Title: REG IV: A TARGET FOR CANCER DIAGNOSIS AND THERAPY

Abstract: The present invention provides methods of diagnosis, providing a prognosis and a therapeutic target for the treatment of cancers that overexpress Reg IV, including prostate and bladder cancers. The invention further provides methods of drug discovery to identify pharmaceutical agents that inhibit or prevent the binding of Reg IV to its receptor, which are useful when used alone or in combination with known chemotherapeutics, immunotherapeutics, and radiotherapy for the reversal of resistance, tumor progression, and metastasis of cancers associated with the overexpression of Reg IV.
REG IV: A TARGET FOR CANCER DIAGNOSIS AND THERAPY

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C.119(e) of U.S. Provisional Application No. 60/662,540, filed March 15, 2005, which is incorporated herein by reference in its entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] This invention was made with Government support under Grant No. PC 001588, awarded by the US Department of Defense. The US Government has certain rights in this invention.

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED ON A COMPACT DISK.

[0003] NOT APPLICABLE

BACKGROUND OF THE INVENTION

INTRODUCTION

[0004] Prostate cancer is the most common malignancy and the second leading cause of cancer-related death in American men. Prostate cancer is a biologically and clinically heterogeneous disease. A majority of men with this malignancy harbor slow-growing tumors that may not impact an individual's natural lifespan, while others are struck by rapidly progressive, metastatic tumors. PSA screening is limited by a lack of specificity and an inability to predict which patients are at risk to develop hormone refractory metastatic disease. Recent studies advocating a lower PSA threshold for diagnosis may increase the number of prostate cancer diagnoses and further complicate the identification of patients with indolent vs. aggressive cancers (Punglia et al., *N Engl J Med*, 349: 335-342 (2003)). New serum and tissue markers that correlate with clinical outcome or identify patients with
potentially aggressive disease are urgently needed (Welsh et al., *Proc Natl Acad Sci U S A*, 100: 3410-3415 (2003)).

[0005] Recent expression profiling studies suggest that expression signatures for metastatic vs. non-metastatic tumors may reside in the primary tumor (Ramaswamy et al., *Nat Genet*, 33: 49-54 (2003); Sotiriou et al., *Proc Natl Acad Sci U S A*, 100: 10393-10398 (2003)). Additional features that predispose tumors to metastasize to specific organs may also be present at some frequency in the primary tumor (Kang et al., *Cancer Cell*, 3: 537-549 (2003)). These recent observations suggest that novel markers of pre-metastatic or pre-hormone refractory prostate cancer may be identified in early stage disease. These markers may also play a role in the biology of metastatic or hormone refractory prostate cancer progression. Recent examples of genes present in primary tumors that correlate with outcome and play a role in the biology of prostate cancer progression include EZH2 and LIM kinase (Varambally et al., *Nature*, 419: 624-629 (2002); Yoshioka et al., *Proc Natl Acad Sci U S A*, 100: 7247-7252 (2003)). However, neither of these two genes is secreted.

[0006] In order to identify new candidate serum or tissue markers of hormone refractory prostate cancer, we compared gene expression profiles of paired hormone dependent and hormone refractory prostate cancer xenografts. The LAPC-9 xenograft was established from an osteoblastic bone metastasis and progresses from androgen dependence to independence following castration in immune deficient mice (Craft et al., *Cancer Research*, In Press (1999)). It has been used previously to identify candidate therapeutic targets in prostate cancer. Differentially expressed genes were validated and then examined for sequence homology to secreted or cell surface proteins. We report here on the identification, characterization and initial validation of one such candidate gene, Reg IV, a new member of the regenerating family of secreted C-lectin proteins (Hartupee et al., *Biochim Biophys Acta*, 1518: 287-293 (2001)). Reg proteins are normally expressed in the gastrointestinal (GI) tract and are induced in inflammatory bowel disease and some GI malignancies. Their pleiotropic functions include promoting tissue regeneration, proliferation and resistance to apoptosis (Macadam et al., *Br J Cancer*, 83: 188-195 (2000)). We demonstrate that Reg IV encodes a secreted protein, which is not expressed in the normal prostate. Reg IV is expressed at low levels in a subset of primary tumors and is moderately or highly expressed in a majority of hormone refractory and metastatic tumors. These results demonstrate that Reg IV is a marker of prostate cancer metastasis or hormone refractory growth as well as a marker with respect to other urogenital cancers, including bladder cancer.
BRIEF SUMMARY OF THE INVENTION

[0007] The present invention provides methods of diagnosis and providing a prognosis for individuals at risk for cancers that overexpress a Reg IV protein or mRNA transcript, particularly urogenital cancers including prostate and/or bladder cancer. The methods generally comprise contacting a test tissue sample from an individual at risk of having a cancer that overexpresses a Reg IV protein or mRNA transcript with an antibody that specifically binds to a Reg IV protein; and determining the presence or absence of a Reg IV protein in the test tissue sample in comparison to a control tissue sample from an individual known to be negative for cancer. Typically, the tissue sample is serum, but can also be biopsy tissue, particularly urogenital tissues including prostate tissue or bladder tissue.

[0008] The present invention further provides methods of inhibiting the growth of and promoting the regression of a cancerous tumor that overexpresses a Reg IV protein or mRNA transcript, the method comprising inhibiting the binding of Reg IV protein to a Reg IV receptor on a cell of the tumor tissue. The methods find particular use in treating any cancer that overexpresses a Reg IV protein or mRNA transcript, particularly urogenital cancers including prostate and bladder cancers.

[0009] The present invention also provides methods of identifying compounds that inhibit the binding of a Reg IV protein to a Reg IV receptor, wherein said compounds find use in inhibiting the growth of and promoting the regression of a cancerous tumor that overexpresses Reg IV protein, for example, a tumor of a urogenital tissue including a prostate or bladder cancer tumor. The screening methods can be carried out in vitro (i.e., by ELISA) and in vivo.

[0010] In some embodiments, the invention provides methods of diagnosing a cancer in a subject by determining the level of Reg IV protein expression or activity in a biological sample or biopsy of the cancer or tumor from the subject wherein an increased level of Reg IV protein expression or activity in the sample or biopsy is indicative of cancer. In some embodiments, determining the Reg IV protein levels involves steps of (a) contacting a tissue sample or biopsy from the subject with an antibody that specifically binds to Reg IV protein; and (b) determining whether or not the Reg IV protein is overexpressed in the sample or biopsy; thereby diagnosing the cancer. In a further embodiment of such, the cancer can be a prostate cancer, ovarian cancer, renal cancer, breast cancer, colon cancer, lung cancer, leukemia, non-Hodgkin’s lymphoma, multiple myeloma, or hepatocarcinoma. In some
further embodiments, still the tissue sample can be a needle biopsy, a surgical biopsy or a bone marrow biopsy. A tissue sample can be fixed or embedded in paraffin. A tissue sample can be, for instance, from prostate, ovary, bone, blood, lymph node, liver, or kidney. The antibody in some embodiments is a monoclonal antibody. An elevated level of Reg IV protein in a sample is indicative of cancer. In a preferred embodiment, the cancer is a prostate or bladder cancer. In preferred embodiments, the diagnosis of cancer is made upon the basis of the Reg IV levels as well as optionally on other conventional indicators of cancer. For instance, the diagnosis can be based upon both the Reg IV protein findings and the histology or growth characteristics of the cancer cells. In the case of prostate cancer, for instance, the Reg IV protein findings can supplement the Gleason scoring system to provide a more accurate or reliable indicator of carcinogenicity and likelihood of disease progression. In some embodiments of the above, the diagnosis is also based upon serum PSA levels.

[0011] In other embodiments of any of the above, the method alternatively determines the Reg IV protein by measuring mRNA transcript levels by (a) contacting a tissue sample with a primer set of a first oligonucleotide and a second oligonucleotide that each specifically hybridize to Reg IV protein 3 nucleic acid; (b) amplifying Reg IV protein nucleic acid in the sample; and (c) determining whether or not Reg IV protein nucleic acid is overexpressed in the sample; thereby diagnosing the cancer. The first oligonucleotide can comprise a nucleotide sequence of Reg IV and the second oligonucleotide can comprise a nucleotide sequence complementary to that of Reg IV 3 cDNA. Preferably, both nucleotides are less than 50 base pairs in length. In a preferred embodiment, the cancer is a prostate or bladder cancer. In some embodiments of the above, the diagnosis is also based upon serum PSA levels.

[0012] In some aspects, the invention provides a method of prognosis for a cancer that overexpresses Reg IV by assessing the likelihood that the cancer will be invasive, metastasize, recur or be resistant to therapy. In a first embodiment in this aspect, the invention provides a method of further diagnosing a cancer that overexpresses Reg IV or has increased Reg IV transcriptional activity and therefore has an increased likelihood of invasiveness, metastasizing, recurrence or resistance to therapy. The method comprises the steps of (a) contacting a tissue sample with an antibody that specifically binds to Reg IV; and (b) determining whether or not the Reg IV is overexpressed in the sample; thereby diagnosing the cancer that overexpresses Reg IV. The cancer may be diagnosed before or after obtaining and analyzing the sample for Reg IV expression or activity levels. The cancer may have been
initially identified on the basis of histological appearance (e.g., Gleason score in the case of prostate cancer) and not on the basis of the Reg IV level determination. The cancer can have been diagnosed as such with or without, or despite, knowledge of Reg IV level. In a further embodiment of such, the cancer can be a prostate cancer or bladder cancer, renal cancer, breast cancer, colon cancer, lung cancer, leukemia, non-Hodgkin’s lymphoma, multiple myeloma, or hepatocarcinoma. In some further embodiments, still the tissue sample can be a needle biopsy, a surgical biopsy or a bone marrow biopsy. A tissue sample can be fixed or embedded in paraffin. A tissue sample can be, for instance, from prostate, ovary, bone, lymph node, liver, or kidney. The antibody in some embodiments is a monoclonal antibody. An elevated level of Reg IV in a sample is prognostic of, and associated with, an increased risk of recurrence or resistance to therapy for the cancer. In a preferred embodiment, the cancer is a prostate cancer or bladder cancer. In some embodiments of the above, the diagnosis is also based upon PSA levels.

[0013] In other embodiments of the above for this second aspect, the method of diagnosing a cancer that overexpresses Reg IV, comprises the steps of (a) contacting a tissue sample with a primer set of a first oligonucleotide and a second oligonucleotide that each specifically hybridize to Reg IV nucleic acid; (b) amplifying Reg IV nucleic acid in the sample; and (c) determining whether or not Reg IV nucleic acid is overexpressed in the sample; thereby diagnosing the cancer that overexpresses Reg IV. The first oligonucleotide can comprise a nucleotide sequence of Reg IV cDNA and the second oligonucleotide can comprise a nucleotide sequence complementary to that of Reg IV cDNA. Preferably, both nucleotides are less than 50 base pairs in length. In the above methods, a increased level of Reg IV in a sample is, prognostic for, and associated with, an increased risk of recurrence, metastasis, hormone independene or resistance to therapy for the cancer. In a preferred embodiment, the cancer is a prostate or bladder cancer. In some embodiments of the above, the diagnosis is also based upon serum PSA levels.

[0014] In yet other embodiments, the invention provides a method of targeting patients for more aggressive or alternative cancer therapy or increased surveillance for a cancer recurrence based upon an elevated level of Reg IV in a tissue sample from the patient taken before, during, or after surgical removal of the cancerous tissue (e.g., prosectomy) or before, during, or after another cancer treatment. The Reg IV activity or expression levels can be determined as described above. In some embodiments of the above, the diagnosis is also based upon serum PSA levels. The cancer that overexpresses Reg IV can be, for instance, a
prostate cancer, ovarian cancer, renal cancer, lung cancer, breast cancer, colon cancer, leukemia, non-Hodgkin’s lymphoma, multiple myeloma or hepatocarcinoma. In a preferred embodiment, the cancer is a prostate or bladder cancer. Patients identified as having raised Reg IV levels and accordingly being at high risk of metastasis, recurrence or a therapy resistant cancer can be treated with exogenous or endogenous hormone ablation, optionally supplemented with chemotherapy and/or radiation. In the case of prostate cancer, the hormone ablation is androgen ablation (e.g., treatment with finasteride and other anti-tesosterone or anti-DHT agents).

[0015] In some embodiments, the invention provides a method of treating or inhibiting a cancer, a therapy resistant cancer, a metastasis of cancer, or recurrence of cancer, that overexpresses Reg IV in a subject comprising administering to the subject a therapeutically effective amount of one or more inhibitors of Reg IV or Reg IV expression. The cancer that overexpresses Reg IV can be, for instance, a prostate cancer, bladder cancer, ovarian cancer, renal cancer, lung cancer, breast cancer, colon cancer, leukemia, non-Hodgkin’s lymphoma, multiple myeloma or hepatocarcinoma. In a preferred embodiment, the cancer is a prostate or bladder cancer. The compound can be a compound as identified in the following aspect. The overexpression can be identified as described in the previous aspects. The compound can be administered concurrently with another cancer therapy.

[0016] The invention also provides a method of identifying a compound that inhibits cancer, therapy resistant cancer, or metastasis, or a recurrence of cancer, the method comprising the steps of contacting a cell with a compound; and determining the effect of the compound on the expression or activity of the Reg IV polypeptide in the cell; wherein compounds which decrease the Reg IV expression or activity levels are identified as being able to inhibit cancer, its metastasis, or progression to a hormone-independent or treatment resistant state. In some embodiments, the compound decreases the expression of Reg IV in the cell. In yet other embodiments, the cell is a cancer cell and, more particularly, may be cancer cell of a particular tissue type or origin (e.g., prostate, ovary, kidney, lung, breast, colon, leukemia, non-Hodgkin’s lymphoma, multiple myeloma or hepatocarcinoma) which has overexpression of Reg IV. In still further embodiments, the cancer that overexpresses Reg IV is prostate cancer, bladder cancer, ovarian cancer, renal cancer, lung cancer, breast cancer, colon cancer, leukemia, non-Hodgkin’s lymphoma, multiple myeloma or hepatocarcinoma. In a preferred embodiment, the cancer is a prostate cancer or bladder cancer.
The invention also provides a method of localizing a cancer that overexpresses Reg IV \textit{in vivo}, and is therefore likely to be invasive, likely to metastasize, become hormone independent, or refractory to treatment, the method comprising the step of imaging in a subject a cell overexpressing Reg IV wherein the cancer that overexpresses Reg IV is selected from the group consisting of prostate cancer, bladder cancer, ovarian cancer, renal cancer, breast cancer, lung cancer, colon cancer, leukemia, non-Hodgkin's lymphoma, multiple myeloma and hepatocarcinoma.

In addition, Reg IV proteins and Reg IV -encoding nucleic acid molecules may be used in various immunotherapeutic methods to promote immune-mediated destruction of cancers (e.g., prostate or bladder tumors), particularly, when such tumors are invasive.

In some embodiments, the invention provides methods of treating cancer, particularly an invasive cancer or a metastasis, or preventing the progression of a cancer to a treatment resistant, hormone-independent, or metastasizing state by administering antibodies that bind to Reg IV to reduce their respective activity in the patient. Additionally, in some other embodiments, the antibodies are conjugated to effector moieties which thereby are preferentially cytotoxic to cells overexpressing the Reg IV. In some embodiments, the antibodies are humanized monoclonal antibodies.

In some embodiments, the invention provides RNA interference (RNAi)-based methods of treating cancer or preventing the progression of a cancer to a treatment resistant, hormone-independent, or metastasizing state by administration of short interfering RNA molecule (siRNA) or an antisense molecule specific for Reg IV and which accordingly are capable of inhibit the expression of Reg IV. In some embodiments, the siRNA molecule is a short hairpin RNAi.

In any of the above aspects and embodiments, the tissue, cancer, subject, or patient to be treated or diagnosed can be human or mammalian.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Reg IV expression in prostate cancer xenografts and in normal tissues and Reg IV secretion. Northern analysis of Reg IV in (a) prostate xenografts, showing overexpression in two hormone refractory (AI) sublines of LAPC 9, and (b) multiple normal tissues, with notable expression in colon. Expression was also seen in pancreas and
small bowel in a multiple tissue dot blot (not shown here). (c) A Myc-tagged Reg IV cDNA was transiently transfected into 293 T cells and recovered from the conditioned media with an anti-Myc antibody. A control antibody did not identify this band, indicating that it is Reg IV. Likewise, a Myc antibody did not pull down a specific protein from a vector-only transfectant control (not shown).

[0023] **Figure 2: In situ expression analysis of Reg IV expression.** (A) The antisense probe (right panel) shows Reg IV (brown) expression in an androgen-independent tumor but not in the adjacent normal tissue to the left of the tumor. The sense control, which is negative, is in the left panel. (B) Progression of Reg IV expression. In the upper left panel (a) is normal prostate, which is not staining. To the right (b) is a negative Gleason 6 cancer. On the bottom left (c) is a Gleason 9/10 primary tumor staining strongly for Reg IV (note intense purple color). To the right (d) is a strongly staining lymph node metastasis.

[0024] **Figure 3: Reg IV expression in normal, primary and metastatic prostate cancer.** The mean expression score +/- standard deviation for Reg IV expression is shown for normal prostate, primary prostate cancer and metastatic prostate cancer. The results summarize a prostate cancer tissue array representing the gamut of prostate tissue.

[0025] **Figure 4: Association of Reg IV expression with Gleason score.** The mean expression score +/- standard deviation for Reg IV expression is shown for Gleason 5-6 and 7-10 prostate tumors.

[0026] **Figure 5: Reg IV mRNA and amino acid sequences from LAPC 9 hormone refractory xenograft.**

**DETAILED DESCRIPTION OF THE INVENTION**

**General**

[0027] The diagnosis and management of prostate cancer is hampered by the absence of markers capable of identifying patients with metastatic disease. In order to identify potential new markers for prostate cancer, we compared gene expression signatures of matched androgen dependent and hormone refractory prostate cancer xenografts. One candidate gene overexpressed in a hormone refractory xenograft was homologous to the regenerating protein gene family, a group of secreted proteins expressed in the gastrointestinal tract and overexpressed in inflammatory bowel disease and cancer. This gene, Reg IV, was confirmed
to be differentially expressed in the LAPC 9 hormone refractory xenograft. Consistent with its upregulation in a hormone refractory xenograft, the RegIV protein and/or mRNA transcripts are overexpressed in a number of prostate tumors after neoadjuvant hormone ablation therapy. As predicted by its sequence homology, it is secreted from transiently transfected cells. Message RNA transcripts are over-expressed in a majority of hormone refractory metastases represented on two high-density tissue microarrays. In comparison, it is not expressed by any normal prostate specimens and only at low levels in ~40% of primary tumors. These data support Reg IV as a candidate marker and cancer associated antigen for hormone refractory metastatic prostate cancer and urogenital cancers.

Definitions

[0028] "Reg IV" refers to nucleic acids, e.g., gene, pre-mRNA, mRNA, and polypeptides, polymorphic variants, alleles, mutants, and interspecies homologs that: (1) have an amino acid sequence that has greater than about 60% amino acid sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a region of over a region of at least about 25, 50, 100, 200, 500, 1000, or more amino acids, to a polypeptide encoded by a referenced nucleic acid or an amino acid sequence described herein, for example, as depicted in Figure 5; (2) specifically bind to antibodies, e.g., polyclonal antibodies, raised against an immunogen comprising a referenced amino acid sequence as depicted in Figure 5, immunogenic fragments thereof, and conservatively modified variants thereof; (3) specifically hybridize under stringent hybridization conditions to a nucleic acid encoding a referenced amino acid sequence as depicted in Figure 5, and conservatively modified variants thereof; (4) have a nucleic acid sequence that has greater than about 95%, preferably greater than about 96%, 97%, 98%, 99%, or higher nucleotide sequence identity, preferably over a region of at least about 25, 50, 100, 150, 200, 250, 500, 1000, or more nucleotides, to a reference nucleic acid sequence (e.g., as shown in Figure 5). A polynucleotide or polypeptide sequence is typically from a mammal including, but not limited to, primate, e.g., human; rodent, e.g., rat, mouse; hamster; cow, pig, horse, sheep, or any mammal. The nucleic acids and proteins of the invention include both naturally occurring or recombinant molecules.

[0029] "Cancer" refers to human cancers and carcinomas, sarcomas, adenocarcinomas, lymphomas, leukemias, etc., including solid tumors and lymphoid cancers, kidney, breast, lung, kidney, bladder, colon, ovarian, prostate, pancreas, stomach, brain, head and neck, skin, uterine, testicular, esophagus, and liver cancer, lymphoma, including non-Hodgkins and
Hodgkins lymphoma, leukemia, and multiple myeloma. "Urogenital cancer" refers to human cancers of urinary tract and genital tissues, including but not limited to kidney, bladder, urinary tract, urethra, prostate, penis, testicle, vulva, vagina, cervical and ovary tissues.

[0030] The cancer will generally comprise Reg IV-expressing cells, such that the anti-Reg IV antibody herein is able to bind to the cancer. While the cancer may be characterized by overexpression of the Reg IV receptor, the present application further provides a method for treating cancer which is not considered to be an Reg IV overexpressing cancer.

[0031] The cancer to be treated herein may be one characterized by excessive activation of Reg IV. In one embodiment of the invention, a diagnostic or prognostic assay will be performed to determine whether the patient's cancer is characterized by overexpression of Reg IV. Various assays for determining such amplification/overexpress ion are contemplated and include the immunohistochemistry, FISH and shed antigen assays, southern blotting, or PCR techniques. Moreover, the Reg IV overexpression or amplification may be evaluated using an in vivo diagnostic assay, e.g. by administering a molecule (such as an antibody) which binds the molecule to be detected and is tagged with a detectable label (e.g. a radioactive isotope) and externally scanning the patient for localization of the label.

[0032] "Therapy resistant" cancers, tumor cells, and tumors refers to cancers that have become resistant or refractory to either or both apoptosis-mediated (e.g., through death receptor cell signaling, for example, Fas ligand receptor, TRAIL receptors, TNF-R1, chemotherapeutic drugs, radiation) and non-apoptosis mediated (e.g., toxic drugs, chemicals) cancer therapies, including chemotherapy, hormonal therapy, radiotherapy, and immunotherapy.

[0033] "Overexpression" generally refers to RNA or protein expression of Reg IV in a test tissue sample that is significantly higher that RNA or protein expression of Reg IV in a control tissue sample from a normal individual. In one embodiment, the tissue sample is autologous. Cancerous test tissue samples typically have at least one fold higher expression of Reg IV, mRNA or protein, often up to two-, three-, four-, five-, eight-, or ten-fold or more fold higher expression of Reg IV in comparison to normal tissue samples. The terms "overexpress," "overexpression" or "overexpressed" interchangeably refer to a gene that is transcribed or translated at a detectably greater level, usually in a cancer cell, in comparison to a normal cell. Overexpression therefore refers to both overexpression of protein and RNA (due to increased transcription, post transcriptional processing, translation, post translational
processing, altered stability, and altered protein degradation), as well as local overexpression due to altered protein traffic patterns (increased nuclear localization), and augmented functional activity, e.g., as in an increased enzyme hydrolysis of substrate. Overexpression can be 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more in comparison to a normal cell or comparison cell (e.g., a BPH cell).

[0034] The terms “cancer that overexpresses Reg IV” and “cancer associated with the overexpression of Reg IV” interchangeably refer to cancer cells or tissues that overexpress Reg IV in accordance with the above definition.

[0035] The terms “cancer-associated antigen” or “tumor-specific marker” or “tumor marker” interchangeably refer to a molecule (typically protein, carbohydrate or lipid) that is preferentially expressed in a cancer cell in comparison to a normal cell, and which is useful for the preferential targeting of a pharmacological agent to the cancer cell. A marker or antigen can be expressed on the cell surface or intracellularly. Oftentimes, a cancer-associated antigen is a molecule that is overexpressed or stabilized with minimal degradation in a cancer cell in comparison to a normal cell, for instance, 2-fold overexpression, 3-fold overexpression or more in comparison to a normal cell. Oftentimes, a cancer-associated antigen is a molecule that is inappropriately synthesized in the cancer cell, for instance, a molecule that contains deletions, additions or mutations in comparison to the molecule expressed on a normal cell. Oftentimes, a cancer-associated antigen will be expressed exclusively in a cancer cell and not synthesized or expressed in a normal cell. Exemplified cell surface tumor markers include the proteins c-erbB-2 and human epidermal growth factor receptor (HER) for breast cancer, PSMA for prostate cancer, and carbohydrate mucins in numerous cancers, including breast, ovarian and colorectal. Exemplified intracellular tumor markers include, for example, mutated tumor suppressor or cell cycle proteins, including p53.

[0036] An “agonist” refers to an agent that binds to a polypeptide or polynucleotide of the invention, stimulates, increases, activates, facilitates, enhances activation, sensitizes or up regulates the activity or expression of a polypeptide or polynucleotide of the invention.

[0037] An “antagonist” refers to an agent that inhibits expression of a polypeptide or polynucleotide of the invention or binds to, partially or totally blocks stimulation, decreases, prevents, delays activation, inactivates, desensitizes, or down regulates the activity of a polypeptide or polynucleotide of the invention.
"Inhibitors," "activators," and "modulators" of expression or of activity are used to refer to inhibitory, activating, or modulating molecules, respectively, identified using in vitro and in vivo assays for expression or activity, e.g., ligands, agonists, antagonists, and their homologs and mimetics. The term "modulator" includes inhibitors and activators. Inhibitors are agents that, e.g., inhibit expression of a polypeptide or polynucleotide of the invention or bind to, partially or totally block stimulation or enzymatic activity, decrease, prevent, delay activation, inactivate, desensitize, or down regulate the activity of a polypeptide or polynucleotide of the invention, e.g., antagonists. Activators are agents that, e.g., induce or activate the expression of a polypeptide or polynucleotide of the invention or bind to, stimulate, increase, open, activate, facilitate, enhance activation or enzymatic activity, sensitize or up regulate the activity of a polypeptide or polynucleotide of the invention, e.g., agonists. Modulators include naturally occurring and synthetic ligands, antagonists, agonists, small chemical molecules and the like. Assays to identify inhibitors and activators include, e.g., applying putative modulator compounds to cells, in the presence or absence of a polypeptide or polynucleotide of the invention and then determining the functional effects on a polypeptide or polynucleotide of the invention activity. Samples or assays comprising a polypeptide or polynucleotide of the invention that are treated with a potential activator, inhibitor, or modulator are compared to control samples without the inhibitor, activator, or modulator to examine the extent of effect. Control samples (untreated with modulators) are assigned a relative activity value of 100%. Inhibition is achieved when the activity value of a polypeptide or polynucleotide of the invention relative to the control is about 80%, optionally 50% or 25-1%. Activation is achieved when the activity value of a polypeptide or polynucleotide of the invention relative to the control is 110%, optionally 150%, optionally 200-500%, or 1000-3000% higher.

The term "test compound" or "drug candidate" or "modulator" or grammatical equivalents as used herein describes any molecule, either naturally occurring or synthetic, e.g., protein, oligopeptide (e.g., from about 5 to about 25 amino acids in length, preferably from about 10 to 20 or 12 to 18 amino acids in length, preferably 12, 15, or 18 amino acids in length), small organic molecule, polysaccharide, lipid, fatty acid, polynucleotide, RNAi, oligonucleotide, etc. The test compound can be in the form of a library of test compounds, such as a combinatorial or randomized library that provides a sufficient range of diversity. Test compounds are optionally linked to a fusion partner, e.g., targeting compounds, rescue compounds, dimerization compounds, stabilizing compounds, addressable compounds, and
other functional moieties. Conventionally, new chemical entities with useful properties are generated by identifying a test compound (called a “lead compound”) with some desirable property or activity, e.g., inhibiting activity, creating variants of the lead compound, and evaluating the property and activity of those variant compounds. Often, high throughput screening (HTS) methods are employed for such an analysis.

0040 A “small organic molecule” refers to an organic molecule, either naturally occurring or synthetic, that has a molecular weight of more than about 50 Daltons and less than about 2500 Daltons, preferably less than about 2000 Daltons, preferably between about 100 to about 1000 Daltons, more preferably between about 200 to about 500 Daltons.

0041 Cytotoxic agents include “cell-cycle-specific” or “antimitotic” or “cytoskeletal-interacting” drugs. These terms interchangeably refer to any pharmacological agent that blocks cells in mitosis. Such agents are useful in chemotherapy. Generally, cell-cycle-specific drugs bind to the cytoskeletal protein tubulin and block the ability of tubulin to polymerize into microtubules, resulting in the arrest of cell division at metaphase.

Exemplified cell-cycle-specific drugs include vinca alkaloids, taxanes, colchicine, and podophyllotoxin. Exemplified vinca alkaloids include vinblastine, vincristine, vindesine and vinorelbine. Exemplified taxanes include paclitaxel and docetaxel. Another example of a cytoskeletal-interacting drug includes 2-methoxyestradiol.

0042 An “siRNA” or "RNAi" molecule refers to a nucleic acid that forms a double stranded RNA, which double stranded RNA has the ability to reduce or inhibit expression of a gene or target gene when the siRNA or RNAi molecule is expressed in the same cell as the gene or target gene. “siRNA” or "RNAi" thus refers to the double stranded RNA formed by the complementary strands. The complementary portions of the siRNA that hybridize to form the double stranded molecule typically have substantial or complete identity. In one embodiment, an siRNA refers to a nucleic acid that has substantial or complete identity to a target gene and forms a double stranded siRNA. Typically, the siRNA is at least about 15-50 nucleotides in length (e.g., each complementary sequence of the double stranded siRNA is 15-50 nucleotides in length, and the double stranded siRNA is about 15-50 base pairs in length, preferably about preferably about 20-30 base nucleotides, preferably about 20-25 or about 24-29 nucleotides in length, e.g., 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

0043 The design and making of siRNA molecules and vectors are well known to those of ordinary skill in the art. For instance, an efficient process for designing a suitable siRNA is
to start at the AUG start codon of the mRNA transcript (e.g., see, Figure 5) and scan for AA
5

dinucleotide sequences (see, Elbashir et al. EMBO J 20: 6877-6888 (2001). Each AA and
the 3' adjacent nucleotides are potential siRNA target sites. The length of the adjacent site
sequence will determine the length of the siRNA. For instance, 19 adjacent sites would give

a 21 Nucleotide long siRNA. siRNAs with 3' overhanging UU dinucleotides are often the

most effective. This approach is also compatible with using RNA pol III to transcribe hairpin

siRNAs. RNA pol III terminates transcription at 4-6 nucleotide poly(T) tracts to create RNA

molecules having a short poly(U) tail. However, siRNAs with other 3' terminal dinucleotide

overhangs can also effectively induce RNAi and the sequence may be empirically selected.

For selectivity, target sequences with more than 16-17 contiguous base pairs of homology to

other coding sequences can be avoided by conducting a BLAST search (see,


[0044] The siRNA can be administered directly or an siRNA expression vectors can be
used to induce RNAi can have different design criteria. A vector can have inserted two

inverted repeats separated by a short spacer sequence and ending with a string of T's which

serve to terminate transcription. The expressed RNA transcript is predicted to fold into a
short hairpin siRNA. The selection of siRNA target sequence, the length of the inverted

repeats that encode the stem of a putative hairpin, the order of the inverted repeats, the length

and composition of the spacer sequence that encodes the loop of the hairpin, and the presence

or absence of 5'-overhangs, can vary. A preferred order of the siRNA expression cassette is

sense strand, short spacer, and antisense strand. Hairp siRNAs with these various stem

lengths (e.g., 15 to 30) can be suitable. The length of the loops linking sense and antisense

strands of the hairpin siRNA lcan have varying lengths (e.g., 3 to 9 nucleotides, or longer).

The vectors may contain promoters and expression enhancers or other regulatory elements

which are operably linked to the nucleotide sequence encoding the siRNA. The expression

"control sequences" refers to DNA sequences necessary for the expression of an operably

linked coding sequence in a particular host organism. The control sequences that are suitable

for prokaryotes, for example, include a promoter, optionally an operator sequence, and a

ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation

signals, and enhancers. These control elements may be designed to allow the clinician to turn

off or on the expression of the gene by adding or controlling external factors to which the

regulatory elements are responsive.
[0045] Construction of suitable vectors containing the desired therapeutic gene coding and control sequences employs standard ligation and restriction techniques, which are well understood in the art (see Maniatis et al., in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1982)). Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

[0046] Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are near each other, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

[0047] "Determining the functional effect" refers to assaying for a compound that increases or decreases a parameter that is indirectly or directly under the influence of a polynucleotide or polypeptide of the invention, e.g., measuring physical and chemical or phenotypic effects. Such functional effects can be measured by any means known to those skilled in the art, e.g., changes in spectroscopic (e.g., fluorescence, absorbance, refractive index), hydrodynamic (e.g., shape), chromatographic, or solubility properties for the protein; measuring inducible markers or transcriptional activation of the protein; measuring binding activity or binding assays, e.g. binding to antibodies; measuring changes in ligand binding affinity; measurement of calcium influx; measurement of the accumulation of an enzymatic product of a polypeptide of the invention or depletion of an substrate; changes in enzymatic activity, e.g., kinase activity, measurement of changes in protein levels of a polypeptide of the invention; measurement of RNA stability; G-protein binding; GPCR phosphorylation or dephosphorylation; signal transduction, e.g., receptor-ligand interactions, second messenger concentrations (e.g., cAMP, IP3, or intracellular Ca2+); identification of downstream or reporter gene expression (CAT, luciferase, β-gal, GFP and the like), e.g., via
chemiluminescence, fluorescence, colorimetric reactions, antibody binding, inducible markers, and ligand binding assays.

[0048] Samples or assays comprising a nucleic acid or protein disclosed herein that are treated with a potential activator, inhibitor, or modulator are compared to control samples without the inhibitor, activator, or modulator to examine the extent of inhibition. Control samples (untreated with inhibitors) are assigned a relative protein activity value of 100%. Inhibition is achieved when the activity value relative to the control is about 80%, preferably 50%, more preferably 25-0%. Activation is achieved when the activity value relative to the control (untreated with activators) is 110%, more preferably 150%, more preferably 200-500% (i.e., two to five fold higher relative to the control), more preferably 1000-3000% higher.

[0049] “Biological sample” includes sections of tissues such as biopsy and autopsy samples, and frozen sections taken for histologic purposes. Such samples include blood and blood fractions or products (e.g., serum, plasma, platelets, red blood cells, and the like), sputum, tissue, cultured cells, e.g., primary cultures, explants, and transformed cells, stool, urine, etc. A biological sample is typically obtained from a eukaryotic organism, most preferably a mammal such as a primate e.g., chimpanzee or human; cow; dog; cat; a rodent, e.g., guinea pig, rat, Mouse; rabbit; or a bird; reptile; or fish.

[0050] A “biopsy” refers to the process of removing a tissue sample for diagnostic or prognostic evaluation, and to the tissue specimen itself. Any biopsy technique known in the art can be applied to the diagnostic and prognostic methods of the present invention. The biopsy technique applied will depend on the tissue type to be evaluated (i.e., prostate, lymph node, liver, bone marrow, blood cell), the size and type of the tumor (i.e., solid or suspended (i.e., blood or ascites)), among other factors. Representative biopsy techniques include excisional biopsy, incisional biopsy, needle biopsy, surgical biopsy, and bone marrow biopsy. An “excisional biopsy” refers to the removal of an entire tumor mass with a small margin of normal tissue surrounding it. An “incisional biopsy” refers to the removal of a wedge of tissue that includes a cross-sectional diameter of the tumor. A diagnosis or prognosis made by endoscopy or fluoroscopy can require a “core-needle biopsy” of the tumor mass, or a “fine-needle aspiration biopsy” which generally obtains a suspension of cells from within the tumor mass. Biopsy techniques are discussed, for example, in Harrison's Principles of Internal Medicine, Kasper, et al., eds., 16th ed., 2005, Chapter 70, and throughout Part V.
The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 60% identity, preferably 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region, when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection (see, e.g., NCBI web site http://www.ncbi.nlm.nih.gov/BLAST/ or the like). Such sequences are then said to be "substantially identical." This definition also refers to, or may be applied to, the complement of a test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 50-100 amino acids or nucleotides in length.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Preferably, default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer

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Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, e.g., *Current Protocols in Molecular Biology* (Ausubel et al., eds. 1995 supplement)).

A preferred example of algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nuc. Acids Res.* 25:3389-3402 (1977) and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

“Nucleic acid” refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form, and complements thereof. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring,
which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

[0056] Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzel et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); Rossolini et al., Mol. Cell. Probes 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

[0057] A particular nucleic acid sequence also implicitly encompasses “splice variants.”

Similarly, a particular protein encoded by a nucleic acid implicitly encompasses any protein encoded by a splice variant of that nucleic acid. “Splice variants,” as the name suggests, are products of alternative splicing of a gene. After transcription, an initial nucleic acid transcript may be spliced such that different (alternate) nucleic acid splice products encode different polypeptides. Mechanisms for the production of splice variants vary, but include alternate splicing of exons. Alternate polypeptides derived from the same nucleic acid by read-through transcription are also encompassed by this definition. Any products of a splicing reaction, including recombinant forms of the splice products, are included in this definition. An example of potassium channel splice variants is discussed in Leicher et al., J. Biol. Chem. 273(52):35095-35101 (1998).

[0058] The terms “polypeptide,” “peptide” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

[0059] The term “amino acid” refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the
genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0060] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0061] “Conservatively modified variants” applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are “silent variations,” which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence with respect to the expression product, but not with respect to actual probe sequences.

[0062] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded
sequence is a “conservatively modified variant” where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[0063] The following eight groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (see, e.g., Creighton, Proteins (1984)).

[0064] A “label” or a “detectable moiety” is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, useful labels include $^{32}$P, fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins which can be made detectable, e.g., by incorporating a radiolabel into the peptide or used to detect antibodies specifically reactive with the peptide.

[0065] The term “recombinant” when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

[0066] The term “heterologous” when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g., a fusion protein).
The phrase “stringent hybridization conditions” refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, “Overview of principles of hybridization and the strategy of nucleic acid assays” (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary “moderately stringent hybridization conditions” include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous reference, e.g., and *Current Protocols in Molecular Biology*, ed. Ausubel, *et al.*, John Wiley & Sons.

For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about
65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C - 95°C for 30 sec - 2 min., an annealing phase lasting 30 sec. - 2 min., and an extension phase of about 72°C for 1 - 2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, e.g., in Innis et al. (1990) PCR Protocols, A Guide to Methods and Applications, Academic Press, Inc. N.Y.).

[0070] “Antibody” refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. Typically, the antigen-binding region of an antibody will be most critical in specificity and affinity of binding. A Reg IV antibody or anti-Reg IV antibody is one which specifically binds and recognizes a Reg IV protein or Reg IV antigen.

[0071] An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one “light” (about 25 kD) and one “heavy” chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

[0072] Antibodies exist, e.g., as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'2, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The F(ab)'2 may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the F(ab)'2 dimer into an Fab’ monomer. The Fab’ monomer is essentially Fab with part of the hinge region (see Fundamental Immunology (Paul ed., 3d ed. 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized de novo either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole
antibodies, or those synthesized de novo using recombinant DNA methodologies (e.g., single chain Fv) or those identified using phage display libraries (see, e.g., McCafferty et al., Nature 348:552-554 (1990))

[0073] For preparation of antibodies, e.g., recombinant, monoclonal, or polyclonal antibodies, many technique known in the art can be used (see, e.g., Kohler & Milstein, Nature 256:495-497 (1975); Kozbor et al., Immunology Today 4: 72 (1983); Cole et al., pp. 77-96 in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. (1985); Coligan, Current Protocols in Immunology (1991); Harlow & Lane, Antibodies, A Laboratory Manual (1988); and Goding, Monoclonal Antibodies: Principles and Practice (2d ed. 1986)). The genes encoding the heavy and light chains of an antibody of interest can be cloned from a cell, e.g., the genes encoding a monoclonal antibody can be cloned from a hybridoma and used to produce a recombinant monoclonal antibody. Gene libraries encoding heavy and light chains of monoclonal antibodies can also be made from hybridoma or plasma cells. Random combinations of the heavy and light chain gene products generate a large pool of antibodies with different antigenic specificity (see, e.g., Kuby, Immunology (3rd ed. 1997)). Techniques for the production of single chain antibodies or recombinant antibodies (U.S. Patent 4,946,778, U.S. Patent No. 4,816,567) can be adapted to produce antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized or human antibodies (see, e.g., U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, Marks et al., Bio/Technology 10:779-783 (1992); Lonberg et al., Nature 368:856-859 (1994); Morrison, Nature 368:812-13 (1994); Fishwild et al., Nature Biotechnology 14:845-51 (1996); Neuberger, Nature Biotechnology 14:826 (1996); and Lonberg & Huszar, Intern. Rev. Immunol. 13:65-93 (1995)). Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (see, e.g., McCafferty et al., Nature 348:552-554 (1990); Marks et al., Biotechnology 10:779-783 (1992)). Antibodies can also be made bispecific, i.e., able to recognize two different antigens (see, e.g., WO 93/08829, Traunecker et al., EMBO J. 10:3655-3659 (1991); and Suresh et al., Methods in Enzymology 121:210 (1986)). Antibodies can also be heteroconjugates, e.g., two covalently joined antibodies, or immunotoxins (see, e.g., U.S. Patent No. 4,676,980, WO 91/00360; WO 92/200373; and EP 03089).

[0074] Methods for humanizing or primatizing non-human antibodies are well known in the art (see, U.S. Patent No. 6,949,245). Generally, a humanized antibody has one or more
amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers (see, e.g., Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-327 (1988); Verhoeyen et al., Science 239:1534-1536 (1988) and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[0075] A “chimeric antibody” is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

[0076] In one embodiment, the antibody is conjugated to an “effector” moiety. The effector moiety can be any number of molecules, including labeling moieties such as radioactive labels or fluorescent labels, or can be a therapeutic moiety. In one aspect the antibody modulates the activity of the protein.

[0077] The phrase “specifically (or selectively) binds” to an antibody or “specifically (or selectively) immunoreactive with,” when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein, often in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and more typically more than 10 to 100 times background. Specific binding to an antibody under such conditions requires an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with the selected antigen and not with other proteins. This selection may be achieved by subtracting out antibodies that cross-react with other
molecules. A variety of immunoassay formats may be used to select antibodies specifically
immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays
are routinely used to select antibodies specifically immunoreactive with a protein (see, e.g.,
Harlow & Lane, Using Antibodies, A Laboratory Manual (1998) for a description of
immunoassay formats and conditions that can be used to determine specific
immunoreactivity).

[0078] By “therapeutically effective dose or amount” herein is meant a dose that produces
effects for which it is administered. The exact dose will depend on the purpose of the
treatment, and will be ascertainable by one skilled in the art using known techniques (see,
e.g., Lieberman, Pharmaceutical Dosage Forms (vols. 1-3, 1992); Lloyd, The Art, Science
and Technology of Pharmaceutical Compounding (1999); Remington: The Science and
Practice of Pharmacy, 20th Edition, Gennaro, Editor (2003), and Pickar, Dosage
Calculations (1999)).

[0079] The term “pharmaceutically acceptable salts” or “pharmaceutically acceptable
carrier” is meant to include salts of the active compounds which are prepared with relatively
nontoxic acids or bases, depending on the particular substituents found on the compounds
described herein. When compounds of the present invention contain relatively acidic
functionalities, base addition salts can be obtained by contacting the neutral form of such
compounds with a sufficient amount of the desired base, either neat or in a suitable inert
solvent. Examples of pharmaceutically acceptable base addition salts include sodium,
potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When
compounds of the present invention contain relatively basic functionalities, acid addition salts
can be obtained by contacting the neutral form of such compounds with a sufficient amount
of the desired acid, either neat or in a suitable inert solvent. Examples of pharmaceutically
acceptable acid addition salts include those derived from inorganic acids like hydrochloric,
hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric,
monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric,
hydrionic, or phosphorous acids and the like, as well as the salts derived from relatively
nontoxic organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic,
suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric,
methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the
like, and salts of organic acids like glucuronic or galacturonic acids and the like (see, e.g.,
compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts. Other pharmaceutically acceptable carriers known to those of skill in the art are suitable for the present invention.

[0080] The neutral forms of the compounds may be regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents, but otherwise the salts are equivalent to the parent form of the compound for the purposes of the present invention.

[0081] In addition to salt forms, the present invention provides compounds which are in a prodrug form. Prodrugs of the compounds described herein are those compounds that readily undergo chemical changes under physiological conditions to provide the compounds of the present invention. Additionally, prodrugs can be converted to the compounds of the present invention by chemical or biochemical methods in an ex vivo environment. For example, prodrugs can be slowly converted to the compounds of the present invention when placed in a transdermal patch reservoir with a suitable enzyme or chemical reagent.

[0082] Certain compounds of the present invention can exist in unsolvated forms as well as solvated forms, including hydrated forms. In general, the solvated forms are equivalent to unsolvated forms and are intended to be encompassed within the scope of the present invention. Certain compounds of the present invention may exist in multiple crystalline or amorphous forms. In general, all physical forms are equivalent for the uses contemplated by the present invention and are intended to be within the scope of the present invention.

[0083] Certain compounds of the present invention possess asymmetric carbon atoms (optical centers) or double bonds; the racemates, diastereomers, geometric isomers and individual isomers are all intended to be encompassed within the scope of the present invention.

**Detailed Embodiments**

[0084] The present invention provides methods of diagnosis and providing a prognosis for individuals at risk for a cancer that overexpresses a Reg IV protein or mRNA transcript, particularly urogenital cancers including prostate and/or bladder cancer. The methods generally comprise contacting a test tissue sample from an individual at risk of having a cancer that overexpresses a Reg IV protein or mRNA transcript with an antibody that
specifically binds to a Reg IV protein; and determining the presence or absence of a Reg IV protein in the test tissue sample in comparison to a control tissue sample from an individual known to be negative for a cancer that overexpresses a Reg IV protein or mRNA transcript. Typically, the tissue sample is serum, but can also be a tissue from a biopsy, particularly from a urogenital tissue including prostate tissue or bladder tissue. Usually, the antibody is a monoclonal antibody. A positive diagnosis for a cancer that overexpresses a Reg IV protein or mRNA transcript is indicated when a higher level of Reg IV protein is detected in a test tissue sample in comparison to a control tissue sample from an individual known not to have cancer, for example, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2-fold, 3-fold, 4-fold, five-fold, eight-fold, ten-fold higher or more. The detection methods can be carried out, for example, using standard ELISA techniques known in the art (reviewed in Gosling, *Immunoassays: A Practical Approach*, 2000, Oxford University Press). Detection is accomplished by labeling a primary antibody or a secondary antibody with, for example, a radioactive isotope, a fluorescent label, an enzyme or any other detectable label known in the art.

[0085] In another embodiment, invention provides methods of diagnosis and providing a prognosis for individuals at risk for a cancer that overexpresses a Reg IV protein or mRNA transcript, particularly a urogenital cancer including prostate and/or bladder cancer, by contacting a test tissue sample from an individual at risk of having a cancer that overexpresses a Reg IV protein or mRNA transcript with a primer set of a first oligonucleotide and a second oligonucleotide that each specifically hybridize to a Reg IV nucleic acid; amplifying the Reg IV nucleic acid in the sample; and determining the presence or absence of the Reg IV nucleic acid in the test tissue sample in comparison to a control tissue sample from an individual known to be negative for a cancer that overexpresses a Reg IV protein or mRNA transcript. Again, usually the tissue sample is serum, but can also be a tissue from a biopsy, particularly a urogenital tissue including a prostate or bladder tissue. A positive diagnosis for a cancer that overexpresses a Reg IV protein or mRNA transcript is indicated when a higher level of Reg IV transcribed RNA is detected in a test tissue sample in comparison to a control tissue sample from an individual known not to have cancer.

[0086] The invention also provides methods for improving the response to cancer therapy in a cancer that overexpresses a Reg IV protein or mRNA transcript by administering a therapeutically effective amount of a compound that inhibits the binding of Reg IV protein to
a Reg IV receptor on a cell of the cancer tumor tissue. In some embodiments the methods of inhibiting Reg IV binding to its receptor are carried out concurrently with another anticancer therapy, including, for example, known chemotherapeutics, immunotherapeutics, and radiotherapy for the reversal of resistance, tumor progression, and metastasis.

[0087] The present invention further provides methods of inhibiting the growth of and promoting the regression of a tumor that overexpresses Reg IV protein, the methods comprising inhibiting the binding of Reg IV protein to a Reg IV receptor on a cell of the tumor tissue. The methods can be carried out by administering to an individual in need thereof a sufficient amount of a compound that inhibits the binding of a Reg IV protein to a Reg IV receptor. In some embodiments, the compound specifically binds to a Reg IV protein. In some embodiments, the compound specifically binds to a Reg IV receptor. In some embodiments, the compound prevents the transcription or the translation of a Reg IV protein. The methods find particular use in treating urogenital cancers, including prostate and bladder cancers. In some embodiments, the compound comprises a polypeptide, including an antibody or an analog or fragment of a Reg IV polypeptide.

[0088] The methods find particular application in the diagnosis, prognosis and treatment of prostate and bladder cancers. In certain embodiments the methods are applied to hormone refractory or therapy resistant cancers. In certain embodiments the methods are applied to metastatic cancers. For example comparisons of differential expression of a Reg IV protein and/or mRNA can be used to determine the stage of cancer of an individual having a cancer that overexpresses a Reg IV protein or mRNA transcript.

[0089] Treatment will generally involve the repeated administration of the anti-RegIV antibodies, immunoconjugates, inhibitors, and siRNA preparations via an acceptable route of administration such as intravenous injection (IV), at an effective dose. Dosages will depend upon various factors generally appreciated by those of skill in the art, including without limitation the type of cancer and the severity, grade, or stage of the cancer, the binding affinity and half life of the agents used, the degree of Reg IV expression in the patient, the extent of circulating shed Reg IV antigen, the desired steady-state antibody concentration level, frequency of treatment, and the influence of chemotherapeutic agents used in combination with the treatment method of the invention. Typical daily doses may range from about 0.1 to 100 mg/kg. Doses in the range of 10-500 mg of the mAb or immunoconjugates per week may be effective and well tolerated, although even higher weekly doses may be
appropriate and/or well tolerated. The principal determining factor in defining the appropriate dose is the amount of a particular agent necessary to be therapeutically effective in a particular context. Repeated administrations may be required in order to achieve tumor inhibition or regression. Initial loading doses may be higher. The initial loading dose may be administered as an infusion. Periodic maintenance doses may be administered similarly, provided the initial dose is well tolerated.

[0090] Direct administration of the agents is also possible and may have advantages in certain contexts. For example, for the treatment of bladder carcinoma, the agents may be injected directly into the bladder. Because agents administered directly to bladder will be cleared from the patient rapidly, it may be possible to use non-human or chimeric antibodies effectively without significant complications of antigenicity.

[0091] The invention further provides vaccines formulated to contain a Reg IV protein or fragment thereof. The use of a tumor antigen in a vaccine for generating humoral and cell-mediated immunity for use in anti-cancer therapy is well known in the art and, for example, has been employed in prostate cancer using human PSMA and rodent PAP immunogens (Hodge et al., 1995, Int. J. Cancer 63: 231-237; Fong et al., 1997, J. Immunol. 159: 3113-3117). Such methods can be readily practiced by employing a Reg IV protein, or fragment thereof, or a Reg IV-encoding nucleic acid molecule and recombinant vectors capable of expressing and appropriately presenting the Reg IV immunogen.

[0092] For example, viral gene delivery systems may be used to deliver a Reg IV-encoding nucleic acid molecule. Various viral gene delivery systems which can be used in the practice of this aspect of the invention include, but are not limited to, vaccinia, fowlpox, canarypox, adenovirus, influenza, poliovirus, adeno-associated virus, lentivirus, and sindbis virus (Rustifo, 1996, Curr. Opin. Immunol. 8: 658-663). Non-viral delivery systems may also be employed by using naked DNA encoding a Reg IV protein or fragment thereof introduced into the patient (e.g., intramuscularly) to induce an anti-tumor response. In one embodiment, the full-length human Reg IV cDNA may be employed. In another embodiment, Reg IV nucleic acid molecules encoding specific cytotoxic T lymphocyte (CTL) epitopes may be employed. CTL epitopes can be determined using specific algorithms (e.g., Epimer, Brown University) to identify peptides within a Reg IV protein which are capable of optimally binding to specified HLA alleles.
Various ex vivo strategies may also be employed. One approach involves the use of dendritic cells to present Reg IV antigen to a patient's immune system. Dendritic cells express MHC class I and II, B7 costimulator, and IL-12, and are thus highly specialized antigen presenting cells. In prostate cancer, autologous dendritic cells pulsed with peptides of the Reg IV can be used to stimulate prostate cancer patients' immune systems (Tjoa et al., 1996, Prostate 28: 65-69; Murphy et al., 1996, Prostate 29: 371-380). Dendritic cells can be used to present Reg IV peptides to T cells in the context of MHC class I and II molecules. In one embodiment, autologous dendritic cells are pulsed with Reg IV peptides capable of binding to MHC molecules. In another embodiment, dendritic cells are pulsed with the complete Reg IV protein. Yet another embodiment involves engineering the overexpression of the Reg IV gene in dendritic cells using various implementing vectors known in the art, such as adenovirus (Arthur et al., 1997, Cancer Gene Ther. 4: 17-25), retrovirus (Henderson et al., 1996, Cancer Res. 56: 3763-3770), lentivirus, adeno-associated virus, DNA transfection (Ribas et al., 1997, Cancer Res. 57: 2865-2869), and tumor-derived RNA transfection (Ashley et al., 1997, J.

Anti-idiotypic anti-Reg IV antibodies can also be used in anti-cancer therapy as a vaccine for inducing an immune response to cells expressing a Reg IV protein. Specifically, the generation of anti-idiotypic antibodies is well known in the art and can readily be adapted to generate anti-idiotypic anti-Reg IV antibodies that mimic an epitope on a Reg IV protein (see, for example, Wagner et al., 1997, Hybridoma 16: 33-40; Foon et al., 1995, J Clin Invest 96: 334-342; Herlyn et al., 1996, Cancer Immunol Immunother 43: 65-76). Such an anti-idiotypic antibody can be used in anti-idiotypic therapy as presently practiced with other anti-idiotypic antibodies directed against tumor antigens.

Genetic immunization methods may be employed to generate prophylactic or therapeutic humoral and cellular immune responses directed against cancer cells expressing Reg IV. Using the Reg IV -encoding DNA molecules described herein, constructs comprising DNA encoding a Reg IV protein/immunogen and appropriate regulatory sequences may be injected directly into muscle or skin of an individual, such that the cells of the muscle or skin take-up the construct and express the encoded Reg IV protein/immunogen. The Reg IV protein/immunogen may be expressed as a cell surface protein or be secreted. Expression of the Reg IV protein/immunogen results in the generation of prophylactic or therapeutic humoral and cellular immunity against prostate cancer. Various prophylactic and therapeutic
genetic immunization techniques known in the art may be used (for review, see information
and references published at internet address www.genweb.com).

[0096] The invention further provides methods for inhibiting cellular activity (e.g., cell
proliferation, activation, or propagation) of a cell expressing multiple Reg IV antigens on its
cell surface. This method comprises reacting the immunoconjugates of the invention (e.g., a
heterogeneous or homogenous mixture) with the cell so that the Reg IV antigens on the cell
surface forms a complex with the immunoconjugates. The greater the number of Reg IV
antigens on the cell surface, the greater the number of Reg IV -antibody complexes can be
used. The greater the number of Reg IV -antibody complexes the greater the cellular activity
that is inhibited.

[0097] A heterogeneous mixture includes Reg IV antibodies that recognize different or the
same epitope, each antibody being conjugated to the same or different therapeutic agent. A
homogenous mixture includes antibodies that recognize the same epitope, each antibody
being conjugated to the same therapeutic agent.

[0098] The invention further provides methods for inhibiting the biological activity of Reg
IV by blocking Reg IV from binding its receptor. The methods comprises contacting an
amount of Reg IV with an antibody or immunoconjugate of the invention under conditions
that permit a Reg IV -immunoconjugate or Reg IV -antibody complex thereby blocking Reg
IV from binding its ligand and inhibiting the activity of Reg IV.

[0099] In some embodiments, the invention provides a method of treating cancer,
particularly a cancer which overexpresses Reg IV, or of inhibiting the growth of a cancer cell
overexpressing a Reg IV protein by treating a subject or contacting the cancer cell with an
antibody or fragment thereof that recognizes and binds the Reg IV protein in an amount
effective to inhibit the growth of the cancer cell. In some embodiments, the cancer cell is a
prostate cancer cell or a bladder cancer cell. The contacting antibody can be a monoclonal
antibody and/or a chimeric antibody. In some embodiments, the chimeric antibody comprises
a human immunoglobulin constant region. In some embodiments, the antibody is a human
antibody or comprises a human immunoglobulin constant region. In further embodiments,
the antibody fragment comprises an Fab, F(ab)_2, or Fv. In other embodiments, the fragment
comprises a recombinant protein having an antigen-binding region.

[0100] In another embodiment, the invention provides methods for treating cancer,
particularly, a cancer overexpressing Reg IV or selectively inhibiting a cell expressing or
overexpressing a Reg IV antigen by reacting any one or a combination of the
immunoconjugates of the invention with the cell in an amount sufficient to inhibit the cell.
Such amounts include an amount to kill the cell or an amount sufficient to inhibit cell growth
or proliferation. As discussed supra the dose and dosage regimen will depend on the nature of
the disease or disorder to be treated associated with Reg IV, its population, the site to which
the antibodies are to be directed, the characteristics of the particular immunotoxin, and the
patient. For example, the amount of immunoconjugate can be in the range of 0.1 to 200
mg/kg of patient weight. The immunoconjugate can comprise the anti-Reg IV antibody or
the fragment linked to a therapeutic agent. The therapeutic agent can be cytotoxic agent. The
cytotoxic agent can be selected from a group consisting of ricin, ricin A-chain, doxorubicin,
daunorubicin, taxol, ethidiuim bromide, mitomycin, etoposide, tenoposide, vincristine,
vindesine, colchicine, dihydroxy anthracin dione, actinomycin D, diphtheria toxin,
Pseudomonas exotoxin (PE) A, PE40, abrin, arbin A chain, modeccin A chain, alpha-sarcin,
gelonin mitogellin, retstrictocin, phenomycin, enomycin, curicin, crotin, calicheamicin,
sapoaaria officiinalis inhibitor, maytansinoids, and glucocorticoidricin. The therapeutic
agent can be a radioactive isotope. The therapeutic isotope can be selected from the group
consisting of $^{212}$Bi, $^{131}$I, $^{111}$In, $^{90}$Y and $^{186}$Re.

[0101] In any of the embodiments above, a chemotherapeutic drug and/or radiation therapy
can be administered further. In some embodiments, the patient also receives hormone
antagonist therapy. The contacting of the patient with the antibody or antibody fragment, can
be by administering the antibody to the patient intravenously, intraperitoneally,
imtramuscularly, intratumorally, or intradermally. In some embodiments, the patient has a
urogenital cancer (e.g., bladder cancer, prostate cancer). In some embodiments of the above,
the patient suffers from prostate cancer and optionally further receives patient hormone
ablation therapy. In some embodiments, the contacting comprises administering the antibody
directly into the cancer or a metastasis of the cancer.

[0102] In some embodiments, the immunoconjugate has a cytotoxic agent which is a small
molecule. Toxins such as maytansin, maytansinoids, saporin, gelonin, ricin or calicheamicin
and analogs or derivatives thereof are also suitable. Other cytotoxic agents that can be
conjugated to the anti-Reg IV antibodies include BCNU, streptozocin, vincristine and 5-
fluorouracil. Enzymatically active toxins and fragments thereof can also be used. The radio-
effecter moieties may be incorporated in the conjugate in known ways (e.g., bifunctional
linkers, fusion proteins). The antibodies of the present invention may also be conjugated to an

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effect of a moiety which is an enzyme which converts a prodrug to an active chemotherapeutic agent. See, WO 88/07378; U.S. Patent No. 4,975,278; and U.S. Patent No. 6,949,245. The antibody or immunoconjugate may optionally be linked to nonprotein polymers (e.g., polyethylene glycol, polypropylene glycol, polyoxalkylenes, or copolymers of polyethylene glycol and polypropylene glycol).

[0103] Conjugates of the antibody and cytotoxic agent may be made using methods well known in the art. For instance, the conjugates may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolylene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. Science 238: 1098 (1987).

Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyl diethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Chari et al. Cancer Research 52: 127-131 (1992)) may be used.

Methods of Administration and Formulation

[0104] The anti-Reg IV antibodies or immunoconjugates are administered to a human patient in accord with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous or subcutaneous administration of the antibody is preferred. The administration may be local or systemic.

[0105] The compositions for administration will commonly comprise an agent as described herein (e.g., Reg IV inhibitors, Reg IV antibodies and immunoconjugates, Reg IV siRNA and vectors thereof) dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., buffered saline and the like. These
solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of active agent in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

[0106] Thus, a typical pharmaceutical composition for intravenous administration will vary according to the agent. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pa. (1980).

[0189] The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include, but are not limited to, powder, tablets, pills, capsules and lozenges. It is recognized that antibodies when administered orally, should be protected from digestion. This is typically accomplished either by complexing the molecules with a composition to render them resistant to acidic and enzymatic hydrolysis, or by packaging the molecules in an appropriately resistant carrier, such as a liposome or a protection barrier. Means of protecting agents from digestion are well known in the art.

[0107] Pharmaceutical formulations, particularly, of the antibodies and immunoconjugates and inhibitors for use with the present invention can be prepared by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers. Such formulations can be lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations used. Acceptable carriers, excipients or stabilizers can be acetate, phosphate, citrate, and other organic acids; antioxidants (e.g., ascorbic acid) preservatives low molecular weight polypeptides; proteins, such as serum albumin or gelatin, or hydrophilic polymers such as polyvinylpyrrolidone; and amino acids, monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating
agents; and ionic and non-ionic surfactants (e.g., polysorbate); salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants. The antibody can be formulated at a concentration of between 0.5 - 200 mg/ml, or between 10-50 mg/ml.

[0108] The formulation may also provide additional active compounds, including, chemotherapeutic agents, cytotoxic agents, cytokines, growth inhibitory agent, and anti-hormonal agent. The active ingredients may also be prepared as sustained-release preparations (e.g., semi-permeable matrices of solid hydrophobic polymers (e.g., polyesters, hydrogels (for example, poly (2-hydroxyethyl-methacrylate), or poly (vinylalcohol)), polylactides. The antibodies and immunoconjugates may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions.

[0109] The compositions can be administered for therapeutic or prophylactic treatments. In therapeutic applications, compositions are administered to a patient suffering from a disease (e.g., cancer) in a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health. Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient. A "patient" or "subject" for the purposes of the present invention includes both humans and other animals, particularly mammals. Thus the methods are applicable to both human therapy and veterinary applications. In the preferred embodiment the patient is a mammal, preferably a primate, and in the most preferred embodiment the patient is human. Other known cancer therapies can be used in combination with the methods of the invention. For example, the compositions for use according to the invention may also be used to target or sensitize a cell to other cancer therapeutic agents such as 5FU, vinblastine, actinomycin D, cisplatin, methotrexate, and the like.

[0110] In other embodiments, the methods of the invention with other cancer therapies (e.g., radical prostatectomy), radiation therapy (external beam or brachytherapy), hormone therapy (e.g., orchiectomy, LHRH-analog therapy to suppress testosterone production, anti-androgen
therapy), or chemotherapy. Radical prostatectomy involves removal of the entire prostate gland plus some surrounding tissue. This treatment is used commonly when the cancer is thought not to have spread beyond the tissue. Radiation therapy is commonly used to treat prostate cancer that is still confined to the prostate gland, or has spread to nearby tissue. If the disease is more advanced, radiation may be used to reduce the size of the tumor. Hormone therapy is often used for patients whose prostate cancer has spread beyond the prostate or has recurred. The objective of hormone therapy is to lower levels of the male hormones, androgens and thereby cause the prostate cancer to shrink or grow more slowly. Luteinizing hormone-releasing hormone (LHRH) agonists decrease the production of testosterone. These agents may be injected either monthly or longer. Two such analogs are leuprolide and goserelin. Anti-androgens (e.g., flutamide, bicalutamide, and nilutamide) may also be used. Total androgen blockade refers to the use of anti-androgens in combination with orchiectomy or LHRH analogs, the combination is called. Chemotherapy is an option for patients whose prostate cancer has spread outside of the prostate gland and for whom hormone therapy has failed. It is not expected to destroy all of the cancer cells, but it may slow tumor growth and reduce pain. Some of the chemotherapy drugs used in treating prostate cancer that has returned or continued to grow and spread after treatment with hormonal therapy include doxorubicin (Adriamycin), estramustine, etoposide, mitoxantrone, vinblastine, and paclitaxel. Two or more drugs are often given together to reduce the likelihood of the cancer cells becoming resistant to chemotherapy. Small cell carcinoma is a rare type of prostate cancer that is more likely to respond to chemotherapy than to hormonal therapy.

[0111] In some embodiments, a "cardioprotectant" is also administered with the antibody Reg IV binding inhibitor or siRNA molecule for use to according to the invention. A cardioprotectant is a compound or composition which prevents or reduces myocardial dysfunction (i.e. cardiomyopathy and/or congestive heart failure) associated with administration of a drug, such as an anthracycline antibiotic to a patient. The cardioprotectant may, for example, block or reduce a free-radical-mediated cardiotoxic effect and/or prevent or reduce oxidative-stress injury. Examples of cardioprotectants encompassed by the present definition include the iron-chelating agent dexrazoxane (ICRF-187) (Seifert et al. The Annals of Pharmacotherapy 28:1063-1072 (1994)); a lipid-lowering agent and/or anti-oxidant such as probucol (Singal et al. J. Mol. Cell Cardiol. 27:1055-1063 (1995)); amifostine (aminothiol 2-[(3-aminopropyl)amino]ethanethiol-dihydrogen phosphate ester, also called WR-2721, and the dephosphorylated cellular uptake form thereof called WR-1065) and S-3-(3-
methylaminopropylamino)propylphosphoro-thioic acid (WR-151327), see Green et al. Cancer Research 54:738-741 (1994); digoxin (Bristow, M. R. In: Bristow M R, ed. Drug-Induced Heart Disease. New York: Elsevier 191-215 (1980)); beta-blockers such as metoprolol (Hjalmarsen et al. Drugs 47:Suppl 4:31-9 (1994)); and Shaddy et al. Am. Heart J. 129:197-9 (1995)); vitamin E; ascorbic acid (vitamin C); free radical scavengers such as oleanolic acid, ursolic acid and N-acetylcysteine (NAC); spin trapping compounds such as alpha-phenyl-tert-butyl nitrate (PBN); (Paracchini et al., Anticancer Res. 13:1607-1612 (1993)); selenoorganic compounds such as P251 (Elbesen); and the like.

[0112] The combined administrations contemplates coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities.

[0113] Molecules and compounds identified that indirectly or directly modulate the expression and/or function of a Reg IV protein can be useful in treating cancers that overexpress Reg IV. Reg IV protein modulators can be administered alone or co-administered in combination with conventional chemotherapy, radiotherapy or immunotherapy as well as currently developed therapeutics.

[0114] Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the packaged nucleic acid suspended in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, e.g., sucrose, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art.

[0115] The compound of choice, alone or in combination with other suitable components, can be made into aerosol formulations (i.e., they can be “nebulized”) to be administered via
inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[0116] Suitable formulations for rectal administration include, for example, suppositories, which consist of the packaged nucleic acid with a suppository base. Suitable suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the compound of choice with a base, including, for example, liquid triglycerides, polyethylene glycols, and paraffin hydrocarbons.

[0117] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intratumoral, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. Parenteral administration, oral administration, and intravenous administration are the preferred methods of administration. The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials.

[0118] Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Cells transduced by nucleic acids for ex vivo therapy can also be administered intravenously or parenterally as described above.

[0119] The pharmaceutical preparation is preferably in unit dosage form. In such form the preparation is subdivided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packeted tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself, or it can be the appropriate number of any of these in packaged form. The composition can, if desired, also contain other compatible therapeutic agents.

[0120] Preferred pharmaceutical preparations deliver one or more active Reg IV protein modulators, optionally in combination with one or more chemotherapeutic agents or immunotherapeutic agents, in a sustained release formulation. Typically, the Reg IV
modulator is administered therapeutically as a sensitizing agent that increases the susceptibility of tumor cells to other cytotoxic cancer therapies, including chemotherapy, radiation therapy, immunotherapy and hormonal therapy.

[0121] In therapeutic use for the treatment of cancer, the Reg IV modulators or inhibitors utilized in the pharmaceutical method of the invention are administered at the initial dosage of about 0.001 mg/kg to about 1000 mg/kg daily. A daily dose range of about 0.01 mg/kg to about 500 mg/kg, or about 0.1 mg/kg to about 200 mg/kg, or about 1 mg/kg to about 100 mg/kg, or about 10 mg/kg to about 50 mg/kg, can be used. The dosages, however, may be varied depending upon the requirements of the patient, the severity of the condition being treated, and the compound being employed. For example, dosages can be empirically determined considering the type and stage of cancer diagnosed in a particular patient. The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector, or transduced cell type in a particular patient. Determination of the proper dosage for a particular situation is within the skill of the practitioner. Generally, treatment is initiated with smaller dosages which are less than the optimum dose of the compound. Thereafter, the dosage is increased by small increments until the optimum effect under circumstances is reached. For convenience, the total daily dosage may be divided and administered in portions during the day, if desired.

[0122] The pharmaceutical preparations (e.g., siRNAs, Reg IV antibodies, Reg IV vaccines, Reg IV inhibitors, and immunoconjugates) for use according to the invention are typically delivered to a mammal, including humans and non-human mammals. Non-human mammals treated using the present methods include domesticated animals (i.e., canine, feline, murine, rodentia, and lagomorpha) and agricultural animals (bovine, equine, ovine, porcine).

**Assays for modulators of Reg IV protein**

[0123] Modulation of a Reg IV protein, and corresponding modulation of cellular, e.g., tumor cell, proliferation, can be assessed using a variety of *in vitro* and *in vivo* assays, including cell-based models. Such assays can be used to test for inhibitors and activators of a Reg IV protein, and, consequently, inhibitors and activators of cellular proliferation, including modulators of chemotherapeutic sensitivity and toxicity. Such modulators of a Reg IV protein are useful for treating disorders related to pathological cell proliferation, e.g.,
cancer. Modulators of Reg IV protein are tested using either recombinant or naturally occurring Reg IV, preferably human Reg IV.

[0124] Measurement of cellular proliferation modulation with a Reg IV protein or a cell expressing a Reg IV protein, either recombinant or naturally occurring, can be performed using a variety of assays, *in vitro*, *in vivo*, and *ex vivo*, as described herein. A suitable physical, chemical or phenotypic change that affects activity, e.g., enzymatic activity such as kinase activity, cell proliferation, or ligand binding (e.g., a Reg IV protein receptor) can be used to assess the influence of a test compound on the polypeptide of this invention. When the functional effects are determined using intact cells or animals, one can also measure a variety of effects, such as, ligand binding, kinase activity, transcriptional changes to both known and uncharacterized genetic markers (e.g., northern blots), changes in cell metabolism, changes related to cellular proliferation, cell surface marker expression, DNA synthesis, marker and dye dilution assays (e.g., GFP and cell tracker assays), contact inhibition, tumor growth in nude mice, etc.

*In vitro assays*

[0125] Assays to identify compounds with Reg IV modulating activity can be performed *in vitro*. Such assays can use a full length Reg IV protein or a variant thereof (see, e.g., Figure 5), or a mutant thereof, or a fragment of a Reg IV protein. Purified recombinant or naturally occurring Reg IV protein can be used in the *in vitro* methods of the invention. In addition to purified Reg IV protein, the recombinant or naturally occurring Reg IV protein can be part of a cellular lysate or a cell membrane. As described below, the binding assay can be either solid state or soluble. Preferably, the protein or membrane is bound to a solid support, either covalently or non-covalently. Often, the *in vitro* assays of the invention are substrate or ligand binding or affinity assays, either non-competitive or competitive. Other *in vitro* assays include measuring changes in spectroscopic (e.g., fluorescence, absorbance, refractive index), hydrodynamic (e.g., shape), chromatographic, or solubility properties for the protein. Other *in vitro* assays include enzymatic activity assays, such as phosphorylation or autophosphorylation assays.

[0126] In one embodiment, a high throughput binding assay is performed in which the Reg IV protein or a fragment thereof is contacted with a potential modulator and incubated for a suitable amount of time. In one embodiment, the potential modulator is bound to a solid support, and the Reg IV protein is added. In another embodiment, the Reg IV protein is
bound to a solid support. A wide variety of modulators can be used, as described below, including small organic molecules, peptides, antibodies, and Reg IV ligand analogs. A wide variety of assays can be used to identify Reg IV-modulator binding, including labeled protein-protein binding assays, electrophoretic mobility shifts, immunoassays, enzymatic assays such as kinase assays, and the like. In some cases, the binding of the candidate modulator is determined through the use of competitive binding assays, where interference with binding of a known ligand or substrate is measured in the presence of a potential modulator.

[0127] In one embodiment, microtiter plates are first coated with either a Reg IV protein or a Reg IV protein receptor, and then exposed to one or more test compounds potentially capable of inhibiting the binding of a Reg IV protein to a Reg IV protein receptor. A labeled (i.e., fluorescent, enzymatic, radioactive isotope) binding partner of the coated protein, either a Reg IV protein receptor or a Reg IV protein, is then exposed to the coated protein and test compounds. Unbound protein is washed away as necessary in between exposures to a Reg IV protein, a Reg IV protein receptor, or a test compound. An absence of detectable signal indicates that the test compound inhibited the binding interaction between a Reg IV protein and a Reg IV protein receptor. The presence of detectable signal (i.e., fluorescence, colorimetric, radioactivity) indicates that the test compound did not inhibit the binding interaction between a Reg IV protein and a Reg IV protein receptor. The presence or absence of detectable signal is compared to a control sample that was not exposed to a test compound, which exhibits uninhibited signal. In some embodiments the binding partner is unlabeled, but exposed to a labeled antibody that specifically binds the binding partner.

Cell-based in vivo assays

[0128] In another embodiment, Reg IV protein is expressed in a cell, and functional, e.g., physical and chemical or phenotypic, changes are assayed to identify Reg IV and modulators of cellular proliferation, e.g., tumor cell proliferation. Cells expressing Reg IV proteins can also be used in binding assays and enzymatic assays. Any suitable functional effect can be measured, as described herein. For example, cellular morphology (e.g., cell volume, nuclear volume, cell perimeter, and nuclear perimeter), ligand binding, kinase activity, apoptosis, cell surface marker expression, cellular proliferation, GFP positivity and dye dilution assays (e.g., cell tracker assays with dyes that bind to cell membranes), DNA synthesis assays (e.g., \(^3\)H-thymidine and fluorescent DNA-binding dyes such as BrdU or Hoechst dye with FACS
analysis), are all suitable assays to identify potential modulators using a cell based system. Suitable cells for such cell based assays include both primary cancer or tumor cells and cell lines, as described herein, e.g., A549 (lung), MCF7 (breast, p53 wild-type), H1299 (lung, p53 null), Hela (cervical), PC3 (prostate, p53 mutant), MDA-MB-231 (breast, p53 wild-type). Cancer cell lines can be p53 mutant, p53 null, or express wild type p53. The Reg IV protein can be naturally occurring or recombinant. Also, fragments of Reg IV or chimeric Reg IV proteins can be used in cell based assays.

[0129] Cellular Reg IV polypeptide levels can be determined by measuring the level of protein or mRNA. The level of Reg IV protein or proteins related to Reg IV are measured using immunoassays such as western blotting, ELISA and the like with an antibody that selectively binds to the Reg IV polypeptide or a fragment thereof. For measurement of mRNA, amplification, e.g., using PCR, LCR, or hybridization assays, e.g., northern hybridization, RNase protection, dot blotting, are preferred. The level of protein or mRNA is detected using directly or indirectly labeled detection agents, e.g., fluorescently or radioactively labeled nucleic acids, radioactively or enzymatically labeled antibodies, and the like, as described herein.

[0130] Alternatively, Reg IV expression can be measured using a reporter gene system. Such a system can be devised using an Reg IV protein promoter operably linked to a reporter gene such as chloramphenicol acetyltransferase, firefly luciferase, bacterial luciferase, β-galactosidase and alkaline phosphatase. Furthermore, the protein of interest can be used as an indirect reporter via attachment to a second reporter such as red or green fluorescent protein (see, e.g., Mistili & Spector, Nature Biotechnology 15:961-964 (1997)). The reporter construct is typically transfected into a cell. After treatment with a potential modulator, the amount of reporter gene transcription, translation, or activity is measured according to standard techniques known to those of skill in the art.

Animal models

[0131] Animal models of cellular proliferation also find use in screening for modulators of cellular proliferation. Similarly, transgenic animal technology including gene knockout technology, for example as a result of homologous recombination with an appropriate gene targeting vector, or gene overexpression, will result in the absence or increased expression of the Reg IV protein. The same technology can also be applied to make knock-out cells. When desired, tissue-specific expression or knockout of the Reg IV protein may be
necessary. Transgenic animals generated by such methods find use as animal models of cellular proliferation and are additionally useful in screening for modulators of cellular proliferation.

[0132] Knock-out cells and transgenic mice can be made by insertion of a marker gene or other heterologous gene into an endogenous Reg IV gene site in the mouse genome via homologous recombination. Such mice can also be made by substituting an endogenous Reg IV with a mutated version of the Reg IV gene, or by mutating an endogenous Reg IV, e.g., by exposure to carcinogens.

[0133] A DNA construct is introduced into the nuclei of embryonic stem cells. Cells containing the newly engineered genetic lesion are injected into a host mouse embryo, which is re-implanted into a recipient female. Some of these embryos develop into chimeric mice that possess germ cells partially derived from the mutant cell line. Therefore, by breeding the chimeric mice it is possible to obtain a new line of mice containing the introduced genetic lesion (see, e.g., Capecchi et al., *Science* 244:1288 (1989)). Chimeric targeted mice can be derived according to Hogan et al., *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory (1988), *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, Robertson, ed., IRL Press, Washington, D.C., (1987), and Pinkert, *Transgenic Animal Technology: A Laboratory Handbook*, Academic Press (2003).

**Exemplary assays**

*Soft agar growth or colony formation in suspension*

[0134] Normal cells require a solid substrate to attach and grow. When the cells are transformed, they lose this phenotype and grow detached from the substrate. For example, transformed cells can grow in stirred suspension culture or suspended in semi-solid media, such as semi-solid or soft agar. The transformed cells, when transfected with tumor suppressor genes, regenerate normal phenotype and require a solid substrate to attach and grow.

[0135] Soft agar growth or colony formation in suspension assays can be used to identify Reg IV modulators. Typically, transformed host cells (e.g., cells that grow on soft agar) are used in this assay. For example, RKO or HCT116 cell lines can be used. Techniques for soft agar growth or colony formation in suspension assays are described in Freshney, *Culture of Animal Cells a Manual of Basic Technique*, 3rd ed., Wiley-Liss, New York (1994), herein
incorporated by reference. See also, the methods section of Garkavtsev et al. (1996), supra, herein incorporated by reference.

Contact inhibition and density limitation of growth

[0136] Normal cells typically grow in a flat and organized pattern in a petri dish until they touch other cells. When the cells touch one another, they are contact inhibited and stop growing. When cells are transformed, however, the cells are not contact inhibited and continue to grow to high densities in disorganized foci. Thus, the transformed cells grow to a higher saturation density than normal cells. This can be detected morphologically by the formation of a disoriented monolayer of cells or rounded cells in foci within the regular pattern of normal surrounding cells. Alternatively, labeling index with $[^3]$H-thymidine at saturation density can be used to measure density limitation of growth. See Freshney (1994), supra. The transformed cells, when contacted with cellular proliferation modulators, regenerate a normal phenotype and become contact inhibited and would grow to a lower density.

[0137] Contact inhibition and density limitation of growth assays can be used to identify Reg IV modulators which are capable of inhibiting abnormal proliferation and transformation in host cells. Typically, transformed host cells (e.g., cells that are not contact inhibited) are used in this assay. For example, RKO or HCT116 cell lines can be used. In this assay, labeling index with $[^3]$H-thymidine at saturation density is a preferred method of measuring density limitation of growth. Transformed host cells are contacted with a potential Reg IV modulator and are grown for 24 hours at saturation density in non-limiting medium conditions. The percentage of cells labeling with $[^3]$H-thymidine is determined autoradiographically. See, Freshney (1994), supra. The host cells contacted with a Reg IV modulator would give arise to a lower labeling index compared to control (e.g., transformed host cells transfected with a vector lacking an insert).

Growth factor or serum dependence

[0138] Growth factor or serum dependence can be used as an assay to identify Reg IV modulators. Transformed cells have a lower serum dependence than their normal counterparts (see, e.g., Temin, J. Natl. Cancer Inst. 37:167-175 (1966); Eagle et al., J. Exp. Med. 131:836-879 (1970)); Freshney, supra. This is in part due to release of various growth factors by the transformed cells. When transformed cells are contacted with a Reg IV
modulator, the cells would reacquire serum dependence and would release growth factors at a lower level.

*Tumor specific markers levels*

[0139] Tumor cells release an increased amount of certain factors (hereinafter “tumor specific markers”) than their normal counterparts. For example, plasminogen activator (PA) is released from human glioma at a higher level than from normal brain cells (see, e.g., Gullino, *Angiogenesis, tumor vascularization, and potential interference with tumor growth.* In Mihich (ed.): “Biological Responses in Cancer.” New York, Academic Press, pp. 178-184 (1985)). Similarly, tumor angiogenesis factor (TAF) is released at a higher level in tumor cells than their normal counterparts. See, e.g., Folkman, *Angiogenesis and cancer, Sem Cancer Biol.* (1992)).

[0140] Tumor specific markers can be assayed to identify Reg IV modulators which decrease the level of release of these markers from host cells. Typically, transformed or tumorigenic host cells are used. Various techniques which measure the release of these factors are described in Freshney (1994), supra. Also, see, Unkless et al., *J. Biol. Chem.* 249:4295-4305 (1974); Strickland & Beers, *J. Biol. Chem.* 251:5694-5702 (1976); Whur et al., *Br. J. Cancer* 42:305-312 (1980); Gulino, *Angiogenesis, tumor vascularization, and potential interference with tumor growth.* In Mihich, E. (ed): “Biological Responses in Cancer.” New York, Plenum (1985); Freshney *Anticancer Res.* 5:111-130 (1985).

*Invasiveness into Matrigel*

[0141] The degree of invasiveness into Matrigel or some other extracellular matrix constituent can be used as an assay to identify Reg IV modulators which are capable of inhibiting abnormal cell proliferation and tumor growth. Tumor cells exhibit a good correlation between malignancy and invasiveness of cells into Matrigel or some other extracellular matrix constituent. In this assay, tumorigenic cells are typically used as host cells. Therefore, Reg IV modulators can be identified by measuring changes in the level of invasiveness between the host cells before and after the introduction of potential modulators. If a compound modulates Reg IV, its expression in tumorigenic host cells would affect invasiveness.

[0142] Techniques described in Freshney (1994), supra, can be used. Briefly, the level of invasion of host cells can be measured by using filters coated with Matrigel or some other
extracellular matrix constituent. Penetration into the gel, or through to the distal side of the filter, is rated as invasiveness, and rated histologically by number of cells and distance moved, or by prelabeling the cells with $^{125}$I and counting the radioactivity on the distal side of the filter or bottom of the dish. See, e.g., Freshney (1984), *supra*.

$G_0/G_1$ cell cycle arrest analysis

[0143] $G_0/G_1$ cell cycle arrest can be used as an assay to identify Reg IV modulators. In this assay, cell lines, such as RKO or HCT116, can be used to screen Reg IV modulators. The cells can be co-transfected with a construct comprising a marker gene, such as a gene that encodes green fluorescent protein, or a cell tracker dye. Methods known in the art can be used to measure the degree of $G_1$ cell cycle arrest. For example, a propidium iodide signal can be used as a measure for DNA content to determine cell cycle profiles on a flow cytometer. The percent of the cells in each cell cycle can be calculated. Cells contacted with a Reg IV modulator would exhibit, e.g., a higher number of cells that are arrested in $G_0/G_1$ phase compared to control.

Tumor growth in vivo

[0144] Effects of Reg IV modulators on cell growth can be tested in transgenic or immune-suppressed mice. Knock-out transgenic mice can be made, in which the endogenous Reg IV gene is disrupted. Such knock-out mice can be used to study effects of Reg IV, e.g., as a cancer model, as a means of assaying in vivo for compounds that modulate Reg IV, and to test the effects of restoring a wild-type or mutant Reg IV to a knock-out mouse.

[0145] Knock-out cells and transgenic mice can be made by insertion of a marker gene or other heterologous gene into the endogenous Reg IV gene site in the mouse genome via homologous recombination. Such mice can also be made by substituting the endogenous Reg IV with a mutated version of Reg IV, or by mutating the endogenous Reg IV, e.g., by exposure to carcinogens.

[0146] A DNA construct is introduced into the nuclei of embryonic stem cells. Cells containing the newly engineered genetic lesion are injected into a host mouse embryo, which is re-implanted into a recipient female. Some of these embryos develop into chimeric mice that possess germ cells partially derived from the mutant cell line. Therefore, by breeding the chimeric mice it is possible to obtain a new line of mice containing the introduced genetic lesion (see, e.g., Capecchi et al., *Science* 244:1288 (1989)). Chimeric targeted mice can be

Alternatively, various immune-suppressed or immune-deficient host animals can be used. For example, genetically athymic “nude” mouse (see, e.g., Giovanella et al., *J. Natl. Cancer Inst.* 52:921 (1974)), a SCID mouse, a thymectomized mouse, or an irradiated mouse (see, e.g., Bradley et al., *Br. J. Cancer* 38:263 (1978); Selby et al., *Br. J. Cancer* 41:52 (1980)) can be used as a host. Transplantable tumor cells (typically about $10^6$ cells) injected into isogenic hosts will produce invasive tumors in a high proportions of cases, while normal cells of similar origin will not. Hosts are treated with Reg IV modulators, e.g., by injection. After a suitable length of time, preferably 4–8 weeks, tumor growth is measured (e.g., by volume or by its two largest dimensions) and compared to the control. Tumors that have statistically significant reduction (using, e.g., Student’s T test) are said to have inhibited growth. Using reduction of tumor size as an assay, Reg IV modulators which are capable, e.g., of inhibiting abnormal cell proliferation can be identified.

**Screening Methods**

The present invention also provides methods of identifying compounds that inhibit the binding of a Reg IV protein to a Reg IV receptor, wherein said compounds find use in inhibiting the growth of and promoting the regression of a tumor that overexpresses Reg IV protein, for example a urogenital cancer tumor, including a prostate or bladder cancer tumor.

Using the assays described herein, one can identify lead compounds that are suitable for further testing to identify those that are therapeutically effective modulating agents by screening a variety of compounds and mixtures of compounds for their ability to decrease, inhibit the binding of a Reg IV protein to a Reg IV receptor. Compounds of interest can be either synthetic or naturally occurring.

Screening assays can be carried out *in vitro* or *in vivo*. Typically, initial screening assays are carried out *in vitro*, and can be confirmed *in vivo* using cell based assays or animal models. For instance, proteins of the regenerating gene family are involved with cell proliferation. Therefore, compounds that inhibit the binding of a Reg IV protein to a Reg IV receptor can inhibit cell proliferation resulting from this binding interaction in comparison to cells unexposed to a test compound. Also, the binding of a Reg IV protein to a Reg IV
receptor is involved with tissue injury responses, inflammation, and dysplasia. In animal models, compounds that inhibit the binding of a Reg IV protein to a Reg IV receptor can, for example, inhibit wound healing or the progression of dysplasia in comparison to an animal unexposed to a test compound. See, for example, Zhang, et al., World J Gastroenter (2003) 9:2635-41.

[0151] Usually a compound that inhibits the binding of Reg IV to a Reg IV receptor is synthetic. The screening methods are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (e.g., in microtiter formats on microtiter plates in robotic assays).

[0152] The invention provides in vitro assays for inhibiting Reg IV binding to its receptor in a high throughput format. For each of the assay formats described, “no modulator” control reactions which do not include a modulator provide a background level of Reg IV binding interaction to its receptor or receptors. In the high throughput assays of the invention, it is possible to screen up to several thousand different modulators in a single day. In particular, each well of a microtiter plate can be used to run a separate assay against a selected potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 100 (96) modulators. If 1536 well plates are used, then a single plate can easily assay from about 100-1500 different compounds. It is possible to assay many different plates per day; assay screens for up to about 6,000-20,000, and even up to about 100,000-1,000,000 different compounds is possible using the integrated systems of the invention. The steps of labeling, addition of reagents, fluid changes, and detection are compatible with full automation, for instance using programmable robotic systems or “integrated systems” commercially available, for example, through BioTX Automation, Conroe, TX; Qiagen, Valencia, CA; Beckman Coulter, Fullerton, CA; and Caliper Life Sciences, Hopkinton, MA.

[0153] Essentially any chemical compound can be tested as a potential inhibitor of Reg IV binding to its receptor for use in the methods of the invention. Most preferred are generally compounds that can be dissolved in aqueous or organic (especially DMSO-based) solutions are used. It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-Biochemica Analytika (Buchs Switzerland), as well as providers of small
organic molecule and peptide libraries ready for screening, including Chembridge Corp. (San
Diego, CA), Discovery Partners International (San Diego, CA), Triad Therapeutics (San
Diego, CA), Nanosyn (Menlo Park, CA), Affymax (Palo Alto, CA), ComGenex (South San
Francisco, CA), and Tripos, Inc. (St. Louis, MO).

In one preferred embodiment, inhibitors of Reg IV-Reg IV receptor binding interaction are identified by screening a combinatorial library containing a large number of potential therapeutic compounds (potential modulator compounds). Such “combinatorial chemical or peptide libraries” can be screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional “lead compounds” or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical “building blocks” such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.


**EXAMPLES**

The following example is offered to illustrate, but not to limit the claimed invention.

**EXAMPLE 1:**

**MATERIALS AND METHODS**

[0158] **Microarray Analysis of Gene Expression.** Tumor samples from a matched pair of androgen dependent and independent LAPC 9 xenografts were grown and prepared as described previously (Craft et al., *Cancer Research*, In Press (1999)). Total RNA was isolated by using Ultraspec RNA isolation systems (Biotecx). mRNA was purified using Oligotex mRNA Midi Kit (Qiagen). 2 micrograms of mRNA was reverse transcribed, and cDNA then labeled with Cy-5. Labeled tumor cDNA was combined with a Cy-3 labeled common reference RNA derived from 11 different cell lines and hybridized to cDNA microarrays containing 22,648 elements representing 17,083 genes, as reported previously (Eisen, M. B. and Brown, P. O., *Methods Enzymol.*, 303: 179-205 (1999)). The slides were scanned with a GenePix microarray scanner (Axon Instruments) and were analyzed with Genepix software. Spots of insufficient quality by visual inspection were excluded from analysis. Data files were entered into the Stanford Microarray Database where spot intensity was correlated with gene identification⁴. Only features with a signal intensity >50% above
background in either Cy5 or Cy3 channel and whose expression varied at least four-fold between the paired samples were retrieved from SMD. Detailed descriptions of array manufacture, hybridization protocols, and data analysis are available on the worldwide web at cmgm.Stanford.EDU/pbrown.

5 **Construction of myc-His Tagged Reg IV Expression Vector.** The Reg IV coding sequence was subcloned into the multiple cloning site of pcDNA3.1/myc-His expression vector (Invitrogen) at the BamHI and EcoRI sites. The reading frame was confirmed by sequencing.

**[0160] RNA Probes and in situ Hybridization.** A 399 by DNA fragment from the 3' untranslated region of Reg IV (GeneBank AI 732541) was inserted into the pCR2.1 vector (Invitrogen) in both sense and antisense orientations under the control of the T7 promoter. Plasmids were linearized and digoxigenin labeled riboprobes were generated using the DIG RNA Labeling Kit (Roche Applied Science). Automated ISH was performed on the Discovery System (Ventana Medical Systems, Tucson, AZ, USA). After deparaffinization, slides were soaked in 2X SSC for 5 min and digested with proteinase K (Gibco BRL, Life Technologies) (final concentration 10 μg/ml) for 30 minutes at 37°C. Sense and anti-sense riboprobes were diluted at 1:100 (1 μg of probe/ml) in hybridization solution (50% deionized formamide, 10% polyethylene glycol, 0.3M NaCl, 10mM Tris pH 8.0, 1mM EDTA, Denhardt's solution 1X, yeast tRNA 500 μg/ml, 50mM DTT). The hybridization was performed for 6 hours at 65°C with Dark blue cytoplasmic staining was scored as positive 100:1 of hybridization solution. After hybridization, slides were washed twice at 70°C for 6 minutes in 1.0X SSC. The hybridization was followed by a 30 minute incubation with a biotinylated anti digoxigenin (Sigma Bio Sciences, St Louis, MO, USA), followed by alkaline phosphatase conjugated streptavidin for 16 minutes. Visualization was in NBT/BCIP (Ventana mapBlue) for 5 hours, and hematoxylin counterstain.

**[0161] Recombinant Reg IV Expression and Immunoprecipitation.** 293T cells were transiently transfected with a myc-His tagged Reg IV expression vector by calcium phosphate precipitation for 48 hours. Cell labeling and immunoprecipitation was performed as described (Reiter et al., Proc Natl Acad Sci U S A, 95: 1735-1740 (1998)). Briefly, cells were labeled with 500 microCi of trans 35S label (ICN) in methionine and lysine free DMEM (Invitrogen) containing 5% of dialysed fetal bovine serum for 4 hours. Cell lysates and conditioned medium were incubated with 3 micrograms of anti-myc monoclonal antibody
(9E10, ATCC) and 20 microliters of protein G-Sepharose CL-4B (Amersham) for 2 hours at 4°C. Samples were washed and boiled in SDS sample buffer for 5 min and separated on 12.5% SDS-PAGE. The gel was treated with Amplify (Amersham) before drying and autoradiographed.

**[0162] Northern Blot Analysis of Gene Expression.** RNAs were extracted as described above. 10 micrograms of RNA was separated on a 1.2% agarose denaturing gel, transferred to nitrocellulose filters, and hybridized with RT-PCR-prepared DNA fragments of Reg IV (GeneBank AI732541). Probes were labeled with alpha $^{32}$PdCTP by random priming using the Random Primer Labeling System (Amersham) and hybridization was carried out at 62°C in 6xSSC overnight, followed by washing with 2xSSC-0.1% SDS and 0.2xSSC-0.1% SDS at 62°C. For multiple tissue northern analysis, the hybridization was performed as described by the manufacture (Clontech).

**[0163] Case Selection for Tissue Microarray.** In order to evaluate Reg IV, we used a prostate cancer progression TMA. This TMA is composed of benign prostate tissue, localized prostate cancer, and hormone refractory metastatic prostate cancer. These cases came from well-fixed radical prostatectomy specimens from the University of Michigan (Ann Arbor, Michigan), the University Hospital Ulm (Ulm, Germany), and the rapid autopsy program from the University of Michigan Specialized Program of Research Excellence in Prostate Cancer (Rubin et al., Clin Cancer Res, 6:1038-1045 (2000)). All samples were collected with prior Institutional Review Board approval at each respective institution. This TMA was composed of classic acinar prostate cancers and areas demonstrating foamy gland features from the same cases. Benign tissue samples were also placed in the TMA to serve as a negative control. A second array containing predominately metastatic cases was also stained and scored.

**[0164] Scoring of Reg IV Expression.** Reg IV expression was determined using a validated scoring method (Varambally et al., Nature, 419: 624-629 (2002); Rubin et al., Jama, 287: 1662-1670 (2002); Dhanasekaran et al., Nature, 412: 822-826 (2001); Kuefner et al., Am J Pathol, 161: 841-848 (2002)) where staining was evaluated for intensity. Benign epithelial glands and prostate cancer cells were scored for Reg IV staining intensity on a 4 tiered system ranging from negative to strong expression. A score of 1 was negative. A score of 2 was considered low expression. A score of 3 indicated moderate expression, and a
score of 4 correlated with strong expression. Slides were read independently by two pathologists (MAR and ML) with >90% interobserver agreement.

[0165] **Construction and production of Lentivirus Expressing Reg IV-Myc.His.** A Myc.His tagged Reg IV construct was PCR amplified from a pcDNA Reg IV-Myc.His vector and inserted into the lentiviral vector CCR through restriction sites of EcoRI and Nhel (Barry et al., *Hum Gene Therapy*, 12: 1103-1108 (2001)). Lentivirus stocks were generated by calcium phosphate mediated transfection of 293T cells. The titer of the virus was checked with 293T cells using CCR EGFP as a positive control and indicator.

[0166] **LNCaP and LAPC-9 Prostate Xenograft models.** 5 x 10^6 LNCaP or LNCaP-Reg IV.Myc.His cells were mixed with equal volume of Matrigel and inoculated into SCID mice subcutaneously. In the case of LAPC 9, explanted tumor is digested with pronase and cultured in 10% FBS-RPMI 1640 for 16 hours. Cells are then transduced by CCR lentivirus vector alone or lentivirus Reg. IV.Myc.His at M.O.I. of 5 for 2 hours. Cells are then washed with culture media, mixed with Matrigel, and inoculated back to SCID mice (1x10^6 cells/mice).

[0167] **Elisa Detection of Serum Reg IV.Myc.His.** 25 microliters of mouse serum was mixed with 75 microliters of PBS and incubated in Ni-NTA HisSorb strips (Qiagen) for 3 hours. The strips were washed with PBS-0.1% Tween-20 (PBST) and then incubated with anti-Myc mAb (Invitrogen) for 45 min. The anti-mouse IgG antibodies conjugated with alkaline phosphatase (Promega) were then incubated for 45 min. The color was developed using 1-step NBT/BCIP (Pierce) for 20 min and read at 450 nm.

[0168] **Statistics.** The Pearson chi-square statistic was used to test whether the rows (e.g. tumor type) and columns (e.g. reg expression) of a table were independent. To test for differences of reg expression across different tumor types, we also used the Kruskal-Wallis test, which is a non-parametric multi-group comparison tests. Statistical analyses were performed using the freely-available R software (http://www.r-project.org/).

**RESULTS**

**Cloning and Characterization of Reg IV**

[0169] The LAPC 9 xenograft was established from metastatic prostate tumors and progresses in vivo from androgen dependence to independence. In order to identify novel candidate markers of prostate cancer progression, RNAs from paired androgen dependent
(AD) and androgen independent (AI) LAPC 9 tumors were labeled and hybridized to 24,000 spot cDNA arrays with common reference RNA. 204 clones representing 101 named genes and 59 ESTs showed expression variation of at least 4-fold between the AD and AI samples. Out of the 101 named genes, 75 have been characterized functionally to some degree and most can be categorized into six biological processes according to Gene Ontology (GO) annotations: cell-cell signaling/signal transduction, cell adhesion/motility, structural molecule/cytoskeleton, immune response, cell proliferation/cell cycle, and metabolism (Table 1). Eleven androgen responsive genes, including well-known androgen targets such as *kk3* (Prostate Specific Antigen), were identified by comparison to the expression profiles of 567 androgen-regulated transcripts we had identified previously (Table 1). With one exception (*WWP1*), all of the genes normally up-regulated by androgens showed decreased expression in the AI tumor growing in the castrated animal, confirming that androgens modulate their expression in vivo.
<table>
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<th>Up-regulated in AI</th>
<th>Down-regulated in AI</th>
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We focused our attention on uncharacterized genes that were differentially expressed between the AD and AI samples. Two of the most highly upregulated transcripts in the AI tumor have extensive homology to the Reg family of secreted C type lectins, a family of proteins normally expressed in the upper gastrointestinal tract and believed to play important roles in response to tissue injury, islet cell regeneration and tumorigenesis. On Northern blot, a single 1.2 kb band was present in multiple independently derived hormone refractory LAPC 9 tumors, but not in the paired parental androgen dependent LAPC 9 tumors or other xenografts (Figure 1a). The microarray result was also confirmed by quantitative PCR, which showed an average 70-fold increase in expression of these ESTs in androgen independent LAPC9 tumors compared with androgen dependent ones. These results demonstrate that two ESTs related to the Reg gene family are reproducibly upregulated during androgen independent progression of LAPC 9.

A full length cDNA was obtained by 5' and 3' RACE PCR, sequenced and found to be identical to Reg IV, a newly described member of the Reg gene family. Reg IV has an open reading frame of 474 bp, predicting a peptide of 158 amino acids with an N terminal signal sequence of 22 amino acids. It is 39% similar to Reg I and Reg III, the other two members of this gene family in humans.

A multiple tissue Northern blot was probed and demonstrated that Reg IV expression is restricted to the gastrointestinal tract, most prominently the colon (Figure 1b). Expression was also seen in pancreas and small intestine (duodenum and jejunum) on a 76-tissue dot blot, suggesting that there may be inter-individual variations in the level and location of Reg IV expression. No expression was seen in prostate on either blot. Digital Northern analysis using the Cancer Genome Anatomy Project (NIH) database confirmed this normal tissue distribution, and also showed that Reg IV ESTs were present in a number of prostate, gastric and colon cancers (UniGene cluster Hs. 105484), suggesting that Reg IV expression may be expressed more broadly in prostate cancer and not limited to LAPC 9.

Reg IV Encodes a Secreted Protein of ~ 20 kd and is Detectable in Serum of Tumor-Bearing Animals

Reg IV is predicted to be a secreted protein based on the presence of a putative signal sequence and on its homology to Reg I and III. To confirm this prediction, we transiently expressed a Myc-tagged Reg IV cDNA construct in 293T cells and harvested the cell pellets and conditioned media. As shown in Figure 1c, the majority of Reg IV protein
was found in the culture medium, consistent with the conclusion that Reg IV is a secreted protein. A single band of -20 kd was identified, again consistent with the predicted molecular weight of Reg IV.

[0174] In order to determine if secreted Reg IV can be detected in the serum of prostate cancer-bearing mice, LNCaP and LAPC-9 prostate cancer cells were stably transduced with lentivirus constructs expressing Myc.His-tagged human Reg IV. Expression of tagged Reg IV was confirmed by Western blot and then tumors were established subcutaneously in SCID mice. Non-Reg IV expressing tumors were also established as controls. Once tumors reached an average size of 1 cm, serum was obtained and the mice were sacrificed. An ELISA assay was developed to detect the presence of the Myc.His tagged protein as described in the Methods section. Control sera were used to normalize for background signal. The sera from animals containing His.Myc. Reg IV positive LNCaP and LAPC 9 tumors were positive, while all control animals were negative. These results suggest that Reg IV is secreted and that it is released into and detectable in serum.

15 **Reg IV is Expressed by High-Risk Tumors Treated with Neoadjuvant Hormone Ablation Therapy**

[0175] Reg IV was identified in hormone refractory LAPC-9 sublines, suggesting that Reg IV is involved in hormone refractory prostate cancer progression. In order to test this hypothesis preliminarily, sense and antisense Reg IV probes were generated and hybridized to four radical prostatectomy specimens obtained from patients with high-risk (high grade, locally advanced) tumors treated with neoadjuvant hormone ablation therapy for three to eight months. All four cases had residual disease, which stained specifically with the antisense Reg IV probe, but not the control sense probe. No staining was seen in residual adjacent normal tissue (Figure 2A). These results demonstrate that Reg IV is expressed in residual hormone refractory prostate cancer. The pre-treatment sample for these patients was not available to test the hypothesis that Reg IV expression was induced by androgen ablation.

[0176] To determine if Reg IV expression is androgen regulated, LAPC 4 and LNCaP cell lines were grown in the absence of androgen and assayed for Reg IV expression. No Reg IV induction was seen after androgen starvation in tissue culture or in hormone refractory variants of these cell lines in vivo (Figure 1A), suggesting that the Reg IV expression seen in hormone refractory LAPC 9 tumors and in tumors treated with neoadjuvant hormone ablation is not regulated simply by the removal of androgen.
[0177] **Reg IV is Strongly Expressed by a Majority of Metastatic Prostate Cancers:** In order to study Reg IV expression further, a tissue array spanning the gamut of prostate histology (n = 211 tissue microarray elements) was evaluated by RNA in situ hybridization (Figure 2B). The percentage of samples staining positive for Reg IV increased from benign to clinically localized to metastatic prostate cancer. None of 48 evaluable benign specimens expressed Reg IV, whereas 44.6% (25/56) of primary tumors and 62.5% (40/64) of metastatic tumors stained positively (Table 2). These differences (between normal and primary tumors, and between primary tumors and metastases) were statistically significant (p = 0.00000038 and one sided p = 0.038, respectively) and show that the prevalence of Reg IV expression increases as prostate cancers progress.

| Table 2: Distribution of Reg IV Expression on a Prostate Cancer Tissue Array |
|-----------------------------------|--------|-------|-------|--------|
| None                | Weak    | Moderate | Strong |
| Benign              | 48      | 0      | 0      | 0      |
| Primary             | 31 (55.4%) | 15 (26.7%) | 8 (14%) | 2 (3.6%) |
| Metastatic          | 24 (37.5%) | 5 (7.8%)  | 11 (17%) | 24 (37.5%) |

[0178] **We also evaluated the relative level of Reg IV expression in benign, localized and metastatic tumors.** As shown in Figure 3, the overall intensity of Reg IV staining increased from benign to clinically localized to metastatic prostate cancer, with a median staining intensity of 1.0, 1.7 and 2.5, respectively (Kruskal Wallace test; p < 0.001) (note that a score of 1 means no detectable expression). Whereas a majority of positive localized tumors expressed only weak levels of Reg IV, a majority of positive metastatic tumors stained strongly (Table 2). The increase in Reg IV staining intensity between benign prostate tissue and localized prostate cancer was statistically significant (p = 0.00000016). Likewise, metastatic prostate cancer had statistically higher expression of Reg IV than localized prostate cancer (Kruskal Wallace test; p < 0.00033). These differences show that the level of Reg IV expression increases as prostate cancers progress, particularly in metastatic cancer.

[0179] **We also asked whether Reg IV expression is associated with tumor grade in localized tumors.** As shown in Figure 4, Reg IV expression was significantly more intense among high grade tumors (i.e. Gleason 7-10) than in low grade ones (i.e. Gleason 5-6) (Mann-
Whitney test p=0.03). There was no association of Reg IV expression with recurrence or survival.

[0180] To confirm the high intensity expression in metastatic prostate cancer, we also evaluated an array containing 259 metastases obtained from 24 patients who died of hormone-refractory metastatic prostate cancer. The mean staining intensity in autopsy cases was 3.2, similar to that in the "progression" array. Benign prostate tissue on this array was negative. Among positive tumors, almost all cells stained positive, again similar to the "progression" array. These results confirm that as prostate cancer progresses, there is increasing expression of Reg IV. Expression is highest in hormone refractory metastatic tumors.

DISCUSSION

[0181] The two seminal events in the natural history of prostate cancer are metastasis and progression to androgen independence. The ability to predict at diagnosis the clinical course of an individual tumor is currently suboptimal. Thirty percent of clinically localized tumors recur after local therapy and a subset of these go on to metastasize and kill their host. The association of Reg IV expression with androgen independence and metastasis raises the possibility that expression of Reg IV may correlate with the risk of progression to hormone refractory metastasis. Expression of the Reg IV homologues Reg Iα and PAP has been reported to predict for reduced survival from colon cancer (Macadam et al., Br J Cancer, 83: 188-195 (2000)). Indeed, we found that increasing Reg IV expression did correlate with higher grade primary tumors, suggesting that Reg IV expression may have prognostic utility in primary tumors. However, there was no association with recurrence in this initial small series. Analysis of Reg IV expression in a larger patient cohort with long-term follow-up will be necessary to determine its relationship to recurrence and prostate cancer survival. None of the patients with localized tumors in our database went on to die from prostate cancer.

[0182] Because Reg IV is secreted, it is also useful as a serum marker to identify patients with metastasis or at risk to develop metastases. This possibility is supported by the ability to detect Reg IV in the serum of tumor-bearing animals. Antibodies against Reg IV are currently being generated to assess Reg IV protein expression in tissue samples and to measure circulating Reg IV levels in normal and cancer patients. An important issue will be to determine if Reg IV expression in the gastrointestinal tract interferes with the detection of Reg IV from tumor tissue.
[0183] Reg IV was cloned from a hormone refractory xenograft and is expressed by both androgen resistant local tumors and metastases. It is not known whether Reg IV expression is related specifically to androgen independence and/or metastasis, since all of the metastases were obtained from hormone refractory patients. Reg IV expression does not appear to be regulated by androgen, since androgen starvation of both LAPC-4 and LNCaP prostate cancer cell lines in tissue culture did not result in Reg IV expression. Nor did androgen independent sublines of LNCaP or LAPC-4 express Reg IV in vivo.

[0184] Reg proteins have been associated with proliferation and regeneration, cell survival, resistance to apoptosis and cell adhesion. Hartupee and associates reported that Reg IV is highly expressed in ulcerative colitis and hypothesized that it might be related to the high rate of colon cancer in individuals with this disease (Hartupee et al., Biochim Biophys Acta, 1518: 287-293 (2001)). Violette et al. found a consistent relationship between Reg IV expression and chemotherapy resistance in colon cancer cell lines (Violette et al., Int J Cancer, 103: 185-193 (2003)). They found that Reg IV is expressed by 5/7 chemoresistant lines, but is absent from all chemosensitive lines. Importantly, they noted that Reg IV is expressed by LS513, a cell line that survives but does not proliferate in the presence of chemotherapy, suggesting that Reg IV may be a survival factor rather than a mitogen. Similarly, recent studies have demonstrated that Reg I alpha is a signaling intermediate in a survival pathway in motoneurons (Nishimune et al., Nat Cell Biol, 2: 906-914 (2000)). The hypothesis that Reg IV plays a role in cell survival is consistent with its expression in hormone refractory prostate cancer. The association of Reg IV expression with chemotherapy resistance is also consistent with the fact that a majority of patients with lethal prostate cancer metastases on our tissue array received chemotherapy during their clinical course.

[0185] Reg IV is the second gastrointestinal secreted protein that we have identified in prostate cancer. Intestinal trefoil factor (ITF/TFF3) was initially identified in prostate cancer arrays and has since been reported to be expressed by ~40% of localized prostate cancers and a higher percentage of metastases (Luo et al., Cancer Res, 61: 4683-4688 (2001); Garraway et al., Prostate. 61: 209-214 (2004); Faith et al., Prostate, 61: 215-227 (2004)). Trefoil factors are known to play an important role in intestinal protection and restitution, a process in which mucosal continuity is reestablished following tissue injury, whereas Reg proteins are believed to play a role in tissue regeneration (Taupin et al., Nat Rev Mol Cell Biol, 4: 721-732 (2003)). Both Reg and trefoil proteins are overexpressed in inflammatory bowel disease. Both are also overexpressed in malignancy. TFF3, for example, is an adverse prognostic
factor in gastric cancer (Taupin et al., Nat Rev Mol Cell Biol, 4: 721-732 (2003)). It will be important to understand the reasons why a number of related gastrointestinal proteins are expressed in prostate cancer, their regulation and their functions as paracrine or autocrine factors. The data are consistent with the conclusion that Reg I, Reg IV, TFF3 and their receptors are useful therapeutic targets for the management of prostate cancer (see also, Kobayashi et al., J Biol Chem, 275: 10723-10726 (2000)).

[0186] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.
WHAT IS CLAIMED IS:

1. A method of diagnosing a urogenital cancer that overexpresses a Reg IV protein, the method comprising the steps of:
   (a) obtaining a test tissue sample from an individual at risk of having a cancer that overexpresses a Reg IV protein; and
   (b) determining the presence or absence or amount of the Reg IV protein in the test tissue sample in comparison to a control tissue sample from an individual known to be negative for the cancer; thereby diagnosing said cancer that overexpresses a Reg IV protein.

2. The method of claim 1, wherein the test tissue is contacted with an antibody that specifically binds to a Reg IV protein, whereby the overexpression of the Reg IV protein is determined.

3. The method of claim 1, wherein Reg IV mRNA is also overexpressed and the test tissue sample is contacted with a primer set of a first oligonucleotide and a second oligonucleotide that each specifically hybridize to the Reg IV mRNA nucleic acid to amplify the Reg IV mRNA nucleic acid; whereby the overexpression of the Reg IV protein is also determined.

4. The method of claim 1, wherein said tissue sample is a serum or a blood sample.

5. The method of claim 1, wherein said tissue sample is prostate or bladder tissue.

6. The method of claim 1, wherein said cancer is a prostate cancer.

7. The method of claim 6, wherein said cancer is a bladder cancer.

8. The method of claim 1, wherein said cancer is a hormone refractory prostate cancer.

9. The method of claim 1, wherein said cancer is a metastatic cancer.

10. The method of claim 1, wherein said antibody is a monoclonal antibody.
11. The method of claim 1, wherein the overexpression is by at least 50% greater than levels in the control sample.

12. The method of claim 3, wherein the determining of the presence or absence or amount of the mRNA transcript is by PCR.

13. A method of providing a cancer prognosis, the method comprising the steps of:
(a) contacting a test tissue sample from an individual at risk of having the cancer; and
(b) determining the presence or absence or amount of the Reg IV protein in the test tissue sample in comparison to a control tissue sample from an individual known to be negative for the cancer; thereby identifying the cancer as overexpressing a Reg IV mRNA transcript.

14. The method of claim 13, wherein the test tissue sample is contacted with a primer set of a first oligonucleotide and a second oligonucleotide that each specifically hybridize to a Reg IV mRNA nucleic acid to amplify the Reg IV mRNA nucleic acid in the sample; whereby the presence or absence or the amount of the Reg IV protein is determined.

15. The method of claim 13, wherein the test tissue sample is contacted with an antibody that specifically binds to the Reg IV protein whereby the comparative presence or absence or the amount of the Reg IV protein is determined.

16. The method of claim 13, wherein the cancer is a urogenital cancer.

17. The method of claim 13, wherein the cancer is prostate cancer.

18. The method of claim 13, wherein the cancer is bladder cancer.

19. The method of claim 13, wherein an overexpression of Reg IV indicates that the cancer is likely to become invasive, metastasize, become hormone independent, or become refractory treatment.

20. The method of claim 19, wherein the overexpression is by at least two-fold over the control sample.
21. A method of identifying a compound that inhibits a cancer associated with the overexpression of Reg IV, the method comprising the steps of:
   (a) contacting a cell expressing Reg IV protein and a Reg IV receptor with a compound; and
   (b) determining whether said compound inhibits the binding of Reg IV protein to the Reg IV receptor; thereby identifying a compound that inhibits a cancer associated with the overexpression of Reg IV.

22. The method of claim 21, wherein said cancer is a urogenital cancer.

23. The method of claim 22, wherein said cancer is a prostate cancer or a bladder cancer.

24. A method of treating cancer overexpressing Reg IV, the method comprising administering a therapeutically effective amount of a compound that inhibits the binding of Reg IV protein to a Reg IV receptor in a prostate tissue cell.

25. The method of claim 24, wherein said cancer is a urogenital cancer.

26. The method of claim 25, wherein said cancer is a prostate cancer or a bladder cancer.

27. The method of treating cancer, said method comprising administration of a therapeutically effective amount of an antibody which binds to Reg IV.

28. The method of claim 27, wherein the method inhibits the invasiveness or metastasis of the cancer.

29. The method of claim 27, wherein the cancer is prostate or bladder cancer.

30. The method of any of claims 27 to 29, wherein the cancer overexpresses Reg IV.

31. A method of treating cancer, said method comprising administration of a therapeutically effective amount of an antibody which binds to Reg IV, wherein the antibody is conjugated to an effector moiety.
32. The method of claim 31, wherein the method inhibits the invasiveness or metastasis of the cancer.

33. The method of claim 31, wherein the cancer is prostate or bladder cancer.

34. The method of any of claims 31 to 33, wherein the cancer overexpresses Reg IV.

35. The method of claim 31, wherein the effector molecule is a cytotoxic agent.

36. The method of claim 35, wherein the cytotoxic agent is selected from the group consisting of ricin, ricin A-chain, doxorubicin, daunorubicin, taxol, ethidium bromide, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicine, dihydroxy anthracin dione, actinomycin D, diphteria toxin, Pseudomonas exotoxin (PE) A, PE40, abrin, abrin A chain, modeccin A chain, alpha-sarcin, gelonin mitogellin, restrictocin, phenomycin, enomycin, curicin, crotin, calicheamicin, sapaonaria officinals inhibitor, maytansinoids, and glucocorticoidricin.

37. A method of treating cancer, said method comprising administration of an siRNA which is capable of inhibiting or silencing the expression of Reg IV.

38. The method of claim 37, wherein the expression of Reg IV is inhibited and the RNAi has a sequence which is identical to a nucleic acid sequence of Figure 5.

39. The method of claim 37, wherein the cancer is prostate or bladder cancer.

40. The method of any of claims 37 to 39, wherein the cancer overexpresses Reg IV.

41. The method of any one of claims claim 37 to 39, wherein the siRNA is short hairpin RNA.
42. The method of any one of claims 37 to 41, wherein the method inhibits the invasiveness or metastasis of the cancer.

43. A method of treating a cancer patient, comprising determining whether a cancer is likely to become invasive, metastasize, hormone independent, or refractory treatment according to the method of claim 13, and administering a chemotherapeutic agent, an immunotherapeutic agent, hormonal therapy, or radiotherapy according to whether there is an increased likelihood of the cancer becoming invasive, metastasizing, hormone independent, or refractory to treatment.

44. The method of claim 43, wherein the cancer is a urogenital cancer.

45. The method of claim 43, wherein the cancer is prostate cancer.

46. The method of claim 43, wherein the chemotherapeutic agent is selected from the group consisting of ricin, ricin A-chain, doxorubicin, daunorubicin, taxol, ethidium bromide, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicine, dihydroxy anthracin dione, actinomycin D, diphteria toxin, Pseudomonas exotoxin (PE) A, PE40, abrin, abrin A chain, modeccin A chain, alpha-sarcin, gelonin mitogellin, retstrictocin, phenomycin, enomycin, curcin, crotin, calicheamicin, sapaonaria officinalis inhibitor, maytansinoids, and glucocorticoidricin.

47. The method of claim 45, wherein the patient is treated by radical prostatectomy, radiation therapy, hormone therapy, or chemotherapy.

48. The method of claims 43 to 47, wherein the overexpression of Reg IV is by at least four-fold over the control sample.

49. The method of claim 43, wherein a Reg IV inhibitor, Reg IV siRNA, or anti-Reg IV antibody is also administered.

50. The use of Reg IV protein or mRNA as a target in the diagnosis, prognosis, or treatment of cancer.

51. The use of claim 50, wherein the cancer is prostate cancer or bladder cancer.
52. The use of claim 50, wherein the cancer overexpresses Reg IV.

53. The use of claim 51, wherein the cancer overexpresses Reg IV by at least four-fold.

54. The use of claim 50, wherein the Reg IV protein is contacted with an anti-Reg IV antibody.

55. The use of claim 50, wherein siRNA which is capable of interfering with the expression of the mRNA is administered to a subject having a cancer which overexpresses Reg IV.

56. The use of claim 50 or 51, wherein the cancer is invasive or refractory to treatment.
FIG. 3
FIG. 4
Homo sapiens regenerating gene type IV (Reg IV) mRNA:

actggagcgcaagtgaagcccttagatgctggttgcacaacagattttcagatcagataaggaacaggtgctgctctctgttagtacagggtctgagatccttgcatagctacatctcaggttagaggaagagatggtttcagagacaagcagtccgtcctgtgtgtttacacaaatgcaattgctaggttatcaggaagctgaggaactgtctgtatgtgctcgccagaagttcgtctgcgcagctgatcctgtggtactctagagaagccctgtttacgagagaagcctccctccacggatattatatatataa

Total length: 882 bp

REG IV PROTEIN SEQUENCE

MASRSMRLLLCLSCLAKTGVLDIIMRPSCAPGWYHKSNACYGFRLNWSDAEL
ECQSYGNAGAHLISLKEASTAYISGQRSQIPWGLHDPQKRQWOQWIDGAMY
LYRWSGKSMGNNKHCAEMSSNNFITWSNENCNKRQHFLCKYRP

Total length: 158 amino acids

FIG. 5

SUBSTITUTE SHEET (RULE 26)
# INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**

| IPC: | A61K 38/00(2006.01),31/70(2006.01),31/415(2006.01),31/34(2006.01);A01N 43/04(2006.01),43/56(2006.01),43/08(2006.01) |

**USPC: 514/12, 44, 406, 471**

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

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## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S.: 514/12, 44, 406, 471

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)

MEDLINE, WEST

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## C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>3-9, 11, 16-20, 22-30, 33, 37-39, 41, 43-45 and 51</td>
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</table>

Further documents are listed in the continuation of Box C.

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Date of the actual completion of the international search

19 June 2006 (19.06.2006)

Date of mailing of the international search report

26 JUL 2006

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Facsimile No. (571) 272-3201

Authorized officer

Alana M. Harris, Ph.D. 

Telephone No. (571) 272-1600

Form PCT/ISA/210 (second sheet) (April 2005)
<table>
<thead>
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<th>Category</th>
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<th>Relevant to claim No.</th>
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<tbody>
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<td>US 2004/0242526 A1 (DIECKGRAEF et al) 02 December 2004 (02.12.2004), see abstract; page 2, section 0007, 0011-0015; page 3, sections 0021-0023; page 4, section 0026; pages 13 and 14, the claims.</td>
<td>14, 37-53, 55 and 56</td>
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<td>1-13, 15-36 and 54</td>
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</tbody>
</table>
INTERNATIONAL SEARCH REPORT

Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 42
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest  ☐ The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.