FURIN-KNOCKDOWN AND GM-CSF-AUGMENTED (FANG) CANCER VACCINE

TECHNICAL FIELD OF THE INVENTION

The present invention relates in general to the field of vaccine development, and more particularly, to the development of compositions and methods for making and using an autologous cancer vaccine genetically modified for Furin knockdown and GM-CSF expression.

BACKGROUND OF THE INVENTION

Without limiting the scope of the invention, its background is described in connection with the development of genetically modified whole cell cancer vaccines. More specifically, the present invention relates to vaccines capable of augmenting tumor antigen expression, presentation, and processing through expression of the GM-CSF transgene and attenuating secretory immunosuppressive activity of TGF-β via furin bi-functional shRNA transgene induced knockdown.

The prevailing hypothesis for immune tolerance to cancer vaccines include the low immunogenicy of the tumor cells, the lack of appropriate presentation by professional antigen presenting cells, immune selection of antigen-loss variants, tumor induced immunosuppression, and tumor induced privileged site. Whole cancer cell vaccines can potentially solicit broad-based, polyvalent immune responses to both defined and undefined tumor antigens, thereby addressing the possibility of tumor resistance through downregulation and/or selection for antigen-loss variants.

Granulocyte-macrophage colony-stimulating factor, often abbreviated to GM-CSF, is a protein secreted by macrophages, T cells, mast cells, endothelial cells and fibroblasts. When integrated as a cytokine transgene, GM-CSF enhances presentation of cancer vaccine peptides, tumor cell lysates, or whole tumor cells from either autologous patient tumor cells or established allogeneic tumor cell lines. GM-CSF induces the differentiation of hematopoietic precursors and attracts them to the site of vaccination. GM-CSF also functions as an adjuvant for dendritic cell maturation and activational processes. However, GM-CSF-mediated immunosensitization can be suppressed by different isoforms of transforming growth factor beta (TGF-β) produced and/or secreted by tumor cells. The TGF-β family of multifunctional proteins possesses well known immunosuppressive activities. The three

known TGF-β ligands (TGF-β1, β2, and β3) are ubiquitous in human cancers. TGF-β overexpression correlates with tumor progression and poor prognosis. Elevated TGF-β levels within the tumor microenvironment are linked to an anergic tumor response. TGF-β directly and indirectly inhibits GM-CSF induced maturation of dendritic cells and their expression of MHC class II and co-stimulatory molecules. This negative impact of TGF-β on GM-CSF-mediated immune activation supports the rationale of depleting TGF-β secretion in GM-CSF-based cancer cell vaccines.

All mature isoforms of TGF-β require furin-mediated limited proteolytic cleavage for proper activity. Furin, a calcium-dependent serine endoprotease, is a member of the subtilisin-like proprotein convertase family. Furin is best known for the functional activation of TGF-β with corresponding immunoregulatory ramifications. Apart from the previously described immunosuppressive activities of tumor secreted TGF-β, conditional deletion of endogenously expressed furin in T lymphocytes has been found to allow for normal T-cell development, but impaired function of regulatory and effector T cells, which produced less TGF-β1. Furin expression by T cells appears to be indispensable in maintaining peripheral tolerance, which is due, at least in part, to its non-redundant, essential function in regulating TGF-β1 production.

High levels of furin have been demonstrated in virtually all cancer lines. The inventors and others have found that up to a 10-fold higher level of TGF-β1 may be produced by human colorectal, lung cancer, and melanoma cells, and likely impact the immune tolerance state by a higher magnitude. The presence of furin in tumor cells likely contributes significantly to the maintenance of tumor directed, TGF-β-mediated peripheral immune tolerance. Hence furin knockdown represents a novel and attractive approach for optimizing GM-CSF-mediated immunosensitization.

Interferon-gamma (γIFN) is a key immunoregulatory cytokine that plays a critical role in the host innate and adaptive immune response and in tumor control. Also known as type II interferon, γIFN is a single-copy gene whose expression is regulated at multiple levels. γIFN coordinates a diverse array of cellular programs through transcriptional regulation of immunologically relevant genes. Initially, it was believed that CD4+ T helper cell type 1 (Th1) lymphocytes, CD8+ cytotoxic lymphocytes, and NK cells exclusively produced γIFN. However, there is now evidence that other cells, such as B cells, NKT cells, and professional

antigen-presenting cells (APCs) secrete γIFN. γIFN production by professional APCs [monocyte/macrophage, dendritic cells (DCs)] acting locally may be important in cell self-activation and activation of nearby cells. γIFN secretion by NK cells and possibly professional APCs is likely to be important in early host defense against infection, whereas T lymphocytes become the major source of γIFN in the adaptive immune response. Furthermore, a role for γIFN in preventing development of primary and transplanted tumors has been identified. γIFN production is controlled by cytokines secreted by APCs, most notably interleukin (IL)-12 and IL-18. Negative regulators of γIFN production include IL-4, IL-10, glucocorticoids, and TGF-β.

SUMMARY OF THE INVENTION

The present invention also provides an autologous (i.e., patient specific) cancer vaccine composition (FANG vaccine), comprising a therapeutically effective amount of cells with an shRNAfurin / GM-CSF expression vector. This vector comprises a first nucleic acid encoding GM-CSF, which may be human GM-CSF, and a second nucleic acid insert encoding one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of an mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference. Both nucleic acid inserts are operably linked to a promoter. The shRNA may be bi-functional, incorporating both (cleavage dependent) RISC (RNA induced silencing complex) formatted) siRNA (cleavage dependent) and (cleavage-independent RISC formatted) either miRNA or miRNA-like motifs simultaneously. In one embodiment of the present invention, the shRNA is both the RISC cleavage dependent and RISC cleavage independent inhibitor of furin expression. Furthermore, the expression vector may contain a picornaviral 2A ribosomal skip peptide intercalated between the first and the second nucleic acid inserts, and the promoter may be CMV mammalian promoter which could contain an enhancer sequence and intron. The mRNA sequences targeted by the bi-functional shRNA are not limited to coding sequences; in one embodiment, the shRNA may target the 3' untranslated region (3'-UTR) sequence of the furin mRNA transcript, and in one embodiment may target both the coding sequence and the 3' UTR sequence of the furin mRNA transcript simultaneously.. The cells used to produce the vaccine may be autologous tumor cells, but xenograft expanded autologous tumor cells, allogeneic tumor cells, xenograft expanded allogeneic tumor cells, or combinations of them may also be used. The vaccine dosage administered to patients contains 1 $x10^7$ cells to 2.5 x 10^7 cells. The FANG vaccine can be given in conjunction with a

therapeutically effective amount of γIFN (gamma interferon). The dosage range of γIFN may be 50 or 100 μg/m^2.

The present invention describes an autologous cell vaccine composition comprising: a bishRNA^{furin}/GMCSF expression vector plasmid and one or more optional vaccine. The vector plasmid comprises a first nucleic acid insert operably linked to a promoter, wherein the first insert encodes a Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) cDNA and a second nucleic acid insert operably linked to the promoter, wherein the second insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of a mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference. In one aspect the GM-CSF is human. In another aspect the shRNA incorporates siRNA (cleavage dependent RISC formatted) and either miRNA or miRNA-like (cleavage-independent RISC formatted) motifs. The shRNA as described herein is both the cleavage dependent RISC formatted and cleavage independent RISC formatted inhibitor of furin expression and is further defined as a bi-functional shRNA.

In another aspect, a picornaviral 2A ribosomal skip peptide is intercalated between the first and the second nucleic acid inserts. In yet another aspect the promoter is a CMV mammalian promoter containing a CMV IE 5' UTR enhancer sequence and a CMV IE Intron A. In a related aspect the CMV mammalian promoter. In other aspects the region targeted by the shRNA is the 3' UTR region sequence of the furin mRNA transcript and the region targeted by the shRNA is the coding region of the furin mRNA transcript.

The present invention provides a method of preventing, treating and/or ameliorating symptoms of a cancer in a patient by comprising the steps of: identifying the patient in need of prevention, treatment, and/or amelioration of the symptoms of the cancer and administering an autologous cell vaccine comprising a bishRNA^{furin}/GMCSF expression vector plasmid, wherein the vector plasmid comprises a first nucleic acid insert operably linked to a promoter, wherein the first insert encodes a Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) cDNA, a second nucleic acid insert operably linked to the promoter, wherein the second insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of a mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference, and one or more optional vaccine adjuvants.

The method further comprises the steps of monitoring the progression of the therapy by measuring a level of a transforming growth factor beta (TGF-beta or TGF-β) and the GM-

CSF in the one or more cancer cells, wherein a reduction in the level of TGF-β and an elevation in the level of the GM-CSF is indicative of a successful therapy and altering the administration of the autologous cell vaccine based on the levels of the TGF-β and the GM-CSF. As per the method of the present invention the TGF-β is selected from at least one of TGF-β1, TGF-β2, or TGF-β3. In one aspect the cancer is selected from the group consisting of melanoma, non-small-cell lung cancer, gall bladder cancer, colorectal cancer, breast cancer, ovarian, liver cancer, liver cancer metastases, and Ewing's sarcoma as well as other patient derived TGF-β producing cancers. In another aspect the shRNA incorporates siRNA (cleavage dependent RISC formatted) and either miRNA or miRNA-like (cleavage-independent RISC formatted) motifs and the shRNA is both the cleavage dependent RISC formatted and cleavage independent RISC formatted inhibitor of furin expression. In yet another aspect the shRNA is further defined as a bi-functional shRNA.

In another embodiment the present invention discloses an autologous furin-knockdown and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) augmented (FANG) cancer vaccine composition comprising: a bishRNA^{furin}/GMCSF expression vector plasmid, wherein the vector plasmid comprises a first nucleic acid insert operably linked to a promoter, wherein the first insert encodes the GM-CSF cDNA and a second nucleic acid insert operably linked to the promoter, wherein the second insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of a mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference and one or more optional vaccine adjuvants. The composition of the present invention is used to prevent, treat, and/or ameliorate the symptoms of a cancer, wherein the cancer is selected from the group consisting of melanoma, non-small-cell lung cancer, gall bladder cancer, colorectal cancer, breast cancer, ovarian, liver cancer, liver cancer metastases, and Ewing's sarcoma as well as other patient derived TGF-β producing cancers.

In yet another embodiment the present invention is a method of treating, preventing, and/or ameliorating the symptoms of a non small cell lung cancer (NSCLC) in a patient by an administration of a furin-knockdown and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) augmented (FANG) cancer vaccine comprising the steps of: identifying the patient in need of prevention, treatment, and/or amelioration of the symptoms of the NSCLC and administering the FANG vaccine comprising a bishRNA^{furin}/GMCSF expression vector plasmid, wherein the vector plasmid comprises a first nucleic acid insert operably

linked to a promoter, wherein the first insert encodes the GM-CSF cDNA, a second nucleic acid insert operably linked to the promoter, wherein the second insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of a mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference, and one or more optional vaccine adjuvants. The method of the instant invention further comprising the steps of: monitoring the progression of the therapy by measuring a level of a transforming growth factor beta (TGF-beta or TGF-β) and the GM-CSF in the one or more NSCLC cells, wherein a reduction in the level of TGF-β and an elevation in the level of the GM-CSF is indicative of a successful therapy and altering the administration of the autologous cell vaccine based on the levels of the TGF-β and the GM-CSF. In one aspect of the present invention the TGF-β is selected from at least one of TGF-β1, TGF-β2, or TGF-β3.

The present invention in a further embodiment describes a method of making a furin-knockdown and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) augmented (FANG) cancer vaccine comprising the steps of: (i) harvesting one or more cancer cells from a patient aseptically, (ii) placing the harvested cells in an antibiotic solution in a sterile container, (iii) forming a cell suspension from the harvested solution, wherein the formation of the cell, (iv) suspension is achieved by enzymatic dissection, mechanical disaggregation or both, (v) modifying the cells genetically by electroporating the cell suspension to make the vaccine with a bishRNA^{furin}/GMCSF expression vector plasmid, wherein the vector plasmid comprises a first nucleic acid insert operably linked to a promoter, wherein the first insert encodes the GM-CSF cDNA, a second nucleic acid insert operably linked to the promoter, wherein the second insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of a mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference, (vi) harvesting the vaccine, (vii) irradiating the vaccine and (viii) freezing the vaccine.

In one aspect of the method the one or more cancer cells are harvested from a patient suffering from a cancer selected from the group consisting of melanoma, non-small-cell lung cancer, gall bladder cancer, colorectal cancer, breast cancer, ovarian, liver cancer, liver cancer metastases, and Ewing's sarcoma as well as other patient derived TGF-β producing cancers. In another aspect the genetically modified cells have been rendered proliferation-incompetent by irradiation. In yet another aspect the genetically modified cells are autologous, allogenic, or xenograft expanded cells.

In one aspect the allogenic cells are established cell lines. In another aspect the genetically modified cells are administered to the subject once a month for up 12 doses, wherein the dose of genetically modified cells administered to the subject is 1 x 10^7 cells/injection to 5 x 10^7 cell/injection and the administration of the genetically modified cells is part of a combination therapy with an additional therapeutic agent. In yet another aspect the additional therapeutic agent used in the combination therapy is γIFN, wherein the dose of γIFN administered to the subject in the combination therapy is 50 or 100 μg/m^2. The method of the present invention further comprises the step of incubating the genetically modified cells with γIFN after transfection, wherein the dose of γIFN applied to the genetically modified cells after transfection is approximately 250 U/ml (500 U/ml over 24 hours to 100 U/ml over 48 hours).

Another embodiment of the invention is a siRNA-mediated method to inhibit the expression of transforming growth factor beta (TGF-β) via furin knockdown. This method comprises the steps of selecting a target cell and transfecting the target cell with an expression vector comprising a promoter and a nucleic acid insert operably linked to the promoter. The insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of an mRNA transcript encoding furin, consequently inhibiting furin expression via RNA interference. The shRNA may be bi-functional , i.e., it may simultaneously incorporate siRNA (cleavage dependent RISC formatted) and either miRNA or miRNA-like (cleavage-independent RISC formatted) motifs, and inhibit furin expression in both a cleavage dependent RISC formatted and cleavage independent RISC formatted manner. Additionally, the expression vector may target the coding region of the furin mRNA transcript, or it may target the 3' UTR region sequence of the furin mRNA transcript, or it may target both the coding sequence and the 3' UTR sequence of the furin mRNA transcript simultaneously.

The present invention also provides a method to augment antigen expression, presentation, and processing, and to attenuate secretory immunosuppressive activity of transforming growth factor beta (TGF-beta or TGF-β) in target cells. This method comprises the steps of selecting a target cell and transfecting the target cell with an expression vector comprising two inserts. The technique used to transfect the target cells may be plasmid vector electroporation. The first nucleic acid insert encodes GM-CSF, whereas the second insert encodes one or more short hairpin RNAs (shRNAs) capable of hybridizing to a region of an mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference. Both inserts are operably linked to a promoter. The TGF-β isoforms whose activation would

be precluded by knocking down furin expression include TGF-β-1, TGF-β-2, and TGF-β-3. Target cells may include autologous or allogeneic cells, which may be established human cell lines.

The present invention also includes a method of preventing, treating and/or ameliorating symptoms of cancer by administering the FANG vaccine to patients. This method comprises the steps of: (i) identifying a subject in need of treatment; (ii) harvesting a cancer tissue sample from the subject; (iii) genetically modifying the cancer cells in the harvested cancer sample; and (iv) administering a therapeutically effective dose of genetically modified cells to the subject. The expression vector used to transfect the cells comprises two nucleic acid inserts. The first nucleic acid insert encodes GM-CSF and it is operably linked to a promoter. The second nucleic acid insert is also operably linked to the promoter, and it encodes one or more short hairpin RNAs (shRNAs) capable of hybridizing to a region of an mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference. In one embodiment of the present invention, the cancer targeted for treatment is a human melanoma or a non-small-cell lung cancer. To render the genetically modified cells proliferation-incompetent, they may be irradiated. The genetically modified cells in the FANG vaccine may be autologous cells, allogeneic cells, xenograft expanded cells, established human cell lines, or combinations of these cellular types. For vaccination, cells are administered to the subject once a month for up 12 doses, each one containing 1 x 10^7 cells to 2.5 x 10^7 cells. Dose escalation to 5 x 10^7 has been shown to be safe.

The genetically modified cells can be administered as a stand-alone therapy; however, they may also be administered as part of a combination therapy. In this embodiment of the invention, the FANG vaccine may be combined with another therapeutic agent[s], such as, but not limited to, IL-12, IL-15 and/or γIFN. When including γIFN in the treatment with FANG vaccine, the method comprises the further step of incubating the genetically modified cells with approximately 100 U/ml of γIFN for 48 hours or 500 U/ml for 24 hours, respectively, after transfection. For combination therapy, cells are administered to the subject once a month for up to 12 doses, each one containing typically 1 x 10^7 cells to 2.5 x 10^7 cells (although doses up to 5 x 10^7 have been shown to be safe) plus a dose of γIFN of 50 or 100 μg/m^2. The method further comprises the step of incubating the genetically modified cells with γIFN after transfection, wherein the dose of γIFN applied to the genetically modified cells after transfection is approximately 250U/ml.

The present invention includes a unique method of inhibiting TGF-β through RNA interference with furin, a proprotein convertase involved critically in the functional processing of all TGF-β isoforms. The FANG vector uniquely incorporates a bi-functional small hairpin construct (shRNAfurin) specific for the knockdown of furin. The bi-functional shRNAfurin of the present invention comprises a two stem-loop structure with a miR-30a backbone. The first stem-loop structure is the siRNA precursor component, while the second stem-loop structure is the miRNA-like precursor component. In this embodiment, the strategy is to use a single targeted site for both cleavage and sequestering mechanisms of RNA interference. In one embodiment, the strategy is to use two different targeted sites, one for the cleavage and one for the sequestering component comprised of, but not limited to, the coding region of the mRNA transcript and the 3' UTR region of the mRNA transcript, respectively. In this embodiment, the bi-functional shRNAfurin is comprised of two stem-loop structures with miR-30a backbone; the first stem-loop structure has complete complementary guiding strand and passenger strand, while the second stem-loop structure has three basepair (bp) mismatches at positions 9 to 11 of the passenger strand. In one embodiment, the bi-functional shRNAfurin is comprised of two stem-loop structures with miR-30a backbone; the first stem-loop structure has complete complementary guiding strand and passenger strand, while the second stem-loop structure has three basepair (bp) mismatches at positions 9 to 11 of the guide strand. In other embodiments, basepair (bp) mismatches will occupy positions preventing Ago 2 mediated cleavage and make it thermodynamically favorable for passenger strand departure. In other embodiments the basepair mismatches will occupy positions of the guide strand. The FANG construct contains GM-CSF and the bi-functional shRNAfurin transcripts under the control of a mammalian promoter (CMV) that drives the entire cassette. This construct is used to generate an autologous (i.e., patient specific) cancer vaccine genetically modified for furin knockdown and GM-CSF expression.

The construct used to produce the FANG vaccine in the present invention includes a bi-functional shRNAfurin/GMCSF expression vector plasmid comprising two nucleic acid inserts. The first nucleic acid insert is linked operably to a promoter, and it encodes a Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) cDNA. The second nucleic acid insert is also linked operably to the promoter, and it encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of an mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference. The bi-functional shRNA of the

present invention has two mechanistic pathways of action, that of the siRNA and that of the miRNA. Thus, the bi-functional shRNA of the present invention is different from a traditional shRNA, i.e., a DNA transcription derived RNA acting by the siRNA mechanism of action or from a "doublet shRNA" that refers to two shRNAs, each acting against the expression of two different genes but in the traditional siRNA mode. In one embodiment of the invention, the GM-CSF is human. The shRNA is bi-functional, incorporating both siRNA (cleavage dependent RISC formatted) and either miRNA or miRNA-like (cleavage-independent RISC formatted) motifs simultaneously. In one embodiment of the present invention, the shRNA is both the cleavage dependent RISC formatted and cleavage independent RISC formatted inhibitor of furin expression. The expression vector may contain a picornaviral 2A ribosomal skip peptide intercalated between the first and the second nucleic acid inserts, and the promoter may be CMV mammalian promoter which could contain a CMV IE 5' UTR enhancer sequence and a CMV IE Intron A. The mRNA sequences targeted by the bi-functional shRNA are not limited to coding sequences; in one embodiment, the shRNA may target the 3' untranslated region (UTR) sequence of the furin mRNA transcript and, in one embodiment, target both the coding sequence and the 3' UTR sequence of the furin mRNA transcript simultaneously

The present invention also includes a vector that may be used to specifically knock down the expression of furin in target cells. This shRNAfurin expression vector comprises a nucleic acid insert linked operably to a promoter. Such insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of an mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference. The bi-functional shRNA may simultaneously incorporate siRNA (cleavage dependent RISC formatted) and either miRNA or mi-RNA-like (cleavage-independent RISC formatted) motifs, and inhibit furin expression in both a cleavage dependent RISC formatted and cleavage independent RISC formatted manner. Additionally, the expression vector may target the coding region of the furin mRNA transcript, or it may target the 3' UTR region sequence of the furin mRNA transcript, or it may target both the coding sequence and the 3' UTR sequence of the furin mRNA transcript simultaneously.

BRIEF DESCRIPTION OF THE DRAWINGS

For a more complete understanding of the features and advantages of the present invention, reference is now made to the detailed description of the invention along with the accompanying figures and in which:

FIGS. 1A-1C are plots showing the summary of: (1A) TGF-β1, (1B)-β2, and (1C) GMCSF protein production pre and post FANG plasmid transfection. ELISA values from Day 4 of the 14-day determinations of cytokine production in manufactured autologous cancer cells. Data represents autologous vaccines independently generated from 10 patients who underwent FANG processing (FANG 001 – 010).

FIGS. 2A-2F are plots showing: (2A) TGF-β1, (2B) TGF-β2, and (2C) GMCSF expression in FANG-004 tumor cells pre and post FANG cGMP plasmid transfection and (2D) TGF-β1, (2E) TGF-β2, and (2F) GMCSF expression in TAG-004 tumor cells pre and post TAG cGMP plasmid transfection. FANG-004 and TAG-004 are from the same tumor and processed sequentially on the same two days as a demonstration of comparative expression profiles;

FIGS. 3A-3C are plots showing that the side-by-side comparison of electroporation of FANG plasmid (the cGMP vaccine manufacturing process) versus the TAG plasmid into patient tumor cells demonstrated (3C) GMCSF protein production and concomitantly; (3A) TGF-β1 expression knocked down by FANG but not TAG and (3B) TGF–β2 knockdown by both FANG and TAG (coincident line);

FIG. 4A is a schematic showing the bi-shRNA^{furin} comprising two stem-loop structures with miR-30a backbone (SEQ ID NO.: 1); the first stem-loop structure has complete complementary guiding strand and passenger strand, while the second stem-loop structure has three basepair (bp) mismatches at positions 9 to 11 of the passenger strand;

FIG. 4B shows the siRNA targeted regions of furin mRNA. Prospective siRNA targeting regions in 3'-UTR and encoding regions of furin mRNA and the targeted sequence by each siRNA;

FIG. 5 is an image shows PET CT 11 cycles in a patient after TAG demonstrating significant response. Residual uptake at L 2 was followed up with a MRI scan and biopsy which revealed no malignancy;

FIGS. 6A-6C show an assessment of GMCSF expression and TGF-β1 and -β2 knockdown, summarizing: (6A) TGF-β1, (6B) TGF-β2, and (6C) GM-CSF protein production before and after FANG or TAG (TAG 004) plasmid transfection. Values represent ELISA

determinations of cytokine production in harvested autologous cancer cells transfected with FANG. Data represents autologous vaccines independently generated from 9 patients who underwent FANG processing (FANG 001 – 009). One patient had sufficient tissue to construct both a FANG (blue) and TAG vaccine (red) (FANG 004/TAG 004);

FIG. 7 shows the GMCSF mRNA by RT-qPCR in Pre and Post FANG Transfected / Irradiated Tumor Cells. Absent bands in some of the lanes is due to degraded RNA. (The extra band in FANG-009 is Day 0 sample loaded twice);

FIG. 8 shows FANG Vaccine cells from a patient pre-transfection and post-transfection, irradiation mRNA by PCR. No signal was detected in pre- and post- samples for TGF-β2;

FIG. 9 shows FANG-009 Vaccine cells pre-transfection and post-transfection /irradiation mRNA by PCR;

FIG. 10 shows the overall survival for Cohort 1 versus Cohorts 2 and 3 for advanced-stage patients (n = 61; P = .0186);

FIG. 11 shows a schematic diagram of GM-CSF-TGF-β2 antisense (TAG) plasmid;

FIG. 12 shows the expression of GM-CSF in NCI-H-460 Squamous Cell and NCI-H-520, Large Cell (NSCLC) containing the pUMVC3-GM-CSF-2A-TGF-β2 antisense vector, *in vitro*;

FIG. 13 shows that TGF-β2 levels are reduced in NCI-H-460 Squamous Cell and NCI-H-520, Large Cell (NSCLC) with the pUMVC3-GM-CSF-2A-TGF-β2 antisense (TAG) vector;

FIG. 14 shows that a 251 base pair probe specifically detects the GM-CSF-2A-TGF-β2 (TAG) transcript expressed in vitro in NCI-H-460 and NCI-H-520 cells (lanes 6 and 10);

FIG. 15 shows the GM-CSF expression in TAG vaccines;

FIG. 16 shows the TGF-β1 expression in TAG vaccines

FIG. 17 shows the TGF-β2 expression in TAG vaccines;

FIGS. 18A and 18B show expression of: (14A) TGF-β1 and (14B) TGF-β2 in human cancer lines following siRNAfurin knockdown; and

FIG. 19 shows the plasmid construct of FANG.

DETAILED DESCRIPTION OF THE INVENTION

While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not delimit the scope of the invention.

To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as "a", "an" and "the" are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims.

It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method, kit, reagent, or composition of the invention, and vice versa. Furthermore, compositions of the invention can be used to achieve methods of the invention.

Table 1: Abbreviations Table

Abbreviation	**Term**
AE	Adverse event
ALT	Alanine transaminase (also referred to as SGPT)
ANC	Absolute neutrophil count
APC	Antigen Presenting Cells
AST	Aspartate transaminase (also referred to as SGOT)
BUN	Blood urea nitrogen
CBC	Complete blood count
CD	Cluster of differentiation
CMV	Cytomegalovirus
CO_2	Total carbon dioxide
CR	Complete response
CRF	Case report form
CTCAE	Common Toxicity Criteria for Adverse Events
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell(s)
DTH	Delayed-type hypersensitivity
ECOG PS	Eastern Cooperative Oncology Group Performance Score
ELISA	Enzyme-Linked ImmunoSorbent Assay
ELISPOT	Enzyme-Linked ImmunoSorbent Spot
ER	Endoplasmic reticulum
FANG	bishRNA^{furin} and GMCSF Augmented Autologous Tumor Cell

Abbreviation	Term
	Vaccine
FL	Flt-3-Ligand
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor (Accession No. NM_000758)
GMP	Good manufacturing practice
GVAX	GMCSF Secreting autologous or allogenic tumor cells
HLA	Human Leukocyte Antigen
IBC	Institutional Biosafety Committee
IEC	Independent Ethics Committee
IL	Infiltrating lymphocytes
IRB	Institutional Review Board
LAK	Lymphokine-activated killer
LD	Longest diameter
LLC	Large latent complex
MHC	Major histocompatability complex
MLR	Mixed lymphocyte reaction
MR	Mannose receptor
NK	Natural Killer
NKT	Natural Killer T cell(s)
NSCLC	Non small cell lung cancer
PCR	Polymerase chain reaction
PD	Progressive disease
PR	Partial response
PS	Performance Status
RECIST	Response Evaluation Criteria in Solid Tumors
SCLC	Small cell lung cancer
SD	Stable disease
SLC	Small latent complex
STMN1	Stathmin 1
TAP	transporter associated with Ag processing
TGF-β	Transforming growth factor-β
TIL	Tumor infiltrating lymphocytes
TNF	Tumor necrosis factor
ULN	Upper limits of normal
WNL	Within normal limits

Furin is a member of the subtilisin-like proprotein convertase family (human Furin Accession No. NM_002569). The members of this family are proprotein convertases (PCs) that process latent precursor proteins into their biologically active products. Furin, a calcium-dependent serine endoprotease, efficiently cleaves precursor proteins at their paired basic amino acid processing sites by the consensus sequence –Arg-X-K/Arg-Arg (RXK/RR), with -RXXR- constituting the minimal cleavage site. Like many other proteases, PCs are synthesized as

inactive zymogens with an N-terminal prosegment extension, which is autocatalytically removed in the endoplasmic reticulum to achieve functionality.

High levels of furin have been demonstrated in virtually all cancer lines. A 10-fold higher level of TGF-β1 may be produced by human colorectal, lung cancer and melanoma cells, and likely impact the immune tolerance state by a higher magnitude. Transforming growth factors betas (TGF-β) are a family of multifunctional proteins with well known immunosuppressive activities. The three known TGF-β ligands (TGF-β1-3, Accession Nos. NM_000660, NM_003238, NM_003239.2, respectively) are ubiquitous in human cancers. TGF-β overexpression correlates with tumor progression and poor prognosis. Elevated TGF-β levels within the tumor microenvironment are linked to an anergic antitumor response. The presence of furin in tumor cells likely contributes significantly to the maintenance of tumor directed TGF-β1 mediated peripheral immune tolerance. Hence, furin knockdown represents a novel and attractive approach for optimizing immunosensitization.

A Furin-knockdown and GM-CSF-augmented (FANG) Autologous Cancer Vaccine for Human Melanoma and Lung Cancer: FANG uniquely incorporates a bi-functional small hairpin RNA (shRNA) construct specific for the knockdown of furin, a proprotein convertase critically involved in the functional processing of all TGF-β isoforms. Prior work by the inventors has demonstrated the effectiveness of FANG in generating GM-CSF expression and TGF-β1 and TGF-β2 depletion in human cancer lines. The incorporation of a bi-functional shRNAfurin in combination with hGM-CSF into an autologous cell vaccine is demonstrated herein to promote and enhance the immune response based on its effect on the afferent limb of that immune response.

As used herein the term "bi-functional" refers to a shRNA having two mechanistic pathways of action, that of the siRNA and that of the miRNA (the guide strand being non-complementary to the mRNA transcript) or miRNA-like (the guide strand being complementary to the mRNA transcript). The term "traditional" shRNA refers to a DNA transcription derived RNA acting by the siRNA mechanism of action. The term "doublet" shRNA refers to two shRNAs, each acting against the expression of two different genes but in the "traditional" siRNA mode.

Survival of patients with advanced NSCLC, the most common cancer involving both men and women, is 7 months or less following treatment with second line chemotherapy. Limited

survival benefit and toxicity related to the cancer and the treatment commonly forces patients to decline further therapy. Demonstration of safety and extensive clinical justification including examples of dramatic response related to "targeted" immune stimulation and suppression of endogenous immune inhibition using the novel, mature technology of the present invention described herein provides an opportunity for safe and potentially effective clinical assessment. The commercial expansion of the RNA interference technology and vaccine manufacturing of the present invention will provide a gateway opportunity into management of NSCLC and likely other solid tumors, notably melanoma, ovary, prostate cancer, colon cancer and breast cancer

Overcoming immune tolerance with cancer vaccines is a promising but difficult quest. The prevailing hypotheses for immune tolerance, based primarily on animal studies, include the low immunogenicity of the tumor cells, the lack of appropriate presentation by professional antigen presenting cells, immune selection of antigen-loss tumor variants, tumor induced immunosuppression, and tumor-induced privileged site [1]. Nevertheless, recent clinical trials that are based on transgene-expressing whole cancer cell vaccines have yielded promising results [2-5]. Whole cancer cell vaccines can potentially elicit broad-based, polyvalent immune responses to both defined and undefined tumor antigens, thereby addressing the possibility of tumor resistance through downregulation and/or selection for antigen-loss variants [6, 7].

Dranoff and Jaffee have shown in animal models [8], that tumor cells genetically modified to secrete GM-CSF, as compared to other cytokines, consistently demonstrated the most potent induction of anti-tumor immunity. When integrated as a cytokine transgene, GM-CSF enhances presentation of cancer vaccine peptides, tumor cell lysates, or whole tumor cells from either autologous or established allogeneic tumor cell lines [9]. GM-CSF induces the differentiation of hematopoietic precursors into professional antigen presenting (APC) dendritic cells (DC) and attracts them to the site of vaccination [8, 10]. GM-CSF also functions as an adjuvant for the DC maturation and activational processes of tumor antigen capture, process and presentation, upregulates their expression of costimulatory molecules, and their ability to migrate to secondary lymphoid tissues for activation of CD4+, CD8+ T cells, CD1d restricted invariant natural killer T (NKT) cells, and antibody producing B cells [11].

Recently, Hodi [12] reported that GVAX vaccination, followed by periodic infusions of anti-CTLA-4 antibodies to modulate effector and T regulatory cell functions, can generate clinically meaningful antitumor immunity in a majority of metastatic melanoma patients. These findings are consistent with the thesis that vaccination with a GM-CSF-augmented autologous cancer vaccine can successfully generate an immune mediated tumor destruction, particularly when coupled with an adjuvant treatment that depletes FoxP3+ Tregs activity, enhances tumor expression of MHC class I A chain (MICA) thereby activating natural killer (NK) and T cells, and enhances central memory T-cell CD4+ and CD8+ response.

The FANG approach of the present invention is supported by the findings of the inventors in 10 patients' autologous vaccines, which demonstrated consistently TGF-β1 and TGF-β2 reductions and elevated GM-CSF levels (FIGS.1A-1C and FIGS. 2A-2F). Soundness of the furin-depletion approach has been confirmed by proof of principle documentation with the furin inhibitor decanoyl-Arg-Val-Lys-Arg-CMK (Dec-RVKR-CMK) in cancer cell lines (CCL-247 colorectal, CRL-1585 melanoma lines). Dec-RVKR-CMK is a peptidyl chloromethylketone that binds irreversibly to the catalytic site of furin and blocks its activity [59]. Dec-RVKR-CMK either completely or partially reduces the activity of furin substrates BASE (β-site APP-cleaving enzyme), MT5-MMP, and Boc-RVRR-AMC [60]. The present inventors found both TGF-β1 and TGF-β2 activity to be significantly reduced in CCL-247 and CRL-1585 cancer lines by specific immunoassay, confirming the effectiveness of furin blockade on TGF-β isoform expression.

The FANG plasmid (FIG. 19) used to transfect the autologous cells is derived from the TAG plasmid [74] by replacing the human TGFβ2 antisense sequence with the bi-shRNAfurin DNA sequence. Otherwise these two plasmids are identical (confirmed by DNA sequencing). The bi-shRNAfurin consists of two stem-loop structures with miR-30a backbone; the first stem-loop structure has complete complementary guiding strand and passenger strand, while the second stem-loop structure has three bp mismatches at positions 9 to 11 of the passenger strand (FIG. 4A). The inventors adopted a strategy of using a single targeted site for both cleavage and sequestration. The encoding shRNA is able to accommodate mature shRNA loaded onto more than one types of RISC [65]. The rationale for focusing on a single site is that multi-site targeting may increase the chance for "seed sequence" induced off-target effect [66]. The two stem-loop double stranded DNA sequence was assembled with 10 pieces of synthetic complementing and interconnecting oligonucleotides through DNA

ligation. The completed 241 base pairs DNA with Bam HI sites at both ends was inserted into the Bam HI site of the TAG expression vector in place of the TGFβ2 antisense sequence. Orientation of the inserted DNA was screened by PCR primer pairs designed to screen for the shRNA insert and orientation. The FANG construct has a single mammalian promoter (CMV) that drives the entire cassette, with an intervening 2A ribosomal skip peptide between the GM-CSF and the furin bifunctional shRNA transcript, followed by a rabbit polyA tail. There is a stop codon at the end of the GM-CSF transcript. Insertion of picornaviral 2A sequences into mRNAs causes ribosomes to skip formation of a peptide bond at the junction of the 2A and downstream sequence, leading to the production of two proteins from a single open reading frame [67]. However, in the instances in which shRNA or antisense are being expressed as the second transcript (as examples), only the first transcript is translated. The inventors found that the 2A linker to be effective for generating approximately equal levels of GM-CSF and anti-TFG-β transcripts with the TAG vaccine, and elected to use the same design for FANG.

The inventors validated the applicability of siRNA-mediated furin-knockdown for inhibiting human TGF-β isoform expression. Prospective siRNA targeting sites (FIG. 4B) in the furin mRNA sequence were determined by the published recommendations of Tuschl and colleagues and the additional selection parameters that integrates BLAST searches of the human and mouse genome databases [73]. siRNAs targeting eligible coding and 3'UTRs sites (FIG. 4B) were tested. Following lipofection of CCL-247, CRL-1585 U87 and H460 cells, each of the 6 $siRNA^{furin}$ constructs was shown to markedly reduce TGF-β1 and TGF-β2 levels in culture supernatants without adversely affecting cell survival. Thus siRNA-mediated furin knockdown is effective for the depletion of TGF-β1 and -β2 isoforms.

The present inventors attempted to detect endogenous Furin protein in cell lines via Western Blot and Flow Cytometry. Five different commercial antibodies were screened for Western Blot and one pre-labeled antibody was screened for Flow Cytometry. All studies yielded negative results. Upon further study of the commercially available antibodies, all idiotypes were developed against fragments (or peptides) of the Furin protein. The Western Blot studies demonstrated that the 60kDa variant was preferentially detected in 4 of the 5 antibodies screened. The last antibody did not detect Furin protein under the Western Blot conditions tested. Control lysates provided by the commercial vendors produced similar results to in-house cell line samples. The pre-labeled antibody for Flow Cytometry did not

demonstrate a significant shift in Furin staining (i.e., no positive Furin population identified). Therefore, the Flow Cytometry could not be used to demonstrate Furin knockdown.

As an alternative to Furin protein detection, the inventors also screened samples for Furin enzyme activity. Using a fluorometric based assay, cell lines were screened for the conversion of substrate (Pyr-Arg-Thr-Lys-Arg-AMC) by Furin to release the fluorophore (AMC). However, the detected signal of released AMC was too low to accurately demonstrate significant knockdown of Furin enzyme activity. A second barrier to the assay is that the substrate is cleaved by all serine proteases in the subtilisin-like prohormone convertase (PC) family. Therefore, similar proteases that are not targeted by our FANG shRNA product would remain active and cleave the fluorogenic substrate in the assay, thus reducing the capability to detect Furin knockdown.

Other applications for the bi-functional shRNAfurin include: (1) Systemic delivery via a tumor (± tumor extracellular matrix (ECM)) selective decorated (targeted), stealthed bilamellar invaginated liposome (BIV) to enhance the efferent limb of the immune response; (2) Systemic delivery via a tumor selective decorated (targeted), stealthed bilamellar invaginated liposome (BIV) to directly subvert the tumor promoting/maintaining effects of furin target molecules including, but not limited to human, TGFβ1, TGFβ2, TGFβ3, IGF-II, IGF-1R, PDGF A, and, in some tumor types, MT1-MMP; (3) Systemic delivery via a tumor selective decorated (targeted), stealthed bilamellar invaginated liposome (BIV) to directly subvert the NOTCH/p300 pathway in putative cancer stem cells; (4) Systemic delivery via a tumor selective decorated (targeted), stealthed bilamellar invaginated liposome (BIV) to inhibit activation of toxins associated with anthrax, Shiga, diphtheria, tetanus, botulism and Ebola and Marburg viruses and/or (5) Systemic and/or inhalational delivery of a bilamellar invaginated liposome (BIV) (±decoration and reversible masking/stealthing) to inhibit Pseudomonas exotoxin A production as an adjunct to antibiotic therapy in patients with diseases with heightened risk of Pseudomonas mediated morbidity and mortality, e.g., cystic fibrosis.

TGF-β Knockdown: Transforming growth factors beta (TGF-β) are a family of multifunctional proteins with well known immunosuppressive activities [13]. The three known TGF-β ligands (TGF-β1, β2, and β3) are ubiquitous in human cancers. TGF-β overexpression correlates with tumor progression and poor prognosis [14, 15]. Elevated TGF-β levels within the tumor microenvironment are linked to an anergic antitumor

response [14, 16-21]. TGF-β inhibits GM-CSF induced maturation of DCs [22] and their expression of MHC class II and co-stimulatory molecules [23]. Ardeshna [24] showed that lipopolysaccharide (LPS)-induced maturation of monocyte-derived DCs involved activation of p38 stress-activated protein kinase (p38SAPK), extracellular signal-regulated protein kinase (ERK), phosphoinositide 3-OH- kinase (PI3 kinase)/Akt, and nuclear factor (NF)-κB pathways. GM-CSF can exert parallel activities of stimulating myeloid hematopoietic cell and leukemia cell line proliferation through rapid, transient phosphorylation of MAP kinase 1/2 and ERK 1/2, whereas TGF-β turns off GM-CSF-induced ERK signaling via PI3-kinase-Akt pathway inhibition [25].

At the efferent level, antigen presentation by immature DCs contributes to T cell anergy [26]. TGF-β similarly inhibits macrophage activation [27] and their antigen presenting function [28, 29]. TGF-β inhibits the activation of cytotoxic T cells by impairing high affinity IL-2 receptor expression and function [30, 31]. TGF-β2 also converts naïve T cells to Treg cells by induction of the transcription factor FOXP3 [32], with emergence of Treg leading to the shutdown of immune activation [33]. According to Polak [34], tolerogenic DCs and suppressor T lymphocytes were present in all stages of melanoma. These immune cell types expressed TGF-β receptor I, and tolerogenic activity was dependent on TGF-β1 or -β2 binding.

At the innate immune response level, TGF-β is antagonistic on NK cells and down-regulates lymphokine activated killer (LAK) cell induction and proliferation [30, 35-39]. Penafuerte [40] recently showed that tumor-secreted TGF-β suppressed GM-CSF+IL2 (GIFT2) mediated immunosensitization of NK cells in the immunocompetent B16 melanoma model. In vivo blockade of B16 production of TGF-β improved survival otherwise compromised by the growth of non-GIFT2 expressing bystander tumors. These findings further validate the negative impact of TGF-β on GM-CSF-mediated immune activation *in vivo*, and by extension, support the rationale of depleting TGF-β secretion in GM-CSF-based cancer cell vaccines.

Trials conducted by the present inventors utilizing a tumor cell vaccine with TGF-β 2 knockdown activity (Belagenpumatucel-L) in patients with non-small cell lung cancer demonstrated acceptable safety, and a dose-related survival improvement in response to randomized control patients and historical experience. The two-year survival for the late stage (IIIB/IV) patients was 52% for patients who received > 2.5×10^7 cells/injection, which

compares favorably with similar patient historical data of less than 10% survival at 2 years. The study patients also displayed significantly elevated cytokine production (IFN-γ, p=0.006; IL-6, p=0.004; IL4, p=0.007) and antibody titers to vaccine HLA antigens (p=0.014), suggesting an immune activating outcome. [41].

TGF-β-knockdown and GM-CSF Expressive Cancer Cell Vaccine (TAG): Thirty six patients were harvested for TAG vaccine. GM-CSF expression and TGF-β2 knockdown met product release criteria. Three (all gastrointestinal tumors with luminal access) had bacterial contaminants and could not be released. One had insufficient cells. Nineteen advanced refractory cancer patients were treated [42-44]. No Grade 3 toxic effects related to therapy were observed. Eleven of 17 (65%) evaluable patients maintained stable disease for at least 3 months. One patient achieved CR by imaging criteria (FIG. 4; melanoma). Thus the TAG vaccine appears to be safe and has evidence of clinical efficacy.

A potential limitation of TAG vaccine, however, is the restricted specificity for TGF-β2, given that all three known isoforms of TGF-β ligand (TGF-β1, -β2, and -β3) are ubiquitously produced in human cancers. In particular, up to a 10-fold higher level of TGF-β1 may be produced by human colorectal, lung cancer, and melanoma cells. The tolerogenic role of TGF-β1 in antigen presenting dendritic cells (DC) and regulatory T cells (Treg) is well established, and this activity is not impacted by TGF-β2 antisense treatment.

Furin: All mature isoforms of TGF-β require limited proteolytic cleavage for proper activity. The essential function of proteolytic activation of TGF-β is mediated by furin. Furin is a member of the subtilisin-like proprotein convertase family. The members of this family are proprotein convertases (PCs) that process latent precursor proteins into their biologically active products. Furin, a calcium-dependent serine endoprotease, efficiently cleaves precursor proteins at their paired basic amino acid processing sites by the consensus sequence –Arg-X-K/Arg-Arg (RXK/RR), with -RXXR- constituting the minimal cleavage site [53]. Like many other proteases, PCs are synthesized as inactive zymogens with an N-terminal prosegment extension, which is autocatalytically removed in the endoplasmic reticulum to achieve functionality [52].

Furin is best known for the functional activation of TGF-β with corresponding immunoregulatory ramifications [54, 55]. Apart from the previously described immunosuppressive activities of tumor secreted TGF-β, conditional deletion of endogenous-

expressing furin in T lymphocytes was found to allow for normal T-cell development, but impaired the function of regulatory and effector T cells, which produced less TGF-β1. Furin-deficient Tregs were less protective in a T-cell transfer colitis model and failed to induce Foxp3 in normal T cells. Additionally, furin-deficient effector cells were inherently over-active and were resistant to suppressive activity of wild-type Treg cells. In APCs, cytotoxic T lymphocyte-sensitive epitopes in the trans-Golgi compartment were processed by furin and the less frequented TAP independent pathway [56]. Thus furin expression by T cells appears to be indispensable in maintaining peripheral tolerance, which is due, at least in part, to its non-redundant, essential function in regulating TGF-β1 production.

High levels of furin have been demonstrated in virtually all cancer lines [45-52]. The present inventors and others have found that up to a 10-fold higher level of TGF-β1 may be produced by human colorectal, lung cancer, and melanoma cells, and likely impact the immune tolerance state by a higher magnitude [34, 57, 58]. The presence of furin in tumor cells likely contributes significantly to the maintenance of tumor directed, TGF-β1 mediated peripheral immune tolerance [54]. Hence furin knockdown represents a novel and attractive approach for optimizing immunosensitization.

FANG (furin shRNA and GMCSF) vaccine: The present inventors constructed the next generation vaccine termed FANG. The novelty of the FANG vaccine lies in the combined approach of depleting multiple immunosuppressive TGF-β isoforms by furin knockdown, in order to maximize the immune enhancing effects of the incorporated GM-CSF transgene on autologous tumor antigen sensitization.

All mature isoforms of TGF-β require proteolytic activation by furin. The feasibility of achieving concomitant depletion of multiple TGF-β isoform activity in several cancer cell lines (H460, CCL-247, CRL-1585, U87) was determined using furin-knockdown and the present inventors have successfully completed GMP manufacturing of FANG vaccine in 9 cancer patients (breast -1; colon – 2; melanoma – 4; gallbladder – 1; NSCLC – 1). Assessment of GMCSF expression and TGF-β1 and -β2 knockdown is shown in FIGS. 1A-1C and FIGS. 2A-2F.

Electroporation of FANG plasmid into patient tumor cells demonstrated GMCSF protein production and concomitantly TGF-β1 and –β2 knockdown as predicted. FIGS. 3A-3C depicts Day 7 assay data of a FANG-transfected NSCLC tumor's expression profile (FANG-

004) versus tissue from the same the tumor processed by the cGMP TAG vaccine method (denoted TAG-004). There are similar reductions in TGFβ2 (FIG. 3B; single line shown due to coincident data) and similar increases in GMCSF (FIG. 3C) expression. However, while TGFβ1 expression is completely inhibited by FANG, it is unaffected by TAG as the TGFβ2 antisense cannot block TGFβ1 expression (FIG. 3A).

FIG. 5 is a PET CT image of an advanced melanoma patient after 11 TAG vaccine treatments demonstrating a significant clinical response. Residual uptake at L 2 was followed up with a MRI scan and biopsy which revealed no malignancy. The patient has consequently become a complete response (no evidence of disease) for greater than seven months. The capability of FANG to knockdown both TGF-β1 and -β2 is supported yb findings in the first 9 patients (FIGS. 6A-6C) who underwent vaccine construction. All 9 vaccine preparations demonstrated significantly elevated levels of GM-CSF (80-1870 pg/ml at day 4 of culture, median of 739 pg/ml). All 9 patients demonstrated >50% reductions of TGF-β2, and 6 of 7 patients with >100 pg of endogenous TGF-β1 production also demonstrated >50% reduction of this cytokine. The expanded target effectiveness of FANG is best demonstrated in one patient (NSCLC) who had adequate tumor tissue to generate both TAG (TAG-004A) and FANG (FANG-004) versions of autologous vaccine, TGF-β1 (as well as TGF-β2) was depleted to below detectable levels using the FANG preparation (FANG-004) from an initial concentration of 1840 pg/ml whereas this high level of TGF-β1 was unchanged with the TAG preparation (TAG-004) albeit with the expected depletion of TGF-β2. These findings support the potential advantage of the FANG vaccine preparation.

Validation of bioactivity of personalized cGMP FANG vaccines: Gene modification will be achieved by the use of a plasmid vector encoding for GM-CSF and a bi-functional short hairpin (bi-sh) RNA optimized for furin knockdown. Cancer patient autologous FANG vaccine has already been generated under cGMP conditions for clinical trial of patients with advanced solid cancers. GM-CSF and TGF-β1, -β2, and -β3 mRNA and protein expression were measured as part of the quality assurance process. Cytokine bioactivity following FANG modification was determined by growth outcome in a GM-CSF and TGF-β dependent cell line utilized by the present inventors in previous studies. Processed vaccine will undergo proteogenomic screening to verify antigenic integrity following FANG modification.

To characterize the augmenting effect of CTLA-4 blockade: Given that FANG immunization only impacts the afferent immunosensitization process, additional approaches that promote

tumor-specific immune effector responses may further promote antitumor outcome. Disrupting Treg suppression and/or enhancing T effectors (Teff) by blockade of the cytotoxic T lymphocyte-4 (CTLA-4) function may enhance the likelihood of clinical success of the FANG vaccine.

RT-qPCR analysis was performed on ten FANG vaccine samples (FANG-003 did not have adequate mRNA for analysis). Samples were cultured pre-electroporation and post-electroporation, post-irradiation for up to 14 days. Total RNA was extracted from each sample at various time points and converted into cDNA via reverse transcription (RT). Quantitative PCR (qPCR) was performed to assess the amount of template present in each sample, at each time point. Furin, TGFβ1, and TGFβ2 qPCR samples were normalized to endogenous GapDH to produce a relative cycle threshold (Ct) value. GMCSF was quantified against an external standard curve to produce an absolute Ct value, relative to the standard curve. The GMCSF mRNA detection is shown in FIG. 7. Post-transfection, GMCSF mRNA is detected in all vaccines but the values are variable depending on mRNA quality – a persistent issue. Table 1 illustrates representative data from two FANG vaccines (FIGS. 8 and 9). All samples were calculated as normalized pre-electroporation Ct values minus normalized post-electroporation, post-irradiation Ct values (pre-post) to calculate the delta Ct (Δ Ct). A calculated Δ Ct < 0.00 represents a decrease in template DNA and a calculated Δ Ct > 0.00 represents an increase in template DNA. The Δ Ct value is used to estimate the percent change in expression (% expression). Values less than 100% represent a decrease in DNA (from pre to post) and values greater then 100% represent an increase in DNA (from pre to post). The nature of shRNA / siRNA silencing can optimally reduce the template DNA 90%, which is equivalent to a Δ Ct = -3.3. (A Δ Ct = -1.0 is equivalent to a 50% knockdown.) Therefore, the data below demonstrate that the FANG plasmid DNA is able to reduce endogenous Furin down 80 – 26% (average = 48%) and the downstream targets TGFβ1 and TGFβ2 are reduced down 98 – 30% (average = 75%). The mechanisms of action of the Furin bifunctional shRNA are to block Furin protein production at the post-transcriptional and translational levels. The reduced levels of Furin protein also impact (by feedback regulation) the expression of TGFβ1 and TGFβ2 mRNA, the conversion of the proform of TGFβ1 and TGFβ2 protein into the mature (active) form of their respective proteins [75], and, by interfering with the TGFβ→furin amplification loop, further dampen the expression of furin itself [76]. It is also possible that accumulation of the proform of the TGF protein may feedback inhibit the transcription of its TGF gene.

Table 1: RT-qPCR Analysis of FANG Vaccines (Pre Versus Post Electroporation)

FANG-008

Furin		
Time Point	Δ Ct	% Expression
day 0	-1.52	35%
day 1	-1.50	35%
day 2	-1.48	36%
day 4	-1.50	35%
day 7	-1.22	43%
day 10	-1.41	38%

TGFB1		
Time Point	Δ Ct	% Expression
day 0	-0.09	94%
day 1	-0.10	93%
day 2	-0.08	95%
day 4	-0.05	97%
day 7	-0.08	95%
day 10	-0.11	93%

TGFB2		
Time Point	Δ Ct	% Expression
day 0	0.00	n/a *
day 1	0.00	n/a *
day 2	0.00	n/a *
day 4	0.00	n/a *
day 7	0.00	n/a *
day 10	0.00	n/a *

FANG-009

Furin		
Ident.	Δ Ct	% Expression
day 0	-0.66	63%
day 1	-0.69	62%
day 2	-0.34	79%
day 4	-0.31	80%
day 7	-1.93	26%
day 10	0.00	n/a *

TGFB1		
Ident.	Δ Ct	% Expression
day 0	-0.54	69%
day 1	-0.49	71%
day 2	-0.42	75%
day 4	-0.31	81%
day 7	-0.04	98%
day 10	-1.29	41%

TGFB2		
Ident.	Δ Ct	% Expression
day 0	-0.53	69%
day 1	-0.47	72%
day 2	-0.45	73%
day 4	-0.52	70%
day 7	-1.70	31%
day 10	-1.74	30%

Δ Ct baseline = 0.00
% expression baseline = 100%

* n/a = not applicable because template was below detection limits

The FANG system was used with 9 patient autologous vaccines, which consistently demonstrated TGF-β1 and TGF-β2 reductions and elevated GM-CSF levels (FIGS. 6A-6C). Both TGF-β1 and TGF-β2 activity by specific immunoassay was also demonstrated to be significantly reduced in these cancer lines, confirming the effect of furin blockade on TGF-β isoform expression. The inventors validated the applicability of siRNA-mediated furin-knockdown for inhibiting TGF-β isoform expression. Prospective siRNA targeting sites in the furin mRNA sequence (FIG. 4B) were determined by the published recommendations of

Tuschl and colleagues and the additional selection parameters that integrated BLAST searches of the human and mouse genome databases (jura.wi.mit.edu/bioc/siRNAext). siRNAs targeting eligible translated and 3'UTRs sites (FIG. 4B) were tested. Demonstration of FANG plasmid DNA knockdown of furin mRNA is shown in FIGS 8 and 9. This could only be detected in two of the vaccines because readily detectable furin mRNA was present in only these two tumors pretransfection. The mechanisms of action of the bi-shRNA^{furin} are the blockade of furin protein production at the post transcriptional and translational levels. The reduced levels of furin protein also impact (by feed back regulation) the expression of TGF-β1 and TGF-β2 mRNA, the conversion of the proform TGF-β1 and TGF-β2 protein into the mature (active) form of their respective proteins [75] and by interfering with the TGF-β→furin loop, further dampening the expression of furin itself [76]. The possibility that the accumulation of the proform of the TGF protein may feedback and inhibit the transcription of its TGF gene should not be in any way construed as a limitation of the present invention. The expanded target effectiveness of FANG is best demonstrated in one patient (NSCLC) who had adequate tumor tissue to generate both TAG (TAG-004) and FANG (FANG-004) versions of autologous vaccine. TGF-β1 (as well as TGF-β2) was depleted to below detectable levels using the FANG preparation (FANG-004) from an initial concentration of 1840 pg/ml. This high level of TGF-β1 was unchanged with the TAG preparation (TAG-004) albeit with the expected depletion of TGF-β2 (FIGS. 1A-1C and FIGS. 2A-2F). These findings support the mechanistic advantage of the FANG vaccine preparation.

Following lipofection of CCL-247, CRL-1585 U87 and H460 cells, each of the 6 siRNAfurin constructs was shown to markedly reduce TGF-β1 and TGF-β2 levels in culture supernatants without adversely affecting cell survival. Thus siRNA-mediated furin knockdown is effective for the depletion of TGF-β1 and -β2 isoforms.

Design and construction of FANG: A "bi-functional " vector was used that incorporates both siRNA and miRNA-like functional components for optimizing gene knockdown [61]. The siRNA component is encoded as a hairpin and encompasses complete matching sequences of the passenger and guide strands. Following cleavage of the passenger strand by the Argonaute-2 (Ago 2) of the RNA-induced silencing complex (RISC), an endonuclease with RNase H like activity, the guide strand binds to and cleaves the complementary target mRNA sequence. In distinction, the miRNA-like component of the "bi-functional" vector

incorporates mismatches between the passenger and guide strands within the encoding shRNA hairpin in order to achieve lower thermodynamic stability. This configuration allows the passenger strand to dissociate from RISC without cleavage (cleavage-independent process) independent of Ago 2 [62, 63], and the miRNA guide component to downregulate its target through translational repression, mRNA degradation, and sequestration of the partially complementary target mRNA in the cytoplasmic processing bodies (P-body).

The inventors have previously demonstrated the enhanced effectiveness of a bi-functional shRNA to knockdown stathmin (STMN1; oncoprotein 18), a protein that regulates rapid microtubule remodeling of the cytoskeleton and found to be upregulated in a high proportion of patients with solid cancers [64]. The bi-functional shRNA construct achieved effective knockdown against STMN-1 resulting in a 5-log dose enhanced potency of tumor cell killing as compared with siRNA oligonucleotides directed against the same gene target.

A similarly designed bi-functional shRNA was used to effect furin knockdown. The bi-sh-furin consists of two stem-loop structures with a miR-30a backbone; the first stem-loop structure has complete complementary guiding strand and passenger strand, while the second stem-loop structure has three bp mismatches at positions 9 to 11 of the passenger strand. The inventors adopted a strategy of using a single targeted site for both cleavage and sequestration processes. The encoding shRNAs are proposed to allow mature shRNA to be loaded onto more than one type of RISC [65]. The inventors focused on a single site since multi-site targeting may increase the chance for "seed sequence" induced off-target effects [66].

The two stem-loop structure was put together with 10 pieces of complementing and interconnecting oligonucleotides through DNA ligation. Orientation of the inserted DNA was screened by PCR primer pairs designed to screen for the shRNA insert and orientation. Positive clones were selected and sequence confirmed at SeqWright, Inc. (Houston, TX). Based on siRNA findings, three bi-functional shRNA's were constructed. The optimal targeting sequence was identified.

The FANG construct has a single mammalian promoter (CMV) that drives the entire cassette, with an intervening 2A ribosomal skip peptide between the GM-CSF and the furin bi-functional shRNA transcripts, followed by a rabbit polyA tail. There is a stop codon at the end of the GM-CSF transcript.

Insertion of picornaviral 2A sequences into mRNAs causes ribosomes to skip formation of a peptide bond at the junction of the 2A and downstream sequences, leading to the production of two proteins from a single open reading frame [67]. The inventors found that the 2A linker to be effective for generating approximately equal levels of GM-CSF and anti-TGF-β transcripts with the TAG vaccine, and elected to use the same design for FANG.

Manufacturing the FANG vaccine: The patient's tumor was aseptically collected in the surgical field, placed in a gentamycin saline solution in a sterile specimen container and packaged for shipment on wet ice to the cGMP manufacturing facility. The specimen was brought into the manufacturing suite, dissected, enzymatically and mechanically disaggregated to form a cell suspension and then washed to remove debris. After the tumor cells are enumerated, QC aliquots are taken and the remaining cells are electroporated with the FANG plasmid and incubated overnight to allow vector transgene expression. Cells are harvested and gamma irradiated to arrest cell growth, then enumerated prior to removal of final QC aliquots and vaccine controlled rate freezing. The two day manufacturing process was followed by an almost three week QC testing phase after which all of vaccine assay data are evaluated prior to releasing the vaccine for patient treatment. All 9 initial patients who underwent FANG manufacturing passed all QC testing criteria.

cGMP FANG vaccines: Cancer patient autologous FANG vaccines were generated under cGMP conditions for use in clinical trials. GM-CSF and TGF-β1, -β2, and -β3 mRNA and protein expression were measured before and after FANG modification, and cytokine bioactivity determined by growth outcome on a GM-CSF and TGF-β dependent human cell line we have previously characterized. Each patient's processed vaccine will undergo proteogenomic screening to verify antigenic integrity following FANG modification.

cGMP production of FANG: FANG vaccine was generated by plasmid vector electroporation of established human cell lines. The selected FANG plasmid vector represents a construct containing the furin shRNA that has been prevalidated for optimal TGF-β downregulation.

Before being injected into patients, a frozen vial (dose) was thawed at room temperature and processed in a biosafety hood. The cell suspension will be delivered in a capped 1mL syringe. The prepared vaccine will be injected intradermally into patient at a dose of 1×10^7 or 2.5×10^7 cells per injection.

Two full scale preclinical manufacturing processes and eight clinical manufacturing processes were prepared and studies by the present inventors. Table 2 depicts the types of tumors processed (tumors 3 through 10 are the clinical vaccines).

Table 2: Tumor mass versus cell yield.

Tumor Processed	Vaccine ID	Tissue Weight (grams)	Cell # / dose	Number of Vials
1	FANG-001	12.72	1.0×10^7	40
2	FANG-002	27.41	1.0×10^7	28
3	FANG-003	6.04	2.5×10^7	9
4	FANG-004	41.08	2.5×10^7	11
5	FANG-005	6.96	2.5×10^7	8
6	FANG-006	12.48	1.0×10^7	8
7	FANG-007	10.90	2.5×10^7	15
8	FANG-008	9.80	2.5×10^7	13
9	FANG-009	6.80	1.0×10^7	6
10	FANG-010	13.00	2.5×10^7	12

The tumors processed range in size, as well as type, and the resulting viable cell yield varies greatly as shown in Table 3. All vaccines are vialed at either 1.0 $x10^7$ cells (dose Cohort 1) or 2.5 $x10^7$ cells (dose Cohort 2) depending on the total viable cell count on Day 2 of manufacturing. Patients with multiple tumor harvests were allowed to combine vials to qualify for minimum clinical dose requirement. A maximum of 12 doses at Cohort 2 dose level will be made available for patient treatment. Because tumor cell yield is highly variable due to tumor mass, cellularity, and processing compatibility, the minimum dose number and lower dose cohort (Cohort 1) were included.

Table 3: Final product viability (Day 2, Pre Irradiation)

Tumor Processed	Vaccine ID	% Viability
1	FANG-001	78
2	FANG-002	90
3	FANG-003	94
4	FANG-004	89
5	FANG-005	94
6	FANG-006	96
7	FANG-007	96
8	FANG-008	95
9	FANG-009	95
10	FANG-010	93

The Day 4 expression profiles of the 10 tumors processed are depicted in FIGS. 1A-1C. Note that the y-axis scales are different for all three cytokines. These data are representative of the 14 day assay (remainder of data not shown). The mean pretransfection TGFβ1 is 1251± 1544 pg/1x10^6 cells/ml; median 778pg. The mean posttransfection TGFβ1 is 191±455 pg/1x10^6 cells/ml; median 13 pg. The average percent knockdown of TGFβ1 was 85%. The mean pretransfection TGFβ2 is 232±164 pg/1x10^6 cells/ml; median 225pg. The mean posttransfection TGFβ2 is 1319 pg/1x10^6 cells/ml; median 5pg. The average percent knockdown of TGFβ2 was 94%. The average GM-CSF expression post transfection is 543±540 pg/1x10^6 cells/ml; median 400 pg. These data indicate that the GMCSF expression is consistent with the TAG vaccine as is the TGF β2 knockdown. In contrast, FANG vaccines have reduced the TGFβ1 expression more than fivefold. The minimum detectable quantity of TGFβ1 is approximately 4.6 pg/ml (R&D Systems, Quantikine Human TGFβ1). The minimum detectable quantity of TGFβ2 is approximately 7 pg/ml (R&D Systems, Quantikine Human TGFβ2). The minimum detectable quantity of GMCSF is approximately 3 pg/ml (R&D Systems, Quantikine Human GMCSF).

The protocol for setting up cultures pre and post Transfection for Autologous tumor cell vaccine to test for the expression of GMCSF, TGFβ1 and TGFβ2 has been previously described (Maples, et al., 2009). Briefly, GMCSF, TGFβ1 and TGFβ2 expression were

determined by commercially available ELISA kits (R & D Systems). The ELISA assays were performed according to manufacturer's instruction. The pretransfection sample (4 x 106 cells) is taken on Day 1. After manufacturing is completed, the sample is removed from the manufacturing facility so that the cell cultures can be set up for generating the sample for ELISA. On Day 2, the post-transfection, post-irradiation, pre-freeze sample (4 x 106 cells) is taken. After manufacturing is completed, the sample is removed from the manufacturing facility so that the cell cultures can be set up for generating the sample for ELISA.

Ten (10) vaccines (FANG -001 to -010) have been manufactured as part of the preclinical qualification process. These vaccines have been evaluated for GM-CSF, TGFβ1 and TGFb2 mRNA and protein expression using post-transfection, post-irradiation samples compared with pre-transfection, pre-irradiation samples (per FDA review, TAG vaccine, BB-IND 13650). In addition, Furin protein detection was attempted by several methods. Furin mRNA was detected by qRT-PCR.

The present inventors detected endogenous Furin protein in cell lines via Western Blot and Flow Cytometry. Five (5) different antibodies (from 3 different vendors) were screened for Western Blot and one (1) pre-labeled antibody was screened for Flow Cytometry. All experiments yielded negative results (data not shown).

A summary of all ELISA data for all manufacturing processes (Table 4) indicates that the median level of GMCSF expression is about 400 picogram/ml and the average is 543 picograms/ml. Further, the level of GMCSF tends to increase with time. In all manufactured products, GMCSF expression is observed although the level of expression is variable between manufacturing processes (tumor types). In addition to documented variability in the level of GMCSF expression between manufacturing processes, the levels of expression achieved with the FANG vaccine are deemed clinically relevant as 1) use of a plasmid rather than a viral vector obviates the obfuscating effects of elicited anti-viral neutralizing antibodies, 2) use of a plasmid likewise prevents the development of elicited antibodies interfering with long-term gene expression, and 3) concurrent suppression of furin, TGFβ1, and TGFβ2 will minimize tumor associated inhibition of GMCSF induced dendritic cell maturation [25].

Table 4: FANG vaccines 1-10 TGFβ1, TGFβ2 and GM-CSF expression in the 14 Day post manufacturing expression assay.

	TGFβ1 pg/ml Pre			TGFβ1 pg/ml Post			TGFβ2 pg/ml Pre			TGFβ2 pg/ml Post			GMCSF pg/ml Pre			GMCSF pg/ml Post		
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median
Day 0	625	678	416	105	202	7	78	116	35	9	22	0	2	2	2	157	277	28
Day 1	1154	1266	760	53	187	11	138	135	113	9	19	0	3	4	3	359	465	201
Day 2	998	1014	620	180	446	0	198	107	197	12	21	4	3	3	2	467	418	310
Day 3	1632	3221	879	173	384	4	247	156	229	12	18	8	3	4	2	582	531	475
Day 4	1241	1115	1035	211	421	20	293	185	257	9	12	4	4	6	3	657	556	602
Day 7	1726	1735	778	264	723	3	292	153	235	14	18	8	5	8	3	683	681	471
Day 10	1367	894	1629	243	530	21	335	135	310	23	21	28	5	8	4	745	546	673
Day 14	1108	892	867	281	681	15	308	158	225	17	23	12	18	24	4	821	531	645
Overall	1251	1544	778	191	456	13	232	164	225	13	19	5	5	16	3	543	548	400

Quantification of GM-CSF and TGF-β expressions: GM-CSF and TGF-β1 and -β2 expression was determined by cytokine specific colorimetric assay [68].

Validation of bioactivity: GM-CSF-induced proliferative activity similar to that of myeloid hematopoietic cells has been observed in myeloid leukemia cell lines, as mediated by the rapid and transient phosphorylation of MAP kinase 1/2 and ERK 1/2. By contrast, TGF-β turns off GM-CSF-mediated ERK signaling by inhibition of the PI3-kinase-Akt pathway [25]. The growth regulatory effects of GM-CSF and TGF-β on myeloid leukemic cells were used as an *in vitro* surrogate model to validate cytokine bioactivity in prepared FANG vaccine culture supernatants.

Cytokine activities in the FANG (or control-transfected) vaccine culture supernatants were validated by co-culture studies with erythroleukemic CD34+ TF-1a cells [69] and, if necessary, confirmed with the biphenotypic B myelomonocytic leukemic CD10+CD15+ MV4-11 cells [70] (ATCC, Rockville, MD). Both of these cell lines have been shown respond to the positive proliferative effects of GM-CSF and the negative inhibitory activity of TGF-β at ng/ml amounts [25]. Proliferative activity will be determined by Easycount Viasure assay (Immunicon) and MTT assay [68].

Phenotypic profile analysis of FANG modification: Furin knockdown likely impacts the expression of other protein substrates with the target sequence in addition to TGF-β downregulation [51]. The antigenic profile of the FANG-processed autologous vaccines were determined from cancer patients, in the event that this information may be useful towards the understanding any differential clinical outcome in vaccinated patients.

High throughput genetic profiling was used to develop individualized therapeutics for cancer patients. High throughput, gene expression array analysis was carried out to compare the differential gene expression profile of FANG-transfected vs. control vector-transfected cancer cells.

Differentially labeled FANG and control preparations are combined and fractionated by high performance liquid chromatography (Dionex), using a strong cation exchange column (SCX) and a 2nd dimension RP nano column. The fractions are spotted onto Opti-TOF™ LC/MALDI Insert (123 x 81 mm) plates (Applied Biosystems) in preparation for mass spectrometry analysis using the Applied Biosystems 4800 MALDI TOF/TOF™ Analyzer. Both protein and gene expression data were then evaluated by the GeneGo, MetaCore software suite.

Proteogenomic analysis was carried out for the purpose of determining the antigen repertoire of the autologous cancer vaccine before and after FANG process. In addition to the validation of furin knockdown, particular attention was focused on 1) baseline and differential expression of furin-substrate proteins; 2) expression of landmark tumor-associated antigens (TAAs; such as gp100, Mart1, MAGE-1, tyrosinase, for melanoma; MAGE-3, MUC-1 for non-small cell lung cancer) [71, 72] and other reported TAAs; 3) HLA antigens and co-stimulatory molecules (CD80/86) expression; 4) proteins unrelated to the above categories that are differentially expressed by 2-fold or higher following FANG transfection.

FIG. 10 shows the overall survival for Cohort 1 versus Cohorts 2 and 3 for advanced-stage patients (n = 61; P = .0186). A schematic diagram of GM-CSF-TGF-β2 antisense plasmid is shown in FIG. 11. The expression of GM-CSF in NCI-H-460 Squamous Cell and NCI-H-520, Large Cell (NSCLC) containing the pUMVC3-GM-CSF-2A-TGF-β2 antisense vector, *in vitro* is depicted in FIG. 12. TGF-β2 levels are reduced in NCI-H-460 Squamous Cell and NCI-H-520, Large Cell (NSCLC) with the pUMVC3-GM-CSF-2A-TGF-β2 antisense vector. This reduction is seen in the date presented in FIG. 13. FIG. 14 shows that a 251 base pair probe specifically detects the GM-CSF-2A-TGF-β2 transcript expressed in vitro in NCI-H-460 and NCI-H-520 cells (lanes 6 and 10); GM-CSF and TGF-β2 expression in TAG vaccines are shown in FIGS. 12 and 13, respectively. Expression of TGF-β1 and TGF-β2 in human cancer lines following siRNAfurin knockdown is shown in FIGS. 18A and 18B. Finally FIG. 19 is a schematic of the plasmid construct of FANG.

TGFβ1 expression data (FIG. 16) generated from TAG vaccine manufacturing data (n=33 vaccines; Day 7 values, TGFβ1 assay post vaccine manufacturing) clearly demonstrate that TAG does not interfere with TGFβ1 expression. The clinical significance of blocking TGFβ1 and TGFβ2 (FIG. 17), as well as TGFβ3 (data not shown) is that they are postulated to be significant negative immunomodulators expressed by the tumor. GM-CSF expression in TAG vaccines is shown in FIG. 15. These TGFβ isoforms are ubiquitous and expressed in the majority of tumors [77]. Many tumors, including breast, colon, esophageal, gastric, hepatocellular, pancreatic, SCLC and NSCLC produce high levels of one or more active TGFβ isoforms [78, 14, 15, 79-84]. Furthermore, overexpression of TGFβ has been correlated with tumor progression and poor prognosis [14, 15]. Elevated TGFβ levels have also been linked with immunosuppression in both afferent efferent limbs [14, 16-21]. Additionally, TGFβ has antagonistic effects on Natural Killer (NK) cells as well as the induction and proliferation of lymphokine-activated killer (LAK) cells [30, 35-39].

The immune suppressor functions of TGFβ are therefore likely to play a major role in modulating the effectiveness of cancer cell vaccines. TGFβ inhibits GMCSF induced maturation of bone marrow derived dendritic cells (DCs) [22] as well as expression of MHC class II and co-stimulatory molecules [23]. It has been shown that antigen presentation by immature DCs result in T cell unresponsiveness [26]. TGFβ also inhibits activated macrophages [27] including their antigen presenting function [28, 29]. Hence both the ubiquity of expression as well as the inhibitory effects of TGFβ on GMCSF immunomodulatory function support the knockdown of all tumor TGFβ expression in the autologous cancer vaccine treatment approach of the present invention.

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It will be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the claims.

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one." The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

The term "or combinations thereof" as used herein refers to all permutations and combinations of the listed items preceding the term. For example, "A, B, C, or combinations thereof" is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, MB, BBC, AAABCCCC, CBBAAA,

CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

As used herein, words of approximation such as, without limitation, “about”, "substantial" or "substantially" refers to a condition that when so modified is understood to not necessarily be absolute or perfect but would be considered close enough to those of ordinary skill in the art to warrant designating the condition as being present. The extent to which the description may vary will depend on how great a change can be instituted and still have one of ordinary skilled in the art recognize the modified feature as still having the required characteristics and capabilities of the unmodified feature. In general, but subject to the preceding discussion, a numerical value herein that is modified by a word of approximation such as “about” may vary from the stated value by at least ±1, 2, 3, 4, 5, 6, 7, 10, 12 or 15%.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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siRNA-MEDIATED METHOD TO INHIBIT TRANSFORMING GROWTH FACTOR BETA

TECHNICAL FIELD OF THE INVENTION

The present invention relates in general to the field of vaccine development, and more particularly, to the development of compositions and methods for making and using an autologous cancer vaccine genetically modified for Furin knockdown and GM-CSF expression.

BACKGROUND OF THE INVENTION

The background of the invention is described in connection with the development of genetically modified whole cell cancer vaccines. More specifically, the present invention relates to vaccines capable of augmenting tumor antigen expression, presentation, and processing through expression of the GM-CSF transgene and attenuating secretory immunosuppressive activity of TGF-β via furin bi-functional shRNA transgene induced knockdown.

The prevailing hypothesis for immune tolerance to cancer vaccines include the low immunogenicy of the tumor cells, the lack of appropriate presentation by professional antigen presenting cells, immune selection of antigen-loss variants, tumor induced immunosuppression, and tumor induced privileged site. Whole cancer cell vaccines can potentially solicit broad-based, polyvalent immune responses to both defined and undefined tumor antigens, thereby addressing the possibility of tumor resistance through downregulation and/or selection for antigen-loss variants.

Granulocyte-macrophage colony-stimulating factor, often abbreviated to GM-CSF, is a protein secreted by macrophages, T cells, mast cells, endothelial cells and fibroblasts. When integrated as a cytokine transgene, GM-CSF enhances presentation of cancer vaccine peptides, tumor cell lysates, or whole tumor cells from either autologous patient tumor cells or established allogeneic tumor cell lines. GM-CSF induces the differentiation of hematopoietic precursors and attracts them to the site of vaccination. GM-CSF also functions as an adjuvant for dendritic cell maturation and activational processes. However, GM-CSF-mediated immunosensitization can be suppressed by different isoforms of transforming growth factor beta (TGF-β) produced and/or secreted by tumor cells. The TGF-β family of multifunctional proteins possesses well known immunosuppressive activities. The three known TGF-β ligands (TGF-β1, β2, and β3) are ubiquitous in human cancers. TGF-β

overexpression correlates with tumor progression and poor prognosis. Elevated TGF-β levels within the tumor microenvironment are linked to an anergic tumor response. TGF-β directly and indirectly inhibits GM-CSF induced maturation of dendritic cells and their expression of MHC class II and co-stimulatory molecules. This negative impact of TGF-β on GM-CSF-mediated immune activation supports the rationale of depleting TGF-β secretion in GM-CSF-based cancer cell vaccines.

All mature isoforms of TGF-β require furin-mediated limited proteolytic cleavage for proper activity. Furin, a calcium-dependent serine endoprotease, is a member of the subtilisin-like proprotein convertase family. Furin is best known for the functional activation of TGF-β with corresponding immunoregulatory ramifications. Apart from the previously described immunosuppressive activities of tumor secreted TGF-β, conditional deletion of endogenously expressed furin in T lymphocytes has been found to allow for normal T-cell development, but impaired function of regulatory and effector T cells, which produced less TGF-β1. Furin expression by T cells appears to be indispensable in maintaining peripheral tolerance, which is due, at least in part, to its non-redundant, essential function in regulating TGF-β1 production.

High levels of furin have been demonstrated in virtually all cancer lines. The inventors and others have found that up to a 10-fold higher level of TGF-β1 may be produced by human colorectal, lung cancer, and melanoma cells, and likely impact the immune tolerance state by a higher magnitude. The presence of furin in tumor cells likely contributes significantly to the maintenance of tumor directed, TGF-β-mediated peripheral immune tolerance. Hence furin knockdown represents a novel and attractive approach for optimizing GM-CSF-mediated immunosensitization.

Interferon-gamma (γIFN) is a key immunoregulatory cytokine that plays a critical role in the host innate and adaptive immune response and in tumor control. Also known as type II interferon, γIFN is a single-copy gene whose expression is regulated at multiple levels. γIFN coordinates a diverse array of cellular programs through transcriptional regulation of immunologically relevant genes. Initially, it was believed that CD4+ T helper cell type 1 (Th1) lymphocytes, CD8+ cytotoxic lymphocytes, and NK cells exclusively produced γIFN. However, there is now evidence that other cells, such as B cells, NKT cells, and professional antigen-presenting cells (APCs) secrete γIFN. γIFN production by professional APCs [monocyte/macrophage, dendritic cells (DCs)] acting locally may be important in cell self-

activation and activation of nearby cells. γIFN secretion by NK cells and possibly professional APCs is likely to be important in early host defense against infection, whereas T lymphocytes become the major source of γIFN in the adaptive immune response. Furthermore, a role for γIFN in preventing development of primary and transplanted tumors has been identified. γIFN production is controlled by cytokines secreted by APCs, most notably interleukin (IL)-12 and IL-18. Negative regulators of γIFN production include IL-4, IL-10, glucocorticoids, and TGF-β.

United States Patent Application No. 0070224194 by McColl et al. entitled "Methods for Inhibiting Angiogenesis and/or Lymphangiogenesis" teaches that a proprotein convertase inhibitor has been found to block proteolytic processing and activation of VEGF-C and VEGF-D and inhibit angiogenesis and/or lymphangiogenesis. Method and composition are disclosed using a simple shRNA for inhibiting angiogenesis and/or lymphangiogenesis, and for treating conditions associated with excessive angiogenesis, such as tumors and/or retinopathies, as well as conditions associated with lymphangiogenesis, such as the metastatic spread of malignancies, macular degeneration, inflammatory mediated diseases, rheumatoid arthritis, diabetic retinopathy and psoriasis in a patient. In contrast, the present invention is a bifunctional shRNA that targets both an siRNA (cleavage dependent) and miRNA (cleavage-independent) motifs that provide inhibitory activity against furin.

Pesu et al., entitled "T cell-expressed proprotein convertase furin is essential for maintenance of peripheral tolerance", Nature. 2008 September 11; 455(7210): 246–250, teaches that proprotein convertases (PC) are a family of proteases that cleave target proproteins at basic amino acids, generating mature, biologically active polypeptides, but does not teach knocking down furin with any type of siRNA.

Rao et al., is a review entitled "siRNA vs. shRNA: Similarities and differences", and discussed the differences between siRNA, shRNA and bifunctional shRNA. Nothing in this review article teaches anything about knocking down any targets of the present invention.

Scamuffa et al., is entitled, "Selective inhibition of proprotein convertases represses the metastatic potential of human colorectal tumor cells", The Journal of Clinical Investigation, Volume 118, Number, 1 January 2008, is directed to the role of proprotein convertases (PCs) in the activation of various precursor proteins that play an important role in tumor cell metastasis. However, it does not teach or disclose the inhibition of TGF-β.

SUMMARY OF THE INVENTION

The present invention also provides an autologous (i.e., patient specific) cancer vaccine composition (FANG vaccine), comprising a therapeutically effective amount of cells with an shRNAfurin / GM-CSF expression vector. This vector comprises a first nucleic acid encoding GM-CSF, which may be human GM-CSF, and a second nucleic acid insert encoding one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of an mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference. Both nucleic acid inserts are operably linked to a promoter. The shRNA may be bi-functional, incorporating both (cleavage dependent) RISC (RNA induced silencing complex) formatted) siRNA (cleavage dependent) and (cleavage-independent RISC formatted) either miRNA or miRNA-like motifs simultaneously. In one embodiment of the present invention, the shRNA is both the RISC cleavage dependent and RISC cleavage independent inhibitor of furin expression. Furthermore, the expression vector may contain a picornaviral 2A ribosomal skip peptide intercalated between the first and the second nucleic acid inserts, and the promoter may be CMV mammalian promoter which could contain an enhancer sequence and intron. The mRNA sequences targeted by the bi-functional shRNA are not limited to coding sequences; in one embodiment, the shRNA may target the 3' untranslated region (3'-UTR) sequence of the furin mRNA transcript, and in one embodiment may target both the coding sequence and the 3' UTR sequence of the furin mRNA transcript simultaneously.. The cells used to produce the vaccine may be autologous tumor cells, but xenograft expanded autologous tumor cells, allogeneic tumor cells, xenograft expanded allogeneic tumor cells, or combinations of them may also be used. The vaccine dosage administered to patients contains 1 $x10^7$ cells to 2.5 x 10^7 cells. The FANG vaccine can be given in conjunction with a therapeutically effective amount of γIFN (gamma interferon). The dosage range of γIFN may be 50 or 100 $\mu g/m^2$.

The present invention describes an autologous cell vaccine composition comprising: a $bishRNA^{furin}$/GMCSF expression vector plasmid and one or more optional vaccine. The vector plasmid comprises a first nucleic acid insert operably linked to a promoter, wherein the first insert encodes a Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) cDNA and a second nucleic acid insert operably linked to the promoter, wherein the second insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of a mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference. In one aspect the GM-CSF is human. In another aspect the shRNA incorporates

siRNA (cleavage dependent RISC formatted) and either miRNA or miRNA-like (cleavage-independent RISC formatted) motifs. The shRNA as described herein is both the cleavage dependent RISC formatted and cleavage independent RISC formatted inhibitor of furin expression and is further defined as a bi-functional shRNA.

In another aspect, a picornaviral 2A ribosomal skip peptide is intercalated between the first and the second nucleic acid inserts. In yet another aspect the promoter is a CMV mammalian promoter containing a CMV IE 5' UTR enhancer sequence and a CMV IE Intron A. In a related aspect the CMV mammalian promoter. In other aspects the region targeted by the shRNA is the 3' UTR region sequence of the furin mRNA transcript and the region targeted by the shRNA is the coding region of the furin mRNA transcript.

The present invention provides a method of preventing, treating and/or ameliorating symptoms of a cancer in a patient by comprising the steps of: identifying the patient in need of prevention, treatment, and/or amelioration of the symptoms of the cancer and administering an autologous cell vaccine comprising a bishRNA^{furin}/GMCSF expression vector plasmid, wherein the vector plasmid comprises a first nucleic acid insert operably linked to a promoter, wherein the first insert encodes a Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) cDNA, a second nucleic acid insert operably linked to the promoter, wherein the second insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of a mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference, and one or more optional vaccine adjuvants.

The method further comprises the steps of monitoring the progression of the therapy by measuring a level of a transforming growth factor beta (TGF-beta or TGF-β) and the GM-CSF in the one or more cancer cells, wherein a reduction in the level of TGF-β and an elevation in the level of the GM-CSF is indicative of a successful therapy and altering the administration of the autologous cell vaccine based on the levels of the TGF-β and the GM-CSF. As per the method of the present invention the TGF-β is selected from at least one of TGF-β1, TGF-β2, or TGF-β3. In one aspect the cancer is selected from the group consisting of melanoma, non-small-cell lung cancer, gall bladder cancer, colorectal cancer, breast cancer, ovarian, liver cancer, liver cancer metastases, and Ewing's sarcoma as well as other patient derived TGF-β producing cancers. In another aspect the shRNA incorporates siRNA (cleavage dependent RISC formatted) and either miRNA or miRNA-like (cleavage-independent RISC formatted) motifs and the shRNA is both the cleavage dependent RISC

formatted and cleavage independent RISC formatted inhibitor of furin expression. In yet another aspect the shRNA is further defined as a bi-functional shRNA.

In another embodiment the present invention discloses an autologous furin-knockdown and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) augmented (FANG) cancer vaccine composition comprising: a bishRNA^{furin}/GMCSF expression vector plasmid, wherein the vector plasmid comprises a first nucleic acid insert operably linked to a promoter, wherein the first insert encodes the GM-CSF cDNA and a second nucleic acid insert operably linked to the promoter, wherein the second insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of a mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference and one or more optional vaccine adjuvants. The composition of the present invention is used to prevent, treat, and/or ameliorate the symptoms of a cancer, wherein the cancer is selected from the group consisting of melanoma, non-small-cell lung cancer, gall bladder cancer, colorectal cancer, breast cancer, ovarian, liver cancer, liver cancer metastases, and Ewing's sarcoma as well as other patient derived TGF-β producing cancers.

In yet another embodiment the present invention is a method of treating, preventing, and/or ameliorating the symptoms of a non small cell lung cancer (NSCLC) in a patient by an administration of a furin-knockdown and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) augmented (FANG) cancer vaccine comprising the steps of: identifying the patient in need of prevention, treatment, and/or amelioration of the symptoms of the NSCLC and administering the FANG vaccine comprising a bishRNA^{furin}/GMCSF expression vector plasmid, wherein the vector plasmid comprises a first nucleic acid insert operably linked to a promoter, wherein the first insert encodes the GM-CSF cDNA, a second nucleic acid insert operably linked to the promoter, wherein the second insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of a mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference, and one or more optional vaccine adjuvants. The method of the instant invention further comprising the steps of: monitoring the progression of the therapy by measuring a level of a transforming growth factor beta (TGF-beta or TGF-β) and the GM-CSF in the one or more NSCLC cells, wherein a reduction in the level of TGF-β and an elevation in the level of the GM-CSF is indicative of a successful therapy and altering the administration of the autologous cell vaccine based on the levels of the TGF-β and the GM-CSF. In one aspect of the present invention the TGF-β is selected from at least one of TGF-β1, TGF-β2, or TGF-β3.

The present invention in a further embodiment describes a method of making a furin-knockdown and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) augmented (FANG) cancer vaccine comprising the steps of: (i) harvesting one or more cancer cells from a patient aseptically, (ii) placing the harvested cells in an antibiotic solution in a sterile container, (iii) forming a cell suspension from the harvested solution, wherein the formation of the cell, (iv) suspension is achieved by enzymatic dissection, mechanical disaggregation or both, (v) modifying the cells genetically by electroporating the cell suspension to make the vaccine with a bishRNA^{furin}/GMCSF expression vector plasmid, wherein the vector plasmid comprises a first nucleic acid insert operably linked to a promoter, wherein the first insert encodes the GM-CSF cDNA, a second nucleic acid insert operably linked to the promoter, wherein the second insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of a mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference, (vi) harvesting the vaccine, (vii) irradiating the vaccine and (viii) freezing the vaccine.

In one aspect of the method the one or more cancer cells are harvested from a patient suffering from a cancer selected from the group consisting of melanoma, non-small-cell lung cancer, gall bladder cancer, colorectal cancer, breast cancer, ovarian, liver cancer, liver cancer metastases, and Ewing's sarcoma as well as other patient derived TGF-β producing cancers. In another aspect the genetically modified cells have been rendered proliferation-incompetent by irradiation. In yet another aspect the genetically modified cells are autologous, allogenic, or xenograft expanded cells.

In one aspect the allogenic cells are established cell lines. In another aspect the genetically modified cells are administered to the subject once a month for up 12 doses, wherein the dose of genetically modified cells administered to the subject is 1 x 10^7 cells/injection to 5 x 10^7 cell/injection and the administration of the genetically modified cells is part of a combination therapy with an additional therapeutic agent. In yet another aspect the additional therapeutic agent used in the combination therapy is γIFN, wherein the dose of γIFN administered to the subject in the combination therapy is 50 or 100 μg/m^2. The method of the present invention further comprises the step of incubating the genetically modified cells with γIFN after transfection, wherein the dose of γIFN applied to the genetically modified cells after transfection is approximately 250 U/ml (500 U/ml over 24 hours to 100 U/ml over 48 hours).

Another embodiment of the invention is a siRNA-mediated method to inhibit the expression of transforming growth factor beta (TGF-β) via furin knockdown. This method comprises the steps of selecting a target cell and transfecting the target cell with an expression vector comprising a promoter and a nucleic acid insert operably linked to the promoter. The insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of an mRNA transcript encoding furin, consequently inhibiting furin expression via RNA interference. The shRNA may be bi-functional , i.e., it may simultaneously incorporate siRNA (cleavage dependent RISC formatted) and either miRNA or miRNA-like (cleavage-independent RISC formatted) motifs, and inhibit furin expression in both a cleavage dependent RISC formatted and cleavage independent RISC formatted manner. Additionally, the expression vector may target the coding region of the furin mRNA transcript, or it may target the 3' UTR region sequence of the furin mRNA transcript, or it may target both the coding sequence and the 3' UTR sequence of the furin mRNA transcript simultaneously.

The present invention also provides a method to augment antigen expression, presentation, and processing, and to attenuate secretory immunosuppressive activity of transforming growth factor beta (TGF-beta or TGF-β) in target cells. This method comprises the steps of selecting a target cell and transfecting the target cell with an expression vector comprising two inserts. The technique used to transfect the target cells may be plasmid vector electroporation. The first nucleic acid insert encodes GM-CSF, whereas the second insert encodes one or more short hairpin RNAs (shRNAs) capable of hybridizing to a region of an mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference. Both inserts are operably linked to a promoter. The TGF-β isoforms whose activation would be precluded by knocking down furin expression include TGF-β-1, TGF-β-2, and TGF-β-3. Target cells may include autologous or allogeneic cells, which may be established human cell lines.

The present invention also includes a method of preventing, treating and/or ameliorating symptoms of cancer by administering the FANG vaccine to patients. This method comprises the steps of: (i) identifying a subject in need of treatment; (ii) harvesting a cancer tissue sample from the subject; (iii) genetically modifying the cancer cells in the harvested cancer sample; and (iv) administering a therapeutically effective dose of genetically modified cells to the subject. The expression vector used to transfect the cells comprises two nucleic acid inserts. The first nucleic acid insert encodes GM-CSF and it is operably linked to a promoter. The second nucleic acid insert is also operably linked to the promoter, and it encodes one or

more short hairpin RNAs (shRNAs) capable of hybridizing to a region of an mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference. In one embodiment of the present invention, the cancer targeted for treatment is a human melanoma or a non-small-cell lung cancer. To render the genetically modified cells proliferation-incompetent, they may be irradiated. The genetically modified cells in the FANG vaccine may be autologous cells, allogeneic cells, xenograft expanded cells, established human cell lines, or combinations of these cellular types. For vaccination, cells are administered to the subject once a month for up 12 doses, each one containing 1 x 10^7 cells to 2.5 x 10^7 cells. Dose escalation to 5 x 10^7 has been shown to be safe.

The genetically modified cells can be administered as a stand-alone therapy; however, they may also be administered as part of a combination therapy. In this embodiment of the invention, the FANG vaccine may be combined with another therapeutic agent[s], such as, but not limited to, IL-12, IL-15 and/or γIFN. When including γIFN in the treatment with FANG vaccine, the method comprises the further step of incubating the genetically modified cells with approximately 100 U/ml of γIFN for 48 hours or 500 U/ml for 24 hours, respectively, after transfection. For combination therapy, cells are administered to the subject once a month for up to 12 doses, each one containing typically 1 x 10^7 cells to 2.5 x 10^7 cells (although doses up to 5 x 10^7 have been shown to be safe) plus a dose of γIFN of 50 or 100 μg/m^2. The method further comprises the step of incubating the genetically modified cells with γIFN after transfection, wherein the dose of γIFN applied to the genetically modified cells after transfection is approximately 250U/ml.

The present invention includes a unique method of inhibiting TGF-β through RNA interference with furin, a proprotein convertase involved critically in the functional processing of all TGF-β isoforms. The FANG vector uniquely incorporates a bi-functional small hairpin construct (shRNAfurin) specific for the knockdown of furin. The bi-functional shRNAfurin of the present invention comprises a two stem-loop structure with a miR-30a backbone. The first stem-loop structure is the siRNA precursor component, while the second stem-loop structure is the miRNA-like precursor component. In this embodiment, the strategy is to use a single targeted site for both cleavage and sequestering mechanisms of RNA interference. In one embodiment, the strategy is to use two different targeted sites, one for the cleavage and one for the sequestering component comprised of, but not limited to, the coding region of the mRNA transcript and the 3' UTR region of the mRNA transcript, respectively. In this embodiment, the bi-functional shRNAfurin is comprised of two stem-

loop structures with miR-30a backbone; the first stem-loop structure has complete complementary guiding strand and passenger strand, while the second stem-loop structure has three basepair (bp) mismatches at positions 9 to 11 of the passenger strand. In one embodiment, the bi-functional shRNAfurin is comprised of two stem-loop structures with miR-30a backbone; the first stem-loop structure has complete complementary guiding strand and passenger strand, while the second stem-loop structure has three basepair (bp) mismatches at positions 9 to 11 of the guide strand. In other embodiments, basepair (bp) mismatches will occupy positions preventing Ago 2 mediated cleavage and make it thermodynamically favorable for passenger strand departure. In other embodiments the basepair mismatches will occupy positions of the guide strand. The FANG construct contains GM-CSF and the bi-functional shRNAfurin transcripts under the control of a mammalian promoter (CMV) that drives the entire cassette. This construct is used to generate an autologous (i.e., patient specific) cancer vaccine genetically modified for furin knockdown and GM-CSF expression.

The construct used to produce the FANG vaccine in the present invention includes a bi-functional shRNAfurin/GMCSF expression vector plasmid comprising two nucleic acid inserts. The first nucleic acid insert is linked operably to a promoter, and it encodes a Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) cDNA. The second nucleic acid insert is also linked operably to the promoter, and it encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of an mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference. The bi-functional shRNA of the present invention has two mechanistic pathways of action, that of the siRNA and that of the miRNA. Thus, the bi-functional shRNA of the present invention is different from a traditional shRNA, i.e., a DNA transcription derived RNA acting by the siRNA mechanism of action or from a “doublet shRNA” that refers to two shRNAs, each acting against the expression of two different genes but in the traditional siRNA mode. In one embodiment of the invention, the GM-CSF is human. The shRNA is bi-functional, incorporating both siRNA (cleavage dependent RISC formatted) and either miRNA or miRNA-like (cleavage-independent RISC formatted) motifs simultaneously. In one embodiment of the present invention, the shRNA is both the cleavage dependent RISC formatted and cleavage independent RISC formatted inhibitor of furin expression. The expression vector may contain a picornaviral 2A ribosomal skip peptide intercalated between the first and the second nucleic acid inserts, and the promoter may be CMV mammalian promoter which could contain a

CMV IE 5' UTR enhancer sequence and a CMV IE Intron A. The mRNA sequences targeted by the bi-functional shRNA are not limited to coding sequences; in one embodiment, the shRNA may target the 3' untranslated region (UTR) sequence of the furin mRNA transcript and, in one embodiment, target both the coding sequence and the 3' UTR sequence of the furin mRNA transcript simultaneously

The present invention also includes a vector that may be used to specifically knock down the expression of furin in target cells. This shRNAfurin expression vector comprises a nucleic acid insert linked operably to a promoter. Such insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of an mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference. The bi-functional shRNA may simultaneously incorporate siRNA (cleavage dependent RISC formatted) and either miRNA or mi-RNA-like (cleavage-independent RISC formatted) motifs, and inhibit furin expression in both a cleavage dependent RISC formatted and cleavage independent RISC formatted manner. Additionally, the expression vector may target the coding region of the furin mRNA transcript, or it may target the 3' UTR region sequence of the furin mRNA transcript, or it may target both the coding sequence and the 3' UTR sequence of the furin mRNA transcript simultaneously.

BRIEF DESCRIPTION OF THE DRAWINGS

For a more complete understanding of the features and advantages of the present invention, reference is now made to the detailed description of the invention along with the accompanying figures and in which:

FIGS. 1A-1C are plots showing the summary of: (1A) TGF-β1, (1B)-β2, and (1C) GMCSF protein production pre and post FANG plasmid transfection. ELISA values from Day 4 of the 14-day determinations of cytokine production in manufactured autologous cancer cells. Data represents autologous vaccines independently generated from 10 patients who underwent FANG processing (FANG 001 – 010).

FIGS. 2A-2F are plots showing: (2A) TGF-β1, (2B) TGF-β2, and (2C) GMCSF expression in FANG-004 tumor cells pre and post FANG cGMP plasmid transfection and (2D) TGF-β1, (2E) TGF-β2, and (2F) GMCSF expression in TAG-004 tumor cells pre and post TAG cGMP plasmid transfection. FANG-004 and TAG-004 are from the same tumor and processed sequentially on the same two days as a demonstration of comparative expression profiles;

FIGS. 3A-3C are plots showing that the side-by-side comparison of electroporation of FANG plasmid (the cGMP vaccine manufacturing process) versus the TAG plasmid into patient tumor cells demonstrated (3C) GMCSF protein production and concomitantly; (3A) TGF-β1 expression knocked down by FANG but not TAG and (3B) TGF–β2 knockdown by both FANG and TAG (coincident line);

FIG. 4A is a schematic showing the bi-shRNA^{furin} comprising two stem-loop structures with miR-30a backbone (SEQ ID NO.: 1); the first stem-loop structure has complete complementary guiding strand and passenger strand, while the second stem-loop structure has three basepair (bp) mismatches at positions 9 to 11 of the passenger strand;

FIG. 4B shows the siRNA targeted regions of furin mRNA. Prospective siRNA targeting regions in 3'-UTR and encoding regions of furin mRNA and the targeted sequence by each siRNA;

FIG. 5 is an image shows PET CT 11 cycles in a patient after TAG demonstrating significant response. Residual uptake at L 2 was followed up with a MRI scan and biopsy which revealed no malignancy;

FIGS. 6A-6C show an assessment of GMCSF expression and TGF-β1 and -β2 knockdown, summarizing: (6A) TGF-β1, (6B) TGF-β2, and (6C) GM-CSF protein production before and after FANG or TAG (TAG 004) plasmid transfection. Values represent ELISA determinations of cytokine production in harvested autologous cancer cells transfected with FANG. Data represents autologous vaccines independently generated from 9 patients who underwent FANG processing (FANG 001 – 009). One patient had sufficient tissue to construct both a FANG (blue) and TAG vaccine (red) (FANG 004/TAG 004);

FIG. 7 shows the GMCSF mRNA by RT-qPCR in Pre and Post FANG Transfected / Irradiated Tumor Cells. Absent bands in some of the lanes is due to degraded RNA. (The extra band in FANG-009 is Day 0 sample loaded twice);

FIG. 8 shows FANG Vaccine cells from a patient pre-transfection and post-transfection, irradiation mRNA by PCR. No signal was detected in pre- and post- samples for TGF-β2;

FIG. 9 shows FANG-009 Vaccine cells pre-transfection and post-transfection /irradiation mRNA by PCR;

FIG. 10 shows the overall survival for Cohort 1 versus Cohorts 2 and 3 for advanced-stage patients (n = 61; P = .0186);

FIG. 11 shows a schematic diagram of GM-CSF-TGF-β2 antisense (TAG) plasmid;

FIG. 12 shows the expression of GM-CSF in NCI-H-460 Squamous Cell and NCI-H-520, Large Cell (NSCLC) containing the pUMVC3-GM-CSF-2A-TGF-β2 antisense vector, *in vitro*;

FIG. 13 shows that TGF-β2 levels are reduced in NCI-H-460 Squamous Cell and NCI-H-520, Large Cell (NSCLC) with the pUMVC3-GM-CSF-2A-TGF-β2 antisense (TAG) vector;

FIG. 14 shows that a 251 base pair probe specifically detects the GM-CSF-2A-TGF-β2 (TAG) transcript expressed in vitro in NCI-H-460 and NCI-H-520 cells (lanes 6 and 10);

FIG. 15 shows the GM-CSF expression in TAG vaccines;

FIG. 16 shows the TGF-β1 expression in TAG vaccines

FIG. 17 shows the TGF-β2 expression in TAG vaccines;

FIGS. 18A and 18B show expression of: (14A) TGF-β1 and (14B) TGF-β2 in human cancer lines following siRNAfurin knockdown; and

FIG. 19 shows the plasmid construct of FANG.

DETAILED DESCRIPTION OF THE INVENTION

While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific ways to make and use the invention.

To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as “a”, “an” and “the” are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims.

It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method, kit, reagent, or composition of the invention, and vice versa. Furthermore, compositions of the invention can be used to achieve methods of the invention.

Table 1: Abbreviations Table

Abbreviation	Term
AE	Adverse event
ALT	Alanine transaminase (also referred to as SGPT)
ANC	Absolute neutrophil count
APC	Antigen Presenting Cells
AST	Aspartate transaminase (also referred to as SGOT)
BUN	Blood urea nitrogen
CBC	Complete blood count
CD	Cluster of differentiation
CMV	Cytomegalovirus
CO_2	Total carbon dioxide
CR	Complete response
CRF	Case report form
CTCAE	Common Toxicity Criteria for Adverse Events
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell(s)
DTH	Delayed-type hypersensitivity
ECOG PS	Eastern Cooperative Oncology Group Performance Score
ELISA	Enzyme-Linked ImmunoSorbent Assay
ELISPOT	Enzyme-Linked ImmunoSorbent Spot
ER	Endoplasmic reticulum
FANG	bishRNA^{furin} and GMCSF Augmented Autologous Tumor Cell Vaccine
FL	Flt-3-Ligand
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor (Accession No. NM_000758)
GMP	Good manufacturing practice
GVAX	GMCSF Secreting autologous or allogenic tumor cells
HLA	Human Leukocyte Antigen
IBC	Institutional Biosafety Committee
IEC	Independent Ethics Committee
IL	Infiltrating lymphocytes
IRB	Institutional Review Board
LAK	Lymphokine-activated killer
LD	Longest diameter
LLC	Large latent complex
MHC	Major histocompatability complex
MLR	Mixed lymphocyte reaction
MR	Mannose receptor
NK	Natural Killer
NKT	Natural Killer T cell(s)
NSCLC	Non small cell lung cancer
PCR	Polymerase chain reaction
PD	Progressive disease
PR	Partial response
PS	Performance Status
RECIST	Response Evaluation Criteria in Solid Tumors
SCLC	Small cell lung cancer

Abbreviation	Term
SD	Stable disease
SLC	Small latent complex
STMN1	Stathmin 1
TAP	transporter associated with Ag processing
TGF-β	Transforming growth factor-β
TIL	Tumor infiltrating lymphocytes
TNF	Tumor necrosis factor
ULN	Upper limits of normal
WNL	Within normal limits

Furin is a member of the subtilisin-like proprotein convertase family (human Furin Accession No. NM_002569). The members of this family are proprotein convertases (PCs) that process latent precursor proteins into their biologically active products. Furin, a calcium-dependent serine endoprotease, efficiently cleaves precursor proteins at their paired basic amino acid processing sites by the consensus sequence –Arg-X-K/Arg-Arg (RXK/RR), with -RXXR- constituting the minimal cleavage site. Like many other proteases, PCs are synthesized as inactive zymogens with an N-terminal prosegment extension, which is autocatalytically removed in the endoplasmic reticulum to achieve functionality.

High levels of furin have been demonstrated in virtually all cancer lines. A 10-fold higher level of TGF-β1 may be produced by human colorectal, lung cancer and melanoma cells, and likely impact the immune tolerance state by a higher magnitude. Transforming growth factors betas (TGF-β) are a family of multifunctional proteins with well known immunosuppressive activities. The three known TGF-β ligands (TGF-β1-3, Accession Nos. NM_000660, NM_003238, NM_003239.2, respectively) are ubiquitous in human cancers. TGF-β overexpression correlates with tumor progression and poor prognosis. Elevated TGF-β levels within the tumor microenvironment are linked to an anergic antitumor response. The presence of furin in tumor cells likely contributes significantly to the maintenance of tumor directed TGF-β1 mediated peripheral immune tolerance. Hence, furin knockdown represents a novel and attractive approach for optimizing immunosensitization.

A Furin-knockdown and GM-CSF-augmented (FANG) Autologous Cancer Vaccine for Human Melanoma and Lung Cancer: FANG uniquely incorporates a bi-functional small hairpin RNA (shRNA) construct specific for the knockdown of furin, a proprotein convertase critically involved in the functional processing of all TGF-β isoforms. Prior work by the inventors has demonstrated the effectiveness of FANG in generating GM-CSF expression

and TGF-β1 and TGF-β2 depletion in human cancer lines. The incorporation of a bi-functional shRNAfurin in combination with hGM-CSF into an autologous cell vaccine is demonstrated herein to promote and enhance the immune response based on its effect on the afferent limb of that immune response.

As used herein the term "bi-functional" refers to a shRNA having two mechanistic pathways of action, that of the siRNA and that of the miRNA (the guide strand being non-complementary to the mRNA transcript) or miRNA-like (the guide strand being complementary to the mRNA transcript). The term "traditional" shRNA refers to a DNA transcription derived RNA acting by the siRNA mechanism of action. The term "doublet" shRNA refers to two shRNAs, each acting against the expression of two different genes but in the "traditional" siRNA mode.

Survival of patients with advanced NSCLC, the most common cancer involving both men and women, is 7 months or less following treatment with second line chemotherapy. Limited survival benefit and toxicity related to the cancer and the treatment commonly forces patients to decline further therapy. Demonstration of safety and extensive clinical justification including examples of dramatic response related to "targeted" immune stimulation and suppression of endogenous immune inhibition using the novel, mature technology of the present invention described herein provides an opportunity for safe and potentially effective clinical assessment. The commercial expansion of the RNA interference technology and vaccine manufacturing of the present invention will provide a gateway opportunity into management of NSCLC and likely other solid tumors, notably melanoma, ovary, prostate cancer, colon cancer and breast cancer

Overcoming immune tolerance with cancer vaccines is a promising but difficult quest. The prevailing hypotheses for immune tolerance, based primarily on animal studies, include the low immunogenicity of the tumor cells, the lack of appropriate presentation by professional antigen presenting cells, immune selection of antigen-loss tumor variants, tumor induced immunosuppression, and tumor-induced privileged site [1]. Nevertheless, recent clinical trials that are based on transgene-expressing whole cancer cell vaccines have yielded promising results [2-5]. Whole cancer cell vaccines can potentially elicit broad-based, polyvalent immune responses to both defined and undefined tumor antigens, thereby addressing the possibility of tumor resistance through downregulation and/or selection for antigen-loss variants [6, 7].

Dranoff and Jaffee have shown in animal models [8], that tumor cells genetically modified to secrete GM-CSF, as compared to other cytokines, consistently demonstrated the most potent induction of anti-tumor immunity. When integrated as a cytokine transgene, GM-CSF enhances presentation of cancer vaccine peptides, tumor cell lysates, or whole tumor cells from either autologous or established allogeneic tumor cell lines [9]. GM-CSF induces the differentiation of hematopoietic precursors into professional antigen presenting (APC) dendritic cells (DC) and attracts them to the site of vaccination [8, 10]. GM-CSF also functions as an adjuvant for the DC maturation and activational processes of tumor antigen capture, process and presentation, upregulates their expression of costimulatory molecules, and their ability to migrate to secondary lymphoid tissues for activation of CD4+, CD8+ T cells, CD1d restricted invariant natural killer T (NKT) cells, and antibody producing B cells [11].

Recently, Hodi [12] reported that GVAX vaccination, followed by periodic infusions of anti-CTLA-4 antibodies to modulate effector and T regulatory cell functions, can generate clinically meaningful antitumor immunity in a majority of metastatic melanoma patients. These findings are consistent with the thesis that vaccination with a GM-CSF-augmented autologous cancer vaccine can successfully generate an immune mediated tumor destruction, particularly when coupled with an adjuvant treatment that depletes FoxP3+ Tregs activity, enhances tumor expression of MHC class I A chain (MICA) thereby activating natural killer (NK) and T cells, and enhances central memory T-cell CD4+ and CD8+ response.

The FANG approach of the present invention is supported by the findings of the inventors in 10 patients' autologous vaccines, which demonstrated consistently TGF-β1 and TGF-β2 reductions and elevated GM-CSF levels (FIGS.1A-1C and FIGS. 2A-2F). Soundness of the furin-depletion approach has been confirmed by proof of principle documentation with the furin inhibitor decanoyl-Arg-Val-Lys-Arg-CMK (Dec-RVKR-CMK) in cancer cell lines (CCL-247 colorectal, CRL-1585 melanoma lines). Dec-RVKR-CMK is a peptidyl chloromethylketone that binds irreversibly to the catalytic site of furin and blocks its activity [59]. Dec-RVKR-CMK either completely or partially reduces the activity of furin substrates BASE (β-site APP-cleaving enzyme), MT5-MMP, and Boc-RVRR-AMC [60]. The present inventors found both TGF-β1 and TGF-β2 activity to be significantly reduced in CCL-247 and CRL-1585 cancer lines by specific immunoassay, confirming the effectiveness of furin blockade on TGF-β isoform expression.

The FANG plasmid (FIG. 19) used to transfect the autologous cells is derived from the TAG plasmid [74] by replacing the human TGFβ2 antisense sequence with the bi-shRNAfurin DNA sequence. Otherwise these two plasmids are identical (confirmed by DNA sequencing). The bi-shRNAfurin consists of two stem-loop structures with miR-30a backbone; the first stem-loop structure has complete complementary guiding strand and passenger strand, while the second stem-loop structure has three bp mismatches at positions 9 to 11 of the passenger strand (FIG. 4A). The inventors adopted a strategy of using a single targeted site for both cleavage and sequestration. The encoding shRNA is able to accommodate mature shRNA loaded onto more than one types of RISC [65]. The rationale for focusing on a single site is that multi-site targeting may increase the chance for "seed sequence" induced off-target effect [66]. The two stem-loop double stranded DNA sequence was assembled with 10 pieces of synthetic complementing and interconnecting oligonucleotides through DNA ligation. The completed 241 base pairs DNA with Bam HI sites at both ends was inserted into the Bam HI site of the TAG expression vector in place of the TGFβ2 antisense sequence. Orientation of the inserted DNA was screened by PCR primer pairs designed to screen for the shRNA insert and orientation. The FANG construct has a single mammalian promoter (CMV) that drives the entire cassette, with an intervening 2A ribosomal skip peptide between the GM-CSF and the furin bifunctional shRNA transcript, followed by a rabbit polyA tail. There is a stop codon at the end of the GM-CSF transcript. Insertion of picornaviral 2A sequences into mRNAs causes ribosomes to skip formation of a peptide bond at the junction of the 2A and downstream sequence, leading to the production of two proteins from a single open reading frame [67]. However, in the instances in which shRNA or antisense are being expressed as the second transcript (as examples), only the first transcript is translated. The inventors found that the 2A linker to be effective for generating approximately equal levels of GM-CSF and anti-TFG-β transcripts with the TAG vaccine, and elected to use the same design for FANG.

The inventors validated the applicability of siRNA-mediated furin-knockdown for inhibiting human TGF-β isoform expression. Prospective siRNA targeting sites (FIG. 4B) in the furin mRNA sequence were determined by the published recommendations of Tuschl and colleagues and the additional selection parameters that integrates BLAST searches of the human and mouse genome databases [73]. siRNAs targeting eligible coding and 3'UTRs sites (FIG. 4B) were tested. Following lipofection of CCL-247, CRL-1585 U87 and H460 cells, each of the 6 $siRNA^{furin}$ constructs was shown to markedly reduce TGF-β1 and TGF-β2

levels in culture supernatants without adversely affecting cell survival. Thus siRNA-mediated furin knockdown is effective for the depletion of TGF-β1 and -β2 isoforms.

The present inventors attempted to detect endogenous Furin protein in cell lines via Western Blot and Flow Cytometry. Five different commercial antibodies were screened for Western Blot and one pre-labeled antibody was screened for Flow Cytometry. All studies yielded negative results. Upon further study of the commercially available antibodies, all idiotypes were developed against fragments (or peptides) of the Furin protein. The Western Blot studies demonstrated that the 60kDa variant was preferentially detected in 4 of the 5 antibodies screened. The last antibody did not detect Furin protein under the Western Blot conditions tested. Control lysates provided by the commercial vendors produced similar results to in-house cell line samples. The pre-labeled antibody for Flow Cytometry did not demonstrate a significant shift in Furin staining (i.e., no positive Furin population identified). Therefore, the Flow Cytometry could not be used to demonstrate Furin knockdown.

As an alternative to Furin protein detection, the inventors also screened samples for Furin enzyme activity. Using a fluorometric based assay, cell lines were screened for the conversion of substrate (Pyr-Arg-Thr-Lys-Arg-AMC) by Furin to release the fluorophore (AMC). However, the detected signal of released AMC was too low to accurately demonstrate significant knockdown of Furin enzyme activity. A second barrier to the assay is that the substrate is cleaved by all serine proteases in the subtilisin-like prohormone convertase (PC) family. Therefore, similar proteases that are not targeted by our FANG shRNA product would remain active and cleave the fluorogenic substrate in the assay, thus reducing the capability to detect Furin knockdown.

Other applications for the bi-functional shRNAfurin include: (1) Systemic delivery via a tumor (± tumor extracellular matrix (ECM)) selective decorated (targeted), stealthed bilamellar invaginated liposome (BIV) to enhance the efferent limb of the immune response; (2) Systemic delivery via a tumor selective decorated (targeted), stealthed bilamellar invaginated liposome (BIV) to directly subvert the tumor promoting/maintaining effects of furin target molecules including, but not limited to human, TGFβ1, TGFβ2, TGFβ3, IGF-II, IGF-1R, PDGF A, and, in some tumor types, MT1-MMP; (3) Systemic delivery via a tumor selective decorated (targeted), stealthed bilamellar invaginated liposome (BIV) to directly subvert the NOTCH/p300 pathway in putative cancer stem cells; (4) Systemic delivery via a tumor selective decorated (targeted), stealthed bilamellar invaginated liposome (BIV) to

inhibit activation of toxins associated with anthrax, Shiga, diphtheria, tetanus, botulism and Ebola and Marburg viruses and/or (5) Systemic and/or inhalational delivery of a bilamellar invaginated liposome (BIV) (±decoration and reversible masking/stealthing) to inhibit Pseudomonas exotoxin A production as an adjunct to antibiotic therapy in patients with diseases with heightened risk of Pseudomonas mediated morbidity and mortality, e.g., cystic fibrosis.

TGF-β Knockdown: Transforming growth factors beta (TGF-β) are a family of multifunctional proteins with well known immunosuppressive activities [13]. The three known TGF-β ligands (TGF-β1, β2, and β3) are ubiquitous in human cancers. TGF-β overexpression correlates with tumor progression and poor prognosis [14, 15]. Elevated TGF-β levels within the tumor microenvironment are linked to an anergic antitumor response [14, 16-21]. TGF-β inhibits GM-CSF induced maturation of DCs [22] and their expression of MHC class II and co-stimulatory molecules [23]. Ardeshna [24] showed that lipopolysaccharide (LPS)-induced maturation of monocyte-derived DCs involved activation of p38 stress-activated protein kinase (p38SAPK), extracellular signal-regulated protein kinase (ERK), phosphoinositide 3-OH- kinase (PI3 kinase)/Akt, and nuclear factor (NF)-κB pathways. GM-CSF can exert parallel activities of stimulating myeloid hematopoietic cell and leukemia cell line proliferation through rapid, transient phosphorylation of MAP kinase 1/2 and ERK 1/2, whereas TGF-β turns off GM-CSF-induced ERK signaling via PI3-kinase-Akt pathway inhibition [25].

At the efferent level, antigen presentation by immature DCs contributes to T cell anergy [26]. TGF-β similarly inhibits macrophage activation [27] and their antigen presenting function [28, 29]. TGF-β inhibits the activation of cytotoxic T cells by impairing high affinity IL-2 receptor expression and function [30, 31]. TGF-β2 also converts naïve T cells to Treg cells by induction of the transcription factor FOXP3 [32], with emergence of Treg leading to the shutdown of immune activation [33]. According to Polak [34], tolerogenic DCs and suppressor T lymphocytes were present in all stages of melanoma. These immune cell types expressed TGF-β receptor I, and tolerogenic activity was dependent on TGF-β1 or -β2 binding.

At the innate immune response level, TGF-β is antagonistic on NK cells and down-regulates lymphokine activated killer (LAK) cell induction and proliferation [30, 35-39]. Penafuerte [40] recently showed that tumor-secreted TGF-β suppressed GM-CSF+IL2 (GIFT2)

mediated immunosensitization of NK cells in the immunocompetent B16 melanoma model. In vivo blockade of B16 production of TGF-β improved survival otherwise compromised by the growth of non-GIFT2 expressing bystander tumors. These findings further validate the negative impact of TGF-β on GM-CSF-mediated immune activation *in vivo*, and by extension, support the rationale of depleting TGF-β secretion in GM-CSF-based cancer cell vaccines.

Trials conducted by the present inventors utilizing a tumor cell vaccine with TGF-β2 knockdown activity (Belagenpumatucel-L) in patients with non-small cell lung cancer demonstrated acceptable safety, and a dose-related survival improvement in response to randomized control patients and historical experience. The two-year survival for the late stage (IIIB/IV) patients was 52% for patients who received $> 2.5 \times 10^7$ cells/injection, which compares favorably with similar patient historical data of less than 10% survival at 2 years. The study patients also displayed significantly elevated cytokine production (IFN-γ, p=0.006; IL-6, p=0.004; IL4, p=0.007) and antibody titers to vaccine HLA antigens (p=0.014), suggesting an immune activating outcome. [41].

TGF-β-knockdown and GM-CSF Expressive Cancer Cell Vaccine (TAG): Thirty six patients were harvested for TAG vaccine. GM-CSF expression and TGF-β2 knockdown met product release criteria. Three (all gastrointestinal tumors with luminal access) had bacterial contaminants and could not be released. One had insufficient cells. Nineteen advanced refractory cancer patients were treated [42-44]. No Grade 3 toxic effects related to therapy were observed. Eleven of 17 (65%) evaluable patients maintained stable disease for at least 3 months. One patient achieved CR by imaging criteria (FIG. 4; melanoma). Thus the TAG vaccine appears to be safe and has evidence of clinical efficacy.

A potential limitation of TAG vaccine, however, is the restricted specificity for TGF-β2, given that all three known isoforms of TGF-β ligand (TGF-β1, -β2, and -β3) are ubiquitously produced in human cancers. In particular, up to a 10-fold higher level of TGF-β1 may be produced by human colorectal, lung cancer, and melanoma cells. The tolerogenic role of TGF-β1 in antigen presenting dendritic cells (DC) and regulatory T cells (Treg) is well established, and this activity is not impacted by TGF-β2 antisense treatment.

Furin: All mature isoforms of TGF-β require limited proteolytic cleavage for proper activity. The essential function of proteolytic activation of TGF-β is mediated by furin. Furin is a member of the subtilisin-like proprotein convertase family. The members of this family are

proprotein convertases (PCs) that process latent precursor proteins into their biologically active products. Furin, a calcium-dependent serine endoprotease, efficiently cleaves precursor proteins at their paired basic amino acid processing sites by the consensus sequence –Arg-X-K/Arg-Arg (RXK/RR), with -RXXR- constituting the minimal cleavage site [53]. Like many other proteases, PCs are synthesized as inactive zymogens with an N-terminal prosegment extension, which is autocatalytically removed in the endoplasmic reticulum to achieve functionality [52].

Furin is best known for the functional activation of TGF-β with corresponding immunoregulatory ramifications [54, 55]. Apart from the previously described immunosuppressive activities of tumor secreted TGF-β, conditional deletion of endogenous-expressing furin in T lymphocytes was found to allow for normal T-cell development, but impaired the function of regulatory and effector T cells, which produced less TGF-β1. Furin-deficient Tregs were less protective in a T-cell transfer colitis model and failed to induce Foxp3 in normal T cells. Additionally, furin-deficient effector cells were inherently over-active and were resistant to suppressive activity of wild-type Treg cells. In APCs, cytotoxic T lymphocyte-sensitive epitopes in the trans-Golgi compartment were processed by furin and the less frequented TAP independent pathway [56]. Thus furin expression by T cells appears to be indispensable in maintaining peripheral tolerance, which is due, at least in part, to its non-redundant, essential function in regulating TGF-β1 production.

High levels of furin have been demonstrated in virtually all cancer lines [45-52]. The present inventors and others have found that up to a 10-fold higher level of TGF-β1 may be produced by human colorectal, lung cancer, and melanoma cells, and likely impact the immune tolerance state by a higher magnitude [34, 57, 58]. The presence of furin in tumor cells likely contributes significantly to the maintenance of tumor directed, TGF-β1 mediated peripheral immune tolerance [54]. Hence furin knockdown represents a novel and attractive approach for optimizing immunosensitization.

FANG (furin shRNA and GMCSF) vaccine: The present inventors constructed the next generation vaccine termed FANG. The novelty of the FANG vaccine lies in the combined approach of depleting multiple immunosuppressive TGF-β isoforms by furin knockdown, in order to maximize the immune enhancing effects of the incorporated GM-CSF transgene on autologous tumor antigen sensitization.

All mature isoforms of TGF-β require proteolytic activation by furin. The feasibility of achieving concomitant depletion of multiple TGF-β isoform activity in several cancer cell lines (H460, CCL-247, CRL-1585, U87) was determined using furin-knockdown and the present inventors have successfully completed GMP manufacturing of FANG vaccine in 9 cancer patients (breast -1; colon – 2; melanoma – 4; gallbladder – 1; NSCLC – 1). Assessment of GMCSF expression and TGF-β1 and -β2 knockdown is shown in FIGS. 1A-1C and FIGS. 2A-2F.

Electroporation of FANG plasmid into patient tumor cells demonstrated GMCSF protein production and concomitantly TGF-β1 and –β2 knockdown as predicted. FIGS. 3A-3C depicts Day 7 assay data of a FANG-transfected NSCLC tumor's expression profile (FANG-004) versus tissue from the same the tumor processed by the cGMP TAG vaccine method (denoted TAG-004). There are similar reductions in TGFβ2 (FIG. 3B; single line shown due to coincident data) and similar increases in GMCSF (FIG. 3C) expression. However, while TGFβ1 expression is completely inhibited by FANG, it is unaffected by TAG as the TGFβ2 antisense cannot block TGFβ1 expression (FIG. 3A).

FIG. 5 is a PET CT image of an advanced melanoma patient after 11 TAG vaccine treatments demonstrating a significant clinical response. Residual uptake at L 2 was followed up with a MRI scan and biopsy which revealed no malignancy. The patient has consequently become a complete response (no evidence of disease) for greater than seven months. The capability of FANG to knockdown both TGF-β1 and -β2 is supported yb findings in the first 9 patients (FIGS. 6A-6C) who underwent vaccine construction. All 9 vaccine preparations demonstrated significantly elevated levels of GM-CSF (80-1870 pg/ml at day 4 of culture, median of 739 pg/ml). All 9 patients demonstrated >50% reductions of TGF-β2, and 6 of 7 patients with >100 pg of endogenous TGF-β1 production also demonstrated >50% reduction of this cytokine. The expanded target effectiveness of FANG is best demonstrated in one patient (NSCLC) who had adequate tumor tissue to generate both TAG (TAG-004A) and FANG (FANG-004) versions of autologous vaccine, TGF-β1 (as well as TGF-β2) was depleted to below detectable levels using the FANG preparation (FANG-004) from an initial concentration of 1840 pg/ml whereas this high level of TGF-β1 was unchanged with the TAG preparation (TAG-004) albeit with the expected depletion of TGF-β2. These findings support the potential advantage of the FANG vaccine preparation.

Validation of bioactivity of personalized cGMP FANG vaccines: Gene modification will be achieved by the use of a plasmid vector encoding for GM-CSF and a bi-functional short hairpin (bi-sh) RNA optimized for furin knockdown. Cancer patient autologous FANG vaccine has already been generated under cGMP conditions for clinical trial of patients with advanced solid cancers. GM-CSF and TGF-β1, -β2, and -β3 mRNA and protein expression were measured as part of the quality assurance process. Cytokine bioactivity following FANG modification was determined by growth outcome in a GM-CSF and TGF-β dependent cell line utilized by the present inventors in previous studies. Processed vaccine will undergo proteogenomic screening to verify antigenic integrity following FANG modification.

To characterize the augmenting effect of CTLA-4 blockade: Given that FANG immunization only impacts the afferent immunosensitization process, additional approaches that promote tumor-specific immune effector responses may further promote antitumor outcome. Disrupting Treg suppression and/or enhancing T effectors (Teff) by blockade of the cytotoxic T lymphocyte-4 (CTLA-4) function may enhance the likelihood of clinical success of the FANG vaccine.

RT-qPCR analysis was performed on ten FANG vaccine samples (FANG-003 did not have adequate mRNA for analysis). Samples were cultured pre-electroporation and post-electroporation, post-irradiation for up to 14 days. Total RNA was extracted from each sample at various time points and converted into cDNA via reverse transcription (RT). Quantitative PCR (qPCR) was performed to assess the amount of template present in each sample, at each time point. Furin, TGFβ1, and TGFβ2 qPCR samples were normalized to endogenous GapDH to produce a relative cycle threshold (Ct) value. GMCSF was quantified against an external standard curve to produce an absolute Ct value, relative to the standard curve. The GMCSF mRNA detection is shown in FIG. 7. Post-transfection, GMCSF mRNA is detected in all vaccines but the values are variable depending on mRNA quality – a persistent issue. Table 1 illustrates representative data from two FANG vaccines (FIGS. 8 and 9). All samples were calculated as normalized pre-electroporation Ct values minus normalized post-electroporation, post-irradiation Ct values (pre-post) to calculate the delta Ct (Δ Ct). A calculated Δ Ct < 0.00 represents a decrease in template DNA and a calculated Δ Ct > 0.00 represents an increase in template DNA. The Δ Ct value is used to estimate the percent change in expression (% expression). Values less than 100% represent a decrease in DNA (from pre to post) and values greater then 100% represent an increase in DNA (from pre to post). The nature of shRNA / siRNA silencing can optimally reduce the template

DNA 90%, which is equivalent to a Δ Ct = -3.3. (A Δ Ct = -1.0 is equivalent to a 50% knockdown.) Therefore, the data below demonstrate that the FANG plasmid DNA is able to reduce endogenous Furin down 80 – 26% (average = 48%) and the downstream targets TGFβ1 and TGFβ2 are reduced down 98 – 30% (average = 75%). The mechanisms of action of the Furin bifunctional shRNA are to block Furin protein production at the post-transcriptional and translational levels. The reduced levels of Furin protein also impact (by feedback regulation) the expression of TGFβ1 and TGFβ2 mRNA, the conversion of the proform of TGFβ1 and TGFβ2 protein into the mature (active) form of their respective proteins [75], and, by interfering with the TGFβ→furin amplification loop, further dampen the expression of furin itself [76]. It is also possible that accumulation of the proform of the TGF protein may feedback inhibit the transcription of its TGF gene.

Table 1: RT-qPCR Analysis of FANG Vaccines (Pre Versus Post Electroporation)

FANG-008

Furin		
Time Point	Δ Ct	% Expression
day 0	-1.52	35%
day 1	-1.50	35%
day 2	-1.48	36%
day 4	-1.50	35%
day 7	-1.22	43%
day 10	-1.41	38%

TGFB1		
Time Point	Δ Ct	% Expression
day 0	-0.09	94%
day 1	-0.10	93%
day 2	-0.08	95%
day 4	-0.05	97%
day 7	-0.08	95%
day 10	-0.11	93%

TGFB2		
Time Point	Δ Ct	% Expression
day 0	0.00	n/a *
day 1	0.00	n/a *
day 2	0.00	n/a *
day 4	0.00	n/a *
day 7	0.00	n/a *
day 10	0.00	n/a *

FANG-009

Furin		
Ident.	Δ Ct	% Expression
day 0	-0.66	63%
day 1	-0.69	62%
day 2	-0.34	79%
day 4	-0.31	80%
day 7	-1.93	26%
day 10	0.00	n/a *

TGFB1		
Ident.	Δ Ct	% Expression
day 0	-0.54	69%
day 1	-0.49	71%
day 2	-0.42	75%
day 4	-0.31	81%
day 7	-0.04	98%
day 10	-1.29	41%

TGFB2		
Ident.	Δ Ct	% Expression
day 0	-0.53	69%
day 1	-0.47	72%
day 2	-0.45	73%
day 4	-0.52	70%
day 7	-1.70	31%
day 10	-1.74	30%

Δ Ct baseline = 0.00
% expression baseline = 100%

* n/a = not applicable because template was below detection limits

The FANG system was used with 9 patient autologous vaccines, which consistently demonstrated TGF-β1 and TGF-β2 reductions and elevated GM-CSF levels (FIGS. 6A-6C). Both TGF-β1 and TGF-β2 activity by specific immunoassay was also demonstrated to be significantly reduced in these cancer lines, confirming the effect of furin blockade on TGF-β isoform expression. The inventors validated the applicability of siRNA-mediated furin-knockdown for inhibiting TGF-β isoform expression. Prospective siRNA targeting sites in the furin mRNA sequence (FIG. 4B) were determined by the published recommendations of Tuschl and colleagues and the additional selection parameters that integrated BLAST

searches of the human and mouse genome databases (jura.wi.mit.edu/bioc/siRNAext). siRNAs targeting eligible translated and 3'UTRs sites (FIG. 4B) were tested. Demonstration of FANG plasmid DNA knockdown of furin mRNA is shown in FIGS 8 and 9. This could only be detected in two of the vaccines because readily detectable furin mRNA was present in only these two tumors pretransfection. The mechanisms of action of the bi-shRNA^{furin} are the blockade of furin protein production at the post transcriptional and translational levels. The reduced levels of furin protein also impact (by feed back regulation) the expression of TGF-β1 and TGF-β2 mRNA, the conversion of the proform TGF-β1 and TGF-β2 protein into the mature (active) form of their respective proteins [75] and by interfering with the TGF-β→furin loop, further dampening the expression of furin itself [76]. The possibility that the accumulation of the proform of the TGF protein may feedback and inhibit the transcription of its TGF gene should not be in any way construed as a limitation of the present invention. The expanded target effectiveness of FANG is best demonstrated in one patient (NSCLC) who had adequate tumor tissue to generate both TAG (TAG-004) and FANG (FANG-004) versions of autologous vaccine. TGF-β1 (as well as TGF-β2) was depleted to below detectable levels using the FANG preparation (FANG-004) from an initial concentration of 1840 pg/ml. This high level of TGF-β1 was unchanged with the TAG preparation (TAG-004) albeit with the expected depletion of TGF-β2 (FIGS. 1A-1C and FIGS. 2A-2F). These findings support the mechanistic advantage of the FANG vaccine preparation.

Following lipofection of CCL-247, CRL-1585 U87 and H460 cells, each of the 6 siRNAfurin constructs was shown to markedly reduce TGF-β1 and TGF-β2 levels in culture supernatants without adversely affecting cell survival. Thus siRNA-mediated furin knockdown is effective for the depletion of TGF-β1 and -β2 isoforms.

Design and construction of FANG: A "bi-functional " vector was used that incorporates both siRNA and miRNA-like functional components for optimizing gene knockdown [61]. The siRNA component is encoded as a hairpin and encompasses complete matching sequences of the passenger and guide strands. Following cleavage of the passenger strand by the Argonaute-2 (Ago 2) of the RNA-induced silencing complex (RISC), an endonuclease with RNase H like activity, the guide strand binds to and cleaves the complementary target mRNA sequence. In distinction, the miRNA-like component of the "bi-functional" vector incorporates mismatches between the passenger and guide strands within the encoding shRNA hairpin in order to achieve lower thermodynamic stability. This configuration allows

the passenger strand to dissociate from RISC without cleavage (cleavage-independent process) independent of Ago 2 [62, 63], and the miRNA guide component to downregulate its target through translational repression, mRNA degradation, and sequestration of the partially complementary target mRNA in the cytoplasmic processing bodies (P-body).

The inventors have previously demonstrated the enhanced effectiveness of a bi-functional shRNA to knockdown stathmin (STMN1; oncoprotein 18), a protein that regulates rapid microtubule remodeling of the cytoskeleton and found to be upregulated in a high proportion of patients with solid cancers [64]. The bi-functional shRNA construct achieved effective knockdown against STMN-1 resulting in a 5-log dose enhanced potency of tumor cell killing as compared with siRNA oligonucleotides directed against the same gene target.

A similarly designed bi-functional shRNA was used to effect furin knockdown. The bi-sh-furin consists of two stem-loop structures with a miR-30a backbone; the first stem-loop structure has complete complementary guiding strand and passenger strand, while the second stem-loop structure has three bp mismatches at positions 9 to 11 of the passenger strand. The inventors adopted a strategy of using a single targeted site for both cleavage and sequestration processes. The encoding shRNAs are proposed to allow mature shRNA to be loaded onto more than one type of RISC [65]. The inventors focused on a single site since multi-site targeting may increase the chance for “seed sequence” induced off-target effects [66].

The two stem-loop structure was put together with 10 pieces of complementing and interconnecting oligonucleotides through DNA ligation. Orientation of the inserted DNA was screened by PCR primer pairs designed to screen for the shRNA insert and orientation. Positive clones were selected and sequence confirmed at SeqWright, Inc. (Houston, TX). Based on siRNA findings, three bi-functional shRNA’s were constructed. The optimal targeting sequence was identified.

The FANG construct has a single mammalian promoter (CMV) that drives the entire cassette, with an intervening 2A ribosomal skip peptide between the GM-CSF and the furin bi-functional shRNA transcripts, followed by a rabbit polyA tail. There is a stop codon at the end of the GM-CSF transcript.

Insertion of picornaviral 2A sequences into mRNAs causes ribosomes to skip formation of a peptide bond at the junction of the 2A and downstream sequences, leading to the production of two proteins from a single open reading frame [67]. The inventors found that the 2A linker

to be effective for generating approximately equal levels of GM-CSF and anti-TGF-β transcripts with the TAG vaccine, and elected to use the same design for FANG.

Manufacturing the FANG vaccine: The patient's tumor was aseptically collected in the surgical field, placed in a gentamycin saline solution in a sterile specimen container and packaged for shipment on wet ice to the cGMP manufacturing facility. The specimen was brought into the manufacturing suite, dissected, enzymatically and mechanically disaggregated to form a cell suspension and then washed to remove debris. After the tumor cells are enumerated, QC aliquots are taken and the remaining cells are electroporated with the FANG plasmid and incubated overnight to allow vector transgene expression. Cells are harvested and gamma irradiated to arrest cell growth, then enumerated prior to removal of final QC aliquots and vaccine controlled rate freezing. The two day manufacturing process was followed by an almost three week QC testing phase after which all of vaccine assay data are evaluated prior to releasing the vaccine for patient treatment. All 9 initial patients who underwent FANG manufacturing passed all QC testing criteria.

cGMP FANG vaccines: Cancer patient autologous FANG vaccines were generated under cGMP conditions for use in clinical trials. GM-CSF and TGF-β1, -β2, and -β3 mRNA and protein expression were measured before and after FANG modification, and cytokine bioactivity determined by growth outcome on a GM-CSF and TGF-β dependent human cell line we have previously characterized. Each patient's processed vaccine will undergo proteogenomic screening to verify antigenic integrity following FANG modification.

cGMP production of FANG: FANG vaccine was generated by plasmid vector electroporation of established human cell lines. The selected FANG plasmid vector represents a construct containing the furin shRNA that has been prevalidated for optimal TGF-β downregulation.

Before being injected into patients, a frozen vial (dose) was thawed at room temperature and processed in a biosafety hood. The cell suspension will be delivered in a capped 1mL syringe. The prepared vaccine will be injected intradermally into patient at a dose of 1×10^7 or 2.5×10^7 cells per injection.

Two full scale preclinical manufacturing processes and eight clinical manufacturing processes were prepared and studies by the present inventors. Table 2 depicts the types of tumors processed (tumors 3 through 10 are the clinical vaccines).

Table 2: Tumor mass versus cell yield.

Tumor Processed	Vaccine ID	Tissue Weight (grams)	Cell # / dose	Number of Vials
1	FANG-001	12.72	1.0×10^7	40
2	FANG-002	27.41	1.0×10^7	28
3	FANG-003	6.04	2.5×10^7	9
4	FANG-004	41.08	2.5×10^7	11
5	FANG-005	6.96	2.5×10^7	8
6	FANG-006	12.48	1.0×10^7	8
7	FANG-007	10.90	2.5×10^7	15
8	FANG-008	9.80	2.5×10^7	13
9	FANG-009	6.80	1.0×10^7	6
10	FANG-010	13.00	2.5×10^7	12

The tumors processed range in size, as well as type, and the resulting viable cell yield varies greatly as shown in Table 3. All vaccines are vialed at either 1.0×10^7 cells (dose Cohort 1) or 2.5×10^7 cells (dose Cohort 2) depending on the total viable cell count on Day 2 of manufacturing. Patients with multiple tumor harvests were allowed to combine vials to qualify for minimum clinical dose requirement. A maximum of 12 doses at Cohort 2 dose level will be made available for patient treatment. Because tumor cell yield is highly variable due to tumor mass, cellularity, and processing compatibility, the minimum dose number and lower dose cohort (Cohort 1) were included.

Table 3: Final product viability (Day 2, Pre Irradiation)

Tumor Processed	Vaccine ID	% Viability
1	FANG-001	78
2	FANG-002	90
3	FANG-003	94
4	FANG-004	89
5	FANG-005	94
6	FANG-006	91
7	FANG-007	96
8	FANG-008	95
9	FANG-009	95
10	FANG-010	93

The Day 4 expression profiles of the 10 tumors processed are depicted in FIGS. 1A-1C. Note that the y-axis scales are different for all three cytokines. These data are representative of the 14 day assay (remainder of data not shown). The mean pretransfection TGFβ1 is 1251± 1544 pg/1x10^6 cells/ml; median 778pg. The mean posttransfection TGFβ1 is 191±455 pg/1x10^6 cells/ml; median 13 pg. The average percent knockdown of TGFβ1 was 85%. The mean pretransfection TGFβ2 is 232±164 pg/1x10^6 cells/ml; median 225pg. The mean posttransfection TGFβ2 is 1319 pg/1x10^6 cells/ml; median 5pg. The average percent knockdown of TGFβ2 was 94%. The average GM-CSF expression post transfection is 543±540 pg/1x10^6 cells/ml; median 400 pg. These data indicate that the GMCSF expression is consistent with the TAG vaccine as is the TGF β2 knockdown. In contrast, FANG vaccines have reduced the TGFβ1 expression more than fivefold. The minimum detectable quantity of TGFβ1 is approximately 4.6 pg/ml (R&D Systems, Quantikine Human TGFβ1). The minimum detectable quantity of TGFβ2 is approximately 7 pg/ml (R&D Systems, Quantikine Human TGFβ2). The minimum detectable quantity of GMCSF is approximately 3 pg/ml (R&D Systems, Quantikine Human GMCSF).

The protocol for setting up cultures pre and post Transfection for Autologous tumor cell vaccine to test for the expression of GMCSF, TGFβ1 and TGFβ2 has been previously described (Maples, et al., 2009). Briefly, GMCSF, TGFβ1 and TGFβ2 expression were

determined by commercially available ELISA kits (R & D Systems). The ELISA assays were performed according to manufacturer's instruction. The pretransfection sample (4 x 106 cells) is taken on Day 1. After manufacturing is completed, the sample is removed from the manufacturing facility so that the cell cultures can be set up for generating the sample for ELISA. On Day 2, the post-transfection, post-irradiation, pre-freeze sample (4 x 106 cells) is taken. After manufacturing is completed, the sample is removed from the manufacturing facility so that the cell cultures can be set up for generating the sample for ELISA.

Ten (10) vaccines (FANG -001 to -010) have been manufactured as part of the preclinical qualification process. These vaccines have been evaluated for GM-CSF, TGFβ1 and TGFb2 mRNA and protein expression using post-transfection, post-irradiation samples compared with pre-transfection, pre-irradiation samples (per FDA review, TAG vaccine, BB-IND 13650). In addition, Furin protein detection was attempted by several methods. Furin mRNA was detected by qRT-PCR.

The present inventors detected endogenous Furin protein in cell lines via Western Blot and Flow Cytometry. Five (5) different antibodies (from 3 different vendors) were screened for Western Blot and one (1) pre-labeled antibody was screened for Flow Cytometry. All experiments yielded negative results (data not shown).

A summary of all ELISA data for all manufacturing processes (Table 4) indicates that the median level of GMCSF expression is about 400 picogram/ml and the average is 543 picograms/ml. Further, the level of GMCSF tends to increase with time. In all manufactured products, GMCSF expression is observed although the level of expression is variable between manufacturing processes (tumor types). In addition to documented variability in the level of GMCSF expression between manufacturing processes, the levels of expression achieved with the FANG vaccine are deemed clinically relevant as 1) use of a plasmid rather than a viral vector obviates the obfuscating effects of elicited anti-viral neutralizing antibodies, 2) use of a plasmid likewise prevents the development of elicited antibodies interfering with long-term gene expression, and 3) concurrent suppression of furin, TGFβ1, and TGFβ2 will minimize tumor associated inhibition of GMCSF induced dendritic cell maturation [25].

Table 4: FANG vaccines 1-10 TGFβ1, TGFβ2 and GM-CSF expression in the 14 Day post manufacturing expression assay.

	TGFβ1 pg/ml Pre			TGFβ1 pg/ml Post			TGFβ2 pg/ml Pre			TGFβ2 pg/ml Post			GMCSF pg/ml Pre			GMCSF pg/ml Post		
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median
Day 0	625	678	416	105	202	7	70	116	25	9	22	0	2	2	2	157	277	29
Day 1	1154	1266	760	93	187	11	138	139	113	9	19	0	3	4	3	359	469	281
Day 2	998	1014	620	180	446	0	199	107	197	12	21	4	3	3	3	407	418	310
Day 3	1832	3221	879	173	394	4	247	156	229	12	16	8	3	4	2	580	531	475
Day 4	1241	1115	1039	211	421	20	293	189	257	9	12	4	4	6	3	657	550	602
Day 7	1729	1735	778	264	723	3	292	150	235	14	16	8	5	9	3	683	681	471
Day 10	1367	994	1629	243	530	21	335	135	310	23	21	28	5	8	4	745	546	673
Day 14	1108	892	887	281	661	19	308	158	229	17	23	12	18	24	4	821	631	645
Overall	1251	1544	778	191	455	13	232	164	225	13	19	5	5	10	3	543	540	400

Quantification of GM-CSF and TGF-β expressions: GM-CSF and TGF-β1 and -β2 expression was determined by cytokine specific colorimetric assay [68].

Validation of bioactivity: GM-CSF-induced proliferative activity similar to that of myeloid hematopoietic cells has been observed in myeloid leukemia cell lines, as mediated by the rapid and transient phosphorylation of MAP kinase 1/2 and ERK 1/2. By contrast, TGF-β turns off GM-CSF-mediated ERK signaling by inhibition of the PI3-kinase-Akt pathway [25]. The growth regulatory effects of GM-CSF and TGF-β on myeloid leukemic cells were used as an *in vitro* surrogate model to validate cytokine bioactivity in prepared FANG vaccine culture supernatants.

Cytokine activities in the FANG (or control-transfected) vaccine culture supernatants were validated by co-culture studies with erythroleukemic CD34+ TF-1a cells [69] and, if necessary, confirmed with the biphenotypic B myelomonocytic leukemic CD10+CD15+ MV4-11 cells [70] (ATCC, Rockville, MD). Both of these cell lines have been shown respond to the positive proliferative effects of GM-CSF and the negative inhibitory activity of TGF-β at ng/ml amounts [25]. Proliferative activity will be determined by Easycount Viasure assay (Immunicon) and MTT assay [68].

Phenotypic profile analysis of FANG modification: Furin knockdown likely impacts the expression of other protein substrates with the target sequence in addition to TGF-β downregulation [51]. The antigenic profile of the FANG-processed autologous vaccines were determined from cancer patients, in the event that this information may be useful towards the understanding any differential clinical outcome in vaccinated patients.

High throughput genetic profiling was used to develop individualized therapeutics for cancer patients. High throughput, gene expression array analysis was carried out to compare the differential gene expression profile of FANG-transfected vs. control vector-transfected cancer cells.

Differentially labeled FANG and control preparations are combined and fractionated by high performance liquid chromatography (Dionex), using a strong cation exchange column (SCX) and a 2nd dimension RP nano column. The fractions are spotted onto Opti-TOF™ LC/MALDI Insert (123 x 81 mm) plates (Applied Biosystems) in preparation for mass spectrometry analysis using the Applied Biosystems 4800 MALDI TOF/TOF™ Analyzer. Both protein and gene expression data were then evaluated by the GeneGo, MetaCore software suite.

Proteogenomic analysis was carried out for the purpose of determining the antigen repertoire of the autologous cancer vaccine before and after FANG process. In addition to the validation of furin knockdown, particular attention was focused on 1) baseline and differential expression of furin-substrate proteins; 2) expression of landmark tumor-associated antigens (TAAs; such as gp100, Mart1, MAGE-1, tyrosinase, for melanoma; MAGE-3, MUC-1 for non-small cell lung cancer) [71, 72] and other reported TAAs; 3) HLA antigens and co-stimulatory molecules (CD80/86) expression; 4) proteins unrelated to the above categories that are differentially expressed by 2-fold or higher following FANG transfection.

FIG. 10 shows the overall survival for Cohort 1 versus Cohorts 2 and 3 for advanced-stage patients (n = 61; P = .0186). A schematic diagram of GM-CSF-TGF-β2 antisense plasmid is shown in FIG. 11. The expression of GM-CSF in NCI-H-460 Squamous Cell and NCI-H-520, Large Cell (NSCLC) containing the pUMVC3-GM-CSF-2A-TGF-β2 antisense vector, *in vitro* is depicted in FIG. 12. TGF-β2 levels are reduced in NCI-H-460 Squamous Cell and NCI-H-520, Large Cell (NSCLC) with the pUMVC3-GM-CSF-2A-TGF-β2 antisense vector. This reduction is seen in the date presented in FIG. 13. FIG. 14 shows that a 251 base pair probe specifically detects the GM-CSF-2A-TGF-β2 transcript expressed in vitro in NCI-H-460 and NCI-H-520 cells (lanes 6 and 10); GM-CSF and TGF-β2 expression in TAG vaccines are shown in FIGS. 12 and 13, respectively. Expression of TGF-β1 and TGF-β2 in human cancer lines following siRNAfurin knockdown is shown in FIGS. 18A and 18B. Finally FIG. 19 is a schematic of the plasmid construct of FANG.

TGFβ1 expression data (FIG. 16) generated from TAG vaccine manufacturing data (n=33 vaccines; Day 7 values, TGFβ1 assay post vaccine manufacturing) clearly demonstrate that TAG does not interfere with TGFβ1 expression. The clinical significance of blocking TGFβ1 and TGFβ2 (FIG. 17), as well as TGFβ3 (data not shown) is that they are postulated to be significant negative immunomodulators expressed by the tumor. GM-CSF expression in TAG vaccines is shown in FIG. 15. These TGFβ isoforms are ubiquitous and expressed in the majority of tumors [77]. Many tumors, including breast, colon, esophageal, gastric, hepatocellular, pancreatic, SCLC and NSCLC produce high levels of one or more active TGFβ isoforms [78, 14, 15, 79-84]. Furthermore, overexpression of TGFβ has been correlated with tumor progression and poor prognosis [14, 15]. Elevated TGFβ levels have also been linked with immunosuppression in both afferent efferent limbs [14, 16-21]. Additionally, TGFβ has antagonistic effects on Natural Killer (NK) cells as well as the induction and proliferation of lymphokine-activated killer (LAK) cells [30, 35-39].

The immune suppressor functions of TGFβ are therefore likely to play a major role in modulating the effectiveness of cancer cell vaccines. TGFβ inhibits GMCSF induced maturation of bone marrow derived dendritic cells (DCs) [22] as well as expression of MHC class II and co-stimulatory molecules [23]. It has been shown that antigen presentation by immature DCs result in T cell unresponsiveness [26]. TGFβ also inhibits activated macrophages [27] including their antigen presenting function [28, 29]. Hence both the ubiquity of expression as well as the inhibitory effects of TGFβ on GMCSF immunomodulatory function support the knockdown of all tumor TGFβ expression in the autologous cancer vaccine treatment approach of the present invention.

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All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. As used in this

specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

The term "or combinations thereof" as used herein refers to all permutations and combinations of the listed items preceding the term. For example, "A, B, C, or combinations thereof" is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, MB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention.

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What is claimed is:

1. An siRNA-mediated method to inhibit transforming growth factor beta (TGF-beta or TGF-β) expression via furin knockdown, the method comprising the steps of:

selecting a target cell; and

transfecting the target cell with an expression vector comprising a promoter and a nucleic acid insert operably linked to the promoter, wherein the insert encodes one or more short hairpin RNAs (shRNA) capable of hybridizing to a region of a mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference.

2. The method of claim 1, wherein the shRNA incorporates siRNA (cleavage dependent) and miRNA (cleavage-independent) motifs.

3. The method of claim 1, wherein the shRNA is both the cleavage dependent and cleavage independent inhibitor of furin expression.

4. The method of claim 1, wherein the shRNA is further defined as a bi-functional shRNA.

5. The method of claim 1 wherein the region targeted by the shRNA is the 3' UTR region sequence of the furin mRNA transcript.

6. The method of claim 1, wherein the region targeted by the shRNA is the coding region of the furin mRNA transcript.

Claims

1. An siRNA-mediated method to inhibit transforming growth factor beta (TGF-beta or TGF-β) expression via furin knockdown, the method comprising the steps of:

selecting a target cell; and

transfecting the target cell with an expression vector comprising a promoter and a nucleic acid insert operably linked to the promoter, wherein the insert encodes one or more short hairpin bifunctional RNAs (shRNA) capable of hybridizing to a region of a mRNA transcript encoding furin, wherein the transcript incorporates siRNA (cleavage dependent) and miRNA (cleavage-independent) motifs, thereby inhibiting furin expression via RNA interference.

2. The method of claim 1, wherein the region targeted by the shRNA is the 3' UTR region sequence of the furin mRNA transcript.

3. The method of claim 1, wherein the region targeted by the shRNA is the coding region of the furin mRNA transcript.

4. The method of claim 1, wherein the shRNA is defined further as comprising a picornaviral 2A ribosomal skip peptide intercalated between a first and a second nucleic acid insert in the expression vector.

5. The method of claim 1, wherein the promoter is a CMV mammalian promoter.

6. The method of claim 5, wherein the CMV mammalian promoter contains a CMV IE 5' UTR enhancer sequence and a CMV IE Intron A.

7. The method of claim 1, wherein the bi-functional shRNA furin is defined further as comprising a first stem-loop structure comprising a complete complementary guide strand and a passenger strand and a second stem-loop structure comprising one or more basepair mismatches of the guide strand, wherein the bi-functional shRNA furin hybridized to a region of a mRNA transcript encoding furin, thereby inhibiting furin expression via RNA interference.

8. The method of claim 7, wherein the second stem-loop structure comprises three base pair mismatches.

9. The method of claim 7, wherein the basepair mismatches are at positions 9 to 11 of the guide strand.