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(54) Title: COMPOSITIONS FOR THE TREATMENT OF CANCER

(57) Abstract: Provided herein are methods and uses relating to transcription factors involved in regulating the epithelial-to-mesenchymal transition during vertebrate development, such as the Brachyury protein, and compositions comprising poxvirus vectors encoding such transcription factors, for the treatment of cancer.



COMPOSITIONS FOR THE TREATMENT OF CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This patent application claims the benefit of U.S. Provisional Patent Application No. 61/097,101, filed September 15, 2008, which is incorporated by reference.

PARTIES TO JOINT RESEARCH AGREEMENT

[0002] The inventions described herein were made during the course of activities undertaken within the scope of a joint research agreement between the Government of the United States of America, as represented by the National Institutes of Health (“NIH”), and BN ImmunoTherapeutics, Inc. (“BNIT”).

SEQUENCE LISTING

[0003] Incorporated by reference in its entirety herein is a nucleotide/amino acid sequence listing submitted concurrently herewith.

FIELD

[0004] This application relates to the field of cancer vaccines and active immunotherapies. In particular, this application relates to the use of transcription factors involved in regulating the epithelial-to-mesenchymal transition during vertebrate development, such as the Brachyury protein, and poxvirus vectors encoding such transcription factors, for the treatment of cancer.

BACKGROUND

[0005] There remains a substantial unmet medical need for non-invasive cancer treatments, particularly those with the ability to reduce tumor burden and/or increase overall survival with few of the common side effects associated with most currently approved chemotherapeutics, such as nausea, vomiting, diarrhea, kidney damage, loss of appetite, cachexia, and the like.

[0006] Active immunotherapies targeting tumor-associated or tumor-specific antigens show tremendous promise in this area. The use of active immunotherapy (i.e., so-called ‘cancer vaccines’) to treat different types of cancer requires the identification of tumor-specific antigens capable of eliciting host immune responses against tumor cells expressing those antigens. Antigens essential for malignant transformation, tumor progression, or metastasis that are selectively expressed by malignant cells present ideal targets for such therapies. Because the epithelial-to-mesenchymal transition (“EMT”) is a key step during the progression of primary tumors into metastases, molecules involved in regulating the EMT, such as the transcription factors Snail, Twist, Slug, and Brachyury, may also provide potentially useful targets for therapeutic intervention. Thiery et al., *Nat. Rev. Cancer* 2:442–54 (2002); Yang et al., *Cell* 117:927–39 (2004); Cano et al., *Nature Cell Biol.* 2:76–83 (2000); and Huber et al., *Curr. Opin. Cell Biol.* 17:548–58 (2005).

SUMMARY

[0007] In one aspect, provided herein are poxvirus vectors comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence 95% identical to the amino acid sequence of SEQ ID NO:1. In certain embodiments, the poxvirus vectors comprise a nucleic acid encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:1. In certain embodiments, the poxvirus vector is an orthopox virus, an avipox virus, a capripox virus, a suipox virus, a raccoon pox virus, or a rabbit pox virus. In certain embodiments, the orthopox virus is a vaccinia virus. In certain embodiments, the vaccinia virus is a modified vaccinia virus Ankara (“MVA”) or an MVA-Bavarian Nordic (“MVA-BN”). In certain embodiments, the vector is an avipox virus. In certain embodiments, the avipox virus is a canarypox virus or a fowlpox virus. In certain embodiments, the orthopoxvirus vector further comprises nucleic acids encoding a B7-1 polypeptide, an ICAM-1 polypeptide, and an LFA-3 polypeptide. In certain embodiments, the avipoxvirus vector further comprises nucleic acids encoding a B7-1 polypeptide, an ICAM-1 polypeptide, and an LFA-3 polypeptide.

[0008] In another aspect, provided herein are compositions comprising any of the orthopoxvirus vectors provided herein and a pharmaceutically acceptable carrier or any of the avipoxvirus vectors provided herein and a pharmaceutically acceptable carrier.

[0009] In another aspect, provided herein are methods of eliciting an immune response against Brachyury in a subject, the method comprising administering a therapeutically effective amount of any of the orthopox virus compositions provided herein or any of the avipox virus compositions provided herein to the subject, thereby eliciting the immune response. In certain embodiments, the methods further comprise administering one or more additional therapeutically effective amounts of any of the orthopox virus compositions provided herein or any of the avipox virus compositions to the subject, thereby eliciting the immune response. In certain embodiments, the subject is a human. In certain embodiments, the subject has a Brachyury-expressing tumor. In certain embodiments, the Brachyury-expressing tumor is a cancer of the brain, breast, lung, esophagus, stomach, small intestine, colon, liver, kidney, bladder, uterus, Fallopian tube, ovary, testes, ureter, prostate, or pancreas.

[0010] In another aspect, provided herein are methods of treating or preventing cancer in a subject, the method comprising administering a therapeutically effective amount of any of the orthopox virus compositions provided herein or any of the avipox virus compositions provided herein to the subject, thereby treating or preventing the cancer. In certain embodiments, the methods further comprise administering one or more additional therapeutically effective amounts of any of the orthopox virus compositions provided herein or any of the avipox virus compositions provided herein to the subject, thereby treating or preventing the cancer. In certain embodiments, treating the cancer comprises reducing the size of the primary tumor. In certain embodiments, treating the cancer comprises reducing the number of metastatic lesions. In certain embodiments, the subject is a human. In certain embodiments, the subject has a Brachyury-expressing tumor. In certain embodiments, the Brachyury-expressing tumor is a cancer of the brain, breast, lung, esophagus, stomach, small intestine, colon, liver, kidney, bladder, uterus, Fallopian tube, ovary, testes, ureter, prostate, or pancreas.

[0011] In certain embodiments, the methods further comprise administering a therapeutically effective amount of a second agent to the subject. In certain embodiments, the second agent is selected from the group consisting of cytokines, chemotherapeutics, and radiotherapeutics. In certain embodiments, the second agent is a cytokine. In certain embodiments, the cytokine is selected from the group consisting of interleukin (IL)-2, IL-3, IL-6, IL-10, IL-12, IL-15, GM-CSF, interferon (IFN)- α , IFN- β , IFN- γ , and IFN- ω . In certain

embodiments, the second agent is a chemotherapeutic. In certain embodiments, the chemotherapeutic is selected from the group consisting of alkylating agents, antimetabolites, natural products, hormones and hormone antagonists, targeted therapeutics, and miscellaneous agents. In certain embodiments, the chemotherapeutic is an alkylating agent. In certain embodiments, the alkylating agent is selected from the group consisting of nitrogen mustards, alkyl sulfonates, and nitrosoureas. In certain embodiments, the chemotherapeutic is an antimetabolite. In certain embodiments, the antimetabolite is selected from the group consisting of folic acid analogs, pyrimidine analogs, purine analogs, and topoisomerase inhibitors. In certain embodiments, the chemotherapeutic is a natural product. In certain embodiments, the natural product is selected from the group consisting of vinca alkaloids, epipodophyllotoxins, antibiotics, and enzymes. In certain embodiments, the chemotherapeutic is a hormone or hormone antagonist. In certain embodiments, the hormone or hormone antagonist is selected from the group consisting of adrenocorticosteroids, progestins, estrogens, antiestrogens, and androgens. In certain embodiments, the chemotherapeutic agent is a targeted therapeutic. In certain embodiments, the targeted therapeutic is selected from the group consisting of selective estrogen receptor modulators (SERMs), aromatase inhibitors, topoisomerase inhibitors, tyrosine kinase inhibitors, serine/threonine kinase inhibitors, histone deacetylase (HDAC) inhibitors, retinoid receptor activators, apoptosis stimulators, angiogenesis inhibitors, and poly (ADP-ribose) polymerase (PARP) inhibitors. In certain embodiments, the chemotherapeutic is a miscellaneous agent. In certain embodiments, the miscellaneous agent is selected from the group consisting of platinum coordination complexes, substituted ureas, methyl hydrazine derivatives, and adrenocortical suppressants.

[0012] In another aspect, provided herein are methods treating cancer in a subject, wherein the subject has a tumor that is resistant to chemotherapy or ionizing radiation, the method comprising (a) selecting a subject having a tumor that is resistant to chemotherapy or ionizing radiation and (b) administering a therapeutically effective amount of any of the orthopox virus compositions provided herein or any of the avipox virus compositions provided herein to the subject, thereby treating the cancer. In certain embodiments, the methods further comprise administering a therapeutically effective amount of any of the orthopox virus compositions provided herein or any of the avipox virus compositions provided herein to the

subject, thereby treating the resistant cancer. In certain embodiments, treating the resistant cancer comprises reducing the size of the primary tumor. In certain embodiments, treating the resistant cancer comprises reducing the number of metastatic lesions. In certain embodiments, the subject is a human. In certain embodiments, the subject has a Brachyury-expressing tumor. In certain embodiments, the Brachyury-expressing tumor is a cancer of the brain, breast, lung, esophagus, stomach, small intestine, colon, liver, kidney, bladder, uterus, Fallopian tube, ovary, testes, ureter, prostate, or pancreas.

[0013] In certain embodiments, the methods further comprise administering a therapeutically effective amount of a second agent to the subject. In certain embodiments, the second agent is selected from the group consisting of cytokines, chemotherapeutics, and radiotherapeutics. In certain embodiments, the second agent is a cytokine. In certain embodiments, the cytokine is selected from the group consisting of interleukin (IL)-2, IL-3, IL-6, IL-10, IL-12, IL-15, GM-CSF, interferon (IFN)- α , IFN- β , IFN- γ , and IFN- ω . In certain embodiments, the second agent is a chemotherapeutic. In certain embodiments, the chemotherapeutic is selected from the group consisting of alkylating agents, antimetabolites, natural products, hormones and hormone antagonists, targeted therapeutics, and miscellaneous agents. In certain embodiments, the chemotherapeutic is an alkylating agent. In certain embodiments, the alkylating agent is selected from the group consisting of nitrogen mustards, alkyl sulfonates, and nitrosoureas. In certain embodiments, the chemotherapeutic is an antimetabolite. In certain embodiments, the antimetabolite is selected from the group consisting of folic acid analogs, pyrimidine analogs, purine analogs, and topoisomerase inhibitors. In certain embodiments, the chemotherapeutic is a natural product. In certain embodiments, the natural product is selected from the group consisting of vinca alkaloids, epipodophyllotoxins, antibiotics, and enzymes. In certain embodiments, the chemotherapeutic is a hormone or hormone antagonist. In certain embodiments, the hormone or hormone antagonist is selected from the group consisting of adrenocorticosteroids, progestins, estrogens, antiestrogens, and androgens. In certain embodiments, the chemotherapeutic agent is a targeted therapeutic. In certain embodiments, the targeted therapeutic is selected from the group consisting of selective estrogen receptor modulators (SERMs), aromatase inhibitors, topoisomerase inhibitors, tyrosine kinase inhibitors, serine/threonine kinase inhibitors, histone deacetylase (HDAC) inhibitors,

retinoid receptor activators, apoptosis stimulators, angiogenesis inhibitors, and poly (ADP-ribose) polymerase (PARP) inhibitors. In certain embodiments, the chemotherapeutic is a miscellaneous agent. In certain embodiments, the miscellaneous agent is selected from the group consisting of platinum coordination complexes, substituted ureas, methyl hydrazine derivatives, and adrenocortical suppressants.

[0014] Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The objects and advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[0015] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

[0016] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate various embodiments of the invention and together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] **Figure 1** shows that Brachyury protein is predominantly expressed in human tumor tissues. Expression of Brachyury and GAPDH was analyzed by RT-PCR in multiple commercially available cDNA samples. **Figure 1(A)** – human carcinoma tissues (individual cases); **Figure 1(B)** –corresponding normal human tissues; **Figure 1(C)** – normal human immune tissues; and **Figure 1(D)** – normal immune cell fractions.

[0018] **Figure 2** shows Brachyury expression data obtained by RT-PCR for a selected group of normal human tissues compared to tumors derived from the same tissue.

[0019] **Figure 3** compares expression levels of Brachyury, Twist, and Snail in multiple commercially available cDNA samples derived from various human breast and lung tumor tissues relative to glyceraldehyde 3-phosphate dehydrogenase (“GAPDH”), measured by RT-PCR. Expression was also evaluated in histologically normal tissues derived from cancer patients (designated as “normal”). Like Brachyury, both Twist and Snail are involved in regulating the

epithelial-to-mesenchymal transition (“EMT”). Unlike Brachyury, neither Twist nor Snail are predominantly expressed in human tumor tissues.

[0020] **Figure 4** shows expression of the EMT regulators Snail and Twist analyzed relative to that of GAPDH by RT-PCR analysis in multiple cDNA samples commercially available from normal tissues (left panels) and various human tumor cell lines derived from lung, colon, and prostate carcinoma (right panels).

[0021] **Figure 5** shows relative mRNA expression of three EMT regulators in normal mouse tissues and mouse tumor cell lines. RT-PCR was performed for Brachyury (panel A), Snail (panel B), and Twist (panel C) on a panel of cDNAs isolated from normal mouse tissues (Clontech, Mountain View, CA) and several murine tumor cell lines. The following TaqMan probes (Life Technologies, Foster City, CA) were used: for Brachyury (Mm01318249_m1); for Snail (Mm00441533_g1); and for Twist (Mm00442036_m1). All values are expressed as a ratio of the target gene to the endogenous control gene GAPDH.

[0022] **Figure 6** shows expression of Brachyury analyzed by immunohistochemistry analysis with a Brachyury-specific monoclonal Ab in multiple normal tissues derived from non-cancerous patients and in various lung tumor tissues. Indicated is the percentage of Brachyury positive tissues detected.

[0023] **Figure 7** shows that Brachyury expression is associated with increased resistance to ionizing radiation. Human lung carcinoma A549 cells stably transfected with a control pCMV vector or a vector encoding for the full length human Brachyury protein (phBrachyury) (**Figure 7A**) and human lung carcinoma H226 cells stably transfected with a control shRNA (shControl) or a Brachyury-specific shRNA vector (shBrachyury) (**Figure 7B**) were compared for their sensitivity to various doses of ionizing radiation. Cells were left untreated or treated with various doses of ionizing radiation as indicated in a Cs-137 irradiator and immediately plated in triplicate on 96-well plates. Seventy-two hours post-treatment, cells were evaluated for survival by performing an MTT assay using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Results are expressed as percentage of survival for each cell line normalized to the untreated group.

[0024] **Figure 8** shows that Brachyury expression is associated with increased resistance to chemotherapy. Human lung carcinoma A549 cells stably transfected with a control pCMV

vector or a vector encoding for the full length human Brachyury protein (phBrachyury) (**Figure 8A**) and human lung carcinoma H226 cells stably transfected with a control shRNA (shControl) or a Brachyury-specific shRNA vector (shBrachyury) (**Figure 8B**) were compared for their sensitivity to treatment with various chemotherapeutic agents. Cells were plated in triplicate on 96-well plates, allowed to attach overnight, and subsequently left untreated or treated with Taxotere, Vinorelbine, Cisplatin, or a combination of Cisplatin and Vinorelbine, at the doses (in ng/ml) indicated in the figure. After 6 hours of treatment with chemotherapeutic agents, fresh culture medium was added and cells were incubated for additional 72 hours. Survival of cells was evaluated by performing an MTT assay. Results are expressed as percentage of survival for each cell line normalized to the untreated group. Populations of A549 cells were generated by single-cell cloning (**Figure 8C**). Each population (designated with numbers) was evaluated for levels of Brachyury expression by RT-PCR (panel (i)), tumor cell growth over a 5-day period (panel (ii)), as well as susceptibility to the chemotherapeutics Docetaxel (panel (iii)), Cisplatin (panel (iv)), Vinorelbine (panel (v)), and the combination of Cisplatin with Vinorelbine (panel (vi)). The results demonstrated that the levels of Brachyury in tumor cells negatively correlated with tumor growth and positively correlated with survival in response to treatment with chemotherapy. Numbers between parentheses indicate Pearson's correlation coefficients calculated in relation to Brachyury expression.

[0025] **Figure 9A** shows expression of TRICOM following infection of human Dendritic Cells ("DCs") *in vitro* with a recombinant MVA vector encoding Brachyury and a Triad of Costimulatory Molecules (B7.1, LFA-3, and ICAM-1; "MVA-Brachyury-TRICOM"). **Figure 9B** shows expression of Brachyury following infection of human DCs *in vitro* with MVA-Brachyury-TRICOM.

[0026] **Figure 10A** shows expression of TRICOM by flow cytometry following infection of monkey mammary tumor cells ("CMMT"; ATCC No. CRL-6299) with recombinant fowlpox expressing Brachyury and TRICOM ("rF-Brachyury-TRICOM"). Controls are uninfected cells and CMMT cells infected with fowlpox expressing prostate-specific antigen and TRICOM ("PROSTVAC-F"). **Figure 10B** shows expression of Brachyury by flow cytometry following infection of CMMT cells with rF-Brachyury-TRICOM (panels marked 231B-B6 and 249A-A11) or MVA-Brachyury-TRICOM (panels marked 240B-A5). **Figure 10C** shows expression of

Brachyury by Western Blot following infection of CMMT cells with rF-Brachyury-TRICOM (panels marked 231B-B6 and 249A-A11) or MVA-Brachyury-TRICOM (panels marked 240B-A5).

[0027] **Figure 11** shows that human DCs infected with MVA-Brachyury-TRICOM expand Brachyury-specific CD4⁺ T-cells from the blood of normal human donors. **Figure 11A** shows the results with human DCs isolated from normal human donor 1228. **Figure 11B** shows the results with human DCs isolated from normal human donor 635.

[0028] **Figure 12** shows that MVA-Brachyury-TRICOM-infected DCs stimulate Brachyury-specific CD8⁺ T-cells.

[0029] **Figure 13A** shows the experimental design for an experiment testing the anti-tumor efficacy of vaccination with a recombinant MVA expressing the EMT regulator Twist and TRICOM ("MVA-Twist-TRICOM") in mice. **Figure 13B** shows that vaccination with MVA-Twist-TRICOM was able to significantly reduce primary tumor volume as well as to markedly reduce the number of metastatic 4T1 cells in the lungs.

BRIEF DESCRIPTION OF THE SEQUENCES

[0030] The nucleic acid and amino acid sequences listed are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. § 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood to be included by any reference to the displayed strand.

[0031] SEQ ID NO: 1 is an exemplary amino acid sequence of a human Brachyury protein.

[0032] SEQ ID NO: 2 is an exemplary nucleic acid sequence encoding the human Brachyury protein of SEQ ID NO:1.

[0033] SEQ ID NO: 3 is an exemplary amino acid sequence of a mouse Brachyury protein.

[0034] SEQ ID NO: 4 is an exemplary nucleic acid sequence encoding the mouse Brachyury protein of SEQ ID NO:3.

[0035] SEQ ID NO:5 is the amino acid sequence of Tp2 peptide.

[0036] SEQ ID NO:6 is the Brachyury-specific forward PCR primer E7F.

- [0037] SEQ ID NO:7 is the Brachyury-specific reverse PCR primer E7R.
- [0038] SEQ ID NO:8 is a GAPDH-specific forward PCR primer.
- [0039] SEQ ID NO:9 is a GAPDH-specific reverse PCR primer.
- [0040] SEQ ID NO:10 is an exemplary amino acid sequence of mouse Twist protein.
- [0041] SEQ ID NO:11 is an exemplary nucleic acid encoding the mouse Twist protein of SEQ ID NO:10.
- [0042] SEQ ID NO:12 is an exemplary amino acid sequence of a human B7.1 protein.
- [0043] SEQ ID NO:13 is an exemplary nucleic acid encoding the human B7.1 protein of SEQ ID NO:12.
- [0044] SEQ ID NO:14 is an exemplary amino acid sequence of a mouse B7.1 protein.
- [0045] SEQ ID NO:15 is an exemplary nucleic acid encoding the mouse B7.1 protein of SEQ ID NO:14.
- [0046] SEQ ID NO:16 is an exemplary amino acid sequence of a human ICAM-1 protein.
- [0047] SEQ ID NO:17 is an exemplary nucleic acid encoding the human ICAM-1 protein of SEQ ID NO:16.
- [0048] SEQ ID NO:18 is an exemplary amino acid sequence of a mouse ICAM-1 protein.
- [0049] SEQ ID NO:19 is an exemplary nucleic acid encoding the mouse ICAM-1 protein of SEQ ID NO:18.
- [0050] SEQ ID NO:20 is an exemplary amino acid sequence of a human LFA-3 protein.
- [0051] SEQ ID NO:21 is an exemplary nucleic acid encoding the human LFA-3 protein of SEQ ID NO:20.
- [0052] SEQ ID NO:22 is an exemplary amino acid sequence of a mouse LFA-3 protein.
- [0053] SEQ ID NO:23 is an exemplary nucleic acid encoding the mouse LFA-3 protein of SEQ ID NO:22.

DESCRIPTION

[0054] *Metastasis.* Tumor metastasis is a complex, multistep process by which tumor cells detach from a primary tumor, invade surrounding tissue, enter the bloodstream or lymphatic system, home to distant organs, establish micro-metastases and colonize macroscopic metastases at one or more secondary sites. Yang et al., “Exploring a New Twist on Tumor Metastasis,”

Cancer Res. 66:4549-4552 (2006). Despite the fact that metastatic progression is responsible for the majority of all human cancer deaths, not all of the complex biological processes involved in metastasis are well understood. One key step in the process involves the phenotypic conversion of epithelial tumor cells into motile mesenchymal cells, accomplished at least in part by a reversible process known as the epithelial-to-mesenchymal transition. Fernando et al., "The T-box Transcription Factor Brachyury Promotes Epithelial-Mesenchymal Transition in Human Tumor Cells," *J. Clin. Invest.* 120(2):533-544 (2010).

[0055] *The Epithelial-to-Mesenchymal Transition ("EMT")*. The EMT is a process by which epithelial cell layers lose polarity and cell-cell contacts, during which they also undergo a significant remodeling of their cytoskeleton. Kang et al., "Epithelial-Mesenchymal Transitions: Twist in Development and Metastasis," *Cell* 118:277-279 (2004). At the same time epithelial cells undergoing the EMT lose cell adhesion and cytoskeletal components, they also begin to express various mesenchymal components and to display a migratory phenotype. Under normal circumstances, such cell migration underlies essential developmental processes such as gastrulation and the subsequent formation of various tissues and organs including, for example, the neural crest, heart, musculoskeletal system, craniofacial structures, and the peripheral nervous system. Kang et al., (2004). In a cancer patient, however, cell migration eventually results in the establishment of micro-metastases, colonization of macroscopic metastases at one or more secondary sites and disease progression.

[0056] One major hallmark of the EMT is the down-regulation of expression of various epithelial proteins, including E-cadherin and cytokeratins, as well as the up-regulation of expression of various mesenchymal proteins, including fibronectin, N-cadherin, vimentin, and a variety of matrix metalloproteases. Fernando et al., (2010). E-cadherin is a central component of adherens junctions, the cell-cell adhesion junctions required both to form epithelia in embryos and to maintain epithelial homeostasis in adults. Kang et al., (2004). Loss of E-cadherin expression increases tumor cell invasiveness *in vitro* and contributes to the transition of adenoma to carcinoma in animal models. Kang et al., (2004). In addition, E-cadherin expression levels often inversely correlate with tumor grade and stage. Kang et al., (2004). Finally, the switch from expressing E-cadherin (a hallmark of epithelial tissues) to expressing N-cadherin (a hallmark of

mesenchymal tissues) is associated with cancer progression and cancer-related death. Fernando et al., (2010).

[0057] *Regulators of the EMT in Cancer.* The EMT program triggered during tumor progression appears to be controlled by a number of developmentally important genes normally expressed in the early embryo, including *Brachyury*, *Gooseoid*, *SIP1*, *Slug*, *Snail*, and *Twist*. Fernando et al., (2010); and Palena et al., “The Human T-Box Mesodermal Transcription Factor Brachyury Is a Candidate Target for T-Cell-Mediated Cancer Immunotherapy,” *Clin. Cancer Res.* 13(8):2471-2478 (2007). The proteins encoded by those genes impart mesenchymal characteristics to tumor cells in part by down-regulating expression of proteins involved in formation of adherens junctions such as E-cadherin, and in part by up-regulating expression of mesenchymal proteins such as N-cadherin. For example, both *Snail* and *SIP1* bind to so-called ‘E box’ motifs in certain promoters, including the E-cadherin promoter. Kang et al., (2004). In vertebrates, *Snail* and the closely-related protein *Slug* are essential for gastrulation and emergence of the neural crest from the neural tube. Moreover, *Snail* has been shown to repress transcription of E-cadherin and mediate the EMT in invasive carcinoma cells in both mouse and human. Kang et al., (2004).

[0058] Similarly, the *Mus musculus* protein Twist1 (“Twist”), a basic helix-loop-helix (“bHLH”) transcription factor, regulates cell movement and tissue reorganization during early embryogenesis by promoting the EMT. *See, e.g.*, Yang et al., “Twist, a Master Regulator of Morphogenesis, Plays an Essential Role in Tumor Metastasis,” *Cell* 117:927-939 (2004). In vertebrates, *Twist* is mainly expressed in neural crest cells and plays a role in proper migration and differentiation of neural crest and head mesenchymal cells. Yang et al., (2006). Both mesoderm formation and neural crest development require the EMT, which converts a tightly attached sheet of epithelial cells into highly mobile mesenchymal or neural crest cells. Yang et al., (2006). Ectopic expression of *Twist* in kidney and mammary epithelial cells resulted in the loss of E-cadherin-mediated cell-cell adhesion, activation of mesenchymal markers, and gain of cell motility, indicating that, like other transcription factors involved in regulating the EMT, *Twist* contributes to invasion and metastasis by promoting the EMT. Yang et al., (2006).

[0059] Like *Twist* in mice, the protein Brachyury regulates cell movement and tissue reorganization during early embryogenesis in humans by promoting the EMT. Palena et al.,

(2007). Brachyury is involved in the formation and organization of mesoderm during vertebrate embryogenesis; formation of mesoderm during embryogenesis requires epithelial cells to change into mesenchymal cells, a change accomplished by triggering an EMT. Palena et al., (2007).

[0060] In humans, Brachyury is predominantly expressed in multiple carcinoma types, including lung, breast, ovary, colon, prostate, small intestine, stomach, kidney, bladder, uterus, and testis, in human cell lines derived from lung, colon, and prostate carcinomas, as well as in chronic lymphocytic leukemia and EBV-positive tumor cells. *See, e.g.*, Figures 1-2. Tumor specificity for Brachyury has been confirmed by demonstrating the absence of Brachyury expression in most normal human adult tissues that have been tested, with the exception of testes. *See, e.g.*, Figures 1-2. Comparison of expression of Brachyury and other transcription factors that regulate the EMT in human tissues demonstrates the high tumor specificity of Brachyury expression. For example, unlike Brachyury, the expression of two other EMT regulators, Twist and Snail, is high in most normal human tissues tested. *See, e.g.*, Figures 3-4. Therefore, an active immunotherapy directed to Brachyury (*i.e.*, a recombinant poxvirus vaccine expressing Brachyury) would provide a specific way of targeting and eliminating tumor cells, including metastatic cells that have already converted into a mesenchymal-like phenotype without impacting healthy, non-cancerous tissues.

[0061] A number of additional experimental findings suggest that Brachyury is an ideal target for an active immunotherapy, such as a poxvirus-based cancer vaccine. First, Brachyury overexpression drives the acquisition of an aggressive, metastatic phenotype by epithelial tumor cells, which exhibit a mesenchymal-like, migratory and invasive phenotype *in vitro*. Second, in xenograft models *in vivo*, the reduction of Brachyury expression in human tumor cells correlated with a decreased ability to disseminate from the primary to the metastatic site and a reduced ability to form experimental lung metastases. Third, Brachyury expression in tumor cells is also associated with enhanced resistance to cell death induced by various genotoxic agents, such as radiation and chemotherapy, including the commonly-used chemotherapeutics Taxotere, Cisplatin, Vinorelbine, and combinations of Cisplatin and Vinorelbine. Therefore, an active immunotherapy directed to Brachyury (*i.e.*, a recombinant poxvirus-based cancer vaccine expressing Brachyury) would provide a specific way of targeting and eliminating Brachyury-expressing tumor cells and cells with the potential to express Brachyury, including both

metastatic tumor cells that have already converted into a mesenchymal-like phenotype and tumor cells resistant to commonly-used first line cancer treatments including various chemotherapies and radiation, without impacting healthy, non-cancerous tissues. Consequently, Brachyury is an attractive candidate for a tumor-specific antigen for use with recombinant poxvirus vaccine therapy aimed at preventing tumor formation and/or interfering with tumor progression and metastasis for a wide range of human cancers.

[0062] Previous work with Brachyury used an *in silico* approach to identify putative peptides able bind the major histocompatibility complex ("MHC") on APCs and to expand CD4+ and CD8+ T-cells. *See, e.g.*, Palena et al. (2007). Yet in spite of the computer models, only one of the four putative MHC-binding peptides so identified was able successfully to bind APCs and to expand CD4+ and CD8+ T-cells.

[0063] Although the ability of antigen-presenting cells expressing human Brachyury to expand pre-existing populations of CD4+ and CD8+ T-cells has been tested *in vitro*, a human Brachyury-specific cancer vaccine has not yet been tested and shown to have anti-tumor efficacy in a well-accepted vertebrate model system. Given the complexity of the vertebrate immune system, however, it could not be predicted whether a particular tumor-associated antigen (*e.g.*, Brachyury) could be processed and presented by antigen-presenting cells ("APCs") at levels sufficient to expand antigen-specific CD4+ and CD8+ T-cells, and whether the antigen was sufficiently immunogenic to generate Brachyury-specific antibody responses. *See, e.g.*, Palena et al., (2007) and Example 4.

[0064] Until the work presented herein, the full-length human Brachyury protein had never been cloned into a vaccinia virus-derived vector, expressed in human APCs, and shown to be properly processed and presented such that it can expand CD4+ and CD8+ T-cells, all of which are essential prerequisites for a successful active immunotherapy and cancer vaccine. It had not previously been contemplated to express full-length Brachyury protein from a poxvirus vector backbone because there has been no accepted vertebrate model system to study an active immunotherapy targeting Brachyury. Nor had another protein involved in regulating the EMT been cloned into a vaccinia virus-derived vector and shown to have the ability to reduce both tumor volume and the number of metastatic lesions in lung in a well-established vertebrate tumor model like the mouse 4T1 tumor model, as disclosed herein. The present inventors have now

demonstrated that targeting regulators of the EMT with an active immunotherapy can successfully treat cancer in a vertebrate model system using Twist, a mouse protein which, like human Brachyury, also regulates the EMT.

[0065] Because the mouse homolog of Brachyury is neither highly expressed in normal mouse tissues nor predominantly expressed in mouse tumor tissues, the efficacy of Brachyury as a target for an active immunotherapy cannot be studied effectively in a mouse model system. *See, e.g.,* Figure 5. Fortunately, however, like Brachyury, the mouse homolog of the EMT regulator Twist both promotes the EMT during development by down-regulating E-cadherin-mediated cell-cell adhesion and up-regulating mesenchymal markers and is predominantly expressed in mouse tumor tissue. *See, e.g.,* Figure 5. Therefore, the study of a Twist-specific cancer vaccine in mice is very likely to have strong predictive value regarding the efficacy of a Brachyury-specific cancer vaccine in humans. *See, e.g.,* Example 8.

[0066] Accordingly, provided herein are compositions comprising poxvirus vectors comprising nucleic acids encoding Brachyury, and such compositions further comprising nucleic acids encoding one or more immunostimulatory molecules. Also provided herein are methods and uses of such compositions for eliciting an immune response against Brachyury, for the treatment of cancer, and for the treatment of chemotherapy- and radiation-resistant cancer.

Definitions

[0067] Unless otherwise noted, technical terms herein are used according to conventional usage by one of ordinary skill in the art of molecular biology. For common terms in molecular biology, conventional usage may be found in standard textbooks such as, for example, *Genes V* by Benjamin Lewin, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.); *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and *Molecular Biology and Biotechnology: a Comprehensive Desk Reference* edited by Robert A. Meyers, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

[0068] As used herein, the singular forms “a”, “an”, and “the” include plural references unless the context clearly indicates otherwise. Thus, for example, reference to “an epitope” includes reference to one or more epitopes and reference to “the method” includes reference to

equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

[0069] Unless otherwise indicated, the term “at least” preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are encompassed by the present invention.

[0070] Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising” mean “includes”, and therefore include a stated integer or step or group of integers or steps and do exclude any other integer or step or group of integers or steps. When used herein the term “comprising” can be substituted with the term “containing”, “including” or “having”. Any of the aforementioned terms (comprising, containing, including, having), whenever used herein in the context of an aspect or embodiment of the present invention can be substituted with the term “consisting of”.

[0071] When used herein, the term “consisting of” excludes any element, step, or ingredient not specified in the claim. When used herein, “consisting essentially of” excludes any materials or steps “which would affect the basic and novel characteristics” of the product or method defined in the rest of the claim. *Water Techs. Corp. v. Calco Ltd.*, 7 U.S.P.Q.2d 1097, 1102 (Fed. Cir. 1988).

[0072] As used herein, the conjunctive “and/or” between multiple recited elements is understood as encompassing both individual and combined options. For instance, where two elements are conjoined by “and/or”, a first option refers to the applicability of the first element without the second. A second option refers to the applicability of the second element without the first. A third option refers to the applicability of the first and second elements together. Any one of these options is understood to fall within the meaning, and therefore to satisfy the requirement of the term “and/or” as used herein. Concurrent applicability of more than one of the options is also understood to fall within the meaning, and therefore satisfy the requirement of the term “and/or.”

[0073] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including all definitions, will control.

[0074] **Adjuvant:** A vehicle used to enhance antigenicity. Adjuvants can include: (1) suspensions of minerals (alum, aluminum hydroxide, or phosphate) on which antigen is adsorbed; (2) water-in-oil emulsions in which an antigen solution is emulsified in mineral oil (Freund's incomplete adjuvant), sometimes with the inclusion of killed mycobacteria (Freund's complete adjuvant) to further enhance antigenicity by inhibiting degradation of antigen and/or causing an influx of macrophages; (3) immunostimulatory oligonucleotides such as, for example, those including a CpG motif can also be used as adjuvants (for example see U.S. Patent No. 6,194,388; and U.S. Patent No. 6,207,646); and (4) purified or recombinant proteins such as costimulatory molecules. Exemplary adjuvants include, but are not limited to, B7-1, ICAM-1, LFA-3, and GM-CSF.

[0075] **Antigen; antigenic determinant; epitope:** A compound, composition, or substance that can stimulate the production of antibodies or a CD4⁺ or CD8⁺ T-cell response in an animal, including compositions that are injected or absorbed into an animal. An antigen reacts with the immune system to produce an antigen-specific humoral or cellular immune response. The term "antigen" includes all related epitopes of a particular compound, composition or substance. The term "epitope" or "antigenic determinant" refers to a site on an antigen to which B- and/or T-cells respond, either alone or in conjunction with another protein such as, for example, a major histocompatibility complex ("MHC") protein or a T-cell receptor. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by secondary and/or tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents, while epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 5, 6, 7, 8, 9, 10 or more amino acids—but generally less than 20 amino acids—in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. *See, e.g., "Epitope Mapping Protocols" in Methods in Molecular Biology, Vol. 66, Glenn E. Morris, Ed (1996).*

[0076] An antigen can be a tissue-specific antigen or a disease-specific antigen. Those terms are not mutually exclusive, because a tissue-specific antigen can also be a disease-specific antigen. A tissue-specific antigen is expressed in a limited number of tissues. Tissue-specific antigens include, for example, prostate-specific antigen (“PSA”). A disease-specific antigen is expressed coincidentally with a disease process, where antigen expression correlates with or is predictive of development of a particular disease. Disease-specific antigens include, for example, HER-2, which is associated with certain types of breast cancer, or PSA, which is associated with prostate cancer. A disease-specific antigen can be an antigen recognized by T-cells or B-cells.

[0077] **Brachyury:** The Brachyury gene encodes the founding member of the T-box family of transcription factors, characterized by a conserved DNA-binding domain (Papaioannou and Silver, *Bioessays* 20(1):9-19, 1998) that has an essential role in the formation and organization of mesoderm in vertebrates. Exemplary human brachyury amino acid and nucleic acid sequences are set forth in GENBANK® Accession No. NP_003172 (SEQ ID NO:1) and GENBANK® Accession No. NM_003181 (SEQ ID NO:2), as available on August 2, 2012, and incorporated herein by reference.

[0078] **Cancer; Neoplasm; Tumor:** A malignant growth arising from a particular body tissue that has undergone characteristic loss of structural differentiation, generally accompanied by increased capacity for cell division, invasion of surrounding tissue, and the capacity for metastasis. Tumors may be benign or malignant. For example, prostate cancer is a malignant neoplasm that arises in or from prostate tissue, ovarian cancer is a malignant neoplasm that arises in or from ovarian tissue, colon cancer is a malignant neoplasm that arises in or from colon tissue, and lung cancer is a malignant neoplasm that arises in or from lung tissue. Residual cancer is cancer that remains in a subject after treatment given to the subject to reduce or eradicate the cancer. Metastatic cancer is a cancer at one or more sites in the body other than the site of origin of the original (primary) cancer from which the metastatic cancer is derived.

[0079] **cDNA (complementary DNA):** A piece of DNA lacking internal non-coding segments (introns) and regulatory sequences that determine the timing and location of transcription initiation and termination. cDNA can be synthesized in the laboratory by reverse transcription of messenger RNA (“mRNA”) extracted from cells.

[0080] Chemotherapy; chemotherapeutic agents: Any therapeutically useful chemical agent, including radioactive substances, for the treatment of diseases characterized by abnormal cell growth. Such diseases include tumors, neoplasms and cancer as well as diseases characterized by hyperplastic growth such as psoriasis. One of skill in the art can readily identify a chemotherapeutic agent of use (e.g. see Slapak and Kufe, *Principles of Cancer Therapy*, Chapter 86 in Harrison's Principles of Internal Medicine, 14th edition; Perry et al., *Chemotherapy*, Ch. 17 in Abeloff, Clinical Oncology 2nd ed., © 2000 Churchill Livingstone, Inc; Baltzer L, Berkery R (eds): Oncology Pocket Guide to Chemotherapy, 2nd ed. St. Louis, Mosby-Year Book, 1995; Fischer DS, Knobf MF, Durivage HJ (eds): The Cancer Chemotherapy Handbook, 4th ed. St. Louis, Mosby-Year Book, 1993). Any of the poxviral vectors encoding the Brachyury protein provided herein can be used in combination with one or more chemotherapeutic agents.

[0081] Conservative variant: A “conservative” variant is a variant protein or polypeptide having one or more amino acid substitutions that do not substantially affect or decrease an activity or antigenicity of the protein or an antigenic epitope thereof. Generally conservative substitutions are those in which a particular amino acid is substituted with another amino acid having the same or similar chemical characteristics. For example, replacing a basic amino acid such as lysine with another basic amino acid such as arginine or glutamine is a conservative substitution. The term conservative variant also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid, provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide, and/or that the substituted polypeptide retains the function of the unsubstituted polypeptide. Non-conservative substitutions are those that replace a particular amino acid with one having different chemical characteristics, and typically reduce an activity or antigenicity of the protein or an antigenic epitope thereof.

[0082] Specific, non-limiting examples of conservative substitutions include the following examples:

Original Residue	Conservative Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu

Cys	Ser
Gln	Asn
Glu	Asp
His	Asn; Gln
Ile	Leu, Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

[0083] CD4: Cluster of differentiation factor 4, a T-cell surface protein that mediates interaction with the MHC Class II molecule. Cells that express CD4, referred to as “CD4+” cells, are often helper T (“T_H”) cells.

[0084] CD8: Cluster of differentiation factor 8, a T-cell surface protein that mediates interaction with the MHC Class I molecule. Cells that express CD8, referred to as “CD8+” cells, are often cytotoxic T (“CTL”) cells.

[0085] Chemotherapy; chemotherapeutic agents: Any therapeutically useful chemical agent for the treatment of diseases characterized by abnormal cell growth, including tumors, neoplasms and cancer. Commonly used classes of chemotherapeutics include alkylating agents, antimetabolites, natural products, or hormones and their antagonists. Alkylating agents include nitrogen mustards, alkyl sulfonates, and nitrosoureas. Antimetabolites include folic acid analogs, pyrimidine analogs, and purine analogs. Natural products include vinca alkaloids, epipodophyllotoxins, antibiotics, and enzymes. Miscellaneous agents include platinum coordination complexes, substituted ureas, methyl hydrazine derivatives, and adrenocortical suppressants. Hormones and hormone antagonists include adrenocorticosteroids, progestins, estrogens, antiestrogens, and androgens.

[0086] Costimulatory molecule: T-cell activation typically requires binding of the T-cell receptor (“TCR”) with a peptide-MHC complex as well as a second signal delivered via the interaction of a costimulatory molecule with its ligand. Costimulatory molecules are molecules that, when bound to their ligand, deliver the second signal required for T-cell activation. The

most well-known costimulatory molecule on the T-cell is CD28, which binds to either B7-1 or B7-2. Other costimulatory molecules that can also provide the second signal necessary for activation of T-cells include intracellular adhesion molecule-1 (“ICAM-1”), intracellular adhesion molecule-2 (“ICAM-2”), leukocyte function associated antigen-1 (“LFA-1”), leukocyte function associated antigen-2 (“LFA-2”), and leukocyte function associated antigen-3 (“LFA-3”).

[0087] Degenerate variant: A polynucleotide encoding a protein or fragment thereof that includes a sequence that contains codons that differ from the native or wild-type gene sequence but still specify the same amino acid. The genetic code includes 20 natural amino acids, most of which are specified by more than one codon. All degenerate nucleotide sequences are encompassed in this disclosure provided the amino acid sequence of the Brachyury protein encoded by the degenerate polynucleotide remains unchanged.

[0088] Dendritic cell (DC): Dendritic cells are the primary antigen presenting cells (“APCs”) involved in primary immune responses. Dendritic cells include plasmacytoid dendritic cells and myeloid dendritic cells. Their major function is to obtain antigen in tissues, migrate to lymphoid organs and present the antigen in order to activate T-cells. Immature dendritic cells originate in the bone marrow and reside in the periphery as immature cells.

[0089] Epithelial-to-Mesenchymal Transition: An “epithelial-to-mesenchymal” transition is a biological process in which a population of cells converts from a relatively highly-differentiated epithelial phenotype to a relatively less-differentiated mesenchymal phenotype.

[0090] Expression Control Sequences: Nucleic acid sequences that regulate the expression of a heterologous nucleic acid sequence to which they are operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and/or translation of the nucleic acid sequence. Thus, the term “expression control sequences” encompasses promoters, enhancers, transcription terminators, start codons, splicing signals for introns, and stop codons. The term “control sequences” includes, at a minimum, components the presence of which can influence transcription and/or translation of the heterologous nucleic acid sequence and can also include additional components whose presence is advantageous such as, for example, leader sequences and fusion partner sequences.

[0091] The term “**expression control sequences**” encompasses promoter sequences. A promoter is a minimal sequence sufficient to direct transcription of a homologous or heterologous gene. Also included are those promoter elements sufficient to render promoter-dependent gene expression cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the gene. The term “promoter” encompasses both constitutive and inducible promoters. *See, e.g., Bitter et al., Methods in Enzymology* 153:516-544 (1987). Exemplary promoter sequences include, but are not limited to, the retrovirus long terminal repeat (“LTR”), the adenovirus major late promoter, the vaccinia virus 7.5K promoter (“Pr7.5”), the PrSynIIIm promoter, the PrLE1 promoter, or the PrS promoter.

[0092] **Heterologous:** Originating from separate genetic sources or species. A polypeptide that is heterologous to human Brachyury originated from a nucleic acid that does not encode human Brachyury such as, for example, mouse Brachyury, β -galactosidase, maltose binding protein, or human serum albumin.

[0093] **Host cells:** Cells in which a vector can be propagated and its DNA expressed. The cells may be prokaryotic (*e.g.*, bacterial) or eukaryotic (*e.g.*, mammalian or human). The term also encompasses progeny of the original host cell, even though all progeny may not be identical to the parental cell since there may be mutations that occur during replication.

[0094] **Immune response:** An adaptive response of an immune system cell, such as a B-cell, T-cell, or monocyte, to a stimulus. An adaptive response is a response to a particular antigen, and is thus described as “antigen-specific”. An adaptive immune response can include the production of antibodies to a particular antigen by a B-cell, T-cell help by a CD4+ helper T-cell expanding a population of antigen-specific CD8+ T-cells (“CTLs”), cytotoxic activity of CD8+ T-cells directed against cells expressing a particular antigen, or yet another type of antigen-specific immune response.

[0095] **Immunogenic composition; immunogenic Brachyury composition:** As used herein, the term “immunogenic composition” or “immunogenic Brachyury composition” refers to a composition comprising a nucleic acid encoding the Brachyury protein under the control of an expression control sequence or promoter, such as a poxvirus vector, that induces a measurable Brachyury-specific, adaptive immune response. The nucleic acid or poxvirus vector may

optionally include additional nucleic acids encoding, for example, one or more costimulatory molecules as described elsewhere herein. That immune response may be, for example, a CD8+ T-cell or CTL response directed against cells expressing Brachyury protein, or a B-cell response producing Brachyury-specific antibodies. Such compositions may include the isolated nucleic acid or vector, optionally formulated with one or more pharmaceutically acceptable carriers.

[0096] **Lymphocytes:** A type of white blood cell that is involved in the immune defenses of the body. There are two main types of lymphocytes: B-cells and T-cells.

[0097] **Major Histocompatibility Complex (MHC):** A generic designation meant to encompass the histocompatibility antigen systems described in different species, including the human leukocyte antigens ("HLA").

[0098] **Mammal:** This term includes both human and non-human mammals. Similarly, the term "subject" includes both human and veterinary subjects.

[0099] **Open reading frame (ORF):** A series of nucleotide codons specifying a series of amino acids without any internal termination codons that capable of being translated to produce a polypeptide.

[0100] **Operably linked:** A first nucleic acid sequence is operably linked to a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For example, a promoter is operably linked to a coding sequence if the promoter is placed in a position where it can direct transcription of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

[0101] **Pharmaceutically acceptable carriers:** *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations using conventional pharmaceutically acceptable carriers suitable for administration of the vectors and compositions disclosed herein. Generally the nature of the carrier used depends on the particular mode of administration being employed. For example, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like, as a vehicle. For solid compositions (such as powders, pills, tablets, or capsules), conventional non-toxic solid carriers include, for example,

pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. Pharmaceutical compositions can also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, preservatives, pH-buffering agents and the like such as, for example, sodium acetate or sorbitan monolaurate.

[0102] Polynucleotide; nucleic acid: The term polynucleotide refers to a nucleic acid polymer at least 300 bases long composed of ribonucleotides (*i.e.*, RNA) or deoxyribonucleotides (*i.e.*, DNA or cDNA) and capable of encoding a polypeptide or protein. The term includes single- and double-stranded forms of DNA.

[0103] Polypeptide or Protein: The term polypeptide or protein refers to an polymer at least 100 amino acids long, generally greater than 50 amino acids in length.

[0104] Poxvirus: The term poxvirus refers to any of the genera of poxviruses capable of infecting humans (*e.g.*, orthopox viruses, avipox viruses, parapox viruses, yatapox viruses, and molluscipox viruses) whether productively or not, but preferably the orthopox and/or avipox viruses. **Orthopox viruses** include smallpox virus (also known as variola virus), vaccinia virus, cowpox virus, and monkeypox virus. **Avipox viruses** include canarypox virus, fowlpox virus, pigeonpox virus, mynahpox virus, uncopox virus, pigeonpox virus, quailpox virus, peacockpox virus, penguinpox virus, sparrowpox virus, starlingpox virus, and turkeypox virus. The term “**vaccinia virus**” refers to both the wild-type vaccinia virus and any of the various attenuated strains or isolates subsequently isolated including, for example, vaccinia virus-Western Reserve, vaccinia virus-Copenhagen, Dryvax (also known as vaccinia virus-Wyeth), ACAM2000, modified vaccinia virus Ankara (“MVA”), and modified vaccinia virus Ankara-Bavarian Nordic (“MVA-BN”).

[0105] Prime-boost vaccination: The term “prime-boost vaccination” refers to a vaccination strategy using a first, priming injection of a vaccine targeting a specific antigen followed at intervals by one or more boosting injections of the same vaccine. Prime-boost vaccination may be homologous or heterologous. A homologous prime-boost vaccination uses a vaccine comprising the same immunogen and vector for both the priming injection and the one or more boosting injections. A heterologous prime-boost vaccination uses a vaccine comprising the same immunogen for both the priming injection and the one or more boosting injections but different vectors for the priming injection and the one or more boosting injections. For example,

a homologous prime-boost vaccination may use an MVA vector comprising nucleic acids expressing Brachyury and TRICOM for both the priming injection and the one or more boosting injections. In contrast, a heterologous prime-boost vaccination may use an MVA vector comprising nucleic acids expressing Brachyury and TRICOM for the priming injection and a fowlpox vector comprising nucleic acids expressing Brachyury and TRICOM for the one or more boosting injections. Heterologous prime-boost vaccination also encompasses various combinations such as, for example, use of a plasmid encoding an immunogen in the priming injection and use of a poxvirus vector encoding the same immunogen in the one or more boosting injections, or use of a recombinant protein immunogen in the priming injection and use of a plasmid or poxvirus vector encoding the same protein immunogen in the one or more boosting injections.

[0106] Recombinant; recombinant nucleic acid; recombinant vector; recombinant poxvirus: The term “recombinant” when applied to a nucleic acid, vector, poxvirus and the like refers to a nucleic acid, vector, or poxvirus made by an artificial combination of two or more otherwise heterologous segments of nucleic acid sequence, or to a nucleic acid, vector or poxvirus comprising such an artificial combination of two or more otherwise heterologous segments of nucleic acid sequence. The artificial combination is most commonly accomplished the artificial manipulation of isolated segments of nucleic acids, using well-established genetic engineering techniques.

[0107] Resistant: The term “resistant” used in relation to chemotherapy or radiation (*i.e.*, “chemotherapy-resistant” or “radiation-resistant” tumor) refers to a tumor that is less susceptible to treatment with a chemotherapeutic agent or radiation than a non-resistant cancer or tumor. That is, a chemotherapy-resistant tumor is not affected by a dose of chemotherapy (*i.e.*, not reduced in size or eliminated) to the same degree by a dose of chemotherapy as a non-resistant tumor receiving an identical dose of chemotherapy would be. Similarly, a radiation-resistant tumor is not affected by a dose of radiation (*i.e.*, not reduced in size or eliminated) to the same degree by a dose of radiation as a non-resistant tumor receiving an identical dose of radiation would be.

[0108] Sequence identity: The term “sequence identity” refers to the degree of similarity between the nucleic acid or amino acid sequences. Sequence identity is frequently measured in

terms of percent identity (often described as sequence “similarity” or “homology”). The higher the percent sequence identity, the more similar the two sequences are. Homologs or variants of a Brachyury protein will have a relatively high degree of sequence identity when aligned using standard methods.

[0109] Methods of aligning sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith and Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman and Wunsch, *J. Mol. Biol.* 48:443, 1970; Higgins and Sharp, *Gene* 73:237, 1988; Higgins and Sharp, *CABIOS* 5:151, 1989; Corpet et al., *Nucl. Acids Res.* 16:10881, 1988; and Pearson and Lipman, *Proc. Nat’l Acad. Sci. USA* 85:2444, 1988. In addition, Altschul et al., *Nature Genet.* 6:119, 1994, presents a detailed consideration of sequence alignment methods and homology calculations. The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., *J. Mol. Biol.* 215:403, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD; *see also* <http://blast.ncbi.nlm.nih.gov/Blast.cgi>), for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx.

[0110] Homologs and variants of a human Brachyury protein typically have at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity over a full-length alignment with the amino acid sequence of wild-type human Brachyury prepared with NCBI Blast v2.0, using blastp set to the default parameters. For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to the default parameters (gap existence cost of 11, and a per residue gap cost of 1).

[0111] Subject: Living multi-cellular vertebrate organisms, including, for example, humans, non-human mammals and birds. The term “subject” may be used interchangeably with the term “animal” herein.

[0112] T-Cell: A lymphocyte or white blood cell essential to the adaptive immune response. T-cells include, but are not limited to, CD4⁺ T-cells and CD8⁺ T-cells. A CD4⁺ T-cell is an immune cell that carries a marker on its surface known as “cluster of differentiation 4” (“CD4”). These cells, also known as helper T-cells, help orchestrate the immune response, including both

antibody and CTL responses. CD8⁺ T-cells carry the “cluster of differentiation 8” (“CD8”) marker. CD8⁺ T-cells include both CTLs and suppressor T-cells.

[0113] Therapeutically active polypeptide: An agent composed of amino acids, such as a Brachyury protein, that induces an adaptive immune response, as measured by clinical response (*e.g.*, an increase in CD4⁺ T-cells, CD8⁺ T-cells, or B-cells, an increase in Brachyury-specific cytolytic activity, a measurable reduction in tumor size, or a reduction in number of metastases). Therapeutically active molecules can also be made from nucleic acids such as, for example, a poxvirus vector comprising a nucleic acid encoding human Brachyury operably linked to an expression control sequence.

[0114] Therapeutically effective amount: A “therapeutically effective amount” is a quantity of a composition or a cell sufficient to achieve a desired therapeutic or clinical effect in a subject being treated. For example, a therapeutically effective amount of a poxviral vector comprising a nucleic acid encoding human Brachyury protein operably linked to an expression control sequence would be an amount sufficient to elicit a Brachyury-specific immune response, to reduce tumor size or burden, to reduce the number of tumor metastases, to delay progression of a cancer, or to increase overall survival of a patient or population of patients having cancer. A therapeutically effective amount of the poxvirus vectors and compositions comprising the poxvirus vectors described herein is an amount sufficient to raise an immune response to Brachyury-expressing cells and cells with the potential to express Brachyury. The immune response must be of sufficient magnitude to slow the proliferation of Brachyury-expressing cells and cells with the potential to express Brachyury, to inhibit their growth, to reduce a sign or a symptom of the tumor, to provide subjective relief of one or more symptoms associated with the tumor or to provide objectively identifiable improvement in one or more symptoms noted by the attending clinician such as, for example, a reduction in tumor size, a decrease in the number of metastatic lesions, a delay in disease progression, or an increase in overall survival, and the like.

[0115] Transduced or Transformed: The term “transduced” or “transformed” refers to a cell into which a recombinant nucleic acid has been introduced by standard molecular biological methods. As used herein, the term transduction encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including infection with viral vectors,

transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, or particle gun acceleration.

[0116] Treating cancer: The term “treating cancer” refers to a therapeutic intervention intended to reduce or eliminate a sign or symptom of the cancer or to delay progression of the disease and increase overall survival of a subject having cancer. Cancer may be treated by standard chemotherapy, radiation, or active immunotherapy such as, for example, administration of a recombinant vaccinia virus comprising a nucleic acid encoding a Brachyury protein. Reducing or eliminating a sign or symptom of the cancer encompasses a wide variety of effects including, for example reducing signs or symptoms of a tumor, reducing tumor volume, reducing the number of metastases, increasing response duration, increasing time to progression, increasing disease-free survival, increasing progression-free survival, or increasing the overall survival of patients having the disease.

[0117] TRICOM: A Triad of Costimulatory Molecules consisting of B7-1 (also known as CD80), intracellular adhesion molecule-1 (ICAM-1, also known as CD54) and lymphocyte function-associated antigen-3 (LFA-3, also known as CD58), commonly included in recombinant viral vectors (*e.g.*, poxviral vectors) expressing a specific antigen in order to increase the antigen-specific immune response. The individual components of TRICOM can be under the control of the same or different promoters, and can be provided on the same vector with the specific antigen or on a separate vector. Exemplary vectors are disclosed, for example, in Hodge et al., “A Triad of Costimulatory Molecules Synergize to Amplify T-Cell Activation,” *Cancer Res.* 59:5800-5807 (1999) and U.S. Patent No. 7,211,432 B2, both of which are incorporated herein by reference.

[0118] Twist: The Twist gene encodes the protein Twist1 (“Twist”), a basic helix-loop-helix (“bHLH”) transcription factor, which regulates cell movement and tissue reorganization during early embryogenesis by promoting the EMT. *See, e.g.*, Yang et al., “Twist, a Master Regulator of Morphogenesis, Plays an Essential Role in Tumor Metastasis,” *Cell* 117:927-939 (2004). In vertebrates, *Twist* is mainly expressed in neural crest cells and plays a role in proper migration and differentiation of neural crest and head mesenchymal cells. Yang et al., (2006). Exemplary mouse Twist amino acid and nucleic acid sequences are set forth in GENBANK®

Accession No. AAH83139.1 and GENBANK® Accession No. NM_011658.2, as available on August 2, 2012, and incorporated herein by reference.

[0119] Vector: A nucleic acid molecule introduced into a host cell, thereby producing a transduced or transformed host cell. Vectors generally include nucleic acid sequences enabling them to replicate in a host cell, such as an origin of replication, as well as one or more selectable marker genes, expression control sequences, restriction endonuclease recognition sequences, primer sequences and a variety of other genetic elements known in the art. Commonly used vector types include plasmids for expression in bacteria (*e.g.*, *E. coli*) or yeast (*e.g.*, *S. cerevisiae*), shuttle vectors for constructing recombinant poxviruses, cosmids, bacterial artificial chromosomes, yeast artificial chromosomes, and viral vectors. Viral vectors include poxvirus vectors, retrovirus vectors, adenovirus vectors, herpes virus vectors, baculovirus vectors, Sindbis virus vectors, and poliovirus vectors, among others. Poxvirus vectors include, but are not limited to orthopox viruses, avipox viruses, parapox viruses, yatapox viruses, and molluscipox viruses, but preferably the orthopox and/or avipox viruses. **Orthopox viruses** include smallpox virus (also known as variola virus), vaccinia virus, cowpox virus, and monkeypox virus. **Avipox viruses** include canarypox virus, fowlpox virus, canarypox virus, fowlpox virus, pigeonpox virus, mynahpox virus, uncopox virus, pigeonpox virus, quailpox virus, peacockpox virus, penguinpox virus, sparrowpox virus, starlingpox virus, and turkeypox virus. The term “**vaccinia virus**” refers to both the wild-type vaccinia virus and any of the various attenuated strains or isolates subsequently isolated including, for example, vaccinia virus-Western Reserve, vaccinia virus-Copenhagen, Dryvax (also known as vaccinia virus-Wyeth), ACAM2000, modified vaccinia virus Ankara (“MVA”), and modified vaccinia virus Ankara-Bavarian Nordic (“MVA-BN”).

Tumor Antigens and Costimulatory Molecules

[0120] In one aspect, provided herein are nucleic acids encoding a cell-associated polypeptide antigen. In certain embodiments, the cell-associated polypeptide antigen is a self-protein antigen related to a pathological process other than a tumor-associated antigen, or a viral antigen, or an antigen derived from an intracellular parasite or bacterium.

[0121] In certain embodiments, the cell-associated polypeptide antigen is a tumor antigen. Many tumor-associated antigens are known in the art. Exemplary tumor-associated antigens

include, but are not limited to, 5- α -reductase, α -fetoprotein ("AFP"), AM-1, APC, April, B melanoma antigen gene ("BAGE"), β -catenin, Bcl12, bcr-abl, Brachyury, CA-125, caspase-8 ("CASP-8", also known as "FLICE"), Cathepsins, CD19, CD20, CD21/complement receptor 2 ("CR2"), CD22/BL-CAM, CD23/Fc ϵ RII, CD33, CD35/complement receptor 1 ("CR1"), CD44/PGP-1, CD45/leucocyte common antigen ("LCA"), CD46/membrane cofactor protein ("MCP"), CD52/CAMPATH-1, CD55/decay accelerating factor ("DAF"), CD59/protectin, CDC27, CDK4, carcinoembryonic antigen ("CEA"), c-myc, cyclooxygenase-2 ("cox-2"), deleted in colorectal cancer gene ("DCC"), DcR3, E6/E7, CGFR, EMBP, Dna78, farnesyl transferase, fibroblast growth factor-8a ("FGF8a"), fibroblast growth factor-8b ("FGF8b"), FLK-1/KDR, folic acid receptor, G250, G melanoma antigen gene family ("GAGE-family"), gastrin 17, gastrin-releasing hormone, ganglioside 2 ("GD2")/ganglioside 3 ("GD3")/ganglioside-monosialic acid-2 ("GM2"), gonadotropin releasing hormone ("GnRH"), UDP-GlcNAc:R₁Man(α 1-6)R₂ [GlcNAc to Man(α 1-6)] β 1,6-N-acetylglucosaminyltransferase V ("GnT V"), GP1, gp100/Pme117, gp-100-in4, gp15, gp75/tyrosine-related protein-1 ("gp75/TRP-1"), human chorionic gonadotropin ("hCG"), heparanase, Her2/neu, human mammary tumor virus ("HMTV"), 70 kiloDalton heat-shock protein ("HSP70"), human telomerase reverse transcriptase ("hTERT"), insulin-like growth factor receptor-1 ("IGFR-1"), interleukin-13 receptor ("IL-13R"), inducible nitric oxide synthase ("iNOS"), Ki67, KIAA0205, K-ras, H-ras, N-ras, KSA, LKLR-FUT, melanoma antigen-encoding family ("MAGE-family", including at least MAGE-1, MAGE-2, MAGE-3, and MAGE-4), mammaglobin, MAP17, Melan-A/melanoma antigen recognized by T-cells-1 ("MART-1"), mesothelin, MIC A/B, MT-MMPs, mucin, testes-specific antigen NY-ESO-1, osteonectin, p15, P170/MDR1, p53, p97/melanotransferrin, PAI-1, platelet-derived growth factor ("PDGF"), μ PA, PRAME, probasin, progenipoiectin, prostate-specific antigen ("PSA"), prostate-specific membrane antigen ("PSMA"), RAGE-1, Rb, RCAS1, SART-1, SSX-family, STAT3, STn, TAG-72, transforming growth factor-alpha ("TGF- α "), transforming growth factor-beta ("TGF- β "), Thymosin-beta-15, tumor necrosis factor-alpha ("TNF- α "), TP1, TRP-2, tyrosinase, vascular endothelial growth factor ("VEGF"), ZAG, p16INK4, and glutathione-S-transferase ("GST").

[0122] One exemplary tumor-associated antigen is Brachyury. Brachyury is a T-box transcription factor which regulates cell movement and tissue reorganization during early

embryogenesis by promoting the EMT. Palena et al., (2007). Brachyury is involved in the formation and organization of mesoderm in during vertebrate embryogenesis, a process which requires epithelial cells to change into mesenchymal cells, a change accomplished by triggering an EMT. Palena et al., (2007).

[0123] In humans, Brachyury is predominantly expressed in multiple carcinoma types, including lung, breast, ovary, colon, prostate, small intestine, stomach, kidney, bladder, uterus, and testis, in cell lines derived from lung, colon, and prostate carcinomas, as well as in chronic lymphocytic leukemia and EBV-positive tumor cells. *See, e.g.*, Figures 1-2. Tumor specificity for human Brachyury has been confirmed by demonstrating the absence of Brachyury expression in most normal human adult tissues that have been tested, with the exception of testes. *See, e.g.*, Figures 1-2. Comparison of expression of Brachyury and other transcription factors that regulate the EMT in humans demonstrates the high tumor specificity of Brachyury expression. For example, unlike Brachyury, the expression of two other EMT regulators, Twist and Snail, is high in most normal human tissues tested. *See, e.g.*, Figures 3-4.

[0124] In certain embodiments, the tumor-associated antigen is Brachyury. In certain embodiments, the Brachyury is human Brachyury. In certain embodiments, the human Brachyury comprises the amino acid sequence of SEQ ID NO:1 encoded by the nucleic acid sequence of SEQ ID NO:2. In certain embodiments, the human Brachyury comprises an amino acid sequence having 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity to the amino acid sequence of SEQ ID NO:1. Positions 41 to 223 of the amino acid sequence set forth as SEQ ID NO:1 span the T-box DNA binding domain of human Brachyury. The T-box DNA binding domain in Brachyury sequences from different organisms can readily be identified by comparison to that segment of SEQ ID NO:1. In certain embodiments, the human Brachyury protein comprises the T-box DNA binding domain of SEQ ID NO:1.

[0125] In certain embodiments, the tumor-associated antigen is mouse Brachyury. In certain embodiments, the mouse Brachyury comprises the amino acid sequence of SEQ ID NO:3 encoded by the nucleic acid sequence of SEQ ID NO:4. In certain embodiments, the mouse Brachyury comprises an amino acid sequence having 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity to the amino acid sequence of SEQ ID NO:3.

Positions 41 to 223 of the amino acid sequence set forth as SEQ ID NO:3 span the T-box DNA binding domain of mouse Brachyury. The T-box DNA binding domain in Brachyury sequences from different organisms can readily be identified by comparison to that segment of SEQ ID NO:3. In certain embodiments, the mouse Brachyury protein comprises the T-box DNA binding domain of SEQ ID NO:3.

[0126] Human Brachyury shares significant nucleotide and amino acid sequence identity with Brachyury from other vertebrate species including, for example, mouse. Mouse Brachyury is approximately 85% identical to human Brachyury at the nucleotide level, and approximately 91% identical to human Brachyury at the amino acid level; furthermore, mouse and human Brachyury differ by two amino acids in the T-box region. In addition, human Brachyury shares 99.5% amino acid sequence identity with Brachyury from *Pan troglodytes*, 90.1% amino acid sequence identity with Brachyury from *Canis lupus familiaris*, 88.5% amino acid sequence identity with Brachyury from *Bos taurus*, 92.2% amino acid sequence identity with Brachyury from *Rattus norvegicus*, and 80.9% amino acid sequence identity to Brachyury from *Gallus gallus*. Therefore nucleic acids encoding those Brachyury proteins can also be used with the poxvirus vectors, compositions and methods disclosed herein, provided those sequences elicit an effective immune response against the target antigen (*i.e.*, native Brachyury expressed by a tumor cell).

[0127] Nucleic acids encoding the Brachyury proteins disclosed herein are also provided, including DNA, cDNA, and/or RNA sequences encoding the Brachyury protein of interest. Such nucleic acids can be incorporated in the poxvirus vectors disclosed herein and used with the methods and uses disclosed herein. Because the genetic code is degenerate, meaning that nearly all amino acids are specified by more than one codon, the various Brachyury proteins disclosed herein can be encoded by many different nucleic acid sequences. The isolation of all possible degenerate nucleic acid sequences encoding Brachyury polypeptides having the requisite degree of amino acid sequence identity is easily accomplished by one of ordinary skill in the art of molecular biology. All such nucleic acid molecules are encompassed within the scope of this disclosure.

[0128] Nucleic acids encoding a Brachyury protein can be cloned or amplified by *in vitro* methods, such as the polymerase chain reaction (PCR), the ligase chain reaction (LCR), the

transcription-based amplification system (TAS), the self-sustained sequence replication system (3SR) and the Q β replicase amplification system (QB). For example, a polynucleotide encoding the protein can be isolated by polymerase chain reaction from a cDNA template using primers based on a DNA sequence encoding the wild-type protein. A wide variety of cloning and *in vitro* amplification methodologies are well known to persons skilled in the art. Exemplary PCR methods are described in, for example, U.S. Patent No. 4,683,195; Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.* 51:263, 1987; and Erlich, ed., *PCR Technology* (Stockton Press, NY, 1989). Polynucleotides also can be isolated by screening genomic or cDNA libraries with probes selected from the sequences of the desired polynucleotide under stringent hybridization conditions.

[0129] Nucleic acids encoding a Brachyury protein can be incorporated into a poxviral vector. The nucleotides can be deoxyribonucleotides or modified forms thereof. DNA sequences encoding a Brachyury protein can be expressed from a poxviral vector *in vitro* by DNA transfer into a suitable host cell, including any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication.

Poxvirus Vectors, Expression Control Sequences, Costimulatory Molecules and Host Cells

[0130] In another aspect, provided herein are poxvirus vectors comprising nucleic acids encoding one or more cell-associated polypeptide antigens. In certain embodiments, the cell-associated polypeptide antigen is a self-protein antigen related to a pathological process other than a tumor-associated antigen, or a viral antigen, or an antigen derived from an intracellular parasite or bacterium.

[0131] In certain embodiments, the cell-associated polypeptide antigen is a tumor-associated antigen. In certain embodiments, the tumor antigen is a human Brachyury protein of SEQ ID NO:1 encoded by the nucleic acid of SEQ ID NO:2. In certain embodiments, the tumor antigen is a mouse Brachyury protein of SEQ ID NO:3 encoded by the nucleic acid of SEQ ID NO:4. In certain embodiments, the poxvirus vector comprises a nucleic acid of SEQ ID NO:2 encoding a human Brachyury protein of SEQ ID NO:1. In certain embodiments, the poxvirus vector comprises a nucleic acid of SEQ ID NO:4 encoding a mouse Brachyury protein of SEQ ID NO:3.

[0132] In certain embodiments, the poxvirus vector is selected from the group consisting of chordopox virus vectors and entomopox virus vectors. In certain embodiments, the poxvirus vector is a chordopox virus vector. In certain embodiments, the chordopox virus vector is selected from the group consisting of avipox virus vectors, molluscipox virus vectors, orthopox virus vectors, capripox virus vectors, suipox virus vectors, leporipox virus vectors, yatapox virus vectors, and parapox virus vectors. In certain embodiments, the poxvirus vector is an entomopox virus vector.

[0133] In certain embodiments, the poxvirus vector is an orthopox virus vector. In certain embodiments, the orthopox virus vector is selected from the group consisting of vaccinia virus vector, variola virus vector, monkeypox virus vector, ectromelia virus vector, camelpox virus vector, raccoonpox virus vector, and cowpox virus vector. In certain embodiments, the vaccinia virus vector is selected from the group consisting of vaccinia virus-Western Reserve, vaccinia virus-EM63, vaccinia virus-Lister, vaccinia virus-New York City Board of Health, vaccinia virus-Temple of Heaven, vaccinia virus-Copenhagen, NYVAC, vaccinia virus-Wyeth, ACAM1000, ACAM2000, and modified vaccinia virus Ankara ("MVA"). In certain embodiments, the MVA is selected from the group consisting of MVA-572, deposited at the European Collection of Animal Cell Cultures ("ECACC"), Health Protection Agency, Microbiology Services, Porton Down, Salisbury SP4 0JG, United Kingdom ("UK"), under the deposit number ECACC 94012707 on January 27, 1994, MVA-575, deposited at the ECACC under deposit number ECACC 00120707 on December 7, 2000, MVA-Bavarian Nordic ("MVA-BN"), deposited at the ECACC under deposit number V00080038 on August 30, 2000, and derivatives of MVA-BN. Additional exemplary poxvirus vectors are described in US Patent No. 7,211,432, which is incorporated herein by reference in its entirety.

[0134] In certain embodiments, the poxvirus vector is an avipox virus vector. In certain embodiments, the avipox vector is selected from the group consisting of canarypox virus vector, fowlpox virus vector, pigeonpox virus vector, mynahpox virus vector, uncopox virus vector, pigeonpox virus vector, quailpox virus vector, peacockpox virus vector, penguinpox virus vector, sparrowpox virus vector, starlingpox virus vector, and turkeypox virus vector. In certain embodiments, the avipox virus vector is a canarypox virus vector. In certain embodiments, the avipox virus vector is a fowlpox virus vector.

[0135] The vaccinia virus MVA was generated by 516 serial passages on chicken embryo fibroblasts of the Ankara strain of Vaccinia virus, referred to as chorioallantois virus Ankara ("CVA"; for review see Mayr, A., et al., *Infection* 3, 6-14 (1975)). The genome of the resulting attenuated MVA lacks approximately 31 kilobase pairs of genomic DNA compared to the parental CVA strain and is highly host-cell restricted to avian cells (Meyer, H. et al., *J. Gen. Virol.* 72, 1031-1038 (1991)). It was shown in a variety of animal models that the resulting MVA was significantly avirulent (Mayr, A., & Danner, K., *Dev. Biol. Stand.* 41:225-34 (1978)). This MVA strain has been tested in clinical trials as a vaccine to immunize against smallpox in humans (Mary et al., *Zbl. Bakt. Hyg. I, Abt. Org. B* 167:375-390 (1987); Stickl et al., *Dtsch. Med. Wschr.* 99:2386-2392 (1974)). Those studies involved over 120,000 humans, including high-risk patients, and proved that compared to vaccinia virus-based vaccines, MVA had diminished virulence or infectiousness while still able to induce a good specific immune response. Although MVA-BN is preferred for its better safety profile because it is less replication competent than other MVA strains, all MVAs are suitable for this invention, including MVA-BN and its derivatives.

[0136] Both MVA and MVA-BN are able to efficiently replicate their DNA in mammalian cells even though they are avirulent. This trait is the result of losing two important host range genes among at least 25 additional mutations and deletions that occurred during its passages through chicken embryo fibroblasts (Meyer et al., *Gen. Virol.* 72:1031-1038 (1991); Antoine et al., *Virol.* 244:365-396 (1998)). In contrast to the attenuated Copenhagen strain (NYVAC) and host range restricted avipox (ALVAC), both-early and late transcription in MVA are unimpaired, which allows for continuous gene expression throughout the viral life cycle (Sutter and Moss, *Proc. Nat'l Acad. Sci. USA* 89:10847-10851 (1992)). In addition, MVA can be used in conditions of pre-existing poxvirus immunity (Ramirez et al., *J. Virol.* 74:7651-7655 (2000)).

[0137] Both MVA and MVA-BN lack approximately 15% (31 kb from six regions) of the genome compared with the ancestral chorioallantois vaccinia virus Ankara ("CVA"). The deletions affect a number of virulence and host range genes, as well as the gene for Type A inclusion bodies. MVA-BN can attach to and enter human cells where virally-encoded genes are expressed very efficiently. However, assembly and release of progeny virus does not occur. MVA-BN is strongly adapted to primary chicken embryo fibroblast (CEF) cells and does not

replicate in human cells. In human cells, viral genes are expressed, and no infectious virus is produced. Despite its high attenuation and reduced virulence, in preclinical studies MVA-BN has been shown to elicit both humoral and cellular immune responses to vaccinia and to heterologous gene products encoded by genes cloned into the MVA genome [E. Harrer et al. (2005), *Antivir. Ther.* 10(2):285-300; A. Cosma et al. (2003), *Vaccine* 22(1):21-9; M. Di Nicola et al. (2003), *Hum. Gene Ther.* 14(14):1347-1360; M. Di Nicola et al. (2004), *Clin. Cancer Res.*, 10(16):5381-5390].

[0138] The reproductive replication of a virus is typically expressed by the amplification ratio. The term “amplification ratio” refers to the ratio of virus produced from an infected cell (“output”) to the amount originally used to infect the cells in the first place (“input”). An amplification ratio of “1” defines an amplification status in which the amount of virus produced from infected cells is the same as the amount initially used to infect the cells, meaning the infected cells are permissive for virus infection and reproduction. An amplification ratio of less than 1 means that infected cells produce less virus than the amount used to infect the cells in the first place, and indicates that the virus lacks the capability of reproductive replication, a measure of virus attenuation.

[0139] Thus, the term “not capable of reproductive replication” means that an MVA or MVA derivative has an amplification ratio of less than 1 in one or more human cell lines, such as, for example, the human embryonic kidney 293 cell line (“HEK293”; deposited at ECACC under deposit number ECACC No. 85120602), the human bone osteosarcoma cell line 143B (“143B”; deposited at ECACC under deposit number ECACC No. 91112502), the human cervix adenocarcinoma cell line HeLa (“HeLa”; deposited at the American Type Culture Collection (“ATCC”) under deposit number ATCC No. CCL-2) and the human keratinocyte cell line HaCat (“HaCat”; Boukamp et al., *J. Cell Biol.* 106(3):761-71 (1988)).

[0140] As described in US Patent Number 6,761,893, US Patent No. 6,193,752, and International Application No. PCT/EP01/013628, MVA-BN does not reproductively replicate in the human cell lines HEK293, 143B, HeLa and HaCat. For example, in one exemplary experiment, MVA-BN exhibited an amplification ratio of 0.05 to 0.2 in HEK293 cells, an amplification ratio of 0.0 to 0.6 in 143B cells, an amplification ratio of 0.04 to 0.8 in HeLa cells, and an amplification ratio of 0.02 to 0.8 in HaCat cells. Thus, MVA-BN does not reproductively

replicate in any of the human cell lines HEK293, 143B, HeLa, and HaCat. In contrast, the amplification ratio of MVA-BN is greater than 1 in primary cultures of chicken embryo fibroblast cells ("CEF") and in baby hamster kidney cells ("BHK"; deposited at ATCC under deposit number ATCC No. CRL-1632), also as described in US Patent Number 6,761,893, US Patent No. 6,193,752, and International Application No. PCT/EP01/013628. Therefore MVA-BN can easily be propagated and amplified in CEF primary cultures with an amplification ratio above 500, and in BHK cells with an amplification ratio above 50.

[0141] As noted above, all MVAs are suitable for this invention, including MVA-BN and its derivatives. The term "derivatives" refers to viruses showing essentially the same replication characteristics as the strain deposited with ECACC on August 30, 2000, under deposit number V00080038 but showing differences in one or more parts of its genome. Viruses having the same "replication characteristics" as the deposited virus are viruses that replicate with similar amplification ratios as the deposited strain in CEF cells, BHK cells, in the human cell lines HEK293, 143B, HeLa, and HaCat.

[0142] Nucleic acids encoding a Brachyury protein can be operatively linked to expression control sequences. An expression control sequence operatively linked to a coding sequence is joined such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. The expression control sequences include, but are not limited to, appropriate promoters, enhancers, transcription terminators, a start codon at the beginning a protein-encoding open reading frame, splicing signals for introns, and in-frame stop codons. Suitable promoters include, but are not limited to, the SV40 early promoter, an RSV promoter, the retrovirus LTR, the adenovirus major late promoter, the human CMV immediate early I promoter, and various poxvirus promoters including, but not limited to the following vaccinia virus or MVA-derived promoters: the 30K promoter, the I3 promoter, the PrS promoter, the Pr7.5K, the 40K promoter, the PrSynIIIm promoter, and the PrLE1 promoter; or the following fowlpox-derived promoters: the Pr7.5K promoter, the I3 promoter, the 30K promoter, or the 40K promoter.

[0143] Additional expression control sequences include, but are not limited to, leader sequences, termination codons, polyadenylation signals and any other sequences necessary for the appropriate transcription and subsequent translation of the nucleic acid sequence encoding

the desired recombinant protein (*e.g.*, Brachyury) in the desired host system. The poxvirus vector may also contain additional elements necessary for the transfer and subsequent replication of the expression vector containing the nucleic acid sequence in the desired host system. It will further be understood by one skilled in the art that such vectors are easily constructed using conventional methods (Ausubel et al., (1987) in "Current Protocols in Molecular Biology," John Wiley and Sons, New York, N.Y.) and are commercially available.

[0144] In certain embodiments, the poxvirus vectors disclosed herein further comprise nucleic acid molecules encoding one or more costimulatory molecules. In certain embodiments, the one or more costimulatory molecules are selected from the group consisting of B7-1, B7-2, ICAM-1, LFA-3, 4-1BBL, CD59, CD40, CD70, VCAM-1 and OX-40L. In certain embodiments, the poxvirus vectors disclosed herein further comprise a nucleic acid molecule encoding one costimulatory molecule. In certain embodiments, the poxvirus vectors disclosed herein further comprise one or more nucleic acid molecules encoding two costimulatory molecules. In certain embodiments, the poxvirus vectors disclosed herein further comprise one or more nucleic acid molecules encoding three costimulatory molecules. In certain embodiments, the three costimulatory molecules are B7-1, ICAM-1, and LFA-3 (*i.e.*, TRICOM). In certain embodiments, the poxvirus vectors comprise one or more nucleic acid molecules having the sequence of SEQ ID NO:13 encoding the amino acid sequence of SEQ ID NO:12 (human B7.1), the sequence of SEQ ID NO:17 encoding the amino acid sequence of SEQ ID NO:16 (human ICAM-1), and the sequence of SEQ ID NO:21 encoding the amino acid sequence of SEQ ID NO:20 (human LFA-3). In certain embodiments, the one or more nucleic acid molecules encoding B7-1, ICAM-1, and LFA-3 are under the control of the same expression control sequences. In certain embodiments, the nucleic acids encoding B7-1, ICAM-1, and LFA-3 are under control of different expression control sequences.

[0145] The use of at least three costimulatory molecules produces a synergistic enhancement of the immune response induced by the poxvirus vector encoding a tumor-associated antigen (*e.g.*, Brachyury), and the synergy is not obtainable using only one or two costimulatory molecules. Effective combinations of costimulatory molecules are selected from the group consisting of: B7-1, ICAM-1, and LFA-3; B7-1, B7-2, ICAM-1, and LFA-3; B7-1, B7-2, ICAM-1, and 4-1BBL; B7-1, B7-2, ICAM-1, LFA-3, and 4-1BBL; CD59 and VCAM-1; B7-1

and B7-2; CD59, CD40, 4-1 BBL, and CD70; VCAM-1, B7-1, and B7-2; and OX-40L and 4-1BBL; and the like, depending on the desired immune response and the disease or condition to be treated (*see, e.g.*, US Patent No. 7,211,432, which is hereby incorporated herein by reference in its entirety).

[0146] Genes or functional portions thereof encoding costimulatory molecules that can be incorporated into the poxvirus vectors disclosed herein include but are not limited to B7-1, B7-2, ICAM-1, LFA-3, 4-1BBL, CD59, CD40, CD70, OX-40L, and their mammalian homologs.

[0147] The term “B7” refers to a family of costimulatory molecules which are members of the immunoglobulin (“Ig”) gene superfamily. The members include B7-1 (also known as “CD80”) and B7-2 (also known as “CD86”), which are the natural ligands of CD28 and CTLA-4 (also known as “CD152”). The gene sequence of mouse B7.1 (SEQ ID NO:14 and SEQ ID NO:15) is deposited in GenBank under Accession No. X60958. *See, e.g.*, Freeman et al., J. Immunol. 143:2714-2722 (1989). The gene sequence of mouse B7.2 is deposited in GenBank under Accession No. L25606. *See, e.g.*, Azuma et al., Nature 366:76-79 (1993). The human homologs of the mouse B7-1 and B7-2 costimulatory molecules include CD80, the homolog of mouse B7.1, and CD86, the homolog of mouse B7.2. The gene sequence of human B7.1 (SEQ ID NO:12 and SEQ ID NO:13) is deposited in GenBank under Accession No. M27533. The gene sequence of human B7.2 (CD86) is deposited in GenBank under Accession Nos. U04343 and AF099105.

[0148] The term “intercellular adhesion molecule” (“ICAM”) refers to a family of costimulatory molecules which are members of the Ig gene superfamily. The members include ICAM-1 (also known as “CD54”), ICAM-2 (also known as “CD102”), ICAM-3 (also known as “CD50”), ICAM-4 (also known as “CD242”), and ICAM-5, which are the natural ligands of the leukocyte integrins CD11a/CD18 (also known as “leukocyte function-associated antigen-1” or “LFA-1”) which are expressed on the surface of lymphocytes and granulocytes. The gene sequence of human ICAM-1 (also known as “CD54”; SEQ ID NO:16 and SEQ ID NO:17) is deposited in GenBank under Accession No. J03132. The gene sequence of mouse ICAM-1 (SEQ ID NO:18 and SEQ ID NO:19) is deposited in GenBank under Accession No. X52264.

[0149] The term “leukocyte function-associated antigen” (“LFA”) refers to a family of costimulatory molecules involved in cell adhesion. The members include LFA-1 (also known as

“CD11a/CD18”, LFA-2 (also known as “CD2”), and LFA-3 (also known as “CD58”). LFA-3, a glycosyl-phosphatidylinositol-linked glycoprotein, is a member of the CD2 family of the Ig gene superfamily. The natural ligand of LFA-3 is CD2 (also known as “LFA-2”) which is expressed on thymocytes, T-cells, B-cells and natural killer (“NK”) cells. The gene sequence of human LFA-3 (SEQ ID NO:20 and SEQ ID NO:21) is deposited in GenBank under Accession No. Y00636. The gene sequence of mouse LFA-3 (SEQ ID NO:22 and SEQ ID NO:23) is deposited in GenBank under Accession No. X53526.

[0150] In certain embodiments, the expression control sequences are selected from the group consisting of the SV40 early promoter, an RSV promoter, the retrovirus LTR, the adenovirus major late promoter, the human CMV immediate early I promoter, and various poxvirus promoters including, but not limited to the following vaccinia virus or MVA-derived promoters: the 30K promoter, the I3 promoter, the PrS promoter, the Pr7.5K, the 40K promoter, the PrSynIIIm promoter, or the PrLE1 promoter; or the following fowlpox-derived promoters: the Pr7.5K promoter, the I3 promoter, the 30K promoter, or the 40K promoter.

[0151] In another aspect, provided herein are host cells transformed or transduced with any of the poxvirus vectors described herein. Host cells can include microbial, yeast, insect, avian and mammalian host cells. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Suitable host cells include, for example, bacterial cells (*e.g.*, *E. coli*), yeast cells (*e.g.*, *S. cerevisiae* or *S. pombe*), insect cells (*e.g.*, *S. frugiperda*), avian cells (*e.g.*, the quail fibroblast cell line QT6, CEF cells), and mammalian cells (*e.g.*, CV1 cells, HeLa cells, BHK cells, Chinese hamster ovary (“CHO”) cells, COS-1 cells). Techniques for the propagation of mammalian cells in culture are well-known (*see, e.g.*, Jakoby & Pastan (eds.), *Cell Culture, Methods in Enzymology*, Volume 58 (1979), Academic Press, Inc., Harcourt Brace Jovanovich, NY, NY). Host cells may be selected for other features, such as the ability to express heterologous nucleic acid sequences at higher levels than other cell lines, to produce recombinant proteins with altered glycosylation patterns, and the like.

[0152] Conventional techniques for transforming or transducing different types of host cells with recombinant DNA vectors are well-known to those of ordinary skill in the art. For example, prokaryotic host cells capable of transformation with recombinant DNA can be prepared from cells harvested in the exponential growth phase and subsequently treated with salt solutions

comprising either CaCl_2 , MgCl_2 , or RbCl . Alternatively, eukaryotic cells can be transfected with recombinant DNA by a variety of standard methods including, for example, calcium phosphate coprecipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or infection with the poxvirus vectors. Eukaryotic cells can also be co-transformed with polynucleotide sequences encoding a desired antigen such as, for example, Brachyury and a second foreign DNA molecule encoding a gene having a selectable phenotype, such as the herpes simplex thymidine kinase gene. Methods for using viral vectors to transform eukaryotic cells are well-established (*see, e.g., Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed. (1982)).

[0153] Standard techniques for preparing recombinant poxvirus vectors comprising a heterologous DNA sequence encoding a protein of interest such as, for example, Brachyury, are well-known in the art. Such techniques involve, for example, shuttle vectors comprising poxvirus sequences sufficient to direct homologous recombination between the shuttle vector and the parental virus such that the protein of interest is inserted into a desired location of the parental pox virus (Mackett et al., *Proc. Nat'l Acad. Sci. USA* 79:7415-7419 (1982)). Recombinant poxvirus vectors can be constructed by well-established methods analogous to those used to produce synthetic recombinants of the fowlpox virus described in US Patent No. 5,093,258, which is hereby incorporated herein by reference in its entirety.

[0154] Generally, a shuttle vector used to prepare a recombinant poxvirus contains the following elements: (i) a prokaryotic origin of replication, so that the vector may be amplified in a prokaryotic host; (ii) a gene encoding a selectable marker expressed in the desired host cells (*e.g.*, a gene encoding antibiotic resistance); (iii) at least one DNA sequence encoding the desired protein (*e.g.*, Brachyury) located adjacent to a transcriptional promoter capable of directing the expression of the sequence; and (iv) DNA sequences homologous to the region of the parent virus genome where the foreign gene will be inserted, flanking the construct of element (iii). Methods for constructing donor plasmids for the introduction of multiple foreign genes into poxviruses are described, for example, in PCT Publication No. WO 91/19803, which is hereby incorporated herein by reference in its entirety.

[0155] Generally, DNA fragments for construction of the shuttle vector, including fragments containing expression control sequences (*e.g.*, promoters) and fragments containing sequences

homologous to the region of the parent virus genome into which foreign DNA sequences are to be inserted, can be obtained from genomic DNA or cloned DNA fragments. The shuttle vector can contain one or more inserted foreign DNA sequences, as well as a selectable marker useful for identification of recombinant viruses containing inserted foreign DNA. Several types of marker genes can be used to permit the identification and isolation of recombinant viruses including, for example, genes that encode antibiotic or chemical resistance (see, e.g., Spyropoulos et al., *J. Virol.* 62:1046 (1988); Falkner and Moss, *J. Virol.* 62:1849 (1988); and Franke et al., *Mol. Cell. Biol.* 5:1918 (1985)), as well as genes such as the *E. coli* lacZ gene, that permit identification of recombinant viral plaques by colorimetric assay (Panicali et al., *Gene* 47:193-199 (1986)).

[0156] The heterologous nucleic acid sequence to be inserted into the recombinant poxvirus can be placed into a shuttle vector, such as an *E. coli* plasmid, into which DNA homologous to the desired insertion point in the poxvirus vector (e.g., MVA) has already been inserted. The desired heterologous nucleic acid sequence is separately ligated to the desired expression control sequence(s) and the resulting construct is placed in the shuttle vector such that it is flanked on both ends by DNA homologous to the desired insertion point in the poxvirus vector. The resulting shuttle vector is then used to transform a host cell such as, for example, the bacterium *E. coli*, amplified and isolated. Next, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into cultured cells such as, for example, chick embryo fibroblast ("CEF") cells, along with the parental virus such as, for example, MVA. Homologous recombination between poxviral sequences in the shuttle vector and the genome of the poxvirus vector (e.g., MVA) produces a recombinant poxvirus comprising the expression construct inserted into its genome at a site that does not affect virus viability.

[0157] Homologous recombination between donor plasmid DNA and viral DNA in an infected cell produces recombinant viruses incorporating expression cassettes capable of directing expression of the desired heterologous sequence such as, for example, the Brachyury protein. Appropriate host cells for *in vivo* recombination are generally eukaryotic cells that can be infected by the virus and transfected by the plasmid vector. Cells suitable for such use include, for example, CEF cells, HuTK143 cells (human), CV-1 cells (monkey), and BSC-40 cells (monkey). Infection of cells with poxvirus and transfection of these cells with plasmid

vectors is accomplished by standard techniques well-known in the art. *See, e.g.*, US Patent No. 4,603,112 and International Publication No. WO 89/03429, both of which are incorporated herein by reference in their entirety.

[0158] Finally, recombinant viral progeny incorporating the expression cassette capable of directing expression of the desired heterologous sequence can be identified by standard procedures selected depending on which insertion site was utilized (*i.e.*, if the expression cassette was inserted into the thymidine kinase (“TK”) gene of the parent virus, the recombinant progeny will be TK[−]; Mackett et al., *Proc. Nat’l Acad. Sci. USA* 79:7415 (1982)) or what selectable marker was included in the shuttle vector (*i.e.*, if the *E. coli* lacZ (β-galactosidase) gene was used, recombinant progeny can be selected using a chromogenic substrate for the enzyme; Panicali et al., *Gene* 47:193 (1986)). After the identification of a recombinant virus, expression of the heterologous gene inserted into the virus vector can be assayed by a number of different standard techniques such as, for example, plaque assays, Western blotting, radioimmunoprecipitation, and enzyme immunoassay (“EIA”).

[0159] The desired heterologous nucleic acid sequence (*e.g.*, a nucleic acid encoding human or mouse Brachyury) is inserted into a site in the genome of the viral vector that does not affect the ability of the resulting recombinant virus vector to infect cells or express native or heterologous genes. Such sites can readily be identified by one skilled in the art by, for example, testing segments of virus DNA for sites that can incorporate heterologous sequences while retaining viability and infectivity. One site present in many viruses that can be used is the thymidine kinase (“TK”) gene. The TK gene has been found in all poxvirus genomes examined so far, including those of leporipoxviruses (Upton et al., *J. Virol.* 60(3):920 (1986)); capripoxviruses (Gershon et al., *J. Gen. Virol.* 70(3):525 (1989)); orthopoxviruses (Weir et al., *J. Virol.* 46(2):530 (1983)), including monkeypox, variola virus (Esposito et al., *Virol.* 135(2):561 (1984)), and vaccinia virus (Hruby et al., *Proc. Nat’l Acad. Sci. USA*, 80(11):3411 (1983)); and avipoxviruses (Binns et al., *J. Gen. Virol.* 69(6):1275 (1988)), including fowlpox (Boyle et al., *Virol.* 156(2):355 (1987)). In vaccinia virus, in addition to the TK region, other potentially useful insertion regions include, for example, the HindIII M fragment. In fowlpox, in addition to the TK region, other potentially useful insertion regions include, for example, the BamHI J fragment (Jenkins et al., *AIDS Res. Hum. Retrovir.* 7:991-998 (1991)) and the EcoRI-HinD III fragment as

set forth in EP Application No. 0 308 220 A1, which is incorporated herein by reference in its entirety. In addition, the intergenic regions of the MVA genome can be used as insertion sites for exogenous proteins (*see, e.g.*, US Patent No. 7,964,374, which is incorporated herein by reference in its entirety), such as a Brachyury protein and one or more costimulatory molecules.

[0160] Poxvirus open reading frames (ORFs) can be identified by their orientation and their position on the different *HinD* III restriction digest fragments of the genome (*see, e.g.*, Goebel et al., *Virol.* 179:247-266 179:517-563 (1990); *see also* Massung et al., *Virol.* 201:215-240 (1994)). The different *HinD* III fragments are named with capital letters in alphabetical order from largest to smallest (*i.e.*, *HinD* III A is the largest *HinD* III fragment, *HinD* III B is the second largest *HinD* III fragment, and so on). The ORFs are numbered from left to right on each *Hind*III fragment and the orientation of the ORF is indicated by a capital L (indicating the ORF is transcribed from right to Left) or R (indicating the ORF is transcribed from left to Right). US Patent No. 7,550,147 and US Patent No. 8,021,669, both of which are hereby incorporated herein by reference in their entireties, disclose that heterologous DNA sequences can be inserted into one or more intergenic regions ("IGRs") between two adjacent ORFs in the MVA genome. Thus, in certain embodiments, a heterologous DNA sequence can be inserted into an intergenic region of a poxvirus vector. In certain embodiments, the poxvirus vector is an orthopox virus vector. In certain embodiments, the orthopox virus vector is selected from the group consisting of vaccinia virus vector, variola virus vector, monkeypox virus vector, ectromelia virus vector, camelpox virus vector, raccoonpox virus vector, and cowpox virus vector. In certain embodiments, the vaccinia virus vector is selected from the group consisting of vaccinia virus-Western Reserve, vaccinia virus-EM63, vaccinia virus-Lister, vaccinia virus-New York City Board of Health, vaccinia virus-Temple of Heaven, vaccinia virus-Copenhagen, NYVAC, vaccinia virus-Wyeth, ACAM1000, ACAM2000, and modified vaccinia virus Ankara ("MVA"). In certain embodiments, the MVA is selected from the group consisting of MVA-572, deposited at the European Collection of Animal Cell Cultures ("ECACC"), Health Protection Agency, Microbiology Services, Porton Down, Salisbury SP4 0JG, United Kingdom ("UK"), under the deposit number ECACC 94012707 on January 27, 1994, MVA-575, deposited at the ECACC under deposit number ECACC 00120707 on December 7, 2000, MVA-Bavarian Nordic ("MVA-BN"), deposited at the ECACC under deposit number V00080038 on August 30, 2000, and

derivatives of MVA-BN. Additional exemplary poxvirus vectors are described in US Patent No. 7,211,432, which is incorporated herein by reference in its entirety.

[0161] In certain embodiments, the poxvirus vector is an avipox virus vector. In certain embodiments, the avipox vector is selected from the group consisting of canarypox virus vector, fowlpox virus vector, pigeonpox virus vector, mynahpox virus vector, uncopox virus vector, pigeonpox virus vector, quailpox virus vector, peacockpox virus vector, penguinpox virus vector, sparrowpox virus vector, starlingpox virus vector, and turkeypox virus vector. In certain embodiments, the avipox virus vector is a canarypox virus vector. In certain embodiments, the avipox virus vector is a fowlpox virus vector.

[0162] In certain embodiments, the poxvirus vector comprises a nucleic acid encoding a Brachyury protein having the amino acid sequence of SEQ ID NO:1 encoded by the nucleic acid of SEQ ID NO:2 or a nucleic acid encoding a Brachyury protein having the amino acid sequence of SEQ ID NO:3 encoded by the nucleic acid of SEQ ID NO:4, wherein the nucleic acid is inserted into an intergenic region selected from the group consisting of 001L-002L, 002L-003L, 005R-006R, 006L-007R, 007R-008L, 008L-009L, 017L-018L, 018L-019L, 019L-020L, 020L-021L, 023L-024L, 024L-025L, 025L-026L, 028R-029L, 030L-031L, 031L-032L, 032L-033L, 035L-036L, 036L-037L, 037L-038L, 039L-040L, 043L-044L, 044L-045L, 046L-047R, 049L-050L, 050L-051L, 051L-052R, 052R-053R, 053R-054R, 054R-055R, 055R-056L, 061L-062L, 064L-065L, 065L-066L, 066L-067L, 077L-078R, 078R-079R, 080R-081R, 081R-082L, 082L-083R, 085R-086R, 086R-087R, 088R-089L, 089L-090R, 092R-093L, 094L-095R, 096R-097R, 097R-098R, 101R-102R, 103R-104R, 105L-106R, 107R-108L, 108L-109L, 109L-110L, 110L-111L, 113L-114L, 114L-115L, 115L-116R, 117L-118L, 118L-119R, 122R-123L, 123L-124L, 124L-125L, 125L-126L, 133R-134R, 134R-135R, 136L-137L, 137L-138L, 141L-142R, 143L-144R, 144R-145R, 145R-146R, 146R-147R, 147R-148R, 148R-149L, 152R-153L, 153L-154R, 154R-155R, 156R-157L, 157L-158R, 159R-160L, 160L-161R, 162R-163R, 163R-164R, 164R-165R, 165R-166R, 166R-167R, 167R-168R, 170R-171R, 173R-174R, 175R-176R, 176R-177R, 178R-179R, 179R-180R, 180R-181R, 183R-184R, 184R-185L, 185L-186R, 186R-187R, 187R-188R, 188R-189R, 189R-190R, and 192R-193R. A nucleic acid molecule encoding a Brachyury protein can be inserted into vaccinia virus (*e.g.*, MVA or MVA-BN) at any of these positions. Similarly, one or more nucleic acid molecules one, two, three, or more costimulatory molecules

described herein can also be inserted into vaccinia virus (*e.g.*, MVA or MVA-BN) at any of these positions.

[0163] Alternatively, as described in US Patent No. 6,440,422, which is hereby incorporated herein by reference in its entirety, heterologous DNA sequences can be inserted into one or more of the six deletion sites present in MVA, designated I, II, III, IV, V, and IV, and derivatives thereof. Thus, a heterologous nucleic acid encoding a human or mouse Brachyury protein can be inserted into an MVA deletion site. Thus, in certain embodiments, the poxvirus vector comprises a nucleic acid encoding a Brachyury protein having the amino acid sequence of SEQ ID NO:1 encoded by the nucleic acid of SEQ ID NO:2 or a nucleic acid encoding a Brachyury protein having the amino acid sequence of SEQ ID NO:3 encoded by the nucleic acid of SEQ ID NO:4, wherein the nucleic acid is inserted into an intergenic region selected from the group consisting of deletion site I, deletion site II, deletion site III, deletion site IV, deletion site V, and deletion site VI. A nucleic acid molecule encoding a Brachyury protein can be inserted into MVA at any of these positions. Similarly, one or more nucleic acid molecules one, two, three, or more costimulatory molecules described herein can also be inserted into MVA at any of these positions.

Compositions

[0164] In another aspect, provided herein are compositions comprising any of the poxvirus vectors disclosed herein and a pharmaceutically acceptable additive or carrier. In certain embodiments, the pharmaceutically acceptable additive is selected from the group consisting of an antibiotic, a preservative, an adjuvant, a diluent and a stabilizer. In certain embodiments, the pharmaceutically acceptable additive is a diluent. In certain embodiments, the diluent is selected from the group consisting of water, saline, glycerol, ethanol, wetting or emulsifying agents, and pH buffering substances. In certain embodiments, the pharmaceutically acceptable additive is a stabilizer. In certain embodiments, the stabilizer is selected from the group consisting of mannitol, dextran, sugar, glycine, lactose, polyvinylpyrrolidone, antioxidants, and inert gas. In certain embodiments, the pharmaceutically acceptable carrier is selected from the group consisting of proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and lipid aggregates. In certain embodiments, the

pharmaceutically acceptable carrier is a protein. In certain embodiments, the protein is human serum albumin.

[0165] Any of the poxvirus vectors disclosed herein can be converted into a physiologically acceptable preparation for use with the methods described herein. In certain embodiments, the poxvirus vector is an orthopox virus vector. In certain embodiments, the orthopox virus vector is selected from the group consisting of vaccinia virus vector, variola virus vector, monkeypox virus vector, ectromelia virus vector, camelpox virus vector, raccoonpox virus vector, and cowpox virus vector. In certain embodiments, the vaccinia virus vector is selected from the group consisting of vaccinia virus-Western Reserve, vaccinia virus-EM63, vaccinia virus-Lister, vaccinia virus-New York City Board of Health, vaccinia virus-Temple of Heaven, vaccinia virus-Copenhagen, NYVAC, vaccinia virus-Wyeth, ACAM1000, ACAM2000, and modified vaccinia virus Ankara ("MVA"). In certain embodiments, the MVA is selected from the group consisting of MVA-572, deposited at the European Collection of Animal Cell Cultures ("ECACC"), Health Protection Agency, Microbiology Services, Porton Down, Salisbury SP4 0JG, United Kingdom ("UK"), under the deposit number ECACC 94012707 on January 27, 1994, MVA-575, deposited at the ECACC under deposit number ECACC 00120707 on December 7, 2000, MVA-Bavarian Nordic ("MVA-BN"), deposited at the ECACC under deposit number V00080038 on August 30, 2000, and derivatives of MVA-BN. In certain embodiments, the poxvirus vector is an avipox virus vector. In certain embodiments, the avipox vector is selected from the group consisting of canarypox virus vector, fowlpox virus vector, pigeonpox virus vector, mynahpox virus vector, uncopox virus vector, pigeonpox virus vector, quailpox virus vector, peacockpox virus vector, penguinpox virus vector, sparrowpox virus vector, starlingpox virus vector, and turkeypox virus vector. In certain embodiments, the avipox virus vector is a canarypox virus vector. In certain embodiments, the avipox virus vector is a fowlpox virus vector. Additional exemplary poxvirus vectors are described in US Patent No. 7,211,432, which is incorporated herein by reference in its entirety.

[0166] In certain embodiments, such preparation is based on experience in the preparation of poxvirus vaccines used for vaccination against smallpox as described, for example, in Stickl, et al., *Dtsch. med. Wschr.* 99:2386-2392 (1974). For example, purified virus is stored at -80°C with a titer of 5×10^8 TCID₅₀/ml formulated in 10 mM Tris, 140 mM NaCl, pH 7.4. To prepare single

doses of vaccine, *e.g.*, 10^2 - 10^8 particles of the virus can be lyophilized in phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human serum albumin in an ampoule, preferably a glass ampoule. Alternatively, the vaccine doses can be prepared by stepwise freeze-drying of the virus in another formulation. In certain embodiments, the formulation contains additional additives such as mannitol, dextran, sugar, glycine, lactose, polyvinylpyrrolidone, antioxidants or inert gases, stabilizers or recombinant proteins (*e.g.*, human serum albumin) suitable for *in vivo* administration. The ampoules are then sealed and stored at a suitable temperature, for example, between 4°C and room temperature for several months. For longer term storage, however, ampoules can be stored preferably at temperatures below -20°C.

[0167] In certain embodiments, the poxvirus lyophilisate is dissolved in 0.1 to 0.5 ml of an aqueous solution. In certain embodiments, the aqueous solution is physiological saline or Tris buffer.

[0168] Compositions comprising any of the poxvirus vectors disclosed herein can be administered by any means known to one of skill in the art (see Banga, A., "Parenteral Controlled Delivery of Therapeutic Peptides and Proteins," in *Therapeutic Peptides and Proteins*, Technomic Publishing Co., Inc., Lancaster, PA, 1995) either locally or systemically, such as by intramuscular, subcutaneous, intraperitoneal or intravenous injection, but even oral, nasal, transdermal or anal administration is contemplated. In certain embodiments, the aqueous solution is administered either systemically or locally. In certain embodiments, the aqueous solution is administered parenterally, subcutaneously, intravenously, intramuscularly, intranasally, intradermally, or by any other path of administration known to a skilled practitioner. In certain embodiments, the composition is administered by subcutaneous or intramuscular injection. Optimization of the mode of administration, dose, and number of administrations is within the level of ordinary skill in the art.

[0169] To extend the time during which the peptide or protein is available to stimulate a response, the compositions disclosed herein can be provided as an implant, an oily injection, or as a particulate system. The particulate system can be a microparticle, a microcapsule, a microsphere, a nanocapsule, or similar particle. (*see, e.g.*, Banga, *supra*). A particulate carrier based on a synthetic polymer has been shown to act as an adjuvant to enhance the immune

response, in addition to providing a controlled release. Aluminum salts can also be used as adjuvants to produce an immune response.

[0170] In certain embodiments, the poxvirus vectors and compositions disclosed herein may optionally be administered in combination with one or more cytokines, growth factors, or costimulatory molecules. In certain embodiments, the one or more cytokines, growth factors, or costimulatory molecules are selected from the group consisting of IL-2, IL-6, IL-12, RANTES, GM-CSF, tumor necrosis factor-alpha ("TNF- α "), interferon-gamma ("IFN- γ "), GM-CSF, G-CSF, B7-1, B7-2, B7-3, ICAM-1, ICAM-2, LFA-1, LFA-2, LFA-3, CD72, OX-40L and 41 BBL (see, e.g., Salgaller et al., *J. Surg. Oncol.* 68(2):122-38 (1998); Lotze et al., *Cancer J. Sci. Am.* 6(Suppl 1):S61-6 (2000); Cao et al., *Stem Cells* 16(Suppl 1):251-60 (1998); Kuiper et al., *Adv. Exp. Med. Biol.* 465:381-90 (2000)). Such molecules can be administered systemically or locally to the subject by any of the routes disclosed above in relation to administration of compositions comprising the poxvirus vectors disclosed herein.

[0171] In certain embodiments, the poxvirus vectors and compositions disclosed herein further comprise or are administered (concurrently, sequentially, or intermittently), with any other agent, compound, composition or protocol useful for preventing or treating cancer generally, or cancers associated with Brachyury-expressing tumors specifically. In addition, poxvirus vectors and compositions described herein can be administered together with other immunotherapeutic compositions, including prophylactic and/or therapeutic immunotherapy. Thus, the poxvirus vectors and compositions disclosed herein can be used to inhibit or reduce chemotherapy resistance or radiation resistance that may occur in certain Brachyury-expressing cancers or cancers with the potential to express Brachyury by inhibiting Brachyury expression in the tumor, thereby inhibiting anti-proliferative influences.

[0172] Additional agents, compositions or protocols (e.g., therapeutic protocols) that are useful for the treatment of cancer include, but are not limited to, chemotherapy, surgical resection of a tumor, radiation therapy, allogeneic or autologous stem cell transplantation, and/or targeted cancer therapies (e.g., small molecule drugs, biologics, or monoclonal antibody therapies that specifically target molecules involved in tumor growth and progression, including, but not limited to, selective estrogen receptor modulators (SERMs), aromatase inhibitors, topoisomerase inhibitors, tyrosine kinase inhibitors, serine/threonine kinase inhibitors, histone

deacetylase (HDAC) inhibitors, retinoid receptor activators, apoptosis stimulators, angiogenesis inhibitors, poly (ADP-ribose) polymerase (PARP) inhibitors, or immunostimulators).

[0173] In certain embodiments, the poxvirus vectors and compositions disclosed herein are administered to a subject in conjunction with chemotherapy or a targeted chemotherapy. In such embodiments, it may be desirable to administer the poxvirus vector or composition during the “holiday” between doses of chemotherapy or targeted cancer therapy, in order to maximize efficacy of the vaccine. Furthermore, surgical resection of a tumor may precede administration of the poxvirus vectors or compositions encoding, but additional or primary surgery may occur during or after administration of the vectors or compositions.

[0174] Thus, the effect of administration of the poxvirus vectors and compositions encoding a Brachyury protein disclosed herein can be augmented by administering one or more additional agents. In certain embodiments, the additional agent is a cytokine. In certain embodiments, the cytokine is selected from the group consisting of interleukin (IL)-2, IL-3, IL-6, IL-10, IL-12, IL-15, GM-CSF, interferon (IFN)- α , IFN- β , IFN- γ , and IFN- ω .

[0175] Topoisomerase I inhibitors: irinotecan, topotecan, camptothecin and lamellarin D all target type IB topoisomerases.

[0176] Topoisomerase II inhibitors: etoposide (VP-16), teniposide, doxorubicin, daunorubicin, mitoxantrone, amsacrine, ellipticines, aurintricarboxylic acid, and HU-331, a quinolone synthesized from cannabidiol.

[0177] In certain embodiments, the additional agent is a radiotherapeutic agent. In certain embodiments, the additional agent is a chemotherapeutic agent. Classes of chemotherapeutic agents include, but are not limited to, alkylating agents, antimetabolites, natural products, or hormones and their antagonists. Exemplary alkylating agents include, but are not limited to, nitrogen mustards (*e.g.*, mechlorethamine, cyclophosphamide, melphalan, uracil mustard or chlorambucil), alkyl sulfonates (*e.g.*, busulfan), nitrosoureas (*e.g.*, carmustine, lomustine, semustine, streptozocin, or dacarbazine). Exemplary antimetabolites include, but are not limited to, folic acid analogs (*e.g.*, methotrexate), pyrimidine analogs (*e.g.*, 5-fluorouracil (5-FU) or cytarabine), and purine analogs (*e.g.*, mercaptopurine or thioguanine). Exemplary natural products include, but are not limited to, vinca alkaloids (*e.g.*, vinblastine, vincristine, or vindesine), epipodophyllotoxins (*e.g.*, etoposide or teniposide), antibiotics (*e.g.*, dactinomycin,

daunorubicin, doxorubicin, bleomycin, plicamycin, or mitocycin C), and enzymes (*e.g.*, L-asparaginase). Exemplary miscellaneous agents include, but are not limited to, platinum coordination complexes (*e.g.*, cis-diamine-dichloroplatinum II also known as cisplatin), substituted ureas (*e.g.*, hydroxyurea), methyl hydrazine derivatives (*e.g.*, procarbazine), and adrenocortical suppressants (*e.g.*, mitotane and aminoglutethimide). Exemplary hormones and hormone antagonists include, but are not limited to, adrenocorticosteroids (*e.g.*, prednisone), progestins (*e.g.*, hydroxyprogesterone caproate, medroxyprogesterone acetate, and magesrol acetate), estrogens (*e.g.*, diethylstilbestrol and ethinyl estradiol), antiestrogens (*e.g.*, tamoxifen), and androgens (*e.g.*, testosterone propionate and fluoxymesterone).

[0178] Commonly used chemotherapeutics that can be concurrently administered with the poxviral vectors and compositions disclosed herein include Adriamycin, Alkeran, Ara-C, BiCNU, Busulfan, CCNU, Carboplatinum, Cisplatinum, Cytosan, Daunorubicin, DTIC, 5-FU, Fludarabine, Hydrea, Idarubicin, Ifosfamide, Methotrexate, Mithramycin, Mitomycin, Mitoxantrone, Nitrogen Mustard, Taxol (or other taxanes, such as docetaxel), Velban, Vincristine, VP-16. Newer chemotherapeutics include Gemcitabine (Gemzar), Herceptin, Irinotecan (Camptosar, CPT-11), Leustatin, Navelbine, Rituxan STI-571, Taxotere, Topotecan (Hycamtin), Xeloda (Capecitabine), Zevelin and calcitriol. Exemplary immunomodulators and/or cytokines include, but are not limited to, AS-101 (Wyeth-Ayerst Labs.), bropirimine (Upjohn), gamma interferon (Genentech), GM-CSF (granulocyte macrophage colony stimulating factor; Genetics Institute), IL-2 (Cetus or Hoffman-LaRoche), human immune globulin (Cutter Biological), IMREG (from Imreg of New Orleans, La.), SK&F 106528, tumor necrosis factor (TNF)- α , and TNF- β .

[0179] In certain embodiments, the chemotherapeutics agent is administered sequentially or simultaneously with the poxviral vectors and compositions disclosed herein. In certain embodiments, the administration is sequential. In certain embodiments, 1, 2, 3, 4, or more additional chemotherapeutic agents is administered in combination with the poxviral vectors and compositions disclosed herein.

Methods and Uses

[0180] In another aspect, provided herein are methods for eliciting a Brachyury-specific immune response, methods for treating cancer, and methods for treating chemotherapy- or

radiation-resistant cancer using the poxvirus vectors and compositions disclosed herein for eliciting a Brachyury-specific immune response, for treating cancer, or for treating chemotherapy- or radiation-resistant cancer. Also provided herein are uses of the poxvirus vectors and compositions disclosed herein for eliciting a Brachyury-specific immune response, for treating cancer, or for treating chemotherapy- or radiation-resistant cancer, as well as uses of the poxvirus vectors and compositions disclosed herein in the preparation of a medicament for eliciting a Brachyury-specific immune response, for treating cancer, or for treating chemotherapy- or radiation-resistant cancer.

[0181] Thus provided herein are methods of eliciting an immune response against Brachyury in a subject. In certain embodiments, the methods comprise administering a therapeutically effective amount of a poxvirus vector comprising a nucleic acid encoding a polypeptide having an amino acid sequence 99% identical to the sequence of SEQ ID NO:1 to a subject, thereby eliciting the immune response against Brachyury. In certain embodiments, the methods comprise administering a therapeutically effective amount of a poxvirus vector comprising a nucleic acid encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:1 encoded by the nucleic acid sequence of SEQ ID NO:2 to a subject, thereby eliciting the immune response against Brachyury.

[0182] Also provided herein are methods of treating cancer in a subject. In certain embodiments, the methods comprise administering a therapeutically effective amount of a poxvirus vector comprising a nucleic acid encoding a polypeptide having an amino acid sequence 99% identical to the sequence of SEQ ID NO:1 to a subject, thereby treating the cancer. In certain embodiments, the methods comprise administering a therapeutically effective amount of a poxvirus vector comprising a nucleic acid encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:1 encoded by the nucleic acid sequence of SEQ ID NO:2 to a subject, thereby treating the cancer.

[0183] Also provided herein are methods of treating cancer in a subject, wherein the subject has a tumor that is resistant to chemotherapy or ionizing radiation. In certain embodiments, the methods comprise (a) selecting a subject having a tumor that is resistant to chemotherapy or ionizing radiation and (b) administering a therapeutically effective amount of a poxvirus vector comprising a nucleic acid encoding a polypeptide having an amino acid sequence 99% identical

to the sequence of SEQ ID NO:1 to a subject, thereby treating the resistant cancer. In certain embodiments, the methods comprise (a) selecting a subject having a tumor that is resistant to chemotherapy or ionizing radiation and (b) administering a therapeutically effective amount of a poxvirus vector comprising a nucleic acid encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:1 encoded by the nucleic acid sequence of SEQ ID NO:2 to a subject, thereby treating the resistant cancer.

[0184] In certain embodiments, treating the resistant cancer comprises reducing the size of the primary tumor. In certain embodiments, treating the resistant cancer comprises reducing the number of metastatic lesions. In certain embodiments, treating the resistant cancer comprises reducing the size of the primary tumor and reducing the number of metastatic lesions. In certain embodiments, treating the resistant cancer comprises reducing the size of the primary tumor or reducing the number of metastatic lesions. In certain embodiments, treating the resistant cancer comprises delaying progression of the cancer. In certain embodiments, treating the resistant cancer comprises improving overall survival of the subject having cancer.

[0185] In certain embodiments, the vector is an orthopox virus, an avipox virus, a capripox virus, a suipox virus, a raccoon pox virus, or a rabbit pox virus. In certain embodiments, the orthopox virus is a vaccinia virus. In certain embodiments, the vaccinia virus is a modified vaccinia virus Ankara ("MVA") or an MVA-Bavarian Nordic ("MVA-BN"). In certain embodiments, the poxvirus vector is an avipox virus. In certain embodiments, the avipox vector is selected from the group consisting of canarypox virus vector, fowlpox virus vector, pigeonpox virus vector, mynahpox virus vector, uncopox virus vector, pigeonpox virus vector, quailpox virus vector, peacockpox virus vector, penguinpox virus vector, sparrowpox virus vector, starlingpox virus vector, and turkeypox virus vector. In certain embodiments, the avipox virus is a canarypox virus. In certain embodiments, the avipox virus is a fowlpox virus. In certain embodiments, the orthopox virus vector further comprises one or more nucleic acids encoding a B7-1 polypeptide, an ICAM-1 polypeptide, and an LFA-3 polypeptide. In certain embodiments, the avipox virus vector further comprises one or more nucleic acids encoding a B7-1 polypeptide, an ICAM-1 polypeptide, and an LFA-3 polypeptide. In certain embodiments, the methods comprise administering a composition comprising any of the poxvirus vectors disclosed herein and a pharmaceutically acceptable carrier.

[0186] In certain embodiments, the methods further comprise administering one or more additional therapeutically effective amounts of a poxvirus vector comprising a nucleic acid encoding a polypeptide having an amino acid sequence 99% identical to the sequence of SEQ ID NO:1 to a subject, thereby eliciting the immune response against Brachyury. In certain embodiments, the methods comprise administering a therapeutically effective amount of a poxvirus vector comprising a nucleic acid encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:1 encoded by the nucleic acid sequence of SEQ ID NO:2 to a subject, thereby eliciting the immune response against Brachyury. In any of the embodiments disclosed herein, the methods comprise administering a composition comprising any one or more of the poxvirus vectors disclosed herein and a pharmaceutically acceptable carrier.

[0187] In certain embodiments, the subject is a mammal. In certain embodiments, the mammal is a human. In certain embodiments, the human is immune-compromised. In certain embodiments, the subject has a Brachyury-expressing tumor. In certain embodiments, the subject has a tumor with the potential to express Brachyury. In certain embodiments, the human has a Brachyury-expressing tumor. In certain embodiments, the human has a tumor with the potential to express Brachyury. In certain embodiments, the immune-compromised human has a Brachyury-expressing tumor. In certain embodiments, the immune-compromised human has a tumor with the potential to express Brachyury. In certain embodiments, the Brachyury-expressing tumor is a cancer of the brain, breast, lung, esophagus, stomach, small intestine, colon, liver, kidney, bladder, uterus, Fallopian tube, ovary, testes, ureter, prostate, or pancreas. In certain embodiments, the tumor with the potential to express Brachyury is a cancer of the brain, breast, lung, esophagus, stomach, small intestine, colon, liver, kidney, bladder, uterus, Fallopian tube, ovary, testes, ureter, prostate, or pancreas.

[0188] In certain embodiments, the one or more additional therapeutically effective amounts of a poxvirus vector comprise the same poxvirus vector as previously administered and the methods comprise a homologous prime-boost vaccination. In certain embodiments, the one or more additional therapeutically effective amounts of a poxvirus vector comprise a different poxvirus vector than previously administered and the methods comprise a heterologous prime-boost vaccination. In certain embodiments, the first poxvirus vector (*i.e.*, the priming vaccination) administered is an orthopox virus vector comprising a nucleic acid encoding a

polypeptide having an amino acid sequence 99% identical to the sequence of SEQ ID NO:1. In certain embodiments, the first poxvirus vector administered is an orthopox virus vector comprising a nucleic acid encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:1 encoded by the nucleic acid sequence of SEQ ID NO:2. In certain embodiments, the orthopox virus vector is an MVA or an MVA-BN. In certain embodiments, the second poxvirus vector (*i.e.*, the one or more boosting vaccinations) administered is an avipox virus vector comprising a nucleic acid encoding a polypeptide having an amino acid sequence 99% identical to the sequence of SEQ ID NO:1. In certain embodiments, the second poxvirus vector (*i.e.*, the one or more boosting vaccinations) administered is an avipox virus vector comprising a nucleic acid encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:1 encoded by the nucleic acid sequence of SEQ ID NO:2. In certain embodiments, the avipox vector is selected from the group consisting of canarypox virus vector, fowlpox virus vector, pigeonpox virus vector, mynahpox virus vector, uncopox virus vector, pigeonpox virus vector, quailpox virus vector, peacockpox virus vector, penguinpox virus vector, sparrowpox virus vector, starlingpox virus vector, and turkeypox virus vector. In certain embodiments, the avipox virus vector is a canary pox virus vector. In certain embodiments, the avipox virus vector is a fowlpox virus vector.

[0189] In certain embodiments, the one or more additional therapeutically effective amounts of a poxvirus vector (*i.e.*, the one or more boosting vaccinations) are administered at intervals comprising days, weeks or months after administration of the initial therapeutically effective amount of a poxvirus vector (*i.e.*, the priming vaccination). In certain embodiments, the one or more additional therapeutically effective amounts of a poxvirus vector (*i.e.*, the one or more boosting vaccinations) are administered at intervals of 1, 2, 3, 4, 5, 6, 7 or more days after administration of the initial therapeutically effective amount of a poxvirus vector (*i.e.*, the priming vaccination). In certain embodiments, the one or more additional therapeutically effective amounts of a poxvirus vector (*i.e.*, the one or more boosting vaccinations) are administered at intervals of 1, 2, 3, 4, 5, 6, 7, 8 or more weeks after administration of the initial therapeutically effective amount of a poxvirus vector (*i.e.*, the priming vaccination). In certain embodiments, the one or more additional therapeutically effective amounts of a poxvirus vector (*i.e.*, the one or more boosting vaccinations) are administered at intervals of 1, 2, 3, 4, 5, 6, 7, 8,

9, 10, 11, 12 or more months after administration of the initial therapeutically effective amount of a poxvirus vector (*i.e.*, the priming vaccination). In certain embodiments, the one or more additional therapeutically effective amounts of a poxvirus vector (*i.e.*, the one or more boosting vaccinations) are administered at any combination of intervals after administration of the initial therapeutically effective amount of a poxvirus vector (*i.e.*, the priming vaccination)(*e.g.*, 1, 2, 3, 4, 5, 6, 7 or more days, 1, 2, 3, 4, 5, 6, 7, 8 or more weeks, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more months) disclosed herein.

[0190] In certain embodiments, the subject has a tumor or cancer in which Brachyury is not detected at the time the poxvirus vector or composition comprising the poxvirus vector is first administered. In general, when Brachyury is not detected in a subject's cancer, the subject may have an earlier stage cancer in which Brachyury expression has not yet manifested, or in which Brachyury expression is not yet detectable in any event (*i.e.*, Brachyury may be expressed at a low level or in a small number of tumor cells, but is not yet readily detectable using standard detection methods). Thus, administration of the poxvirus vectors or compositions comprising the poxvirus vectors by the methods provided herein may prevent, delay or inhibit development of Brachyury-expressing tumor cells or tumor cells with the potential to express Brachyury. As a result, administration of the poxvirus vectors or compositions comprising the poxvirus vectors by the methods provided herein may prevent, delay or inhibit tumor migration and/or metastatic progression of the tumor.

[0191] In certain embodiments, the subject has a tumor in which Brachyury expression is detected at the time the poxvirus vector or composition comprising the poxvirus vector is first administered. Thus, administration of the poxvirus vectors or compositions comprising the poxvirus vectors by the methods provided herein may reduce or eliminate tumors expressing Brachyury. This can result, for example, in a reduction of tumor burden, inhibition of Brachyury-expressing tumor growth, reduction and/or elimination of tumor cells having the potential to express Brachyury, and/or a reduction in or slowing of metastatic progression, thereby increasing the subject's survival.

[0192] In certain embodiments, administration of the poxvirus vector encoding Brachyury or a composition comprising the poxvirus vector to a subject reduces or prevents the development of a resistance to chemotherapy or ionizing radiation. For example, administration of the

poxvirus vector encoding Brachyury or a composition comprising the poxvirus vector to a subject can prevent, delay or inhibit the onset of resistance to chemotherapy or radiation therapy by preventing, delaying, or inhibiting growth of Brachyury-expressing cells in the tumor or of cells in the tumor with the potential to express Brachyury. In certain embodiments, the subject has a cancer that is resistant to chemotherapy or ionizing radiation, and administration of the poxvirus vector encoding Brachyury or compositions comprising the poxvirus vector prevents, delays or inhibits the development of resistance to chemotherapy or radiation therapy and/or enhances the ability of the chemotherapy or radiation therapy to treat the subject.

[0193] Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

EXAMPLES

[0194] Example 1: Brachyury is Predominantly Expressed in Human Tumor Tissues, but Other Regulators of the Epithelial-to-Mesenchymal Transition (“EMT”) are Not

[0195] *Source of cDNA.* Brachyury expression in normal tissues was studied using multiple tissue cDNA (MTC) panels containing sets of normalized cDNAs from pooled normal tissues from several individuals (Clontech, Mountain View, CA). The following panels were used: human MTC panel I, panel II, immune tissues panel and blood fractions panel. Commercially available tumor tissue-derived cDNAs prepared from different individuals having different tumor types were obtained from BioChain Institute Inc. (Hayward, CA). Total RNA from human cancer cell lines and normal CD19+ isolated B-cells were prepared using the RNeasy extraction kit (Qiagen, Inc., Valencia, CA).

[0196] *Cell Culture.* The human carcinoma cell lines used in this study were maintained free of *Mycoplasma* in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and a 1X solution of antibiotic/antimycotic (Invitrogen).

[0197] *PCR Analysis of cDNA panels.* PCR amplification of cDNA panels was carried out with the following primers specific for NCBI Reference Sequence No. NM_003181, corresponding to the complete cDNA sequence of the human Brachyury gene, and for the GAPDH gene: (1) E7F: 5'-GGGTGGCTTCTTCCTGGAAC-3' (SEQ ID NO:6); (2) E7R: 5'-

TTGGAGAATTGTTCCGAT-3' (SEQ ID NO:7); (3) GAPDH-specific forward primer: 5'-TGAAGGGTCTGGAGTCAACGGATTTGGT-3' (SEQ ID NO:8); and (4) GAPDH-specific reverse primer: 5'-CATGTGGGCCATGAGGTCCACCAC-3' (SEQ ID NO:9). Cycle conditions were as follows: 1 minute at 95°C, 35 cycles of 30 seconds at 95°C/30 seconds at 58°C/1 minute at 72°C, and 5 minutes at 72°C. The expected size for the Brachyury and GAPDH PCR products was 172 base pairs ("bp") and 983 bp, respectively.

[0198] *RT-PCR Analysis of Human Cancer Cell Lines.* Total RNA derived from human cancer cell lines and normal CD19+ isolated B-cells were amplified using the TITANIUM One-Step RT-PCR kit (Clontech) according to the manufacturer's instructions, and PCR was performed as described above. Results are shown in Figures 1-4.

[0199] *RT-PCR analysis of Brachyury, Twist, and Snail expression in normal and tumor lung and breast tissues.* Commercially available tumor and normal tissue adjacent to lung and breast tumor cDNA panels were obtained from Origene Technologies, Inc., Rockville, MD. Brachyury, Twist and Snail mRNA expression was analyzed by using the Gene Expression Master Mix and the following Taqman Gene Expression Assays (Applied Biosystems, Carlsbad, CA): *Brachyury* (Hs00610080), *Twist 1* (Hs00361186), *Snail* (Hs00195591), and *GAPDH* (4326317E). PCR was performed as described above. Indicated are the numbers of positive cases (and percentage) within each group.

[0200] *Immunohistochemistry.* Lung tumor tissues were obtained from thirty-nine patients with histologically diagnosed primary lung cancer; normal tissues were obtained from non-cancer subjects. Sections of paraffin-embedded, formalin-fixed tissues were tested for Brachyury antigen expression using a mouse anti-Brachyury MAb (ab57480, Abcam, Cambridge, MA). Immunostaining was carried out using the Vectastaining ABC kit (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions; color was developed with DAB peroxidase substrate (Vector, Burlingame, CA). Two pathologists independently evaluated the tumor and normal tissue samples in a blinded, randomized way. For each slide, three to five random fields were evaluated; for each field, the percentage of DAB-positive-tumor cells was calculated as [(number of DAB-positive tumor cells/total number of tumor cells) x 100]. For normal tissues, the percentage of reactivity was individually evaluated for each cell type and calculated as: [(number of DAB-positive cells/total number of cells of the same type) x 100].

Staining was recorded as negative if <5% of the tumor or parenchymal cells from normal tissues stained positive for Brachyury expression.

[0201] *Results:* The expression of Brachyury was negative among most normal tissues analyzed, with the major exception of testis that was positive in 3 out of 3 cases. Brachyury protein expression was detected in 16/39 (41%) of primary lung tumor tissues analyzed, including 10/21 adenocarcinomas (48%) and 3/12 (25%) squamous carcinomas.

[0202] Example 2: Twist is Predominantly Expressed in Mouse Tumor Tissues, but Other Regulators of the Epithelial-to-Mesenchymal Transition (“EMT”) are Not

[0203] *RT-PCR Analysis of Brachyury, Snail and Twist mRNA expression in murine normal tissues and murine tumor cell lines:* Murine normal tissue cDNAs were commercially obtained from Clontech, CA. Total RNA was purified from various murine tumor cell lines using a Qiagen mRNA extraction kit, following the manufacturer's recommendations. One microgram (1 µg) of RNA was reverse-transcribed using the Advantage RT-for-PCR kit (Clontech). Expression of mRNA encoding Brachyury, Snail and Twist was analyzed by RT-PCR using 10 ng of cDNA and the Taqman Gene Expression Master Mix with the following Taqman Gene Expression Probes (Applied Biosystems, Carlsbad, CA): Brachyury (Mm01318249_m1), Snail (Mm00441533_g1), and Twist (Mm00442036_m1). PCR was performed according to the manufacturer's recommendations on the 7300 Real-Time PCR System (Applied Biosystems). Mean Ct values for target genes were normalized to mean Ct values for the endogenous control GAPDH ($-\Delta Ct = Ct(GAPDH) - Ct(target\ gene)$). The ratio of mRNA expression of target gene vs. GAPDH was defined as $2^{-\Delta Ct}$.

[0204] *Results.* As with normal human tissues, Brachyury mRNA was undetectable or expressed at very low levels in most normal mouse tissues analyzed. *See* Figure 5A. However, unlike with human carcinomas, the majority of mouse tumor cell lines also were negative for the expression of Brachyury. The exceptions were the embryonic teratocarcinoma cell lines NF-1 and P10, which showed significant Brachyury expression, a result consistent with Brachyury normally being expressed during embryonic development.

[0205] Like our results with human tissues and cell lines, Snail mRNA was detected at comparable levels across all normal mouse tissues and tumor cell lines assayed. In contrast, however, Twist mRNA was expressed at relatively low levels in many normal mouse tissues but

at increased levels in most mouse tumor cell lines assayed, with expression increased up to 60-fold over the median expression across all normal mouse tissues examined, particularly in the mouse transgenic adenocarcinoma of the mouse prostate ("TRAMP") prostate cancer cell line.

[0206] The lack of endogenous Brachyury expression in the majority of mouse cancer cell lines assayed suggests that it is not an appropriate target for preclinical studies of cancer vaccine efficacy. Instead, these studies show that Twist is differentially expressed between mouse tumor cell lines and normal mouse tissues, indicating that it can be used as a model target antigen in preclinical studies for assessing efficacy of immunotherapies targeting regulators of the EMT.

[0207] Example 3: Brachyury Expression is Associated with Increased Resistance to Radiation and Chemotherapy

[0208] *Cell Culture, Radiation Treatment, and Chemotherapy.* Lung carcinoma A549 cells were stably transfected with a full-length human Brachyury construct (phBrachyury), or an empty-vector control (pCMV control). The lung carcinoma H226 cells were transfected with a Brachyury-specific shRNA or a control, nonspecific shRNA (Control.shRNA) vector (Origene Technologies Inc.).

[0209] For radiation survival assays, tumor cells were suspended in growth media and exposed to the indicated doses of ionizing radiation using a Cs-137 gamma-irradiator, washed with media and subsequently seeded on 96-well plates. Cultures were maintained for 2-4 days, after which cell survival was evaluated by the MTT assay. For chemotherapy treatment assays, tumor cells were seeded in 96-well plates, allowed to attach overnight, and treated with chemotherapy the following day (Day 0). Chemotherapeutics included docetaxel (Taxotere, Sanofi-Aventis, Bridgewater, NJ), cisplatin (APP Pharmaceuticals, Schaumburg, IL), and vinorelbine (Bedford Laboratories, Bedford, OH). All wells were replaced with fresh media after 6 hours. Survival for treated wells was calculated as a percentage of the values representing wells of untreated cells.

[0210] *Results.* Overexpression of Brachyury in A549 cells resulted in enhanced resistance to gamma radiation, at doses ranging from 200 to 800 Rads, as compared to control A549 cells (Figure 7, left panel). Similarly, it was found that significantly fewer H226 cells inhibited for Brachyury expression (H226 shBrachyury) survived radiotherapy, as compared to H226 shcontrol cells (Figure 7, right panel). Similarly, it was observed that Brachyury induces

resistance to chemotherapy treatment in vitro. As shown in Figure 8A, twice as many A549 pBrachyury cells survived treatment with docetaxel (taxotere), vinorelbine, cisplatin, or the combination cisplatin plus vinorelbine than A549 pCMV cells. In additional experiments, H226 cells inhibited for the expression of Brachyury (h226 shBrachyury) were found to have significantly decreased survival as compared to H226 shcontrol cells, after treatment with indicated chemotherapies (Figure 8B). In Figure 8C, 6 different clones of A549 pBrachyury with various levels of Brachyury expression (Figure 8C-i) demonstrated an inverse correlation between Brachyury and tumor cell growth (Figure 8C-ii), and a positive correlation between the level of Brachyury and the survival of the tumor cells to treatment with docetaxel, cisplatin, vinorelbine, and the combination of cisplatin and vinorelbine (Figures 8C iii-iv).

[0211] Example 4: MVA-Brachyury-TRICOM Expresses both Brachyury and TRICOM in Human Cells

[0212] A recombinant modified vaccinia virus Ankara (“MVA”) comprising a nucleic acid expressing full-length human Brachyury protein (SEQ ID NO:1 (nucleic acid) and SEQ ID NO:2 (amino acid)) and additional nucleic acids expressing the TRIad of COstimulatory Molecules (CD80/B7.1, CD54/ICAM-1, and CD58/LFA-3; “TRICOM”) was constructed by standard procedures well-known to those skilled in the art of molecular biology.

[0213] To assess expression of the three costimulatory molecules, human dendritic cells (“DCs”) were prepared from peripheral blood mononuclear cells (“PBMCs”) of normal donors by culture for six days in the presence of granulocyte-macrophage colony-stimulating factor (“GM-CSF”) and interleukin-4 (“IL-4”). On day 6, DCs were infected with wild-type modified vaccinia virus Ankara (“MVA-WT”) or MVA-Brachyury-TRICOM virus at two different multiplicities of infection (“MOI”): MOI=5 and MOI=10. Infection was carried out for 1 hour at 37°C in Opti-MEM® medium (Life Technologies, Inc., Carlsbad, California), followed by overnight incubation in RPMI medium containing serum. Expression of CD80/B7.1, CD54/ICAM-1, and CD58/LFA-3 was evaluated by FACS analysis. Figure 9A shows the percentage of cells positive for each marker and the mean fluorescence intensity (“MFI”, shown in parentheses) for each marker.

[0214] To assess expression of Brachyury, DCs were prepared from PBMCs of normal donors by culture for six days in the presence of GM-CSF and IL-4. On day 6, DCs were

infected with MVA-WT or MVA-Brachyury-TRICOM (MOI=10). Infection was carried out for 1 hour at 37°C in OptiMEM® medium (Life Technologies, Inc., Carlsbad, California), followed by overnight incubation in RPMI medium containing serum. Figure 9B shows expression of Brachyury evaluated by immunofluorescence analysis using a monoclonal anti-Brachyury Ab (Abcam, Cambridge, Massachusetts). Green fluorescence (which appears white in the black and white figure) indicates Brachyury expression and gray indicates DAPI-stained nuclei.

Example 5: rF-Brachyury-TRICOM Expresses both Brachyury and TRICOM in Monkey Cells

[0215] A recombinant fowlpox (“rF”) comprising a nucleic acid expressing full-length human Brachyury protein and additional nucleic acids expressing the TRIad of COstimulatory Molecules (CD80/B7.1, CD54/ICAM-1, and CD58/LFA-3; “TRICOM”) was constructed by standard procedures well-known to those skilled in the art of molecular biology.

[0216] To assess expression of the three costimulatory molecules, cultivated monkey mammary tumor cells (“CMMT”; ATCC No. CRL-6299) derived from rhesus macaques were cultured according to standard procedures and infected with either rF-Brachyury-TRICOM or MVA-Brachyury-TRICOM. Expression was assessed by fluorescence-activated cell sorting (“FACS”). Results are shown in Figure 10A.

[0217] To assess expression of the three costimulatory molecules, cultivated monkey mammary tumor cells (“CMMT”; ATCC No. CRL-6299) derived from rhesus macaques were cultured according to standard procedures and infected with either rF-Brachyury-TRICOM or MVA-Brachyury-TRICOM. Expression was assessed by fluorescence-activated cell sorting (“FACS”). Results are shown in Figure 10B. Brachyury expression was also assessed by Western Blot performed according to standard procedures. Results are shown in Figure 10C.

[0218] Example 6: Human DCs Infected with MVA-Brachyury-TRICOM *In Vitro* Expand Brachyury-specific CD4+ T-cells from the Blood of Normal Human Donors

[0219] PBMCs derived from four normal human donors (donor numbers 487, 635, 1184, and 1228) were evaluated for CD4+ T-cell responses against full-length Brachyury protein. Two donors (donor numbers 635 and 1228) expanded Brachyury-specific CD4+ T-cells in response to MVA-Brachyury-TRICOM-infected DCs, while the remaining two donors (donor numbers 487 and 1184) did not. Results of these experiments are shown in Figure 11A (donor number 1228) and Figure 11B (donor number 635).

[0220] DCs were prepared from PBMCs obtained from a normal human donor (donor number 1228) by culture for six days in the presence of GM-CSF and IL-4. On day 6, DCs were infected with MVA-Brachyury-TRICOM (MOI=10). Infection was carried out for 1 hour at 37°C in OptiMEM® medium (Life Technologies, Inc., Carlsbad, California). Cells were subsequently cultured overnight in RPMI medium containing serum. Autologous T-cells were stimulated with irradiated DCs infected with MVA-Brachyury-TRICOM at a T-cell:DC ratio of 10:1. After 3 days, interleukin-2 (“IL-2”) at 20 U/ml was added to the cultures. Each stimulation cycle lasted 7 days. After an initial *in vitro* stimulation cycle (“IVS1”), T-cells were re-stimulated with DCs infected as above. At the end of the 2nd *in vitro* stimulation cycle (“IVS2”), CD4+ T-cells were isolated by negative selection with magnetic beads and assayed for proliferation in response to autologous PBMCs pulsed with control human serum albumin (“HSA”) protein or with a purified, Histidine-tagged recombinant Brachyury protein (“His-Brachyury”). Proliferation of CD4+ T-cells was measured on day 5 by ³H incorporation. Supernatants were collected at 96 hours and assayed for production of interferon-gamma (“IFN-γ”) production by enzyme-linked immunosorbent assay (“ELISA”). Results are shown in Figure 11A.

[0221] DCs were prepared from PBMCs obtained from a normal human donor (donor number 635) by culture for six days in the presence of GM-CSF and IL-4. On day 6, DCs were infected with MVA-WT (MOI=10) or MVA-Brachyury-TRICOM (MOI=10). Infection was carried out for 1 hour 37°C in OptiMEM® medium (Life Technologies, Inc., Carlsbad, California). Cells were subsequently cultured overnight in RPMI medium containing serum. Autologous T-cells were stimulated with irradiated DCs at a T-cell:DC ratio of 10:1. After 3 days, IL-2 at 20 U/ml was added to the cultures and replenished every other day. On day 7, T-cells were re-stimulated as above. At the end of the second *in vitro* stimulation cycle (“IVS2”), CD4+ T-cells were isolated by negative selection and assayed for proliferation against 10 µg/ml HSA or 10 µg/ml Brachyury protein. Proliferation was measured by ³H incorporation. Shown are counts per minute after subtraction of background with PBMCs alone. Results are shown in Figure 11B.

[0222] Example 7: Human DCs Infected with MVA-Brachyury-TRICOM *In Vitro* Expand Brachyury-specific CD8+ T-cells from the Blood of Normal Human Donors

[0223] DCs were prepared from PBMCs of a normal donor and infected on day 6 with MVA-WT, MVA-TRICOM, or MVA-Brachyury-TRICOM (MOI=10) for 1 hour at 37°C. Cells were then cultured overnight in RPMI medium containing serum. Allogeneic, Brachyury-specific T-cells (1×10^5 T-cells generated against Tp2, a Brachyury-specific 9-mer peptide (SEQ ID NO:5)) were stimulated with irradiated DCs at a T-cell:DC ratio of 10:1. After 24 hours, supernatants were collected and evaluated for IFN- γ production by ELISA, depicted after subtraction of background in response to MVA-WT-infected DCs. Results are shown in Figure 12.

[0224] Example 8: Anti-tumor responses in Balb/c mice bearing 4T1 tumors vaccinated with MVA-Twist-TRICOM

[0225] *4T1 Tumor Model.* The 4T1 cell line is one of four distinct tumor cell lines derived from a single mammary tumor that arose spontaneously in a wild-type BALB/c mouse. Cells from all four of these tumor cell lines form mammary carcinomas within a month of being implanted into the mammary fat pads of syngeneic BALB/c mice. While these cell lines form primary tumors with equivalent kinetics, they differ dramatically in their metastatic potential. Cells of the 4T1 cell line are able to complete all steps of metastasis and form visible metastatic nodules in lung efficiently. Therefore the 4T1 tumor model is useful for studying immunotherapies targeting regulators of the EMT.

[0226] *Vaccination Protocol.* At day 0, 5×10^4 4T1 cells were injected into the mammary fat pads of 8-10 female BALB/c mice. The mice were randomized into three groups, one receiving injections of phosphate-buffered saline ("PBS"), one receiving 10^8 plaque-forming units ("pfu") of MVA-TRICOM, and one receiving 10^8 pfu of MVA-Twist-TRICOM. The priming injection was delivered on day +4. Two boosting injections were delivered weekly, the first on day +11, the second on day +18. Mice were sacrificed on day +21, and tumor volume plus the number of lung metastases were measured. The vaccination protocol is shown in Figure 13A, and the results are shown in Figure 13B.

[0227] *Results.* Vaccination with MVA-TWIST-TRICOM was able to significantly reduce primary tumor volume in Balb/c mice bearing 4T1 tumors, compared to untreated mice or mice vaccinated with MVA-TRICOM (Figure 13B, left panel). As shown in the right panel, vaccination with MVA-TWIST-TRICOM was also efficient at reducing the number of tumor

cells disseminated to the lungs (lung metastasis), as compared to MVA-TRICOM vaccination or untreated animals.

WE CLAIM:

1. A poxvirus vector comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence 95% identical to the amino acid sequence of SEQ ID NO:1.
2. The poxvirus vector of claim 1, comprising a nucleic acid encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:1.
3. The poxvirus vector of claim 1 or claim 2, wherein the vector is an orthopox virus, an avipox virus, a capripox virus, a suipox virus, a raccoon pox virus, or a rabbit pox virus.
4. The poxvirus vector of claim 3, wherein the orthopox virus is a vaccinia virus.
5. The poxvirus vector of claim 4, wherein the vaccinia virus is a modified vaccinia virus Ankara ("MVA") or an MVA-Bavarian Nordic ("MVA-BN").
6. The poxvirus vector of claim 3, wherein the vector is an avipox virus.
7. The poxvirus vector of claim 6, wherein the avipox virus is a canarypox virus or a fowlpox virus.
8. The poxvirus vector of claim 5, further comprising nucleic acids encoding a B7-1 polypeptide, an ICAM-1 polypeptide, and an LFA-3 polypeptide.
9. The poxvirus vector of claim 7, further comprising nucleic acids encoding a B7-1 polypeptide, an ICAM-1 polypeptide, and an LFA-3 polypeptide.
10. A composition comprising the poxviral vector of claim 8 and a pharmaceutically acceptable carrier.
11. A composition comprising the poxviral vector of claim 9 and a pharmaceutically acceptable carrier.

12. A method of eliciting an immune response against Brachyury in a subject, the method comprising administering a therapeutically effective amount of the composition of claim 10 to the subject, thereby eliciting the immune response.

13. The method of claim 12, further comprising administering one or more additional therapeutically effective amounts of the composition of claim 11 to the subject, thereby eliciting the immune response.

14. The method of claim 13, wherein the subject is a human.

15. The method of claim 14, wherein the subject has a Brachyury-expressing tumor.

16. The method of claim 15, wherein the Brachyury-expressing tumor is a cancer of the brain, breast, lung, esophagus, stomach, small intestine, colon, liver, kidney, bladder, uterus, Fallopian tube, ovary, testes, ureter, prostate, or pancreas.

17. A method of treating or preventing cancer in a subject, the method comprising administering a therapeutically effective amount of the composition of claim 10 to the subject, thereby treating or preventing the cancer.

18. The method of claim 17, further comprising administering one or more additional therapeutically effective amounts of the composition of claim 11 to the subject, thereby treating or preventing the cancer.

19. The method of claim 18, wherein treating the cancer comprises reducing the size of the primary tumor.

20. The method of claim 18, wherein treating the cancer comprises reducing the number of metastatic lesions.

21. The method of claim 18, wherein the subject is a human.

22. The method of claim 21, wherein the subject has a Brachyury-expressing tumor.

23. The method of claim 22, wherein the Brachyury-expressing tumor is a cancer of the brain, breast, lung, esophagus, stomach, small intestine, colon, liver, kidney, bladder, uterus, Fallopian tube, ovary, testes, ureter, prostate, or pancreas.

24. The method of any one of claims 17 to 23, further comprising administering a therapeutically effective amount of a second agent to the subject.

25. The method of claim 19, wherein the second agent is selected from the group consisting of cytokines, chemotherapeutics, and radiotherapeutics.

26. The method of claim 25, wherein the second agent is a cytokine.

27. The method of claim 26, wherein the cytokine is selected from the group consisting of interleukin (IL)-2, IL-3, IL-6, IL-10, IL-12, IL-15, GM-CSF, interferon (IFN)- α , IFN- β , IFN- γ , and IFN- ω .

28. The method of claim 25, wherein the second agent is a chemotherapeutic.

29. The method of claim 28, wherein the chemotherapeutic is selected from the group consisting of alkylating agents, antimetabolites, natural products, hormones and hormone antagonists, targeted therapeutics, and miscellaneous agents.

30. The method of claim 29, wherein the chemotherapeutic is an alkylating agent.

31. The method of claim 30, wherein the alkylating agent is selected from the group consisting of nitrogen mustards, alkyl sulfonates, and nitrosoureas.

32. The method of claim 29, wherein the chemotherapeutic is an antimetabolite.

33. The method of claim 32, wherein the antimetabolite is selected from the group consisting of folic acid analogs, pyrimidine analogs, purine analogs, and topoisomerase inhibitors.

34. The method of claim 29, wherein the chemotherapeutic is a natural product.

35. The method of claim 34, wherein the natural product is selected from the group consisting of vinca alkaloids, epipodophyllotoxins, antibiotics, and enzymes.
36. The method of claim 29, wherein the chemotherapeutic is a hormone or hormone antagonist.
37. The method of claim 36, wherein the hormone or hormone antagonist is selected from the group consisting of adrenocorticosteroids, progestins, estrogens, antiestrogens, and androgens.
38. The method of claim 29, wherein the chemotherapeutic agent is a targeted therapeutic.
39. The method of claim 38, wherein the targeted therapeutic is selected from the group consisting of selective estrogen receptor modulators (SERMs), aromatase inhibitors, topoisomerase inhibitors, tyrosine kinase inhibitors, serine/threonine kinase inhibitors, histone deacetylase (HDAC) inhibitors, retinoid receptor activators, apoptosis stimulators, angiogenesis inhibitors, and poly (ADP-ribose) polymerase (PARP) inhibitors.
40. The method of claim 29, wherein the chemotherapeutic is a miscellaneous agent.
41. The method of claim 40, wherein the miscellaneous agent is selected from the group consisting of platinum coordination complexes, substituted ureas, methyl hydrazine derivatives, and adrenocortical suppressants.
40. A method of treating cancer in a subject, wherein the subject has a tumor that is resistant to chemotherapy or ionizing radiation, the method comprising (a) selecting a subject having a tumor that is resistant to chemotherapy or ionizing radiation and (b) administering a therapeutically effective amount of the composition of claim 10 to the subject, thereby treating the cancer.

41. The method of claim 40, the method further comprising administering a therapeutically effective amount of the composition of claim 11 to the subject, thereby treating the resistant cancer.

42. The method of claim 41, wherein treating the resistant cancer comprises reducing the size of the primary tumor.

43. The method of claim 41, wherein treating the resistant cancer comprises reducing the number of metastatic lesions.

44. The method of claim 40, wherein the subject is a human.

45. The method of claim 44, wherein the subject has a Brachyury-expressing tumor.

46. The method of claim 45, wherein the Brachyury-expressing tumor is a cancer of the brain, breast, lung, esophagus, stomach, small intestine, colon, liver, kidney, bladder, uterus, Fallopian tube, ovary, testes, ureter, prostate, or pancreas.

47. The method of any one of claims 40 to 46, further comprising administering a therapeutically effective amount of a second agent to the subject.

48. The method of claim 47, wherein the second agent is selected from the group consisting of cytokines, chemotherapeutics, and radiotherapeutics.

49. The method of claim 48, wherein the second agent is a cytokine.

50. The method of claim 49, wherein the cytokine is selected from the group consisting of interleukin (IL)-2, IL-3, IL-6, IL-10, IL-12, IL-15, GM-CSF, interferon (IFN)- α , IFN- β , IFN- γ , and IFN- ω .

51. The method of claim 48, wherein the second agent is a chemotherapeutic.

52. The method of claim 51, wherein the chemotherapeutic is selected from the group consisting of alkylating agents, antimetabolites, natural products, hormones and hormone antagonists, targeted therapeutics, and miscellaneous agents.

53. The method of claim 52, wherein the chemotherapeutic is an alkylating agent.

54. The method of claim 53, wherein the alkylating agent is selected from the group consisting of nitrogen mustards, alkyl sulfonates, and nitrosoureas.

55. The method of claim 52, wherein the chemotherapeutic is an antimetabolite.

56. The method of claim 55, wherein the antimetabolite is selected from the group consisting of folic acid analogs, pyrimidine analogs, purine analogs, and topoisomerase inhibitors.

57. The method of claim 52, wherein the chemotherapeutic is a natural product.

58. The method of claim 57, wherein the natural product is selected from the group consisting of vinca alkaloids, epipodophyllotoxins, antibiotics, and enzymes.

59. The method of claim 52, wherein the chemotherapeutic is a hormone or hormone antagonist.

60. The method of claim 59, wherein the hormone or hormone antagonist is selected from the group consisting of adrenocorticosteroids, progestins, estrogens, antiestrogens, and androgens.

61. The method of claim 52, wherein the chemotherapeutic agent is a targeted therapeutic.

62. The method of claim 61, wherein the targeted therapeutic is selected from the group consisting of selective estrogen receptor modulators (SERMs), aromatase inhibitors, topoisomerase inhibitors, tyrosine kinase inhibitors, serine/threonine kinase inhibitors, histone

deacetylase (HDAC) inhibitors, retinoid receptor activators, apoptosis stimulators, angiogenesis inhibitors, and poly (ADP-ribose) polymerase (PARP) inhibitors.

63. The method of claim 52, wherein the chemotherapeutic is a miscellaneous agent.

64. The method of claim 63, wherein the miscellaneous agent is selected from the group consisting of platinum coordination complexes, substituted ureas, methyl hydrazine derivatives, and adrenocortical suppressants.

Figure 1: Brachyury is Predominantly Expressed in Human Tumor Tissues

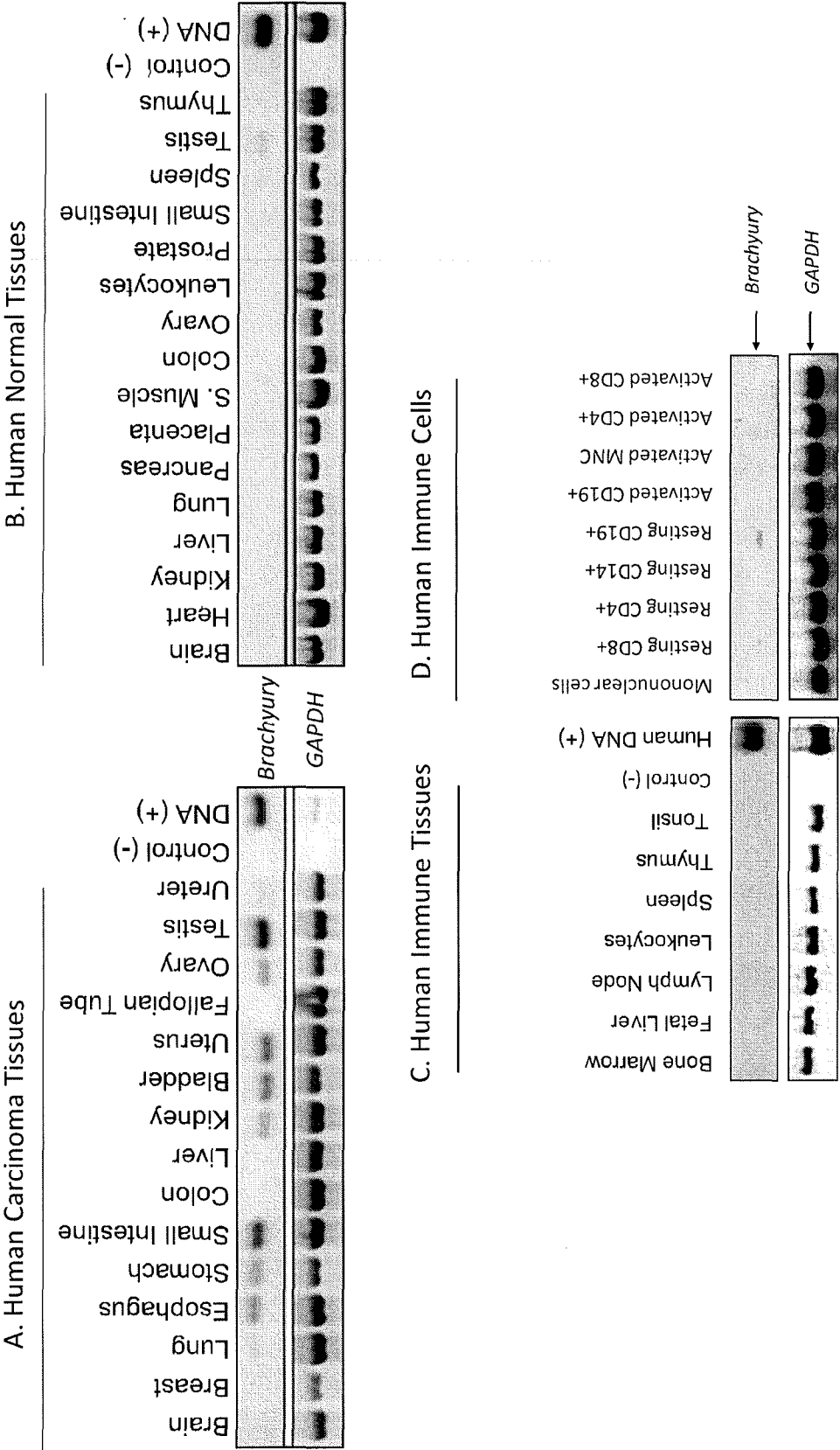


Figure 2: Brachyury Expression in Human Tumor Tissues by RT-PCR

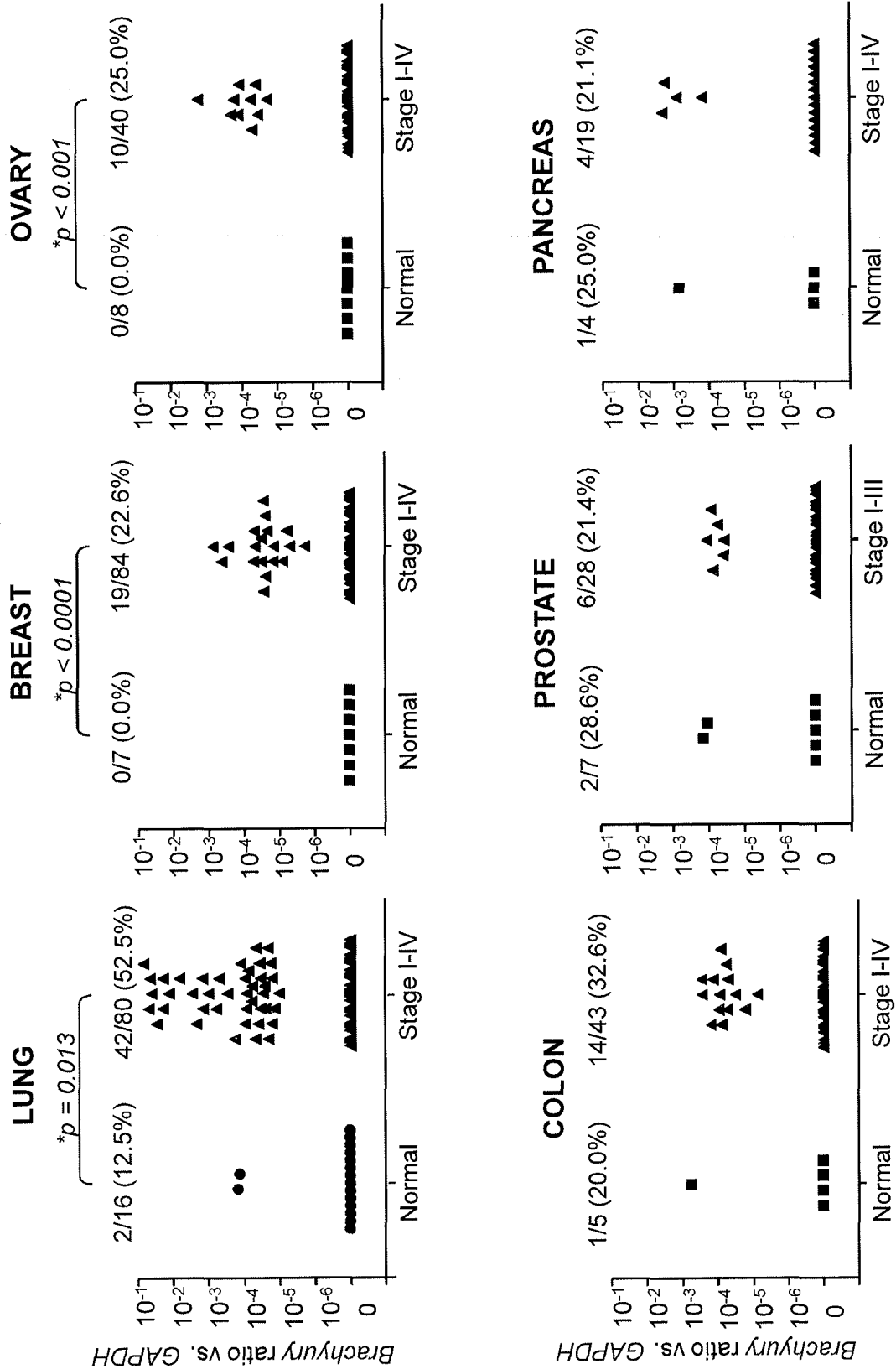


Figure 3: Expression of Brachyury, Twist, and Snail in Human Lung and Breast Tumor Tissues by RT-PCR

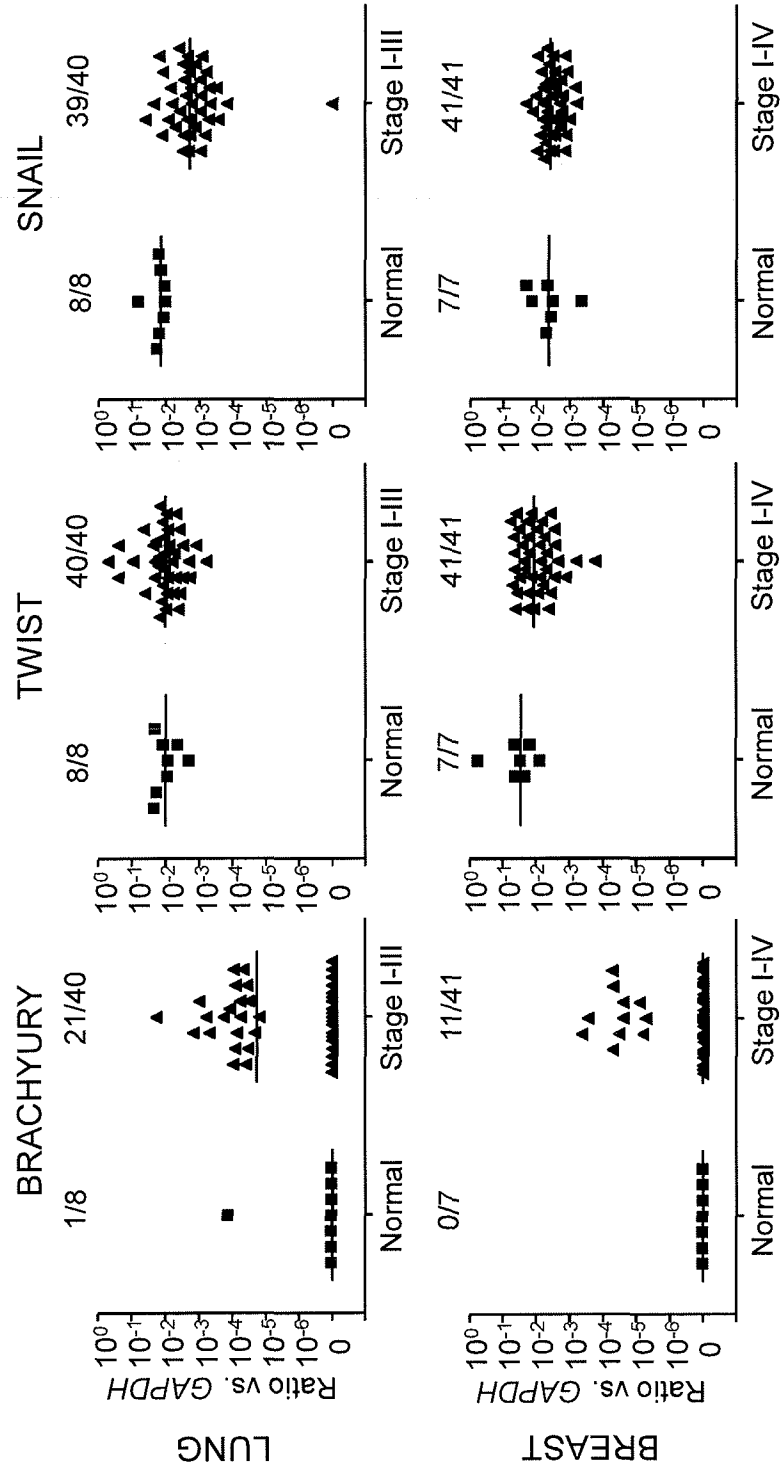
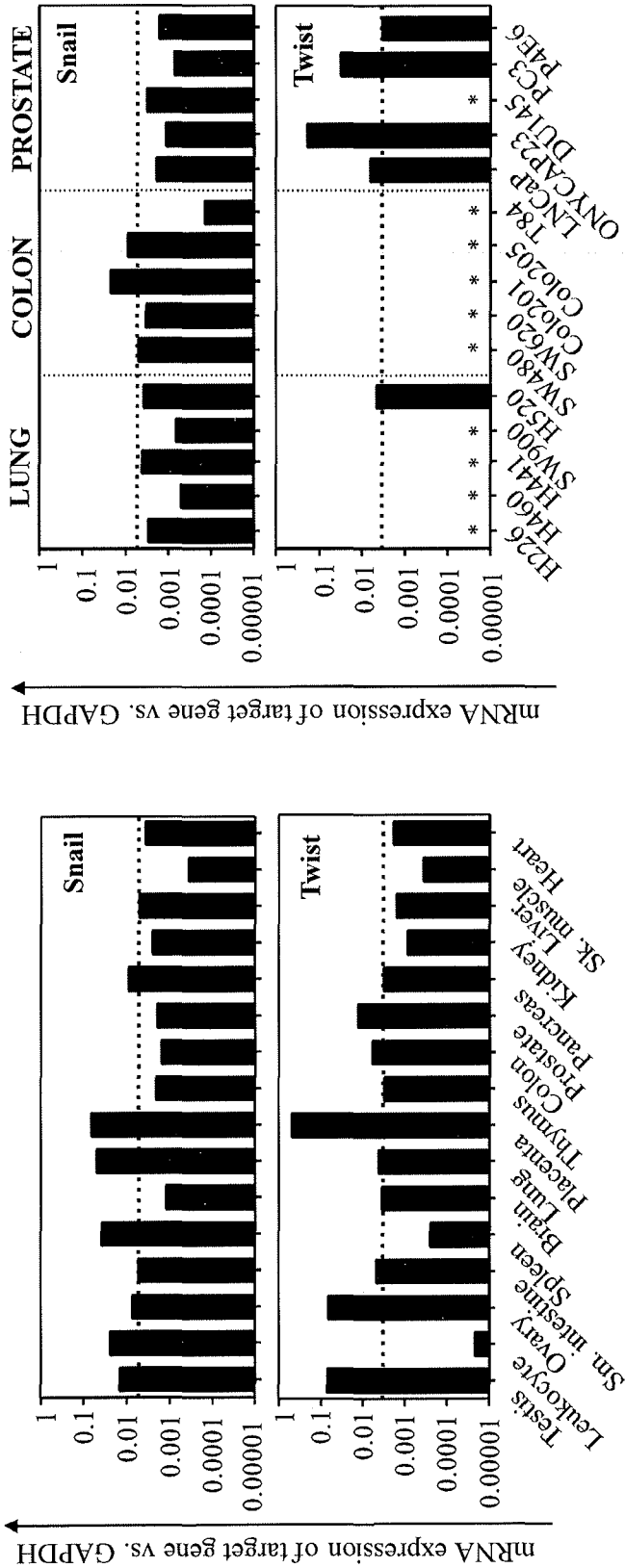


Figure 4: Expression of Snail and Twist in Normal Human Tissues Compared to Tumor Cell Lines as Measured by RT-PCR



The median expression of the target gene by the assayed normal tissues is represented by the dotted line on each graph.
(*) No amplification of target in 40 cycles of real-time PCR.

Figure 5: Expression of Brachyury, Snail and Twist in Normal Mouse Tissues Compared to Tumor Cell Lines as Measured by RT-PCR

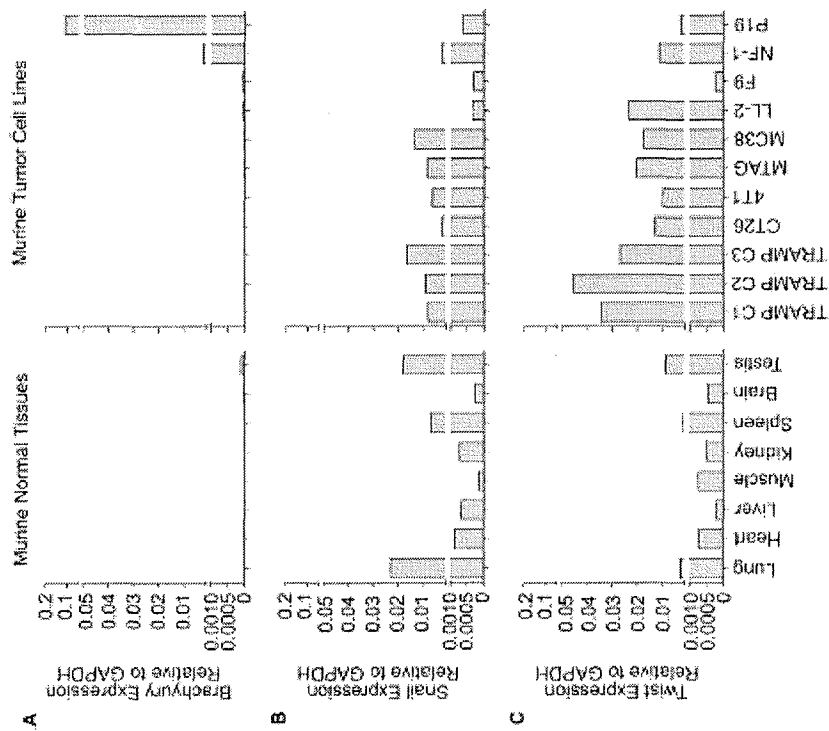


Figure 6: Immunohistochemistry Analysis of Brachyury Expression in Lung Tumors Compared to Normal Tissues

Normal tissue*	Positive samples (%)	Lung tumor	Positive samples (%)
Heart	0/3 (0%)	Adenocarcinoma	10/21 (48%)
Brain	0/3 (0%)	Squamous carcinoma	3/12 (25%)
Liver	0/3 (0%)	Undifferentiated carcinoma	2/4 (50%)
Kidney	0/3 (0%)	Bronchioalveolar, other	1/2 (50%)
Spleen	0/3 (0%)	Total	16/39 (41%)
Adrenal Gland	0/1 (0%)		
Lung	0/3 (0%)		
Skin	0/1 (0%)		
Skeletal Muscle	0/3 (0%)		
Testis	3/3 (100%)		
Total	3/26		

(*) Normal tissues from non-cancerous patients

Figure 7: Brachyury Expression is Associated with Increased Resistance to Ionizing Radiation

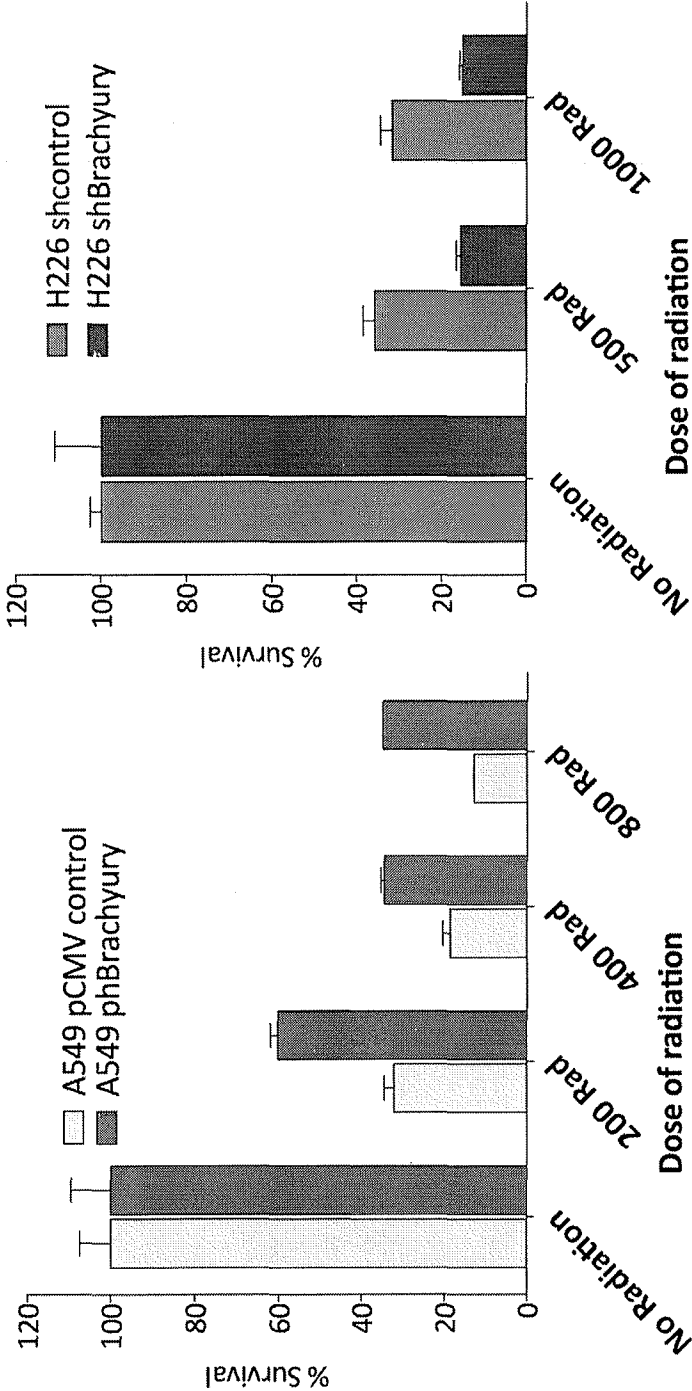


Figure 8A-B: Brachyury Expression is Associated with Increased Resistance to Chemotherapy

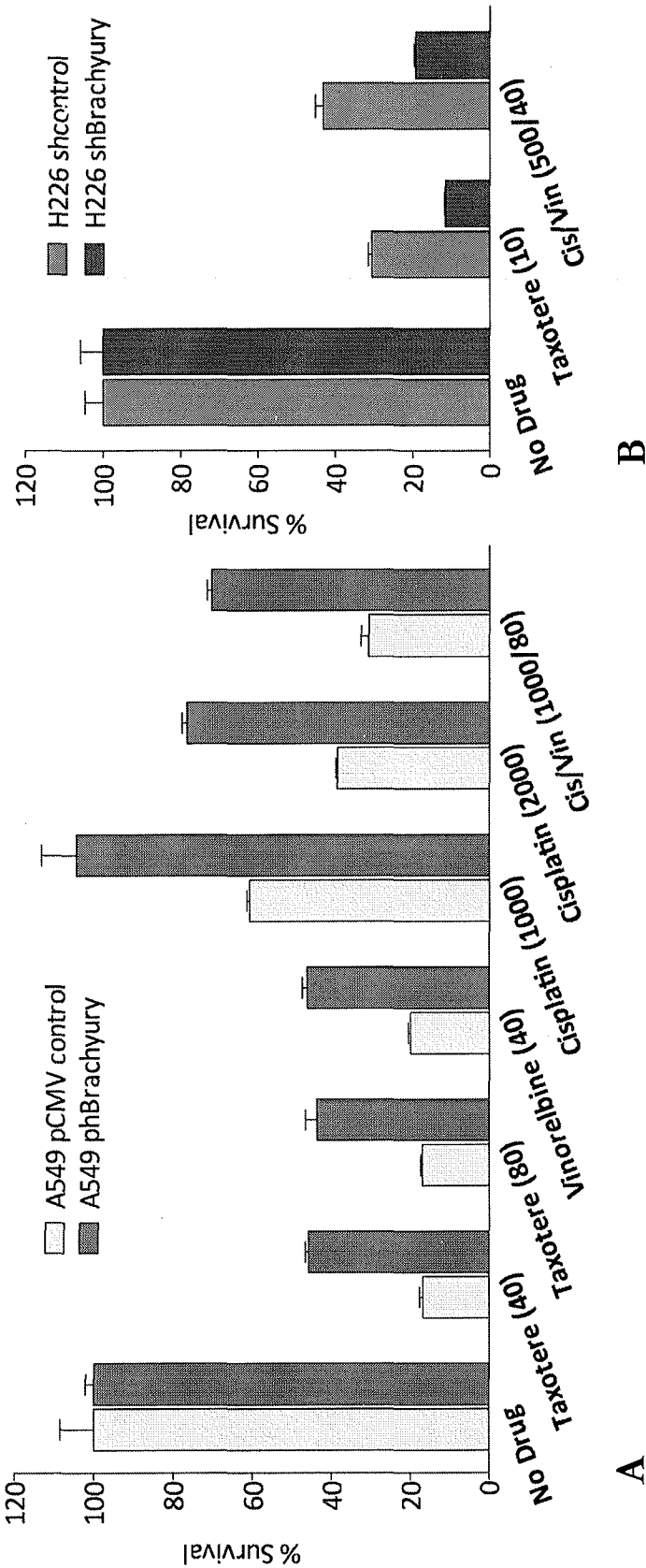


Figure 8C: Brachyury
Expression is Associated with
Increased Resistance to
Chemotherapy

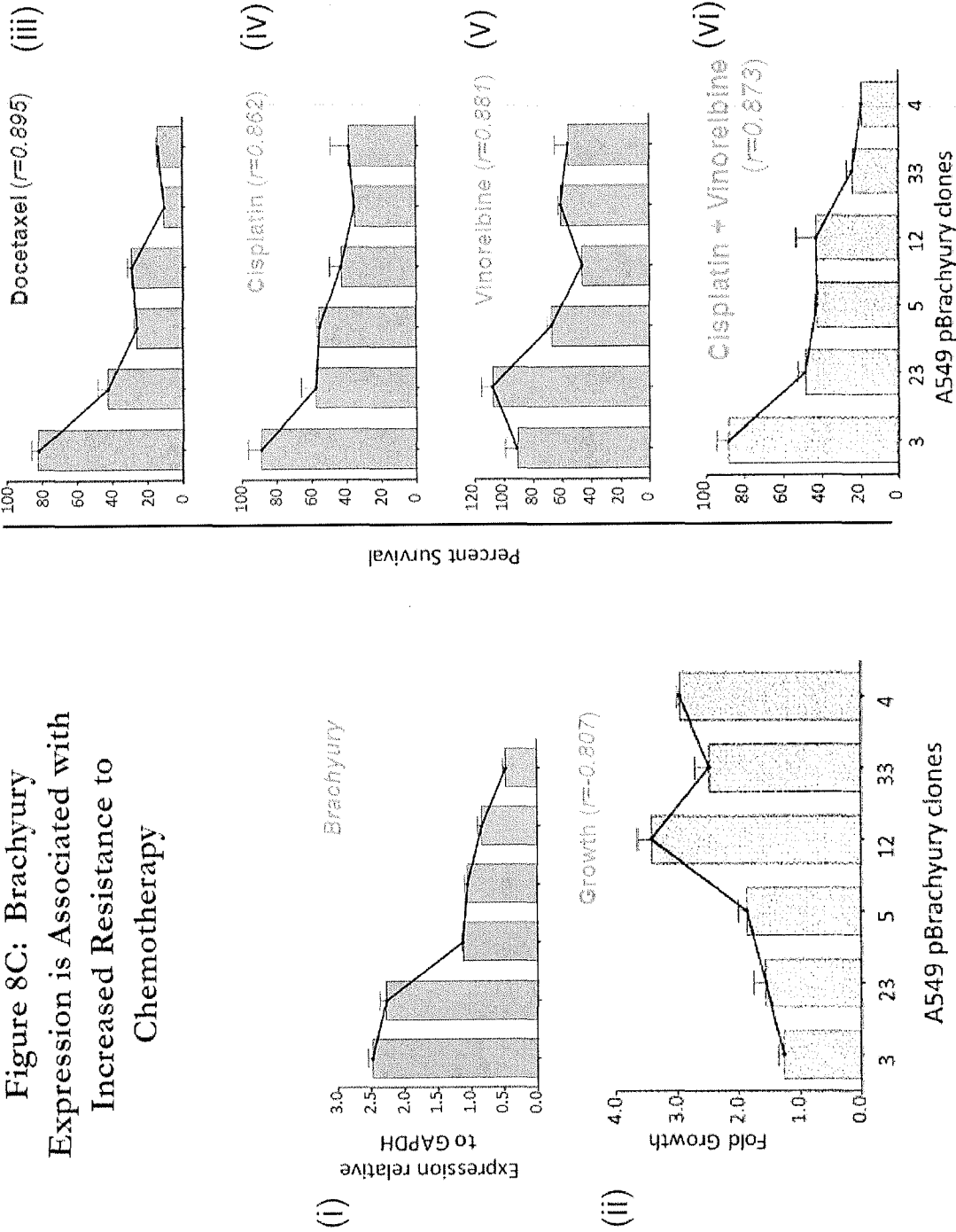


Figure 9A: Detection of TRICOM Expression in Human DCs Infected In Vitro with MVA-Brachyury-TRICOM

Infection with	CD80/B7.1	CD54/ICAM-1	CD58/LFA-3
MVA-WT (control)			
5 MOI	15.6 (23)	86.6 (254)	89.0 (123)
10 MOI	17.3 (21)	81.2 (188)	82.9 (91)
MVA-Brachyury-TRICOM			
5 MOI	83.3 (490)	94.9 (847)	95.4 (394)
10 MOI	81.4 (587)	91.8 (792)	93.9 (381)

Figure 9B: Detection of Brachyury Expression in Human DCs Infected In Vitro with MVA-Brachyury-TRICOM

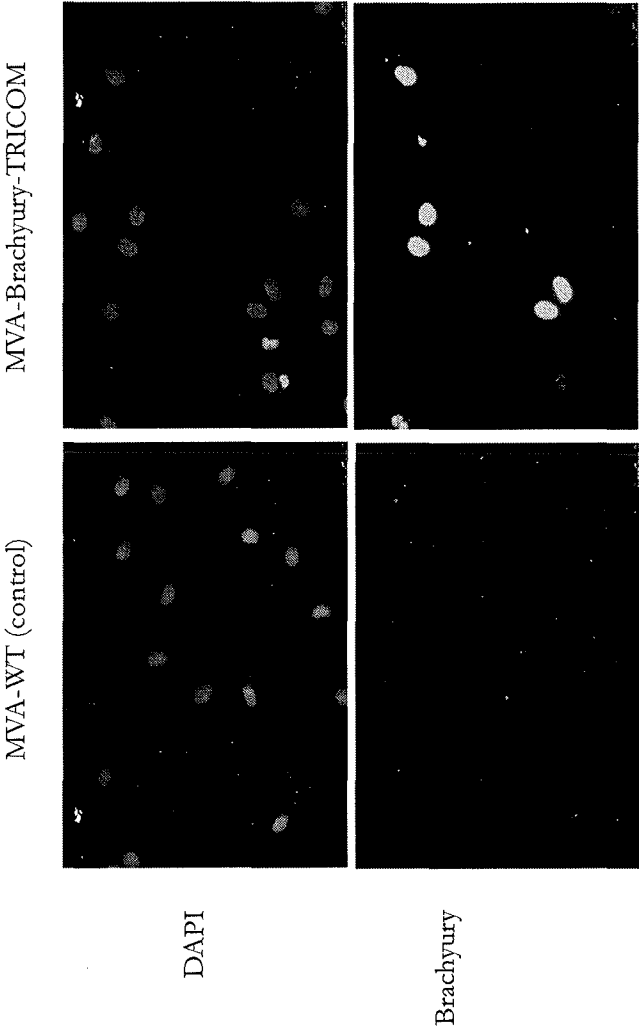


Figure 10A: Detection of TRICOM Expression by Flow Cytometry in Monkey Cells infected In Vitro with rF-Brachyury-TRICOM

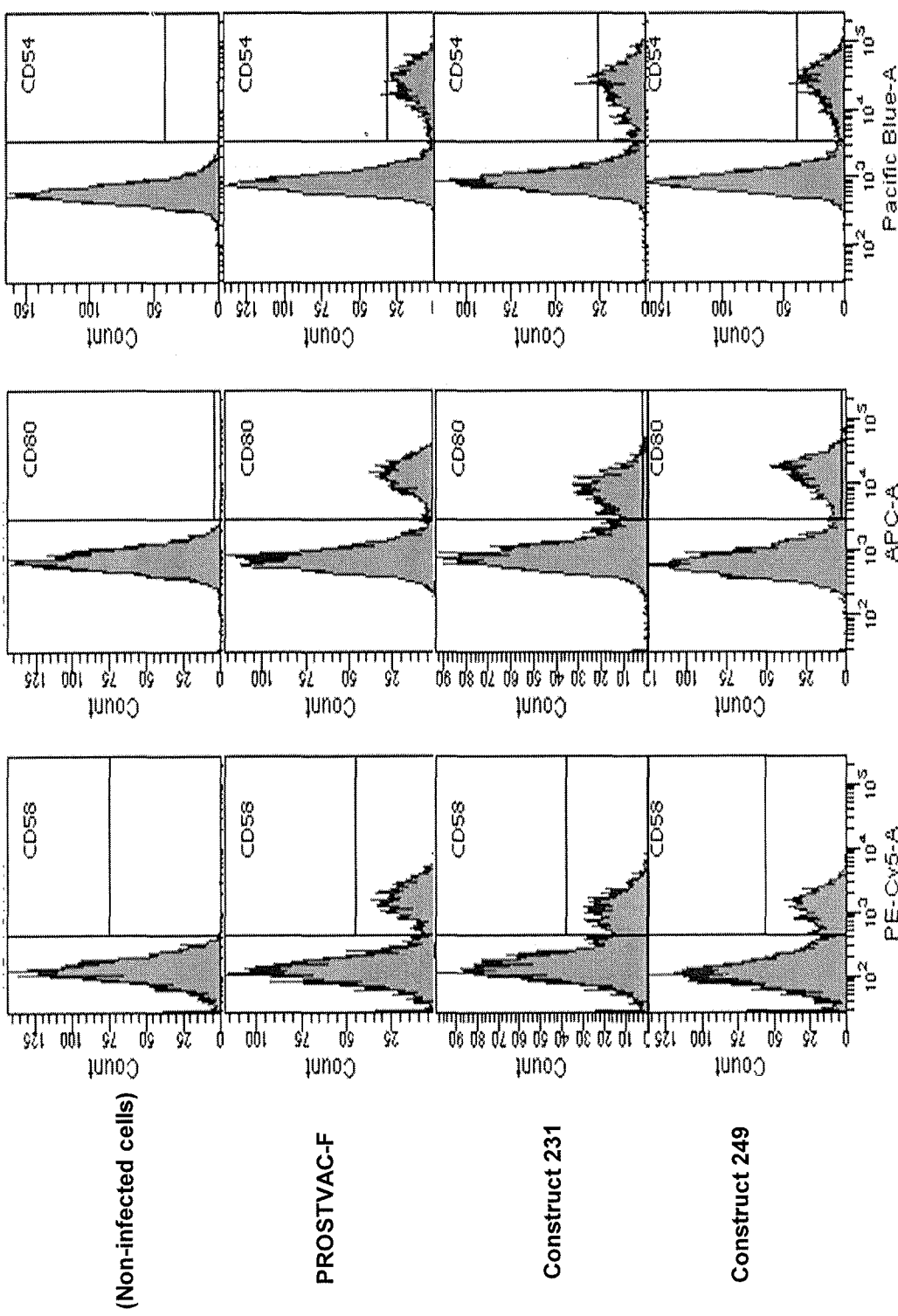


Figure 10B: Detection of Brachyury Expression by Flow Cytometry in Monkey Cells infected In Vitro with rF-Brachyury-TRICOM or MVA-Brachyury-TRICOM

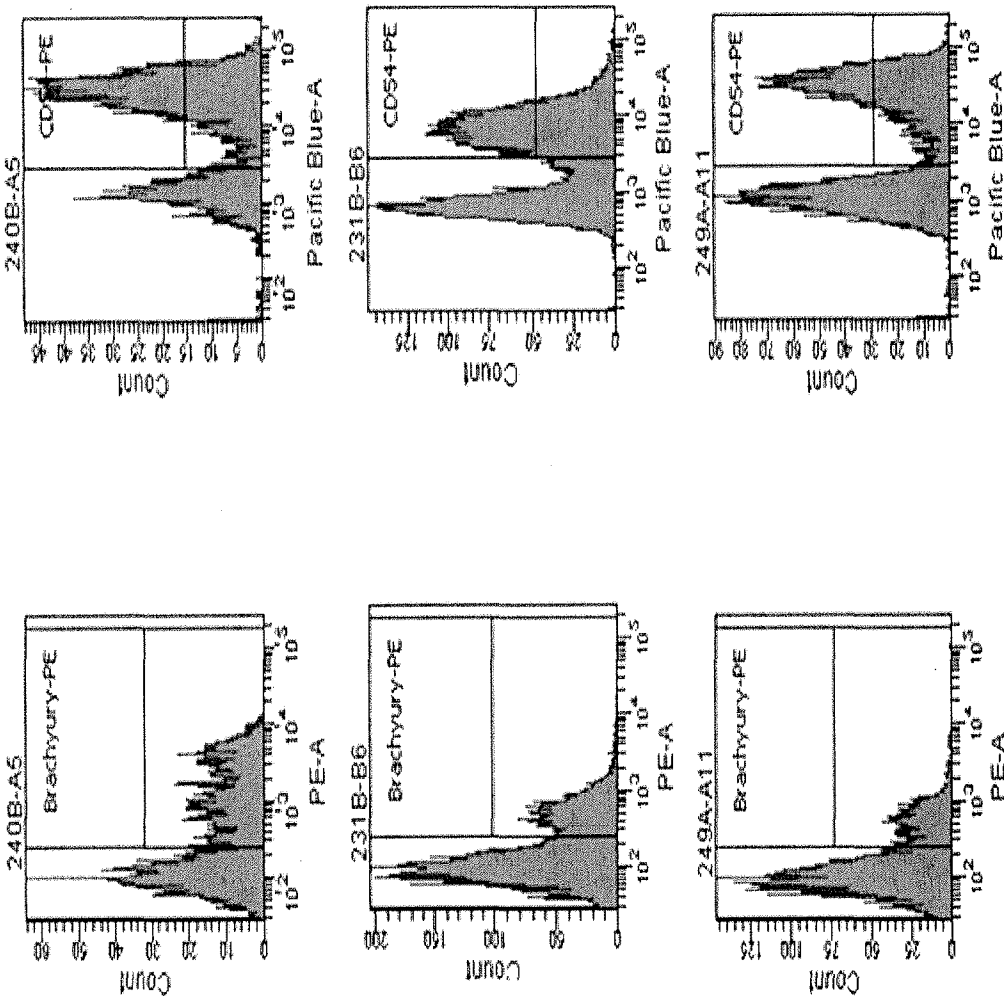


Figure 10C: Detection of Brachyury Expression by Western Blot in Monkey Cells infected In Vitro with rF-Brachyury-TRICOM or MVA-Brachyury-TRICOM

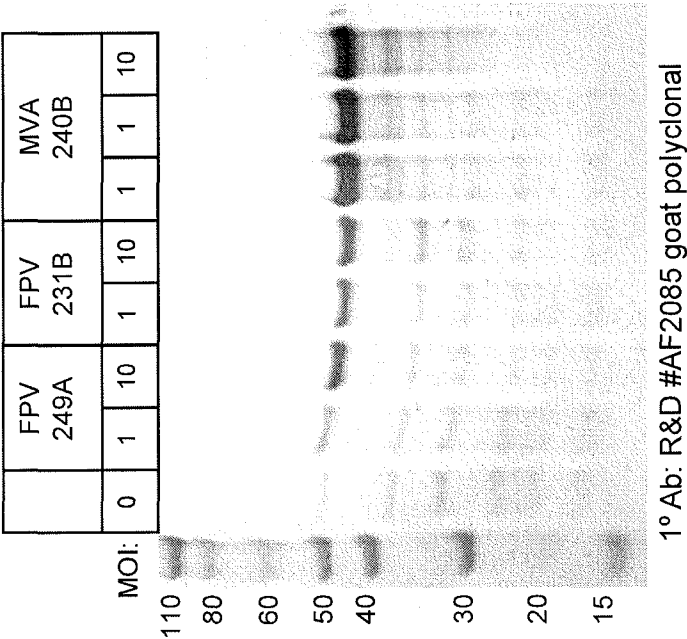


Figure 11: DCs Infected with MVA-Brachyury-TRICOM In Vitro Expand Brachyury-specific CD4+ T-cells from the Blood of Normal Donors

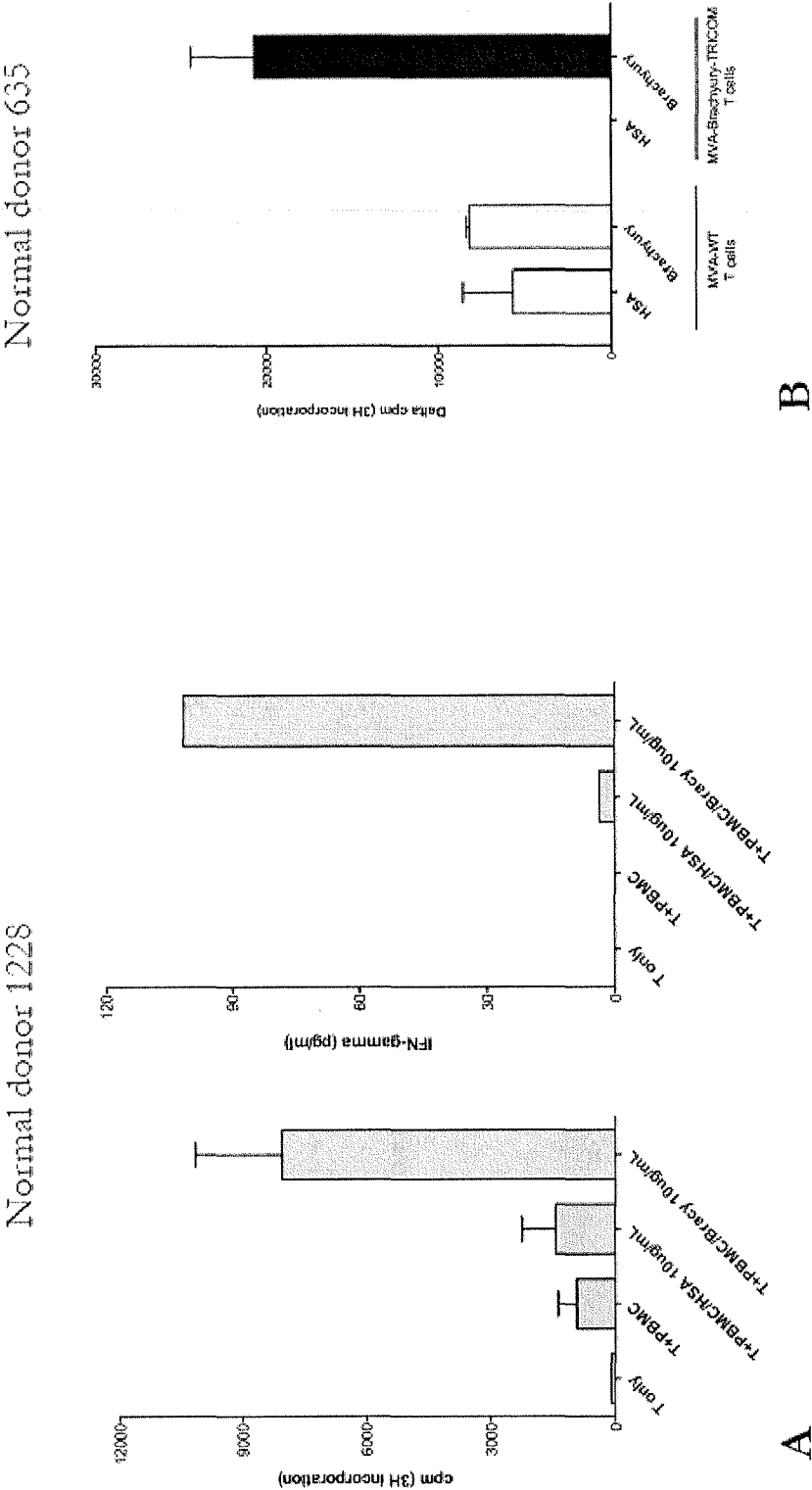


Figure 12: DCs Infected with MVA-Brachyury-TRICOM In Vitro Stimulate
Brachyury-specific CD8+ T-cells

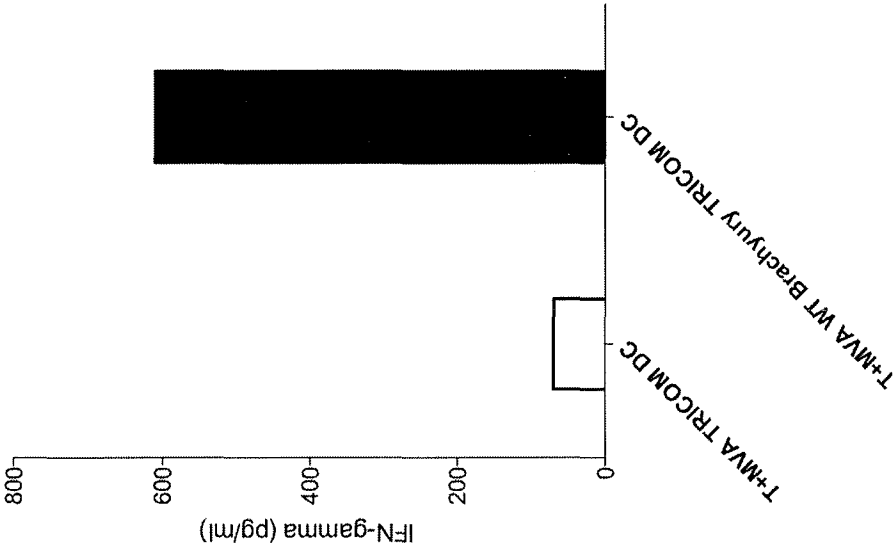
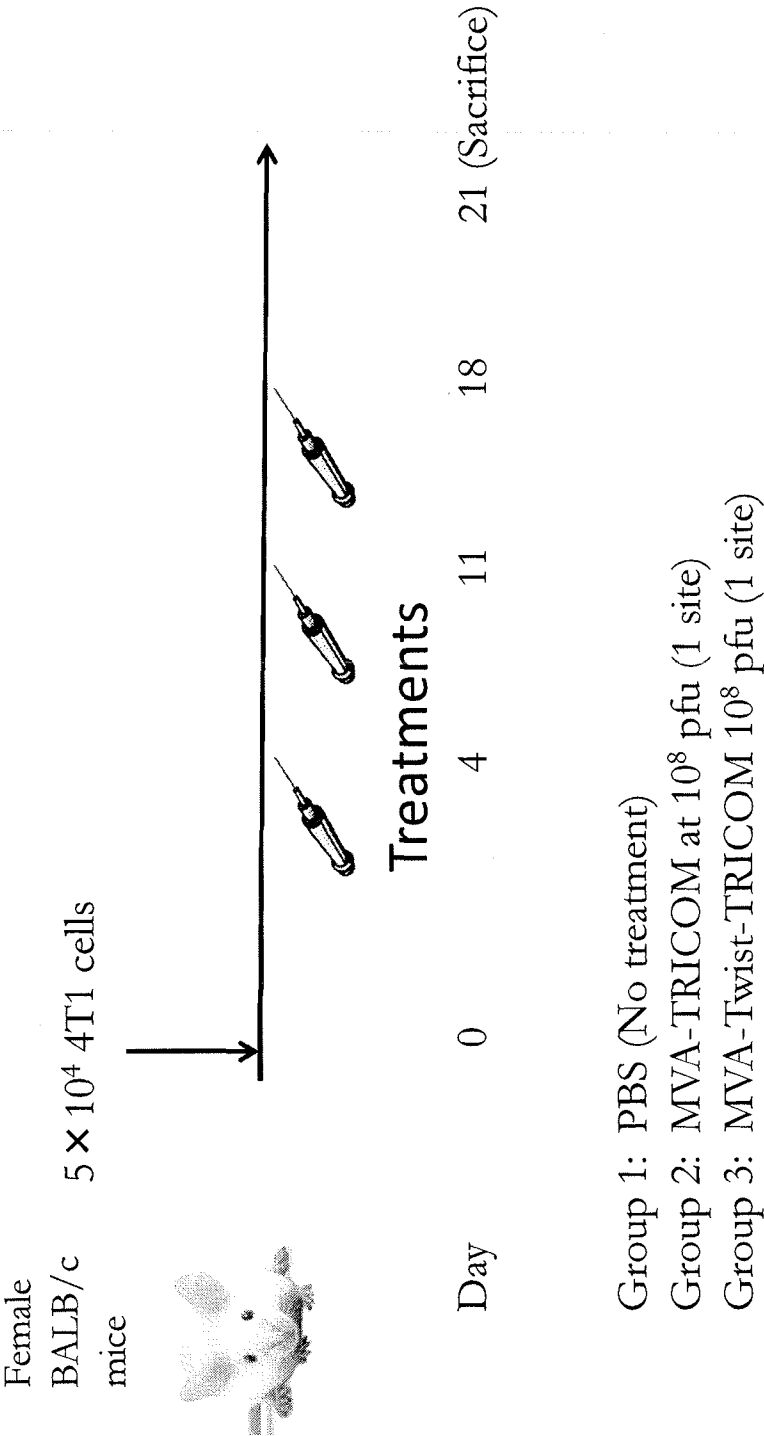


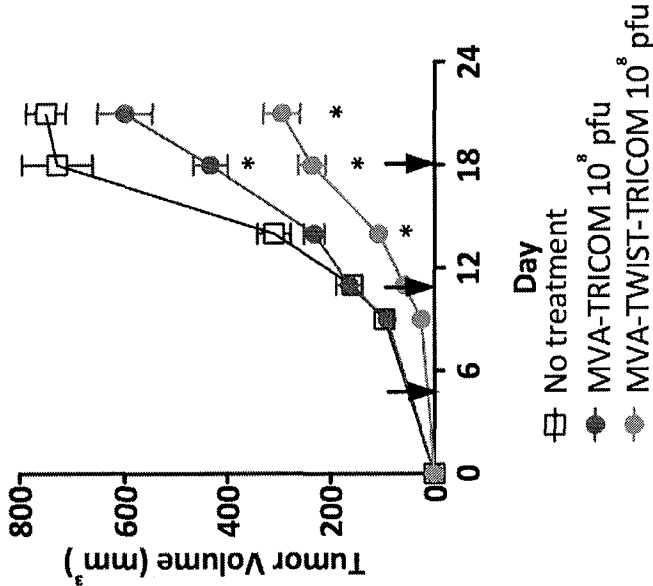
Figure 13: Anti-tumor Responses in BALB/c Mice Bearing 4T1 Tumors
Vaccinated with MVA-Twist-TRICOM



A: Experimental Design

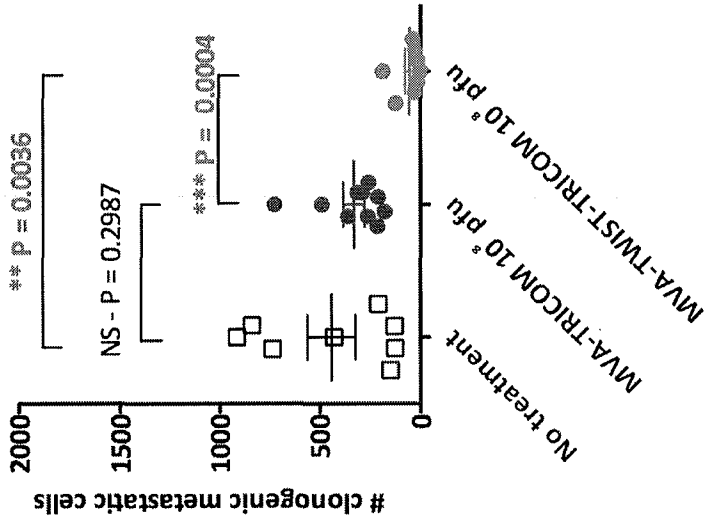
Figure 13: Anti-tumor Responses in BALB/c Mice Bearing 4T1 Tumors
Vaccinated with MVA-Twist-TRICOM

Primary Tumor n = 8-10



B: Anti-tumor Response

Lung Mets



INTERNATIONAL SEARCH REPORT

International application No

PCT/US2013/059761

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/86 A61K35/76 A61K38/18
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	page 4, paragraphs 11, 12 page 10, paragraph 34 - page 11 page 38, paragraph 122 page 41, paragraph 125 page 68 - page 69; example 6 -----	8,9
X	WO 2008/106551 A2 (US GOV HEALTH & HUMAN SERV [US]; SCHLOM JEFFREY [US]; PALENA CLAUDIA M) 4 September 2008 (2008-09-04)	1-7, 10-16
Y	page 2, line 22 - page 3, line 28 page 29, line 8 - line 21 page 39, line 8 - page 40, line 9 ----- -/-	8,9



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

14 November 2013

Date of mailing of the international search report

28/11/2013

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INTERNATIONAL SEARCH REPORT

International application No

PCT/US2013/059761

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	----- HAMILTON DUANE H ET AL: "Cancer vaccines targeting the epithelial-mesenchymal transition: tissue distribution of brachyury and other drivers of the mesenchymal-like phenotype of carcinomas", SEMINARS IN ONCOLOGY, USA, vol. 39, no. 3, 1 June 2012 (2012-06-01), pages 358-366, XP009174100, ISSN: 1532-8708, DOI: 10.1053/J.SEMINONCOL.2012.02.005 page 358 abstract page 5, paragraph 2 page 6, paragraph 3 -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2013/059761

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