

FURIN-KNOCKDOWN BI-FUNCTIONAL RNA**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 immunogenicity 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 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 activation processes. However, GM-CSF-mediated immunosensitization can be suppressed by tumor produced and/or secreted different isoforms of transforming growth factor beta (TGF- β). The TGF- β family of multifunctional proteins possesses well known immunosuppressive activities.

The three known TGF- β ligands (TGF- β 1, β 2, and β 3) 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. TGF- β 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- β 1. 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- β 1 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- β 1 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 peripheral immune tolerance. Hence furin knockdown represents a novel and attractive approach for optimizing GM-CSF-mediated immunosensitization.

Interferon-gamma (γ IFN) 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, γ IFN is a single-copy gene whose expression is regulated at multiple levels. γ IFN 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 γ IFN. However, there is now evidence that other cells, such as B cells, NKT cells, and professional antigen-presenting cells

(APCs) secrete γ IFN. γ IFN production by professional APCs [monocyte/macrophage, dendritic cells (DCs)] acting locally may be important in cell self-activation and activation of nearby cells. γ IFN 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 γ IFN in the adaptive immune response. Furthermore, a role for γ IFN in preventing development of primary and transplanted tumors has been identified. γ IFN production is controlled by cytokines secreted by APCs, most notably interleukin (IL)-12 and IL-18. Negative regulators of γ IFN production include IL-4, IL-10, glucocorticoids, and TGF- β .

SUMMARY OF THE INVENTION

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 structures with a miR-30a backbone. The first stem-loop structure is the siRNA component, while the second stem-loop structure is the miRNA-like component. The strategy is to use a single targeted site for both cleavage and sequestering mechanisms of RNA interference. 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 operably linked to a promoter, and it encodes a Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) cDNA. The second nucleic acid insert is also operably linked 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) and miRNA-like (cleavage-independent) motifs simultaneously. In one embodiment of the present invention, the shRNA is both the cleavage dependent and cleavage independent 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 the coding region of the furin mRNA transcript; in one embodiment, the shRNA may target the 3' untranslated region (UTR) sequence of the furin mRNA transcript.

The present invention also includes a vector that may be used to specifically knock down the expression of furin in target cells. This shRNA_{furin} expression vector comprises a nucleic acid insert operably linked 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) and miRNA (cleavage-independent) motifs, and inhibit furin expression in both a cleavage dependent and cleavage independent manner. Additionally, the expression vector may target the coding region of the furin mRNA transcript, or in the alternative it may target the 3' UTR region sequence of the furin mRNA transcript.

The present invention further provides a composition comprising a therapeutically effective amount of cells with an expression vector. The expression of the composition comprises a cell transfected with a first nucleic acid insert operably linked to a promoter, wherein the first insert encodes GM-CSF 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. The encoded shRNA incorporates siRNA (cleavage dependent) and miRNA (cleavage-independent) motifs and functions as both a

cleavage dependent and cleavage independent inhibitor of furin expression. The shRNA is further defined as a bi-functional shRNA.

A picornaviral 2A ribosomal skip peptide is intercalated between the first and the second nucleic acid inserts of the promoter, wherein the promoter is a CMV mammalian promoter, enhancer, and intron. The region targeted by the shRNA of the present invention is the 3' UTR region sequence of the furin mRNA transcript. Alternatively, the shRNA can also target the coding region of the furin mRNA transcript. As per the present invention the cells are autologous tumor cells, xenograft expanded autologous tumor cells or allogeneic tumor cells. In specific aspects of the present invention the cells are xenograft expanded allogeneic tumor cells and comprises 1×10^7 cells to 2.5×10^7 cells. The composition described herein further comprises a therapeutically effective dose of γ IFN (gamma interferon), wherein the therapeutically effective dose of γ IFN is 50 or 100 $\mu\text{g}/\text{m}^2$.

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 show an assessment of GM-CSF expression and TGF- β 1 and - β 2 knockdown, summarizing (FIG. 1A) TGF- β 1, (FIG. 1B) TGF- β 2, and (FIG. 1C) 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. 2 shows the siRNA targeted regions of furin mRNA (Accession No. NM_002569). Prospective siRNA targeting regions in 3'-UTR and encoding regions of furin mRNA and the targeted sequence by each siRNA;

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

FIG. 4 shows a schematic diagram of GM-CSF-TGF- β 2 antisense plasmid;

FIG. 5 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- β 2 antisense vector, *in vitro*;

FIG. 6 shows that TGF- β 2 levels are reduced in NCI-H-460 Squamous Cell and NCI-H-520, Large Cell (NSCLC) with the pUMVC3-GM-CSF-2A-TGF- β 2 antisense vector;

FIG. 7 shows that a 251 base pair probe specifically detects the GM-CSF-2A-TGF- β 2 transcript expressed *in vitro* in NCI-H-460 and NCI-H-520 cells (lanes 6 and 10);

FIG. 8 shows the GM-CSF expression in TAG vaccines;

FIG. 9 shows the TGF- β 2 expression in TAG vaccines;

FIGS. 10A and 10B show expression of (FIG. 10A) TGF- β 1 and (FIG. 10B) TGF- β 2 in human cancer lines following siRNAfurin knockdown; and

FIG. 11 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 Vaccine
FL	Flt-3-Ligand
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
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 histocompatibility 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
PI	Principal Investigator

Abbreviation	Term
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. 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- β 1 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 human TGF- β ligands (TGF- β 1-3, 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- β 1 peripheral immune tolerance. Hence, furin knockdown represents a novel and attractive approach for optimizing immunosensitization.

The incorporation of a bi-functional shRNA_{furin} 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.

Other applications for the bi-functional shRNA_{furin} include: (1) Systemic delivery via a tumor ($\pm$ 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, 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) ($\pm$ decoration and reversible masking/stealth) 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.

A Furin-knockdown and GM-CSF-augmented (FANG) Autologous Cancer Vaccine for Human Melanoma and Lung Cancer: FANG uniquely incorporates a proprietary 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- β 1 and - β 2 depletion in human cancer lines.

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 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.

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.

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- β 1, β 2, and β 3) 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]. Ardeshtna [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)- κ B 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- β 2 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- β 1 or - β 2 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 $> 2.5 \times 10^7$ 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- β 2 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. 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- β 2, given that all three known isoforms of TGF- β ligand (TGF- β 1, - β 2, and - β 3) are ubiquitously produced in human cancers. In particular, up to a 10-fold higher level of TGF- β 1 may be produced by human colorectal, lung cancer, and melanoma cells. The tolerogenic role of TGF- β 1 in antigen presenting dendritic cells (DC) and T regulatory cells (Treg) is well established, and this activity is not impacted by TGF- β 2 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 (RXXK/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- β 1. 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- β 1 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- β 1 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- β 1 peripheral immune tolerance [54]. Hence furin knockdown represents a novel and attractive approach for optimizing immunosensitization.

FANG (furin shRNA and GM-CSF) 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 GM-CSF expression and TGF- β 1 and - β 2 knockdown is shown in FIGS. 1A -1C.

The capability of FANG to knockdown both TGF- β 1 and - β 2 is supported by findings in the first 9 patients (FIGS. 1A and 1B) 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- β 2, and 6 of 7 patients with >100 pg of endogenous TGF- β 1 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- β 1 (as well as TGF- β 2) 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- β 1 was unchanged with the TAG preparation (TAG-004) albeit with the expected depletion of TGF- β 2. 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- β 1, - β 2, and - β 3 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.

The FANG approach was supported by the findings from 9 patient autologous vaccines, which consistently demonstrated TGF- β 1 and TGF- β 2 reductions and elevated GM-CSF levels (FIGS. 1A to 1C). Both TGF- β 1 and TGF- β 2 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 (FIG. 2) in the human furin mRNA sequence 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 (<http://jura.wi.mit.edu/bioc/siRNAext>). siRNAs targeting eligible translated and 3'UTRs sites (FIG. 2) 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- β 1 and TGF- β 2 levels in culture supernatants without adversely affecting cell survival. Thus siRNA-mediated furin knockdown is effective for the depletion of TGF- β 1 and - β 2 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 (cleavage-dependent process). 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 two bp mismatches at positions 11 and 12 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.

cGMP FANG vaccines: Cancer patient autologous FANG vaccines were generated under cGMP conditions for use in clinical trials. Human GM-CSF and human TGF- β 1, - β 2, and - β 3 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.

Quantification of GM-CSF and TGF- β expressions: GM-CSF and TGF- β 1 and - β 2 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.

In FIG. 3 the overall survival for Cohort 1 versus Cohorts 2 and 3 for advanced-stage patients (n = 61; P = .0186) is shown. A schematic diagram of GM-CSF-TGF-β2 antisense plasmid is represented by FIG. 4. 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. 5.

Data presented in FIG. 6 shows the reduction in TGF-β2 levels in NCI-H-460 Squamous Cell and NCI-H-520, Large Cell (NSCLC) with the pUMVC3-GM-CSF-2A-TGF-β2 antisense vector. FIG. 7 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). FIGS. 8 and 9 shows the GM-CSF and TGF-β2 expression in TAG vaccines, respectively.

FIG. 10A shows the expression TGF- β 1 in human cancer lines following siRNAfurin knockdown. Similar expression profile for TGF- β 2 is shown in FIG. 10B. FIG. 11 shows the plasmid construct of FANG

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 $\pm 1, 2, 3, 4, 5, 6, 7, 10, 12$ 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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What is claimed is:

1. A bi-shRNA^{furin}/GMCSF expression vector plasmid comprising:
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.
2. The expression vector of claim 1, wherein the GM-CSF is human.
3. The expression vector of claim 1, wherein the shRNA incorporates siRNA (cleavage dependent) and miRNA (cleavage-independent) motifs.
4. The expression vector of claim 1, wherein the shRNA is both the cleavage dependent and cleavage independent inhibitor of furin expression.
5. The expression vector of claim 1, wherein the shRNA is further defined as a bi-functional shRNA.
6. The expression vector of claim 1, wherein a picornaviral 2A ribosomal skip peptide is intercalated between the first and the second nucleic acid inserts.
7. The expression vector of claim 1, wherein the promoter is a CMV mammalian promoter.
8. The expression vector of claim 7, wherein the CMV mammalian promoter contains a CMV IE 5' UTR enhancer sequence and a CMV IE Intron A.
9. The expression vector of claim 1, wherein the region targeted by the shRNA is the 3' UTR region sequence of the furin mRNA transcript.
10. The expression vector of claim 1, wherein the region targeted by the shRNA is the coding region of the furin mRNA transcript.
11. A bishRNA^{furin} expression vector comprising:
a nucleic acid insert operably linked to a 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.

12. The expression vector of claim 11, wherein the shRNA incorporates siRNA (cleavage dependent) and miRNA (cleavage-independent) motifs.
13. The expression vector of claim 11, wherein the shRNA is both the cleavage dependent and cleavage independent inhibitor of furin expression.
14. The expression vector of claim 11, wherein the shRNA is further defined as a bi-functional shRNA.
15. The expression vector of claim 11, wherein the region targeted by the shRNA is the 3' UTR region sequence of the furin mRNA transcript.
16. The expression vector of claim 11, wherein the region targeted by the shRNA is the coding region of the furin mRNA transcript.
17. A composition comprising a therapeutically effective amount of cells with an expression vector comprising:

a cell transfected with a first nucleic acid insert operably linked to a promoter, wherein the first insert encodes GM-CSF 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.
18. The composition of claim 17, wherein the GM-CSF is human.
19. The composition of claim 17, wherein the shRNA incorporates siRNA (cleavage dependent) and miRNA (cleavage-independent) motifs.
20. The composition of claim 17, wherein the shRNA is both the cleavage dependent and cleavage independent inhibitor of furin expression.
21. The composition of claim 17, wherein the shRNA is further defined as a bi-functional shRNA.
22. The composition of claim 17, wherein a picornaviral 2A ribosomal skip peptide is intercalated between the first and the second nucleic acid inserts.

23. The composition of claim 17, where the promoter is a CMV mammalian promoter, enhancer and intron.
24. The composition of claim 17, wherein the region targeted by the shRNA is the 3' UTR region sequence of the furin mRNA transcript.
25. The composition of claim 17, wherein the region targeted by the shRNA is the coding region of the furin mRNA transcript.
26. The composition of claim 17, wherein the cells are autologous tumor cells.
27. The composition of claim 17, wherein the cells are xenograft expanded autologous tumor cells.
28. The composition of claim 17, wherein the cells are allogeneic tumor cells.
29. The composition of claim 17, wherein the cells are xenograft expanded allogeneic tumor cells.
30. The composition of claim 17, wherein the composition comprises 1×10^7 cells to 2.5×10^7 cells.
31. The composition of claim 17, wherein the composition further comprises a therapeutically effective dose of γ IFN (gamma interferon).
32. The composition of claim 31, wherein the therapeutically effective dose of γ IFN is 50 or $100 \mu\text{g}/\text{m}^2$.