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(54) Titre : AMELIORATION DE LA REPONSE AU STRESS CELLULAIRE  
(54) Title: AMELIORATION OF CELLULAR STRESS RESPONSE

(57) **Abrégé/Abstract:**

The present invention is directed to compositions and methods useful for treating cancer and for amelioration of cellular stress response triggered by chemotherapy.



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**(57) Abstract:** The present invention is directed to compositions and methods useful for treating cancer and for amelioration of cellular stress response triggered by chemotherapy.

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**AMELIORATION OF CELLULAR STRESS RESPONSE**RELATED APPLICATIONS

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This application claims priority to and the benefit of United States Provisional Application Serial No. 60/892,806, filed March 2, 2007, which is incorporated by reference in its entirety herein.

FIELD OF THE INVENTION

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The present invention is directed to compositions of matter useful for the treatment of cancer in mammals and to methods of using those compositions of matter for the same.

BACKGROUND OF THE INVENTION

20

Malignant tumors (cancers) are the second leading cause of death in the United States, after heart disease (Boring et al., *CA Cancer J. Clin.* 43:7 (1993)). Cancer is characterized by the increase in the number of abnormal, or neoplastic, cells derived from a normal tissue which proliferate to form a tumor mass, the invasion of adjacent tissues by these neoplastic tumor cells, and the generation of malignant cells which eventually spread via the blood or lymphatic system to regional lymph nodes and to distant sites via a process called metastasis. In a cancerous state, a cell proliferates under conditions in which normal cells would not grow. Cancer manifests itself in a wide variety of forms, characterized by different degrees of invasiveness and aggressiveness.

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In attempts to discover effective cellular targets for cancer therapy, researchers have sought to identify transmembrane or otherwise membrane-associated polypeptides that are specifically expressed on the surface of one or more particular type(s) of cancer cell as compared to on one or more normal non-cancerous cell(s). Often, such membrane-associated polypeptides are more abundantly expressed on the surface of the cancer cells as compared to on the surface of the non-cancerous cells. The identification of such tumor-associated cell surface antigen polypeptides has given rise to the ability to specifically target cancer cells for destruction via antibody-based therapies. In this regard, it is noted that antibody-based therapy has proved very effective in the treatment of certain cancers. For example, HERCEPTIN® and RITUXAN® (both from Genentech Inc., South San Francisco, California) are antibodies that have been used successfully to treat breast cancer and non-Hodgkin's lymphoma, respectively. More specifically, HERCEPTIN® is a recombinant DNA-derived humanized monoclonal antibody that selectively binds to the extracellular domain of the human epidermal growth factor receptor 2 (HER2) proto-oncogene. HER2 protein overexpression is observed in 25-30% of primary breast cancers.

RITUXAN® is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes. Both these antibodies are recombinantly produced in CHO cells.

In other attempts to discover effective cellular targets for cancer therapy, researchers have sought to identify (1) non-membrane-associated polypeptides that are specifically produced by one or more particular type(s) of cancer cell(s) as compared to by one or more particular type(s) of non-cancerous normal cell(s), (2) polypeptides that are produced by cancer cells at an expression level that is significantly higher than that of one or more normal non-cancerous cell(s), or (3) polypeptides whose expression is specifically limited to only a single (or very limited number of different) tissue type(s) in both the cancerous and non-cancerous state (e.g., normal prostate and prostate tumor tissue). Such polypeptides may remain intracellularly located or may be secreted by the cancer cell. Moreover, such polypeptides may be expressed not by the cancer cell itself, but rather by cells which produce or secrete polypeptides having a potentiating or growth-enhancing effect on cancer cells. Such secreted polypeptides are often proteins that provide cancer cells with a growth or survival advantage over normal cells and include such things as, for example, angiogenic factors, cellular adhesion factors, growth factors, and the like. Identification of antagonists of such non-membrane associated polypeptides would be expected to serve as effective therapeutic agents for the treatment of such cancers. Furthermore, identification of the expression pattern of such polypeptides would be useful for the diagnosis of particular cancers in mammals. Despite the above-identified advances in mammalian cancer therapy, there is a great need for additional therapeutic agents capable of detecting the presence of tumor in a mammal and for effectively inhibiting neoplastic cell growth or survival, respectively, especially in prostate cancer.

Prostate cancer is a leading cause of cancer mortality among males in the United States, and is the most common malignancy in this population (Landis et al., CA Cancer J. Clin. 49: 8-31, 1999). The initial stage of this class of cancers is generally characterized by androgen-dependent tumor cells, and is treated by androgen depletion. As the cancer progresses, it generally becomes androgen-refractory, and no effective specific therapy is yet available.

Traditional treatment of cancer using chemotherapy often has unintended consequences that interfere with the success of the treatment. Of particular importance is the triggering of the cellular stress response by administration of certain chemotherapeutic agents. The cellular stress response may provide a survival advantage to the cancer cells targeted by the chemotherapy.

### SUMMARY OF THE INVENTION

The present invention relates to the role of RA1c in the stress responses of cancerous cells. In one aspect, the invention provides a method of ameliorating cellular stress response in a cell comprising contacting the cell, preferably a cancer cell, more preferably a prostate cancer tumor cell, with a compound that decreases or blocks RA1c expression or activity in the cell. In one embodiment, the cellular stress response is induced by a chemotherapeutic agent. Preferably, the compound increases the sensitivity of the cell to the chemotherapeutic agent.

Another aspect of the invention provides a method of ameliorating cellular stress response in prostate cancer tumor cells comprising contacting the tumor cells with a compound that decreases or blocks the expression or activity of RA1c. In one embodiment, the tumor cells are undergoing chemotherapy with a chemotherapeutic agent that induces cellular stress response. Preferably, the compound increases the sensitivity of the tumor cells to the chemotherapeutic agent. Tumor cells that are sensitive to the chemotherapeutic agent can, for example, undergo apoptosis at a higher rate than cells not contacted with the compound or undergo growth arrest at a higher rate than cells not contacted with the compound.

Yet another aspect provides a method of ameliorating cellular stress response in the cells of a patient. This method comprises administering to the patient an effective amount of a compound that decreases or blocks the expression or activity of RA1c. In one embodiment, the patient is a cancer patient. In another embodiment, the cells are prostate cancer tumor cells. In yet another embodiment, the tumor cells are undergoing chemotherapy with a chemotherapeutic agent that induces cellular stress response. Preferably, the compound increases the sensitivity of the tumor cells to the chemotherapeutic agent. Tumor cells that are sensitive to the chemotherapeutic agent can, for example, undergo apoptosis at a higher rate than cells not contacted with the compound or undergo growth arrest at a higher rate than cells not contacted with the compound.

In these aspects of the invention, the chemotherapeutic agent is preferably an mTor inhibitor or an Akt1/2 inhibitor. Examples of suitable mTor inhibitors include rapamycin, CCI-779, RAD001, AP23573, XL7F65, and TAFA93, and active derivatives or analogs thereof. Examples of suitable Akt1/2 inhibitors include GSK690693, perifosine, and XL418, and active derivatives or analogs thereof.

The compound used in some embodiments of the various aspects of the invention is an RA1c antagonist, such as an antibody, an antibody fragment, an antibody conjugate, an aptamer, a small molecule, or an oligopeptide. Preferably, the RA1c antagonist specifically blocks RA1c activity. In other embodiments, the compound decreases expression of RA1c. In yet other embodiments, the compound blocks the expression of RA1c. Compounds useful in decreasing or

blocking expression of RA1c include antisense polynucleotides, silencing RNA molecules, catalytic RNA molecules, and RNA-DNA chimeras.

Another aspect of the invention provides a composition comprising a RA1c antagonist, such as an anti-RA1c antibody, and a compound that increases the expression of RA1c, such as rapamycin, CCI-779, RAD001, AP23573, XL7F65, TAFA93, SK690693, perifosine, and XL418, or active derivatives or analogs thereof, optionally further comprising a pharmaceutically acceptable carrier.

Yet another aspect of the invention provides a method of determining whether a chemotherapeutic agent induces cellular stress response in a cancer cell, preferably a prostate cancer cell, more preferably an LNCaP cell. The method comprises contacting the cancer cell with the chemotherapeutic agent and determining the level of RA1c expression in the cancer cell. An increase in RA1c expression over a control cell, or over the level of RA1c expression in the cancer cell prior to contacting with the chemotherapeutic agent, indicates induction of cellular stress response. The chemotherapeutic agent is preferably an mTor inhibitor or an Akt1/2 inhibitor, and active derivatives or analogs thereof.

A further aspect of the invention provides a method of treating cancer with a combination therapy. The method comprises the steps of contacting a cancer cell with a chemotherapeutic agent that increases expression of RA1c, and contacting the cancer cell with an effective amount of a compound that specifically binds to the RA1c polypeptide, such as an anti-RA1c antibody. The antibody is preferably conjugated to a cytotoxic agent. The cancer treating effect of the combination of the chemotherapeutic agent and the compound is synergistic over the effect of either the chemotherapeutic agent or compound alone. In one embodiment, the chemotherapeutic agent is an mTOR or an Akt1/2 inhibitor.

Yet further embodiments of the present invention will be evident to the skilled artisan upon a reading of the present specification.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B depict the results of oligo array analyses of tissue and cell line expression of RA1c, as described in Example 1. In Figure 1A, normal tissue is the leftmost result for each tissue sample and cancer/other diseases are the rightmost result for each tissue sample.

Figures 2A-2C show the results of quantitative real-time RT-PCR (Q-PCR) analyses of RA1c expression in LNCaP cells in response to stimulation by certain cytokines, as described in Example 2.

Figures 3A-3B show the results of experiments testing the effects of serum deprivation on RA1c expression in LNCaP cells in the absence or presence of IL-6, as described in Example

2. Figure 3A depicts Q-PCR data, as described in Example 2(b). Figure 3B depicts a FACS analysis of cell cycle state of treated or untreated LNCaP cells, as described in Example 2(c).

Figures 4A-4B show the results of Q-PCR analyses of RA1c expression in LUCaP77 ex vivo cells and LNCaP cells, as described in Example 2(d).

5 Figure 5 depicts western blots detecting the presence of phosphorylated intracellular signaling pathway components, as described in Example 3a.

Figure 6 depicts Q-PCR data to assess the effect of the addition of a PI3K inhibitor to IL-6-treated LNCaP cells.

10 Figure 7 depicts Q-PCR data to assess the effect of the addition of a P38 inhibitor to IL-6-treated LNCaP cells.

Figure 8 is a schematic diagram of the major signaling pathways expected to be activated by IL-6 treatment or serum deprivation.

Figure 9 depicts Q-PCR data showing the effect of the addition of an mTor inhibitor to IL-6-treated LNCaP cells.

15 Figure 10 depicts Q-PCR data showing the effect of the addition of an Akt1/2 inhibitor to IL-6-treated LNCaP cells.

Figure 11 depicts an assay showing expression of constructs carrying a predicted STAT3 binding site and the effect of mutations of the STAT3 site.

Figure 12 depicts mutagenesis of the STAT3 site.

20 Figure 13 depicts data showing the effect of the addition of a JAK inhibitor to IL-6-treated LNCaP cells.

Figures 14A-14B show the results of Q-PCR studies to assess the effects of DHT on IL-6-induced or serum deprivation-induced expression of RA1c in LNCaP cells, as described in Example 4. Figure 14C presents a diagram showing one potential scheme for intracellular RA1c expression regulation in LNCaP cells, based on the data described herein.

Figure 15 depicts a FACS analysis of RA1c cell surface expression in 293S cells, as described in Example 5.

## MODES FOR CARRYING OUT THE INVENTION

### I. Definitions

The terms "RA1c polypeptide" and "RA1c protein" as used herein encompass native sequence polypeptides, polypeptide variants and fragments of a native sequence polypeptide and polypeptide variants (which are further defined herein). The RA1c polypeptide described herein may be that which is isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods. The terms "RA1c polypeptide" and "RA1c protein" also include variants of an RA1c polypeptide as disclosed herein.

A "native sequence RA1c polypeptide" comprises a polypeptide having the same amino acid sequence as the corresponding RA1c polypeptide derived from nature. In one embodiment, a native sequence RA1c polypeptide comprises the amino acid sequence of SEQ ID NO:2:  
 MSSCNFTHATFVLIGIPGLEKAHFWVGFPLLSMYVVAMFGNCIVVFIVRTERSLHAPMYLFLCML  
 AAIDLALSTSTMPKILALFWFDSREISFEACLTQMFFIHALSAIESTILLAMAFDRYVAICHPLR  
 HAAVLNNTVTAQIGIVAVVRGSLFFFPLPLLIKRLAFCHSNVLSHSCVHQDVMKLAYADTLPNV  
 VYGLTAILLVMGVDVMFISLSYFLIIRTVLQLPSKSERAKAFGTCVSHIGVVLAIFYVPLIGLSVV  
 HRFGNSLHPIVRVVMGDIYLLLPPVINPIIYGAKTKQIRTRVLAMFKISCDKDLQAVGGK. In another embodiment, a native sequence RA1c polypeptide comprises an amino acid sequence comprising the mature protein sequence and further comprising a signal peptide, for example, SEQ ID NO:3:

MGGTAARLGAVILFVVIVGLHGVRGKYALADASLKMADPNRFRGKDLPLVDQLLEMSSCNFTHAT  
 FVLIGIPGLEKAHFWVGFPLLSMYVVAMFGNCIVVFIVRTERSLHAPMYLFLCMLAAIDLALSTS  
 TMPKILALFWFDSREISFEACLTQMFFIHALSAIESTILLAMAFDRYVAICHPLRHAAVLNNTVT  
 AQIGIVAVVRGSLFFFPLPLLIKRLAFCHSNVLSHSCVHQDVMKLAYADTLPNVVYGLTAILLV  
 MGVDVMFISLSYFLIIRTVLQLPSKSERAKAFGTCVSHIGVVLAIFYVPLIGLSVVHRFGNSLHPI  
 VRVVMGDIYLLLPPVINPIIYGAKTKQIRTRVLAMFKISCDKDLQAVGGK. In another

embodiment, a native sequence RA1c polypeptide is encoded by the polynucleotide sequence set forth in GenBank Accession No. NM\_030774. In another embodiment, a native sequence RA1c polypeptide comprises an amino acid sequence lacking a signal peptide. In yet another embodiment, a native sequence RA1c polypeptide comprises an amino acid sequence resulting from enzymatic cleavage of the sequence of SEQ ID NO:2 with a proprotein convertase. Such native sequence RA1c polypeptide can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence RA1c polypeptide" specifically encompasses naturally-occurring truncated or secreted forms of the specific RA1c polypeptide (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the polypeptide. Native sequence RA1c

is also reported in Xu et al., Cancer Res. 60: 6568-6572 (2000), Yuan et al., Gene 278: 41-51 (2001), Xia et al., Oncogene 20: 5903-5907 (2001), and in US Patent No. 6,372,891).

"RA1c polypeptide variant" means an RA1c polypeptide, preferably an active RA1c polypeptide, as defined herein having at least about 80% amino acid sequence identity with any of the native sequence RA1c polypeptide sequences as disclosed herein. Such RA1c polypeptide variants include, for instance, RA1c polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of a native amino acid sequence. Ordinarily, an RA1c polypeptide variant will have at least about 80% amino acid sequence identity, alternatively at least about 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity, to a native sequence RA1c polypeptide sequence as disclosed herein. Ordinarily, RA1c variant polypeptides are at least about 10 amino acids in length, alternatively at least about 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320 amino acids in length, or more. Optionally, RA1c variant polypeptides will have no more than one conservative amino acid substitution as compared to a native RA1c polypeptide sequence, alternatively no more than 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative amino acid substitution as compared to the native RA1c polypeptide sequence.

"Percent (%) amino acid sequence identity" with respect to the RA1c polypeptide sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the specific RA1c polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using a sequence comparison computer program. As a nonlimiting example, the ALIGN-2 sequence comparison computer program authored by Genentech, Inc., the source code for which has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available through Genentech, Inc., South San Francisco, California or may be compiled from the source code provided in any of numerous publications, for example in US Patent No. 7,087,738. The ALIGN-2 program

should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

$$100 \text{ times the fraction } X/Y$$

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

" RA1c variant polynucleotide" or "RA1c variant nucleic acid sequence" means a nucleic acid molecule which encodes an RA1c polypeptide, preferably an active RA1c polypeptide, as defined herein and which has at least about 80% nucleic acid sequence identity with a nucleotide acid sequence encoding a native sequence RA1c polypeptide sequence as disclosed herein. Ordinarily, an RA1c variant polynucleotide will have at least about 80% nucleic acid sequence identity, alternatively at least about 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity with a nucleic acid sequence encoding a native sequence RA1c polypeptide sequence as disclosed herein. Variants do not encompass the native nucleotide sequence.

Ordinarily, RA1c variant polynucleotides are at least about 5 nucleotides in length, alternatively at least about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000, 1010, 1020, 1030, 1040, 1050, 1060, 1070, 1080, 1090, 1100, 1110, 1120, 1130, 1140, 1150, 1160, 1170, 1180, 1190, 1200, 1201, or 1204 nucleotides in length, wherein in this context the term "about" means the referenced nucleotide sequence length plus or minus 10% of that referenced length.

"Percent (%) nucleic acid sequence identity" with respect to an RA1c nucleic acid sequence (encoding a native sequence RA1c polypeptide, for example the nucleic acid sequence of SEQ ID NO:1:

ATGAGTTCCTGCAACTTCACACATGCCACCTTTGTGCTTATTGGTATCCCAGGATTAGAGAAAGC  
 5 CCATTTCTGGGTTGGCTTCCCCCTCCTTTCCATGTATGTAGTGGCAATGTTTGGAAACTGCATCG  
 TGGTCTTCATCGTAAGGACGGAACGCAGCCTGCACGCTCCGATGTACCTCTTTCTCTGCATGCTT  
 GCAGCCATTGACCTGGCCTTATCCACATCCACCATGCCTAAGATCCTTGCCCTTTTCTGGTTTGA  
 TTCCCGAGAGATTAGCTTTGAGGCCTGTCTTACCCAGATGTTCTTTATTCATGCCCTCTCAGCCA  
 TTGAATCCACCATCCTGCTGGCCATGGCCTTTGACCGTTATGTGGCCATCTGCCACCCACTGCGC  
 10 CATGCTGCAGTGCTCAACAATACAGTAACAGCCCAGATTGGCATCGTGGCTGTGGTCCGCGGATC  
 CCTCTTTTTTTTTCCCACTGCCTCTGCTGATCAAGCGGCTGGCCTTCTGCCACTCCAATGTCCTCT  
 CGCACTCCTATTGTGTCCACCAGGATGTAATGAAGTTGGCCTATGCAGACACTTTGCCCAATGTG  
 GTATATGGTCTTACTGCCATTCTGCTGGTCATGGGCGTGGACGTAATGTTTCATCTCCTTGTCCTA  
 TTTTCTGATAATACGAACGGTTCTGCAACTGCCTTCCAAGTCAGAGCGGGCCAAGGCCTTTGGAA  
 15 CCTGTGTGTCACACATTGGTGTGGTACTCGCCTTCTATGTGCCACTTATTGGCCTCTCAGTTGTA  
 CACCGCTTTGGAAACAGCCTTCATCCCATTGTGCGTGTTGTCATGGGTGACATCTACCTGCTGCT  
 GCCTCCTGTCATCAATCCCATCATCTATGGTGCCAAAACCAAACAGATCAGAACACGGGTGCTGG  
 CTATGTTCAAGATCAGCTGTGACAAGGACTTGCAGGCTGTGGGAGGCAAGTGA

comprising the RA1c coding region is defined as the percentage of nucleotides in a candidate  
 20 sequence that are identical with the nucleotides in the RA1c nucleic acid sequence of interest,  
 after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent  
 sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity  
 can be achieved in various ways that are within the skill in the art, for instance, using publicly  
 available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR)  
 25 software. For purposes herein, however, % nucleic acid sequence identity values are generated  
 using the sequence comparison computer program ALIGN-2 (described above). The ALIGN-2  
 sequence comparison computer program was authored by Genentech, Inc. and the source code for  
 which has been filed with user documentation in the U.S. Copyright Office, Washington D.C.,  
 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2  
 30 program is publicly available through Genentech, Inc., South San Francisco, California or may be  
 compiled from the source code provided in any of numerous publications, for example in US  
 Patent No. 7,087,738. The ALIGN-2 program should be compiled for use on a UNIX operating  
 system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the  
 ALIGN-2 program and do not vary.

35 In situations where ALIGN-2 is employed for nucleic acid sequence comparisons, the %  
 nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given

nucleic acid sequence D (which can alternatively be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence D) is calculated as follows:

5 100 times the fraction  $W/Z$

where W is the number of nucleotides scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of C and D, and where Z is the total number of nucleotides in D. It will be appreciated that where the length of nucleic acid sequence C is not  
10 equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C. As examples of % nucleic acid sequence identity calculations, Tables 3 and 4, demonstrate how to calculate the % nucleic acid sequence identity of the nucleic acid sequence designated "Comparison DNA" to the nucleic acid sequence designated "RA1c DNA", wherein " RA1c DNA" represents a hypothetical RA1c  
15 nucleic acid sequence of interest, "Comparison DNA" represents the nucleotide sequence of a nucleic acid molecule against which the " RA1c DNA" nucleic acid molecule of interest is being compared, and "N", "L" and "V" each represent different hypothetical nucleotides. Unless specifically stated otherwise, all % nucleic acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

20 In other embodiments, RA1c variant polynucleotides are nucleic acid molecules that encode an RA1c polypeptide and which are capable of hybridizing, preferably under stringent hybridization and wash conditions, to nucleotide sequences encoding an RA1c polypeptide as disclosed herein. RA1c variant polypeptides may be those that are encoded by an RA1c variant polynucleotide.

25 "Isolated," means a molecule/compound (such as a polypeptide) that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with therapeutic uses for the molecule/compound (such as a polypeptide), and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, a  
30 polypeptide or oligopeptide will be purified (1) to a degree sufficient to obtain at least 10-15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by gel electrophoresis, for example SDS-PAGE under non-reducing or reducing conditions using, for example, Coomassie blue or silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the RA1c

polypeptide natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" polynucleotide is a polypeptide or oligopeptide-encoding nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the polypeptide or oligopeptide-encoding nucleic acid. An isolated polypeptide or oligopeptide-encoding nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated polypeptide or oligopeptide-encoding nucleic acid molecules therefore are distinguished from the specific polypeptide or oligopeptide-encoding nucleic acid molecule as it exists in natural cells. However, an isolated polypeptide or oligopeptide-encoding nucleic acid molecule includes polypeptide or oligopeptide-encoding nucleic acid molecules contained in cells that ordinarily express the polypeptide or oligopeptide where, for example, the nucleic acid molecule is in a chromosomal or extrachromosomal location different from that of natural cells.

The term "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.

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 contiguous, 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.

"Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature which can be

used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995).

"Stringent conditions" or "high stringency conditions", as defined herein, may be identified by those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) overnight hybridization in a solution that employs 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with a 10 minute wash at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) followed by a 10 minute high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.

"Moderately stringent conditions" may be identified as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Press, 1989, and include the use of washing solution and hybridization conditions (e.g., temperature, ionic strength and %SDS) less stringent than those described above. An example of moderately stringent conditions is overnight incubation at 37°C in a solution comprising: 20% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1 x SSC at about 37-50°C. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

The term "aptamer" refers to a nucleic acid molecule that is capable of binding to a target molecule, such as a polypeptide. For example, an aptamer of the invention can specifically bind to an RA1c polypeptide, or to a molecule in a signaling pathway that modulates the expression of RA1c. The generation and therapeutic use of aptamers are well established in the art. See, e.g., U.S. Pat. No. 5,475,096, and the therapeutic efficacy of Macugen® (Eyetechnology, New York) for treating age-related macular degeneration.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising an RA1c polypeptide or anti-RA1c antibody fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the polypeptide to which it is fused. The tag

polypeptide preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 and 50 amino acid residues (preferably, between about 10 and 20 amino acid residues). Nonlimiting examples of tag polypeptides include detectable markers such as polyhistidine and human placenta alkaline phosphatase.

"Active" or "activity" for the purposes herein refers to form(s) of an RA1c polypeptide which retain a biological and/or an immunological activity of native or naturally-occurring RA1c polypeptide, wherein "biological" activity refers to a biological function (either inhibitory or stimulatory) caused by a native or naturally-occurring RA1c polypeptide other than the ability to induce the production of an antibody against an antigenic epitope possessed by a native or naturally-occurring RA1c polypeptide and an "immunological" activity refers to the ability to induce the production of an antibody against an antigenic epitope possessed by a native or naturally-occurring RA1c polypeptide.

The term "antagonist" is used in the broadest sense, and includes any molecule that decreases, partially or fully blocks, inhibits, or neutralizes a biological activity of a native RA1c polypeptide disclosed herein. Suitable antagonist molecules specifically include antagonist antibodies or antibody fragments, fragments or amino acid sequence variants of native RA1c polypeptides, peptides, antisense oligonucleotides, small organic or inorganic small molecules, etc. Methods for identifying antagonists of an RA1c polypeptide may comprise contacting an RA1c polypeptide with a candidate antagonist molecule and measuring a detectable change in one or more biological activities normally associated with the RA1c polypeptide.

"Treating" or "treatment" or "alleviation" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented. A subject or mammal is successfully "treated" for a cancer if, after receiving a therapeutic amount of at least one RA1c antagonist or compound that modulates RA1c expression, or a combination of one or more of such compounds according to the methods of the invention, the patient shows observable and/or measurable reduction in or absence of one or more of the following: reduction in the number of cancer cells or absence of the cancer cells; reduction in the tumor size; inhibition (i.e., slow to some extent and preferably stop) of cancer cell infiltration into peripheral organs including the spread of cancer into soft tissue and bone; inhibition (i.e., slow to some extent and preferably stop) of tumor metastasis; inhibition, to some extent, of tumor growth; and/or relief to some extent, of one or more of the symptoms associated with the specific cancer; reduced morbidity and mortality, and improvement in quality of life

issues. To the extent the RA1c antagonist may prevent growth of and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. Reduction of these signs or symptoms may also be felt by the patient.

The above parameters for assessing successful treatment and improvement in the disease are readily measurable by routine procedures familiar to a physician. For cancer therapy, efficacy can be measured, for example, by assessing the time to disease progression (TTP) and/or determining the response rate (RR). Metastasis can be determined by staging tests and by bone scan and tests for calcium level and other enzymes to determine spread to the bone. CT scans can also be done to look for spread to the pelvis and lymph nodes in the area. Chest X-rays and measurement of liver enzyme levels by known methods are used to look for metastasis to the lungs and liver, respectively. Other routine methods for monitoring the disease include transrectal ultrasonography (TRUS) and transrectal needle biopsy (TRNB).

"Chronic" administration refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain the initial therapeutic effect (activity) for an extended period of time. "Intermittent" administration is treatment that is not consecutively done without interruption, but rather is cyclic in nature.

"Mammal" for purposes of the treatment of, alleviating the symptoms of a cancer refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, etc.

Preferably, the mammal is human.

"Patient" refers to any animal, preferably a mammal, more preferably a human.

Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

"Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; or nonionic surfactants such as TWEEN®, polyethylene glycol (PEG), and PLURONICS®.

By "solid phase" or "solid support" is meant a non-aqueous matrix to which an antibody, RA1c polypeptide binding oligopeptide or RA1c polypeptide binding organic or inorganic small molecule of the invention can adhere or attach. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain  
5 embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Patent No. 4,275,149.

10 A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as an RA1c polypeptide, an antibody thereto or an RA1c polypeptide binding oligopeptide) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

15 A "small" molecule or "small" organic small molecule is defined herein to have a molecular weight below about 500 Daltons.

An "effective amount" of an RA1c antagonist or compound that modulates RA1c expression, or a combination of one or more of such compounds, is an amount sufficient to carry out a specifically stated purpose. An "effective amount" may be determined empirically and in a  
20 routine manner, in relation to the stated purpose.

The term "therapeutically effective amount" refers to an amount of an RA1c antagonist or compound that modulates RA1c expression or a combination of one or more of such compounds, effective to "treat" a disease or disorder in a subject or mammal. In the case of cancer, the therapeutically effective amount of the RA1c antagonist or compound that modulates  
25 RA1c expression, or the combination of one or more of such compounds, may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. See the definition herein of "treating". To  
30 the extent the RA1c antagonist, compound that modulates RA1c expression, compound that modulates cellular stress, or combination of one or more of such compounds may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic.

A "growth inhibitory amount" of an RA1c antagonist or compound that modulates RA1c expression, or combination of one or more of such compounds, is an amount capable of  
35 inhibiting the growth of a cell, especially tumor, e.g., cancer cell, either *in vitro* or *in vivo*. A

“growth inhibitory amount” of an RA1c antagonist or compound that modulates RA1c expression, or combination of one or more of such compounds for purposes of inhibiting neoplastic cell growth may be determined empirically and in a routine manner.

A “cytotoxic amount” of an RA1c antagonist or compound that modulates RA1c expression, or a combination of one or more of such compounds, is an amount capable of causing the destruction of a cell, especially tumor, e.g., cancer cell, either *in vitro* or *in vivo*. A “cytotoxic amount” of an RA1c antagonist or compound that modulates RA1c expression, or a combination of one or more of such compounds, for purposes of inhibiting neoplastic cell growth may be determined empirically and in a routine manner.

The term "antibody" is used in the broadest sense and specifically covers, for example, single anti-RA1c polypeptide monoclonal antibodies (including antagonist, binding and/or neutralizing antibodies), anti- RA1c polypeptide antibody compositions with polyepitopic specificity, polyclonal antibodies, single chain anti- RA1c polypeptide antibodies, and fragments of anti- RA1c polypeptide antibodies (see below) as long as they exhibit the desired biological or immunological activity. The term “immunoglobulin” (Ig) is used interchangeably with antibody herein.

An antibody useful in methods of the invention is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by gel electrophoresis such as SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain.

The basic 4-chain antibody unit is a heterotetrameric glycoprotein composed of two identical light (L) chains and two identical heavy (H) chains (an IgM antibody consists of 5 of the basic heterotetramer unit along with an additional polypeptide called J chain, and therefore contain 10 antigen binding sites, while secreted IgA antibodies can polymerize to form polyvalent assemblages comprising 2-5 of the basic 4-chain units along with J chain). In the case of IgGs, the 4-chain unit is generally about 150,000 daltons. Each L chain is linked to a H chain by one covalent disulfide bond, while the two H chains are linked to each other by one or more disulfide bonds depending on the H chain isotype. Each H and L chain also has regularly spaced intrachain disulfide bridges. Each H chain has at the N-terminus, a variable domain ( $V_H$ ) followed by three

constant domains ( $C_H$ ) for each of the  $\alpha$  and  $\gamma$  chains and four  $C_H$  domains for  $\mu$  and  $\epsilon$  isotypes. Each L chain has at the N-terminus, a variable domain ( $V_L$ ) followed by a constant domain ( $C_L$ ) at its other end. The  $V_L$  is aligned with the  $V_H$  and the  $C_L$  is aligned with the first constant domain of the heavy chain ( $C_{H1}$ ). Particular amino acid residues are believed to form an interface  
5 between the light chain and heavy chain variable domains. The pairing of a  $V_H$  and  $V_L$  together forms a single antigen-binding site. For the structure and properties of the different classes of antibodies, see, e.g., Basic and Clinical Immunology, 8th edition, Daniel P. Stites, Abba I. Terr and Tristram G. Parslow (eds.), Appleton & Lange, Norwalk, CT, 1994, page 71 and Chapter 6.

The L chain from any vertebrate species can be assigned to one of two clearly distinct  
10 types, called kappa and lambda, based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains ( $C_H$ ), immunoglobulins can be assigned to different classes or isotypes. There are five classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, having heavy chains designated  $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$ , and  $\mu$ , respectively. The  $\gamma$  and  $\alpha$  classes are further divided into subclasses on the basis of relatively  
15 minor differences in  $C_H$  sequence and function, e.g., humans express the following subclasses: IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2.

The term "variable" refers to the fact that certain segments of the variable domains differ extensively in sequence among antibodies. The V domain mediates antigen binding and define specificity of a particular antibody for its particular antigen. However, the variability is not  
20 evenly distributed across the 110-amino acid span of the variable domains. Instead, the V regions consist of relatively invariant stretches called framework regions (FRs) of 15-30 amino acids separated by shorter regions of extreme variability called "hypervariable regions" that are each 9-12 amino acids long. The variable domains of native heavy and light chains each comprise four FRs, largely adopting a  $\beta$ -sheet configuration, connected by three hypervariable regions, which  
25 form loops connecting, and in some cases forming part of, the  $\beta$ -sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are  
30 not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen binding. The hypervariable region generally comprises amino acid residues from a "complementarity determining region" or "CDR" (e.g.  
35 around about residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the  $V_L$ , and around about 1-35

(H1), 50-65 (H2) and 95-102 (H3) in the V<sub>H</sub>; Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (*e.g.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the V<sub>L</sub>, and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the V<sub>H</sub>; Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier "monoclonal" is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies useful in the present invention may be prepared by the hybridoma methodology first described by Kohler et al., Nature, 256:495 (1975), or may be made using recombinant DNA methods in bacterial, eukaryotic animal or plant cells (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., Nature, 352:624-628 (1991) and Marks et al., J. Mol. Biol., 222:581-597 (1991), for example.

The monoclonal antibodies herein include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (see U.S. Patent No. 4,816,567; and Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984)). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (*e.g.* Old World Monkey, Ape etc), and human constant region sequences.

An "intact" antibody is one which comprises an antigen-binding site as well as a C<sub>L</sub> and at least heavy chain constant domains, C<sub>H</sub>1, C<sub>H</sub>2 and C<sub>H</sub>3. The constant domains may be native sequence constant domains (*e.g.* human native sequence constant domains) or amino acid sequence variant thereof. Preferably, the intact antibody has one or more effector functions.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')<sub>2</sub>, and Fv fragments; diabodies; linear antibodies (see U.S. Patent No. 5,641,870, Example 2; Zapata et al., Protein Eng. 8(10): 1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, and a residual "Fc" fragment, a designation reflecting the ability to crystallize readily. The Fab fragment consists of an entire L chain along with the variable region domain of the H chain (V<sub>H</sub>), and the first constant domain of one heavy chain (C<sub>H</sub>1). Each Fab fragment is monovalent with respect to antigen binding, i.e., it has a single antigen-binding site. Pepsin treatment of an antibody yields a single large F(ab')<sub>2</sub> fragment which roughly corresponds to two disulfide linked Fab fragments having divalent antigen-binding activity and is still capable of cross-linking antigen. Fab' fragments differ from Fab fragments by having additional few residues at the carboxy terminus of the C<sub>H</sub>1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')<sub>2</sub> antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The Fc fragment comprises the carboxy-terminal portions of both H chains held together by disulfides. The effector functions of antibodies are determined by sequences in the Fc region, which region is also the part recognized by Fc receptors (FcR) found on certain types of cells.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This fragment consists of a dimer of one heavy- and one light-chain variable region domain in tight, non-covalent association. From the folding of these two domains emanate six hypervariable loops (3 loops each from the H and L chain) that contribute the amino acid residues for antigen binding and confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

"Single-chain Fv" also abbreviated as "sFv" or "scFv" are antibody fragments that comprise the V<sub>H</sub> and V<sub>L</sub> antibody domains connected into a single polypeptide chain. Preferably, the sFv polypeptide further comprises a polypeptide linker between the V<sub>H</sub> and V<sub>L</sub> domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv, see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994); Borrebaeck 1995, *infra*.

The term "diabodies" refers to small antibody fragments prepared by constructing sFv fragments (see preceding paragraph) with short linkers (about 5-10 residues) between the V<sub>H</sub> and V<sub>L</sub> domains such that inter-chain but not intra-chain pairing of the V domains is achieved, resulting in a bivalent fragment, i.e., fragment having two antigen-binding sites. Bispecific  
5 diabodies are heterodimers of two "crossover" sFv fragments in which the V<sub>H</sub> and V<sub>L</sub> domains of the two antibodies are present on different polypeptide chains. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993).

"Humanized" forms of non-human (*e.g.*, rodent) antibodies are chimeric antibodies that  
10 contain minimal sequence derived from the non-human antibody. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or non-human primate having the desired antibody specificity, affinity, and capability. In some instances, framework region (FR)  
15 residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops  
20 correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992).

25 A "species-dependent antibody," *e.g.*, a mammalian anti-human IgE antibody, is an antibody which has a stronger binding affinity for an antigen from a first mammalian species than it has for a homologue of that antigen from a second mammalian species. Normally, the species-dependent antibody "bind specifically" to a human antigen (*i.e.*, has a binding affinity (K<sub>d</sub>) value of no more than about  $1 \times 10^{-7}$  M, preferably no more than about  $1 \times 10^{-8}$  and most preferably no  
30 more than about  $1 \times 10^{-9}$  M) but has a binding affinity for a homologue of the antigen from a second non-human mammalian species which is at least about 50 fold, or at least about 500 fold, or at least about 1000 fold, weaker than its binding affinity for the human antigen. The species-dependent antibody can be of any of the various types of antibodies as defined above, but preferably is a humanized or human antibody.

A "RA1c polypeptide binding oligopeptide" is an oligopeptide that binds, preferably specifically, to an RA1c polypeptide as described herein. RA1c polypeptide binding oligopeptides may be chemically synthesized using known oligopeptide synthesis methodology or may be prepared and purified using recombinant technology. RA1c polypeptide binding oligopeptides are usually at least about 5 amino acids in length, alternatively at least about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 amino acids in length or more, wherein such oligopeptides that are capable of binding, preferably specifically, to an RA1c polypeptide as described herein. RA1c polypeptide binding oligopeptides may be identified without undue experimentation using well known techniques. In this regard, it is noted that techniques for screening oligopeptide libraries for oligopeptides that are capable of specifically binding to a polypeptide target are well known in the art (see, e.g., U.S. Patent Nos. 5,556,762, 5,750,373, 4,708,871, 4,833,092, 5,223,409, 5,403,484, 5,571,689, 5,663,143; PCT Publication Nos. WO 84/03506 and WO84/03564; Geysen et al., Proc. Natl. Acad. Sci. U.S.A., 81:3998-4002 (1984); Geysen et al., Proc. Natl. Acad. Sci. U.S.A., 82:178-182 (1985); Geysen et al., in Synthetic Peptides as Antigens, 130-149 (1986); Geysen et al., J. Immunol. Meth., 102:259-274 (1987); Schoofs et al., J. Immunol., 140:611-616 (1988), Cwirla, S. E. et al. (1990) Proc. Natl. Acad. Sci. USA, 87:6378; Lowman, H.B. et al. (1991) Biochemistry, 30:10832; Clackson, T. et al. (1991) Nature, 352: 624; Marks, J. D. et al. (1991), J. Mol. Biol., 222:581; Kang, A.S. et al. (1991) Proc. Natl. Acad. Sci. USA, 88:8363, and Smith, G. P. (1991) Current Opin. Biotechnol., 2:668).

A "RA1c polypeptide binding organic or inorganic small molecule" is an organic or inorganic small molecule other than an oligopeptide or antibody as defined herein that binds, preferably specifically, to an RA1c polypeptide as described herein. A small molecule RA1c antagonist is preferably an organic small molecule. RA1c polypeptide binding organic may be identified and chemically synthesized using known methodology (see, e.g., PCT Publication Nos. WO00/00823 and WO00/39585). RA1c polypeptide binding organic small molecules are usually less than about 2000 daltons in size, alternatively less than about 1500, 750, 500, 250 or 200 daltons in size, wherein such organic small molecules that are capable of binding, preferably specifically, to an RA1c polypeptide as described herein may be identified without undue experimentation using well known techniques. In this regard, it is noted that techniques for screening organic small molecule libraries for molecules that are capable of binding to a polypeptide target are well known in the art (see, e.g., PCT Publication Nos. WO00/00823 and WO00/39585).

An antibody, oligopeptide, or other organic or inorganic small molecule “which binds” an antigen of interest, e.g. an RA1c polypeptide, is one that binds the antigen with sufficient affinity such that the antibody, oligopeptide, or other organic or inorganic small molecule is useful as a therapeutic agent in targeting a cell or tissue expressing the antigen, and does not significantly cross-react with other proteins. In such embodiments, the extent of binding of the antibody, oligopeptide, or other organic or inorganic small molecule to a “non-target” protein will be less than about 10% of the binding of the antibody, oligopeptide, or other organic or inorganic small molecule to its particular target protein as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA). With regard to the binding of an antibody, oligopeptide, or other organic or inorganic small molecule to a target molecule, the term “specific binding” or “specifically binds to” or is “specific for” a particular polypeptide or an epitope on a particular polypeptide target means binding that is measurably different from a non-specific interaction. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding can be determined by competition with a control molecule that is similar to the target, for example, an excess of non-labeled target. In this case, specific binding is indicated if the binding of the labeled target to a probe is competitively inhibited by excess unlabeled target. The term “specific binding” or “specifically binds to” or is “specific for” a particular polypeptide or an epitope on a particular polypeptide target as used herein can be exhibited, for example, by a molecule having a  $K_d$  for the target of at least about  $10^{-4}$  M, alternatively at least about  $10^{-5}$  M, alternatively at least about  $10^{-6}$  M, alternatively at least about  $10^{-7}$  M, alternatively at least about  $10^{-8}$  M, alternatively at least about  $10^{-9}$  M, alternatively at least about  $10^{-10}$  M, alternatively at least about  $10^{-11}$  M, alternatively at least about  $10^{-12}$  M, or greater. In one embodiment, the term “specific binding” refers to binding where a molecule binds to a particular polypeptide or epitope on a particular polypeptide without substantially binding to any other polypeptide or polypeptide epitope.

An antibody, oligopeptide, or other organic or inorganic small molecule, other RA1c antagonist, compound that modulates RA1c expression, or combination of one or more of such compounds that “inhibits the growth of tumor cells expressing RA1c polypeptide” or a “growth inhibitory” antibody, oligopeptide, or other organic or inorganic small molecule, other RA1c antagonist, compound that modulates RA1c expression, compound that modulates cellular stress, or combination of one or more of such compounds is one which results in measurable growth inhibition of prostate cancer cells expressing or overexpressing the RA1c polypeptide. The RA1c polypeptide may be bound to the surface of a cell or may be in an extracellular environment. In certain embodiments, growth inhibitory anti-RA1c polypeptide antibodies, oligopeptides, organic

or inorganic small molecules, other RA1c antagonists, compounds that modulate RA1c expression, or combinations of one or more of such compounds inhibit growth of RA1c polypeptide-expressing tumor cells by greater than about 20%, alternatively from about 20% to about 50%, or alternatively by greater than 50% (e.g., from about 50% to about 100%) as compared to the appropriate control, the control typically being tumor cells not treated with the antibody, oligopeptide, other organic or inorganic small molecule, other RA1c antagonist, compound that modulates RA1c expression, compound that modulates cellular stress, or combination of one or more of such compounds being tested. In one embodiment, growth inhibition can be measured at an antibody concentration of about 0.1 to 30 µg/ml or about 0.5 nM to 200 nM in cell culture, where the growth inhibition is determined 1-10 days after exposure of the tumor cells to the antibody. The antibody is growth inhibitory in vivo if administration of the anti- RA1c polypeptide antibody at about 1 µg/kg to about 100 mg/kg body weight results in reduction in tumor size or tumor cell proliferation within about 5 days to 3 months from the first administration of the antibody, or within about 5 to 30 days.

To “induce cellular stress response” means to cause a cell to initiate at least one cellular process, such as signaling pathway or cascade, that is triggered by an acute or chronic shift from usual cellular conditions and homeostasis and aimed at counteracting the insult, repairing the damage, and protecting the cell.

To “ameliorate cellular stress response” means any reduction, inhibition, or prevention of any physiological conditions associated with or caused by cellular stress response. In one embodiment, amelioration of cellular stress response results in the cells becoming more sensitive to a chemotherapeutic agent that induces cellular stress response. An increase in sensitivity to a chemotherapeutic agent can be monitored, for example, by determining if the cells undergo an increase in the rate of apoptosis when contacted with the agent as compared to the rate of apoptosis prior to amelioration of the cellular stress response. Apoptosis, or programmed cell death, can be determined by monitoring binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). Induction of apoptosis may also be determined by FACS analysis of a sample cell population. The cell is usually one which overexpresses RA1c polypeptide. Preferably the cell is a tumor cell of a prostate cancer. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. The increase in apoptosis is one which results in about 2 to 50 fold, alternatively about 5 to 50 fold, or alternatively about 10

to 50 fold, or greater, induction of annexin binding relative to that observed in untreated cells in an annexin binding assay. An increase in sensitivity to a chemotherapeutic agent can also be monitored by determining if the cells undergo an increase in growth arrest, for example, G0/G1 arrest, when contacted with the agent as compared to the growth arrest of the cells prior to amelioration of the cellular stress response. Methods for determining G0/G1 arrest are found in Example 2. The increase in growth arrest is one which results in about 2 to 50 fold, alternatively about 5 to 50 fold, or alternatively about 10 to 50 fold, or greater increase in cells in G0/G1 arrest relative to that observed in untreated cells.

Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g., B cell receptor); and B cell activation.

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g., Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic cells and are absolutely required for such killing. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. (USA) 95:652-656 (1998).

"Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an

immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see review M. in Daëron, Annu. Rev. Immunol. 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, Annu. Rev. Immunol. 9:457-492 (1991); Capel et al., Immunomethods 4:25-34 (1994); and de Haas et al., J. Lab. Clin. Med. 126:330-41 (1995). Other FcRs, including those to be  
 5 identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol. 117:587 (1976) and Kim et al., J. Immunol. 24:249 (1994)).

“Human effector cells” are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector  
 10 function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source, e.g., from blood.

“Complement dependent cytotoxicity” or “CDC” refers to the lysis of a target cell in the  
 15 presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass) which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g., as described in Gazzano-Santoro et al., J. Immunol. Methods 202:163 (1996), may be performed.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated increase in cell number (generally referred to herein as cell growth), which can be due to abnormal increase in cell proliferation, abnormal decrease of cell death, or an imbalance of amounts of cell proliferation and cell death. Examples of cancer include, but are not limited to, prostate cancer. Examples of prostate cancer  
 20 include, but are not limited to, prostate tumors, adenocarcinoma, and prostate intraepithelial neoplasia.

The terms “cell proliferative disorder” and “proliferative disorder” refer to disorders that are associated with some degree of abnormal cell proliferation. In one embodiment, the cell proliferative disorder is cancer.

30 "Tumor", as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms “cancer”, “cancerous”, “cell proliferative disorder”, “proliferative disorder” and “tumor” are not mutually exclusive as referred to herein.

An RA1c antagonist or compound that modulates RA1c expression, or a combination of  
 35 one or more of such compounds, which “induces cell death” is one which causes a viable cell to

become nonviable. The cell is one which expresses an RA1c polypeptide and is of a cell type which specifically expresses or overexpresses an RA1c polypeptide. The cell may be cancerous or normal cells of the particular cell type. The RA1c polypeptide may be a transmembrane polypeptide expressed on the surface of a cancer cell or may be a polypeptide that is produced and secreted by a cancer cell. The cell may be a cancer cell, e.g., a prostate cancer cell. Cell death *in vitro* may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody-dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (i.e., in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody, oligopeptide or other organic small molecule, other RA1c antagonist, compound that modulates RA1c expression, compound that modulates cellular stress, or combination of one or more of such compounds is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore et al. Cytotechnology 17:1-11 (1995)) or 7AAD can be assessed relative to untreated cells.

Preferred cell death-inducing antibodies, oligopeptides or other organic small molecules, other RA1c antagonists, compounds that modulate RA1c expression, or combinations of one or more of such compounds are those which induce PI uptake in the PI uptake assay in, in a nonlimiting example of a prostate cancer cell model,, LNCaP cells.

A “RA1c polypeptide-expressing cell” is a cell which expresses an endogenous or transfected RA1c polypeptide, for example in a secreted form. RA1c polypeptide expression may be determined in a detection or prognostic assay by evaluating levels of the RA1c protein present in and/or on the surface of a cell, and/or secreted by the cell (e.g., via an immunohistochemistry assay using anti- RA1c polypeptide antibodies prepared against an isolated RA1c polypeptide which may be prepared using recombinant DNA technology from an isolated nucleic acid encoding the RA1c polypeptide; FACS analysis, etc.). Alternatively, or additionally, one may measure levels of RA1c polypeptide-encoding nucleic acid or mRNA in the cell, e.g., via fluorescent *in situ* hybridization using a nucleic acid based probe corresponding to an RA1c polypeptide-encoding nucleic acid or the complement thereof; (FISH; see WO98/45479 published October, 1998), Southern blotting, Northern blotting, or polymerase chain reaction (PCR) techniques, such as real time quantitative PCR (RT-PCR). One may also study RA1c polypeptide expression by measuring shed antigen in a biological fluid such as serum, e.g, using antibody-based assays (see also, e.g., U.S. Patent No. 4,933,294 issued June 12, 1990; WO91/05264 published April 18, 1991; U.S. Patent 5,401,638 issued March 28, 1995; and Sias et al., J. Immunol. Methods 132:73-80 (1990)). Aside from the above assays, various *in vivo* assays are available to the skilled practitioner. For example, one may expose cells within the body of the

patient to an antibody which is optionally labeled with a detectable label, e.g., a radioactive isotope, and binding of the antibody to cells in the patient can be evaluated, e.g., by external scanning for radioactivity or by analyzing a biopsy taken from a patient previously exposed to the antibody.

5 As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the binding specificity of a heterologous protein (an "adhesin") with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of an amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site of an antibody (i.e., is "heterologous"), and an  
10 immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding site of a receptor or a ligand. The immunoglobulin constant domain sequence in the immunoadhesin may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA (including IgA-1 and IgA-2), IgE, IgD or IgM.

15 The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody, oligopeptide, or other organic or inorganic small molecule so as to generate a "labeled" antibody, oligopeptide, or other organic or inorganic small molecule. The label may be detectable by itself (e.g. radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a  
20 substrate compound or composition which is detectable.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., At<sup>211</sup>, I<sup>131</sup>, I<sup>125</sup>, Y<sup>90</sup>, Re<sup>186</sup>, Re<sup>188</sup>, Sm<sup>153</sup>, Bi<sup>212</sup>, P<sup>32</sup> and radioactive isotopes of Lu), chemotherapeutic agents e.g. methotrexate, adriamycin, vinca alkaloids  
25 (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents, enzymes and fragments thereof such as nucleolytic enzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, and the various antitumor or anticancer agents disclosed below. Other cytotoxic agents are described  
30 below. A tumoricidal agent causes destruction of tumor cells.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Specific examples of chemotherapeutic agents include agents that inhibit the mTor or the AKT pathway, including but limited to rapamycin/sirolimus, CCI-779/temsirolimus (Wyeth), RAD001/everolimus (Novartis), AP23573 (Ariad), XL7F65, TAFA93 (Isotechnika), GSK690693

(GlaxoSmithKline), perifosine (Æterna Zentaris), and XL418 (Exelixis), and active derivatives and analogs thereof.

Further examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and  
 5 piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramidate, triethylenethiophosphoramidate and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan  
 10 (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scoplectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin;  
 15 spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (*e. g.*, calicheamicin, especially calicheamicin gammaII and  
 20 calicheamicin omegaII (see, *e.g.*, Agnew, *Chem Intl. Ed. Engl.*, 33: 183-186 (1994)); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN®  
 25 doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU);  
 30 folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiothane, testolactone; anti- adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic  
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acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoids, *e.g.*, TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumburg, Illinois), and TAXOTERE® doxetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; gemcitabine (GEMZAR®); 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine (VELBAN®); platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); oxaliplatin; leucovovin; vinorelbine (NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine (XELODA®); pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU and leucovovin.

Also included in this definition are anti-hormonal agents that act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of cancer, and are often in the form of systemic, or whole-body treatment. They may be hormones themselves. Examples include anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), EVISTA® raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON® toremifene; anti-progesterones; estrogen receptor down-regulators (ERDs); agents that function to suppress or shut down the ovaries, for example, leutinizing hormone-releasing hormone (LHRH) agonists such as LUPRON® and ELIGARD® leuprolide acetate, goserelin acetate, buserelin acetate and triptorelin; other anti-androgens such as flutamide, nilutamide and bicalutamide; and aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestanie, fadrozole, RIVISOR® vorozole, FEMARA® letrozole,

and ARIMIDEX® anastrozole. In addition, such definition of chemotherapeutic agents includes bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), DIDROCAL® etidronate, NE-58095, ZOMETA® zoledronic acid/zoledronate, FOSAMAX® alendronate, AREDIA® pamidronate, SKELID® tiludronate, or ACTONEL® risedronate; as well as  
 5 troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in adherent cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine;  
 10 LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; lapatinib ditosylate (an ErbB-2 and EGFR dual tyrosine kinase small-molecule inhibitor also known as GW572016); and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of an RA1c polypeptide-expressing cancerous cell, either *in vitro* or *in vivo*. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of  
 15 RA1c polypeptide-expressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topoisomerase II inhibitors such as doxorubicin, epirubicin,  
 20 daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders:  
 25 Philadelphia, 1995), especially p. 13. The taxanes (paclitaxel and docetaxel) are anticancer drugs both derived from the yew tree. Docetaxel (TAXOTERE®, Rhone-Poulenc Rorer), derived from the European yew, is a semisynthetic analogue of paclitaxel (TAXOL®, Bristol-Myers Squibb). Paclitaxel and docetaxel promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization, which results in the inhibition of mitosis in cells.

30 "Doxorubicin" is an anthracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy- $\alpha$ -L-lyxo-hexapyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines,  
 35 monokines, and traditional polypeptide hormones. Included among the cytokines are growth

hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor- $\alpha$  and - $\beta$ ; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor (VEGF); integrin; thrombopoietin (TPO); nerve growth factors such as NGF- $\beta$ ; platelet-growth factor; transforming growth factors (TGFs) such as TGF- $\alpha$  and TGF- $\beta$ ; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon - $\alpha$ , - $\beta$ , and - $\gamma$ ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1a, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF- $\alpha$  or TNF- $\beta$ ; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

## II. Compositions and Methods of the Invention

RA1c is highly expressed in prostate cancer cells and is not substantially expressed in most other commonly tested normal and diseased tissues as described herein and, for example, in US Published Patent Application No. 20050106644, Xu et al., Cancer Res. 60: 6568-6572 (2000), Yuan et al., Gene 278: 41-51 (2001), Xia et al., Oncogene 20: 5903-5907 (2001), and in US Patent No. 6,372,891. RA1c has sequence homology to the family of seven-transmembrane G-protein-coupled receptor proteins.

Herein for the first time it is shown that RA1c is located on the extracellular membrane of prostate cancer cells, and is thus accessible as a target for identification and treatment of prostate cancer.

It is also shown herein that RA1c expression in prostate cancer cell models is upregulated in response to IL-6 treatment or serum deprivation, suggesting that RA1c expression is stimulated by an increase in cellular stress. The cellular stress response is evolutionarily conserved in all living organisms and results in molecular responses that dictate whether the organism undergoing the stress adapts, survives, or dies. The cellular response involves a number of specific signalling

events which prepare for the specific cellular adaptation steps in the metabolism, membranes, cytoskeleton, and for those of other cellular elements and functions. Szalay, et al., FEBS Letters, 581 (19):3675-3680 (2007). These molecular responses to stress are for the most part beneficial to the organisms. However, if the cellular stress response is induced in cancer cells it may interfere with the efficacy of treatment with chemotherapeutic agents. Herr, I., and Debatin, K-M, Blood, 98:2603-2614 (2001); Tiligada, E., Endocrine-Related Cancer, 13:S115-S124 (2006). In general, chemotherapeutic agents inhibit cell growth and/or induce apoptosis in sensitive tumor cells. These agents may cause an imbalance of the cellular homeostasis resulting in cellular stress. In response, the cell undergoes cellular stress response in an attempt to return the cell to its previous state. The type and dose of stress within the cellular context appears to dictate the outcome of the cellular response. The cellular stress response may determine if the cells are sensitive or resistant to treatment with the chemotherapeutic agent. Blood, 98:2603-2614.

Studies with inhibitors of certain intracellular signaling pathways thought to be involved in IL-6 or cellular stress responses described herein demonstrated that inhibition of either p38 MAP kinase or inhibition of PI3K resulted in decreased RA1c expression in the treated cells. Inhibition of constitutively active mTOR and Akt 1/2, however, had the opposite effect, stimulating RA1c expression, and this effect was at least additive to the observed effects of IL-6 alone.

The data presented in the Examples herein indicates that RA1c is a stress-induced gene that could provide a survival advantage to cancer cells. Figure 14C. Under normal and replete growth conditions, RA1c expression is suppressed while RA1c expression is upregulated in response to conditions conducive to cellular stress (IL-6 treatment or serum deprivation). Furthermore, the elevation of RA1c expression in response to cellular stress can be reversed or mitigated by pro-growth pathway activation (DHT) and amplified when pro-growth pathways are further inhibited (rapamycin).

The observation that inhibition of Akt1/2 or mTOR is at least additive with IL-6 suggests that the combination of these inhibitors with an anti-RA1c molecule could have therapeutic benefit in the treatment of cancer. Several agents targeting mTOR are under clinical evaluation, e.g., rapamycin/sirolimus, CCI-779/temsirolimus, RAD001/everolimus, AP23573, XL7F65, and TAFA93, and active derivatives or analogs thereof. Inhibitors of Akt have recently entered clinical development as well, including GSK690693, perifosine, and XL418, and active derivatives or analogs thereof.

Accordingly, the invention provides methods of ameliorating cellular stress response, particularly in prostate cancer cells, by decreasing or blocking RA1c expression or RA1c activity in those cells. Compounds that decrease or block RA1c expression or activity in the cell include

RA1c antagonists and compounds that modulate RA1c expression. Examples of compounds that decrease or block RA1c expression or activity in the cell include, but are not limited to, antibodies, antibody fragments, antibody conjugates, aptamers, small molecules, oligopeptides, antisense polynucleotides, silencing RNA molecules, catalytic RNA molecules, and RNA-DNA  
5 chimeras, and are described further herein.

The cellular stress response can be induced by a variety of factor or factors. For example, cellular stress response can be induced in a cell by placing the cell under serum deprivation conditions. Cellular stress response can also be induced by exposing the cell to IL-6.

Chemotherapeutic agents often induce cellular stress response in the cells targeted by the agents.

10 The cellular stress response can be successful in allowing the cells to survive treatment with the chemotherapeutic agent. As a result, the cells are less sensitive to the agent and efficacy of the chemotherapeutic treatment is adversely impacted. Amelioration of the cellular stress response increases sensitivity of the cells to treatment with the chemotherapeutic agent. In one embodiment, the cells in which the cellular stress response has been ameliorated undergo  
15 apoptosis upon contact with the chemotherapeutic agent at a higher rate than cells not contacted with the compound. In another embodiment, the cells in which the cellular stress response has been ameliorated undergo growth arrest upon contact with the chemotherapeutic agent at a higher rate than cells not contacted with the compound.

One aspect of the invention provides for methods of ameliorating cellular stress response  
20 in a cancer patient undergoing chemotherapy with a chemotherapeutic agent that induces cellular stress response comprising administering to the patient at least one compound that decreases or blocks the expression or activity of RA1c. In a particular embodiment, the cancer patient is suffering from prostate cancer. A patient undergoing this method of treatment has cancer cells that are more sensitive to the chemotherapeutic treatment.

25 Chemotherapeutic agents that induce cellular stress response include, but are not limited to, inhibitors of the mTOR or the Akt1/2 pathway.

The invention further provides combination therapies for treating cancer. In one embodiment the combination therapy comprises contacting a cancer cell with a chemotherapeutic agent that increases RA1c expression and a compound that specifically binds to the RA1c  
30 polypeptide. Examples of chemotherapeutic agents that increase expression of RA1c include agents that induce cellular stress response. Specific examples of chemotherapeutic agents that increase RA1c expression include, but are not limited to, inhibitors of the mTOR or the Akt1/2 pathway. Compounds that specifically bind to RA1c polypeptide include, but are not limited to, anti-RA1c antibodies or fragments thereof, RA1c-binding oligopeptides, small molecules that  
35 specifically bind RA1c, and other RA1c antagonists such as aptamers. An increase in RA1c

polypeptide expression provides an upregulated target for compounds that recognize and bind to RA1c. Preferably, the compound that specifically binds to RA1c polypeptide is conjugated to a cytotoxic agent. The combination treatment of a chemotherapeutic agent and a compound that specifically binds to RA1c provides a synergistic effect resulting in an improved cancer treatment. In a particular embodiment, the cancer cells are prostate cancer cells.

In another embodiment the combination therapy comprises contacting a cancer cell with a chemotherapeutic agent that increases RA1c expression and a compound that decreases or blocks RA1c expression or activity. Examples of chemotherapeutic agents that increase expression of RA1c include agents that induce cellular stress response. Specific examples of chemotherapeutic agents that increase RA1c expression include, but are not limited to, inhibitors of the mTOR or the Akt1/2 pathway. An increase in RA1c expression provides an upregulated target for compounds that recognize RA1c. The combination treatment of a chemotherapeutic agent and a compound that decreases or blocks RA1c expression or activity provides a synergistic effect resulting in an improved cancer treatment. In a particular embodiment, the cancer cells are prostate cancer cells.

In the context of treating a cancer patient, the combination therapies involve administering to the cancer patient a chemotherapeutic agent that increases expression of RA1c and a compound that decreases or blocks RA1c expression or activity and/or specifically binds to the RA1c polypeptide.

The invention also provides compositions comprising the above compounds, both singly and in combination, as described further herein.

Methods of diagnosing and staging cancers are also provided by the invention. Because RA1c is accessible at the surface of cells and is a specific marker for prostate cells, particularly cancerous prostate cells, use of any of the RA1c-binding molecules of the invention (including, but not limited to, anti-RA1c antibodies or fragments thereof, RA1c-binding oligopeptides, RA1c antibody conjugates, inorganic or organic small molecules that specifically bind RA1c, and other RA1c antagonists such as aptamers) should permit identification of cells expressing RA1c in vivo or in vitro. One or more appropriate labels (including, but not limited to, radiolabels, fluorescent labels, enzymatic labels, colorimetric labels, spin labels, and any other appropriate label as is well known in the art) may be attached to the RA1c-binding molecule to assist visualization.

Another aspect of the invention relates to methods of determining whether a chemotherapeutic agent causes a cellular stress response in a cancer cell. Such methods are useful in determining the need to treat a patient undergoing chemotherapy with a compound that decreases or blocks the expression or activity of RA1c.

Accordingly, the invention provides a method of determining whether a

chemotherapeutic agent induces a cellular stress response in a cancer cell comprising contacting the cancer cell with the chemotherapeutic agent and determining the level of RA1c expression in the cancer cell. An increase in RA1c expression level over a control cell, or over the level of RA1c expression in the cancer cell prior to contacting with the chemotherapeutic agent, indicates  
5 induction of cellular stress response. Suitable control cells include untreated cancer cells of the same kind as the cancer cell being assayed. The cancer cell used in this assay is preferably a prostate cancer cell, such as an LNCaP cell.

The chemotherapeutic agent assayed in these methods is any agent suspected of being capable of causing cellular stress response. In one embodiment, the chemotherapeutic agent is an  
10 mTor inhibitor. In another embodiment, the chemotherapeutic agent is an Akt1/2 inhibitor.

Kits and articles of manufacture are also provided by the invention, as described further herein.

These methods and compositions and their specific aspects are described in greater detail in the following sections.

#### 15 A. Anti-RA1c Antibodies

In one embodiment, the present invention provides anti-RA1c polypeptide antibodies which may find use herein as therapeutic or diagnostic agents. Exemplary antibodies  
20 include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

##### 1. Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen (especially when synthetic peptides are used) to a protein that is  
25 immunogenic in the species to be immunized. For example, the antigen can be conjugated to keyhole limpet hemocyanin (KLH), serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor, using a bifunctional or derivatizing agent, e.g., maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride,  $\text{SOCl}_2$ , or  $\text{R}^1\text{N}=\text{C}=\text{NR}$ , where R and  $\text{R}^1$  are different alkyl  
30 groups. In certain embodiments, the animals used to raise antibodies may be transgenic animals. In certain such embodiments, the animals may be engineered such that they exhibit a complete absence of a polynucleotide encoding RA1c, such that they have no RA1c expression (referred to as “knockout” animals). Methods of generating such animals are well-known in the art, see, e.g., Snouwaert et al., Science 257:1083, 1992; Lowell et al., Nature 366:740-42, 1993; Capecchi, M.  
35 R., Science 244: 1288-1292, 1989. Raising antibodies to a particular protein or peptide in a knockout animal for that protein or peptide may be advantageous because anti-self reactions in

the animal that may reduce production or yields of the antibody should not occur, as is well known in the art.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later, the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later, the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

## 2. Monoclonal Antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., Nature, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as described above to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. After immunization, lymphocytes are isolated and then fused with a myeloma cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium which medium preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells (also referred to as fusion partner). For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the selective culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred fusion partner myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a selective medium that selects against the unfused parental cells. Preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 and derivatives e.g., X63-Ag8-653 cells available from the American Type Culture Collection,

Manassas, Virginia, USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 (1984); and Brodeur et al., Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

5 Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).

10 The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis described in Munson et al., Anal. Biochem., 107:220 (1980).

Once hybridoma cells that produce antibodies of the desired specificity, affinity, and/or activity are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103  
15 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal e.g., by i.p. injection of the cells into mice.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such as,  
20 for example, affinity chromatography (e.g., using protein A or protein G-Sepharose) or ion-exchange chromatography, hydroxylapatite chromatography, gel electrophoresis, dialysis, etc.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma  
25 cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include  
30 Skerra et al., Curr. Opinion in Immunol., 5:256-262 (1993) and Plückthun, Immunol. Revs. 130:151-188 (1992).

In a further embodiment, monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990). Clackson et al., Nature, 352:624-628 (1991) and Marks et al., J. Mol. Biol., 222:581-597 (1991) describe the isolation of murine and human antibodies,  
35

respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., Bio/Technology, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse et al., Nuc. Acids. Res. 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA that encodes the antibody may be modified to produce chimeric or fusion antibody polypeptides, for example, by substituting human heavy chain and light chain constant domain ( $C_H$  and  $C_L$ ) sequences for the homologous murine sequences (U.S. Patent No. 4,816,567; and Morrison, et al., Proc. Natl Acad. Sci. USA, 81:6851 (1984)), or by fusing the immunoglobulin coding sequence with all or part of the coding sequence for a non-immunoglobulin polypeptide (heterologous polypeptide). The non-immunoglobulin polypeptide sequences can substitute for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

### 3. Human and Humanized Antibodies

The anti-RA1c antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. 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  
5 performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], 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  
10 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.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity and HAMA response (human anti-  
15 mouse antibody) when the antibody is intended for human therapeutic use. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human V domain sequence which is closest to that of the rodent is identified and the human framework region (FR) within it accepted for the humanized antibody (Sims et al., J. Immunol. 151:2296 (1993); Chothia et al., J. Mol. Biol., 196:901 (1987)). Another method uses a particular framework region derived  
20 from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol. 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high binding  
25 affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which  
30 illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that  
35 the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved.

In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

Various forms of a humanized anti-RA1c polypeptide antibody are contemplated. For example, the humanized antibody may be an antibody fragment, such as a Fab, which is optionally conjugated with one or more cytotoxic agent(s) in order to generate an immunoconjugate. Alternatively, the humanized antibody may be an intact antibody, such as an intact IgG1 antibody.

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region ( $J_H$ ) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array into such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggemann et al., Year in Immuno. 7:33 (1993); U.S. Patent Nos. 5,545,806, 5,569,825, 5,591,669 (all of GenPharm); 5,545,807; and WO 97/17852.

Alternatively, phage display technology (McCafferty et al., Nature 348:552-553 [1990]) can be used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats, reviewed in, e.g., Johnson, Kevin S. and Chiswell, David J., Current Opinion in Structural Biology 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al., Nature, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., J. Mol. Biol. 222:581-597 (1991), or Griffith et al., EMBO J. 12:725-734 (1993). See, also, U.S. Patent Nos. 5,565,332 and 5,573,905.

As discussed above, human antibodies may also be generated by *in vitro* activated B cells (see U.S. Patents 5,567,610 and 5,229,275).

#### 4. Antibody fragments

In certain circumstances there are advantages of using antibody fragments, rather than whole antibodies. The smaller size of the fragments allows for rapid clearance, and may lead to improved access to solid tumors.

Various techniques have been developed for the production of antibody fragments.

Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., Journal of Biochemical and Biophysical Methods 24:107-117 (1992); and

Brennan et al., Science, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. Fab, Fv and ScFv antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of these fragments.

Antibody fragments can be isolated from the antibody phage libraries discussed above.

Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled

to form F(ab')<sub>2</sub> fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to

another approach, F(ab')<sub>2</sub> fragments can be isolated directly from recombinant host cell culture.

Fab and F(ab')<sub>2</sub> fragment with increased in vivo half-life comprising a salvage receptor binding epitope residues are described in U.S. Patent No. 5,869,046. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the

antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Patent No.

5,571,894; and U.S. Patent No. 5,587,458. Fv and sFv are the only species with intact combining sites that are devoid of constant regions; thus, they are suitable for reduced nonspecific binding during in vivo use. sFv fusion proteins may be constructed to yield fusion of an effector protein at either the amino or the carboxy terminus of an sFv. See Antibody Engineering, ed.

Borrebaeck, supra. The antibody fragment may also be a "linear antibody", e.g., as described in U.S. Patent 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

#### 5. Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two

different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of an

RA1c polypeptide as described herein. Other such antibodies may combine an RA1c polypeptide binding site with a binding site for another polypeptide. Alternatively, an anti-RA1c polypeptide

antibody arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD3), or Fc receptors for IgG (FcγR), such as FcγRI

(CD64), FcγRII (CD32) and FcγRIII (CD16), so as to focus and localize cellular defense

mechanisms to the RA1c polypeptide-expressing and/or binding cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express and/or bind RA1c polypeptide. These antibodies possess an RA1c polypeptide binding arm and an arm which binds the cytotoxic agent (e.g., saporin, anti-interferon- $\alpha$ , vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g., F(ab')<sub>2</sub> bispecific antibodies).

WO 96/16673 describes a bispecific anti-ErbB2/anti-Fc $\gamma$ RIII antibody and U.S. Patent No. 5,837,234 discloses a bispecific anti-ErbB2/anti-Fc $\gamma$ RI antibody. A bispecific anti-ErbB2/Fc $\alpha$  antibody is shown in WO98/02463. U.S. Patent No. 5,821,337 teaches a bispecific anti-ErbB2/anti-CD3 antibody.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., Nature 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., EMBO J. 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. Preferably, the fusion is with an Ig heavy chain constant domain, comprising at least part of the hinge, C<sub>H</sub>2, and C<sub>H</sub>3 regions. It is preferred to have the first heavy-chain constant region (C<sub>H</sub>1) containing the site necessary for light chain bonding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host cell. This provides for greater flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yield of the desired bispecific antibody. It is, however, possible to insert the coding sequences for two or all three polypeptide chains into a single expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios have no significant affect on the yield of the desired chain combination.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid

immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology 121:210 (1986).

According to another approach described in U.S. Patent No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C<sub>H</sub>3 domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g., tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g., alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., Science 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')<sub>2</sub> fragments. These fragments are reduced in the presence of the dithiol complexing agent, sodium arsenite, to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., J. Exp. Med. 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody F(ab')<sub>2</sub>

molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

5 Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., J. Immunol. 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region  
10 to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., Proc. Natl. Acad. Sci. USA 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a V<sub>H</sub> connected to a V<sub>L</sub> by a linker which is too short to allow pairing between the two domains on the same  
15 chain. Accordingly, the V<sub>H</sub> and V<sub>L</sub> domains of one fragment are forced to pair with the complementary V<sub>L</sub> and V<sub>H</sub> domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., J. Immunol., 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific  
20 antibodies can be prepared. Tutt et al., J. Immunol. 147:60 (1991).

#### 6. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [U.S. Patent  
25 No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-  
30 mercaptobutyrimidate and those disclosed, for example, in U.S. Patent No. 4,676,980.

#### 7. Multivalent Antibodies

A multivalent antibody may be internalized (and/or catabolized) faster than a bivalent antibody by a cell expressing an antigen to which the antibodies bind. The antibodies of the present invention can be multivalent antibodies (which are other than of the IgM class) with three  
35 or more antigen binding sites (e.g. tetravalent antibodies), which can be readily produced by

recombinant expression of nucleic acid encoding the polypeptide chains of the antibody. The multivalent antibody can comprise a dimerization domain and three or more antigen binding sites. The preferred dimerization domain comprises (or consists of) an Fc region or a hinge region. In this scenario, the antibody will comprise an Fc region and three or more antigen binding sites amino-terminal to the Fc region. The preferred multivalent antibody herein comprises (or consists of) three to about eight, but preferably four, antigen binding sites. The multivalent antibody comprises at least one polypeptide chain (and preferably two polypeptide chains), wherein the polypeptide chain(s) comprise two or more variable domains. For instance, the polypeptide chain(s) may comprise VD1-(X1)<sub>n</sub>-VD2-(X2)<sub>n</sub>-Fc, wherein VD1 is a first variable domain, VD2 is a second variable domain, Fc is one polypeptide chain of an Fc region, X1 and X2 represent an amino acid or polypeptide, and n is 0 or 1. For instance, the polypeptide chain(s) may comprise: VH-CH1-flexible linker-VH-CH1-Fc region chain; or VH-CH1-VH-CH1-Fc region chain. The multivalent antibody herein preferably further comprises at least two (and preferably four) light chain variable domain polypeptides. The multivalent antibody herein may, for instance, comprise from about two to about eight light chain variable domain polypeptides. The light chain variable domain polypeptides contemplated here comprise a light chain variable domain and, optionally, further comprise a CL domain.

#### 8. Effector Function Engineering

It may be desirable to modify the antibody of the invention with respect to effector function, e.g., so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al., J. Exp Med. 176:1191-1195 (1992) and Shopes, B. J. Immunol. 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al., Cancer Research 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al., Anti-Cancer Drug Design 3:219-230 (1989). To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in U.S. Patent 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of

the Fc region of an IgG molecule (e.g., IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, or IgG<sub>4</sub>) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

#### 9. Immunoconjugates

The invention also pertains to immunoconjugates, also known as antibody conjugates or antibody-drug conjugates (ADC), comprising an antibody conjugated to a cytotoxic agent such as a chemotherapeutic agent, a drug, a growth inhibitory agent, a toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

The use of antibody-drug conjugates for the local delivery of cytotoxic or cytostatic agents, i.e. drugs to kill or inhibit tumor cells in the treatment of cancer (Syrigos and Epenetos (1999) *Anticancer Research* 19:605-614; Niculescu-Duvaz and Springer (1997) *Adv. Drg Del. Rev.* 26:151-172; U.S. patent 4,975,278) theoretically allows targeted delivery of the drug moiety to tumors, and intracellular accumulation therein, where systemic administration of these unconjugated drug agents may result in unacceptable levels of toxicity to normal cells as well as the tumor cells sought to be eliminated (Baldwin et al., (1986) *Lancet* pp. (Mar. 15, 1986):603-05; Thorpe, (1985) "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in *Monoclonal Antibodies '84: Biological And Clinical Applications*, A. Pinchera et al. (ed.s), pp. 475-506). Maximal efficacy with minimal toxicity is sought thereby. Both polyclonal antibodies and monoclonal antibodies have been reported as useful in these strategies (Rowland et al., (1986) *Cancer Immunol. Immunother.*, 21:183-87). Drugs used in these methods include daunomycin, doxorubicin, methotrexate, and vindesine (Rowland et al., (1986) *supra*). Toxins used in antibody-toxin conjugates include bacterial toxins such as diphtheria toxin, plant toxins such as ricin, small molecule toxins such as geldanamycin (Mandler et al (2000) *Jour. of the Nat. Cancer Inst.* 92(19):1573-1581; Mandler et al (2000) *Bioorganic & Med. Chem. Letters* 10:1025-1028; Mandler et al (2002) *Bioconjugate Chem.* 13:786-791), maytansinoids (EP 1391213; Liu et al., (1996) *Proc. Natl. Acad. Sci. USA* 93:8618-8623), and calicheamicin (Lode et al (1998) *Cancer Res.* 58:2928; Hinman et al (1993) *Cancer Res.* 53:3336-3342). The toxins may effect their cytotoxic and cytostatic effects by mechanisms including tubulin binding, DNA binding, or topoisomerase inhibition. Some cytotoxic drugs tend to be inactive or less active when conjugated to large antibodies or protein receptor ligands.

ZEVALIN® (ibritumomab tiuxetan, Biogen/Idex) is an antibody-radioisotope conjugate composed of a murine IgG1 kappa monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes and <sup>111</sup>In or <sup>90</sup>Y radioisotope bound by a thiourea linker-chelator (Wiseman et al (2000) *Eur. Jour. Nucl. Med.* 27(7):766-77; Wiseman et al (2002) *Blood* 99(12):4336-42; Witzig et al (2002) *J. Clin. Oncol.* 20(10):2453-63; Witzig et al

(2002) J. Clin. Oncol. 20(15):3262-69). Although ZEVALIN has activity against B-cell non-Hodgkin's Lymphoma (NHL), administration results in severe and prolonged cytopenias in most patients. MYLOTARG™ (gemtuzumab ozogamicin, Wyeth Pharmaceuticals), an antibody drug conjugate composed of a hu CD33 antibody linked to calicheamicin, was approved in 2000 for the treatment of acute myeloid leukemia by injection (Drugs of the Future (2000) 25(7):686; US Patent Nos. 4970198; 5079233; 5585089; 5606040; 5693762; 5739116; 5767285; 5773001). Cantuzumab mertansine (Immunogen, Inc.), an antibody drug conjugate composed of the huC242 antibody linked via the disulfide linker SPP to the maytansinoid drug moiety, DM1, is advancing into Phase II trials for the treatment of cancers that express CanAg, such as colon, pancreatic, gastric, and others. MLN-2704 (Millennium Pharm., BZL Biologics, Immunogen Inc.), an antibody drug conjugate composed of the anti-prostate specific membrane antigen (PSMA) monoclonal antibody linked to the maytansinoid drug moiety, DM1, is under development for the potential treatment of prostate tumors. The auristatin peptides, auristatin E (AE) and monomethylauristatin (MMAE), synthetic analogs of dolastatin, were conjugated to chimeric monoclonal antibodies cBR96 (specific to Lewis Y on carcinomas) and cAC10 (specific to CD30 on hematological malignancies) (Doronina et al (2003) Nature Biotechnology 21(7):778-784) and are under therapeutic development.

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof that can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolacca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated antibodies. Examples include <sup>212</sup>Bi, <sup>131</sup>I, <sup>131</sup>In, <sup>90</sup>Y, and <sup>186</sup>Re. Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 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-methyldiethylene triaminepentaacetic acid (MX-

DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, maytansinoids, a trichothecene, and CC1065, and the derivatives of these toxins that have toxin activity, are also contemplated herein.

#### Maytansine and maytansinoids

In one embodiment, an anti-RA1c polypeptide antibody (full length or fragments) of the invention is conjugated to one or more maytansinoid molecules.

Maytansinoids are mitototic inhibitors which act by inhibiting tubulin polymerization. Maytansine was first isolated from the east African shrub *Maytenus serrata* (U.S. Patent No. 3,896,111). Subsequently, it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters (U.S. Patent No. 4,151,042). Synthetic maytansinol and derivatives and analogues thereof are disclosed, for example, in U.S. Patent Nos. 4,137,230; 4,248,870; 4,256,746; 4,260,608; 4,265,814; 4,294,757; 4,307,016; 4,308,268; 4,308,269; 4,309,428; 4,313,946; 4,315,929; 4,317,821; 4,322,348; 4,331,598; 4,361,650; 4,364,866; 4,424,219; 4,450,254; 4,362,663; and 4,371,533, the disclosures of which are hereby expressly incorporated by reference.

#### Maytansinoid-antibody conjugates

In an attempt to improve their therapeutic index, maytansine and maytansinoids have been conjugated to antibodies specifically binding to tumor cell antigens. Immunoconjugates containing maytansinoids and their therapeutic use are disclosed, for example, in U.S. Patent Nos. 5,208,020, 5,416,064 and European Patent EP 0 425 235 B1, the disclosures of which are hereby expressly incorporated by reference. Liu et al., Proc. Natl. Acad. Sci. USA 93:8618-8623 (1996) described immunoconjugates comprising a maytansinoid designated DM1 linked to the monoclonal antibody C242 directed against human colorectal cancer. The conjugate was found to be highly cytotoxic towards cultured colon cancer cells, and showed antitumor activity in an *in vivo* tumor growth assay. Chari et al., Cancer Research 52:127-131 (1992) describe immunoconjugates in which a maytansinoid was conjugated via a disulfide linker to the murine antibody A7 binding to an antigen on human colon cancer cell lines, or to another murine monoclonal antibody TA.1 that binds the HER-2/*neu* oncogene. The cytotoxicity of the TA.1-maytansinoid conjugate was tested *in vitro* on the human breast cancer cell line SK-BR-3, which expresses  $3 \times 10^5$  HER-2 surface antigens per cell. The drug conjugate achieved a degree of cytotoxicity similar to the free maytansinoid drug, which could be increased by increasing the number of maytansinoid molecules per antibody molecule. The A7-maytansinoid conjugate showed low systemic cytotoxicity in mice.

Anti-RA1c polypeptide antibody-maytansinoid conjugates (immunoconjugates)

Anti-RA1c polypeptide antibody-maytansinoid conjugates are prepared by chemically linking an anti-RA1c polypeptide antibody to a maytansinoid molecule without significantly diminishing the biological activity of either the antibody or the maytansinoid molecule. An average of 3-4 maytansinoid molecules conjugated per antibody molecule has shown efficacy in enhancing cytotoxicity of target cells without negatively affecting the function or solubility of the antibody, although even one molecule of toxin/antibody would be expected to enhance cytotoxicity over the use of naked antibody. Maytansinoids are well known in the art and can be synthesized by known techniques or isolated from natural sources. Suitable maytansinoids are disclosed, for example, in U.S. Patent No. 5,208,020 and in the other patents and nonpatent publications referred to hereinabove. Preferred maytansinoids are maytansinol and maytansinol analogues modified in the aromatic ring or at other positions of the maytansinol molecule, such as various maytansinol esters.

There are many linking groups known in the art for making antibody-maytansinoid conjugates, including, for example, those disclosed in U.S. Patent No. 5,208,020 or EP Patent 0 425 235 B1, and Chari et al., Cancer Research 52:127-131 (1992). The linking groups include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups, or esterase labile groups, as disclosed in the above-identified patents, disulfide and thioether groups being preferred.

Conjugates of the antibody and maytansinoid may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) 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 glutaraldehyde), bis-azido compounds (such as bis-(p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). Particularly preferred coupling agents include N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Carlsson et al., Biochem. J. 173:723-737 [1978]) and N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP) to provide for a disulfide linkage.

The linker may be attached to the maytansinoid molecule at various positions, depending on the type of the link. For example, an ester linkage may be formed by reaction with a hydroxyl group using conventional coupling techniques. The reaction may occur at the C-3 position having a hydroxyl group, the C-14 position modified with hydroxymethyl, the C-15 position

modified with a hydroxyl group, and the C-20 position having a hydroxyl group. In a preferred embodiment, the linkage is formed at the C-3 position of maytansinol or a maytansinol analogue.

#### Calicheamicin

Another immunoconjugate of interest comprises an anti-RA1c polypeptide antibody  
 5 conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. For the preparation of conjugates of the calicheamicin family, see U.S. patents 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, 5,877,296 (all to American Cyanamid Company). Structural analogues of calicheamicin which may be used include, but are not limited  
 10 to,  $\gamma_1^I$ ,  $\alpha_2^I$ ,  $\alpha_3^I$ , N-acetyl- $\gamma_1^I$ , PSAG and  $\theta_1^I$  (Hinman et al., Cancer Research 53:3336-3342 (1993), Lode et al., Cancer Research 58:2925-2928 (1998) and the aforementioned U.S. patents to American Cyanamid). Another anti-tumor drug that the antibody can be conjugated is QFA which is an antifolate. Both calicheamicin and QFA have intracellular sites of action and do not readily cross the plasma membrane. Therefore, cellular uptake of these agents through antibody  
 15 mediated internalization greatly enhances their cytotoxic effects.

#### Other cytotoxic agents

Other antitumor agents that can be conjugated to the anti-RA1c polypeptide antibodies of the invention include BCNU, streptozocin, vincristine and 5-fluorouracil, the family of agents known collectively LL-E33288 complex described in U.S. patents 5,053,394, 5,770,710, as well  
 20 as esperamicins (U.S. patent 5,877,296).

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica  
 25 charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published October 28, 1993.

The present invention further contemplates an immunoconjugate formed between an antibody and a compound with nucleolytic activity (e.g., a ribonuclease or a DNA endonuclease  
 30 such as a deoxyribonuclease; DNase).

For selective destruction of the tumor, the antibody may comprise a highly radioactive atom. A variety of radioactive isotopes are available for the production of radioconjugated anti-RA1c antibodies. Examples include  $At^{211}$ ,  $I^{131}$ ,  $I^{125}$ ,  $Y^{90}$ ,  $Re^{186}$ ,  $Re^{188}$ ,  $Sm^{153}$ ,  $Bi^{212}$ ,  $P^{32}$ ,  $Pb^{212}$  and radioactive isotopes of Lu. When the conjugate is used for detection, it may comprise a  
 35 radioactive atom for scintigraphic studies, for example  $tc^{99m}$  or  $I^{123}$ , or a spin label for nuclear

magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

The radio- or other labels may be incorporated in the conjugate in known ways. For example, the peptide may be biosynthesized or may be synthesized by chemical amino acid synthesis using suitable amino acid precursors involving, for example, fluorine-19 in place of hydrogen. Labels such as  $^{99m}\text{Tc}$  or  $^{123}\text{I}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$  and  $^{111}\text{In}$  can be attached via a cysteine residue in the peptide. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker et al (1978) Biochem. Biophys. Res. Commun. 80: 49-57 can be used to incorporate iodine-123. "Monoclonal Antibodies in Immunoscintigraphy" (Chatal, CRC Press 1989) describes other methods in detail.

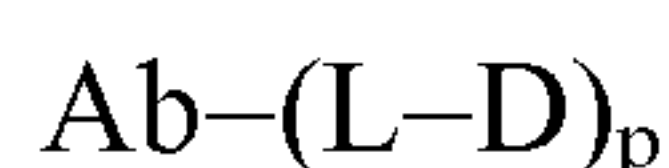
Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 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-methyldiethylene 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, photolabile linker, dimethyl linker or disulfide-containing linker (Chari et al., Cancer Research 52:127-131 (1992); U.S. Patent No. 5,208,020) may be used.

The compounds of the invention expressly contemplate, but are not limited to, ADC prepared with cross-linker reagents: BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from Pierce Biotechnology, Inc., Rockford, IL., U.S.A). See pages 467-498, 2003-2004 Applications Handbook and Catalog.

#### Preparation of antibody drug conjugates

In the antibody drug conjugates (ADC) of the invention, an antibody (Ab) is conjugated to one or more drug moieties (D), e.g. about 1 to about 20 drug moieties per antibody, through a

linker (L). The ADC of Formula I may be prepared by several routes, employing organic chemistry reactions, conditions, and reagents known to those skilled in the art, including: (1) reaction of a nucleophilic group of an antibody with a bivalent linker reagent, to form Ab-L, via a covalent bond, followed by reaction with a drug moiety D; and (2) reaction of a nucleophilic group of a drug moiety with a bivalent linker reagent, to form D-L, via a covalent bond, followed by reaction with the nucleophilic group of an antibody.



Nucleophilic groups on antibodies include, but are not limited to: (i) N-terminal amine groups, (ii) side chain amine groups, e.g. lysine, (iii) side chain thiol groups, e.g. cysteine, and (iv) sugar hydroxyl or amino groups where the antibody is glycosylated. Amine, thiol, and hydroxyl groups are nucleophilic and capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups. Certain antibodies have reducible interchain disulfides, i.e. cysteine bridges. Antibodies may be made reactive for conjugation with linker reagents by treatment with a reducing agent such as DTT (dithiothreitol). Each cysteine bridge will thus form, theoretically, two reactive thiol nucleophiles. Additional nucleophilic groups can be introduced into antibodies through the reaction of lysines with 2-iminothiolane (Traut's reagent) resulting in conversion of an amine into a thiol.

Antibody drug conjugates of the invention may also be produced by modification of the antibody to introduce electrophilic moieties, which can react with nucleophilic substituents on the linker reagent or drug. The sugars of glycosylated antibodies may be oxidized, e.g. with periodate oxidizing reagents, to form aldehyde or ketone groups which may react with the amine group of linker reagents or drug moieties. The resulting imine Schiff base groups may form a stable linkage, or may be reduced, e.g. by borohydride reagents to form stable amine linkages. In one embodiment, reaction of the carbohydrate portion of a glycosylated antibody with either galactose oxidase or sodium meta-periodate may yield carbonyl (aldehyde and ketone) groups in the protein that can react with appropriate groups on the drug (Hermanson, Bioconjugate Techniques). In another embodiment, proteins containing N-terminal serine or threonine residues can react with sodium meta-periodate, resulting in production of an aldehyde in place of the first amino acid (Geoghegan & Stroh, (1992) Bioconjugate Chem. 3:138-146; US 5362852). Such aldehyde can be reacted with a drug moiety or linker nucleophile.

Likewise, nucleophilic groups on a drug moiety include, but are not limited to: amine, thiol, hydroxyl, hydrazide, oxime, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide groups capable of reacting to form covalent bonds with electrophilic groups on

linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups.

Alternatively, a fusion protein comprising the anti-RA1c polypeptide antibody and  
5 cytotoxic agent may be made, e.g., by recombinant techniques or peptide synthesis. The length of DNA may comprise respective regions encoding the two portions of the conjugate either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the conjugate.

In yet another embodiment, the antibody may be conjugated to a "receptor" (such  
10 streptavidin) for utilization in tumor pre-targeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g., avidin) which is conjugated to a cytotoxic agent (e.g., a radionucleotide).

#### 10. Immunoliposomes

15 The anti-RA1c polypeptide antibodies disclosed herein may also be formulated as immunoliposomes. A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. Liposomes containing the antibody are prepared by  
20 methods known in the art, such as described in Epstein et al., Proc. Natl. Acad. Sci. USA 82:3688 (1985); Hwang et al., Proc. Natl. Acad. Sci. USA 77:4030 (1980); U.S. Pat. Nos. 4,485,045 and 4,544,545; and WO97/38731 published October 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method  
25 with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al., J. Biol. Chem. 257:286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally  
30 contained within the liposome. See Gabizon et al., J. National Cancer Inst. 81(19):1484 (1989).

#### B. RA1c polypeptide Binding Oligopeptides

RA1c polypeptide binding oligopeptides of the invention are oligopeptides that bind,  
preferably specifically, to an RA1c polypeptide as described herein. RA1c polypeptide binding oligopeptides may be chemically synthesized using known oligopeptide synthesis methodologies  
35 or may be prepared and purified using recombinant technology. RA1c polypeptide binding

oligopeptides are usually at least about 5 amino acids in length, alternatively at least about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 amino acids in length or more, wherein such oligopeptides that are capable of binding, preferably specifically, to an RA1c polypeptide as described herein. RA1c polypeptide binding oligopeptides may be identified without undue experimentation using well known techniques. In this regard, it is noted that techniques for screening oligopeptide libraries for oligopeptides that are capable of specifically binding to a polypeptide target are well known in the art (see, e.g., U.S. Patent Nos. 5,556,762, 5,750,373, 4,708,871, 4,833,092, 5,223,409, 5,403,484, 5,571,689, 5,663,143; PCT Publication Nos. WO 84/03506 and WO84/03564; Geysen et al., Proc. Natl. Acad. Sci. U.S.A., 81:3998-4002 (1984); Geysen et al., Proc. Natl. Acad. Sci. U.S.A., 82:178-182 (1985); Geysen et al., in *Synthetic Peptides as Antigens*, 130-149 (1986); Geysen et al., J. Immunol. Meth., 102:259-274 (1987); Schoofs et al., J. Immunol., 140:611-616 (1988), Cwirla, S. E. et al. (1990) Proc. Natl. Acad. Sci. USA, 87:6378; Lowman, H.B. et al. (1991) Biochemistry, 30:10832; Clackson, T. et al. (1991) Nature, 352: 624; Marks, J. D. et al. (1991), J. Mol. Biol., 222:581; Kang, A.S. et al. (1991) Proc. Natl. Acad. Sci. USA, 88:8363, and Smith, G. P. (1991) Current Opin. Biotechnol., 2:668).

In this regard, bacteriophage (phage) display is one well known technique which allows one to screen large oligopeptide libraries to identify member(s) of those libraries which are capable of specifically binding to a polypeptide target. Phage display is a technique by which variant polypeptides are displayed as fusion proteins to the coat protein on the surface of bacteriophage particles (Scott, J.K. and Smith, G. P. (1990) Science, 249: 386). The utility of phage display lies in the fact that large libraries of selectively randomized protein variants (or randomly cloned cDNAs) can be rapidly and efficiently sorted for those sequences that bind to a target molecule with high affinity. Display of peptide (Cwirla, S. E. et al. (1990) Proc. Natl. Acad. Sci. USA, 87:6378) or protein (Lowman, H.B. et al. (1991) Biochemistry, 30:10832; Clackson, T. et al. (1991) Nature, 352: 624; Marks, J. D. et al. (1991), J. Mol. Biol., 222:581; Kang, A.S. et al. (1991) Proc. Natl. Acad. Sci. USA, 88:8363) libraries on phage have been used for screening millions of polypeptides or oligopeptides for ones with specific binding properties (Smith, G. P. (1991) Current Opin. Biotechnol., 2:668). Sorting phage libraries of random mutants requires a strategy for constructing and propagating a large number of variants, a procedure for affinity purification using the target receptor, and a means of evaluating the results of binding enrichments. U.S. Patent Nos. 5,223,409, 5,403,484, 5,571,689, and 5,663,143.

Although most phage display methods have used filamentous phage, lambdoid phage display systems (WO 95/34683; U.S. 5,627,024), T4 phage display systems (Ren et al., Gene, 215: 439 (1998); Zhu et al., Cancer Research, 58(15): 3209-3214 (1998); Jiang et al., Infection & Immunity, 65(11): 4770-4777 (1997); Ren et al., Gene, 195(2):303-311 (1997); Ren, Protein Sci., 5: 1833 (1996); Efimov et al., Virus Genes, 10: 173 (1995)) and T7 phage display systems (Smith and Scott, Methods in Enzymology, 217: 228-257 (1993); U.S. 5,766,905) are also known.

Many other improvements and variations of the basic phage display concept have now been developed. These improvements enhance the ability of display systems to screen peptide libraries for binding to selected target molecules and to display functional proteins with the potential of screening these proteins for desired properties. Combinatorial reaction devices for phage display reactions have been developed (WO 98/14277) and phage display libraries have been used to analyze and control bimolecular interactions (WO 98/20169; WO 98/20159) and properties of constrained helical peptides (WO 98/20036). WO 97/35196 describes a method of isolating an affinity ligand in which a phage display library is contacted with one solution in which the ligand will bind to a target molecule and a second solution in which the affinity ligand will not bind to the target molecule, to selectively isolate binding ligands. WO 97/46251 describes a method of biopanning a random phage display library with an affinity purified antibody and then isolating binding phage, followed by a micropanning process using microplate wells to isolate high affinity binding phage. The use of *Staphylococcus aureus* protein A as an affinity tag has also been reported (Li et al. (1998) Mol Biotech., 9:187). WO 97/47314 describes the use of substrate subtraction libraries to distinguish enzyme specificities using a combinatorial library which may be a phage display library. A method for selecting enzymes suitable for use in detergents using phage display is described in WO 97/09446. Additional methods of selecting specific binding proteins are described in U.S. Patent Nos. 5,498,538, 5,432,018, and WO 98/15833.

Methods of generating peptide libraries and screening these libraries are also disclosed in U.S. Patent Nos. 5,723,286, 5,432,018, 5,580,717, 5,427,908, 5,498,530, 5,770,434, 5,734,018, 5,698,426, 5,763,192, and 5,723,323.

### C. RA1c polypeptide-binding small molecules

RA1c polypeptide binding small molecules are preferably organic molecules other than oligopeptides or antibodies as defined herein that bind, preferably specifically, to an RA1c polypeptide as described herein. RA1c polypeptide binding organic small molecules may be identified and chemically synthesized using known methodology (see, e.g., PCT Publication Nos.

WO00/00823 and WO00/39585). RA1c polypeptide binding organic small molecules are usually less than about 2000 daltons in size, alternatively less than about 1500, 750, 500, 250 or 200 daltons in size, wherein such organic small molecules that are capable of binding, preferably specifically, to an RA1c polypeptide as described herein may be identified without undue experimentation using well known techniques. In this regard, it is noted that techniques for screening organic small molecule libraries for molecules that are capable of binding to a polypeptide target are well known in the art (see, e.g., PCT Publication Nos. WO00/00823 and WO00/39585). RA1c polypeptide binding organic small molecules may be, for example, aldehydes, ketones, oximes, hydrazones, semicarbazones, carbazides, primary amines, secondary amines, tertiary amines, N-substituted hydrazines, hydrazides, alcohols, ethers, thiols, thioethers, disulfides, carboxylic acids, esters, amides, ureas, carbamates, carbonates, ketals, thioketals, acetals, thioacetals, aryl halides, aryl sulfonates, alkyl halides, alkyl sulfonates, aromatic compounds, heterocyclic compounds, anilines, alkenes, alkynes, diols, amino alcohols, oxazolidines, oxazolines, thiazolidines, thiazolines, enamines, sulfonamides, epoxides, aziridines, isocyanates, sulfonyl chlorides, diazo compounds, acid chlorides, or the like.

#### D. Compounds that Modulate RA1c Expression

Compounds that modulate RA1c expression of the invention are antisense polynucleotides, silencing RNA molecules, catalytic RNA molecules, RNA-DNA chimeras, aptamers, antibodies, oligopeptides, inorganic or organic small molecules, or other compounds that modulate the expression of an RA1c polypeptide in a cell as described herein. Certain such compounds activate or increase transcription of a RA1c polynucleotide. Certain such compounds activate or increase translation of a RA1c polynucleotide. Certain such compounds activate or increase RA1c polypeptide expression. Certain such compounds decrease or block transcription of a RA1c polynucleotide. Certain such compounds decrease or block translation of a RA1c polynucleotide. Certain such compounds decrease or block RA1c polypeptide expression. Compounds that modulate RA1c expression may act on one or more intracellular signaling pathways involved in RA1c expression. For example, a compound that modulates RA1c expression may modulate a p38 MAP kinase signaling pathway. In another example, a compound that modulates RA1c expression may modulate a PI3K signaling pathway. In another example, a compound that modulates RA1c expression may modulate a signaling pathway normally activated by IL-6.

Compounds that modulate RA1c expression of the invention may be prepared in a manner appropriate for the nature of the compound. An antibody or antibody fragment that modulates RA1c expression, for example, may be prepared using the same techniques described

herein for preparing anti-RA1c antibodies or fragments thereof. An oligopeptide that modulates RA1c expression, in another example, may be prepared using the same techniques as described herein for preparing RA1c polypeptide binding oligopeptides. An organic small molecule that modulates RA1c expression, in another example, may be prepared using the same techniques described herein for preparing RA1c polypeptide binding small molecules. An aptamer that modulates RA1c expression, in another example, may be prepared using the same techniques as described herein for preparing RA1c-binding aptamers.

E. Screening for RA1c Antagonists and Compounds that Modulate RA1c Expression

Techniques for generating RA1c antagonists, such as antibodies, oligopeptides and small molecules that bind to RA1c polypeptides, and compounds that modulate RA1c expression have been described above. One may further select RA1c antagonists or compounds that modulate RA1c expression with certain biological characteristics, as desired.

The growth inhibitory effects of an RA1c antagonist or compound that modulates RA1c expression of the invention may be assessed by methods known in the art, e.g., using cells which express an RA1c polypeptide either endogenously or following transfection with the RA1c polypeptide gene. For example, appropriate tumor cell lines and RA1c polypeptide-transfected cells may be treated with an RA1c antagonist or compound that modulates RA1c expression at various concentrations for a few days (e.g., 2-7) days and stained with crystal violet or MTT or analyzed by some other colorimetric assay. Another method of measuring proliferation would be by comparing <sup>3</sup>H-thymidine uptake by the cells treated in the presence or absence of an RA1c antagonist or compound that modulates RA1c expression. After treatment, the cells are harvested and the amount of radioactivity incorporated into the DNA quantitated in a scintillation counter. Appropriate positive controls include treatment of a selected cell line with a growth inhibitory antibody known to inhibit growth of that cell line. Growth inhibition of tumor cells *in vivo* can be determined in various ways known in the art. The tumor cell may be one that overexpresses an RA1c polypeptide. The RA1c antagonist or compound that modulates RA1c expression will inhibit cell proliferation of an RA1c polypeptide-expressing tumor cell *in vitro* or *in vivo* by about 25-100% compared to the untreated tumor cell, more preferably, by about 30-100%, and even more preferably by about 50-100% or 70-100%, in one embodiment, at an antibody concentration of about 0.5 to 30 µg/ml. In certain embodiments, growth inhibition can be measured at an antibody concentration of about 0.5 to 30 µg/ml or about 0.5 nM to 200 nM in cell culture, where the growth inhibition is determined 1-10 days after exposure of the tumor cells to the antibody. In certain embodiments, an antibody is growth inhibitory *in vivo* if administration

of the anti-RA1c polypeptide antibody at about 1 µg/kg to about 100 mg/kg body weight results in reduction in tumor size or reduction of tumor cell proliferation within about 5 days to 3 months from the first administration of the antibody, preferably within about 5 to 30 days.

To select for an RA1c antagonist or compound that modulates RA1c expression which induces cell death (apoptosis), loss of membrane integrity as indicated by, e.g., propidium iodide (PI), trypan blue or 7AAD uptake may be assessed relative to control. A PI uptake assay can be performed in the absence of complement and immune effector cells. RA1c polypeptide-expressing tumor cells are incubated with medium alone or medium containing the appropriate anti-RA1c polypeptide antibody (e.g, at about 10µg/ml), RA1c polypeptide binding oligopeptide, RA1c polypeptide binding organic small molecule, other RA1c antagonist, or compound that modulates RA1c expression. The cells are incubated for a 3-day time period. Following each treatment, cells are washed and aliquoted into 35 mm strainer-capped 12 x 75 tubes (1ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10 µg/ml). Samples may be analyzed in a nonlimiting example using a FACSCAN® flow cytometer and FACSCONVERT® CellQuest software (Becton Dickinson). Those RA1c antagonists and compounds that modulate RA1c expression that induce statistically significant levels of cell death as determined by PI uptake may be selected as cell death-inducing RA1c antagonists or compounds that modulate RA1c expression.

To screen for antibodies, oligopeptides or other organic small molecules, or other RA1c antagonists which bind to an epitope on an RA1c polypeptide bound by an antibody of interest, a routine cross-blocking assay such as that described in Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. This assay can be used to determine if a test antibody, oligopeptide, other organic small molecule, or other RA1c antagonist binds the same site or epitope as a known anti-RA1c polypeptide antibody.

Alternatively, or additionally, epitope mapping can be performed by methods known in the art . For example, the antibody sequence can be mutagenized such as by alanine scanning, to identify contact residues. The mutant antibody is initially tested for binding with polyclonal antibody to ensure proper folding. In a different method, peptides corresponding to different regions of an RA1c polypeptide can be used in competition assays with the test antibodies or with a test antibody and an antibody with a characterized or known epitope.

#### F. Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (e.g., a peptidyl

chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to convert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as  $\beta$ -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs;  $\beta$ -lactamase useful for converting drugs derivatized with  $\beta$ -lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, e.g., Massey, *Nature* 328:457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-RA1c antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, e.g., Neuberger et al., *Nature* 312:604-608 (1984)).

#### G. Anti-RA1c polypeptide Antibody Variants

In addition to the anti-RA1c polypeptide antibodies described herein, it is contemplated that anti-RA1c polypeptide antibody variants can be prepared. Anti-RA1c polypeptide antibody variants can be prepared by introducing appropriate nucleotide changes into the encoding DNA, or by synthesis of the desired antibody. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the anti-RA1c polypeptide antibody, such as

changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

Variations in the anti-RA1c polypeptide antibodies described herein can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Patent No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the antibody that results in a change in the amino acid sequence as compared with the native sequence antibody or polypeptide. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the anti-RA1c polypeptide antibody. Guidance in determining which amino acid residue may be inserted, substituted or deleted without adversely affecting the desired activity may be found by comparing the sequence of the anti-RA1c polypeptide antibody with that of homologous known protein molecules and minimizing the number of amino acid sequence changes made in regions of high homology. Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural or chemical properties, such as the replacement of a leucine with a serine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of about 1 to 5 amino acids. The variation allowed may be determined by systematically making insertions, deletions or substitutions of amino acids in the sequence and testing the resulting variants for activity exhibited by the parent sequence.

Anti-RA1c polypeptide antibody and RA1c polypeptide fragments are provided herein. Such fragments may be truncated at the N-terminus or C-terminus, or may lack internal residues, for example, when compared with a full length native antibody or protein. Certain fragments lack amino acid residues that are not essential for a desired biological activity of the anti-RA1c antibody or RA1c polypeptide.

Anti-RA1c antibody and RA1c polypeptide fragments may be prepared by any of a number of conventional techniques. Desired peptide fragments may be chemically synthesized. An alternative approach involves generating antibody or polypeptide fragments by enzymatic digestion, e.g., by treating the protein with an enzyme known to cleave proteins at sites defined by particular amino acid residues, or by digesting the DNA with suitable restriction enzymes and isolating the desired fragment. Yet another suitable technique involves isolating and amplifying a DNA fragment encoding a desired antibody or polypeptide fragment, by polymerase chain reaction (PCR). Oligonucleotides that define the desired termini of the DNA fragment are employed at the 5' and 3' primers in the PCR. Preferably, anti-RA1c antibody and RA1c polypeptide fragments share at least one biological or immunological activity with the native anti-RA1c polypeptide antibody or RA1c polypeptide disclosed herein.

In particular embodiments, conservative substitutions of interest are shown in Table 1 under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

Table 1

| <b>Original Residue</b> | <b>Exemplary Substitutions</b>      | <b>Preferred Substitutions</b> |
|-------------------------|-------------------------------------|--------------------------------|
| Ala (A)                 | Val; Leu; Ile                       | Val                            |
| Arg (R)                 | Lys; Gln; Asn                       | Lys                            |
| Asn (N)                 | Gln; His; Asp, Lys; Arg             | Gln                            |
| Asp (D)                 | Glu; Asn                            | Glu                            |
| Cys (C)                 | Ser; Ala                            | Ser                            |
| Gln (Q)                 | Asn; Glu                            | Asn                            |
| Glu (E)                 | Asp; Gln                            | Asp                            |
| Gly (G)                 | Ala                                 | Ala                            |
| His (H)                 | Asn; Gln; Lys; Arg                  | Arg                            |
| Ile (I)                 | Leu; Val; Met; Ala; Phe; Norleucine | Leu                            |
| Leu (L)                 | Norleucine; Ile; Val; Met; Ala; Phe | Ile                            |
| Lys (K)                 | Arg; Gln; Asn                       | Arg                            |
| Met (M)                 | Leu; Phe; Ile                       | Leu                            |
| Phe (F)                 | Trp; Leu; Val; Ile; Ala; Tyr        | Tyr                            |
| Pro (P)                 | Ala                                 | Ala                            |
| Ser (S)                 | Thr                                 | Thr                            |
| Thr (T)                 | Val; Ser                            | Ser                            |
| Trp (W)                 | Tyr; Phe                            | Tyr                            |
| Tyr (Y)                 | Trp; Phe; Thr; Ser                  | Phe                            |
| Val (V)                 | Ile; Leu; Met; Phe; Ala; Norleucine | Leu                            |

Substantial modifications in function or immunological identity of the anti-RA1c antibody or RA1c polypeptide are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of

the molecule at the target site, or (c) the bulk of the side chain. Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, New York (1975)):

(1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)

5 (2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)

(3) acidic: Asp (D), Glu (E)

(4) basic: Lys (K), Arg (R), His(H)

Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

10 (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

15 (6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Such substituted residues also may be introduced into the conservative substitution sites or, more preferably, into the remaining (non-conserved) sites.

20 The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known  
25 techniques can be performed on the cloned DNA to produce the anti-RA1c polypeptide antibody or RA1c polypeptide variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is  
30 typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant [Cunningham and Wells, Science, 244:1081-1085 (1989)]. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol.,

150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Any cysteine residue not involved in maintaining the proper conformation of the anti-RA1c polypeptide antibody or RA1c polypeptide also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the anti-RA1c antibody or RA1c polypeptide to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g., a humanized or human antibody).

Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g., 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g., binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human RA1c polypeptide. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Nucleic acid molecules encoding amino acid sequence variants of the anti-RA1c antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the anti-RA1c antibody.

#### H. Modifications of Anti-RA1c Antibodies and RA1c polypeptides

Covalent modifications of anti-RA1c polypeptide antibodies and RA1c polypeptides are included within the scope of this invention. One type of covalent modification includes reacting

targeted amino acid residues of an anti-RA1c antibody or RA1c polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C- terminal residues of the anti-RA1c polypeptide antibody or RA1c polypeptide. Derivatization with bifunctional agents is useful, for instance, for crosslinking anti-RA1c antibody or RA1c polypeptide to a water-insoluble support matrix or surface for use in the method for purifying anti-RA1c antibodies, and vice-versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)dithio]propioimide.

Other modifications include deamidation of glutamyl and asparagyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the anti-RA1c antibody or RA1c polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the antibody or polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence anti-RA1c antibody or RA1c polypeptide (either by removing the underlying glycosylation site or by deleting the glycosylation by chemical or enzymatic means), or adding one or more glycosylation sites that are not present in the native sequence anti-RA1c antibody or RA1c polypeptide. In addition, the phrase includes qualitative changes in the glycosylation of the native proteins, involving a change in the nature and proportions of the various carbohydrate moieties present.

Glycosylation of antibodies and other polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the anti-RA1c antibody or RA1c polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original anti-RA1c antibody or RA1c polypeptide (for O-linked glycosylation sites). The anti-RA1c antibody or RA1c polypeptide amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the anti-RA1c antibody or RA1c polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the anti-RA1c antibody or RA1c polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the anti-RA1c antibody or RA1c polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Another type of covalent modification of anti-RA1c antibody or RA1c polypeptide comprises linking the antibody or polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol (PEG), polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. The antibody or polypeptide also may 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, nanoparticles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Oslo, A., Ed., (1980).

The anti-RA1c antibody or RA1c polypeptide of the present invention may also be modified in a way to form chimeric molecules comprising an anti-RA1c antibody or RA1c polypeptide fused to another, heterologous polypeptide or amino acid sequence.

In one embodiment, such a chimeric molecule comprises a fusion of the anti-RA1c antibody or RA1c polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl-terminus of the anti-RA1c antibody or RA1c polypeptide. The presence of such epitope-tagged forms of the anti-RA1c antibody or RA1c polypeptide can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the anti-RA1c antibody or RA1c polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag. Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an  $\alpha$ -tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)].

In an alternative embodiment, the chimeric molecule may comprise a fusion of the anti-RA1c antibody or RA1c polypeptide with an immunoglobulin or a particular region of an immunoglobulin. For a bivalent form of the chimeric molecule (also referred to as an “immunoadhesin”), such a fusion could be to the Fc region of an IgG molecule. The Ig fusions preferably include the substitution of a soluble (transmembrane domain deleted or inactivated) form of an anti-RA1c antibody or RA1c polypeptide in place of at least one variable region within an Ig molecule. In a particularly preferred embodiment, the immunoglobulin fusion includes the hinge, CH<sub>2</sub> and CH<sub>3</sub>, or the hinge, CH<sub>1</sub>, CH<sub>2</sub> and CH<sub>3</sub> regions of an IgG1 molecule. For the production of immunoglobulin fusions see also US Patent No. 5,428,130 issued June 27, 1995.

#### I. Preparation of Anti-RA1c Antibodies and RA1c polypeptides

The description below relates primarily to production of anti-RA1c antibodies and RA1c polypeptides by culturing cells transformed or transfected with a vector containing anti-RA1c antibody- and RA1c polypeptide-encoding nucleic acid. It is, of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare anti-RA1c antibodies and RA1c polypeptides. For instance, the appropriate amino acid sequence, or

portions thereof, may be produced by direct peptide synthesis using solid-phase techniques [see, e.g., Stewart et al., Solid-Phase Peptide Synthesis, W.H. Freeman Co., San Francisco, CA (1969); Merrifield, J. Am. Chem. Soc., 85:2149-2154 (1963)]. *In vitro* protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be accomplished, for instance, using an Applied Biosystems Peptide Synthesizer (Foster City, CA) using manufacturer's instructions. Various portions of the anti-RA1c antibody or RA1c polypeptide may be chemically synthesized separately and combined using chemical or enzymatic methods to produce the desired anti-RA1c antibody or RA1c polypeptide.

1. Isolation of DNA Encoding Anti-RA1c Antibody or RA1c Polypeptide

DNA encoding anti-RA1c antibody or RA1c polypeptide may be obtained from a cDNA library prepared from tissue believed to possess the anti-RA1c antibody or RA1c polypeptide mRNA and to express it at a detectable level. Accordingly, human anti-RA1c antibody or RA1c polypeptide DNA can be conveniently obtained from a cDNA library prepared from human tissue. The anti-RA1c antibody- or RA1c polypeptide-encoding gene may also be obtained from a genomic library or by known synthetic procedures (e.g., automated nucleic acid synthesis).

Libraries can be screened with probes (such as oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding anti-RA1c antibody or RA1c polypeptide is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

Techniques for screening a cDNA library are well known in the art. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like <sup>32</sup>P-labeled ATP, biotinylation or enzyme labeling. Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook et al., supra.

Sequences identified in such library screening methods can be compared and aligned to other known sequences deposited and available in public databases such as GenBank or other private sequence databases. Sequence identity (at either the amino acid or nucleotide level) within defined regions of the molecule or across the full-length sequence can be determined using methods known in the art and as described herein.

Nucleic acid having protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., *supra*, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

## 2. Selection and Transformation of Host Cells

Host cells are transfected or transformed with expression or cloning vectors described herein for anti-RA1c antibody or RA1c polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991) and Sambrook et al., *supra*.

Methods of eukaryotic cell transfection and prokaryotic cell transformation are known to the ordinarily skilled artisan, for example,  $\text{CaCl}_2$ ,  $\text{CaPO}_4$ , liposome-mediated and electroporation. Depending on the host cell used, transformation is performed using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., *supra*, or electroporation is generally used for prokaryotes. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) can be employed. General aspects of mammalian cell host system transfections have been described in U.S. Patent No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

Suitable host cells for cloning or expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *E. coli*. Various *E. coli* strains are publicly available, such as *E. coli* K12 strain MM294 (ATCC 31,446); *E. coli* X1776 (ATCC 31,537); *E. coli* strain W3110 (ATCC 27,325) and K5

772 (ATCC 53,635). Other suitable prokaryotic host cells include Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. These examples are illustrative rather than limiting. Strain W3110 is one particularly preferred host or parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell secretes minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins endogenous to the host, with examples of such hosts including *E. coli* W3110 strain 1A2, which has the complete genotype *tonA*; *E. coli* W3110 strain 9E4, which has the complete genotype *tonA ptr3*; *E. coli* W3110 strain 27C7 (ATCC 55,244), which has the complete genotype *tonA ptr3 phoA E15 (argF-lac)169 degP ompT kan<sup>r</sup>*; *E. coli* W3110 strain 37D6, which has the complete genotype *tonA ptr3 phoA E15 (argF-lac)169 degP ompT rbs7 ilvG kan<sup>r</sup>*; *E. coli* W3110 strain 40B4, which is strain 37D6 with a non-kanamycin resistant *degP* deletion mutation; and an *E. coli* strain having mutant periplasmic protease disclosed in U.S. Patent No. 4,946,783 issued 7 August 1990. Alternatively, *in vitro* methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

Full length antibody, antibody fragments, and antibody fusion proteins can be produced in bacteria, in particular when glycosylation and Fc effector function are not needed, such as when the therapeutic antibody is conjugated to a cytotoxic agent (e.g., a toxin) and the immunoconjugate by itself shows effectiveness in tumor cell destruction. Full-length antibodies have greater half life in circulation. Production in *E. coli* is faster and more cost efficient. For expression of antibody fragments and polypeptides in bacteria, see, e.g., U.S. 5,648,237 (Carter et al.), U.S. 5,789,199 (Joly et al.), and U.S. 5,840,523 (Simmons et al.) which describes translation initiation regio (TIR) and signal sequences for optimizing expression and secretion, these patents incorporated herein by reference. After expression, the antibody is isolated from the *E. coli* cell paste in a soluble fraction and can be purified through, e.g., a protein A or G column depending on the isotype. Final purification can be carried out similar to the process for purifying antibody expressed e.g., in CHO cells.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for anti-RA1c antibody- or RA1c polypeptide-encoding vectors. *Saccharomyces cerevisiae* is a commonly used lower eukaryotic host microorganism. Others include *Schizosaccharomyces pombe* (Beach and Nurse, Nature, 290: 140 [1981]; EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. Patent No. 4,943,529; Fleer et al., Bio/Technology, 9:968-975 (1991)) such as, e.g., *K. lactis* (MW98-8C, CBS683, CBS4574;

Louvencourt et al., J. Bacteriol., 154(2):737-742 [1983]), *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickerhamii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilae* (ATCC 36,906; Van den Berg et al., Bio/Technology, 8:135 (1990)), *K. thermotolerans*, and *K. marxianus*; *Yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishna et al., J. Basic Microbiol., 28:265-278 [1988]); *Candida*; *Trichoderma reesei* (EP 244,234); *Neurospora crassa* (Case et al., Proc. Natl. Acad. Sci. USA, 76:5259-5263 [1979]); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31 October 1990); and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium* (WO 91/00357 published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance et al., Biochem. Biophys. Res. Commun., 112:284-289 [1983]; Tilburn et al., Gene, 26:205-221 [1983]; Yelton et al., Proc. Natl. Acad. Sci. USA, 81: 1470-1474 [1984]) and *A. niger* (Kelly and Hynes, EMBO J., 4:475-479 [1985]). Methylotropic yeasts are suitable herein and include, but are not limited to, yeast capable of growth on methanol selected from the genera consisting of *Hansenula*, *Candida*, *Kloeckera*, *Pichia*, *Saccharomyces*, *Torulopsis*, and *Rhodotorula*. A list of specific species that are exemplary of this class of yeasts may be found in C. Anthony, The Biochemistry of Methylotrophs, 269 (1982).

Suitable host cells for the expression of glycosylated anti-RA1c antibody or RA1c polypeptide are derived from multicellular organisms. Examples of invertebrate cells include insect cells such as *Drosophila* S2 and *Spodoptera* Sf9, as well as plant cells, such as cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol. 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34);

buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci. 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

5 Host cells are transformed with the above-described expression or cloning vectors for anti-RA1c antibody or RA1c polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

### 3. Selection and Use of a Replicable Vector

10 The nucleic acid (e.g., cDNA or genomic DNA) encoding anti-RA1c antibody or RA1c polypeptide may be inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an  
15 appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan.

20 The RA1c polypeptide may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the anti-RA1c antibody- or RA1c polypeptide-encoding DNA that is inserted into the  
25 vector. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders, the latter described in U.S. Patent No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179  
30 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression, mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders.

Both expression and cloning vectors contain a nucleic acid sequence that enables the  
35 vector to replicate in one or more selected host cells. Such sequences are well known for a

variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells.

5           Expression and cloning vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

10           An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the anti-RA1c antibody- or RA1c polypeptide-encoding nucleic acid, such as DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). A suitable selection  
15           gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)].

            Expression and cloning vectors usually contain a promoter operably linked to the anti-  
20           RA1c antibody- or RA1c polypeptide-encoding nucleic acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (*trp*) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and  
25           hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding anti-RA1c antibody or RA1c polypeptide.

            Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other  
30           glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657.

Anti-RA1c antibody or RA1c polypeptide transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

Transcription of a DNA encoding the anti-RA1c antibody or RA1c polypeptide by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the vector at a position 5' or 3' to the anti-RA1c antibody or RA1c polypeptide coding sequence, but is preferably located at a site 5' from the promoter.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding anti-RA1c antibody or RA1c polypeptide.

Still other methods, vectors, and host cells suitable for adaptation to the synthesis of anti-RA1c antibody or RA1c polypeptide in recombinant vertebrate cell culture are described in Gething et al., *Nature*, 293:620-625 (1981); Mantei et al., *Nature*, 281:40-46 (1979); EP 117,060; and EP 117,058.

#### 4. Culturing the Host Cells

The host cells used to produce the anti-RA1c antibody or RA1c polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., Meth. Enz. 58:44 (1979), Barnes et al., Anal. Biochem. 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

#### 5. Detecting Gene Amplification/Expression

Gene amplification or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. Antibodies useful for immunohistochemical staining or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence RA1c polypeptide or against a synthetic peptide based on the DNA sequence provided herein or against exogenous sequence fused to RA1c polypeptide DNA and encoding a specific antibody epitope.

## 6. Purification of Anti-RA1c Antibody and RA1c polypeptide

Forms of anti-RA1c antibody and RA1c polypeptide may be recovered from culture medium or from host cell lysates. If membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or by enzymatic cleavage. Cells employed in expression of anti-RA1c antibody and RA1c polypeptide can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents.

It may be desired to purify anti-RA1c antibody and RA1c polypeptide from recombinant cell proteins or polypeptides. The following procedures are exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; protein A Sepharose columns to remove contaminants such as IgG; and metal chelating columns to bind epitope-tagged forms of the anti-RA1c antibody and RA1c polypeptide. Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, Methods in Enzymology, 182 (1990); Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York (1982). The purification step(s) selected will depend, for example, on the nature of the production process used and the particular anti-RA1c antibody or RA1c polypeptide produced.

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Carter et al., Bio/Technology 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is

present in the antibody. Protein A can be used to purify antibodies that are based on human  $\gamma 1$ ,  $\gamma 2$  or  $\gamma 4$  heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human  $\gamma 3$  (Guss et al., *EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other  
5 matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C<sub>H</sub>3 domain, the Bakerbond ABX<sup>TM</sup>resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation,  
10 Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE<sup>TM</sup> chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of  
15 interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g., from about 0-0.25M salt).

J. RA1c polypeptides and RA1c polypeptide-Encoding Nucleic Acids - Specific  
20 forms and applications

Nucleotide sequences (or their complement) encoding RA1c polypeptides have various applications in the art of molecular biology, including uses as hybridization probes, in chromosome and gene mapping and in the generation of anti-sense RNA and DNA probes. RA1c polypeptide-encoding nucleic acid will also be useful for the preparation of RA1c polypeptides  
25 by the recombinant techniques described herein, wherein those RA1c polypeptides may find use, for example, in the preparation of anti-RA1c antibodies as described herein.

A full-length native sequence RA1c polypeptide gene, or portions thereof, may be used as hybridization probes for a cDNA library to isolate other cDNAs (for instance, those encoding naturally-occurring variants of RA1c polypeptide or RA1c polypeptide from other species) which  
30 have a desired sequence identity to a native RA1c polypeptide sequence disclosed herein. Optionally, the length of the probes will be about 20 to about 50 bases. The hybridization probes may be derived from at least partially novel regions of the full length native nucleotide sequence wherein those regions may be determined without undue experimentation or from genomic sequences including promoters, enhancer elements and introns of native sequence RA1c  
35 polypeptide. By way of example, a screening method will comprise isolating the coding region

of the RA1c polypeptide gene using the known DNA sequence to synthesize a selected probe of about 40 bases. Hybridization probes may be labeled by a variety of labels, including radionucleotides such as  $^{32}\text{P}$  or  $^{35}\text{S}$ , or enzymatic labels such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems. Labeled probes having a sequence complementary to that of the RA1c polypeptide gene of the present invention can be used to screen libraries of human cDNA, genomic DNA or mRNA to determine which members of such libraries the probe hybridizes to. Hybridization techniques are described in further detail in the Examples below. Any EST sequences disclosed in the present application may similarly be employed as probes, using the methods disclosed herein.

Other useful fragments of the RA1c polypeptide-encoding nucleic acids include antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target RA1c polypeptide mRNA (sense) or RA1c polypeptide DNA (antisense) sequences. Antisense or sense oligonucleotides, according to the present invention, comprise a fragment of the coding region of RA1c DNA. Such a fragment generally comprises at least about 14 nucleotides, preferably from about 14 to 30 nucleotides. The ability to derive an antisense or a sense oligonucleotide, based upon a cDNA sequence encoding a given protein is described in, for example, Stein and Cohen (Cancer Res. 48:2659, 1988) and van der Krol et al. (BioTechniques 6:958, 1988).

Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of duplexes that block transcription or translation of the target sequence by one of several means, including enhanced degradation of the duplexes, premature termination of transcription or translation, or by other means. Such methods are encompassed by the present invention. The antisense oligonucleotides thus may be used to block expression of an RA1c protein, wherein the RA1c protein may play a role in the induction of cancer in mammals.

Antisense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages, such as those described in WO 91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable *in vivo* (i.e., capable of resisting enzymatic degradation) but retain sequence specificity to be able to bind to target nucleotide sequences.

Preferred intragenic sites for antisense binding include the region incorporating the translation initiation/start codon (5'-AUG / 5'-ATG) or termination/stop codon (5'-UAA, 5'-UAG and 5'-UGA / 5'-TAA, 5'-TAG and 5'-TGA) of the open reading frame (ORF) of the gene. These regions refer to a portion of the mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation or termination codon. Other preferred regions for antisense binding include: introns; exons; intron-exon

junctions; the open reading frame (ORF) or "coding region," which is the region between the translation initiation codon and the translation termination codon; the 5' cap of an mRNA which comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage and includes 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap; the 5' untranslated region (5'UTR), the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene; and the 3' untranslated region (3'UTR), the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene.

Specific examples of preferred antisense compounds useful for inhibiting expression of RA1c polypeptide include oligonucleotides containing modified backbones or non-natural internucleoside linkages. Oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides. Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotri-esters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and borano-phosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included. Representative United States patents that teach the preparation of phosphorus-containing linkages include, but are not limited to, U.S. Pat. Nos.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH<sub>2</sub> component parts. Representative United States patents that teach the preparation of such oligonucleosides include, but are not limited to, U.S. Pat. Nos.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, each of which is herein incorporated by reference.

In other preferred antisense oligonucleotides, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., Science, 1991, 254, 1497-1500.

Preferred antisense oligonucleotides incorporate phosphorothioate backbones or heteroatom backbones, and in particular -CH<sub>2</sub>-NH-O-CH<sub>2</sub>-, -CH<sub>2</sub>-N(CH<sub>3</sub>)-O-CH<sub>2</sub>- [known as a methylene (methylimino) or MMI backbone], -CH<sub>2</sub>-O-N(CH<sub>3</sub>)-CH<sub>2</sub>-, -CH<sub>2</sub>-N(CH<sub>3</sub>)-N(CH<sub>3</sub>)-CH<sub>2</sub>- and -O-N(CH<sub>3</sub>)-CH<sub>2</sub>-CH<sub>2</sub>- [wherein the native phosphodiester backbone is represented as -O-P-O-CH<sub>2</sub>-] described in the above referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above referenced U.S. Pat. No. 5,602,240. Also preferred are antisense oligonucleotides having morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties.

Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-alkyl, S-alkyl, or N-alkyl; O-alkenyl, S-alkenyl, or N-alkenyl; O-alkynyl, S-alkynyl or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C<sub>1</sub> to C<sub>10</sub> alkyl or C<sub>2</sub> to C<sub>10</sub> alkenyl and alkynyl. Particularly preferred are O[(CH<sub>2</sub>)<sub>n</sub>O]<sub>m</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>OCH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>NH<sub>2</sub>, O(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>ONH<sub>2</sub>, and O(CH<sub>2</sub>)<sub>n</sub>ON[(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>]<sub>2</sub>, where n and m are from 1 to about 10. Other preferred antisense oligonucleotides comprise one of the following at the 2' position: C<sub>1</sub> to C<sub>10</sub> lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, SOCH<sub>3</sub>, SO<sub>2</sub>CH<sub>3</sub>, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxy, i.e., a O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub> group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>3</sub>)<sub>2</sub>.

A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene (-CH<sub>2</sub>-)<sub>n</sub> group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Other preferred modifications include 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub> NH<sub>2</sub>), 2'-allyl (2'-CH<sub>2</sub>-CH=CH<sub>2</sub>), 2'-O-allyl (2'-O-CH<sub>2</sub>-CH=CH<sub>2</sub>) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722;

5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl ( $-C\equiv C-CH_3$  or  $-CH_2-C\equiv CH$ ) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, and those disclosed by Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2.degree. C. (Sanghvi et al, Antisense Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications. Representative United States patents that teach the preparation of modified nucleobases include, but are not limited to: U.S. Pat. No. 3,687,808, as well as U.S. Pat. Nos.:

4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; 5,681,941 and 5,750,692, each of which is herein incorporated by reference.

5           Another modification of antisense oligonucleotides involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, cation lipids, phospholipids, cationic phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 6553-6556), cholic acid (Manoharan et al., Bioorg. Med. Chem. Lett., 1994, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., Ann. N.Y. Acad. Sci., 1992, 660, 306-309; Manoharan et al., Bioorg. Med. Chem. Lett., 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., EMBO J., 1991, 10, 1111-1118; Kabanov et al., FEBS Lett., 1990, 259, 327-330; Svinarchuk et al., Biochimie, 1993, 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654; Shea et al., Nucl. Acids Res., 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., Biochim. Biophys. Acta, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety. Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a

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sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in U.S. patent application Ser. No. 09/334,130 (filed Jun. 15, 1999) and United States patents Nos.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide.

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The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides or oligonucleotide mimetics as described above. Preferred chimeric antisense oligonucleotides incorporate at least one 2' modified sugar (preferably 2'-O-(CH<sub>2</sub>)<sub>2</sub>-O-CH<sub>3</sub>) at the 3' terminal to confer nuclease resistance and a region with at least 4 contiguous 2'-H sugars to confer RNase H activity. Such compounds have also been referred to in the art as hybrids or gapmers. Preferred gapmers have a region of 2' modified sugars (preferably 2'-O-(CH<sub>2</sub>)<sub>2</sub>-O-CH<sub>3</sub>) at the 3'-terminal and at the 5' terminal separated

by at least one region having at least 4 contiguous 2'-H sugars and preferably incorporate phosphorothioate backbone linkages. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S. Pat. Nos. 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, each of which is herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives. The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution or absorption. Representative United States patents that teach the preparation of such uptake, distribution or absorption assisting formulations include, but are not limited to, U.S. Pat. Nos. 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

Other examples of sense or antisense oligonucleotides include those oligonucleotides which are covalently linked to organic moieties, such as those described in WO 90/10048, and other moieties that increases affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents or metal complexes may be attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oligonucleotide for the target nucleotide sequence.

Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, CaPO<sub>4</sub>-mediated DNA transfection, electroporation, or by using gene transfer vectors such as Epstein-Barr virus. In a preferred procedure, an antisense or sense oligonucleotide is inserted into a suitable retroviral vector. A cell containing the target nucleic acid sequence is contacted with the recombinant retroviral vector, either *in vivo* or *ex vivo*. Suitable retroviral vectors include, but are not limited

to, those derived from the murine retrovirus M-MuLV, N2 (a retrovirus derived from M-MuLV), or the double copy vectors designated DCT5A, DCT5B and DCT5C (see WO 90/13641).

Sense or antisense oligonucleotides also may be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

Alternatively, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase.

Antisense or sense RNA or DNA molecules are generally at least about 5 nucleotides in length, alternatively at least about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, or 1000 nucleotides in length, wherein in this context the term “about” means the referenced nucleotide sequence length plus or minus 10% of that referenced length.

The probes may also be employed in PCR techniques to generate a pool of sequences for identification of closely related RA1c polypeptide coding sequences.

Nucleotide sequences encoding an RA1c polypeptide can also be used to construct hybridization probes for mapping the gene which encodes that RA1c polypeptide and for the genetic analysis of individuals with genetic disorders. The nucleotide sequences provided herein may be mapped to a chromosome and specific regions of a chromosome using known techniques, such as *in situ* hybridization, linkage analysis against known chromosomal markers, and hybridization screening with libraries.

The RA1c polypeptide can be used in assays to identify other proteins or molecules involved in a binding interaction with the RA1c polypeptide. By such methods, inhibitors of the receptor/ligand binding interaction can be identified. Proteins involved in such binding interactions can also be used to screen for peptide or small molecule inhibitors of the binding

interaction. Screening assays can be designed to find lead compounds that mimic the biological activity of a native RA1c polypeptide or a receptor for RA1c polypeptide. Such screening assays will include assays amenable to high-throughput screening of chemical libraries, making them particularly suitable for identifying small molecule drug candidates. Small molecules  
5 contemplated include synthetic organic or inorganic compounds. The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays and cell based assays, which are well characterized in the art.

Nucleic acids which encode RA1c polypeptide or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the  
10 development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding RA1c polypeptide can be used to clone genomic DNA encoding  
15 RA1c polypeptide in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding RA1c polypeptide. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for RA1c polypeptide transgene  
20 incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding RA1c polypeptide introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding RA1c polypeptide. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with its overexpression. In  
25 accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition.

Alternatively, non-human homologues of RA1c polypeptide can be used to construct an RA1c gene "knock out" animal which has a defective or altered gene encoding RA1c polypeptide  
30 as a result of homologous recombination between the endogenous gene encoding RA1c polypeptide and altered genomic DNA encoding RA1c polypeptide introduced into an embryonic stem cell of the animal. For example, cDNA encoding RA1c polypeptide can be used to clone genomic DNA encoding RA1c polypeptide in accordance with established techniques. A portion of the genomic DNA encoding RA1c polypeptide can be deleted or replaced with another gene,  
35 such as a gene encoding a selectable marker which can be used to monitor integration. Typically,

several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., Cell, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the RA1c polypeptide.

Nucleic acid encoding the RA1c polypeptides may also be used in gene therapy. In gene therapy applications, genes are introduced into cells in order to achieve *in vivo* synthesis of a therapeutically effective genetic product, for example for replacement of a defective gene. "Gene therapy" includes both conventional gene therapy where a lasting effect is achieved by a single treatment, and the administration of gene therapeutic agents, which involves the one time or repeated administration of a therapeutically effective DNA or mRNA. Antisense RNAs and DNAs can be used as therapeutic agents for blocking the expression of certain genes *in vivo*. It has already been shown that short antisense oligonucleotides can be imported into cells where they act as inhibitors, despite their low intracellular concentrations caused by their restricted uptake by the cell membrane. (Zamecnik *et al.*, Proc. Natl. Acad. Sci. USA 83:4143-4146 [1986]). The oligonucleotides can be modified to enhance their uptake, e.g. by substituting their negatively charged phosphodiester groups by uncharged groups.

There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. The currently preferred *in vivo* gene transfer techniques include transfection with viral (typically retroviral) vectors and viral coat protein-liposome mediated transfection (Dzau et al., Trends in Biotechnology 11, 205-210 [1993]). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface

membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting or to facilitate uptake, e.g. capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu et al., J. Biol. Chem. 262, 4429-4432 (1987); and Wagner et al., Proc. Natl. Acad. Sci. USA 87, 3410-3414 (1990). For review of gene marking and gene therapy protocols see Anderson et al., Science 256, 808-813 (1992).

The nucleic acid molecules encoding the RA1c polypeptides or fragments thereof described herein are useful for chromosome identification. In this regard, there exists an ongoing need to identify new chromosome markers, since relatively few chromosome marking reagents, based upon actual sequence data are presently available. Each RA1c nucleic acid molecule of the present invention can be used as a chromosome marker.

Other nucleic acid compounds useful in decreasing or blocking RA1c expression or activity are ribozymes. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. Ribozymes act by sequence-specific hybridization to the complementary target RNA, followed by endonucleolytic cleavage. Specific ribozyme cleavage sites within a potential RNA target can be identified by known techniques. For further details see, e.g., Rossi, Current Biology, 4:469-471 (1994), and PCT publication No. WO 97/33551 (published September 18, 1997).

#### K. Compositions and Pharmaceutical Formulations

The invention also provides a composition comprising an RA1c antagonist or compound that modulates RA1c expression and a carrier. In a further embodiment, the compositions can comprise an RA1c antagonist or compound that modulates RA1c expression in combination with other therapeutic agents such as cytotoxic or growth inhibitory agents, such as chemotherapeutic agents. The invention also provides formulations comprising an RA1c antagonist or compound that modulates RA1c expression and a carrier. In one embodiment, the formulation is a therapeutic formulation comprising at least one pharmaceutically acceptable carrier, excipient and/or stabilizer (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as acetate, Tris, phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium

chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic  
5 polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; tonicifiers such as trehalose and sodium chloride; sugars such as sucrose, mannitol, trehalose or sorbitol; surfactant such as polysorbate; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein  
10 complexes); or non-ionic surfactants such as TWEEN®, PLURONICS® or polyethylene glycol (PEG). The antibody preferably comprises the antibody at a concentration of between 5-200 mg/ml, preferably between 10-100 mg/ml.

The formulations herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do  
15 not adversely affect each other. For example, in addition to an RA1c antagonist or compound that modulates RA1c expression, or combinations of one or more of such compounds, it may be desirable to include in the one formulation, an additional antibody, e.g., a second anti-RA1c antibody which binds a different epitope on the RA1c polypeptide, or an antibody to some other target such as a growth factor that affects the growth of the particular cancer. Alternatively, or  
20 additionally, the composition may further comprise a chemotherapeutic agent, cytotoxic agent, cytokine, growth inhibitory agent, anti-hormonal agent, or cardioprotectant. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or  
25 gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release  
30 preparations include semi-permeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and  $\gamma$  ethyl-L-glutamate, non-degradable ethylene-vinyl acetate,  
35 degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT® (injectable

microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished using standard techniques, for example by filtration through sterile filtration  
5 membranes.

#### L. Therapeutic Applications

RA1c antagonists and compounds that modulate RA1c expression are useful in ameliorating cellular stress response, especially in RA1c expressing cancers such as prostate  
10 cancer. Amelioration of cellular stress response in cancer cells enhances chemotherapeutic treatment of the cancer by increasing the cell's sensitivity to the chemotherapeutic agent.

RA1c antagonists and compounds that modulate RA1c expression are useful in therapies to treat RA1c expressing cancers, such as prostate cancers, especially when combined with chemotherapeutic agents that increase RA1c expression. Examples of chemotherapeutic agents  
15 that increase expression of RA1c include agents that induce cellular stress response. Specific examples of chemotherapeutic agents that increase RA1c expression include, but are not limited to, mTOR or Akt1/2 inhibitors. In one embodiment, the combination therapy comprises a compound that specifically binds to the RA1c polypeptide, such as anti-RA1c antibodies or fragments thereof, RA1c-binding oligopeptides, inorganic or organic small molecules that  
20 specifically bind RA1c, and other RA1c antagonists such as aptamers. In one specific embodiment, the compound is an anti-RA1c antibody. Preferably, the anti-RA1c antibody is conjugated to a cytotoxic agent.

In another embodiment, the combination therapy comprises a compound that decreases or blocks RA1c expression or activity.

25 An increase in RA1c expression provides an increase in the amount RA1c available to interact with the anti-RA1c compounds. The combination treatment of a chemotherapeutic agent and a compound that decreases or blocks RA1c expression or activity provides a synergistic effect resulting in an improved cancer treatment.

For therapeutic applications, the RA1c antagonist or compound that modulates RA1c  
30 expression can be used alone, or in combination therapy with, e.g., hormones, antiangiogens, or radiolabelled compounds, or with surgery, cryotherapy, or radiotherapy.

The RA1c antagonists or compounds that modulate RA1c expression can be administered in conjunction with other forms of conventional therapy, either consecutively with, pre- or post-conventional therapy. Chemotherapeutic drugs such as TAXOTERE® (docetaxel), TAXOL®  
35 (palitaxel), estramustine and mitoxantrone are used in treating cancer, in particular, in good risk

patients. In a therapeutic treatment, the patient can be administered an RA1c antagonist or compound that modulates RA1c expression in conjunction with treatment with the one or more of the chemotherapeutic agents. In particular, combination therapy with mTOR or Akt1/2 inhibitors is contemplated. The RA1c antagonist or compound that modulates RA1c expression will be administered with a therapeutically effective dose of the chemotherapeutic agent. The Physicians' Desk Reference (PDR) discloses dosages of these agents that have been used in treatment of various cancers. The dosing regimen and dosages of these aforementioned chemotherapeutic drugs that are therapeutically effective will depend on the particular cancer being treated, the extent of the disease and other factors familiar to the physician of skill in the art and can be determined by the physician.

The RA1c antagonists or compounds that modulate RA1c expression 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, oligopeptide or organic small molecule is preferred.

Other therapeutic regimens may be combined with the administration of an RA1c antagonist or compound that modulates RA1c expression. The combined administration includes co-administration, 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. Preferably such combined therapy results in a synergistic therapeutic effect.

It may also be desirable to combine administration of an RA1c antagonist or compound that modulates RA1c expression with administration of an antibody directed against another tumor antigen associated with the particular cancer.

The RA1c antagonist or compound that modulates RA1c expression may be combined with an anti-hormonal compound; e.g., an anti-estrogen compound such as tamoxifen; an anti-progesterone such as onapristone (see, EP 616 812); or an anti-androgen such as flutamide, in dosages known for such molecules.

Sometimes, it may be beneficial to also co-administer a cardioprotectant (to prevent or reduce myocardial dysfunction associated with the therapy) or one or more cytokines to the patient. In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells or radiation therapy, before, simultaneously with, or post antibody, oligopeptide or organic/inorganic small molecule therapy. Suitable dosages for any of the above

co-administered agents are those presently used and may be lowered due to the combined action (synergy) of the agent and the RA1c antagonist or compound that modulates RA1c expression.

For the prevention or treatment of disease, the dosage and mode of administration will be chosen by the physician according to known criteria. The appropriate dosage of the RA1c antagonist or compound that modulates RA1c expression will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the RA1c antagonist or compound that modulates RA1c expression is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the RA1c antagonist or compound that modulates RA1c expression, and the discretion of the attending physician. The compound is suitably administered to the patient at one time or over a series of treatments. Preferably, the RA1c antagonist or compound that modulates RA1c expression is administered by intravenous infusion or by subcutaneous injections.

In a particular embodiment, about 1 µg/kg to about 50 mg/kg body weight (e.g., about 0.1-15mg/kg/dose) of anti-RA1c antibody can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A dosing regimen can comprise administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the anti-RA1c antibody. However, other dosage regimens may be useful. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. The progress of this therapy can be readily monitored by conventional methods and assays and based on criteria known to the physician or other persons of skill in the art.

Aside from administration of an antibody protein or antibody fragment protein to the patient, the present application contemplates administration of the antibody by gene therapy. Such administration of nucleic acid encoding the antibody is encompassed by the expression "administering a therapeutically effective amount of an antibody". See, for example, WO96/07321 published March 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

The location of the binding target of an antibody of the invention may be taken into consideration in preparation and administration of the antibody. When the binding target is an intracellular molecule (i.e., for compounds of the invention that modulate RA1c expression), certain embodiments of the invention provide for the antibody or antigen-binding fragment thereof to be introduced into the cell where the binding target is located. In one embodiment, an antibody of the invention can be expressed intracellularly as an intrabody. The term "intrabody,"

as used herein, refers to an antibody or antigen-binding portion thereof that is expressed intracellularly and that is capable of selectively binding to a target molecule, as described in Marasco, Gene Therapy 4: 11-15 (1997); Kontermann, Methods 34: 163-170 (2004); U.S. Patent Nos. 6,004,940 and 6,329,173; U.S. Patent Application Publication No. 2003/0104402, and PCT Publication No. WO2003/077945. Intracellular expression of an intrabody is effected by introducing a nucleic acid encoding the desired antibody or antigen-binding portion thereof (lacking the wild-type leader sequence and secretory signals normally associated with the gene encoding that antibody or antigen-binding fragment) into a target cell. Any standard method of introducing nucleic acids into a cell may be used, including, but not limited to, microinjection, ballistic injection, electroporation, calcium phosphate precipitation, liposomes, and transfection with retroviral, adenoviral, adeno-associated viral and vaccinia vectors carrying the nucleic acid of interest. One or more nucleic acids encoding all or a portion of a compound of the invention can be delivered to a target cell, such that one or more intrabodies are expressed which are capable of intracellular binding to a signaling pathway component polypeptide and modulation of one or more cellular pathways directly or indirectly involved in RA1c expression or activity.

Another approach to getting the nucleic acid (optionally contained in a vector) into the patient's cells is *ex vivo*. For *ex vivo* treatment, the patient's cells are removed, the nucleic acid is introduced into these isolated cells and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are implanted into the patient (see, e.g., U.S. Patent Nos. 4,892,538 and 5,283,187). There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for *ex vivo* delivery of the gene is a retroviral vector.

In another embodiment, internalizing antibodies are provided. Antibodies can possess certain characteristics that enhance delivery of antibodies into cells, or can be modified to possess such characteristics. Techniques for achieving this are known in the art. For example, cationization of an antibody is known to facilitate its uptake into cells (see, e.g., U.S. Patent No. 6,703,019). Lipofections or liposomes can also be used to deliver the antibody into cells. Where antibody fragments are used, the smallest inhibitory fragment that specifically binds to the binding domain of the target protein is generally advantageous. For example, based upon the variable-region sequences of an antibody, peptide molecules can be designed that retain the ability to bind the target protein sequence. Such peptides can be synthesized chemically or

produced by recombinant DNA technology. See, e.g., Marasco et al., Proc. Natl. Acad. Sci. USA, 90: 7889-7893 (1993).

Entry of antibodies into target cells can be enhanced by methods known in the art. For example, certain sequences, such as those derived from HIV Tat or the Antennapedia homeodomain protein are able to direct efficient uptake of heterologous proteins across cell membranes. See, e.g., Chen et al., Proc. Natl. Acad. Sci. USA (1999), 96:4325-4329.

M. Diagnostics and Non-Therapeutic Uses

The RA1c antagonists or compounds that modulate RA1c expression have various non-therapeutic applications. For example, the RA1c antagonists or compounds that modulate RA1c expression can be useful for staging of RA1c polypeptide-expressing cancers (e.g., in radioimaging). The RA1c antagonists or compounds that modulate RA1c expression may be used diagnostically for tissue typing, wherein RA1c polypeptides may be differentially expressed in one tissue as compared to another, preferably in a diseased tissue as compared to a normal tissue of the same tissue type. These compounds are also useful for purification or immunoprecipitation of RA1c polypeptide from cells, for detection and quantitation of RA1c polypeptide *in vitro*, e.g., in an ELISA or a Western blot, to kill and eliminate RA1c polypeptide-expressing cells from a population of mixed cells as a step in the purification of other cells. RA1c nucleic acid molecules will find use for generating probes for PCR, Northern analysis, Southern analysis and Western analysis.

To determine RA1c polypeptide expression in the cancer, various detection assays are available. In one embodiment, RA1c polypeptide overexpression may be analyzed by immunohistochemistry (IHC). Paraffin embedded tissue sections from a tumor biopsy may be subjected to the IHC assay and accorded an RA1c polypeptide staining intensity criteria as follows:

Score 0 - no staining is observed or membrane staining is observed in less than 10% of tumor cells.

Score 1+ - a faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane.

Score 2+ - a weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.

Score 3+ - a moderate to strong complete membrane staining is observed in more than 10% of the tumor cells.

Those tumors with 0 or 1+ scores for RA1c polypeptide expression may be characterized as not overexpressing RA1c polypeptide, whereas those tumors with 2+ or 3+ scores may be characterized as overexpressing RA1c polypeptide.

Alternatively, or additionally, FISH assays such as the INFORM® (sold by Ventana, Arizona) or PATHVISION® (Vysis, Illinois) may be carried out on formalin-fixed, paraffin-embedded tumor tissue to determine the extent (if any) of RA1c polypeptide overexpression in the tumor.

RA1c polypeptide overexpression or amplification may be evaluated using an *in vivo* detection assay, e.g., by administering a molecule (such as an antibody, antibody fragment, antibody conjugate, oligopeptide or organic small molecule, or aptamer) which binds the molecule to be detected and is tagged with a detectable label (e.g., a radioactive isotope or a fluorescent label) and externally scanning the patient for localization of the label.

The invention also provides methods of assaying whether cells are undergoing cellular stress response. These methods find particular use in determining if a particular chemotherapeutic treatment is inducing cellular stress response in cancer cells. Induction of cellular stress response may provide a survival advantage to the cells and render the cells less sensitive, or resistant, to the effects of the chemotherapeutic agent. If a chemotherapeutic agent is found to induce cellular stress response in the target cancer cells, it is desirable to ameliorate the cellular stress response, for example with a compound that decreases or blocks the expression or activity of RA1c, in order to increase the sensitivity of the cells to the chemotherapeutic agent.

The induction of cellular stress response is determined by monitoring the level of RA1c expression in the cell treated with a chemotherapeutic agent. In one embodiment, the method comprises contacting the cancer cell with the chemotherapeutic agent and determining the level of RA1c expression in the cancer cell. The level of RA1c expression in this test cell is compared to the level of RA1c expression in the test cell prior to contacting with the chemotherapeutic agent an increase in expression level in the test cell after contacting with the therapeutic agent as compared to the expression level in the test prior to contacting with the therapeutic agent indicates that the chemotherapeutic agent induces cellular stress response. Alternatively, the expression level of RA1c in the test cell can be compared to the expression level of RA1c in control cells that are of the same type as the test cells. In a particular embodiment, the increase in RA1c expression is about greater than 50 fold, alternatively about 2 to 50 fold, alternatively about 5 to 50 fold, or alternatively about 10 to 50 fold. As is known in the art, it is advantageous to monitor housekeeping genes as a method of calibrating the expression levels of genes in the test and/or control cells. The cancer cell used in this assay is preferably a prostate cancer cell, such as an LNCaP cell.

The assay can monitor the expression level of the RA1c gene or the expression level of the RA1c polypeptide.

The chemotherapeutic agent assayed in these methods is any agent suspected of being capable of causing cellular stress response. In one embodiment, the chemotherapeutic agent is an mTor inhibitor. In another embodiment, the chemotherapeutic agent is an Akt1/2 inhibitor.

This invention further encompasses methods of screening compounds to identify those that prevent the effect of the RA1c polypeptide (antagonists). Screening assays for antagonist drug candidates are designed to identify compounds that bind or complex with the RA1c polypeptides encoded by the genes identified herein, or otherwise interfere with the interaction of the encoded polypeptides with other cellular proteins, including e.g., inhibiting the expression of RA1c polypeptide from cells. Such screening assays will include assays amenable to high-throughput screening of chemical libraries, making them particularly suitable for identifying small molecule drug candidates.

The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays, and cell-based assays, which are well characterized in the art.

All assays for antagonists are common in that they call for contacting the drug candidate with an RA1c polypeptide encoded by a nucleic acid identified herein under conditions and for a time sufficient to allow these two components to interact.

In binding assays, the interaction is binding and the complex formed can be isolated or detected in the reaction mixture. In a particular embodiment, the RA1c polypeptide or the drug candidate is immobilized on a solid phase, e.g., on a microtiter plate, by covalent or non-covalent attachments. Non-covalent attachment generally is accomplished by coating the solid surface with a solution of the RA1c polypeptide and drying. Alternatively, an immobilized antibody, e.g., a monoclonal antibody, specific for the RA1c polypeptide to be immobilized can be used to anchor it to a solid surface. The assay is performed by adding the non-immobilized component, which may be labeled by a detectable label, to the immobilized component, e.g., the coated surface containing the anchored component. When the reaction is complete, the non-reacted components are removed, e.g., by washing, and complexes anchored on the solid surface are detected. When the originally non-immobilized component carries a detectable label, the detection of label immobilized on the surface indicates that complexing occurred. Where the originally non-immobilized component does not carry a label, complexing can be detected, for example, by using a labeled antibody specifically binding the immobilized complex.

If the candidate compound interacts with but does not bind to an RA1c polypeptide, its interaction with that polypeptide can be assayed by methods well known for detecting protein-

protein interactions. Such assays include traditional approaches, such as, e.g., cross-linking, co-immunoprecipitation, and co-purification through gradients or chromatographic columns. In addition, protein-protein interactions can be monitored by using a yeast-based genetic system described by Fields and co-workers (Fields and Song, Nature (London), 340:245-246 (1989); Chien et al., Proc. Natl. Acad. Sci. USA, 88:9578-9582 (1991)) as disclosed by Chevray and Nathans, Proc. Natl. Acad. Sci. USA, 89: 5789-5793 (1991). Many transcriptional activators, such as yeast GAL4, consist of two physically discrete modular domains, one acting as the DNA-binding domain, the other one functioning as the transcription-activation domain. The yeast expression system described in the foregoing publications (generally referred to as the "two-hybrid system") takes advantage of this property, and employs two hybrid proteins, one in which the target protein is fused to the DNA-binding domain of GAL4, and another, in which candidate activating proteins are fused to the activation domain. The expression of a GAL1-*lacZ* reporter gene under control of a GAL4-activated promoter depends on reconstitution of GAL4 activity via protein-protein interaction. Colonies containing interacting polypeptides are detected with a chromogenic substrate for  $\beta$ -galactosidase. A complete kit (MATCHMAKER<sup>TM</sup>) for identifying protein-protein interactions between two specific proteins using the two-hybrid technique is commercially available from Clontech. This system can also be extended to map protein domains involved in specific protein interactions as well as to pinpoint amino acid residues that are crucial for these interactions.

Compounds that interfere with the interaction of a gene encoding an RA1c polypeptide identified herein and other intra- or extracellular components can be tested as follows: usually a reaction mixture is prepared containing the product of the gene and the intra- or extracellular component under conditions and for a time allowing for the interaction and binding of the two products. To test the ability of a candidate compound to inhibit binding, the reaction is run in the absence and in the presence of the test compound. In addition, a placebo may be added to a third reaction mixture, to serve as positive control. The binding (complex formation) between the test compound and the intra- or extracellular component present in the mixture is monitored as described hereinabove. The formation of a complex in the control reaction(s) but not in the reaction mixture containing the test compound indicates that the test compound interferes with the interaction of the test compound and its reaction partner.

To assay for antagonists, the RA1c polypeptide may be added to a cell along with the compound to be screened for a particular activity and the ability of the compound to inhibit the activity of interest in the presence of the RA1c polypeptide indicates that the compound is an antagonist to the RA1c polypeptide. Alternatively, antagonists may be detected by combining the RA1c polypeptide and a potential antagonist with membrane-bound RA1c polypeptide receptors

or encoded receptors under appropriate conditions for a competitive inhibition assay. The RA1c polypeptide can be labeled, such as by radioactivity, such that the number of RA1c polypeptide molecules bound to the receptor can be used to determine the effectiveness of the potential antagonist. The gene encoding the receptor can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting. Coligan et al., Current Protocols in Immun., 1(2): Chapter 5 (1991). Preferably, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to the RA1c polypeptide and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to the RA1c polypeptide. Transfected cells that are grown on glass slides are exposed to labeled RA1c polypeptide. The RA1c polypeptide can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to autoradiographic analysis. Positive pools are identified and sub-pools are prepared and re-transfected using an interactive sub-pooling and re-screening process, eventually yielding a single clone that encodes the putative receptor.

As an alternative approach for receptor identification, labeled RA1c polypeptide can be photoaffinity-linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE and exposed to X-ray film. The labeled complex containing the receptor can be excised, resolved into peptide fragments, and subjected to protein micro-sequencing. The amino acid sequence obtained from micro-sequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the gene encoding the putative receptor.

In another assay for antagonists, mammalian cells or a membrane preparation expressing the receptor would be incubated with labeled RA1c polypeptide in the presence of the candidate compound. The ability of the compound to enhance or block this interaction could then be measured.

#### N. Articles of Manufacture and Kits

Another embodiment of the invention is an article of manufacture containing materials useful for the treatment of an RA1c polypeptide expressing cancer, such as a prostate cancer. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the cancer condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a

stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an RA1c antagonist or compound that modulates RA1c expression. The label or package insert indicates that the composition is used for treating cancer. The label or package insert will further comprise instructions for administering the antibody, oligopeptide or  
5 organic/inorganic small molecule composition to the cancer patient. Additionally, the article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

10 Kits are also provided that are useful for various purposes, e.g., for RA1c polypeptide-expressing cell killing assays, for purification or immunoprecipitation of RA1c polypeptide from cells, and assays to determine induction of cellular stress response. For isolation and purification of RA1c polypeptide, the kit can contain an anti-RA1c antibody, anti-RA1c antibody fragment, anti-RA1c antibody conjugate, oligopeptide, organic/inorganic small molecule, or other RA1c-  
15 binding antagonist coupled to beads (e.g., sepharose beads). Kits can be provided which contain the anti-RA1c antibody, anti-RA1c antibody fragment, anti-RA1c antibody conjugate, oligopeptide, organic/inorganic small molecule, or other RA1c-binding antagonist for detection and quantitation of RA1c polypeptide *in vitro*, e.g., in an ELISA or a Western blot. As with the article of manufacture, the kit comprises a container and a label or package insert on or associated  
20 with the container. The container holds a composition comprising at least one anti-RA1c antibody, anti-RA1c antibody fragment, anti-RA1c antibody conjugate, oligopeptide, organic/inorganic small molecule, or other RA1c antagonist of the invention. Additional containers may be included that contain, e.g., diluents and buffers, control antibodies. The label or package insert may provide a description of the composition as well as instructions for the  
25 intended *in vitro* or detection use.

The following examples are included to demonstrate preferred embodiments of the present invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for  
30 its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention. All references cited throughout the specification are expressly incorporated by reference in their entirety herein.

EXAMPLES**EXAMPLE 1: RA1c EXPRESSION**

The expression of RA1c in several tissues and tumors was examined using the Affymetrix HGU133P oligo array (GeneLogic). RA1c expression was evaluated at GeneLogic, Inc. (Gaithersburg, MD) by interrogating an oligonucleotide microarray (HG-U133, Affymetrix) with a probe designed to hybridize to the 3' UTR of human RA1c (bases 2349-2742 of NM\_030774), as described previously (Shen-Ong et al., Cancer Res. 2003: 63(12): 3296-3301). The results show that RA1c expression was substantially higher in prostate tissue than in any of the other tested tissues (Figure 1A). Further, expression of RA1c in cancerous prostate tissues was substantially greater than expression in normal prostate tissues (Figure 1A). A closer examination of RA1c expression in several normal and diseased prostate samples (Figure 1B) showed that normal prostate tissue and benign prostatic hypertrophy have similar levels of RA1c expression, while the prostatic interepithelial neoplasm sample had a substantially lower amount of RA1c expression than those two tissues, and three different adenocarcinoma samples with Gleason scores of 7 had substantially higher amounts of RA1c expression than those two tissues.

**EXAMPLE 2: MODULATION OF RA1c EXPRESSION****(a) Cytokine Effects on RA1c Expression**

A previous study had found that RA1c expression was enhanced in an immortalized prostate cell line by administration of interleukin 6 (IL-6) to the cells (Weng et al., J. Cell. Biochem. 96: 1034-1048 (2005)). Accordingly, the effects of administering IL-6 and other cytokines to LNCaP cells, an immortalized, androgen-sensitive prostate cancer cell line, were tested. LNCaP cells were plated at equal densities (approximately  $0.5 \times 10^6$  cells/well) in 6-well plates, and grown for 48 hours in DMEM:F12K media containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO<sub>2</sub> before replacement of the media with either complete phenol red-free media or serum-free phenol red-free media containing IL-6, insulin, EGF, or IGF-1 at the indicated concentrations. Treatments with IL-6, EGF, and IGF-1 were for 24 hours, while treatment with insulin was for 7 hours. After treatment, cells were harvested by mechanical lifting. RNA was isolated from the harvested cells using an RNeasy™ kit (Qiagen) and DNase treatment. Quantitative real-time RT-PCR (Q-PCR) was carried out using reagents from PE Biosystems and a Q-PCR system (Taqman™, PE Biosystems) according to the manufacturer's instructions. The Q-PCR primers used were: forward: CCC ATC ATC TAT GGT GCC AAA (SEQ ID NO:4), reverse: TCC TTG TCA CAG CTG ATC TTG AA (SEQ ID NO:5). The probe used was CCA AAC AGA TCA GAA CAC GGG TGC TG (SEQ ID NO: 6). The results are shown in Figure

2A and 2B as the RA1c value normalized by comparison to ribosomal protein L19 (RPL19) using the formula:  $2^{\text{average Ct value for RPL19} / 2^{\text{average Ct value for RA1c}}}$ .

The results showed that, consistent with the earlier report, IL-6 treatment substantially enhanced RA1c expression in complete media-treated cells over control levels (see Figure 2A).

While EGF and IGF-1 treatments on cells grown in serum-free media increased the observed RA1c expression over that seen in control cells grown in complete media, that expression was not substantially different than that observed in control cells grown in serum-free media (see Figure 2A, and compare to Figures 4B and 14A). Similarly, insulin treatment had no significant effect on RA1c expression as compared to media-matched control cells (Figure 2B). Ct values for RPL19 were consistent from sample to sample in each experiment and were also consistent between complete media-treated cells and serum-free media-treated cells. These experiments thus demonstrated that IL-6 treatment increased RA1c expression in treated LNCaP cells over relevant control cells, and also suggested that serum deprivation might also increase RA1c expression in cells independent of IL-6 treatment. Both findings were further investigated.

To assess the timing of upregulation of RA1c expression by IL-6 treatment, a time course was performed in LNCaP cells. Cells were plated in equal densities (approximately  $0.5 \times 10^6$  cells/well) in six-well plates and grown for 72 hours in DMEM:F12K media containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO<sub>2</sub> prior to first treatment with IL-6 (the 24-hour treatment samples). The total time from plating to the termination of the experiment was thus 96 hours. Cell timepoint treatments were staggered such that all samples were harvested at the same time by mechanical lifting. Cells were washed once in phosphate-buffered saline (PBS) before lysis and RNA isolation using the RNeasy™ kit (Qiagen). All samples were treated with DNase during RNA isolation. Q-PCR was performed using the Q-PCR system, primers, and probe described above, and the results were normalized to RPL19 as described above. The results are shown in Figure 2C. RA1c expression increased in response to IL-6 treatment at four hours and peaked at eight hours. The 24-hour treatment was also elevated over control cell RA1c expression, but was similar to the levels observed at the four-hour time point. This result suggested that RA1c is an early target gene of the IL-6 pathway in LNCaP cells.

#### **(b) Effects of Serum Deprivation on RA1c Expression**

Experiments were also performed to further assess the impact of serum deprivation on LNCaP cells. Cells were plated in equal densities (approximately  $0.5 \times 10^6$  cells/well) in six-well plates and left to rest for 24 hours in DMEM:F12K media containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO<sub>2</sub>. Serum-free and phenol red-free media ("50/50") was added to cells. The indicated amounts of fetal bovine serum (FBS) were added to certain samples, and treatment continued for 18 hours. After the incubation, cells were harvested by mechanical lifting, and

RNA was isolated as described above using an RNAeasy™ kit (Qiagen) with DNase treatment during RNA isolation. Q-PCR was carried out as described in Example 2(a) using the Q-PCR system and reagents (PE Biosystems) and the primers and probe described in Example 2(a). Results were normalized to RPL19 as described above. In 50/50 media there is a clear correlation between absence of serum and increased RA1c expression. Figure 3A. As the percentage of fetal bovine serum added to the cells was increased, the amount of RA1c expression decreased. Figure 3A. Thus, RA1c expression appears to be induced by conditions that place stress on normal cellular systems, such as serum deprivation and treatment with IL-6. The results were similar when tested using another kind of serum-free media, RPMI.

Further studies indicated that the elevation in RA1c mRNA under serum deprivation is not solely due to removal of serum-borne androgens. Fetal bovine serum was treated with activated carbon (CDS) to remove androgens and other small organic constituents such as bio-active lipids. LNCaP cells in duplicate wells grown in medium supplemented with CDS expressed RA1c at similar levels compared to cells grown in medium with complete fetal bovine serum. In contrast, cells grown in serum-free medium expressed elevated RA1c, as observed previously. DHT treatment suppressed RA1c expression under all conditions.

#### **(c) Effects of Serum Deprivation on Cell Cycle Regulation in IL-6-Treated LNCaP Cells**

LNCaP cells were plated at a density of  $5 \times 10^6$  cells/well in six-well plates one day prior to IL-6 treatment. One day after plating, media was replaced with either fresh complete media or with serum-free- and phenol red-free media, and certain wells were additionally treated with 10 or 50 ng/mL IL-6. Treatment continued for 48 hours at 37 °C in 5% CO<sub>2</sub>, at which time the cells were removed from the plate using 1 mL of 1X trypsin/10 mM EDTA and washed twice with cold PBS. Cells were then thoroughly resuspended in cold 100% ethanol and kept at 4 °C until analysis. For propidium iodide (PI) staining, cells were spun down and then resuspended in 1 mL PI staining solution (0.4% NP40, 0.05 mg/mL PI, 0.02 mg/mL RNase) and incubated overnight until analysis by fluorescence activated cell sorting (FACS) analysis using a FACScalibur™ system (BD Biosciences). Twenty-five thousand cells were acquired per sample at a medium acquisition setting.

As can be seen in Figure 3B, control cells grown in complete media had a significantly lower proportion of cells in G0/G1 arrest as compared to control cells grown in serum-free media. This result was not unexpected, given that serum contains a number of growth factors and cytokines that stimulate active cell growth and division. With increasing concentrations of IL-6, a shift was observed away from G0/G1-arrested cells towards apoptotic cells. It should be noted that RA1c expression was elevated by much shorter IL-6 exposure (Figure 2C) than that applied

to generate the apoptotic cells shown in Figure 3B. These results suggest that increased RA1c expression may be an early response to cellular stresses that ultimately result in growth arrest or apoptosis.

**(d) Effects of Serum Deprivation in Other Prostate Cancer Models**

5 To assess whether the effects of serum deprivation observed in LNCaP cells in Examples 2(a) and 2(b) were more widely applicable, the experiment was repeated using other prostate tumor models. One such model is the LUCaP77 prostate cancer xenograft model (see Chen et al., Am. J. Pathol. 162: 1349-1354 (2003)). LUCaP77 tumor was homogenized using a screen and glass pestle and then plated in complete media with 20% FBS. The cells were left to grow for 10  
10 days prior to media change with fresh complete or serum and phenol red-free media. LNCaP cells were also treated in the same manner. IL-6 treatments were for 7 hours prior to harvesting by mechanical lifting. The harvested cells were washed once with PBS. RNA was isolated from the harvested cells using an RNeasy™ kit (Qiagen), as described in Example 2(a). All samples were treated with DNase during the RNA isolation. QPCR was performed using the quantitative  
15 real-time PCR reagents, primers, and the probe described in Example 2(a). The results were normalized to RPL19 as described in Example 2(a), and are shown in Figures 4A and 4B.

Similar to the LNCaP results described in Examples 2(a) and 2(b), LUCaP77 cells expressed substantially more RA1c in the absence of serum than when grown in the presence of serum. Notably, treatment with IL-6 in the context of complete media did not result in an  
20 increase in RA1c expression in these cells, though when the cells were serum-starved, a modest additional increase in RA1c expression was observed with increasing IL-6 concentration (see Figure 4A, compare left half of graph to right half of graph). The average Ct values for RA1c and RPL19 expression in the LUCaP77 ex vivo cells were unaffected by the type of media or the IL-6 concentration. LNCaP cells were assayed under the same conditions as the LUCaP77 cells  
25 (see Figure 4B). No significant increase was observed in RA1c production in those cells grown in complete media in response to increasing concentrations of IL-6, but a marked increase in RA1c expression was observed in cells grown in serum-free media, and a further increase was observed when IL-6 was used to treat the serum-free cells (Figure 4B). The increase in RA1c expression in LNCaP cells in response to serum deprivation and IL-6 treatment was substantially  
30 greater than that observed in the LUCaP77 cells, though both cell types clearly demonstrated increased expression in response to both serum deprivation and IL-6 treatment. These results demonstrated that certain aspects of RA1c gene regulation (up-regulation in response to IL-6 treatment and up-regulation in response to serum deprivation) are conserved across in vitro models of prostate cancer.

### EXAMPLE 3: EFFECT OF SIGNALING PATHWAY INHIBITORS ON RA1c EXPRESSION

Like many cytokines, IL-6 may stimulate several different intracellular signaling cascades, and such signaling may in turn be regulated by other intracellular factors. For example, p38 MAP kinase is known to mediate inhibition of IL-6 signaling (Ahmed et al., J. Leukocyte Biol. 72: 154-162 (2002); Chang et al., Am. J. Physiol. Lung Cell. Molec. Physiol. 289: L446-L453 (2005)), and IL-6 is known to activate 4,5-phosphatidyl-inositol 3-kinase (PI3K) in LNCaP cells (Xie et al., Prostate 60: 61-67 (2004)) (see Figure 8). Activation of PI3K is known to have multiple downstream consequences, including activation of the pro-survival Akt/mTOR pathway, but also the protein kinase PDK1, which regulates transcription through the activation of p70S6 kinase. LNCaP cells lack functional PTEN, a tumor suppressor that opposes the effects of PI3K, therefore in LNCaP cells the downstream effectors of PI3K might be constitutively active. The JAK/STAT3 pathway is also activated by IL-6 (Hirano, et al., Oncogene, 19: 2548-2556 (2000)). The effects of several different known signaling pathway inhibitors on IL-6-induced RA1c expression were therefore assessed.

#### (a) Effects of Specific PI3K and p38 MAP kinase Inhibitors

LNCaP cells were plated in equal densities (approximately  $0.5 \times 10^6$  cells/well) in six-well plates and left to rest for 72 hours prior to addition of serum and phenol red-free media and left to rest for 24 hours. Cells were either cultured without inhibitor, or treated with LY294002 (an inhibitor of a broad panel of kinases with particular efficacy against PI3K) or the highly specific p38 MAP kinase inhibitor SB203580, which blocks a major cellular stress response pathway. LY294002 (2  $\mu$ M and 4  $\mu$ M) was added to appropriate wells and incubated for one hour prior to addition of 50 ng/mL IL-6 for an additional 24 hours. SB203580 (5  $\mu$ M and 10  $\mu$ M) was added to appropriate wells and incubated for one hour prior to addition of 50 ng/mL IL-6 for an additional seven hours. The cells were harvested by mechanical lifting and washed once with PBS. RNA was isolated from the harvested cells using an RNeasy™ kit (Qiagen), as described in Example 2(a). All samples were treated with DNase during the RNA isolation. Q-PCR was performed using the Q-PCR system, reagents, primers, and the probe described in Example 2(a). The results were normalized to RPL19 as described in Example 2(a).

The ability of certain molecules within the PI3K and P38 MAP kinase signaling pathways to be activated properly in LNCaP cells upon stimulation with a growth factor known to activate those pathways was also assessed. LNCaP cells were plated at a density of approximately  $0.5 \times 10^6$  cells/well in six-well plates. The cells were grown for 72 hours before being treated for one hour with various inhibitors prior to a ten-minute 10 nM EGF stimulation.

Cells were washed once with cold PBS prior to being lysed in cold 2X sample buffer containing  $\beta$ -mercaptoethanol. Thirty-five microliters of lysate was loaded and run on a 4-20% polyacrylamide gel and then transferred to a PVDF membrane using standard procedures. The presence of activated (i.e., phosphorylated) components of the PI3K and p38 MAP kinase signaling pathways was assessed by western blotting using standard procedures using the following antibodies (all from Cell Signaling Technologies): rabbit polyclonal anti-phosphorylated AKT Ser 473, mouse monoclonal anti-phosphorylated MAP kinase p44/42, mouse monoclonal anti-phosphorylated p38 MAP kinase, and mouse monoclonal anti-phosphorylated mTOR. Anti-beta-actin antibody was used for blot normalization. The results are shown in Figure 5, and demonstrate the presence of the phosphorylated forms of each of those signaling components in LNCaP cells, and the effectiveness of the indicated inhibitors to modulate the population of such phosphorylated (i.e., activated) proteins.

As shown in Figure 6, addition of LY294002 to serum-starved and IL-6-treated cells substantially reduced the overexpression of RA1c observed when serum-starved LNCaP cells were incubated with IL-6 alone. Further, addition of LY294002 to serum-starved cells also reduced the expression of RA1c induced by serum deprivation in the absence of IL-6 (compare rightmost two bars to leftmost bar in Figure 6). Although phosphorylated Akt levels were reduced by LY294002 treatment, activities unrelated to PI3K inhibition could potentially explain the effects of LY294002 as well. Treatment with the p38 MAPK inhibitor SB203580 also inhibited IL-6-induced RA1c expression (Figure 7), though p38 itself is not upregulated by IL-6.

**(b) Effects of Specific mTOR Inhibitor**

LNCaP cells were plated at a density of approximately  $0.5 \times 10^6$  cells/well in a six-well plate and left to grow for 24 hours in complete media at 37 °C and 5% CO<sub>2</sub>. Media was changed and replaced with fresh complete media, and the cells were grown overnight at 37 °C and 5% CO<sub>2</sub>. Rapamycin (100 nM or 500 nM) was added to appropriate wells and incubated for one hour prior to addition of 50 ng/mL IL-6 for an additional seven hours. The cells were harvested by mechanical lifting and were washed once with cold PBS. The harvested, washed cells were stored at -80 °C. Immediately upon removal from -80 °C, RLT lysis buffer from the RNeasy™ kit was added to the cell pellets and the cells were resuspended. Frozen cell pellets were never stored for longer than one week. The resuspended cells were again washed once with PBS. RNA was isolated from the harvested cells using an RNeasy™ kit (Qiagen), as described in Example 2(a). All samples were treated with DNase during the RNA isolation. Q-PCR was performed using the Q-PCR system, reagents, primers, and the probe described in Example 2(a). The results were normalized to RPL19 as described in Example 2(a).

As shown in Figure 9, either rapamycin treatment alone or IL-6 treatment alone caused a measurable increase in RA1c expression in LNCaP cells. However, treatment with both IL-6 and rapamycin together appeared to synergistically enhance RA1c expression in treated cells (Figure 9). Without being limited to any one interpretation, the synergy may be caused by the additive cellular stress from the combination of the IL-6 treatment and inhibition of mTOR activity. IL-6 causes growth arrest in LNCaP cells while the PI3K/Akt/mTOR pathway is also constitutively active in those cells and is important for LNCaP cell growth. Thus, the combined stresses of IL-6 treatment and mTOR inhibition may cause a synergistic upregulation of RA1c expression.

#### (c) Effects of AKT 1/2 Inhibitor

LNCaP cells were plated at a density of approximately  $0.5 \times 10^6$  cells per well and grown for 48 hours. Media was then changed to fresh growth media 24 hours prior to treatment with indicated concentration of Akt1/2 inhibitor (Calbiochem). Akt1/2 inhibitor treatment was for one hour prior to addition of 50ng/mL IL-6. IL-6 treatment proceeded for 7 hours. Cells were then harvested by mechanical lifting, washed once with cold 1X PBS and then snap frozen in liquid nitrogen and stored at -80°C overnight. RNA from cell pellets was then isolated using Qiagen's RNeasy kit. Samples were DNAsed during RNA isolation. Addition of the Akt1/2 inhibitor produced an elevation in levels of RA1c message (Figure 10). More significantly, RA1c message was further increased by the combination of the AKT inhibitor with exogenous IL-6 (Figure 10). These effects were apparently additive. This result, taken with the mTOR inhibition results discussed above, argues against the Akt/mTOR pathway as a positive regulator of RA1c expression in the presence of IL-6.

#### (d) Effects of JAK/STAT3 inhibition

To evaluate the role of the JAK/STAT3 pathway, promoter analysis was performed. Weng, et al. reported that the putative RA1c promoter contains a predicted STAT3 binding site (J. Cell Bioch. 2005; 96: 1034-1048). Luciferase reporter constructs driven by different regions of the putative RA1c promoter were engineered. The construct carrying the predicted STAT3 binding site expressed luciferase above control levels (represented by the pGL3-basic vector), and this expression was further enhanced by the addition of exogenous IL-6 (Figure 11). Mutation of the STAT3 site (Figure 12) severely reduced the constitutive activity and abolished the IL-6 response of this promoter (Figure 11). Also, the JAK inhibitor AG490, while slightly enhancing luciferase activity on its own, prevented further elevation of luciferase activity in combination with IL-6 (Figure 13). Taken together, these results suggest that the primary pathway responsible for increased RA1c expression in response to IL-6 is the JAK/STAT3 pathway.

**EXAMPLE 4: EFFECT OF DHT ON RA1c EXPRESSION**

LNCaP cells are androgen-sensitive, and are stimulated to grow in the presence of testosterone and related molecules. Previous studies have shown that IL-6-mediated induction of neuroendocrine differentiation in LNCaP cells is blocked by androgen administration to those cells (Xie et al., Prostate 60: 61-67 (2004)). Since the studies described above show that IL-6 treatment stimulates RA1c expression, the effect of androgen on IL-6-induced and serum deprivation-induced RA1c expression was investigated.

LNCaP cells were plated in equal densities and grown for three days before replacement of the media with either fresh complete media containing 5 $\alpha$ -dihydrotestosterone (DHT) or serum-free phenol red-free media with DHT. For IL-6 treatments, 10 nM, 25 nM, 100 nM and 1  $\mu$ M DHT doses were used in combination with 10 ng/mL IL-6 in complete and serum-free phenol red-free media. All treatments took place overnight before harvesting of cells by mechanical lifting. Harvested cells were washed once with PBS before isolating RNA using an RNeasy™ kit (Qiagen). All samples were treated with DNase during the RNA isolation. Q-PCR was performed using the Q-PCR system, reagents, primers, and the probe described in Example 2(a). The results were normalized to RPL19 as described in Example 2(a).

DHT treatment was substantially inhibitory to both IL-6-induced expression of RA1c and serum-deprivation-induced expression of RA1c (Figures 14A and 14B). The effect was dose-dependent, and most pronounced in the presence of 100 nM DHT. The Ct values for RPL19 were consistent over the range of DHT treatment, indicating that changes in cell viability were not responsible for the observed decreases in RA1c expression upon DHT treatment. Taken together with the results from Examples 2 and 3, this data suggests that RA1c is a stress-induced gene that could provide a survival advantage to cancer cells (Figure 14C). Under normal and replete growth conditions, RA1c expression is suppressed. The data taken together suggests that elevation of RA1c expression under conditions of stress (for example, IL-6 treatment or serum deprivation) can be reversed or mitigated by pro-growth pathway activation (DHT) and amplified when pro-growth pathways are further inhibited (rapamycin).

**EXAMPLE 5: DEMONSTRATION OF SURFACE EXPRESSION OF RA1c.**

While RA1c has homology to the family of seven-transmembrane G-protein-coupled receptor proteins, evidence that RA1c is accessible on cell surface membranes rather than located on solely intracellular membranes has not previously been provided in the art. To assess the cellular localization of RA1c protein, N-terminal gD-tagged RA1c was overexpressed in human embryonic kidney cells (293S cells) using FuGene® (Roche) transfection reagent. 293S cells were plated at a density of 2 x 10<sup>6</sup> cells/10 cm dish and left to grow at 37 °C and 5% CO<sub>2</sub> in

complete growth media for 24 hours before transfection. Cells were transfected with 5.6 µg RA1c-pRK-N-terminal-gD plasmid using the FuGene® reagent and left to rest for 48 hours prior to lifting from the plates with 5 mM EDTA. Cells were then washed 2X with FACS buffer (3% BSA, 1 mM EDTA in 1X PBS) prior to resuspension in 4 µg/mL anti-gD mAb and incubation on  
5 ice for 1 hour. Cells were then washed three times with cold FACS buffer prior to resuspension in phycoerythrin (PE)-labeled-anti-mouse secondary antibody (1:500 dilution) and incubation on ice for one hour. Cells were washed three times with FACS buffer prior to FACS analysis.

As shown in Figure 15, the FACS analysis detected a large population of cells that specifically bound to the PE-labeled anti-mouse secondary antibody, indicating that RA1c is  
10 accessible at the surface of 293S cells. The results were confirmed using CHO cell expression (results not shown), indicating that cell-surface expression of RA1c is observed in different cell lines from different organisms.

WHAT IS CLAIMED IS:

1. A method of ameliorating cellular stress response in a cell comprising contacting the cell with a compound that decreases or blocks RA1c expression or activity in the cell.
- 5 2. The method of claim 1, wherein the cell is a cancer cell.
3. The method of claim 2, wherein the cancer cell is a prostate tumor cell.
4. The method of claim 2 or 3, wherein the cellular stress response is induced by a chemotherapeutic agent.
- 10 5. The method of claim 4, wherein the chemotherapeutic agent is selected from the group consisting of an mTor inhibitor and an Akt1/2 inhibitor.
6. The method of claim 5, wherein the chemotherapeutic agent is an mTor inhibitor selected from the group consisting of rapamycin, CCI-779, RAD001, AP23573, XL7F65, and TAFA93, and active derivatives or analogs thereof.
- 15 7. The method of claim 5, wherein the chemotherapeutic agent is an Akt1/2 inhibitor selected from the group consisting of GSK690693, perifosine, and XL418, and active derivatives or analogs thereof.
8. The method of claim 1, wherein the compound is an RA1c antagonist.
9. The method of claim 8, wherein the RA1c antagonist is selected from the group consisting of an antibody, an antibody fragment, an antibody conjugate, an aptamer, a small  
20 molecule, and an oligopeptide.
10. The method of claim 9, wherein the RA1c antagonist specifically blocks RA1c activity.
11. The method of claim 1, wherein the compound decreases expression of RA1c.
12. The method of claim 1, wherein the compound blocks the expression of RA1c.
- 25 13. The method of claim 11 or 12, wherein the compound is selected from the group consisting of an antisense polynucleotide, a silencing RNA molecule, a catalytic RNA molecule, and an RNA-DNA chimera.

14. The method of claim 4, wherein the compound increases the sensitivity of the cell to the chemotherapeutic agent.

15. A method of ameliorating cellular stress response in prostate cancer tumor cells comprising contacting the tumor cells with a compound that decreases or blocks the expression or activity of RA1c.

16. The method of claim 15, wherein the tumor cells are undergoing chemotherapy with a chemotherapeutic agent that induces cellular stress response.

17. The method of claim 16, wherein the chemotherapeutic agent is selected from the group consisting of an mTor inhibitor and an Akt1/2 inhibitor.

18. The method of claim 17, wherein the chemotherapeutic agent is an mTor inhibitor selected from the group consisting of rapamycin, CCI-779, RAD001, AP23573, XL7F65, and TAFA93, and active derivatives or analogs thereof.

19. The method of claim 17, wherein the chemotherapeutic agent is an Akt1/2 inhibitor selected from the group consisting of GSK690693, perifosine, and XL418, and active derivatives or analogs thereof.

20. The method of claim 15, wherein the compound is an RA1c antagonist.

21. The method of claim 20 wherein the RA1c antagonist is selected from the group consisting of an antibody, an antibody fragment, an antibody conjugate, an aptamer, a small molecule, and an oligopeptide.

22. The method of claim 21, wherein the RA1c antagonist specifically blocks RA1c activity.

23. The method of claim 15, wherein the compound decreases expression of RA1c.

24. The method of claim 15 wherein the compound blocks the expression of RA1c.

25. The method of claim 23 or claim 24, wherein the compound is selected from the group consisting of an antisense polynucleotide, a silencing RNA molecule, a catalytic RNA molecule, and an RNA-DNA chimera.

26. The method of claim 16, wherein the compound increases the sensitivity of the tumor cells to the chemotherapeutic agent.

27. The method of claim 26, wherein the tumor cells undergo apoptosis at a higher rate than cells not contacted with the compound.

28. The method of claim 26, wherein the tumor cells undergo growth arrest at a higher rate than cells not contacted with the compound.

5 29. A method of ameliorating cellular stress response in the cells of a patient comprising administering to the patient an effective amount of a compound that decreases or blocks the expression or activity of RA1c.

30. The method of claim 29, wherein the patient is a cancer patient.

31. The method of claim 30, wherein the cells are prostate cancer tumor cells.

10 32. The method of claim 31, wherein the tumor cells are undergoing chemotherapy with a chemotherapeutic agent that induces cellular stress response.

33. The method of claim 32, wherein the chemotherapeutic agent is selected from the group consisting of an mTor inhibitor and an Akt1/2 inhibitor.

15 34. The method of claim 33, wherein the chemotherapeutic agent is an mTor inhibitor selected from the group consisting of rapamycin, CCI-779, RAD001, AP23573, XL7F65, and TAFA93, and active derivatives or analogs thereof.

35. The method of claim 33, wherein the chemotherapeutic agent is an Akt1/2 inhibitor selected from the group consisting of GSK690693, perifosine, and XL418, and active derivatives or analogs thereof.

20 36. The method of claim 29, wherein the compound is an RA1c antagonist.

37. The method of claim 36, wherein the RA1c antagonist is selected from the group consisting of an antibody, an antibody fragment, an antibody conjugate, an aptamer, a small molecule, and an oligopeptide.

25 38. The method of claim 37 wherein the RA1c antagonist specifically blocks RA1c activity.

39. The method of claim 29, wherein the compound decreases expression of RA1c.

40. The method of claim 29, wherein the compound blocks the expression of RA1c.

41. The method of claim 39 or claim 40, wherein the compound is selected from the group consisting of an antisense polynucleotide, a silencing RNA molecule, a catalytic RNA molecule, and an RNA-DNA chimera.

42. The method of claim 32, wherein the compound increases the sensitivity of the tumor cells to the chemotherapeutic agent.

43. The method of claim 42, wherein the tumor cells undergo apoptosis at a higher rate than cells not contacted with the compound.

44. The method of claim 42, wherein the tumor cells undergo growth arrest at a higher rate than cells not contacted with the compound.

45. A composition comprising a RA1c antagonist and a compound that increases the expression of RA1c, optionally further comprising a pharmaceutically acceptable carrier.

46. The composition of claim 45, wherein the compound is selected from the group consisting of rapamycin, CCI-779, RAD001, AP23573, XL7F65, TAFA93, SK690693, perifosine, and XL418, and active derivatives or analogs thereof.

47. The composition of claim 46, wherein the RA1c antagonist is an antibody and the compound is selected from the group consisting of rapamycin, CCI-779, RAD001, AP23573, XL7F65, TAFA93, SK690693, perifosine, and XL418, and active derivatives or analogs thereof.

48. A method of determining whether a chemotherapeutic agent induces cellular stress response in a cancer cell comprising:

a) contacting the cancer cell with the chemotherapeutic agent,

b) determining the level of RA1c expression in the cancer cell,

c) comparing the level of RA1c expression in the cancer cell with

1) the level of RA1c expression in the cancer cell prior to contacting with the chemotherapeutic agent, or

2) the level of RA1c expression in a control cell,

where an increase in RA1c expression indicates induction of cellular stress response.

49. The method of claim 48, wherein the cancer cell is a prostate cancer cell.

50. The method of claim 49, wherein the prostate cancer cell is a LNCaP cell.

51. The method of claim 48, wherein the chemotherapeutic agent is selected from the group consisting of an mTor inhibitor and an Akt1/2 inhibitor.

52. A method of treating cancer with a combination therapy comprising:

5 a) contacting a cancer cell with a chemotherapeutic agent that increases expression of RA1c, and

b) contacting the cancer cell with an effective amount of a compound that specifically binds to RA1c polypeptide,

10 whereby the cancer treating effect of the combination of the chemotherapeutic agent and the compound is synergistic over the effect of either the chemotherapeutic agent or compound alone.

53. The method of claim 52, wherein the chemotherapeutic agent is a mTOR or the Akt1/2 inhibitor.

54. The method of claim 52, wherein the compound is an anti-RA1c antibody.

15 55. The method of claim 54, wherein the anti-RA1c antibody is conjugated to a cytotoxic agent.

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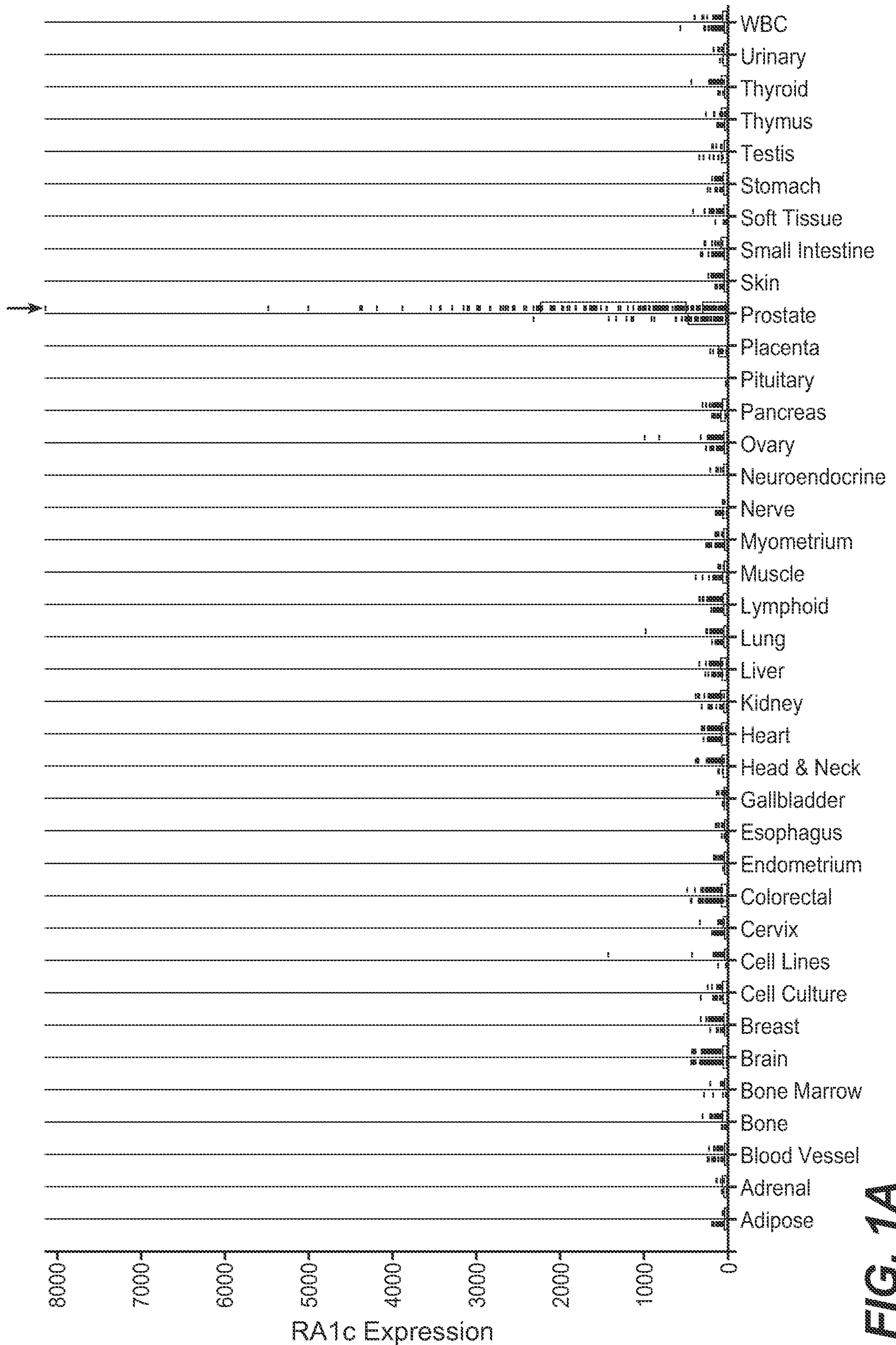
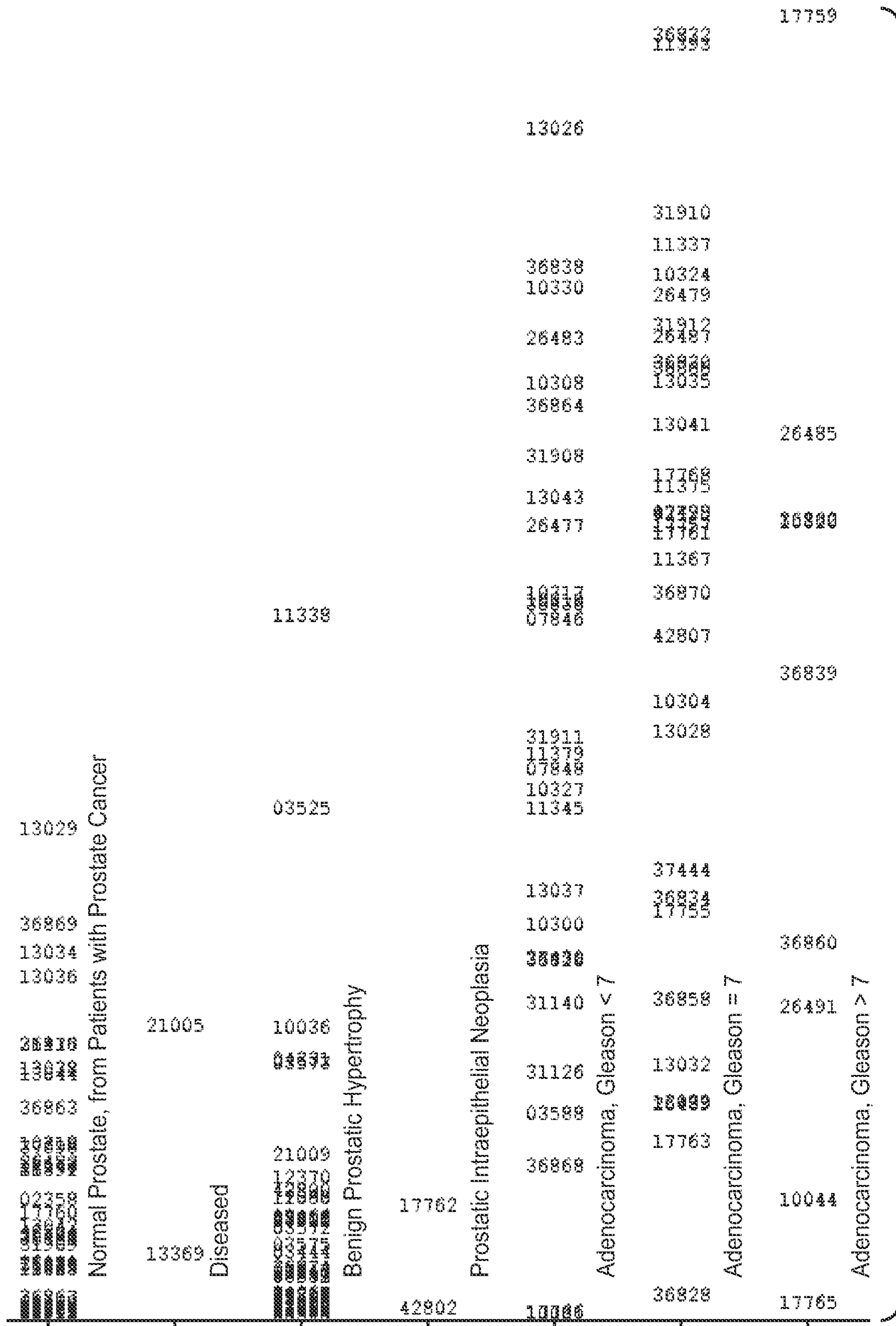
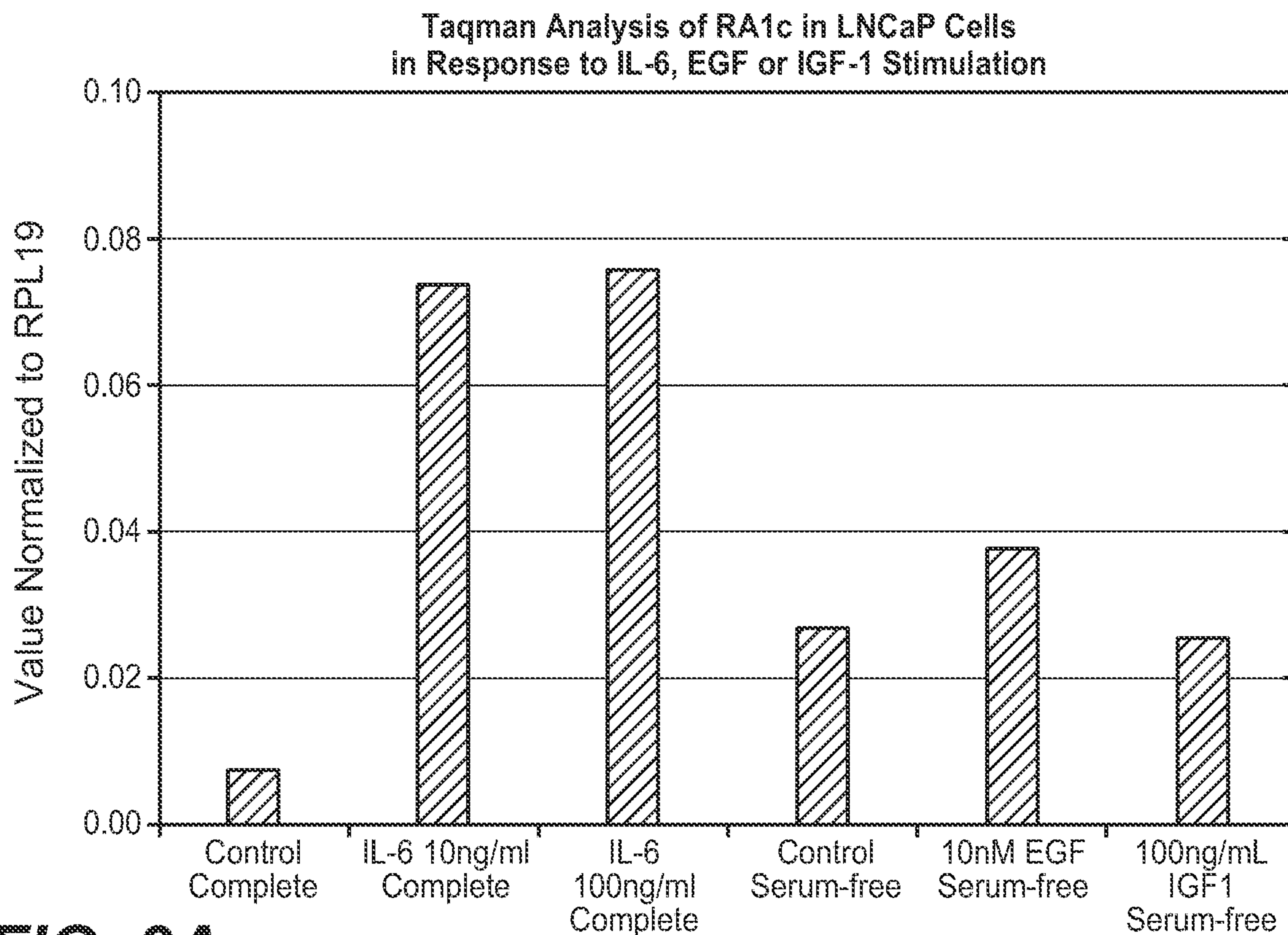
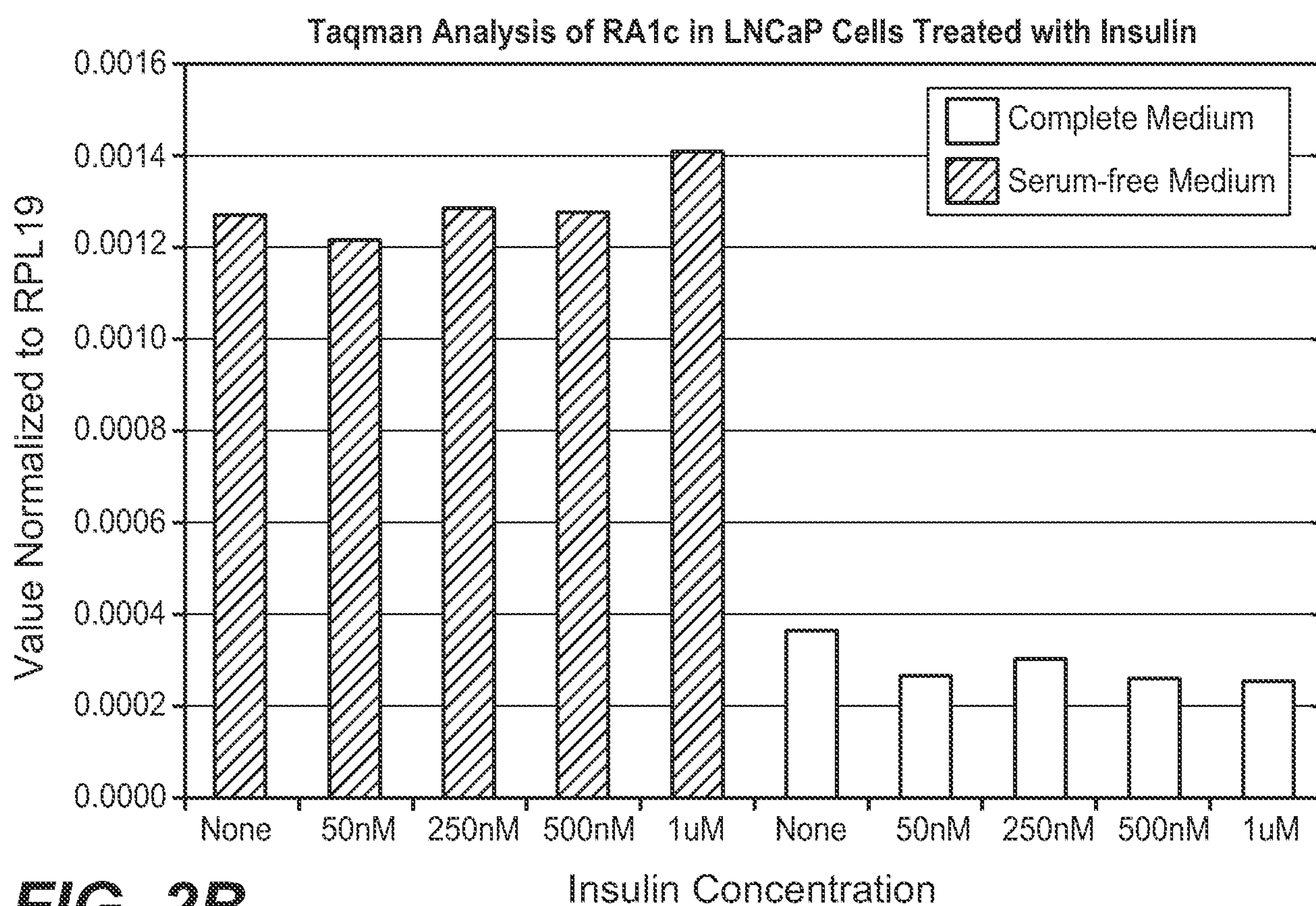


FIG. 1A

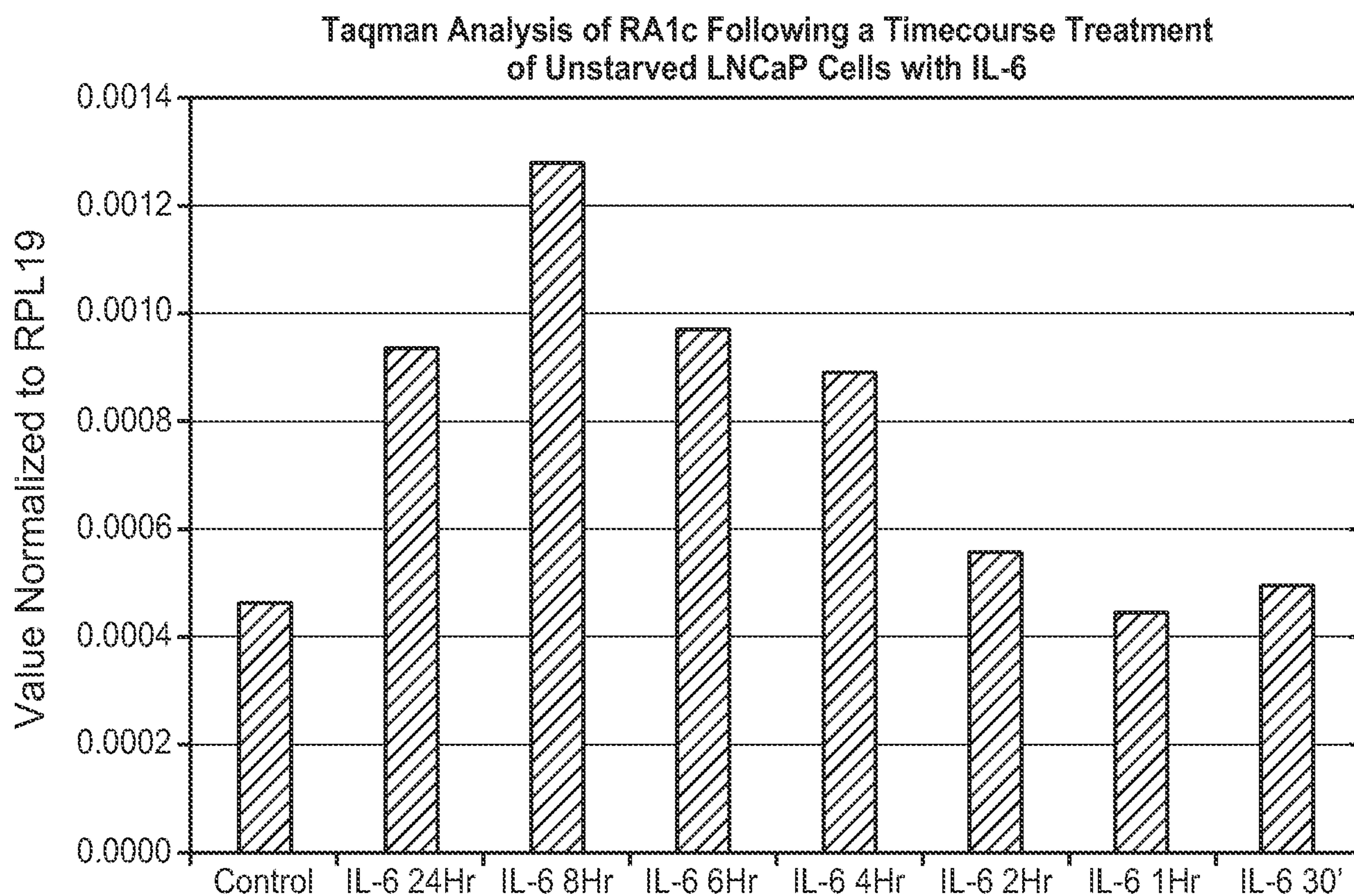
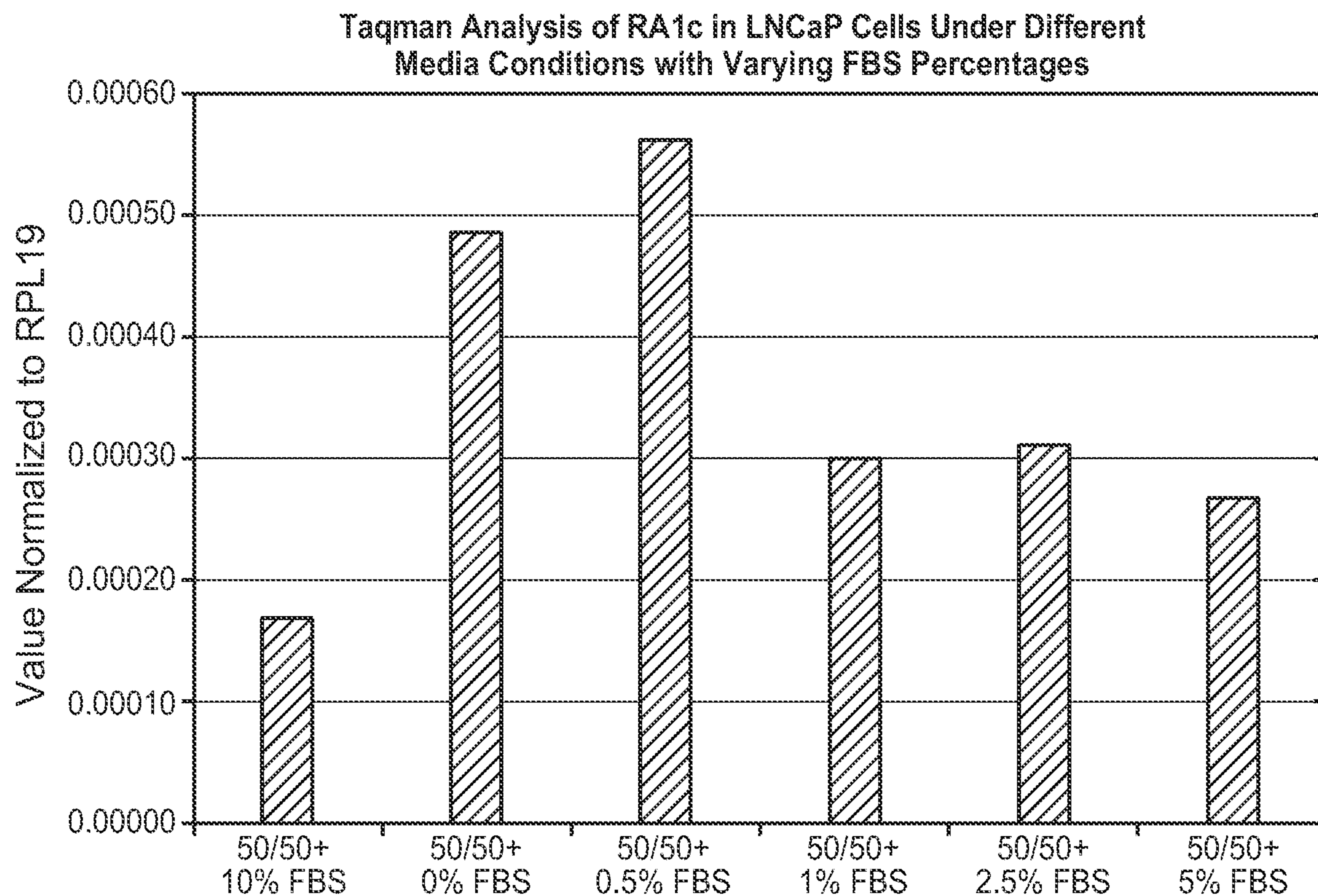
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**FIG. 2A****FIG. 2B**

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**FIG. 2C****FIG. 3A**

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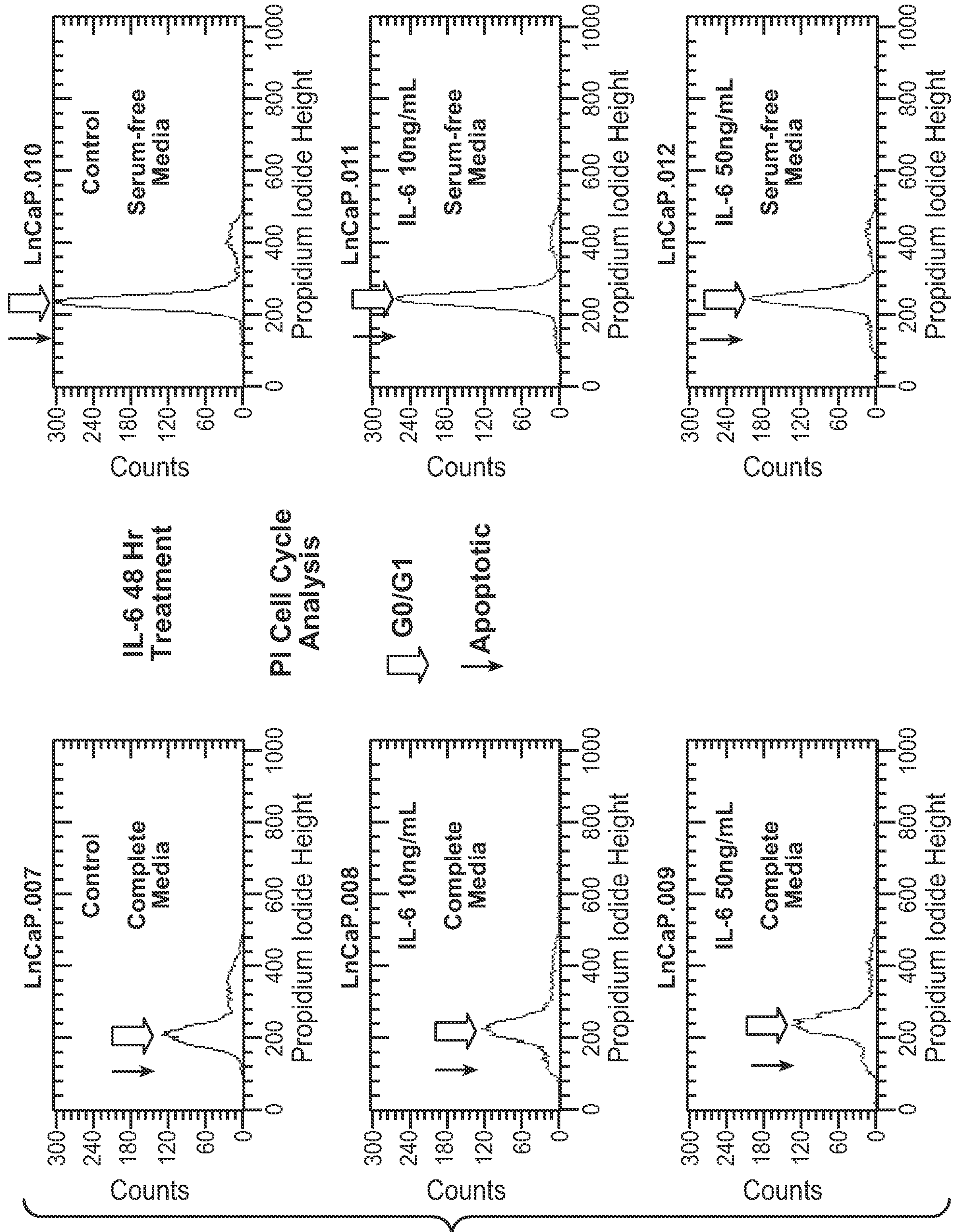
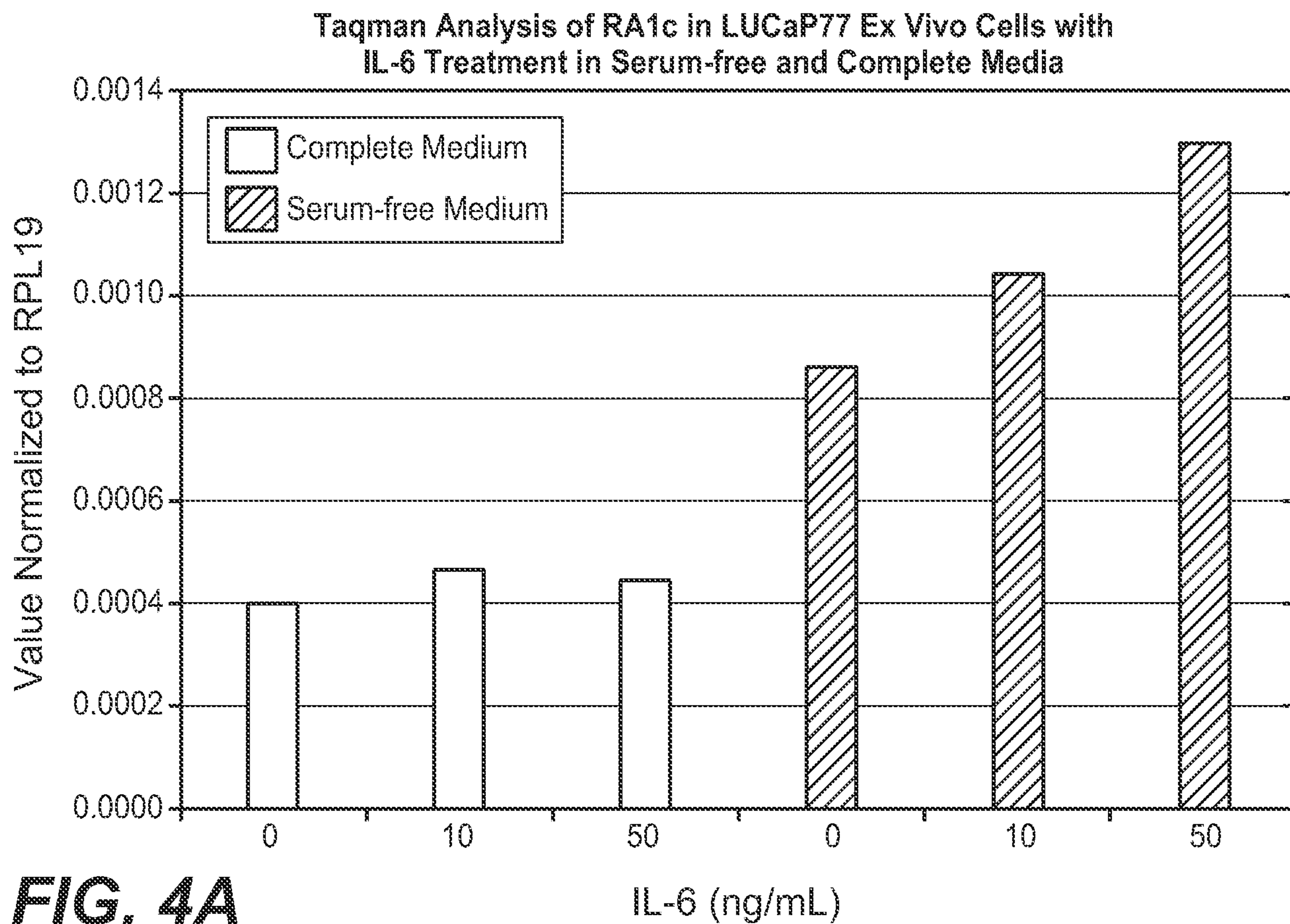
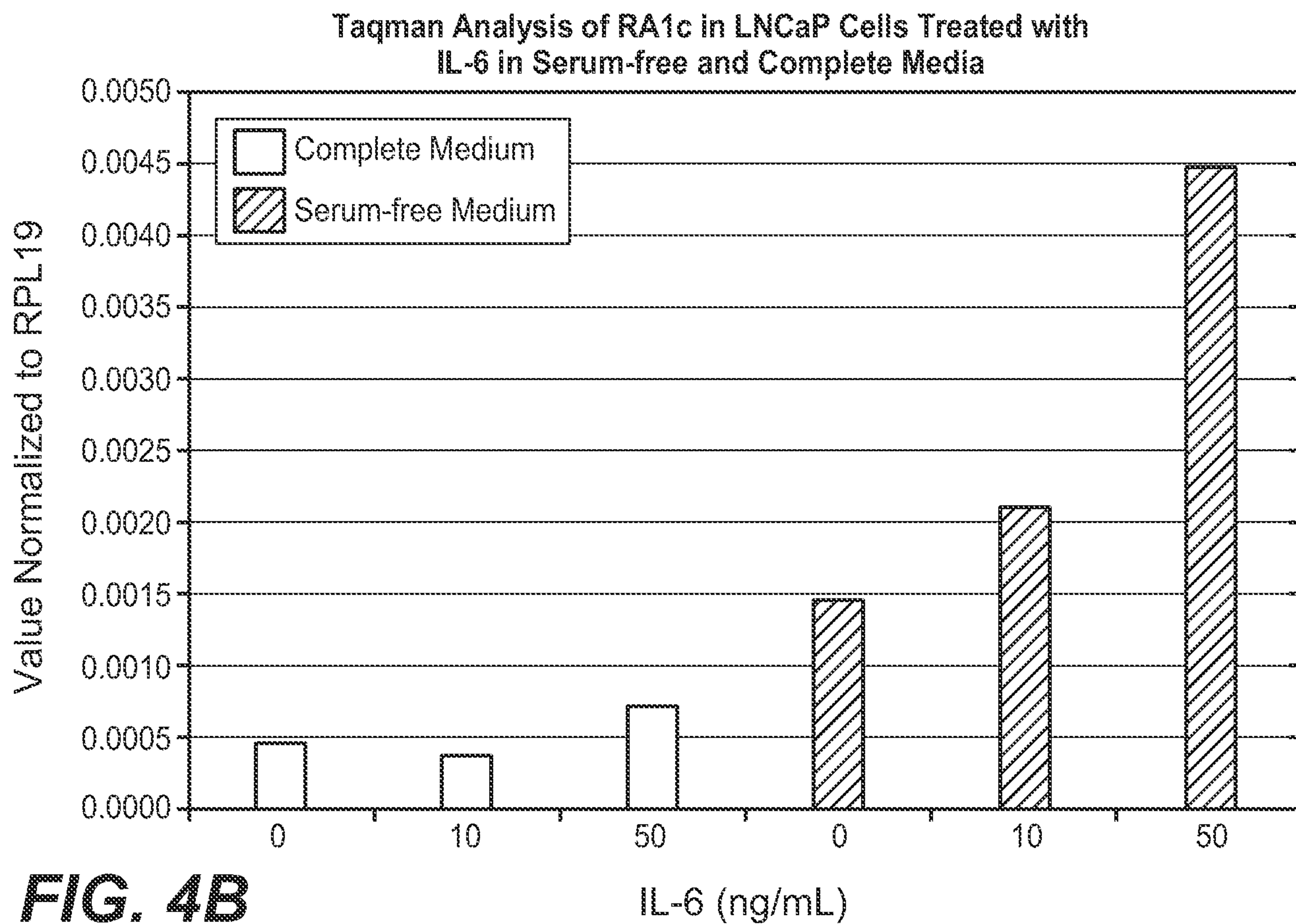
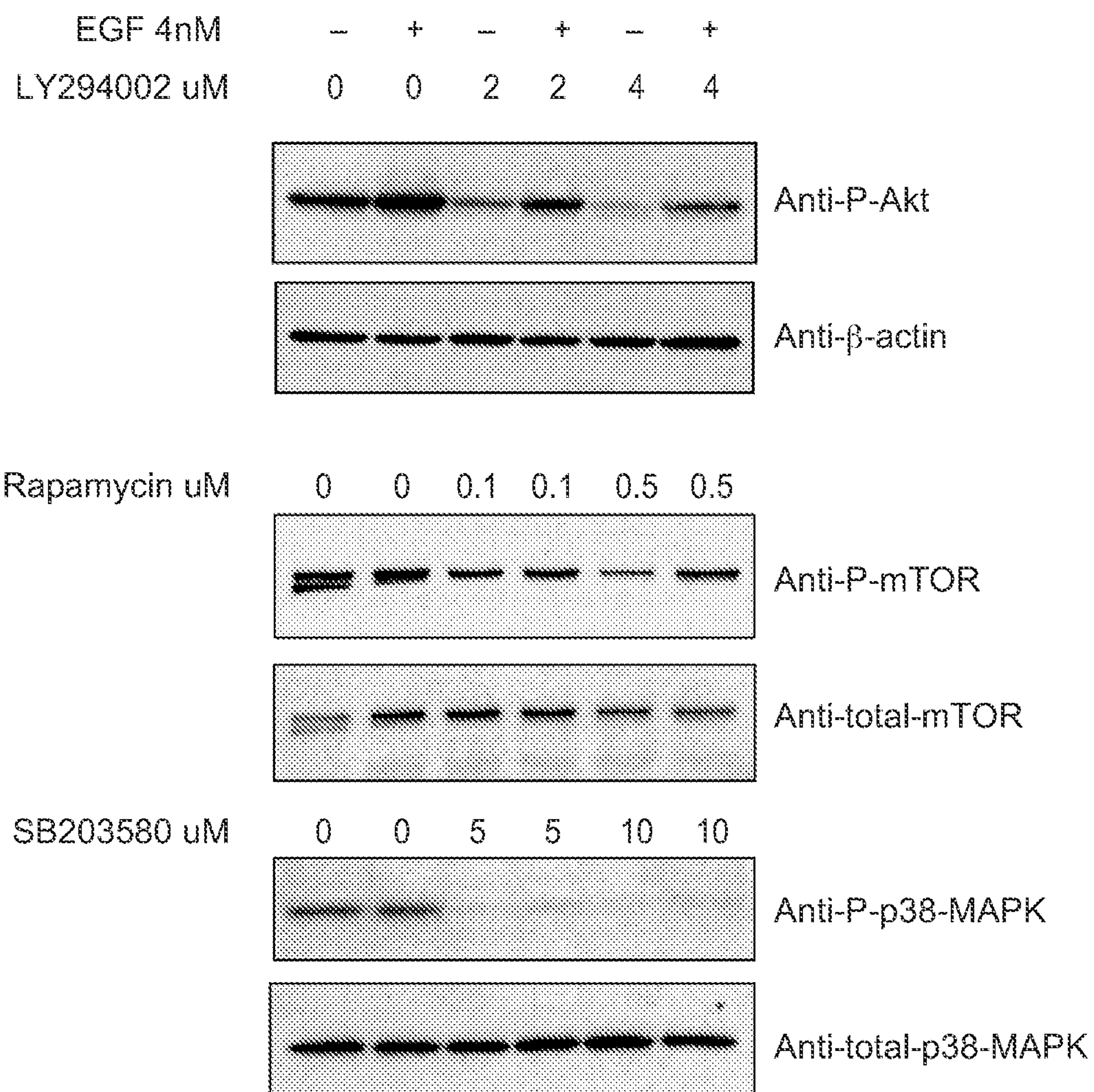


**FIG. 3B**

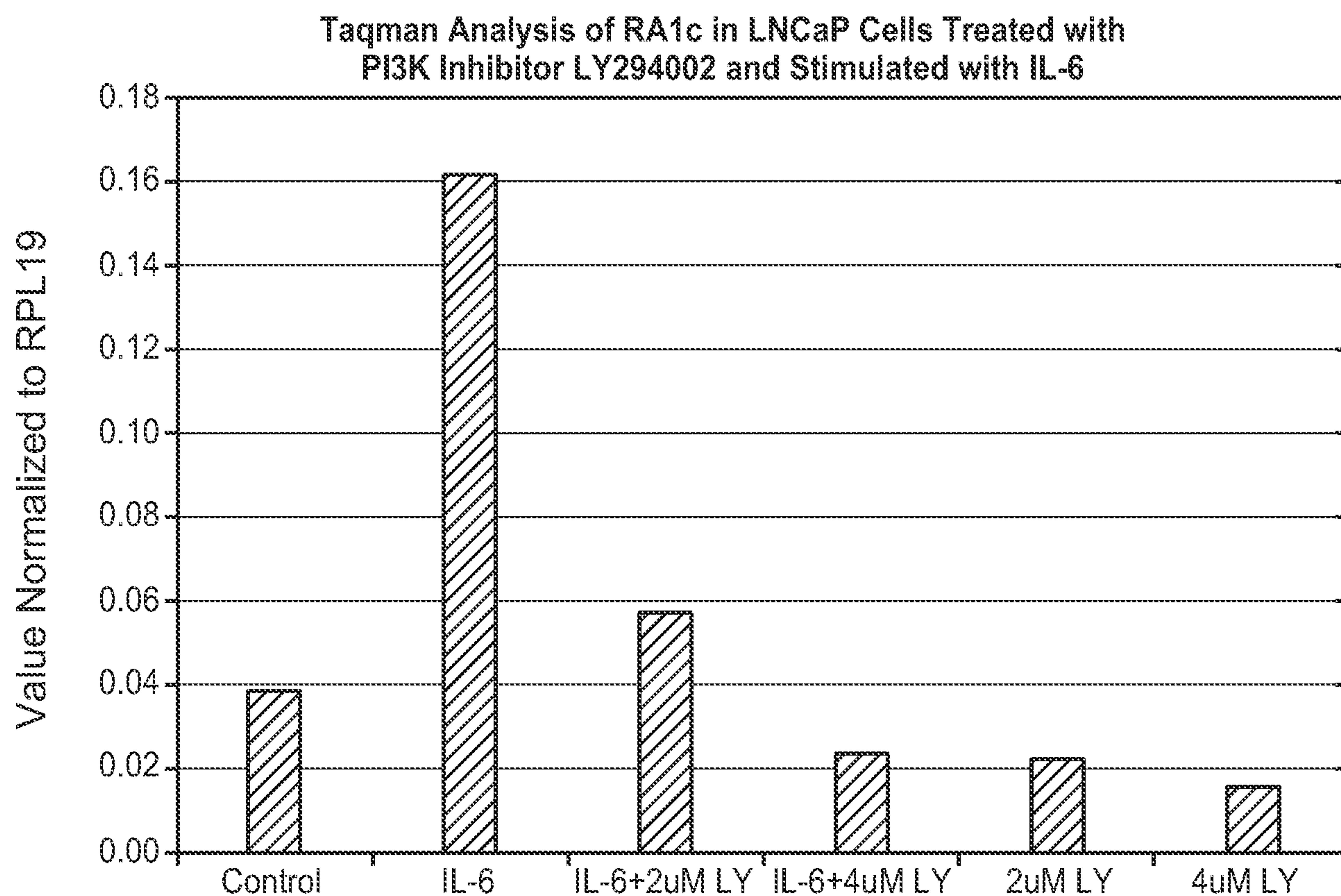
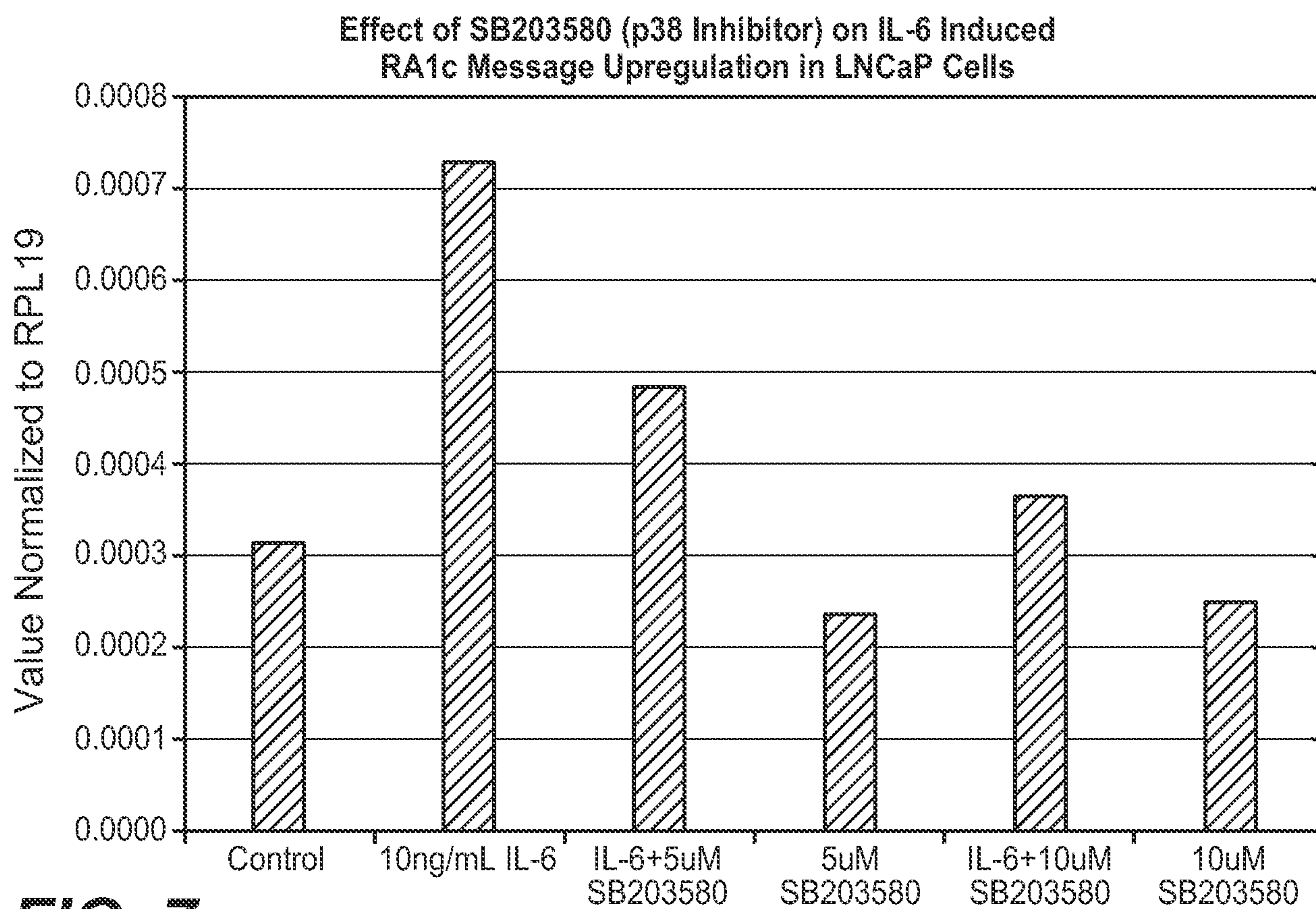
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**FIG. 4A****FIG. 4B**

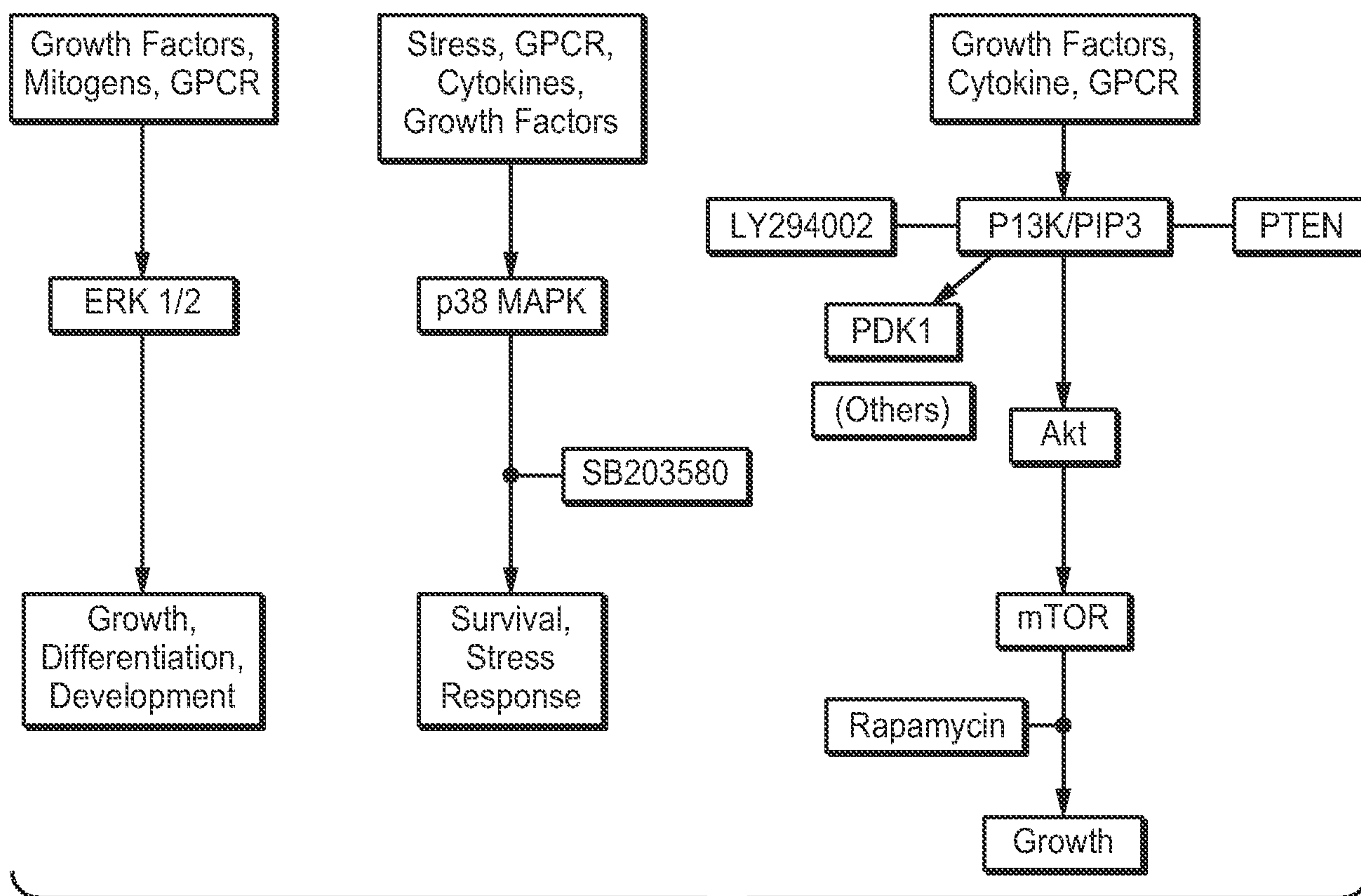
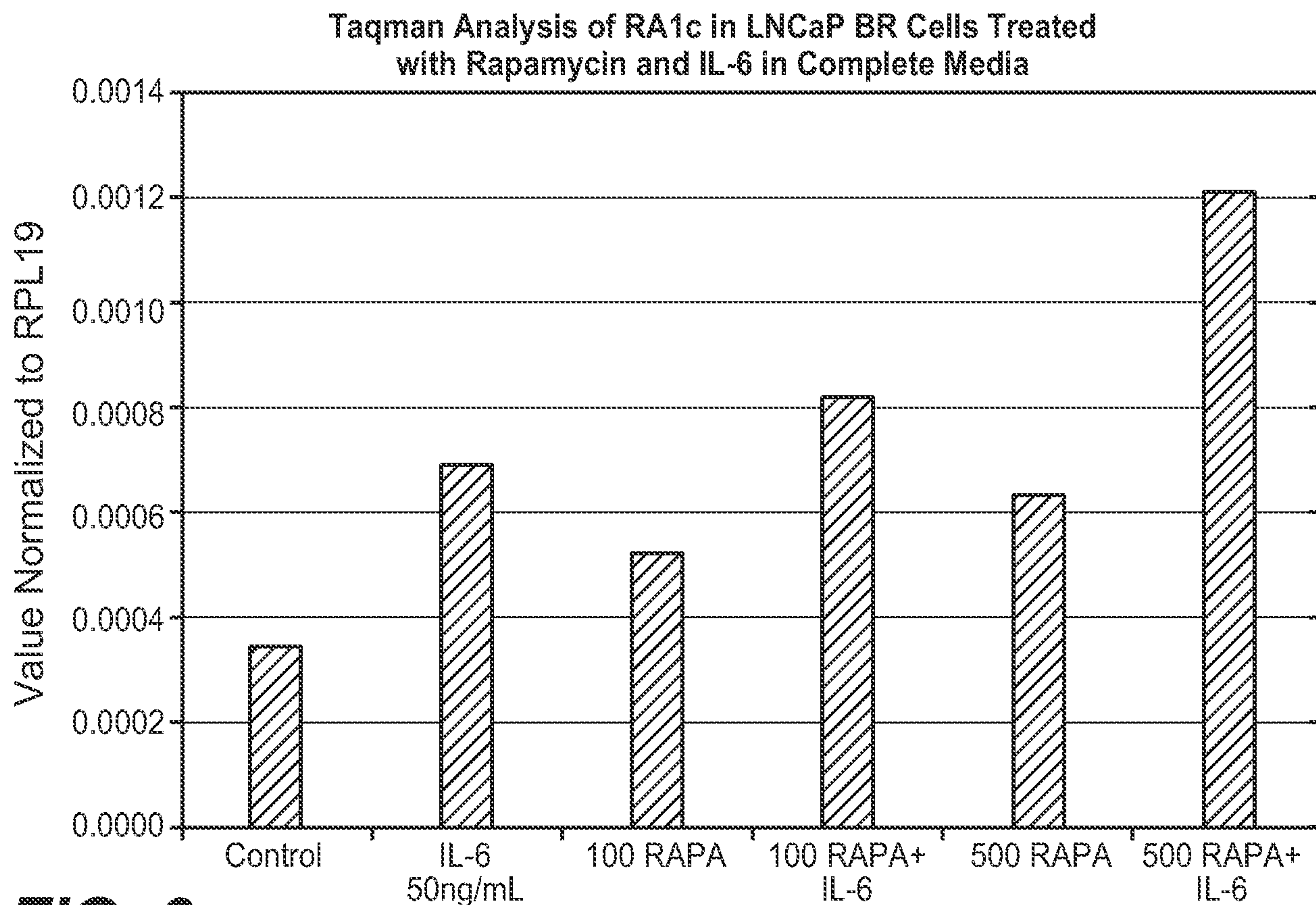
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**FIG. 5**

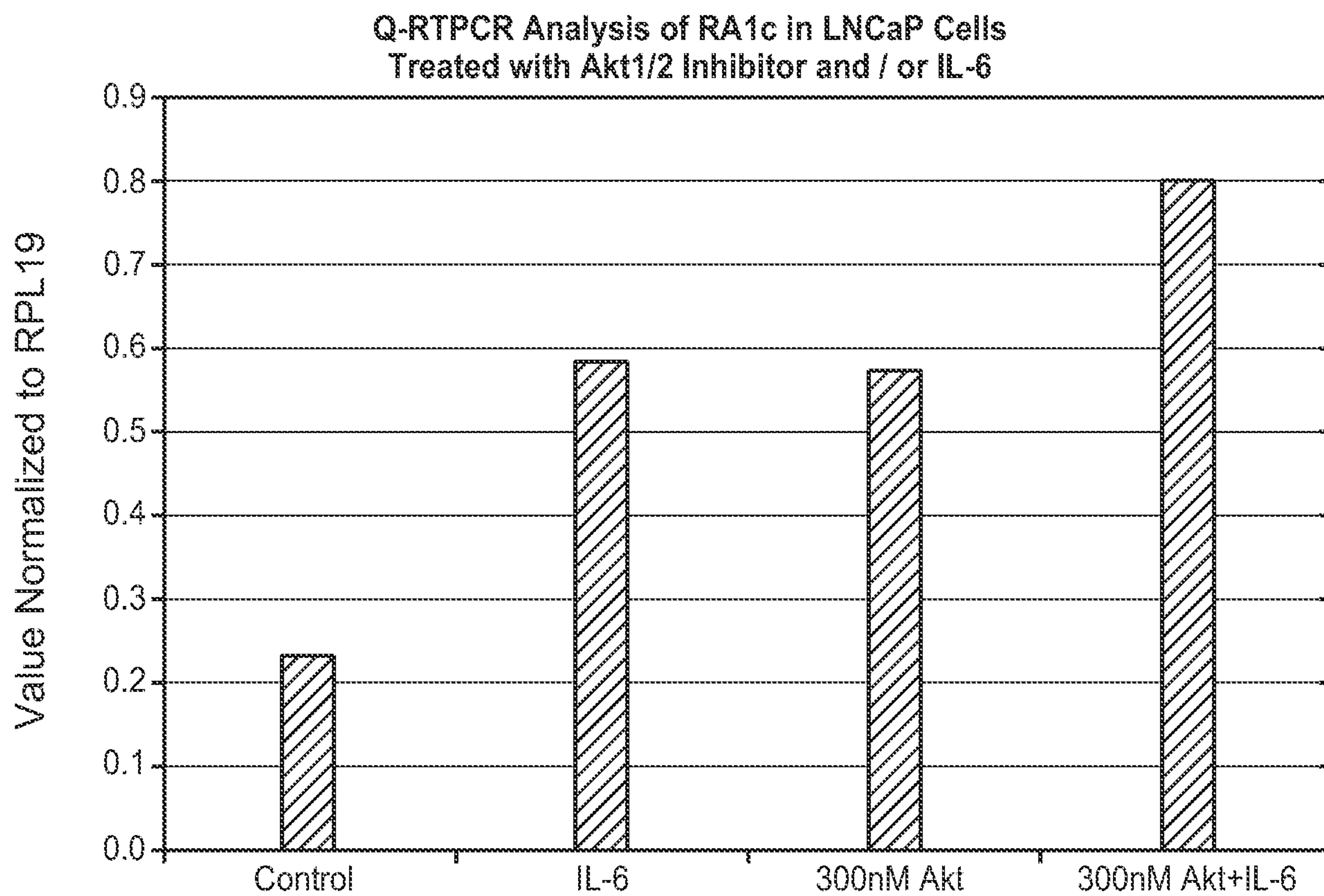
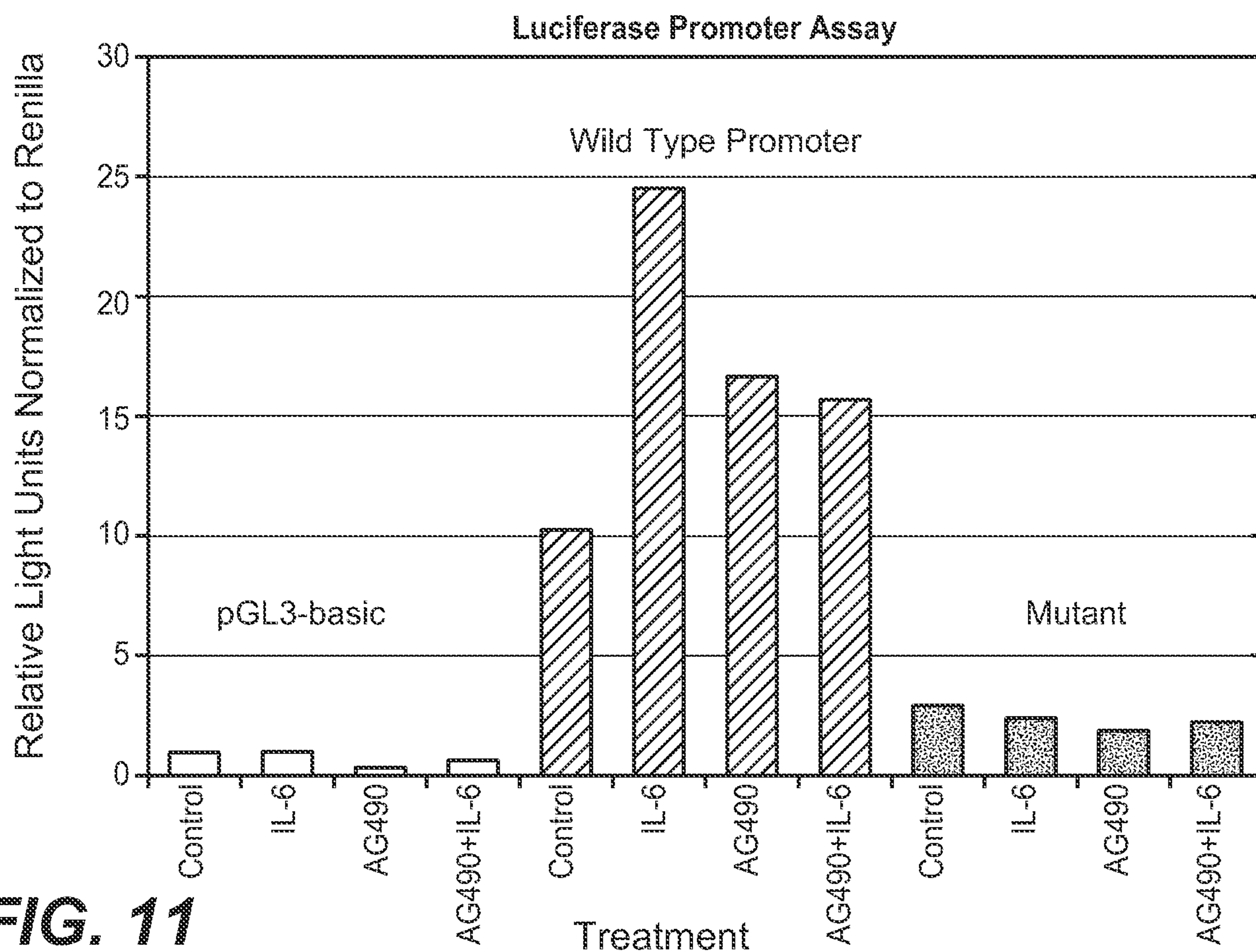
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**FIG. 6****FIG. 7**

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**FIG. 8****FIG. 9**

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**FIG. 10****FIG. 11**

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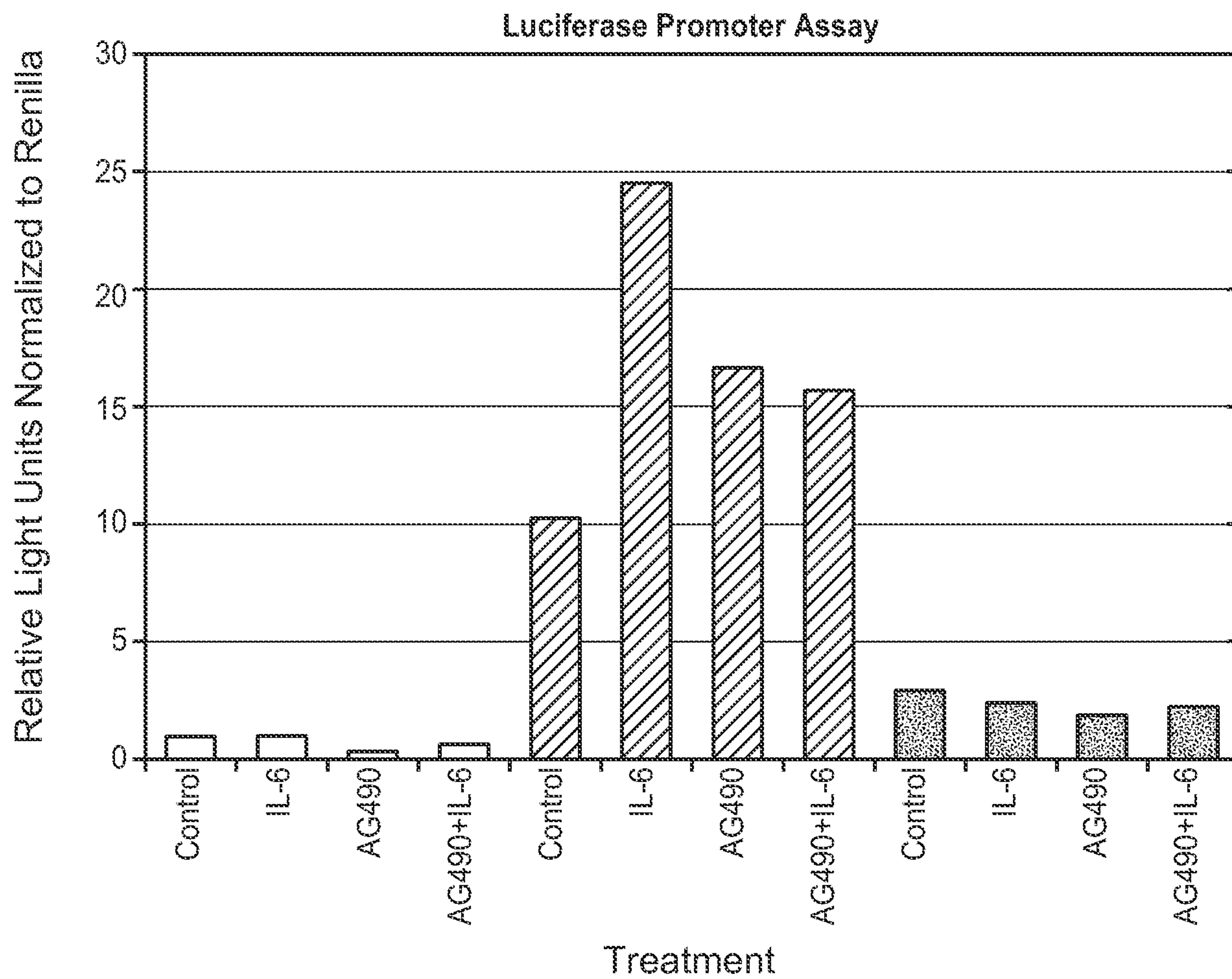
Mutagenesis Schematic

STAT3/NF-kB Binding Site

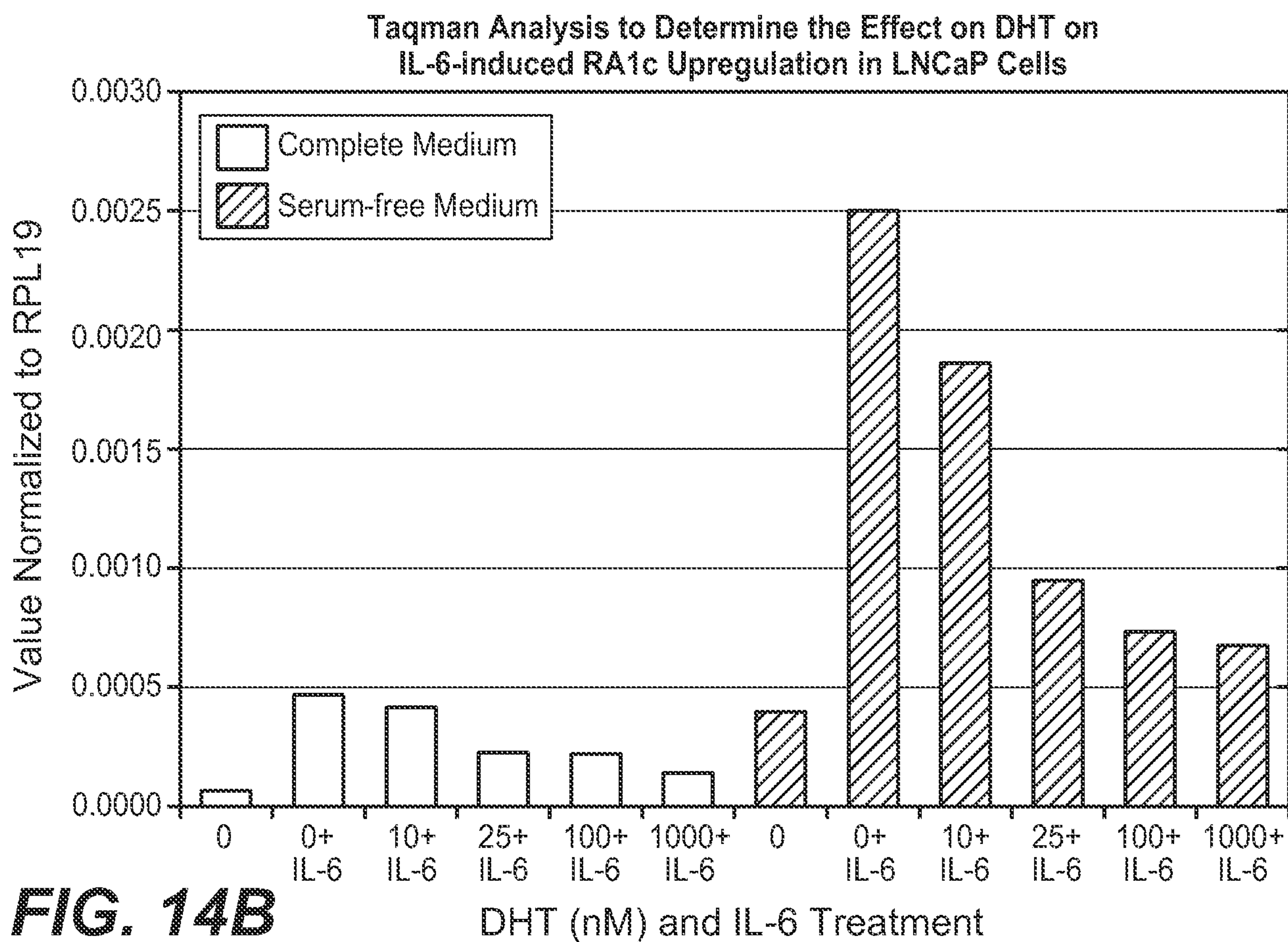
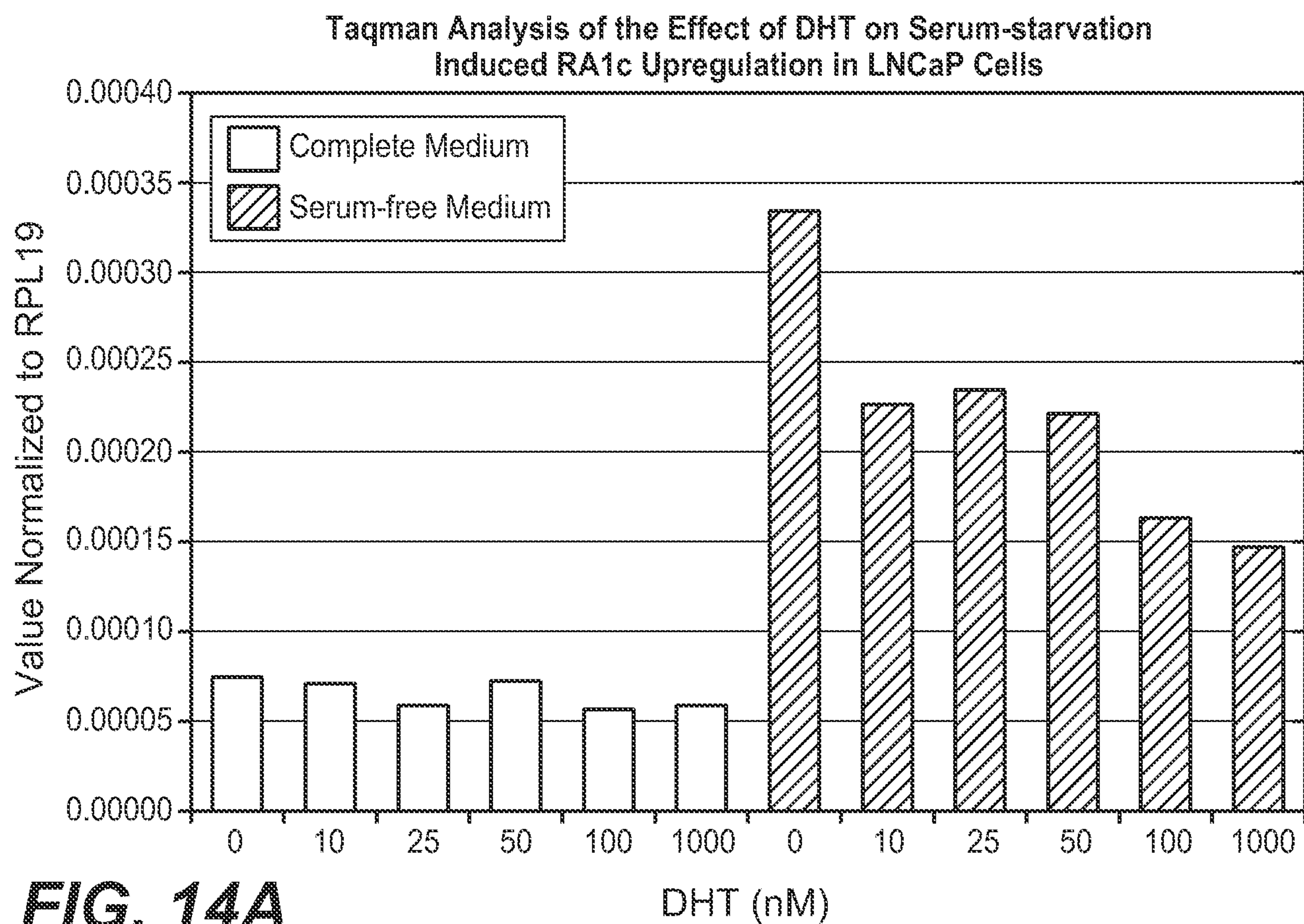
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Site Directed Mutagenesis

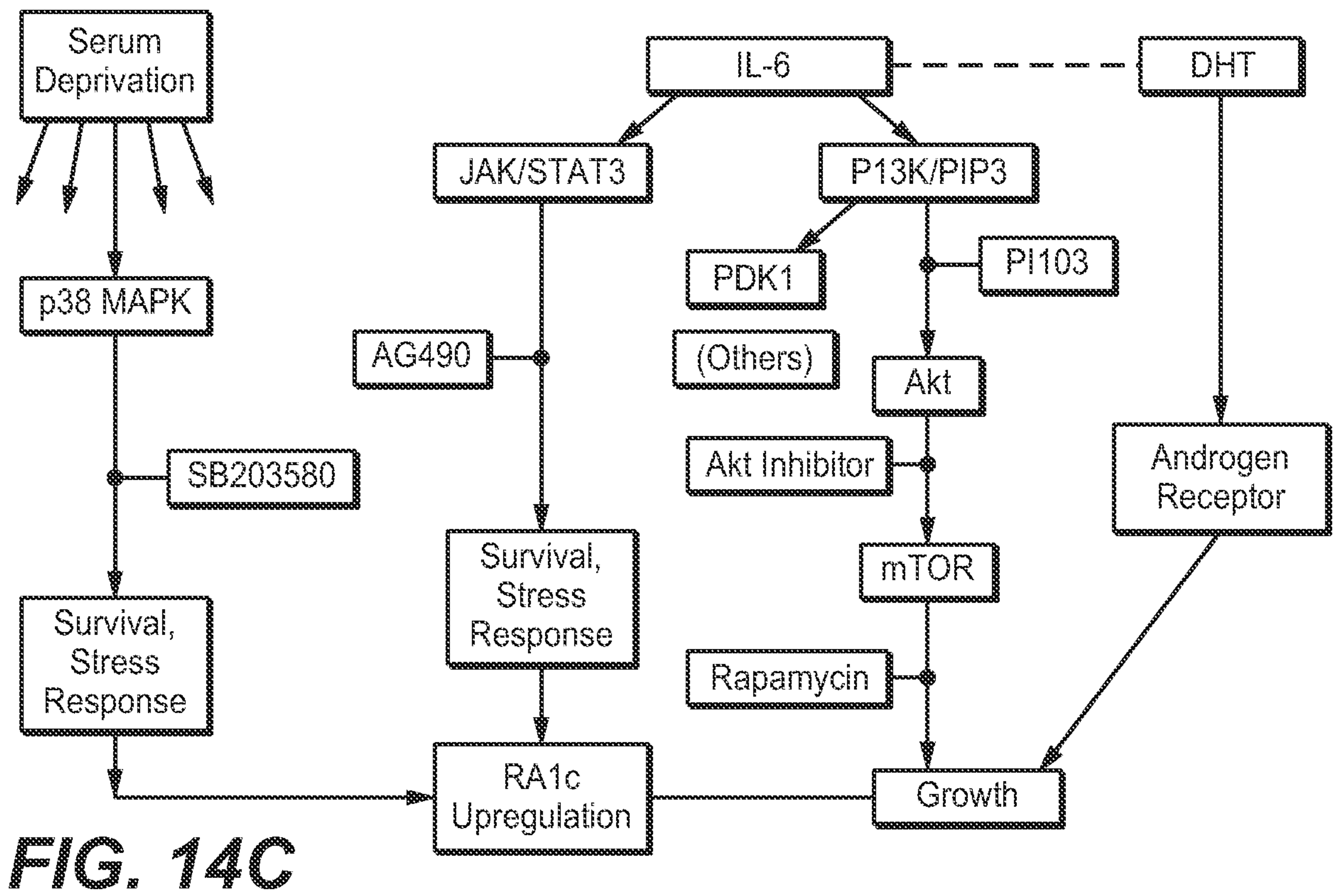
5' — GGGGGCCCCCGGGGACACCC — 3'

**FIG. 12****FIG. 13**

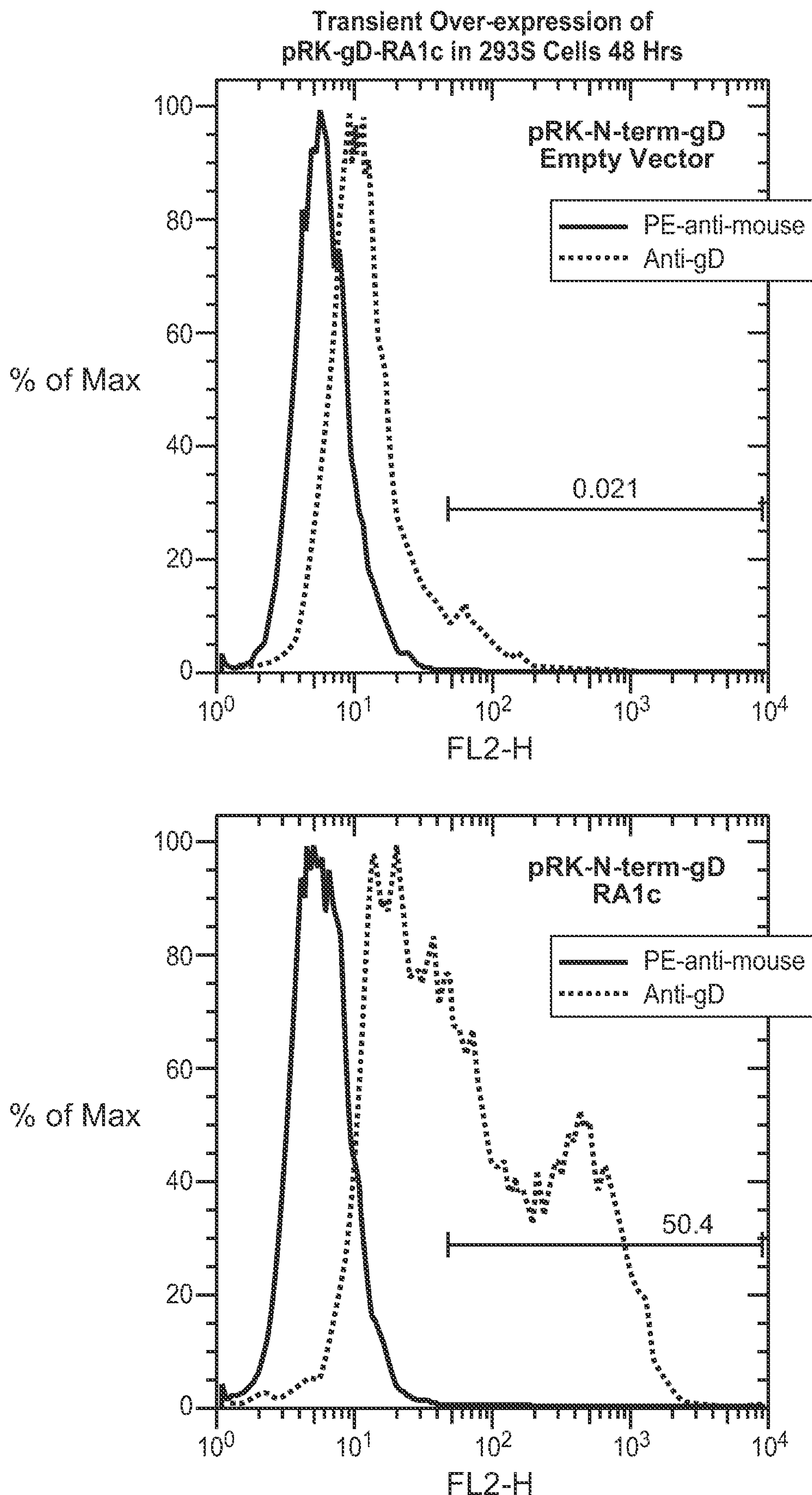
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**FIG. 15**