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(54) Titre : INHIBITEURS DE MAP-KINASE COMME REGULATEURS D'EXPRESSION ANTIGENIQUE ASSOCIE AUX TUMEURS

(54) Title: MAP-KINASE INHIBITORS AS REGULATORS OF TUMOR-ASSOCIATED ANTIGEN EXPRESSION

(57) Abrégé/Abstract:
This invention relates to methods for identifying agents that modulate expression of tumor-associated antigens in tumor cells. The invention also relates to agents and pharmaceutical compositions containing such agents that modulate expression of tumor-associated antigens in tumor cells. The invention, therefore, is particularly useful, inter alia, for treating subjects with autologous, solid tumors having cells that express, or that can be induced to express, tumor-associated antigens. One category of materials according to the invention is tumor-antigen expression up-regulating agents. These agents include modulators of the MAPK/ERK signaling cascade, and more specifically modulators of MEK1/2 and ERK1/2 activity.

## Abstract

This invention relates to methods for identifying agents that modulate expression of tumor-associated antigens in tumor cells. The invention also relates to agents and pharmaceutical compositions containing such agents that modulate expression of tumor-associated antigens in tumor cells. The invention, therefore, is particularly useful, *inter alia*, for treating subjects with autologous, solid tumors having cells that express, or that can be induced to express, tumor-associated antigens. One category of materials according to the invention is tumor-antigen expression up-regulating agents. These agents include modulators of the MAPK/ERK signaling cascade, and more specifically modulators of MEK1/2 and ERK1/2 activity.

# MAP-KINASE INHIBITORS AS REGULATORS OF TUMOR-ASSOCIATED ANTIGEN EXPRESSION

## Field of the Invention

This invention relates to methods and compositions for the treatment of tumors. In particular, the invention relates to methods and agents for the treatment of tumors expressing a tumor-antigen [also known as a tumor-associated antigen (TAA), lineage specific antigen, differentiation antigen, or self-antigen]. The invention also relates to methods for identifying agents that regulate expression of tumor-antigens.

## Background of the Invention

Many solid tumors are presently known to involve the infiltration of autologous lymphocytes. These autologous lymphocytes, known in the art as tumor-infiltrating lymphocytes (TIL), have been shown to recognize specific antigens expressed by cells of the solid tumor. Expression of such tumor-associated antigens (TAA) in combination with appropriate accessory signals leads to a specific cytolytic (cytotoxic) reactivity of the TILs toward the solid tumors.

Several tumor antigens have been identified in association with a variety of tumors Boon T, et al., *Ann Rev Immunol*, 1994, **12:**337-65; Kawakami Y, et al., *Proc Natl Acad Sci USA*, 1994, **91:**3515-9; Bakker A, et al., *J Exp Med*, 1994, **179:**1005-9). In addition to the identification of TAAs, immunodominant epitopes recognized by TILs have also been described for widely-expressed lineage-specific antigens, for example, the HLA-A2-restricted Melan-A/MART-1 in melanomas (Sensi M, et al., *Proc Natl Acad Sci USA*, 1995, **92:**5674-8; Kawakami Y, et al., *J Exp Med*, 1994, **180:**347-52). Melanomas are aggressive, frequently metastatic tumors derived from either melanocytes or melanocyte related nevus cells (*Cellular and Molecular Immunology*, 1991, (eds) Abbas A. K., Lechtman, A. H., Pober, J. S.; W. B. Saunders Company, Philadelphia: pages 340-341).

Although there is mounting evidence that it is possible to induce cell mediated immunity against autologous melanomas, clinical immunotherapy strategies (Kraden, et al., *Cancer Immunol. Immuinother*., 1987, 24:76); Kradin, et al., *Lancet*, 1989, I:577; Rosenberg, et al., *N. Eng. J. Med.*, 1988, 25:1676; Dillman, et al., *Cancer*, 1991, 68:1; Gattoni, et al., *Semin. Oncol.*, 1966, 23:754; Kan-Mitchell, et al., *Cancer Immunol.*

*Immunother.*, 1993, 37:15), have failed to achieve routine efficacy. This failure has been due, at least in part, to the ability of tumors to evade immune destruction (Becker, et al., *Int. Immunol.*, 1993, 5:1501; Jager, et al., *Int. J. Cancer*, 1997, 71:142; Maeurer, et al., *J. Clin. Invest.*, 1996, 98:1633; Marincola, et al., *J. Immunother. Emphasis Tumor Immunol.*, 1996, 9:192).

Recently, it was observed that malignant melanoma cells produce a soluble protein factor(s), which down-regulates melanocyte lineage Melan-A/MART-1 antigen expression by melanoma cells with concomitant loss of recognition by Melan-A/MART-1-specific T cells (Kurnick J T et al., *J. Immunol.* 2001 Aug 1;**167**(3):1204-11). The existence of this autocrine pathway provides an additional novel explanation for melanoma tumor progression in vivo in the presence of cytotoxic T lymphocytes (CTL) specific for this melanocyte lineage Ag. These observations may have important implications for Melan-A/MART-1-specific CTL-mediated immunotherapy of melanoma tumors.

There exists a need to identify additional mechanisms for tumor recognition escape by the TILs and other cells of the immune system.

There also exists a need to identify agents that prevent such immune system recognition escape.

## Summary of the Invention

The invention provides methods for identifying agents that modulate expression of tumor-associated antigens in tumor cells. The invention also provides agents and pharmaceutical compositions containing such agents that modulate expression of tumor-associated antigens in tumor cells. The invention, therefore, is particularly useful, *inter alia*, for treating subjects with autologous, solid tumors having cells that express, or that can be induced to express, tumor-associated antigens.

One category of materials according to the invention is tumor-antigen expression up-regulating agents. These agents include modulators of the MAPK/ERK signaling cascade, and more specifically modulators of MEK1/2 and ERK1/2 activity.

According to one aspect of the invention, a method of upregulating tumor-antigen expression in a tumor cell, is provided. The method invlves contacting a tumor cell with a MEK1/2 inhibitor in an amount effective to increase tumor-antigen expression in the tumor cell. The contacting may occur *in vivo* and/or *in vitro*. In some embodiments, the

origin of the tumor cells may be of: biliary tract cancer; brain cancer, including glioblastomas and medulloblastomas; breast cancer; cervical carcinoma; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; hematological neoplasms, including acute lymphocytic and myelogenous leukemia; multiple myeloma; AIDS associated leukemias and adult T-cell leukemia lymphoma; intraepithelial neoplasms, including Bowen's disease and Paget's disease; liver cancer; lung cancer; lymphomas, including Hodgkin's disease and lymphocytic lymphomas; neuroblastomas; oral cancer, including squamous cell carcinoma; ovarian cancer, including those arising from epithelial cells, stromal cells, germ cells and mesenchymal cells; pancreas cancer; prostate cancer; rectal cancer; sarcomas, including leiomyosarcoma, rhabdomyosarcoma, liposarcoma, fibrosarcoma and osteosarcoma; skin cancer, including melanoma, Kaposi's sarcoma, basocellular cancer and squamous cell cancer; testicular cancer, including germinal tumors (seminoma, non-seminoma[teratomas, choriocarcinomas]), stromal tumors and germ cell tumors; thyroid cancer, including thyroid adenocarcinoma and medullar carcinoma; and renal cancer including adenocarcinoma and Wilms tumor. In certain embodiments, the tumor-antigen can be Melan-A/MART-1, melanoma GP75, PGP 9.5, Annexin I and II, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, Colorectal associated antigen (CRC)--C017-1A/GA733, Ab2 BR3E4, CI17-1A/GA733, HSPPC-96, Hsp96, gp96-associated cellular peptides, G250, Herpes simplex thymidine kinase (HSVtk), Carcinoembryonic Antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, Prostate Specific Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, prostate-specific membrane antigen (PSMA), Ad5-PSA, Parathyroid-hormone-related protein (PTH-rP), Prostatic Acid Phosphatase (PAP), Prostate epithelium-derived Ets transcription factor (PDEF), Oncofetal antigen-immature laminin receptor (OFA-iLR), HP59, Cytochrome oxidase 1, sp100, Ran GTPase activating protein, members of Rab-GAP (Rab GTPase-activating proteins) e.g., PARIS-1, T-cell receptor/CD3-zeta chain, cTAGE-1 and SCP-1, Glycolipid antigens-GM2, GD2, GD3, FucosylGM1, Glycoprotein (mucin) antigens-Tn, sialyl Tn., TF and MUC1, Hsp105, MAGE-family of tumor antigens, GAGE-1,2, BAGE, RAGE, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, ErbB-2/neu, p21ras, RCAS1, α-fetoprotein, E-cadherin, α-catenin, β-catenin and γ-catenin, p120ctn, $gp100^{Pmel117}$, PRAME, GA733/EoCam, thyroglobulin, NY-ESO-1, cdc27, adenomatous polyposis coli

protein (APC), Prp1p/Zer1p, L19H1, MAZ, PINCH, PRAME, Wt-1, PHF3, LAGE-1, SCP-1, SSX-1, SSX-2, SSX-4, fodrin, Smad family of tumor antigens, lmp-1, EBV-encoded nuclear antigen (EBNA)-1, or c-erbB-2. In important embodiments, the MEK1/2 inhibitor is PD98059, U0126, PP1/PP2A, a b-Raf inhibitor, or a c-Raf inhibitor. In certain embodiments, the b-Raf inhibitor is a PK-A inhibitor.

According to another aspect of the invention, a method of upregulating tumor-antigen expression in a tumor cell, is provided. The method involves contacting a tumor cell with a ERK1/2 inhibitor in an amount effective to increase tumor-antigen expression in the tumor cell. The contacting may occur *in vivo* and/or *in vitro*. In some embodiments, the origin of the tumor cells may be of: biliary tract cancer; brain cancer, including glioblastomas and medulloblastomas; breast cancer; cervical carcinoma; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; hematological neoplasms, including acute lymphocytic and myelogenous leukemia; multiple myeloma; AIDS associated leukemias and adult T-cell leukemia lymphoma; intraepithelial neoplasms, including Bowen's disease and Paget's disease; liver cancer; lung cancer; lymphomas, including Hodgkin's disease and lymphocytic lymphomas; neuroblastomas; oral cancer, including squamous cell carcinoma; ovarian cancer, including those arising from epithelial cells, stromal cells, germ cells and mesenchymal cells; pancreas cancer; prostate cancer; rectal cancer; sarcomas, including leiomyosarcoma, rhabdomyosarcoma, liposarcoma, fibrosarcoma and osteosarcoma; skin cancer, including melanoma, Kaposi's sarcoma, basocellular cancer and squamous cell cancer; testicular cancer, including germinal tumors (seminoma, non-seminoma[teratomas, choriocarcinomas]), stromal tumors and germ cell tumors; thyroid cancer, including thyroid adenocarcinoma and medullar carcinoma; and renal cancer including adenocarcinoma and Wilms tumor. In certain embodiments, the tumor-antigen can be Melan-A/MART-1, melanoma GP75, PGP 9.5, Annexin I and II, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, Colorectal associated antigen (CRC)--C017-1A/GA733, Ab2 BR3E4, CI17-1A/GA733, HSPPC-96, Hsp96, gp96-associated cellular peptides, G250, Herpes simplex thymidine kinase (HSVtk), Carcinoembryonic Antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, Prostate Specific Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, prostate-specific membrane antigen (PSMA), Ad5-PSA, Parathyroid-hormone-related protein (PTH-rP), Prostatic Acid

Phosphatase (PAP), Prostate epithelium-derived Ets transcription factor (PDEF), Oncofetal antigen-immature laminin receptor (OFA-iLR), HP59, Cytochrome oxidase 1, sp100, Ran GTPase activating protein, members of Rab-GAP (Rab GTPase-activating proteins) e.g., PARIS-1, T-cell receptor/CD3-zeta chain, cTAGE-1 and SCP-1, Glycolipid antigens-GM2, GD2, GD3, FucosylGM1, Glycoprotein (mucin) antigens-Tn, sialyl Tn., TF and MUC1, Hsp105, MAGE-family of tumor antigens, GAGE-1,2, BAGE, RAGE, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, ErbB-2/neu, p21ras, RCAS1, α-fetoprotein, E-cadherin, α-catenin, β-catenin and γ-catenin, p120ctn, $gp100^{Pmel117}$, PRAME, GA733/EoCam, thyroglobulin, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), Prp1p/Zer1p, L19H1, MAZ, PINCH, PRAME, Wt-1, PHF3, LAGE-1, SCP-1, SSX-1, SSX-2, SSX-4, fodrin, Smad family of tumor antigens, lmp-1, EBV-encoded nuclear antigen (EBNA)-1, or c-erbB-2. In important embodiments, the ERK1/2 inhibitor is MKP-1, MKP-3, or a MEK1/2 inhibitor.

In any of the foregoing embodiments, preferred tumor cells are melanoma cells and a preferred tumor-antigen is Melan-A/MART-1.

According to a further aspect of the invention, a method of enhancing a tumor-specific immune response in a subject with cancer, is provided. The method involves administering to a subject in need of such treatment a MEK-1/2 inhibitor or an ERK-1/2 inhibitor, in an amount effective to enhance a tumor-specific immune response in the subject such as that induced by tumor vaccine or adoptive transfer of tumor-reactive cells. Preferred cancers/tumors are as described elsewhere herein.

According to still another aspect of the invention, a method of enhancing a melanoma-specific immune response in a subject with melanoma, is provided. The method involves administering to a subject in need of such treatment a MEK-1/2 inhibitor or an ERK-1/2 inhibitor, in an amount effective to increase Melan-A/MART-1 expression in malignant melanoma cells and enhance a melanoma-specific immune response in the subject such as that induced by tumor vaccine or adoptive transfer of tumor-reactive cells.

Preferred MEK-1/2 inhibitors and ERK-1/2 inhibitors are as described elsewhere herein.

According to a further aspect, methods for preparing medicaments useful in enhancing a tumor-specific immune response in a subject with cancer and/or a melanoma-specific immune response in a subject with melanoma, are also provided.

According to a further aspect of the invention, a method of identifying genes that modulate tumor-antigen expression in a tumor cell. The method provides (a) contacting a tumor cell known to express a tumor-antigen with a modulator of the MAPK/ERK signaling cascade, (b) measuring tumor-antigen expression on the tumor cell, (c) determining whether tumor-antigen expression on the tumor cell is modulated compared to a control, and (d) identifying genes that modulate expression of tumor-antigen expression in the tumor cell. The genes are identified using differential expression technology and are then isolated and characterized using conventional methodology. In certain embodiments, the tumor cell is a cell selected from the group consisting of of acute lymphoblastic leukemia cells, glioma cells, bladder cancer cells, billiary cancer cells, breast cancer cells, cervical carcinoma cells, colon carcinoma cells, colorectal cancer cells, choriocarcinoma cells, epithelial cancer cells, gastric cancer cells, hepatocellular cancer cells, Hodgkins lymphoma cells, lung cancer cells, lymphoid cell-derived leukemia cells, myeloma cells, non-small cell lung carcinoma cells, nasopharyngeal cancer cells, ovarian cancer cancer cells, prostate cancer cells, pancreatic cancer cells, renal cancer cells, testicular cancer cells, T cell leukemia cells, and melanoma cells, and the tumor-antigen is selected from the group consisting of Melan-A/MART-1, melanoma GP75, PGP 9.5, Annexin I and II, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, Colorectal associated antigen (CRC)--C017-1A/GA733, Ab2 BR3E4, CI17-1A/GA733, HSPPC-96, Hsp96, gp96-associated cellular peptides, G250, Herpes simplex thymidine kinase (HSVtk), Carcinoembryonic Antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, Prostate Specific Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, prostate-specific membrane antigen (PSMA), Ad5-PSA, Parathyroid-hormone-related protein (PTH-rP), Prostatic Acid Phosphatase (PAP), Prostate epithelium-derived Ets transcription factor (PDEF), Oncofetal antigen-immature laminin receptor (OFA-iLR), HP59, Cytochrome oxidase 1, sp100, Ran GTPase activating protein, members of Rab-GAP (Rab GTPase-activating proteins) e.g., PARIS-1, T-cell receptor/CD3-zeta chain, cTAGE-1 and SCP-1, Glycolipid antigens-GM2, GD2, GD3, FucosylGM1, Glycoprotein (mucin) antigens-Tn, sialyl Tn., TF and MUC1, Hsp105, MAGE-family of tumor antigens, GAGE-1,2, BAGE, RAGE, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, ErbB-2/neu, p21ras, RCAS1, α-fetoprotein, E-cadherin, α-catenin, β-catenin and γ-catenin, p120ctn, $gp100^{Pmel117}$,

PRAME, GA733/EoCam, thyroglobulin, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), Prp1p/Zer1p, L19H1, MAZ, PINCH, PRAME, Wt-1, PHF3, LAGE-1, SCP-1, SSX-1, SSX-2, SSX-4, fodrin, Smad family of tumor antigens, lmp-1, EBV-encoded nuclear antigen (EBNA)-1, or c-erbB-2. In important embodiments the contacting occurs in the presence or absence of Oncostatin M. Preferred MAPK/ERK signaling cascade modulators are as described elsewhere herein. In crtain embodiments, wherein the tumor cells are melanoma cells, the cells amy be those of a cell line selected from the group consisiting of 136.2, 453A, MM96L, MU, MU-X, EW, IGR-39D, and A375.

According to a further aspect of the invention, a solid-phase nucleic acid molecule array is provided. The array consists essentially of a set of nucleic acid molecules, expression products thereof, or fragments thereof, fixed to a solid substrate. The set of nucleic acid molecules comprises nucleic acid molecules of a tumor cell known to express a tumor-antigen, said tumor cell previously contacted with a modulator of the MAPK/ERK signaling cascade. In important embodiments, the solid-phase nucleic acid molecule array, further comprises at least one control set of nucleic acid molecules. A preferred control set of nucleic acid molecules consists essentially of nucleic acid molecules, expression products thereof, or fragments thereof, fixed to a solid substrate,. wherein the set of nucleic acid molecules comprises nucleic acid molecules of a tumor cell known to express a tumor-antigen, said tumor cell not previously contacted with a modulator of the MAPK/ERK signaling cascade.

These and other aspects of the invention, as well as various advantages and utilities, will be more apparent with reference to the detailed description of the preferred embodiments.

## Brief Description of the Drawings

Figure 1 shows graphs depicting the down-modulation of Antigen Expression in Melanoma Cell Line MU by OSM. MU tumor cells were cultured for 3 days in control medium or in 20ng/ml of OSM (first and last panels), or in supernatants from EW (contains OSM) ($2^{nd}$ panel) or A375 tumor cells (does not contain OSM) ($3^{rd}$ panel). Cells were stained for cytoplasmic expression of MA/M1 protein (first 3 panels) or gp100 (last

panel) and assayed by flow cytometry. Mean channel of fluorescence is shown within each panel.

Figure 2 is a graph depicting the inhibition of OSM activity on MU tumor cells by MEK inhibitor PD98059. MU tumor cells were cultured for three days in the presence of 10ng/ml of recombinant hOSM (OSM curve) as compared to control medium (CONTROL). In parallel, MU tumor cells were treated with 10mM of PD98059 before adding 10ng/ml of recombinant hOSM (PD98059+OSM). Cultured cells were stained with monoclonal anti-Melan-A/MART-1 antibody (A103) and FITC-anti mouse antibody to detect cytoplasmic antigen. Histograms of the three cultures are overlaid to demonstrate the reversal of OSM effect by the PD98059 treatment.

Figure 3 shows graphs depicting up-regulation of Melan-A/MART-1 expression in melanoma cells by MEK1/2 inhibitors U0126 and PD98059. Both Melan-A/MART-1 positive tumor cells, such as 453A, and Melan-A/ MART-1-deficient cell lines, including EW, IGR-39D and A375 demonstrate up-regulation of this melanocyte-lineage antigen after 3 to 6 Days in the presence of 40-50 μM U0126 or PD98059. Mean channel fluorescence for the Control and MEK-inhibitor-treated cells is shown for each cell line treated. In each case, the treated cells have readily-demonstrable increases in MA/M1 staining, with the curve on the right representing the treated cells in each panel. (Note: Although MA/M1 deficient cells show up-regulation, they do not express as much antigen as the typical Melan-A/MART-1-positive melanoma tumor cells).

Figure 4 shows an RT-PCR product depicting up-regulation of Melan-A/MART-1 mRNA expression in MU tumor cells by PD98059 and thus reversal of OSM-down-modulation.

Figure 5 shows RT-PCR product depicting the effect of UO126 (MEK1/2 inhibitor) on MA/M1 and tyrosinase mRNA levels and on OSM responses. MU tumor cells were treated for 72hrs before extracting mRNA and amplification. 1= OSM 20 ng/ml+10μM UO126; 2= 20μM UO126 only; 3= 10μM UO126 only; 4= OSM 20 ng/ml only; 5= controls (untreated).

Figure 6 shows RT-PCR product depicting the up-regulation of Melan-A/MART-1 mRNA expression in MU-X tumor cells treated with MEK1/2 Inhibitor U0126. Melan-A/MART-1-deficient tumor cells, MU-X were treated for 3 days with 20μM MEK1/2 inhibitor, U0126 (Lane 1), to demonstrate elevated mRNA levels (after PCR amplification

of cDNAs) for Melan-A/MART-1. In contrast no mRNA can be detected in "control" MU-X cells shown in Lane 2, (MW markers in M). As a control, there is no significant impact of U0126 on β-Actin mRNA levels.

Figure 7 is a graph showing the induction of Cytotoxic T Cell Recognition by an MEK1/2 inhibitor. Cloned MA/M1 specific cytotoxic T lymphocytes (CTL) were tested against MA/M1-expressing MU tumor cells vs. low MA/M1-expressing A375 tumor cells. The A375 tumor cells were incubated for 6 days in the presence of MEK 1/2 inhibitor U0126 at 40μM to increase mean channel fluorescence for cytoplasmic MA/M1 staining from 17 to 42, although this level was still lower than that seen in MU cells (mean channel >100).

## Detailed Description of the Invention

We describe herein agents and pharmaceutical compositions containing such agents that modulate expression of tumor-associated antigens in tumor cells. The foregoing can be used, *inter alia, in vivo* or *in vitro,* for the purpose of inhibiting growth of a tumor having cells expressing tumor-associated antigens, and in a variety of screening assays in order to identify additional agents that modulate expression of tumor-associated antigens in tumor cells.

We previously described that tumor cells which normally express or present TAAs (intracellularly or on their surface) "lose" (or down-modulate) such TAA expression/ presentation when cultured at high density (Ramirez-Montagut T, et al., *Clin Exp Immunol.*, 2000, 119(1):11-8; U.S. Provisional App. Ser. No. 60/165,806; and International App. No. PCT/US00/31511). We have discovered, unexpectedly, that such TAA down-modulation is mediated through a mitogen-activated protein kinase (MAPK) pathway. More specifically, we have discovered that TAA down-modulation is mediated through mitogen-activated protein kinase kinase family members MEK-1 and MEK-2 (or MEK1/2 for both), and their substrates ERK-1 and ERK-2 (or ERK1/2 for both, members of the mitogen-activated protein kinase family). The reversal of this antigen loss would have particular importance in the setting of immunotherapeutic attempts to enhance immunity to tumors, by such means as tumor vaccines or adoptive transfer of tumor-reactive cells (Ramirez et al., *Clin. Exp. Immunol.* 119:11-18, 2000).

In certain embodiments of the invention, an immunoreactive cell sample is used in conjunction with the methods of the invention described herein. By "immunoreactive cell" is meant a cell which can mature into an immune cell (such as a B cell, a helper T cell, or a cytolytic T cell) upon appropriate stimulation. Thus immunoreactive cells include $CD34^+$ hematopoietic stem cells, immature T cells and immature B cells. When it is desired to produce cytolytic T cells which recognize a tumor antigen or a tumor-associated antigen, the immunoreactive cell is contacted with a cell which expresses a tumor antigen or a tumor-associated antigen under conditions favoring production, differentiation and/or selection of cytolytic T cells; the differentiation of the T cell precursor into a cytolytic T cell upon exposure to antigen is similar to clonal selection of the immune system.

Some therapeutic approaches are premised on a response by a subject's immune system, leading to lysis of antigen presenting cells, such as cancer cells which present one or more tumor antigens or tumor-associated antigens. One such approach is the administration of autologous CTLs specific to a cancer-associated antigen/MHC complex to a subject with abnormal cells of the phenotype at issue. It is within the ability of one of ordinary skill in the art to develop such CTLs *in vitro*. An example of a method for T cell differentiation is presented in International Application number PCT/US96/05607. Generally, a sample of cells taken from a subject, such as blood cells, are contacted with a cell presenting the complex and capable of provoking CTLs to proliferate. The target cell can be a transfectant, such as a COS cell. These transfectants present the desired complex of their surface and, when combined with a CTL of interest, stimulate its proliferation. COS cells are widely available, as are other suitable host cells. Specific production of CTL clones is well known in the art. The clonally expanded autologous CTLs then are administered to the subject.

Another method for selecting antigen-specific CTL clones has recently been described (Altman et al., *Science* 274:94-96, 1996; Dunbar et al., *Curr. Biol.* 8:413-416, 1998). The reactive CTLs isolated by the method can then be expanded *in vitro* for use as described herein.

To detail a therapeutic methodology, referred to as adoptive transfer (Greenberg, *J. Immunol.* 136(5): 1917, 1986; Riddel et al., *Science* 257: 238, 1992; Lynch et al, *Eur. J. Immunol.* 21: 1403-1410,1991; Kast et al., *Cell* 59: 603-614, 1989), cells presenting the desired complex (e.g., dendritic cells) are combined with CTLs leading to proliferation of

the CTLs specific thereto. The proliferated CTLs are then administered to a subject with a cellular abnormality which is characterized by certain of the abnormal cells presenting the particular complex. The CTLs then lyse the abnormal cells, thereby achieving the desired therapeutic goal.

Many diverse extracellular stimuli -including growth factors, hormones, osmolar shock, stress, and elevated temperature- result in activation of phosphorylation cascades utilizing MAPKs (e.g., ERK1/2). MAPKs comprise a family of related protein kinases that are themselves activated by MAPK-activating enzymes (MAPK/ERK kinases or MEKs) by phosphorylation on their threonine and tyrosine residues. Once activated they can regulate targets in the cytosol and can also translocate to the nucleus where they can phosphorylate a variety of transcription factors regulating gene expression of important cell-cycle and differentiation-specific proteins.

The MAPK-activating enzymes (MAPK/ERK kinases or MEKs) are unusual in their ability to catalyze phosphorylation on both threonine and tyrosine residues. MEKs are themselves activated by phosphorylation of their serine residues by upstream kinases (e.g., c-Raf).

We have discovered, unexpectedly, that modulators of MEK1/2 and ERK1/2 activity, and in particular down-modulators of MEK1/2 and ERK1/2 activity, can up-regulate TAA expression/presentation, thus enhancing recognition/lysis of tumors expressing tumor-associated antigens by tumor-infiltrating lymphocytes (TILs) .

The terms “down-modulation” and “up-modulation” are used interchangeably with the terms “down-regulation” and “up-regulation,” respectively, throughout this application.

“Down-modulating (down-regulating),” as used herein, refers to inhibition of tumor-antigen (or TAA) expression. Inhibition of tumor-antigen expression refers to inhibiting (i.e., reducing to a detectable extent) expression/presentation of the specific antigen intracellularly and/or at the surface of a tumor cell. Such inhibition of tumor-antigen expression can be directly determined by detecting a decrease in the level of mRNA for the gene encoding the antigen, or the level of peptide expression of the tumor-antigen, using any suitable means known to the art, such as nucleic acid hybridization or, preferably, antibody detection methods, respectively (see Examples). Inhibition of tumor-antigen expression can also be determined indirectly, for example, by detecting a change in tumor-cell lysis ability by TILs that specifically recognize the tumor-antigen.

Conversely, "up-modulating (up-regulating)," as used herein, refers to an increase in tumor-antigen (or TAA) expression. Upregulation of tumor-antigen expression refers to increasing (i.e., increasing to a detectable extent) expression/presentation of the specific antigen intracellularly and/or at the surface of a tumor cell. Methods for detecting such increases in tumor-antigen expression are known in the art and are described elsewhere herein (see, e.g., preceding paragraph on "down-modulators")

The present invention relates in one aspect to down-modulators (or inhibitors) of MEK1/2 and/or ERK1/2 activity.

MEK1/2 activity inhibitors are known to those of ordinary skill in the art and include agents that can inhibit the phosphorylation of both threonine and tyrosine residues on MEK1/2 substrates such as ERK1/2 or ATP. In important embodiments, the MEK1/2 inhibitor is selected from the group consisting of small molecule organic compounds, inhibitory antibodies, synthetic kinase substrate peptides, dominant negative MEK1 proteins, antisense nucleic acids, and ribozymes which reduce the expression of translatable MEK1/2 transcripts. Preferably the MEK1/2 inhibitor is a small molecule organic compound, particularly a tricyclic flavone or a (phenylthio)butadiene. In certain preferred embodiments, the MEK1 inhibitor is 2-(2-amino-3-methoxyphenyl)-4-oxo-4H-[1]benzopyran (PD98059), 1,4-diamino-2,3-dicyano-1,4-bis-(phenylthio)butadiene (U0125) or 1,4-diamino-2,3-dicyano- 1,4-bis-(2-aminophenylthio)butadiene (U0126). In certain embodiments, MEK1/2 inhibitors include a compound selected from the group consisting of PP1/PP2A, a b-Raf inhibitor, or a c-Raf inhibitor. In certain embodiments, the b-Raf inhibitor is a PK-A inhibitor.

Variants of the foregoing (phenylthio)butadiene compounds which retain the MEK1/2 inhibitory activity of the (phenylthio)butadiene compounds also can be used in accordance with the invention. Substitutions preferably are made for the phenyl rings, or for one or both of the $NH_2$ group on the phenyl rings of U0126, above. Chemical groups which can be added to one or both ends of the molecule include: hydrido, alkyl, alkenyl, alkynyl, cycloalkyl, heterocycyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, acyl, amino, acyloxy, acylamino, carboalkoxy, carboxyamido, carboxyamido, halo and thio groups. Substitutions also can be made for the terminal groups on the butadiene portion of the molecule (i.e., CN, $NH_2$)

Molecular terms, when used in this application, have their common meaning unless otherwise specified. The term "hydrido" denotes a single hydrogen atom (H). The term "acyl" is defined as a carbonyl radical attached to an alkyl, alkenyl, alkynyl, cycloalkyl, heterocycyl, aryl or heteroaryl group, examples of such radicals being acetyl and benzoyl. The term "amino" denotes a nitrogen radical containing two substituents independently selected from the group consisting of hydrido, alkyl, cycloalkyl, heterocyclyl, aryl, and heteroaryl. The term "acyloxy" denotes an oxygen radical adjacent to an acyl group. The term "acylamino" denotes a nitrogen radical adjacent to an acyl group. The term "carboalkoxy" is defined as a carbonyl radical adjacent to an alkoxy or aryloxy group. The term "carboxyamido" denotes a carbonyl radical adjacent to an amino group. The term "carboxy" embraces a carbonyl radical adjacent to an alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl or heteroaryl group. The term "halo" is defined as a bromo, chloro, fluoro or iodo radical. The term "thio" denotes a radical containing a substituent group independently selected from hydrido, alkyl, cycloalkyl, heterocyclyl, aryl and heteroaryl, attached to a divalent sulfur atom, such as, methylthio and phenylthio.

The term "alkyl" is defined as a linear or branched, saturated radical having one to about ten carbon atoms unless otherwise specified. Preferred alkyl radicals are "lower alkyl" radicals having one to about five carbon atoms. One or more hydrogen atoms can also be replaced by a substitutent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of alkyl groups include methyl, tert-butyl, isopropyl, and methoxymethyl. The term "alkenyl" embraces linear or branched radicals having two to about twenty carbon atoms, preferably three to about ten carbon atoms, and containing at least one carbon-carbon double bond. One or more hydrogen atoms can also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of alkenyl groups include ethylenyl or phenyl ethylenyl. The term "alkynyl" denotes linear or branched radicals having from two to about ten carbon atoms, and containing at least one carbon-carbon triple bond. One or more hydrogen atoms can also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy,

carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of alkynyl groups include propynyl. The term "aryl" denotes aromatic radicals in a single or fused carbocyclic ring system, having from five to twelve ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of aryl groups include phenyl, naphthyl, biphenyl, terphenyl. "Heteroaryl" embraces aromatic radicals which contain one to four hetero atoms selected from oxygen, nitrogen and sulfur in a single or fused heterocyclic ring system, having from five to fifteen ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of heteroaryl groups include, pyridinyl, thiazolyl, thiadiazoyl, isoquinolinyl, pyrazolyl, oxazolyl, oxadiazoyl, triazolyl, and pyrrolyl groups.

The term "cycloalkyl" is defined as a saturated or partially unsaturated carbocyclic ring in a single or fused carbocyclic ring system having from three to twelve ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of a cycloalkyl group include cyclopropyl, cyclobutyl, cyclohexyl, and cycloheptyl. The term "heterocyclyl" embraces a saturated or partially unsaturated ring containing zero to four hetero atoms selected from oxygen, nitrogen and sulfur in a single or fused heterocyclic ring system having from three to twelve ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of a heterocyclyl group include morpholinyl, piperidinyl, and pyrrolidinyl. The term "alkoxy" denotes oxy-containing radicals substituted with an alkyl, cycloalkyl or heterocyclyl group. Examples include methoxy, tert-butoxy, benzyloxy and cyclohexyloxy. The term

"aryloxy" denotes oxy-containing radicals substituted with an aryl or heteroaryl group. Examples include phenoxy. The term "sulfoxy" is defined as a hexavalent sulfur radical bound to two or three substituents selected from the group consisting of oxo, alkyl, cycloalkyl, heterocyclyl, aryl and heteroaryl, wherein at least one of said substituents is oxo.

As mentioned above, the invention embraces the use of antisense oligonucleotides that selectively bind to a nucleic acid molecule encoding a MEK1/2 polypeptide, to decrease MEK1/2 transcription or translation. Antisense molecules, in this manner, can be used to decrease or prevent the effects mediated by MEK1/2.

As used herein, the term "antisense oligonucleotide" or "antisense" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of that gene and, thereby, inhibits the transcription of that gene and/or the translation of that mRNA. The antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene or transcript.

Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions. Based upon the MEK1 cDNA sequence , for example (GenBank accession numbers L02526 (mouse) and L11284 (human)), or upon allelic or homologous genomic and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 10 and, more preferably, at least 15 consecutive bases which are complementary to the target, although in certain cases modified oligonucleotides as short as 7 bases in length have been used successfully as antisense oligonucleotides (Wagner et al., *Nature Biotechnol.* 14:840-844,

1996). Most preferably, the antisense oligonucleotides comprise a complementary sequence of 20-30 bases.

Although oligonucleotides may be chosen which are antisense to any region of the gene or mRNA transcripts, in preferred embodiments the antisense oligonucleotides correspond to N-terminal or 5' upstream sites such as translation initiation, transcription initiation or promoter sites. In addition, 3'-untranslated regions may be targeted. Targeting to mRNA splicing sites has also been used in the art but may be less preferred if alternative mRNA splicing occurs. In addition, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al., *Cell Mol. Neurobiol.* 14(5):439-457, 1994) and at which proteins are not expected to bind. Finally, one of ordinary skill in the art may easily derive the genomic DNAs corresponding to the MEK1/2 cDNAs and thus the present invention also provides for the use of antisense oligonucleotides which are complementary to MEK1/2 genomic DNAs. Similarly, the use of antisense to MEK1/2 cDNAs and genomic DNAs of other species are enabled without undue experimentation.

One implementation of anti-sense inhibitory RNAs is the use of short inhibitory (or small interfering) RNAs (siRNA), sometimes referred to as RNA interference, or RNAi, and "hairpin" or "duplex" RNAs that can bind to specific sequences in mRNAs and result in sequence-specific, post-translational gene silencing. siRNAs are the mediators of mRNA degradation in the process of RNA interference (RNAi). siRNAs are 18-23 nucleotide duplexes. Most frequently, siRNAs are 21-23 nucleotide duplexes.

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. The mediators of sequence-specific messenger RNA degradation are preferably 21- and 22-nucleotide small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from longer dsRNAs (Elbashir SM et al. Nature 2001, May 24, 411, 428-429)

Chemically synthesized siRNA duplexes with overhanging 3' ends mediate efficient target RNA cleavage in the lysate, and the cleavage site is located near the center of the region spanned by the guiding siRNA. The direction of dsRNA processing determines whether sense or antisense target RNA can be cleaved by the siRNA-protein complex. (Elbashir SM et al. Genes Dev 2001 Jan 15;15(2):188-200).

Double-stranded siRNAs also work in concert with single stranded anti-sense RNAs (Martinez et al. Cell 2002, Sep 6; 110(5): 563-574).

In one set of embodiments, the antisense oligonucleotides of the invention may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end of one native nucleotide and the 3' end of another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleotide linkage. These oligonucleotides may be prepared by art recognized methods which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by vectors.

In preferred embodiments, however, the antisense oligonucleotides of the invention also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters and peptides.

The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. Modified oligonucleotides also can include base analogs such as C-5 propyne modified bases (Wagner et al., *Nature Biotechnology* 14:840-844, 1996). The present invention, thus, contemplates the use of pharmaceutical preparations

containing modified antisense molecules that are complementary to and hybridizable with, under physiological conditions, nucleic acids encoding MEK1/2 polypeptides, together with pharmaceutically acceptable carriers.

The invention also provides, in certain embodiments, the use of "dominant negative" MEK1/2 polypeptides. A dominant negative polypeptide is an inactive variant of a protein, which, by interacting with the cellular machinery, displaces an active protein from its interaction with the cellular machinery or competes with the active protein, thereby reducing the effect of the active protein. For example, dominant negative MEK 1 proteins include MEK1/2 proteins having a catalytically-inactive kinase domain which interacts normally with target proteins but does not phosphorylate the target proteins, or which does not interact with normally with target proteins, or both. Dominant negative MEK1/2 proteins include variants in which a portion of the kinase domain has been mutated or deleted to reduce or eliminate substrate binding or kinase activity.

The end result of the expression of a dominant negative MEK1/2 polypeptide in a cell is a reduction in function of active proteins. One of ordinary skill in the art can assess the potential for a dominant negative variant of a protein, and using standard mutagenesis techniques to create one or more dominant negative variant polypeptides. For example, given the nucleotide sequence of MEK1/2, one of ordinary skill in the art can modify the sequence of the MEK1/2 polypeptide by site-specific mutagenesis, scanning mutagenesis, partial gene deletion or truncation, and the like. See, e.g., U.S. Pat. No. 5,580,723 and Sambrook et al., Molecular Cloning. A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989. The skilled artisan then can test the population of mutagenized polypeptides for diminution in a selected activity (e.g., MEK1/2 kinase activity). Other similar methods for creating and testing dominant negative variants of a protein will be apparent to one of ordinary skill in the art.

The invention also embraces MEK1/2 binding agents which can be antibodies or fragments of antibodies having the ability to selectively bind to MEK1/2 polypeptides. Such agents can be used to inhibit the native activity of the MEK1/2 polypeptides by binding to such polypeptides. Antibodies include polyclonal and monoclonal antibodies, prepared according to conventional methodology.

Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in

general, Clark, W. R. (1986) *The Experimental Foundations of Modern Immunology* Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology*, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an $F(ab')_2$ fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

It is now well-established in the art that the non-CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. See, e.g., U.S. Pat. Nos. 4,816,567, 5,225,539, 5,585,089, 5,693,762 and 5,859,205.

Thus, for example, PCT International Publication Number WO 92/04381 teaches the production and use of humanized murine RSV antibodies in which at least a portion of the murine FR regions have been replaced by FR regions of human origin. Such

antibodies, including fragments of intact antibodies with antigen-binding ability, are often referred to as "chimeric" antibodies.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for $F(ab')_2$, Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric $F(ab')_2$ fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. The present invention also includes so-called single chain antibodies.

Thus, the invention involves polypeptides of numerous size and type that bind specifically to MEK1/2 polypeptides, and complexes of both MEK1/2 polypeptides and their binding partners. These polypeptides may be derived also from sources other than antibody technology. For example, such polypeptide binding agents can be provided by degenerate peptide libraries which can be readily prepared in solution, in immobilized form or as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptoids and non-peptide synthetic moieties.

Phage display can be particularly effective in identifying binding peptides useful according to the invention, including human antibodies. Briefly, one prepares a phage library (using e.g. m13, fd, or lambda phage), displaying inserts from 4 to about 80 amino acid residues using conventional procedures. The inserts may represent, for example, a completely degenerate or biased array. One then can select phage-bearing inserts which bind to the MEK1/2 polypeptide. This process can be repeated through several cycles of reselection of phage that bind to the MEK1/2 polypeptide. Repeated rounds lead to enrichment of phage bearing particular sequences. DNA sequence analysis can be conducted to identify the sequences of the expressed polypeptides. The minimal linear portion of the sequence that binds to the MEK1/2 polypeptide can be determined. One can repeat the procedure using a biased library containing inserts containing part or all of the

minimal linear portion plus one or more additional degenerate residues upstream or downstream thereof. Yeast two-hybrid screening methods also may be used to identify polypeptides that bind to the MEK1/2 polypeptides. Thus MEK1/2 polypeptides, or fragments thereof, can be used to screen peptide libraries, including phage display libraries, to identify and select peptide binding partners of MEK1/2. Additional methodologies are described by Zhang et al., Nature Biotechnol. 18:71-74, 2000. Such molecules can be used, as described, for interfering directly with the functioning of MEK1/2 polypeptides and for other purposes that will be apparent to those of ordinary skill in the art.

Several tests can be used to identify compounds which are specific inhibitors of MEK1/2 activity. For example U.S. Pat. No. 5,525,625, the disclosure of which is incorporated herein by reference, describes several assays which are useful for determining the MEK1/2 inhibitory potential of a test compound. The assays include in vitro kinase assays, whole cell kinase assays, and cell growth assays including assays of monolayer growth and growth in soft agar. The Examples below provide an in vivo assay of focal cerebral ischemia and in vitro cell-based assays for testing the activity of MEK1/2 inhibitors. Additional assays are described by Favata et al. (J. Biol. Chem. 273:18623-18632, 1998), the disclosure of which is incorporated by reference. If the test compound is able to inhibit the MEK 1 activity, then it is a compound which is useful in the treatment of ischemia, particularly stroke, and other conditions including hypoxia and glutamate toxicity. The test compound can be determined readily to be a specific inhibitor of MEK1/2 activity.

ERK1/2 are serine/threonine kinases that are activated in the cytosol in response to specific extracellular signals and can be translocated to the nucleus. They are known to phosphorylate many different proteins including transcription factors that regulate expression of important cell-cycle and differentiation-specific proteins.

ERK1/2 activity inhibitors are known to those of ordinary skill in the art and include agents that can inhibit the phosphorylation of a number of proteins, for example, the actin-associated protein caldesmon (CaD) (D'Angelo G, et al., *Am J Physiol Heart Circ Physiol*, 2002, Feb;282:H602-10), or inhibit cell functions such as the attenuation or enhancement of FSH-induced progesterone or estradiol production in granulosa cells (Moore RK, et al., *Biochem Biophys Res Commun.*, 2001, Dec 14; 289:796-800), or the

FGF-induced lens cell proliferation and fibre differentiation (Lovicu FJ, et al., *Development*, 2001, Dec; 128:5075-84). In important embodiments, the ERK1/2 inhibitor is MKP-1, MKP-3, or a MEK1/2 inhibitor.

MEK1/2 and ERK1/2 activities are well established in the art, and as a result, techniques for measuring such activities are also well established and known to those of ordinary skill in the art (see, e.g., Favata MF, et al., *J Biol Chem*., 1998, Jul 17;273:18623-32; Dudley DT, et al., Proc Natl Acad Sci U S A, 1995, Aug 15;92:7686-9).

A tumor cell, according to the invention, may be of a cancer or tumor type thought to escape immune recognition. Such cancers or tumors may be of the folowing origin: biliary tract cancer; brain cancer, including glioblastomas and medulloblastomas; breast cancer; cervical carcinoma; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; hematological neoplasms, including acute lymphocytic and myelogenous leukemia; multiple myeloma; AIDS associated leukemias and adult T-cell leukemia lymphoma; intraepithelial neoplasms, including Bowen's disease and Paget's disease; liver cancer; lung cancer; lymphomas, including Hodgkin's disease and lymphocytic lymphomas; neuroblastomas; oral cancer, including squamous cell carcinoma; ovarian cancer, including those arising from epithelial cells, stromal cells, germ cells and mesenchymal cells; pancreas cancer; prostate cancer; rectal cancer; sarcomas, including leiomyosarcoma, rhabdomyosarcoma, liposarcoma, fibrosarcoma and osteosarcoma; skin cancer, including melanoma, Kaposi's sarcoma, basocellular cancer and squamous cell cancer; testicular cancer, including germinal tumors (seminoma, non-seminoma[teratomas, choriocarcinomas]), stromal tumors and germ cell tumors; thyroid cancer, including thyroid adenocarcinoma and medullar carcinoma; and renal cancer including adenocarcinoma and Wilms tumor. In certain embodiments, the tumor-antigen can be Melan-A/MART-1, melanoma GP75, PGP 9.5, Annexin I and II, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, Colorectal associated antigen (CRC)--C017-1A/GA733, Ab2 BR3E4, CI17-1A/GA733, HSPPC-96, Hsp96, gp96-associated cellular peptides, G250, Herpes simplex thymidine kinase (HSVtk), Carcinoembryonic Antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, Prostate Specific Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, prostate-specific membrane antigen (PSMA), Ad5-PSA, Parathyroid-hormone-related protein (PTH-rP), Prostatic Acid Phosphatase (PAP),

Prostate epithelium-derived Ets transcription factor (PDEF), Oncofetal antigen-immature laminin receptor (OFA-iLR), HP59, Cytochrome oxidase 1, sp100, Ran GTPase activating protein, members of Rab-GAP (Rab GTPase-activating proteins) e.g., PARIS-1, T-cell receptor/CD3-zeta chain, cTAGE-1 and SCP-1, Glycolipid antigens-GM2, GD2, GD3, FucosylGM1, Glycoprotein (mucin) antigens-Tn, sialyl Tn., TF and MUC1, Hsp105, MAGE-family of tumor antigens, GAGE-1,2, BAGE, RAGE, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, ErbB-2/neu, p21ras, RCAS1, α-fetoprotein, E-cadherin, α-catenin, β-catenin and γ-catenin, p120ctn, $gp100^{Pmel117}$, PRAME, GA733/EoCam, thyroglobulin, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), Prp1p/Zer1p, L19H1, MAZ, PINCH, PRAME, Wt-1, PHF3, LAGE-1, SCP-1, SSX-1, SSX-2, SSX-4, fodrin, Smad family of tumor antigens, lmp-1, EBV-encoded nuclear antigen (EBNA)-1, or c-erbB-2. In any of the foregoing embodiments the fraction from the plurality of fractions can be undiluted or concentrated.

In important embodiments, cancers or tumors escaping immune recognition and tumor-antigens associated with such tumors (but not exclusively), include acute lymphoblastic leukemia (etv6; aml1; cyclophilin b), glioma (E-cadherin; α-catenin; β-catenin; γ-catenin; p120ctn; PHF3), bladder cancer (p21ras), billiary cancer (p21ras), breast cancer (MUC family; HER2/neu; ErbB-2/neu; c-erbB-2, PDEF; Cytochrome oxidase 1; sp100; Ran GTPase activating protein; NY-ESO-1; LAGE-1; SCP-1; SSX-1; SSX-2; SSX-4), cervical carcinoma (p53; p21ras), colon carcinoma (p21ras; HER2/neu; c-erbB-2; MUC family; NY-ESO-1; LAGE-1; SCP-1; SSX-1; SSX-2; SSX-4), colorectal cancer (Hsp105, Colorectal associated antigen (CRC)--C017-1A/GA733; APC; Ab2; BR3E4), choriocarcinoma (CEA), epithelial cell-cancer (cyclophilin b), gastric cancer (HER2/neu; c-erbB-2; ga733 glycoprotein), hepatocellular cancer (α-fetoprotein), hodgkins lymphoma (lmp-1; EBNA-1), lung cancer (CEA; MAGE-3; NY-ESO-1), lymphoid cell-derived leukemia (cyclophilin b), myeloma (MUC family; p21ras; Prp1p/Zer1p; L19H1; MAZ; PINCH; PRAME; Wt-1), non-small cell lung carcinoma (HER2/neu; c-erbB-2), nasopharyngeal cancer (lmp-1; EBNA-1), ovarian cancer cancer (MUC family; HER2/neu; c-erbB-2), prostate cancer (Prostate Specific Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3; PSMA; HER2/neu; c-erbB-2, members of Rab-GAP (Rab GTPase-activating proteins), e.g., PARIS-1, Ad5-PSA, PTH-rP, PAP), pancreatic cancer (Hsp105, p21ras; MUC family; HER2/neu; c-erbB-2; ga733

glycoprotein), renal (HER2/neu; c-erbB-2; OFA-iLR; G250), testicular cancer (NY-ESO-1), T cell leukemia (HTLV-1 epitopes, cTAGE-1, SCP-1), and melanoma (Melan-A/MART-1; cdc27; MAGE-3; p21ras; $gp100^{Pmel117}$). Given the teachings of the present invention, a person of ordinary skill in the art can easily identify such *tumor-antigens* and apply the methods of the invention to identify agents which modulate expression of such antigens.

As used herein, a subject is a human, non-human primate, cow, horse, pig, sheep, goat, dog, cat or rodent. In all embodiments, human subjects are preferred.

When used therapeutically, the down-modulation inhibitors of the invention are administered in therapeutically effective amounts. In general, a therapeutically effective amount means that amount necessary to delay the onset of, inhibit the progression of, or halt altogether the particular condition being treated. Generally, a therapeutically effective amount will vary with the subject's age, condition, and sex, as well as the nature and extent of the disease in the subject, all of which can be determined by one of ordinary skill in the art. The dosage may be adjusted by the individual physician or veterinarian, particularly in the event of any complication. A therapeutically effective amount typically varies from 0.01 mg/kg to about 1000 mg/kg, preferably from about 0.1 mg/kg to about 200 mg/kg, and most preferably from about 0.2 mg//kg to about 20 mg/kg, in one or more dose administrations daily, for one or more days.

The therapeutically effective amount of the tumor-antigen expression down-modulation inhibitors of the invention is that amount effective to inhibit tumor-antigen expression down-modulation, and can be determined using, for example, standard tests known in the art. For example, a direct way to measure tumor-antigen (e.g., Melan-A/MART-1) expression the tumor cell (e.g., melanoma) is to use antibodies specific for the *tumor-antigen* and a number of immunocyto- and immunohisto- chemical protocols well known in the art. Antibodies specific for the Melan-A/MART-1 antigen, for example, are fully described in U.S. Patent 5,674,749 to Chen *et al.*, entitled: "Monoclonal antibodies which bind to tumor rejection antigen precursor Melan-A, and uses thereof."

Additionally, the tumor-antigen expression down-modulation inhibitors of the invention (i.e., modulators of the MAPK/ERK signaling cascade, and more specifically down-modulators of MEK1/2 and ERK1/2 activity), may be co-administered with an anti-cancer agent other than an agent of the invention (e.g., other than a MEK1/2 and/or an

ERK1/2 inhibitor), that can act cooperatively, additively or synergistically with an agent of the invention, for treating or preventing cancers that express (and, it is believed, through autocrine secretions down-modulate) tumor-antigens. The term "co-administered," means administered substantially simultaneously with another agent (e.g., in different or same compositions/formulations). By substantially simultaneously, it is meant that a MEK1/2 and/or an ERK1/2 inhibitor of the invention is administered to the subject close enough in time with the administration of the other agent (e.g., an anti-cancer agent) whereby the two agents may exert an additive or even synergistic effect to upregulate tumor-antigen expression and inhibit growth and/or proliferation of the cancer.

Anti-cancer agents other than agents of the invention include, but are not limited to: Acivicin; Aclarubicin; Acodazole Hydrochloride; Acronine; Adozelesin; Aldesleukin; Altretamine; Ambomycin; Ametantrone Acetate; Aminoglutethimide; Amsacrine; Anastrozole; Anthramycin; Asparaginase; Asperlin; Azacitidine; Azetepa; Azotomycin; Batimastat; Benzodepa; Bicalutamide; Bisantrene Hydrochloride; Bisnafide Dimesylate; Bizelesin; Bleomycin Sulfate; Brequinar Sodium; Bropirimine; Busulfan; Cactinomycin; Calusterone; Caracemide; Carbetimer; Carboplatin; Carmustine; Carubicin Hydrochloride; Carzelesin; Cedefingol; Chlorambucil; Cirolemycin; Cisplatin; Cladribine; Crisnatol Mesylate; Cyclophosphamide; Cytarabine; Dacarbazine; Dactinomycin; Daunorubicin Hydrochloride; Decitabine; Dexormaplatin; Dezaguanine; Dezaguanine Mesylate; Diaziquone; Docetaxel; Doxorubicin; Doxorubicin Hydrochloride; Droloxifene; Droloxifene Citrate; Dromostanolone Propionate; Duazomycin; Edatrexate; Eflornithine Hydrochloride; Elsamitrucin; Enloplatin; Enpromate; Epipropidine; Epirubicin Hydrochloride; Erbulozole; Esorubicin Hydrochloride; Estramustine; Estramustine Phosphate Sodium; Etanidazole; Etoposide; Etoposide Phosphate; Etoprine; Fadrozole Hydrochloride; Fazarabine; Fenretinide; Floxuridine; Fludarabine Phosphate; Fluorouracil; Flurocitabine; Fosquidone; Fostriecin Sodium; Gemcitabine; Gemcitabine Hydrochloride; Hydroxyurea; Idarubicin Hydrochloride; Ifosfamide; Ilmofosine; Interferon Alfa-2a; Interferon Alfa-2b; Interferon Alfa-n1; Interferon Alfa-n3; Interferon Beta-I a; Interferon Gamma-I b; Iproplatin; Irinotecan Hydrochloride; Lanreotide Acetate; Letrozole; Leuprolide Acetate; Liarozole Hydrochloride; Lometrexol Sodium; Lomustine; Losoxantrone Hydrochloride; Masoprocol; Maytansine; Mechlorethamine Hydrochloride; Megestrol Acetate; Melengestrol Acetate; Melphalan; Menogaril; Mercaptopurine;

Methotrexate; Methotrexate Sodium; Metoprine; Meturedepa; Mitindomide; Mitocarcin; Mitocromin; Mitogillin; Mitomalcin; Mitomycin; Mitosper; Mitotane; Mitoxantrone Hydrochloride; Mycophenolic Acid; Nocodazole; Nogalamycin; Ormaplatin; Oxisuran; Paclitaxel; Pegaspargase; Peliomycin; Pentamustine; Peplomycin Sulfate; Perfosfamide; Pipobroman; Piposulfan; Piroxantrone Hydrochloride; Plicamycin; Plomestane; Podofilox; Porfimer Sodium; Porfiromycin; Prednimustine; Procarbazine Hydrochloride; Puromycin; Puromycin Hydrochloride; Pyrazofurin; Riboprine; Rogletimide; Safingol; Safingol Hydrochloride; Semustine; Simtrazene; Sparfosate Sodium; Sparsomycin; Spirogermanium Hydrochloride; Spiromustine; Spiroplatin; Streptonigrin; Streptozocin; Sulofenur; Talisomycin; Taxotere; Tecogalan Sodium; Tegafur; Teloxantrone Hydrochloride; Temoporfin; Teniposide; Teroxirone; Testolactone; Thiamiprine; Thioguanine; Thiotepa; Tiazofurin; Tirapazamine; Topotecan Hydrochloride; Toremifene Citrate; Trestolone Acetate; Triciribine Phosphate; Trimetrexate; Trimetrexate Glucuronate; Triptorelin; Tubulozole Hydrochloride; Uracil Mustard; Uredepa; Vapreotide; Verteporfin; Vinblastine Sulfate; Vincristine Sulfate; Vindesine; Vindesine Sulfate; Vinepidine Sulfate; Vinglycinate Sulfate; Vinleurosine Sulfate; Vinorelbine Tartrate; Vinrosidine Sulfate; Vinzolidine Sulfate; Vorozole; Zeniplatin; Zinostatin; Zorubicin Hydrochloride.

The above-described drug therapies are well known to those of ordinary skill in the art and are administered by modes known to those of skill in the art. The drug therapies are administered in amounts which are effective to achieve the physiological goals in combination with the MEK1/2 and/or an ERK1/2 inhibitors of the invention.

A MEK1/2 and/or an ERK1/2 inhibitor may be administered alone or in combination with the above-described drug therapies as part of a pharmaceutical composition. Such a pharmaceutical composition may include the MEK1/2 and/or an ERK1/2 inhibitor in combination with any standard physiologically and/or pharmaceutically acceptable carriers which are known in the art. The compositions can be sterile and contain a therapeutically effective amount of the MEK1/2 and/or an ERK1/2 inhibitor in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration into a human or other animal. The term "carrier" denotes an organic or inorganic

ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy. Pharmaceutically acceptable further means a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular drug selected, the severity of the condition being treated, and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, intradermal, or parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intramuscular, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis. They could, however, be preferred in emergency situations. Oral administration will be preferred for prophylactic treatment because of the convenience to the patient as well as the dosing schedule.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the MEK1/2 and/or an ERK1/2 inhibitor, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of

injectables. Carrier formulations suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the MEK1/2 and/or an ERK1/2 inhibitor into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the MEK1/2 and/or an ERK1/2 inhibitor into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the MEK1/2 and/or an ERK1/2 inhibitor. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the MEK1/2 and/or ERK1/2 inhibitors described above, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include the above-described polymeric systems, as well as polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono- di- and tri-glycerides; hydrogel release systems; sylastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which the MEK1/2 and/or ERK1/2 inhibitor is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,675,189, and 5,736,152, and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,854,480, 5,133,974

and 5,407,686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

Use of a long-term sustained release implant may be particularly suitable for treatment of chronic conditions. Long-term release, are used herein, means that the implant is constructed and arranged to delivery therapeutic levels of the active ingredient for at least 30 days, and preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

The invention also embraces methods for identifying genes that modulate tumor-antigen expression in a tumor cell. A method according to this aspect of the invention typically involves: (a) contacting a tumor cell known to express a tumor-antigen with a modulator of the MAPK/ERK signaling cascade, (b) measuring tumor-antigen expression on the tumor cell, (c) determining whether tumor-antigen expression on the tumor cell is modulated compared to a control, and (d) identifying genes that modulate expression of tumor-antigen expression in the tumor cell. The genes are identified using differential expression technology and are then isolated and characterized using conventional methodology.

A tumor cell known to express a tumor-antigen includes: acute lymphoblastic leukemia (etv6; aml1; cyclophilin b), glioma (E-cadherin; α-catenin; β-catenin; γ-catenin; p120ctn; PHF3), bladder cancer (p21ras), billiary cancer (p21ras), breast cancer (MUC family; HER2/neu; ErbB-2/neu; c-erbB-2, PDEF; Cytochrome oxidase 1; sp100; Ran GTPase activating protein; NY-ESO-1; LAGE-1; SCP-1; SSX-1; SSX-2; SSX-4), cervical carcinoma (p53; p21ras), colon carcinoma (p21ras; HER2/neu; c-erbB-2; MUC family; NY-ESO-1; LAGE-1; SCP-1; SSX-1; SSX-2; SSX-4), colorectal cancer (Hsp105, Colorectal associated antigen (CRC)--C017-1A/GA733; APC; Ab2; BR3E4), choriocarcinoma (CEA), epithelial cell-cancer (cyclophilin b), gastric cancer (HER2/neu; c-erbB-2; ga733 glycoprotein), hepatocellular cancer (α-fetoprotein), hodgkins lymphoma (lmp-1; EBNA-1), lung cancer (CEA; MAGE-3; NY-ESO-1), lymphoid cell-derived leukemia (cyclophilin b), myeloma (MUC family; p21ras; Prp1p/Zer1p; L19H1; MAZ; PINCH; PRAME; Wt-1), non-small cell lung carcinoma (HER2/neu; c-erbB-2), nasopharyngeal cancer (lmp-1; EBNA-1), ovarian cancer cancer (MUC family; HER2/neu; c-erbB-2), prostate cancer (Prostate Specific Antigen (PSA) and its immunogenic epitopes

PSA-1, PSA-2, and PSA-3; PSMA; HER2/neu; c-erbB-2, members of Rab-GAP (Rab GTPase-activating proteins), e.g., PARIS-1, Ad5-PSA, PTH-rP, PAP), pancreatic cancer (Hsp105, p21ras; MUC family; HER2/neu; c-erbB-2; ga733 glycoprotein), renal (HER2/neu; c-erbB-2; OFA-iLR; G250), testicular cancer (NY-ESO-1), T cell leukemia (HTLV-1 epitopes, cTAGE-1, SCP-1), and melanoma (Melan-A/MART-1; cdc27; MAGE-3; p21ras; $gp100^{Pmel117}$).

Determining whether tumor-antigen expression on the tumor cell is down-regulated as a result of such contacting is facilitated by comparison to a control. Typical controls include identically isolated and cultured cells, with the exception, for example, that the supernatant medium in the control cultures is removed at regular intervals during the culture period (e.g. every 2-6 hours), being replaced with fresh culture medium. This media change effectively eliminates any down-regulatory effects a *control-tumor cell* isolate may exert on the *control-tumor cell* tumor-antigen expression. Another control would include identically isolated and cultured cells, with the exception, for example, of the addition of tumor-antigen expression modulators such as OSM and/or MEK1/2, ERK1/2 inhibitors.

Genes that are modulated based upon the up-modulation and/or down-modulation of tumor antigen expression according to the instant invention, can be identified using methods known in the art. For example, differential display is one of the techniques designed to identify genes that are differentially regulated by cells under various physiological or experimental conditions (for example, differentiation, carcinogenesis, pharmacologic treatment). This technique was introduced by Liang and Pardee and described in U.S. Pat. No. 5,262,311. Prior to Liang and Pardee's introduction of this technique, those interested in identifying differentially expressed genes were compelled to resort either to differential hybridization screening (Zimmerman, G. R., et al., Cell, Vol. 21, pp. 709-715 (1980)) or to subtractive hybridization screening (St. John, T. P. et al., Cell, Vol. 16, pp. 443-452 (1979)) of complementary deoxynucleic acid ("cDNA") libraries. Neither of these methods is entirely satisfactory; both are time consuming and labor intensive. Of the two, differential hybridization (also known as +/- screening) is particularly insensitive, being essentially confined to the detection of relatively large differences between high to moderate-abundance transcripts. Subtractive hybridization, while far more sensitive than +/- screening, is also technically far more demanding.

Furthermore, it is necessary to carry out two separate subtractive hybridization experiments in order to identify both up- and down-regulated gene expression.

Differential display offers an attractive alternative to differential and subtractive hybridization screening. Generally, Liang et al. describes a protocol which involves the reverse transcription of a messenger ribonucleic acid ('mRNA') population, in independent reactions, with each of twelve anchor primers ($T_{12}$ MN), where M can be G (guanine), A (adenine) or C (cystosine) and N can be G, A, C or T (thymidine). The resulting single-stranded cDNAs are then amplified by the polymerase chain reaction (hereinafter, 'PCR') using the same anchor primer used for reverse transcription together with an upstream or 5' decamer of arbitrary sequence. The PCR products, which are labeled by incorporation of tracer amounts of a radioactive nucleotide, are resolved for analysis by denaturating polyacrylamide gel electrophoresis (PAGE). This technique permits the visualization of both up- and down-regulated gene expression simultaneously in the same experiment. Liang et al. postulated that each two-primer combination could amplify only a limited subpopulation of cDNAs, and that the twelve anchor primers together with twenty arbitrary decamers (i.e., 240 PCR reactions) should result in the display of the 3' termini of all distinct mRNAs that are theoretically expressed in any given cell type (Liang, P. and Pardee, A. B., Science, Vol. 257, pp. 967-971 (1992)). Improved differential display techniques such as those described by Combates et al. in U.S. Pat. No. 6,045,998, can also be used according to the invention to identify genes that play a role in tumor-antigen expression. Tumor cells and tumor-antigens expressed by the tumor cells are as described above.

The invention also embraces solid-phase nucleic acid molecule arrays. The array consists essentially of a set of nucleic acid molecules, expression products thereof, or fragments (of either the nucleic acid or the polypeptide molecule) thereof. The set of nucleic acid molecules comprises nucleic acid molecules of a tumor cell known to express a tumor-antigen, said tumor cell previously contacted with a modulator of the MAPK/ERK signaling cascade. In important embodiments, the solid-phase nucleic acid molecule array, further comprises at least one control set of nucleic acid molecules. A preferred control set of nucleic acid molecules consists essentially of nucleic acid molecules, expression products thereof, or fragments thereof, fixed to a solid substrate, wherein the set of nucleic acid molecules comprises nucleic acid molecules of a tumor cell known to express a

tumor-antigen, said tumor cell not previously contacted with a modulator of the MAPK/ERK signaling cascade.

According to the invention, standard hybridization techniques of microarray technology are utilized to assess patterns of nucleic acid expression and identify nucleic acid expression. Microarray technology, which is also known by other names including: DNA chip technology, gene chip technology, and solid-phase nucleic acid array technology, is well known to those of ordinary skill in the art and is based on, but not limited to, obtaining an array of identified nucleic acid probes (e.g., sets of antigen-expressing tumor cell-line specific cDNA molecules) on a fixed substrate, labeling target molecules with reporter molecules (e.g., radioactive, chemiluminescent, or fluorescent tags such as fluorescein, Cye3-dUTP, or Cye5-dUTP), hybridizing target nucleic acids to the probes, and evaluating target-probe hybridization. A probe with a nucleic acid sequence that perfectly matches the target sequence will, in general, result in detection of a stronger reporter-molecule signal than will probes with less perfect matches. Many components and techniques utilized in nucleic acid microarray technology are presented in *The Chipping Forecast*, Nature Genetics, Vol.21, Jan 1999, the entire contents of which is incorporated by reference herein.

According to the present invention, microarray substrates may include but are not limited to glass, silica, aluminosilicates, borosilicates, metal oxides such as alumina and nickel oxide, various clays, nitrocellulose, or nylon. In all embodiments a glass substrate is preferred. According to the invention, probes are selected from the group of nucleic acids including, but not limited to: DNA, genomic DNA, cDNA, and oligonucleotides; and may be natural or synthetic. Oligonucleotide probes preferably are 20 to 25-mer oligonucleotides and DNA/cDNA probes preferably are 500 to 5000 bases in length, although other lengths may be used. Appropriate probe length may be determined by one of ordinary skill in the art by following art-known procedures. In one embodiment, preferred probes are a set of nucleic acid molecules (e.g., cDNAs, RNAs, etc.) that comprise nucleic acid molecules of a tumor cell known to express a tumor-antigen, said tumor cell previously contacted with a modulator of the MAPK/ERK signaling cascade. In important embodiments, modulators of the MAPK/ERK signaling cascade include modulators of MEK1/2 and ERK1/2 activity (e.g., MEK1/2 and ERK1/2 inhibitors).

Probes may be purified to remove contaminants using standard methods known to those of ordinary skill in the art such as gel filtration or precipitation.

In one embodiment, the microarray substrate may be coated with a compound to enhance synthesis of the probe on the substrate. Such compounds include, but are not limited to, oligoethylene glycols. In another embodiment, coupling agents or groups on the substrate can be used to covalently link the first nucleotide or olignucleotide to the substrate. These agents or groups may include, but are not limited to: amino, hydroxy, bromo, and carboxy groups. These reactive groups are preferably attached to the substrate through a hydrocarbyl radical such as an alkylene or phenylene divalent radical, one valence position occupied by the chain bonding and the remaining attached to the reactive groups. These hydrocarbyl groups may contain up to about ten carbon atoms, preferably up to about six carbon atoms. Alkylene radicals are usually preferred containing two to four carbon atoms in the principal chain. These and additional details of the process are disclosed, for example, in U.S. Patent 4,458,066, which is incorporated by reference in its entirety.

In one embodiment, probes are synthesized directly on the substrate in a predetermined grid pattern using methods such as light-directed chemical synthesis, photochemical deprotection, or delivery of nucleotide precursors to the substrate and subsequent probe production.

In another embodiment, the substrate may be coated with a compound to enhance binding of the probe to the substrate. Such compounds include, but are not limited to: polylysine, amino silanes, amino-reactive silanes (Chipping Forecast, 1999) or chromium (Gwynne and Page, 2000). In this embodiment, presynthesized probes are applied to the substrate in a precise, predetermined volume and grid pattern, utilizing a computer-controlled robot to apply probe to the substrate in a contact-printing manner or in a non-contact manner such as ink jet or piezo-electric delivery. Probes may be covalently linked to the substrate with methods that include, but are not limited to, UV-irradiation. In another embodiment probes are linked to the substrate with heat.

Targets are nucleic acids selected from the group, including but not limited to: DNA, genomic DNA, cDNA, RNA, mRNA and may be natural or synthetic. In all embodiments, nucleic acid molecules from subjects suspected of developing or having a cardiovascular condition, are preferred. In certain embodiments of the invention, one or

more control nucleic acid molecules are attached to the substrate. Preferably, control nucleic acid molecules allow determination of factors including but not limited to: nucleic acid quality and binding characteristics; reagent quality and effectiveness; hybridization success; and analysis thresholds and success. Control nucleic acids may include, but are not limited to, expression products of genes such as housekeeping genes or fragments thereof.

To select a set of nucleic acids that may modulate tumor antigen expression, the expression data generated by, for example, microarray analysis of gene expression, is preferably analyzed to determine which nucleic acids in differentially treated (e.g., with or without a MAPK/ERK signaling cascade modulator) tumor-antigen expressing cells, are significantly differentially expressed. The significance of gene expression can be determined using Permax computer software, although any standard statistical package that can discriminate significant differences is expression may be used. Permax performs permutation 2-sample t-tests on large arrays of data. For high dimensional vectors of observations, the Permax software computes t-statistics for each attribute, and assesses significance using the permutation distribution of the maximum and minimum overall attributes. The main use is to determine the attributes (genes) that are the most different between two groups (e.g., control untreated cell line and a MAPK/ERK signaling cascade modulator treated cell line, in the presence or absence of another agent, e.g., OSM), measuring "most different" using the value of the t-statistics, and their significance levels.

Expression of nucleic acid molecules can also be determined using protein measurement methods to determine expression of the set of nucleic acids by determining the expression of polypeptides encoded by the set of nucleic acids. Preferred methods of specifically and quantitatively measuring proteins include, but are not limited to: mass spectroscopy-based methods such as surface enhanced laser desorption ionization (SELDI; e.g., Ciphergen ProteinChip System), non-mass spectroscopy-based methods, and immunohistochemistry-based methods such as 2-dimensional gel electrophoresis.

SELDI methodology may, through procedures known to those of ordinary skill in the art, be used to vaporize microscopic amounts of tumor protein and to create a "fingerprint" of individual proteins, thereby allowing simultaneous measurement of the abundance of many proteins in a single sample. Preferably SELDI-based assays may be utilized to characterize cardiovascular conditions as well as stages of such conditions.

Such assays preferably include, but are not limited to the following examples. Gene products discovered by RNA microarrays may be selectively measured by specific (antibody mediated) capture to the SELDI protein disc (e.g., selective SELDI). Gene products discovered by protein screening (e.g., with 2-D gels), may be resolved by "total protein SELDI" optimized to visualize those particular nucleic acids of interest from among the set of nucleic acids.

The use of any of the foregoing microarray methods to determine expression of nucleic acids that may modulate tumor-antigen expression can be done with routine methods known to those of ordinary skill in the art.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention and are not to be construed to limit the scope of the invention.

## Examples

Experimental procedures
Materials and Methods

*Tumor and Tumor Infiltrating Lymphocytes (TIL)*

Melanomas and tumor infiltrating lymphocytes (TIL) from the melanoma tissues were obtained according to approved Massachusetts General Hospital guidelines, and propagated *in vitro* as previously described (Hishii M, et al., *Proc. Natl. Acad Sci USA*, 1997, **94:**1378-1383). Briefly, tumors were cultured in DMEM medium supplemented with 10% FBS, and TIL were propagated in RPMI 1640 supplemented with 5% Human Serum containing recombinant human IL-2 at 100 Units/ml (Cetus, Emoryville, CA). TIL clones were isolated by limiting dilution as previously described (Hishii M, et al., *id*). Briefly, TIL were cloned by limiting dilution using irradiated mononuclear feeder cells together with PHA as a polyclonal stimulus as previously described. Limiting dilution was performed on TIL which had been in culture for two weeks prior to cloning. A minimum of $5 \times 10^7$ cells were utilized for functional assays and PCR analyses.

Tumor cell lines MU, MO, MA and EW were obtained from cutaneous metastatic melanoma deposits and some (MU, MO and MA), were previously described (Pandolfi F, et al., *Clin. Exp. Immunol*, 1994, **95:**141-7; Pandolfi F, et al., *Cancer Res*, 1991, **51:**3164-3170). Melan-A/MART-1 negative variant MU-X were obtained by culture of MU tumor

cells at high density (>5x10$^5$ cells/ml) for several days prior to immunoselection with anti-Melan-A/MART-1 specific TIL. After 1 week of co-culture of tumor and lymphocytes in the presence of recombinant human IL-2, the tumor cells which propagate were collected and maintained in culture in the absence of T cells. The melanoma origin of the lines was confirmed using antibodies to melanoma-associated antigen S-100 and HMB-45 (Ordonez N, et al., *Am J Clin Pathol*, 1988, **90:**385-90). Both MU and MA tumor s expressed HLA-A2; MU tumor cells were also HLA-A1 positive. MO tumor was derived from a patient whose leukocytes expressed HLA-A2, while MO tumor cells did not express this class I MHC antigen. Tumor cells were cultured either in DMEM supplemented with 10% human serum (MU), or in RPMI 1640 supplemented with 5% human serum (MA and MO).

*Expression of Melan-A/MART-1 by tumor cells*

To evaluate the expression of Melan-A/MART-1 antigen in the cytoplasm of melanoma tumor cell lines, the cells were first fixed for 10min in 1% paraformaldehyde, followed by 5min in 0.1% saponin prior to addition of monoclonal antibody specific for Melan-A/MART-1, A-103, (Castelli C, et al., *J Exp Med*, 1995, **181:**363-8)(a kind gift of E. Stockert and L. Old, Ludwig Institute, New York, NY) for 45min at 22°C. Following two washes, the cells were stained for 30min with FITC-conjugated goat-anti-mouse Ig antibody (DAKO, Carpenteria, CA) prior to fixation in 1% paraformaldehyde and analysis by flow cytometry (FACScan, Becton-Dickinson, Mt. View, CA). Histograms of fluorescence staining were generated for comparison of anti-Melan-A/MART-1 staining of various cell populations. Mean channel fluorescence was calculated using the "Consort 30" software provided by the manufacturer.

*Generation of Conditioned Medium*

Conditioned medium from melanoma tumor lines were generated by culturing cells at a starting concentration of 5 x 10$^5$ cells/ml in DMEM medium supplemented with between 1 and 10% FBS. Supernatants were collected after 72 hours by centrifugation of the cell cultures and filtration of the medium through a 0.2 micron filter (Millipore, Bedford, MA). Conditioned medium containing 1% FBS was concentrated between 10 and 20 fold by collecting the retentate from a nominal 30kD YM membrane (Centriprep, Millipore, Bedford, MA).

*Cytotoxicity Assays*

TIL were assayed for the ability to lyse melanoma target cells in 4 hour $^{51}$Cr-release assays as previously described (Hishii M, et al., *Proc. Natl. Acad Sci USA*, 1997, **94:**1378-1383). The melanoma target cells with high constitutive expression of Melan-A/MART-1 were generated by low density culture (1-2 x $10^5$/ml) were compared with respect to their susceptibility to cytolysis with the same cells cultured for 3 to 6 days in the presence of conditioned medium from the Melan-A/MART-1 negative variant, MU-X, to derive target cells with low Melan-A/MART-1 expression. Low Melan-A/MART-1 expressing cells were further assayed after pulsing with Melan-A/MART-1 peptide 27-35, (AAGIGILTV, SEQ ID NO:1)( Boon T, et al., *Ann Rev Immunol*, 1994, **12:**337-65; Sensi M, et al., *Proc Natl Acad Sci USA*, 1995, **92:**5674-8; Mackensen A, et al., *Cancer Res*, 1993, **53:**3569-73; Peoples G, et al., *J Immunol*, 1993, **151:**5472-80), by culturing these target cells at 37$^{o}$ for 2 hours in 1 ml of medium containing 5μg of peptide prior to labeling with $^{51}$Cr for use in cytolytic assays to demonstrate renewed susceptibility to specific T cell recognition (essentially as previously described in Kurnick J, et al., *Clin Immunol Immunopathol*, 1986, **38:**367-380).

In further instances, bulk and cloned TIL progeny was also assayed against autologous tumor (MU), allogeneic melanomas, as well as NK (K562) and LAK (Daudi), and EBV-transformed B lymphocyte targets: EBV-3 (HLA-A1, B8, DR3), EBV-19 (HLA-A2, B18, DR5), using the foregoing $^{51}$Cr-release assay. Pulsing included the following melanocyte lineage-derived peptides: Tyrosinase (Rivoltini, L., et al., *J. Immunol,* 1995, **154**:2257-2265): MLLAVLYCL (SEQ ID NO:2) or YMNGTMSQV(SEQ ID NO:3), MAGE-3 (Gaugler B, et al., *J Exp Med*, 1994, **179:**921-30): EVDPIGHLY (SEQ ID NO:4). Clones were screened for cytotoxic activity at effector to target ratios of 50:1 and below.

**Effect of Tumor-Conditioned Medium on Melan-A/Mart-1 (MA/M1) Expression**

We previously demonstrated that expression of MA/M1 can be down-regulated by culture with supernatants from MA/M1-negative tumors such as EW and A375 (Ramirez-Montagut T, et al., *Clin Exp Immunol.*, 2000, 119(1):11-8; U.S. Provisional App. Ser. No.

60/165,806; and International App. No. PCT/US00/31511) (See also Figure 1). As we described, the Melanoma Antigen Silencing Activity (MASA) of these tumors includes Oncostatin-M (OSM), and at least one additional soluble factor, which we designate MASA2, that is present in EW supernatants following removal of OSM, and is present in A375 cells that do not produce OSM. Oncostatin M is the subject of United States patent 5,618,715 to Shoyab et al., which is expressly incorporated herein by reference. Anti-Oncostatin-M antibodies are known in the art, and include Mouse and Goat anti-human Oncostatin M from Research Diagnostics, Flanders, NJ.

Importantly, the loss of MA/M1 as shown in flow cytometry is associated with a marked diminution in the ability of T cells to lyse tumor cells which have been treated with MASA-containing supernatants or recombinant OSM. The loss of T cell-mediated lysis can be overcome by the addition of the MA/M1-derived peptide, AAGIGILTV, which restores the targets to cytolytic susceptibility. As will be developed further elsewhere herein, the loss of MA/M1 is generally accompanied by diminished gp100 and tyrosinase, as well as other melanocyte lineage proteins, indicating that there is a 'sea-change' in the tumor cells. However, the down-modulation of antigen expression appears to be somewhat selective as the HLA Class I antigen needed for presentation of the melanoma peptide is not down-modulated. Importantly, when the MASA-containing conditioned medium was removed from the MA/M1 expressing tumor cells, there was renewed expression of this antigen. These antigen positive cells are again lysed by MA/M1-specific cytotoxic T cells.

**Down Modulation of Additional Melanocyte Genes**

Of note is that tumors with low or absent MA/M1 are also relatively deficient in tyrosinase and gp100 as well, and 3 of 4 low-MA/M1 melanomas have low Mitf-M, including the MU-X line derived from MA/M1+ MU cells. The sox10 regulator of Mitf-M expression, is deficient in 2 of 4 of the low-MA/M1 melanomas, while another melanocyte-lineage transcription factor, tbx2, was deficient at the mRNA level only in the MA/M1-low EW cell line (see Table 1 below).

OSM induces down-modulation of a variety of melanocyte-related genes, including MA/M1, tyrosinase, gp100, TRP-1 and TRP-2.

It is noteworthy that all four of the MA/M1 deficient melanoma cell lines we have studied produce strong antigen-silencing activity. This suggests a correlation between antigen expression and the production of an antigen-silencing factor. If a tumor mutant had lost the MA/M1 gene, or its promoter, there would be no selective advantage for the cell to continue to produce an antigen silencing factor. The simultaneous loss of tyrosinase and gp100 suggest that any mutations in these cells would be targeting some gene regulatory molecules, as it would be less likely that all of these chromosomally distinct genes would be deleted or mutated simultaneously in several different tumor lines. Whether such a factor is involved in differentiation of the melanocyte lineage, or perhaps maintenance of a less mature phenotype, the active production of MASA seems to be characteristic of antigen-negative melanomas.

**Table 1. Antigen and Transcription Factor mRNA levels among Melanoma cell lines.** A series of 8 melanoma cell lines and a "control" B cell lymphoma (RAMOS) were assessed for a series of markers as indicated. Antigen expression of Melan-A/MART-1 (MA/M1), gp100 and tyrosinase were assessed by cytoplasmic staining with appropriate monoclonal antibodies. In addition, assessment of gross differences in the relative mRNA steady-state levels for these markers between different cell lines was made following PCR amplification. The (++++) designation indicates easily detectable (relatively high level) product formation. In cases where the designation of +/- is assigned, product levels were reproducibly low, often requiring a second round of nested PCR for unequivocal detection. (Comparison of the relative levels between separate markers is not feasible with these assays).

| TUMOR | 136.2 | 453A | MM96L | MU | MU-X | EW | IGR-39D | A375 | RAMOS |
|---|---|---|---|---|---|---|---|---|---|
| OSM | ++ | + | - | ++ | +/- | ++++ | ++ | - | ++++ |
| MA/M1 | ++++ | ++++ | ++++ | ++++ | +/- | +/- | +/- | +/- * | - |
| gp100 | ++++ | ++++ | ++++ | ++++ | + | + | + | + | - |
| tyrosinase | ++++ | ++++ | ++++ | ++++ | - | - | +/- | + | - |
| TRP-1 | ++++ | ++++ | ++++ | ++ | ++ | + | ++ | ++ | - |
| TRP-2 | ++++ | ++++ | ++++ | ++ | + | +/- | - | ++ | - |

*A375 have detectable mRNA for MA/M1, but are relatively deficient in cytoplasmic protein expression.

| TUMOR | 136.2 | 453A | MM96L | MU | MU-X | EW | IGR-39D | A375 | RAMOS |
|---|---|---|---|---|---|---|---|---|---|
| Mitf-M | ++++ | ++++ | ++++ | ++++ | +/- | +/- | +/- | ++ | - |
| Mitf-A | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | - |
| Mitf-B | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | - |
| BRN2 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | - |
| STAT3 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Pax3 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | + |
| SOX 10 | ++++ | ++++ | ++++ | ++++ | + | - | - | ++++ | - |
| Tbx2 | ++++ | ++++ | ++++ | ++++ | ++ | + | ++ | ++++ | - |

**MAP Kinase Pathways**

We began our studies of OSM and MAPK pathways by determining the effect of the MEK1 inhibitor PD98059 on OSM-stimulated down-modulation of MA/M1. As shown in Figure 2, treatment of MU tumor cells with 10mM of PD98059 completely prevented the loss of MA/M1 induced by culture of MU tumor cells with 10ng/ml of

OSM. Similar blocking of OSM activity was noted with another MEK inhibitor (U0126, see also Figure 3).

In addition these agents can upregulate Melan-A/MART-1 expression in Melan-A/MART-1 expressing and deficient melanoma cells. As shown in Figure 3, antigen-positive cell lines, such as 453A show dramatic up-regulation of Melan-A/MART-1, while the relatively deficient tumor cells, such as EW, IGR-39D and A375, show parallel, but more modest levels of Melan-A/MART-1 expression after MEK1/2 inhibitor treatment. The up-regulation of Melan-A/MART-1 by MEK inhibitors is dose-dependent, and cellular toxicity limits the dose at which these inhibitors can be tested.

The up-regulation of Melan-A/MART-1 expression can also be demonstrated at the mRNA level, where both U0126 and PD98059 show a dose-dependent up-regulation of Melan-A/MART-1 mRNA transcription in both antigen-positive cells, such as MU (Figure 4), and Melan-A/MART-1 deficient tumor cells, MU-X (Figure 5). The up-regulation of Melan-A/MART-1 in deficient cells demonstrates that these antigen-negative cells have the necessary components for expression of this lineage-specific antigen, and that the loss of antigen-expression may be reversed with gene-regulatory strategies.

**Effect of MEK inhibitors on MA/M1 mRNA and its modulation by OSM.**

As shown previously, OSM down-modulates MA/M1 expression, but as shown in Figures 2 and 3, PD98059 and UO126 reverse this OSM-mediated diminution of MA/M1 mRNA in MU cells. Additionally, the MEK inhibitors increased the levels of MA/M1 steady-state transcripts in MA/M1-deficient tumor cells (Figure 6). It has been reported that PD98059 and dominant-negative MAPK inhibitors (ras and MEK mutants) strongly enhance tyrosinase promoter activity in murine B16 melanoma cells.

**Induction of Cytotoxicity by U0126.**

As shown in Figure 7, the U0126-stimulated upregulation of mRNA and cytoplasmic protein expression in MA/M1-deficient cell lines, correlates with an increase in cytotoxic T cell recognition of tumor cells. The MA/M1-specific T cell clone is highly effective, so that even at low effector:target ratios (2:1), it still shows almost maximal lysis of MU target cells. However, the A375 are lysed only slightly (10%) at all ratios, while the addition of 40mM U0126 for 6 days enhanced the lysis of A375 cells to approximately

30%. Still, the lysis of treated A375 cells still did not approach the 70% lysis achieved against MA/M1 high expressing MU target cells. The induction of MA/M1 protein expression in A375 cells is still less than that seen in MU cells, and in fact the highest levels of MA/M1 staining we have achieved in A375 cells is approximately the same level to which OSM decreases MA/M1 staining in MU target cells. It is noteworthy that the 30% lysis seen in such targets, is comparable. Our data indicates that upregulation of endogenous antigen to allow enhanced CTL recognition is further evidence that the loss of antigen in melanomas can be the result of reversible gene regulatory events.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All references disclosed herein are incorporated by reference in their entirety.

What is claimed is presented below and is followed by a Sequence Listing.

We claim:

Application number/numéro de demande: 2475206

Figures: 4, 5, 6

Pages:

Drawings

Unscannable items
received with this application
(Request original documents in File Prep. Section on the 10th Floor)

Documents reçus avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au
10ième étage)

SEQUENCE LISTING

<110> The General Hospital Corporation
Kurnick, James T.
Durda, Paul J.

<120> MAP-KINASE INHIBITORS AS REGULATORS OF TUMOR-ASSOCIATED ANTIGEN EXPRESSION

<130> M00765.70056.WO

<150> US 60/357,586
<151> 2002-02-15

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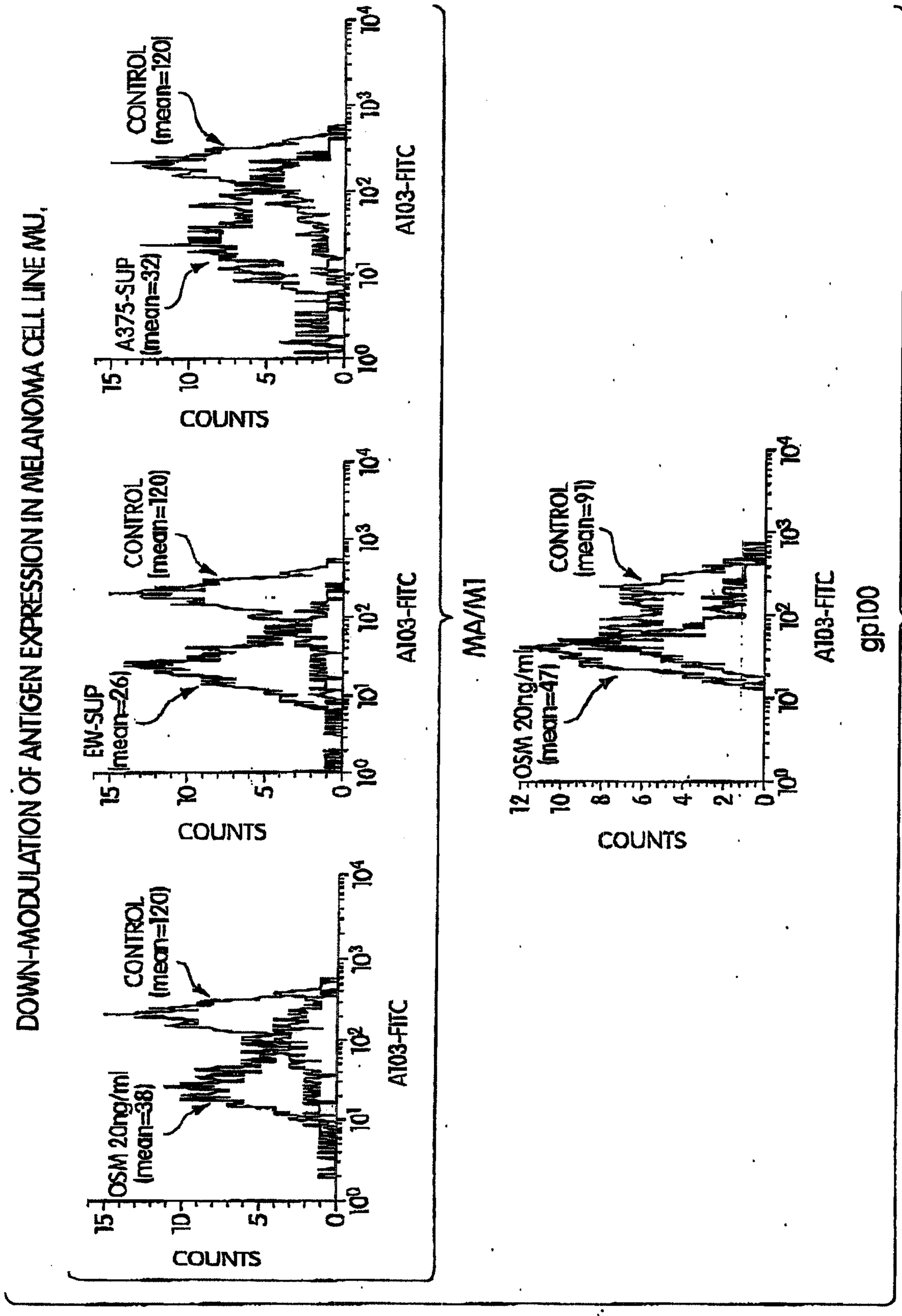

DOWN-MODULATION OF ANTIGEN EXPRESSION IN MELANOMA CELL LINE MU.
CONTROL (mean=120)
A375-SUP (mean=32)
EW-SUP (mean=26)
OSM 20ng/ml (mean=38)
COUNTS
A103-FITC
MA/M1
gp100

Fig. 1

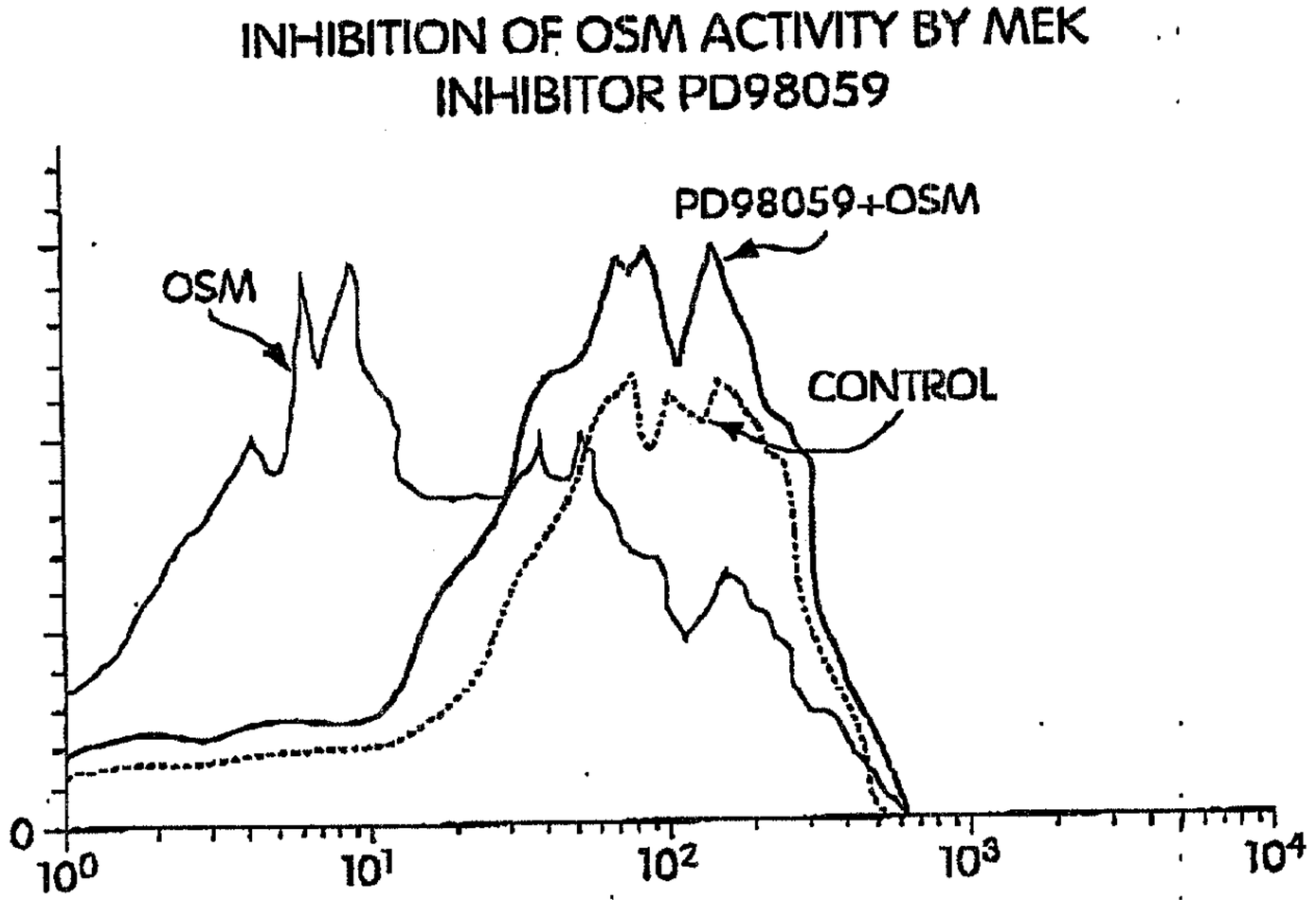
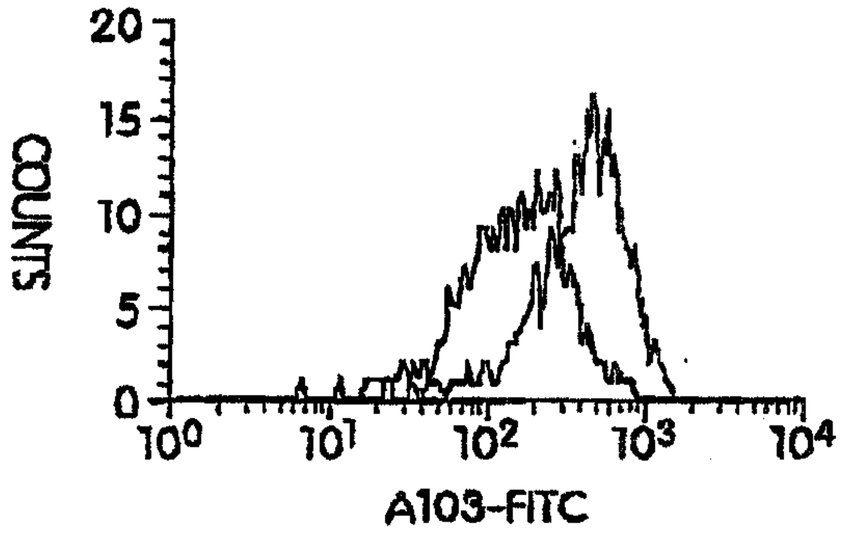
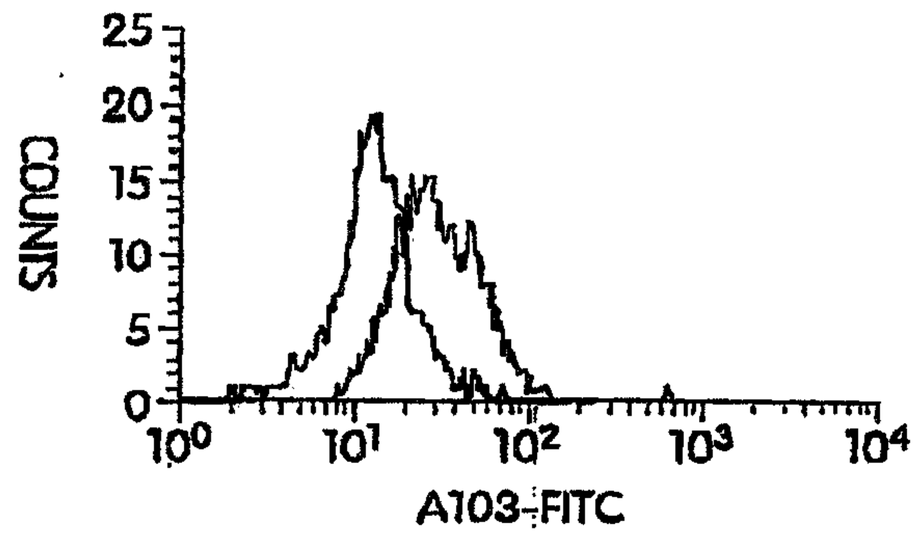

INHIBITION OF OSM ACTIVITY BY MEK INHIBITOR PD98059
PD98059+OSM
OSM
CONTROL
0
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$

Fig. 2

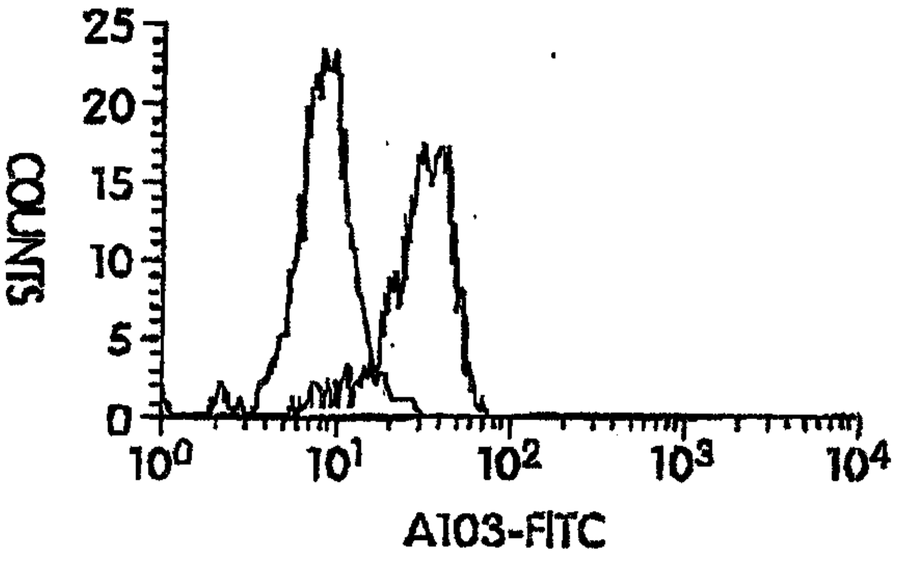
UP-REGULATION OF MELAN-A/MART-1 EXPRESSION IN MELANOMA CELLS BY MEK1/2 INHIBITORS U0126 AND PD98059



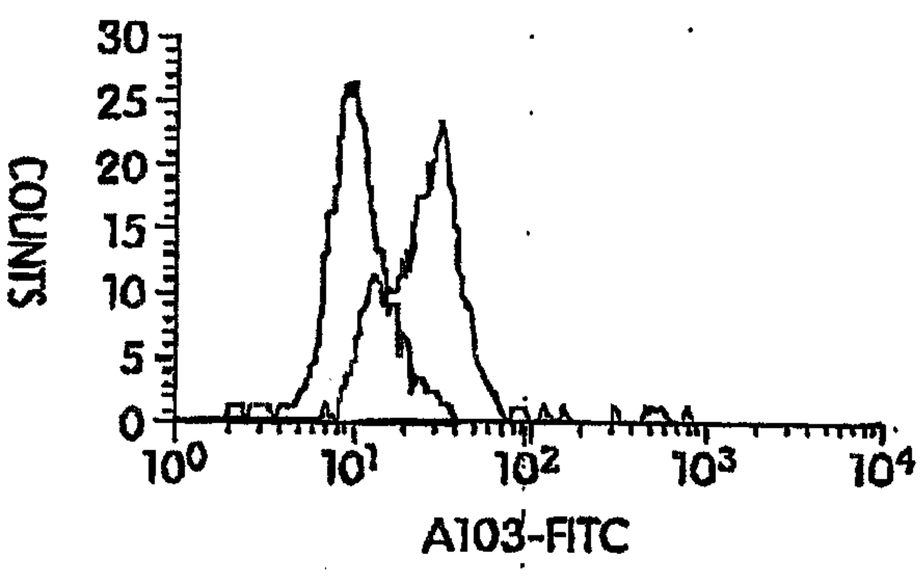

**453A** 3 DAY
CONTROL=138.3
PD98059 40μM=350.5




**IGR-39D** 3 DAY:
CONTROL=12.6; U0126
40μM=30.4




**EW** DAY 6,
CONTROL=8.1;
U0126 50μM=30.9




**A375** 3 DAY:
CONTROL=10.2
U0126 50μM=29.8

Fig. 3

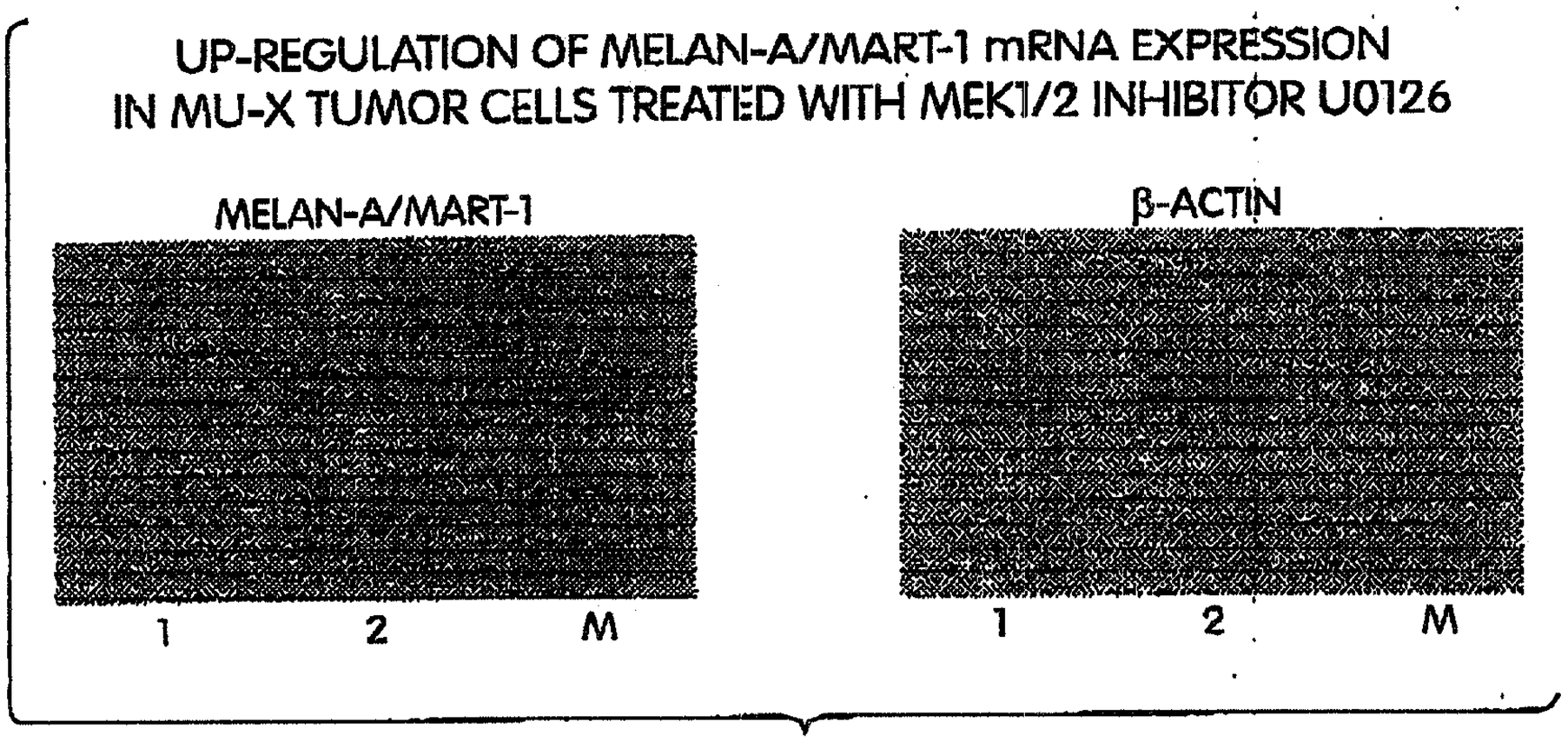

UP-REGULATION OF MELAN-A/MART-1 mRNA EXPRESSION
IN MU-X TUMOR CELLS TREATED WITH MEK1/2 INHIBITOR U0126
MELAN-A/MART-1
β-ACTIN
1
2
M
1
2
M

Fig. 6

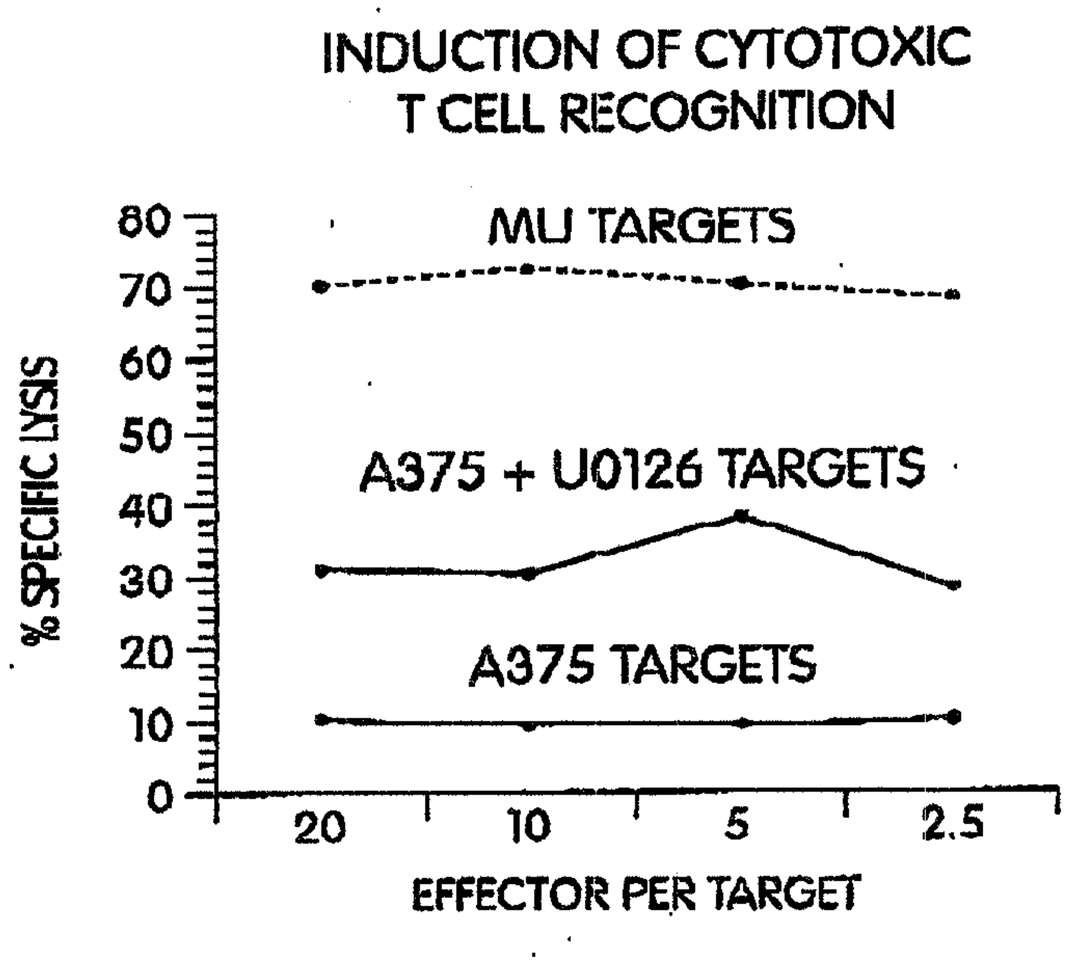

INDUCTION OF CYTOTOXIC
T CELL RECOGNITION
% SPECIFIC LYSIS
80
70
60
50
40
30
20
10
0
MU TARGETS
A375 + U0126 TARGETS
A375 TARGETS
20
10
5
2.5
EFFECTOR PER TARGET

Fig. 7