynucleotide encoding a gene switch comprises a first transcription factor sequence under the control of a first promoter and a second transcription factor sequence under the control of a second promoter, wherein the proteins encoded by said first transcription factor sequence and said second transcription factor sequence interact to form a protein complex which functions as a ligand-dependent transcription factor.

35. The *in vitro* engineered immune cell of claim 29, wherein said polynucleotide encoding the protein having the function of the immunomodulator encodes the human immunomodulator.

36. The *in vitro* engineered immune cell of claim 31, wherein said polynucleotide encoding a protein having the function of IL-12 encodes human IL-12.

37. The *in vitro* engineered immune cell of claim 29, wherein said immune cell is a human dendritic cell.

38. The *in vitro* engineered immune cell of claim 37, wherein said dendritic cell is a bone marrow dendritic cell.

39. A pharmaceutical composition comprising the population of *in vitro* engineered immune cells or TSC according to claim 19.

40. A pharmaceutical composition comprising the *in vitro* engineered immune cell or the TSC of claim 29.

41. The pharmaceutical composition of claim 39 or 40, wherein the pharmaceutical composition is suitable for intratumoral, intraperitoneal or subcutaneous administration.

42. The pharmaceutical composition of claim 39, wherein said population of cells comprises at least 10^4 cells.

43. The pharmaceutical composition of claim 39, wherein said population of cells comprises at least 10^7 cells.

44. A method for treating a tumor in a mammal, comprising:

(a) administering intratumorally to tumor microenvironments a population of an immune cell or TSC, which is *in vitro* engineered to conditionally express one or more proteins having the functions of an immunomodulator; and
(b) administering to said mammal a therapeutically effective amount of one or more activating ligands;
thereby inducing expression of the one or more proteins having the functions of the immunomodulator and treating said tumor, wherein said immunomodulator is selected from IL-1, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10R DN or a subunit thereof, IL-15, IL-18, IL-21, IL-23, IL-24, IL-27, GM-CSF, IFN-alpha, IFN-gamma, IFN-alpha 1, INF-alpha 2, IL-15R alpha, CCL3 (MIP-1a), CCL5

(RANTES), CCL7 (MCP3), XCL1(lymphotactin), CXCL1 (MGSA-alpha), CCR7, CCL19 (MIP-3b), CXCL9 (MIG), CXCL10 (IP-10), CXCL12 (SDF-1), CCL21 (6Ckine), OX40L, 4-1BBL, CD40, CD70, GITRL, LIGHT, b-Defensin, HMGB1, Flt3L, IFN-beta, TNF-alpha, dnFADD, TGF-alpha, PD-L1 RNAi, a PD-L1 antisense oligonucleotide, TGFbRII DN, ICOS-L and S100.

45. The method of claim 44, wherein said tumor is a benign tumor.

46. The method of claim 44, wherein said tumor is a malignant tumor.

47. The method of claim 44, wherein said tumor is a melanoma.

48. The method of claim 44, wherein said tumor is a malignant melanoma skin cancer.

49. The method of claim 44, wherein said *in vitro* engineered immune cells comprise a vector comprising a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, and (2) a polynucleotide encoding a protein having the function of the immunomodulator linked to a promoter which is activated by said ligand-dependent transcription factor.

50. The method of claim 44, wherein said vector is an adenoviral vector.

51. The method of claim 50, wherein said vector further comprises a polynucleotide encoding a protein having the function of IL-12.

52. The method of claim 51, wherein said polynucleotide encoding said protein having the function of the immunomodulator and the polynucleotide encoding said protein having the function of IL-12 are under control of said gene switch.

53. The method of claim 52, wherein said gene switch is an EcR-based gene switch.

54. The method of claim 53, wherein said polynucleotide encoding a gene switch comprises a first transcription factor sequence under the control of a first promoter and a second transcription factor sequence under the control of a second promoter, wherein the proteins encoded by said first transcription factor sequence and said second transcription factor

sequence interact to form a protein complex which functions as a ligand-dependent transcription factor.

55. The method of claim 44, wherein said polynucleotide encoding the protein having the function of the immunomodulator encodes the human immunomodulator.

56. The method of claim 51, wherein said polynucleotide encoding a protein having the function of IL-12 encodes human IL-12.

57. The method of claim 44, wherein said immune cells are human dendritic cells.

58. The method of claim 57, wherein said dendritic cells are bone marrow dendritic cells.

59. The method of claim 44, wherein said ligand is a diacylhydrazine.

60. The method of claim 44, wherein said ligand is selected from RG-115819, RG-115932, and RG-115830.

61. The method of claim 44, wherein said ligand is an amidoketone or oxadiazoline.

62. The method of claim 44, wherein said ligand is administered less than one hour before or after said *in vitro* engineered immune cells.

63. The method of claim 44, wherein said ligand is administered less than 24 hours after said *in vitro* engineered immune cells.

64. The method of claim 44, wherein said ligand is administered less than 48 hours after said *in vitro* engineered immune cells.

65. A method for determining the efficacy of an *in vitro* engineered immune cell based therapeutic regime in a patient comprising:

(a) measuring the level of expression or the level of activity or both of interferon-gamma (IFN-γ) in a first biological sample obtained from said patient in need thereof before administration of *in vitro* engineered immune cells, thereby generating a control level;

(b) administering to a patient in need thereof, *in vitro* engineered immune cells engineered to conditionally express one or more proteins having the functions of an immunomodulator and optionally a protein having the function of IL-12;

(c) administering to said patient in need thereof an effective amount of an activating ligand;

(d) measuring the level of expression or the level of activity or both of IFN-γ in a second biological sample obtained from said patient in need thereof following administration of *in vitro* engineered immune cells and activating ligand, thereby generating a test level; and

(e) comparing the control level to the test level of IFN-γ, wherein an increase in the test level of expression, activity or both of IFN-γ relative to the control level indicates that the therapeutic regime is effective in said patient in need thereof,

wherein the immunomodulator is selected from IL-1, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10R DN or a subunit thereof, IL-15, IL-18, IL-21, IL-23, IL-24, IL-27, GM-CSF, IFN-alpha, IFN-gamma, IFN-alpha 1, INF-alpha 2, IL-15R alpha, CCL3 (MIP-1a), CCL5 (RANTES), CCL7 (MCP3), XCL1(lymphotactin), CXCL1 (MGSA-alpha), CCR7, CCL19 (MIP-3b), CXCL9 (MIG), CXCL10 (IP-10), CXCL12 (SDF-1), CCL21 (6Ckine), OX40L, 4-1BBL, CD40, CD70, GITRL, LIGHT, b-Defensin, HMGB1, Flt3L, IFN-beta, TNF-alpha, dnFADD, TGF-alpha, PD-L1 RNAi, a PD-L1 antisense oligonucleotide, TGFbRII DN, ICOS-L and S100.

66. The method of claim 65, wherein said patient in need thereof is a human patient.

67. The method of claim 65, wherein said patient is a cancer patient.

68. The method of claim 67, wherein said cancer patient is a melanoma patient.

69. The method of claim 65, wherein the level of IFN-γ is measured by ELISA.

70. The method of claim 65, wherein said immune cells are engineered *in vitro* to conditionally express one or more proteins having the functions of an immunomodulators and optionally the protein having the function of IL-12 and comprise a vector comprising a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at

least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, and (2) a polynucleotide encoding one or more proteins having the function of the immunomodulator and, optionally the IL-12 linked to a promoter which is activated by said ligand-dependent transcription factor.

71. The method of claim 70, wherein said vector is an adenoviral vector.

72. The method of claim 70, wherein said gene switch is an EcR-based gene switch.

73. The method of claim 72, wherein said ligand binds to the EcR ligand binding domain.

74. The method of claim 72, wherein said ligand is a diacylhydrazine.

75. The method of claim 74, wherein said ligand is selected from RG-115819, RG-115932, and RG-115830.

76. The method of claim 72, wherein said ligand is an amidoketone or oxadiazoline.

77. The method of claim 72, wherein said polynucleotide encoding a gene switch comprises a first transcription factor sequence under the control of a first promoter and a second transcription factor sequence under the control of a second promoter, wherein the proteins encoded by said first transcription factor sequence and said second transcription factor sequence interact to form a protein complex which functions as a ligand-dependent transcription factor.

78. The method of claim 70, wherein said polynucleotide encoding a protein having the function of IL-12 encodes human IL-12.

79. The method of claim 65, wherein said immune cells are human dendritic cells.

80. The method of claim 79, wherein said dendritic cells are bone marrow dendritic cells.

81. The method of claim 65, wherein said activating ligand is administered intratumorally, orally, intraperitoneally, or subcutaneously.

82. The method of claim 65, wherein said activating ligand is administered less than one hour before or after said *in vitro* engineered dendritic cells.

83. The method of claim 65, wherein said activating ligand is administered less than 24 hours after said *in vitro* engineered dendritic cells.

84. The method of claim 65, wherein said activating ligand is administered less than 48 hours after said *in vitro* engineered dendritic cells.

85. A kit comprising (a) immune cells engineered to contain a vector comprising a polynucleotide encoding a gene switch controlling expression of one or more proteins having the function of an immunomodulator and, optionally, a protein having the function of IL-12, and (b) a ligand that activates the gene switch.

86. The kit of claim 85, wherein the ligand is RG-115819, RG-115830 or RG-115932.

87. The vector of claim 1, wherein said polynucleotide encoding a gene switch comprises a first transcription factor sequence and a second transcription factor sequence under the control of a promoter, wherein the proteins encoded by said first transcription factor sequence and said second transcription factor sequence interact to form a protein complex which functions as a ligand-dependent transcription factor.

88. The vector of claim 87, wherein said first transcription factor sequence and said second transcription factor sequence are connected by an EMCV internal ribosomal entry site (IRES).

89. The *in vitro* engineered immune cell of claim 29, wherein said polynucleotide encoding a gene switch comprises a first transcription factor sequence and a second transcription factor sequence under the control of a promoter, wherein the proteins encoded by said first transcription factor sequence and said second transcription factor sequence interact to form a protein complex which functions as a ligand-dependent transcription factor.

90. The *in vitro* engineered immune cell of claim 89, wherein said first transcription factor sequence and said second transcription factor sequence are connected by an EMCV internal ribosomal entry site (IRES).

91. The method of claim 53, wherein said polynucleotide encoding a gene switch comprises a first transcription factor sequence and a second transcription factor sequence under the control of a promoter, wherein the proteins encoded by said first transcription factor

sequence and said second transcription factor sequence interact to form a protein complex which functions as a ligand-dependent transcription factor.

92. The *in vitro* engineered immune cell of claim 91, wherein said first transcription factor sequence and said second transcription factor sequence are connected by an EMCV internal ribosomal entry site (IRES).

93. The method of claim 72, wherein said polynucleotide encoding a gene switch comprises a first transcription factor sequence and a second transcription factor sequence under the control of a promoter, wherein the proteins encoded by said first transcription factor sequence and said second transcription factor sequence interact to form a protein complex which functions as a ligand-dependent transcription factor.

94. The *in vitro* engineered immune cell of claim 93, wherein said first transcription factor sequence and said second transcription factor sequence are connected by an EMCV internal ribosomal entry site (IRES).

95. A composition comprising a composition comprising two or more populations of *in vitro* engineered immune cells or therapy support cells, wherein each of the populations of *in vitro* engineered cells in the composition comprises a vector comprising a polynucleotide encoding a gene switch, wherein said polynucleotide comprises (1) at least one transcription factor sequence operably linked to a promoter, wherein said at least one transcription factor sequence encodes a ligand-dependent transcription factor, and (2) a polynucleotide encoding a protein having the function of an immunomodulator linked to a promoter which is activated by said ligand-dependent transcription factor, and wherein each population of *in vitro* engineered cells in the composition expresses one or more immunomodulators that are different from the one or more immunomodulators expressed in the other population(s) of *in vitro* engineered cell in the composition.

96. A method for treating a tumor in a mammal, comprising:

(a) administering intratumorally to tumor microenvironments two or more populations of immune cells or TSCs, which are *in vitro* engineered to conditionally express one or more proteins having the function of an immunomodulator,

wherein each population of immune cells or TSCs expresses a different set of one or more immunomodulators; and

(b) administering to said mammal a therapeutically effective amount of one or more activating ligands,

thereby inducing expression of proteins having the function of the immunomodulators and treating said tumor.

97. Amethod for treating a tumor in a mammal, comprising:

(a) administering intratumorally to tumor microenvironments two or more populations of an immune cells or TSCs, which are *in vitro* engineered to conditionally express one or more proteins having the function of an immunomodulator and a protein having the function of IL-12,

wherein each population of immune cells or TSCs expresses a different set of one or more proteins having the function of an immunomodulator,

wherein at least one of the proteins having the function of the immunomodulator or IL-12 is under control of a conditional promoter that is activated by a ligand; and

(b) administering to said mammal a therapeutically effective amount of one or more activating ligands,

thereby inducing expression of a protein having the function of the immunomodulators and/or the protein having the function of IL-12 and treating said tumor.