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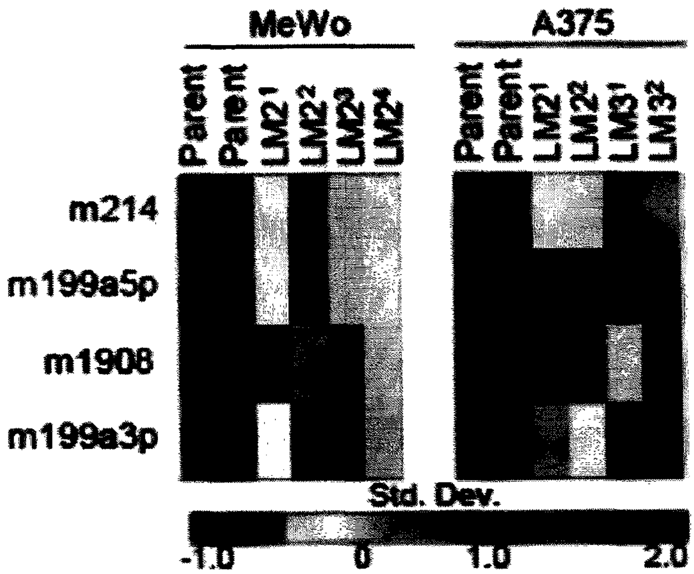

(57) Abrégé/Abstract:
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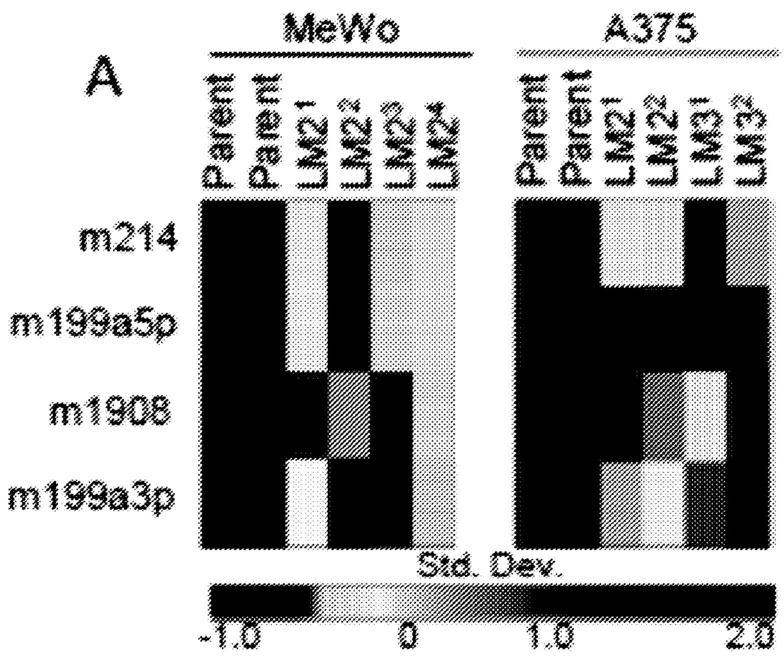

(57) Abstract: The present invention discloses novel agents and methods for diagnosis and treatment of melanoma. Also disclosed are related arrays, kits, and screening methods.

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TREATMENT AND DIAGNOSIS OF MELANOMA

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority of U.S. Provisional Application No. 61/682,339 filed August 13, 2012 and U.S. Provisional Application No. 61/784,057 filed March 14, 2013.

FIELD OF THE INVENTION

This invention relates to diagnosis and treatment of migrating cancers and melanoma.

BACKGROUND OF THE INVENTION

Melanoma, a malignant tumor, develops from abnormal melanocytes in the lower epidermis and can metastasize to distant sites in the body via the blood and lymph systems. Although it accounts for less than 5% of skin cancer cases, melanoma is much more dangerous and responsible for a large majority of the deaths associated with skin cancer. Across the world the incidence of melanoma has been increasing at an alarming rate, with a lifetime risk of developing melanoma as high as 1/58 for males in the U.S. (Jemal et al., 2008, CA: Cancer J. Clin. 58:71-96). The mortality rate of malignant melanoma also continues to rise dramatically throughout the world. According to a 2006 WHO report, about 48,000 melanoma related deaths occur worldwide per year (Lucas et al. (2006) Environmental Burden of Disease Series. 13. World Health Organization. ISBN 92-4-159440-3). In the United States, it was estimated that almost 70,000 people were diagnosed with melanoma during 2010 and approximately 9,000 people would be expected to die from the disease (American Cancer Society; www.cancer.org).

Although some conventional cancer therapies have been used in treating metastatic melanoma, they are not effective. Metastatic melanoma therefore remains one of the most difficult cancers to treat and one of the most feared neoplasms. Accordingly, there is a need for new agents and methods for diagnosis and treatment of melanoma.

SUMMARY OF INVENTION

This invention addresses the above-mentioned need by providing agents and methods for diagnosis and treatment of melanoma. The invention is based, at least in part, on an unexpected discovery of a cooperative miRNA-protein network deregulated in metastatic melanoma. This network includes a number of metastasis suppressor factors and metastasis promoter factors.

In one aspect, the invention features a method for treating cancer, including administering to a subject in need thereof, a LXR agonist, wherein the LXR agonist is administered in an amount sufficient to increase the expression level or activity level of ApoE to a level sufficient to slow the spread of metastasis of the cancer.

In another aspect, the invention features a method for treating cancer, including administering to a subject in need thereof, an ApoE polypeptide in an amount sufficient to treat the cancer.

In another aspect, the invention features a method of slowing the spread of a migrating cancer, comprising administering to a subject in need thereof, a LXR agonist or an ApoE polypeptide.

In some embodiments of any of the aforementioned methods, the LXR agonist is a LXRβ agonist. In certain embodiments, the LXR agonist increases the expression level of ApoE at least 2.5-fold in vitro. In certain embodiments, the LXRβ agonist is selective for LXRβ over LXRα. In other embodiments, the LXRβ agonist has activity for LXRβ that is at least 2.5-fold greater than the activity of said agonist for LXRα. In some embodiments, the LXRβ agonist has activity for LXRβ that is at least 10-fold greater than the activity of said agonist for LXRα. In further embodiments, the LXRβ agonist has activity for LXRβ that is at least 100-fold greater than the activity of said agonist for LXRα. In certain embodiments, the LXR agonist has activity for LXRβ that is at least within 2.5-fold of the activity of said agonist for LXRα.

In some embodiments the migrating cancer is metastatic cancer. The metastatic cancer can include cells exhibiting migration and/or invasion of migrating cells and/or include cells exhibiting endothelial recruitment and/or angiogenesis. In other embodiments, the migrating cancer is a cell migration cancer. In still other embodiments, the cell migration cancer is a non-metastatic cell migration cancer.

The migrating cancer can be a cancer spread via seeding the surface of the peritoneal, pleural, pericardial, or subarachnoid spaces. Alternatively, the migrating cancer can be a cancer spread via the lymphatic system, or a cancer spread hematogenously.

In particular embodiments, the migrating cancer is a cell migration cancer that is a non-metastatic cell migration cancer, such as ovarian cancer, mesothelioma, or primary lung cancer.

In a related aspect, the invention provides a method for inhibiting or reducing metastasis of cancer comprising administering a LXR agonist or an ApoE polypeptide.

In another aspect, the invention provides a method for inhibiting proliferation or growth of cancer stem cells or cancer initiating cells, including contacting the cell with a LXR agonist or an ApoE polypeptide in an amount sufficient to inhibit proliferation or growth of said cell.

In yet another aspect, the invention provides a method of reducing the rate of tumor seeding of a cancer including administering to a subject in need thereof a LXR agonist or an ApoE polypeptide in an amount sufficient to reduce tumor seeding.

In still a further aspect, the invention provides a method of reducing or treating metastatic nodule-forming of cancer including administering to a subject in need thereof a LXR agonist or an ApoE polypeptide in an amount sufficient to treat said metastatic nodule-forming of cancer.

In other embodiments, the cancer is breast cancer, colon cancer, renal cell cancer, non-small cell lung cancer, hepatocellular carcinoma, gastric cancer, ovarian cancer, pancreatic cancer, esophageal cancer, prostate cancer, sarcoma, or melanoma. In some embodiments, the cancer is melanoma. In other embodiments, the cancer is breast cancer. In certain embodiments, the cancer is renal cell cancer. In further embodiments, the cancer is pancreatic cancer. In other embodiments, the cancer is non-small cell lung cancer. In some embodiments the cancer is colon cancer. In further embodiments, the cancer is ovarian cancer.

In other embodiments, the cancer is a drug resistant cancer. In further embodiments, the cancer is resistant to to vemurafenib, dacarbazine, a CTLA4 inhibitor, a PD1 inhibitor, or a PDL1 inhibitor.

In some embodiments, the method comprises administering an LXR agonist selected from the list consisting of a compound of any one of Formula I-IV or any of compound numbers 1-39, or pharmaceutically acceptable salts thereof. In some embodiments, the LXR agonist is compound 1 or a pharmaceutically acceptable salt thereof. In other embodiments, the LXR agonist is compound 2 or a pharmaceutically acceptable salt thereof. In certain embodiments, the LXR agonist is compound 3 or a pharmaceutically acceptable salt thereof. In further embodiments, the LXR agonist is compound 12 or a pharmaceutically acceptable salt thereof. In some embodiments, the LXR agonist is compound 25 or a pharmaceutically acceptable salt thereof. In other embodiments, the LXR agonist is compound 38 or a pharmaceutically acceptable salt thereof. In further embodiments, the LXR agonist is compound 39 or a pharmaceutically acceptable salt thereof.

The method can further include administering an antiproliferative, wherein said LXR agonist and said antiproliferative are administered in an amount that together, is sufficient to slow the progression of migrating cancer. For example, the antiproliferative and LXR agonist can be administered within 28 days of each (e.g., within 21, 14, 10, 7, 5, 4, 3, 2, or 1 days) or within 24 hours (e.g., 12, 6, 3, 2, or 1 hours; or concomitantly) other in amounts that together are effective to treat the subject.

In some embodiments, the method comprises administering an ApoE polypeptide. The ApoE polypeptide fragment can increase the activity level or expression level of LRP1 or LRP8, and/or the ApoE polypeptide can bind to LRP1 or LRP8, the ApoE polypeptide can be the receptor binding region (RBR) of ApoE. The method can further include administering an antiproliferative, wherein said ApoE polypeptide and said antiproliferative are administered in an amount that together, is sufficient to slow the progression of migrating cancer. For example, the antiproliferative and ApoE polypeptide can be administered within 28 days of each (e.g., within 21, 14, 10, 7, 5, 4, 3, 2, or 1 days) or within 24 hours (e.g., 12, 6, 3, 2, or 1 hours; or concomitantly) other in amounts that together are effective to treat the subject.

In some embodiments, the pharmaceutical composition may further comprise an additional compound having antiproliferative activity. The additional compound having antiproliferative activity can be selected from the group of compounds such as chemotherapeutic and cytotoxic agents, differentiation-inducing agents (*e.g.* retinoic acid, vitamin D, cytokines), hormonal agents, immunological agents and anti-angiogenic agents. Chemotherapeutic and cytotoxic agents include, but are not limited to, alkylating agents, cytotoxic antibiotics, antimetabolites, vinca alkaloids, etoposides, and others (*e.g.*, paclitaxel, taxol, docetaxel, taxotere, cis-platinum). A list of additional compounds having antiproliferative activity can be found in L. Brunton, B. Chabner and B. Knollman (eds). Goodman and Gilman's The Pharmacological Basis of Therapeutics, Twelfth Edition, 2011, McGraw Hill Companies, New York, NY.

The method may further include administering a antiproliferative compound selected from the group consisting of alkylating agents, platinum agents, antimetabolites, topoisomerase inhibitors, antitumor antibiotics, antimitotic agents, aromatase inhibitors, thymidylate synthase inhibitors, DNA antagonists, farnesyltransferase inhibitors, pump inhibitors, histone acetyltransferase inhibitors, metalloproteinase inhibitors, ribonucleoside reductase inhibitors, TNF alpha agonists/antagonists, endothelin A receptor antagonist, retinoic acid receptor agonists, immuno-modulators, hormonal and antihormonal agents, photodynamic agents, tyrosine kinase inhibitors, antisense compounds, corticosteroids, HSP90 inhibitors, proteosome inhibitors (for example, NPI-0052), CD40 inhibitors, anti-CSI antibodies, FGFR3 inhibitors, VEGF inhibitors, MEK inhibitors, cyclin D1 inhibitors, NF-kB inhibitors, anthracyclines, histone deacetylases, kinesin inhibitors, phosphatase inhibitors, COX2 inhibitors, mTOR inhibitors, calcineurin antagonists, IMiDs, or other agents used to treat proliferative diseases. Examples of such compounds are provided in Tables 1.

In another aspect, the invention features a method for treating melanoma (e.g., metastatic melanoma) in a subject in need thereof. The method includes (a) increasing in the subject the expression level or activity level of a metastasis suppressor factor selected from the group consisting of DNAJA4, Apolipoprotein E (ApoE), LRP1, LRP8, Liver X Receptor (LXR, e.g., both LXR-alpha and LXR-beta), and miR-7 or (b) decreasing in the subject the expression level or activity level of a metastasis promoter factor selected from the group consisting of miR-199a-3p, miR-199a-5p, miR-1908, and CTGF.

In the method, the increasing step can be carried out by administering to the subject one or more of the followings: (i) a polypeptide having a sequence of DNAJA4, ApoE or an ApoE fragment, LRP1, LRP8, or LXR; (ii) a nucleic acid having a sequence encoding DNAJA4, ApoE, LRP1, LRP8, or LXR; (iii) a ligand for LRP1, LRP8, or LXR; and (iv) an RNAi agent encoding miR-7. Examples of the LRP1 or LRP8 ligand include the receptor binding portion of ApoE, anti-LRP1 or anti-LRP8 antibodies, and small molecule ligands.

In one example, increasing the ApoE expression level can be carried out by increasing the activity level or expression level of LXR. Increasing the DNAJA4 expression level can also be carried out by increasing the activity level or expression level of LXR. The LXR activity level can be increased by administering to the subject a ligand of LXR, such as compounds of Formula I-IV as disclosed below. The increasing step can also be carried out by decreasing the expression level or activity level of a microRNA selected from the group consisting of miR-199a-3p, miR-199a-5p, and miR-1908. To this end, one can use a number of techniques known in the art, including, but not limited to, the miR-Zip technology, Locked Nucleic Acid (LNA), and antagomir technology as described in the examples below.

In a another aspect, the invention provides a method for determining whether a subject has, or is at risk of having, metastatic melanoma. The method includes obtaining from the subject a sample; measuring in the sample (i) a first expression level of a metastasis promoter factor selected from the group consisting of miR-199a-3p, miR-199a-5p, miR-1908, and CTGF, or (ii) a second expression level of a metastasis suppressor factor selected from the group consisting of DNAJA4, ApoE, LRP1, LRP8, LXR, and miR-7; and comparing the first expression level with a first predetermined reference value, or the second expression level with a second predetermined reference value. The subject is determined to have, or to be at risk of having, metastatic melanoma if (a) the first expression level is above a first predetermined reference value or (b) the second expression level is below a second predetermined reference value. The first and second predetermined reference values can be obtained from a control subject that does not have metastatic melanoma. In one embodiment, the measuring step includes measuring both the first expression level and the second expression level. The sample can be a body fluid sample, a tumor sample, a nevus sample, or a human skin sample.

In a another aspect, the invention provides an array having a support having a plurality of unique locations, and any combination of (i) at least one nucleic acid having a sequence that is complementary to a nucleic acid encoding a metastasis promoter factor selected from the group consisting of miR-199a-3p, miR-199a-5p, miR-1908, and CTGF or a complement thereof, or (ii) at least one nucleic acid having a sequence that is complementary to a nucleic acid encoding a metastasis suppressor factor selected from the group consisting of DNAJA4, ApoE, LRP1, LRP8, LXR, and miR-7 or a complement thereof. Preferably, each nucleic acid is immobilized to a unique location of the support. This array can be used for metastatic melanoma diagnosis and prognosis.

Accordingly, the invention also provides a kit for diagnosing a metastatic potential of melanoma in a subject. The kit includes a first reagent that specifically binds to an expression product of a metastasis suppressor gene selected from the group consisting of DNAJA4, ApoE, LRP1, LRP8, LXR, and miR-7; or a second reagent that specifically binds to an expression product of a metastasis promoter gene selected from the group consisting of miR-199a-3p, miR-199a-5p, miR-1908, and CTGF. The second agent can be a probe having a sequence complementary to the suppressor or promoter gene or a complement thereof. The kit can further contain reagents for performing an immunoassay, a hybridization assay, or a PCR assay. In one embodiment, the kit contained the above-mentioned array.

In a another aspect, the invention provides a method of identifying a compound useful for treating melanoma or for inhibiting endothelial recruitment, cell invasion, or metastatic angiogenesis. The method includes (i) obtaining a test cell expressing a reporter gene encoded by a nucleic acid operatively liked to a promoter of a marker gene selected from the group consisting of miR-199a-3p, miR-199a-5p, miR-1908, and CTGF; (ii) exposing the test cell to a test compound; (iii) measuring the expression level of the reporter gene in the test cell; (iv) comparing the expression level with a control level; and (v) selecting the test compound as a candidate useful for treating melanoma or for inhibiting endothelial recruitment, cancer cell invasion, or metastatic angiogenesis, if the comparison indicates that the expression level is lower than the control level.

The invention provides another method of identifying a compound useful for treating melanoma or for inhibiting endothelial recruitment, cell invasion, or metastatic angiogenesis. The method includes (i) obtaining a test cell expressing a reporter gene encoded by a nucleic acid operatively liked to a promoter of a marker gene selected from the group consisting of DNAJA4, ApoE, LRP1, LRP8, LXR, and miR-7; (ii) exposing the test cell to a test compound; (iii) measuring the expression level of the reporter gene in the test cell; (iv) comparing the expression level with a control level; and (v) selecting the test compound as a candidate useful for treating melanoma or for inhibiting endothelial recruitment, cancer cell invasion, or metastatic angiogenesis, if the comparison indicates that the expression level is higher than the control level.

In the above-mentioned identification methods, the reporter gene can be a standard reporter gene (such as LaxZ, GFP, or luciferase gene, or the like), known in the art, or one of the aforementioned metastasis suppressor genes or metastasis promoter genes. In the methods, the control level can be obtained from a control cell that is the same as the test cell except that the control cell has not be exposed to the test compound.

In a another aspect, the invention provides a method for inhibiting endothelial recruitment, inhibiting tumor cell invasion, or treating metastatic cancer in a subject in need thereof, by administering to the subject an agent that inhibits expression or activity of CTGF. The subject can be one having a disorder characterized by pathological angiogenesis, including but not limited to cancer (e.g., metastatic melanoma), an eye disorder, and an inflammatory disorder. An example of the tumor cell is a metastatic melanoma cell. Examples of the agent include an antibody, a nucleic acid, a polypeptide, and a small molecule compound. In a preferred embodiment, the antibody is a monoclonal antibody.

In a another aspect, the invention provides a method for inhibiting endothelial recruitment, inhibiting tumor cell invasion, or treating metastatic cancer in a subject in need thereof, by administering to the subject an agent that increases expression or activity of miR-7. An example of the tumor cell is a metastatic melanoma cell. Examples of the agent include an antibody, a nucleic acid, a polypeptide, and a small molecule compound. In one example, the agent has miR-7 activity. The nucleic acid can be an oligonucleotide. And, the oligonucleotide can include a sequence selected from the group consisting of SEQ ID Nos. 36-38.

As used herein, "migrating cancer" refers to a cancer in which the cancer cells forming the tumor migrate and subsequently grow as malignant implants at a site other than the site of the original tumor. The cancer cells migrate via seeding the surface of the peritoneal, pleural, pericardial, or subarachnoid spaces to spread into the body cavities; via invasion of the lymphatic system through invasion of lymphatic cells and transport to regional and distant lymph nodes and then to other parts of the body; via haematogenous spread through invasion of blood cells; or via invasion of the surrounding tissue. Migrating cancers include metastatic tumors and cell migration cancers, such as ovarian cancer, mesothelioma, and primary lung cancer, each of which is characterized by cellular migration.

As used herein, “slowing the spread of migrating cancer” refers to reducing or stopping the formation of new loci; or reducing, stopping, or reversing the tumor load.

As used herein, “metastatic tumor” refers to a tumor or cancer in which the cancer cells forming the tumor have a high potential to or have begun to, metastasize, or spread from one location to another location or locations within a subject, via the lymphatic system or via haematogenous spread, for example, creating secondary tumors within the subject. Such metastatic behavior may be indicative of malignant tumors. In some cases, metastatic behavior may be associated with an increase in cell migration and/or invasion behavior of the tumor cells.

As used herein, “slowing the spread of metastasis” refers to reducing or stopping the formation of new loci; or reducing, stopping, or reversing the tumor load.

The term “cancer” refers to any cancer caused by the proliferation of malignant neoplastic cells, such as tumors, neoplasms, carcinomas, sarcomas, leukimias, lymphomas, and the like.

As used herein, “drug resistant cancer” refers to any cancer that is resistant to an antiproliferative in Table 2.

Examples of cancers that can be defined as metastatic include but are not limited to non-small cell lung cancer, breast cancer, ovarian cancer, colorectal cancer, biliary tract cancer, bladder cancer, brain cancer including glioblastomas and medullablastomas, cervical cancer, choriocarcinoma, endometrial cancer, esophageal cancer, gastric cancer, hematological neoplasms, multiple myeloma, leukemia, intraepithelial neoplasms, livercancer, lymphomas, neuroblastomas, oral cancer, pancreatic cancer, prostate cancer, sarcoma, skin cancer including melanoma, basocellular cancer, squamous cell cancer, testicular cancer, stromal tumors, germ cell tumors, thyroid cancer, and renal cancer.

“Proliferation” as used in this application involves reproduction or multiplication of similar forms (cells) due to constituting (cellular) elements.

“Cell migration” as used in this application involves the invasion by the cancer cells into the surrounding tissue and the crossing of the vessel wall to exit the vasculature in distal organs of the cancer cell.

By “cell migration cancers” is meant cancers that migrate by invasion by the cancer cells into the surrounding tissue and the crossing of the vessel wall to exit the vasculature in distal organs of the cancer cell.

"Non-metastatic cell migration cancer" as used herein refers to cancers that do not migrate via the lymphatic system or via haematogenous spread.

As used herein, "cell to cell adhesion" refers to adhesion between at least two cells through an interaction between a selectin molecule and a selectin specific ligand. Cell to cell adhesion includes cell migration.

A "cell adhesion related disorder" is defined herein as any disease or disorder which results from or is related to cell to cell adhesion or migration. A cell adhesion disorder also includes any disease or disorder resulting from inappropriate, aberrant, or abnormal activation of the immune system or the inflammatory system. Such diseases include but are not limited to, myocardial infarction, bacterial or viral infection, metastatic conditions, e.g. cancer. The invention further features methods for treating a cell adhesion disorder by administering a LXR agonist or ApoE polypeptide.

As used herein, "cancer stem cells" or "cancer initiating cells" refers to cancer cells that possess characteristics associated with normal stem cells, specifically the ability to give rise to all cell types found in a particular cancer sample. Cancer stem cells are therefore tumorgenic or tumor forming, perhaps in contrast to other non-tumorgenic cancer cells. Cancer stem cells may persist in tumors as a distinct population and cause cancer recurrence and metastasis by giving rise to new tumors.

As used herein, "tumor seeding" refers to the spillage of tumor cell clusters and their subsequent growth as malignant implants at a site other than the site of the original tumor.

As used herein, "metastatic nodule" refers to an aggregation of tumor cells in the body at a site other than the site of the original tumor.

The details of one or more embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Systematic Identification of miR-1908, miR-199a-3p, and miR-199a-5p as Endogenous Promoters of Human Melanoma Metastasis (A) Heat map illustrating variance-normalized microarray expression values of miRNAs up-regulated in independent MeWo and A375 metastatic derivatives relative to their respective parental cells. Standard

deviation changes from the mean of each heat map row are indicated by color map. (B) miRNAs found to be up-regulated by microarray hybridization were validated by qRT-PCR in MeWo-LM2 metastatic derivatives. n=3. (C) Bioluminescence imaging plot of lung metastatic colonization following intravenous injection of 4×10^4 parental MeWo cells over-expressing the precursors for miR-199a, miR-1908, miR-214, or a control hairpin. Lungs were extracted 63 days post-injection and H&E-stained. n=5. (D) Bioluminescence imaging plot and H&E-stained lungs corresponding to lung metastasis following intravenous injection of 4×10^4 LM2 cells expressing a short hairpin (miR-Zip) inhibiting miR-1908 (m1908 KD), miR-199a-3p (m199a3p KD), miR-199a-5p (m199a5p KD), or a control sequence (shCTRL). Lungs were extracted and H&E-stained 49 days post-injection n=5-8. (E) Lung colonization by 2×10^5 A375-LM3 metastatic derivatives with miR-Zip-induced silencing of miR-1908, miR-199a-3p, miR-199a-5p, or a control sequence was quantified at day 42 by bioluminescence imaging. n=5-8 (F) The expression levels of miR-199a-3p, miR-199a-5p, and miR-1908 were determined in a blinded fashion by qRT-PCR in a cohort of non-metastatic (n=38) and metastatic (n=33) primary melanoma skin lesions from MSKCC patients. n=71. All data are represented as mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$. See also Figure 12.

Figure 2. MiR-1908, miR-199a-3p, and miR-199a-5p Display Dual Cell-Autonomous/Non-Cell-Autonomous Roles in Regulating Melanoma Metastatic Progression (A) 1×10^6 parental MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin were injected subcutaneously into immuno-deficient mice, and primary tumor volume was monitored over time. n=4-6. (B) 1×10^5 parental MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin were allowed to invade through a trans-well matrigel-coated insert for 24 hours, and the number of cells invaded into the basal side of each insert was quantified. n=7. (C-D) 1×10^5 highly metastatic MeWo-LM2 (C) and A375-LM3 (D) cells with miR-Zip-induced inhibition of miR-199a-3p, miR-199a-5p, miR-1908, or a control sequence were subjected to the cell invasion assay. n=6-8. (E) 5×10^4 MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin were seeded on the bottom of a well, and 1×10^5 human umbilical vein endothelial cells (HUVEC's) were allowed to migrate towards the cancer cells for 16 hours through a trans-well insert. Endothelial recruitment capacity was measured by quantifying the number of HUVEC's migrated to the basal side of each insert. n=7. (F-G) Endothelial recruitment by 5×10^4 MeWo-LM2 (F) and A375-

LM3 (G) cells inhibited for miR-199a-3p, miR-199a-5p, miR-1908, or a control sequence. n=6-10. (H) Cumulative fraction plot of the percentage blood vessel density distribution for metastatic nodules formed following intravenous injection of 2×10^5 highly metastatic MeWo-LM2 cells depleted for miR-199-3p, miR-199a-5p, miR-1908, or a control sequence. Lung sections were immunohistochemically double-stained for human vimentin (blue) and MECA-32 (red), and the percentage MECA-32 positive area within each metastatic nodule, demarcated based on vimentin staining, was quantified. n=211 nodules (control KD); n=60 nodules (m199a3p KD); n=138 nodules (m199a5p KD); n=39 nodules (m1908 KD). All data are represented as mean ± SEM. Scale bar, 100 µm. See also Figure 13.

Figure 3. Identification of ApoE and DNAJA4 as Common Target Genes of miR-199a and miR-1908 (A) Heat map depicting mRNA levels of ApoE and DNAJA4, measured by qRT-PCR, in poorly metastatic MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin and in highly metastatic MeWo-LM2 cells. Color map illustrates standard deviation changes from the mean of each heat map column. (B) Heterologous luciferase reporter assays measuring the stability of wild-type ApoE and DNAJA4 3'UTR/CDS luciferase fusions or miRNA target-site mutant ApoE and DNAJA4 3'UTR/CDS fusions in parental MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin. n=3-4. (C) Stability of wild-type ApoE and DNAJA4 3'UTR/CDS luciferase fusions in MeWo-LM2 cells with silenced expression of miR-199a-3p, miR-199a-5p, miR-1908, or a control sequence. n=4. (D) Schematic of experimentally derived model of ApoE and DNAJA4 3'UTR/CDS targeting by miR-199a-3p, miR-199a-5p, and miR-1908. (E) Luciferase activity of wild-type and miRNA target-site mutant ApoE and DNAJA4 3'UTR/CDS luciferase fusions in highly metastatic MeWo-LM2 derivatives and their poorly metastatic parental cell line. n=4. (F) Matrigel invasion capacity by 1×10^5 MeWo-LM2 cells expressing a control vector or over-expressing ApoE or DNAJA4. n=4. (G) Endothelial recruitment ability by 5×10^4 MeWo-LM2 cells transduced with a control vector or an over-expression vector for ApoE or DNAJA4. n=6. (H-I) Poorly metastatic parental MeWo cells transduced with lentiviral short hairpins targeting ApoE, DNAJA4, or a control sequence were assessed for their matrigel invasion capacity (H) and ability to recruit endothelial cells (I). n=6-8. All data are represented as mean ± SEM. Scale bar, 100 µm. See also Figure 14.

Figure 4. Direct Targeting of ApoE and DNAJA4 by miR-199a and miR-1908 Promotes Metastatic Invasion, Endothelial Recruitment, and Colonization (A-D) Highly metastatic LM2 cells expressing a control shRNA or shRNAs targeting ApoE or DNAJA4 in the context of miR-1908 inhibition (m1908 KD; A, B) or miR-199a-5p inhibition (m199a5p KD; C, D) were subjected to the cell invasion (A, C) and endothelial recruitment assays (B, D). n=6-8. (E-F) Bioluminescence imaging plot and H&E-stained lungs representative of lung metastasis after intravenous injection of 1×10^5 LM2 cells expressing a control hairpin or hairpins targeting ApoE, DNAJA4, or a control sequence in the setting of miR-1908 silencing (E) or miR-199a-5p silencing (F). n=5. (G-H) Parental MeWo cells over-expressing ApoE or DNAJA4 or expressing a control vector in the context of miR-1908 over-expression were analyzed for the matrigel invasion (G) and endothelial recruitment (H) phenotypes. (I-J) A375-LM3 derivatives expressing a control shRNA or shRNAs targeting ApoE and DNAJA4 were transduced with a cocktail of LNAs targeting miR-199a-3p, miR-199a-5p, and miR-1908 or a control LNA and analyzed in the matrigel invasion (I) and endothelial recruitment (J) assays. n=4. (K) Blood vessel density distribution, represented in a cumulative fraction plot, for metastatic nodules formed by MeWo-LM2 cells inhibited for miR-1908 and transduced with shRNAs targeting ApoE, DNAJA4, or a control sequence. Lung sections from Figure 4E were immunocytochemically double-stained for human vimentin (blue) and the endothelial marker MECA-32 (red). The percentage MECA-32 positive area within each vimentin-positive nodule was quantified. n=39 nodules (shCTRL); n=97 ($shAPOE^1$); n=38 ($shAPOE^2$); n=200 ($shDNAJA4^1$); n=19 ($shDNAJA4^2$). All data are represented as mean ± SEM. Scale bar, 100 μm. See also Figure 15.

Figure 5. Melanoma-Cell Secreted ApoE Inhibits Melanoma Invasion and Endothelial Recruitment, while Genetic Deletion of ApoE Accelerates Metastasis (A-B) Extracellular ApoE levels quantified by ELISA in conditioned media from MeWo-LM2 metastatic derivatives and their parental cells (A) and LM2 cells silenced for miR-199a-5p, miR-1908, or a control sequence (B). n=3. (C) ApoE-neutralizing antibody 1D7 (10-40 μg/mL) or IgG (40 μg/mL) was added to the cell media, and matrigel invasion by parental MeWo cells was assessed. n=4-6. (D) Endothelial recruitment by parental MeWo cells in the presence of 1D7 (40 μg/mL) or a control IgG antibody (40 μg/mL). n=4. (E) The matrigel invasion and endothelial recruitment phenotypes were assessed in LM2 cells in the

presence of bovine serum albumin (BSA) (100 μM) or recombinant ApoE3 (100 μM) added to the cell media. n=7-10. (F-G) LM2 cells with silenced expression of miR-199a-3p, miR-199a-5p, miR-1908, or a control sequence were examined for matrigel invasion capacity (F) and endothelial recruitment ability (G) in the presence of IgG or ApoE-neutralizing 1D7 antibodies (40 μg/mL). n=5-6. (H) ApoE levels quantified by ELISA in conditioned media from parental MeWo cells transduced with shRNAs targeting DNAJA4 or a control sequence. n=3. (I-J) Parental MeWo cells with shRNA-induced silencing of DNAJA4 were analyzed for the matrigel invasion (I) and endothelial recruitment (J) phenotypes in the presence of either BSA (100 μM) or recombinant ApoE3 (100 μM). n=4. (K) Array-based ApoE expression levels in nevi (n=9), primary melanomas (n=6), and distant melanoma metastases samples (n=19). (L) Highly metastatic MeWo-LM2 cells were incubated in the presence of recombinant ApoE3 or BSA at 100 μg/mL. After 24 hours, 4×10^4 cells were intravenously injected into NOD-SCID mice, and lung colonization was monitored by bioluminescence imaging. n=6. (M) Lung metastasis by 5×10^4 B16F10 mouse melanoma cells intravenously injected into ApoE genetically null C57BL/6 mice or their wild-type control littermates. Lung bioluminescence quantification and representative H&E-stained lungs correspond to 19 days post-injection. n=8-18. All data are represented as mean ± SEM. Scale bar, 100 μm.

Figure 6. Identification of Distinct Melanoma and Endothelial Cell Receptors that Mediate the Effects of ApoE on Melanoma Invasion and Endothelial Recruitment (A) Matrigel invasion capacity was examined in 1×10^5 LM2 cells transduced with siRNAs targeting LDLR, VLDLR, LRP8, LRP1, or a control sequence in the presence of either BSA (100 μM) or recombinant ApoE3 (100 μM). n=4-7. (B) 1×10^5 MeWo-LM2 cells transduced with short hairpins targeting miR-1908 or a control sequence were transfected with siRNAs targeting LRP1 or a control siRNA and subjected to the matrigel invasion assay. n=4. (C) Bioluminescence imaging of lung colonization by 1×10^5 LM2 cells transduced with siRNAs targeting LRP1 or a control sequence in the setting of miR-1908 inhibition. n=5. (D) 1×10^5 endothelial cells pre-incubated with BSA (100 μM) or recombinant ApoE3 (100 μM) for 24 hours were analyzed for the endothelial recruitment phenotype by 5×10^5 LM2 cells. n=3-4. (E) 1×10^5 endothelial cells were transduced with siRNAs targeting LDLR, VLDLR, LRP1, LRP8, or a control sequence and allowed to

migrate in a trans-well system towards LM2 cells inhibited for miR-1908 or a control sequence. n=4-12. (F) Trans-well migration by 1×10^5 endothelial cells in the presence of IgG (40 μg/mL) or 1D7 antibodies (40 μg/mL) added to the cell media. n=6-8. (G) Trans-well migration by 1×10^5 endothelial cells transduced with siRNAs targeting LRP8 or a control sequence in the presence of BSA (100 μM) or recombinant ApoE3 (100 μM). n=6-7. (H) 1×10^5 endothelial cells were transduced with siRNAs targeting LRP8 or a control sequence, and trans-well chemotactic migration was assessed along an ApoE gradient. n=6-8. (I) Endothelial recruitment into matrigel plugs, implanted subcutaneously above the ventral flank of mice, containing BSA (10 μg/mL), VEGF (400 ng/mL) + BSA (10 μg/mL), or VEGF (400 ng/mL) + recombinant ApoE3 (10 μg/mL). n=3-6.(J) Blood vessel density within lung metastatic nodules formed following intravenous injection of 5×10^4 B16F10 mouse melanoma cells into wild-type or ApoE genetically null mice. Lung sections from Figure 5M were immunohistochemically stained for MECA-32, and the percentage MECA-32 positive area within each metastatic nodule, outlined based on cell pigmentation, was quantified. n=17-20. All data are represented as mean ± SEM. Scale bar, 100 μm.

Figure 7. Clinical and Therapeutic Cooperativity among miR-199a-3p, miR-199a-5p, and miR-1908 in Melanoma Metastasis (A-D). Kaplan-Meier curves for the MSKCC cohort (N=71) representing metastasis-free survival of patients as a function of their primary melanoma lesion's miR-199a-3p (A), miR-199a-5p (B), miR-1908 (C), or aggregate three miRNA expression levels (D). Patients whose primary tumors' miRNA expression or aggregate miRNA expression levels (sum of the expression values of miR-199a-3p, miR-199a-5p, and miR-1908) were greater than the median for the population were classified as miRNA expression positive (red), while those whose primary tumors expressed the given miRNAs at a level below the median were classified as miRNA expression negative (blue). (E) Lung metastasis by highly metastatic LM2 cells transfected with LNAs individually targeting each miR-1908, miR-199a-3p, or miR-199a-5p, a combination of LNAs targeting all three miRNAs, or a control LNA. 48 hours post-transfection, 1×10^5 cells were intravenously injected into immuno-deficient mice. n=5-6. (F) Systemic metastasis by 1×10^5 MeWo-LM2 cells transfected with a control LNA (LNA-CTRL) or a cocktail of LNAs targeting miR-1908, miR-199a-3p, miR-199a-5p (LNA-3 miRNAs) 48 hours prior to intracardiac injection into athymic nude mice. n=5. (G)

Number of systemic metastatic foci arising from LNA-CTRL and LNA-3 miRNAs LM2 cells at day 28 post-intracardiac injection. n=5. (H-I) Bioluminescence signal quantification of bone metastasis (H) and brain metastasis (I) at day 28 post-intracardiac injection of LNA-CTRL and LNA-3 miRNAs LM2 cells. n=5. (J) 4×10^4 highly metastatic MeWo-LM2 cells were tail-vein injected into immuno-compromised mice, and the mice were intravenously treated with a cocktail of in vivo-optimized LNAs targeting miR-1908, miR-199a-3p, and miR-199a-5p at a total dose of 12.5 mg/kg or a mock PBS control on a bi-weekly basis for four weeks. Lung colonization was assessed by bioluminescence imaging, and representative H&E-stained lungs extracted at day 56 are shown. n=5-6. (K) Model of miRNA-dependent regulation of metastatic invasion, endothelial recruitment, and colonization in melanoma through targeting of ApoE-mediated melanoma cell LRP1 and endothelial cell LRP8 receptor signaling.

Figure 8. MiRNA-dependent targeting of ApoE/LRP1 signaling promotes cancer cell invasion and endothelial recruitment through CTGF induction. (A) A heat-map of variance-normalized CTGF expression levels, determined by qRT-PCR analysis, in (1) MeWo parental and MeWo-LM2 cells, (2) MeWo parental cells over-expressing miR-199a, miR-1908, or a control hairpin, and (3) MeWo parental cells transduced with short hairpins targeting ApoE or a control sequence. Color-map indicates the standard deviations change from the mean. (B) CTGF levels in conditioned media from MeWo parental cells with ApoE knock-down determined by ELISA. n=6; p-values based on a one-sided student's t-test. (C) CTGF levels, quantified by ELISA, in conditioned media from highly metastatic MeWo-LM2 cells treated with recombinant ApoE in the setting of LRP1 knock-down or a control knock-down. n=3-4; p-values based on a one-sided student's t-test. (D-E) Parental MeWo cells with shRNA-induced ApoE knock-down were (1) transfected with independent siRNAs targeting CTGF or a control sequence or (2) incubated in the presence of a CTGF neutralizing antibody (20 μg/mL) or an IgG control antibody (20 μg/mL), and the cells were subjected to cell invasion (D) and endothelial recruitment (E) assays. n=6-8; p-values based on a one-sided student's t-test; scale bar indicates 100 μM. All data are represented as mean ± SEM.

Figure 9. CTGF mediates miRNA-dependent metastatic invasion, endothelial recruitment, and colonization. (A) 1×10^5 parental MeWo cells expressing a control hairpin or over-expressing miR-199a or miR-1908 were subjected to a trans-well cell invasion

assay in the presence of a blocking antibody targeting CTGF (20 μg/mL) or a control IgG antibody (20 μg/mL) as indicated in the figure. n=4-10; p-values based on a one-sided student's t-test. All data are represented as mean ± SEM. (B) Endothelial recruitment by parental MeWo cells expressing a control hairpin or over-expressing miR-199a or miR-1908. At the beginning of the assay, a neutralizing antibody targeting CTGF (20 μg/mL) or a control IgG antibody (20 μg/mL) were added to endothelial cells as indicated, and 1×10^5 endothelial cells were allowed to migrate towards 5×10^4 cancer cells in a trans-well migration assay. n=3-8; p-values based on a one-sided student's t-test. (C) Bioluminescence imaging of lung metastasis by 5×10^4 parental MeWo cells knocked down for CTGF in the setting of miR-199a or miR-1908 over-expression. n=5-6; p-values obtained using a one-way Mann-Whitney t-test. All data are represented as mean ± SEM.

Figure 10. Treatment with the LXR agonist GW3965 elevates melanoma cell ApoE levels and suppresses cancer cell invasion, endothelial recruitment, and metastatic colonization. (A-B) Parental MeWo cells were incubated in the presence of DMSO or GW3965 at the indicated concentrations. After 48 hours, total RNA was extracted, and the levels of ApoE (A) and DNAJA4 (B) were determined by qRT-PCR. n=3. (C) Cell invasion by 1×10^5 parental MeWo cells pre-treated with GW3965 or DMSO for 48 hours. n=6-7. p-values based on a one-sided student's t-test. All data are represented as mean ± SEM. (D) Endothelial recruitment by 5×10^4 parental MeWo cells pre-treated with GW3965 or DMSO for 48 hours. n=6-7. p-values based on a one-sided student's t-test. (E) Mice were fed with grain-based chow diet containing GW3965 (20mg/kg) or a control diet. After 10 days, 4×10^4 parental MeWo cells were tail-vein injected into mice, and the mice were continuously fed with GW3965-containing chow or a control diet throughout the experiment. Lung colonization was assessed by bioluminescence imaging. n=5-6; p-values obtained using a one-way Mann-Whitney t-test All data are represented as mean ± SEM.

Figure 11. Identification of miR-7 as an endogenous suppressor of melanoma metastasis. (A) Bioluminescence imaging plot of lung metastatic colonization following intravenous injection of 4×10^4 parental MeWo cells expressing a short hairpin (miR-Zip) inhibiting miR-7 (miR-7 KD). Lungs were extracted 63 days post-injection and H&E-stained. n=5. (B). Lung metastasis by 4×10^4 LM2 cells over-expressing the precursor for miR-7 or a control hairpin. Lung colonization was monitored weekly by bioluminescence imaging, and lungs were extracted at day 77 post-injection. n=5. All data are represented as

mean ± SEM; p-values were determined using a one-way Mann-Whitney t-test. *p<0.05, **p<0.01.

Figure 12. In Vivo Selection For Highly Metastatic Human Melanoma Cell Line Derivatives and Identification of miR-199a-3p, miR-199a-5p, and miR-1908 as Metastasis-Promoter miRNAs (A-B) Bioluminescence imaging of lung metastasis and representative images of H&E-stained lungs corresponding to MeWo-LM2 (A) and A375-LM3 metastatic derivatives (B) and their respective parental cell lines. 4×10^4 MeWo-Par/MeWo-LM2 cells and 1×10^5 A375-Par/A375-LM3 cells were intravenously injected into NOD-SCID mice, and lungs were extracted and H&E stained on day 72 and day 49, respectively. n=4-5. (C) Expression levels of miR-199a-5p, miR-199a-3p, miR-1908, and miR-214 were determined by qRT-PCR in A375-LM3 metastatic derivatives and their parental cells. n=3. (D) Parental MeWo cells were transduced with retrovirus expressing a control hairpin or a pre-miRNA hairpin construct giving rise to miR-199a (both miR-199a-3p and miR-199a-5p), miR-1908, or miR-214. The expression levels of the target miRNAs were determined by qRT-PCR. n=3. (E) H&E-stained lung sections from Figure 1C were analyzed for the number of metastatic nodules resulting from parental MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin. n=3. (F) The number of metastatic nodules formed by LM2 cells with silenced expression of miR-199a-3p, miR-199a-5p, miR-1908, or a control sequence was analyzed in H&E-stained lung sections from Figure 1D. n=3. All data are represented as mean ± SEM.

Figure 13. MiR-199a and miR-1908 Inhibit Proliferation in vitro and Selectively Promote Cell Invasion and Endothelial Recruitment (A) 2.5×10^4 MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin were seeded in triplicate, and viable cells were counted after 5 days. n=3. (B) 1×10^5 poorly metastatic parental MeWo and highly metastatic LM2 cells were compared for their ability to invade though matrigel in a trans-well assay. n=3-4. (C) 1×10^5 endothelial cells were seeded in a 6-well plate and allowed to form a monolayer. 2×10^5 parental MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin were seeded on top of the endothelial monolayer and incubated for 30 minutes. Each monolayer was subsequently imaged, and the number of cancer cells adhering to endothelial cells was quantified. n=3. (D) 1×10^6 parental MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin were seeded in low adherent plates containing cell media supplemented with 0.2 % methylcellulose. Following

48 hours in suspension, the numbers of dead and viable cells were quantified. n=3. (E) 5 × 10^5 parental MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin were seeded in a 6-well plate and incubated in low-serum media for 48 hours, after which the number of viable cells was quantified. n=4. (F) Colony formation by parental MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin. 50 cells were seeded in a 6-cm plate, and the number of colonies formed was quantified 2 weeks later. n=4. (G) 5 × 10^4 parental MeWo and LM2 cells were seeded on the bottom of a well and assessed for their ability to recruit endothelial cells. n=6-8. (H) Percentage blood vessel density, shown as a cumulative fraction plot, for metastatic nodules formed by parental MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin. Lung sections from Figure 1C were immunohistochemically double-stained for human vimentin and MECA-32, and the MECA-32 positive area relative to the total nodule area, given by human vimentin staining, was quantified using ImageJ. n=43 nodules (control); n=117 nodules (miR-199a OE); n=55 nodules (miR-1908 OE). All data are represented as mean ± SEM. Scale bar, 100 μm.

Figure 14. MiR-199a and miR-1908 Convergently and Cooperatively Target ApoE and DNAJA4 (A) Venn diagram showing the integrative experimental approach that lead to the identification of putative target genes common to miR-199a-3p, miR-199a-5p, and miR-1908. Transcriptomic profiling of genes down-regulated by greater than 1.5-fold upon each miRNA over-expression were overlapped with genes up-regulated by more than 1.5-fold upon each miRNA silencing and with genes down-regulated by more than 1.5-fold in metastatic LM2 cells relative to their parental cell line. (B-D) Expression levels of ApoE and DNAJA4 measured by qRT-PCR in parental MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin (B), in parental MeWo cells and their highly metastatic LM2 derivative cell line (C), and in MeWo-LM2 cells with miR-Zip-based silencing of miR-199a-3p, miR-199a-5p, miR-1908, or a control sequence (D). n=3. (E) Heterologous luciferase reporter assays measuring the stability of miR-199a-3p, miR-199a-5p, or miR-1908 target site mutant ApoE and DNAJA4 3'UTR/CDS luciferase fusions in highly metastatic LM2 cells with inhibition of miR-199a-3p, miR-199a-5p, miR-1908, or a control sequence. n=3-4. (F) MeWo-LM2 cells were transduced with retrovirus expressing a control vector or an over-expression vector giving rise to ApoE or DNAJA4. The expression levels of the target genes were determined by qRT-PCR. (G) Expression levels of ApoE and DNAJA4, determined by qRT-PCR, in parental MeWo cells were transduced

with lentiviral shRNAs targeting ApoE, DNAJA4, or a control sequence. All data are represented as mean ± SEM.

Figure 15. Epistatic Interactions between miR-199a/miR-1908 and ApoE/DNAJA4 (A-D). MeWo-LM2 cells were transduced with lentiviral shRNAs targeting ApoE (A, C), DNAJA4 (B, D), or a control shRNA in the setting of miR-Zip-induced silencing of miR-1908 (A, B), miR-199a-5p (C, D), or a control sequence. The levels of the target genes were analyzed by qRT-PCR. (E) Bioluminescence imaging of lung metastasis by 1×10^5 LM2 cells expressing a control hairpin or shRNAs (independent from the shRNAs used in Figure 4E) targeting ApoE, DNAJA4, or a control sequence in the setting of miR-1908 inhibition. Representative bioluminescence images and H&E-stained lungs correspond to day 42 post-injection. n=5. (F-G) The expression levels of ApoE and DNAJA4 were analyzed by qRT-PCR in parental MeWo cells transduced with retrovirus expressing a control vector or an over-expression vector for ApoE or DNAJA4 in the setting of miR-1908 (F) or miR-199a (G) over-expression. (H-I). Parental MeWo cells over-expressing ApoE or DNAJA4 or expressing a control vector in the setting of miR-199a over-expression were examined for the invasion (H) and endothelial recruitment (I) phenotypes. n=7-8. (J) Bioluminescence imaging of lung metastasis by 4×10^4 parental MeWo cells over-expressing ApoE or DNAJA4 or expressing a control vector in the setting of miR-1908 over-expression. Representative bioluminescence images and H&E-stained lungs correspond to day 56 post-injection n=4-8. (K). Expression levels of ApoE and DNAJA4, determined by qRT-PCR, in highly metastatic A375-LM3 derivatives transduced with lentivirus expressing shRNA constructs targeting ApoE and DNAJA4 or a control sequence. All data are represented as mean ± SEM. Scale bar, 100 μm.

Figure 16. Extracellular ApoE Inhibits Melanoma Invasion and Endothelial Recruitment Phenotypes Independent of Any Effects on Cancer or Endothelial Cell Proliferation and Survival (A) Extracellular ApoE levels were measured by ELISA in conditioned media from MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin. n=3. (B-C) 3×10^4 MeWo-LM2 cells (B) or endothelial cells (C) were cultured in the presence of BSA (100 μM) or APOE (100 μM), and cell proliferation was monitored over time by counting the number of viable cells at each indicated time-point. n=3. (D-E) Survival of MeWo-LM2 cells (D) or endothelial cells (E) in the context of serum starvation in the presence of BSA (100 μM) or APOE (100 μM). n=3. (F-G) The mRNA expression

levels of ApoE were assessed in parental MeWo cells transduced with lentivirus expressing a control hairpin or short hairpin constructs targeting DNAJA4 (F) and in LM2 cells transduced with retrovirus expressing a control vector or an over-expression vector for DNAJA4 (G). n=3. (H-I) LM2 cells transduced with retrovirus expressing a control vector or an over-expression vector for DNAJA4 were assessed for their ability to invade through matrigel (H; n=6-8) and recruit endothelial cells in a trans-well assay (I; n=4) in the presence of IgG (40 μg/mL) or 1D7 (40 μg/mL) ApoE neutralization antibodies. All data are represented as mean ± SEM.

Figure 17. ApoE Inhibits Cell Invasion and Endothelial Recruitment by Targeting Melanoma Cell LRP1 and Endothelial Cell LRP8 Receptors (A) 1×10^5 LM2 cells transduced with siRNAs against LRP1 or a control sequence were analyzed for the ability to invade through matrigel. n=9-12. (B) 1×10^5 MeWo-LM2 cells inhibited for miR-199a-5p or a control sequence were transfected with siRNAs targeting LRP1 or a control siRNA and examined for their matrigel invasion capacity. n=4. (C) Representative H&E-stained lungs extracted at day 56 from NOD-SCID mice injected with MeWo-LM2 miR-1908 KD cells transduced with a control siRNA or siRNAs targeting LRP1 (See Figure 6C). (D-E) 1×10^5 endothelial cells were transfected with siRNAs targeting LRP8 or a control sequence and allowed to trans-well migrate towards 5×10^4 MeWo-LM2 cells expressing a short control hairpin (D; n=8) or 5×10^4 MeWo-LM2 cells inhibited for miR-199a-5p or a control sequence (E; n=4). All data are represented as mean ± SEM. Scale bar, 100 μm.

Figure 18. LNA-Based Inhibition of miR-199a and miR-1908 Suppresses Melanoma Metastasis (A) In vitro cell proliferation by 2.5×10^4 MeWo-LM2 cells transduced with a control LNA or a cocktail of LNAs targeting miR-199a-3p, miR199a-5p and miR-1908. The number of viable cells was quantified after five days. n=3. (B) Lung colonization by highly metastatic A375-LM3 derivatives transfected with a control LNA or a cocktail of LNAs targeting miR-199a-3p, miR199a-5p, and miR-1908. 48 hours post-transfection, 5×10^5 cells were injected intravenously into NOD-SCID mice, and lung colonization was determined by measuring bioluminescence 35 days later. n=5-6. (C) The weight of mice treated with a cocktail of LNAs targeting the three miRNAs or a mock PBS control treatment (Figure 7J) was monitored bi-weekly. n=5-6. All data are represented as mean ± SEM.

Figure 19. Activation of LXRβ Signaling Suppresses Melanoma Cell Invasion and Endothelial Recruitment. (A) Heat-map depicting microarray-based expression levels of *LXR* and *RXR* isoforms in the NCI-60 melanoma cell line collection. The heat map for these genes is extracted from the larger nuclear hormone receptor family heat map (Figure 20). Color-map key indicates the change in standard deviations for the expression value of each receptor relative to the average expression value of all microarray-profiled genes (> 39,000 transcript variants) in each cell line. (B) Cell invasion by 1×10^5 MeWo, 5×10^4 HT-144, 5×10^5 SK-Mel-2, and 5×10^4 SK-Mel-334.2 human melanoma cells. Cells were treated with DMSO, GW3965, T0901317, or Bexarotene at 1 μM for 72 hours and subjected to a trans-well matrigel invasion assay. n=4-8. (C) 5×10^4 MeWo, HT-144, SK-Mel-2, and SK-Mel-334.2 human melanoma cells were tested for their ability to recruit 1×10^5 endothelial cells in a trans-well migration assay, following treatment of the melanoma cells with DMSO, GW3965 , T0901317, or Bexarotene at 1 μM for 72 hours. n=4-8. (D-E) 1×10^5 MeWo (D) and 1×10^5 HT-144 (E) melanoma cells expressing a control shRNA or shRNAs targeting *LXRα* or *LXRβ* were subjected to the cell invasion assay following treatment of the cells with DMSO, GW3965, or T0901317 at 1 μM for 72 hours. n=4-12. (F-G) 5×10^4 MeWo (F) and 5×10^4 HT-144 (G) cells, transduced with lentiviral shRNAs targeting *LXRα* or *LXRβ* or a control shRNA, were treated with DMSO, GW3965, or T0901317 at 1 μM for 72 hours and tested for their ability to recruit 1×10^5 endothelial cells in a trans-well migration assay. n=7-8. All data are represented as mean ± SEM. Scale bar, 50 μm. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 20. Analysis of Nuclear Hormone Receptor Expression in Melanoma and Effects of LXR and RXR Agonists on *In Vitro* Cell Growth, Related to Figure 19(A-G). (A) Heat-map showing microarray-based expression levels of all nuclear hormone receptor family members across the NCI-60 collection of melanoma lines. The expression levels of each receptor is presented as the number of standard deviations below or above the average expression levels of all genes (> 39,000 transcript variants) detected by the microarray in each respective cell line. (B) 2.5×10^4 MeWo, HT-144, or SK-Mel-334.2 human melanoma cells were seeded in 6-well plates and cultured in the presence of DMSO, GW3965, T0901317, or Bexarotene at 1 μM. Viable cells were counted on day 5 post-seeding. n=3-6. (C) 2.5×10^4 MeWo, HT-144, or SK-Mel-334.2 cells were plated in triplicates and incubated in media containing DMSO, GW3965, T0901317, or Bexarotene

at 1 μM for 5 days, after which the number of dead cells was quantified using trypan blue dead cell stain. n=3. (D-G) Relative expression of *LXRα* and *LXRβ*, determined by qRT-PCR, in MeWo (D, E) and HT-144 (F, G) human melanoma cells expressing a control shRNA or shRNAs targeting *LXRα* or *LXRβ*. All data are represented as mean ± SEM.

Figure 21. Therapeutic LXR Activation Inhibits Melanoma Tumor Growth. (A-B) Primary tumor growth by 5×10^4 B16F10 mouse melanoma cells subcutaneously injected into C57BL/6-WT mice. Following tumor growth to 5-10 mm^3 in volume, mice were continuously fed a control chow or a chow supplemented with GW3965 (20 mg/kg/day or 100 mg/kg/day) (A) or T0901317 (20 mg/kg/day) (B). Representative tumor images shown correspond to tumors extracted at the final day (d12). n=10-18 (A), 8-10 (B). (C-E) Primary tumor growth by 1×10^6 MeWo (C), 7.5×10^5 SK-Mel-334.2 (D), and 2×10^6 SK-Mel-2 (E) human melanoma cells subcutaneously injected into immunocompromised mice. Following tumor growth to 5-10 mm^3 in volume, mice were randomly assigned to a control diet or a diet supplemented with GW3965 (20 mg/kg or 100 mg/kg, as indicated). Tumor images shown correspond to last day of measurements. n=6-34 (C), 8 (D), 5 (E). (F) 5×10^4 B16F10 cells were injected subcutaneously into C57BL/6-WT mice. Upon tumor growth to 150 mm^3, mice were fed continuously with a control chow or a chow containing GW3965 (150 mg/kg), and tumor growth was measured daily. n=6-13. (G-I) Mouse overall survival following subcutaneous grafting of 5×10^4 B16F10 (G), 1×10^6 MeWo (H), and 7.5×10^5 SK-Mel-334.2 cells (I) into mice that were administered a normal chow or a chow supplemented with GW3965 (100 mg/kg) upon formation of tumors measuring 5-10 mm^3 in volume. n=6-9 (F), 4-7(H), 3-6 (I). (J-L) Tumor endothelial cell density, determined by immunohistochemical staining for the mouse endothelial cell antigen MECA-32 (J), tumor cell proliferation, determined by staining for the proliferative marker Ki-67 (K), and tumor cell apoptosis, determined by staining for cleaved caspase-3 (L), in subcutaneous melanoma tumors formed by 1×10^6 MeWo human melanoma cells in response to mouse treatment with a control diet or a GW3965-supplemented diet (20 mg/kg) for 35 days. n=5. Tumor volume was calculated as $(\text{small diameter})^2 \times (\text{large diameter})/2$. All data are represented as mean ± SEM. Scale bars, 5 mm (A-D), 50 μm (J, K), 25 μm (L).

Figure 22. LXRβ Agonism Suppresses Melanoma Tumor Growth, Related to Figure 21(A-E). (A) Weight measurements of mice fed a control diet or a diet

supplemented with GW3965 (20 mg/kg/day or 100 mg/kg/day) or T0901317 (20 mg/kg) for 65 days. n=5-6.

Figure 23. LXR Agonism Suppresses Melanoma Metastasis to the Lung and Brain. (A) MeWo cells were pre-treated with DMSO or GW3965 (1 μM) for 48 hours and 4×10^4 cells were intravenously injected via the tail-vein into NOD Scid mice. Lung colonization was monitored by weekly bioluminescence imaging. Representative H&E-stained lungs correspond to the final day (d70) are shown. n=4-5. (B-C) Bioluminescence imaging of lung metastasis by 4×10^4 MeWo cells intravenously injected into NOD Scid mice that were fed a control chow or a chow containing GW3965 (20 mg/kg) or T0901317 (20 mg/kg) starting 10 days prior to cancer cell injection. Representative H&E-stained lungs correspond to final imaging day n=5-6. (B-C) Bioluminescence imaging of lung metastasis by 4×10^4 MeWo cells intravenously injected into NOD Scid mice that were fed a control chow or a chow containing GW3965 (20 mg/kg) or T0901317 (20 mg/kg) starting 10 days prior to cancer cell injection. Representative H&E-stained lungs correspond to final imaging day n=5-6. (F) Systemic and brain photon flux following intracardiac injection of 1×10^5 MeWo brain metastatic derivative cells into athymic nude mice that were fed a control diet or a GW3965-supplemented diet (100 mg/kg) starting on day 0 post-injection. n=7. (G) Schematic of experimental orthotopic metastasis model used to assess the ability of GW3965 treatment to suppress lung metastasis post-tumor excision. (H) Ex-vivo lung photon flux, determined by bioluminescence imaging, in NOD Scid mice that were administered a control chow or a chow containing GW3965 (100 mg/kg) for 1 month following the excision of size-matched (~300-mm^3 in volume) subcutaneous melanoma tumors formed by 1×10^6 MeWo melanoma cells. Representative lungs stained for human vimentin are also shown. n=7-9. (I) 4×10^4 MeWo cells were intravenously injected into NOD Scid mice. Following initiation of metastases, detected by bioluminescence imaging on d42, mice were administered a control diet or a GW3965 diet (100 mg/kg) as indicated, and lung colonization progression was measured weekly. n=6. (J) Number of macroscopic metastatic nodules in H&E-stained lungs extracted at the final day (d77) from NOD Scid mice administered a control diet or a diet supplemented with GW3965 (100 mg/kg), as indicated in (I). n=4-5. (K) Overall mouse survival following intravenous injection of 4×10^4 MeWo cells into NOD-Scid mice that were continuously fed a control chow or a

GW3965-supplemented chow (20 mg/kg) starting 10 days prior to cancer cell injection. n=5-6. All data are represented as mean ± SEM.

Figure 24. Suppression of Genetically-Driven Melanoma Progression by LXR Activation Therapy. (A) Overall survival of *Tyr::CreER; Braf*$^{V600E/+}$*; Pten*$^{lox/+}$ C57BL/6 mice following general melanoma induction by intraperitoneal administration of 4-HT (25 mg/kg) on three consecutive days. After the first 4-HT injection, mice were randomly assigned to a control diet or a diet supplemented with GW3965 (100 mg/kg). n=10-11. (B) Melanoma tumor burden, expressed as the percentage of dorsal skin area, measured on day 35 in *Tyr::CreER; Braf*$^{V600E/+}$*; Pten*$^{lox/lox}$ mice administered a control chow or a chow supplemented with GW3965 (100 mg/kg) upon melanoma induction as described in (A). n=4-5. (C) Number of macroscopic metastatic nodules to the salivary gland lymph nodes detected post-mortem in *Tyr::CreER; Braf*$^{V600E/+}$*; Pten*$^{lox/lox}$ mice that were fed a control chow or a chow containing GW3965 (100 mg/kg) following global induction of melanoma progression as described in (A). n=7-8. (D) Tumor growth following subcutaneous injection of 1×10^5 *Braf*$^{V600E/+}$*; Pten*$^{-/-}$*; CDKN2A*$^{-/-}$ primary melanoma cells into syngeneic C57BL/6-WT mice. Upon tumor growth to 5-10 mm^3 in volume, mice were fed with a control chow or a chow supplemented with GW3965 (100 mg/kg). n=16-18. (E) Overall survival of C57BL/6-WT mice subcutaneously injected with 1×10^5 *Braf*$^{V600E/+}$*; Pten*$^{-/-}$*; CDKN2A*$^{-/-}$ melanoma cells and treated with a GW3965 diet (100 mg/kg) or a control diet following tumor growth to 5-10 mm^3 in volume. n=7-8. (F) Lung colonization by 1×10^5 *Braf*$^{V600E/+}$*; Pten*$^{-/-}$*; CDKN2A*$^{-/-}$ primary melanoma cells intravenously injected into C57BL/6-WT mice. Immediately following cancer cell injection, mice were randomly assigned to a control diet or a GW3965-supplemented diet (100 mg/kg) for the remainder of the experiment. n=14-15. All data are represented as mean ± SEM. Scale bar, 2 mm (B), 5 mm (D).

Figure 25. LXR-Mediated Suppression of Melanoma Progression in a Genetically-Driven Melanoma Mouse Model, Related to Figure 24 (A-C). (A) Overall survival of *Tyr::CreER; Braf*$^{V600E/+}$*; Pten*$^{lox/lox}$ C57BL/6 mice following general melanoma induction by intraperitoneal administration of 4-HT (25 mg/kg) on three consecutive days. After the first 4-HT injection, mice were randomly assigned to a control diet or a diet supplemented with GW3965 (100 mg/kg). n=7. (B) Representative images of *Tyr::CreER; Braf*$^{V600E/+}$*;*

$Pten^{lox/lox}$ C57BL/6 mice fed a control diet of GW3965-supplemented diet (100 mg/kg) taken 43 days following melanoma induction by intraperitoneal 4-HT administration.

Figure 26. A List of the 50 most upregulated genes in MeWo human melanoma cells in response to GW3965 treatment.

Figure 27. LXRβ Activation Induces *ApoE* Expression in Melanoma Cells; ApoE mediates LXRβ-Dependent Suppression of *In Vitro* Melanoma Progression Phenotypes. (A-C) MeWo (A), HT-144 (B), and WM-266-4 (C) human melanoma cells were treated with GW3965 or T0901317 at the indicated concentrations for 48 hours, and the expression levels of *ApoE* were analysed by qRT-PCR. n=3. (D) Extracellular ApoE protein levels, quantified by ELISA, in serum-free conditioned media collected from HT-144 human melanoma cells treated with DMSO, GW3965, or T0901317 at 1 μM for 72 hours. n=3-4. (E-F) 5×10^4 HT-144 cells, treated with DMSO, GW3965, or T0901317 at 1 μM for 72 hours, were tested for the cell invasion (E) and endothelial recruitment phenotypes (F) in the presence of an ApoE neutralization antibody (1D7) or an IgG control antibody added at 40 μg/mL to each trans-well at the start of the assay. n=4. (G-H) Cell invasion (G) and endothelial recruitment (F) by 1×10^5 and 5×10^4 MeWo cells, respectively, expressing a control shRNA or an shRNA targeting *ApoE* and treated with DMSO or GW3965 at 1 μM for 72 hours prior to each assay. n=7-8. (I-J) Relative *ApoE* expression, quantified by qRT-PCR, in MeWo (I) and HT-144 (J) cells transduced with a control shRNA or shRNAs targeting *LXRα* or *LXRβ* and subsequently treated with DMSO, GW3965, or T0901317 at 1 μM for 48 hours. n=3-9. (K) Extracellular ApoE protein levels, measured by ELISA, in serum-free conditioned media harvested from HT-144 cells transduced with a control shRNA or an shRNA targeting *LXRα* or *LXRβ* and treated with DMSO or GW3965 at 1 μM for 72 hours. n=3. All data are represented as mean ± SEM. Scale bar, 50μm.

Figure 28. LXRβ Activation Suppresses Melanoma Invasion and Endothelial Recruitment by Transcriptionally Enhancing Melanoma-Cell *ApoE* Expression. (A) Luciferase activity driven off the *ApoE* promoter fused downstream of multi-enhancer element 1 (ME.1) or multi-enhancer element 2 (ME.2) sequences and transfected into MeWo cells treated with DMSO, GW3965, or T0901317 at 1 μM for 24 hours. n= 4-8. (B) Extracellular ApoE protein levels were quantified by ELISA in serum-free conditioned media harvested from MeWo cells treated with DMSO, GW3965, or T0901317 at 1 μM for 72 hours. n=3-4. (C) Cell invasion by 1×10^5 MeWo cells pre-treated with DMSO,

GW3965, or T0901317 at 1 μM for 72 hours. At the start of the assay, an ApoE neutralization antibody (1D7) or an IgG control antibody was added at 40 μg/mL to each trans-well, as indicated. n=7-8. (D) 5×10^4 MeWo cells, pre-treated with DMSO, GW3965, or T0901317 at 1 μM for 72 hours, were tested for their ability to recruit 1×10^5 endothelial cells in the presence of 1D7 or IgG antibodies at 40 μg/mL. n=6-8. (E) Extracellular ApoE protein levels, quantified by ELISA, in serum-free conditioned media from SK-Mel-334.2 primary human melanoma cells treated with DMSO or GW3965 at 1 μM for 72 hours. n=4. (F-G) 5×10^4 SK-Mel-334.2 cells, pre-treated with GW3965 at 1 μM for 72 hours, were subjected to the cell invasion (F) and endothelial recruitment (G) assays in the presence of 1D7 or IgG antibodies at 40 μg/mL. n=7-8. (H) Activity of the *ApoE* promoter fused to ME.1 or ME.2 enhancer elements was determined through measuring luciferase reporter activity in MeWo cells expressing a control shRNA or shRNAs targeting *LXRα* or *LXRβ* in the presence of DMSO or GW3965 (1μM) for 24 hours. n=3-8. (I) Extracellular ApoE protein levels, quantified by ELISA, were assessed in serum-free conditioned media collected from human MeWo melanoma cells expressing a control shRNA or shRNAs targeting *LXRα* or *LXRβ* in response to treatment with GW3965 or T0901317 (1μM) for 72 hours. n=3-8. All data are represented as mean ± SEM. Scale bar, 50 μm.

Figure 29. Therapeutic Delivery of LXR Agonists Upregulates Melanoma-Derived and Systemic *ApoE* Expression. (A-B) *ApoE* expression levels, quantified by qRT-PCR, in subcutaneous tumors formed by B16F10 mouse melanoma cells injected into C57BL/6 mice. After 5-mm^3 tumor formation, mice were fed a control diet or diet containing GW3965 (20 mg/kg) (A) or T0901317 (20 mg/kg) (B) for 7 days. n=3-4. (C-E) *ApoE* transcript expression in primary tumors (C), lung metastases (D), and brain metastases (E) formed by MeWo human melanoma cells grafted onto NOD Scid mice that were administered control chow or chow supplemented with GW3965 (20 mg/kg). *ApoE* levels were assessed on day 35 (C), day 153 (D), and day 34 (E) post-injection of the cancer cells. n=3-5. (F) Relative expression levels of *LXRα*, *LXRβ*, and *ApoE* were determined by qRT-PCR in B16F10 mouse melanoma cells expressing a control hairpin or an shRNA targeting mouse *LXRα* (sh_m*LXRα*), mouse *LXRβ* (sh_m*LXRβ*), or mouse ApoE (sh_m*ApoE*). (G-H) *ApoE* (G) and *ABCA1* (H) mRNA levels, measured by qRT-PCR, in B16F10 cells expressing a control shRNA or shRNAs targeting mouse *LXRβ* or mouse *ApoE*. The cells were treated with DMSO or GW3965 at 5 μM for 48 hours. n=3. (I) *ABCA1* mRNA levels,

measured by qRT-PCR, in systemic white blood cells extracted from *LXRα* -/- or *LXRβ* -/- mice fed a control diet or a GW3965-supplemented diet (20 mg/kg) for 10 days. n=3-4. (J) Relative expression of *ApoE* mRNA, expressed as the frequency of SAGE tags, in mouse skin and lung tissues was determined using the public mSAGE Expression Matrix database available through the NCI-funded Cancer Genome Anatomy Project (CGAP). (K) Relative expression of *ApoE* mRNA, determined by qRT-PCR, in MeWo melanoma cells dissociated from lung metastatic nodules (LM2) or primary tumors relative to control unselected MeWo parental cells. n=3.

Figure 30. LXRβ Agonism Suppresses Melanoma Tumor Growth and Metastasis by Inducing Melanoma-Derived and Systemic *ApoE* Expression. (A) Western blot measurements of ApoE protein levels in adipose, lung, and brain tissue lysates extracted from wild-type mice fed with a control chow or a chow supplemented with GW3965 (20 mg/kg) or T0901317 (20 mg/kg) for 10 days. (B) Quantification of ApoE protein expression based on western blots shown in (A). Total tubulin was used as an endogenous control for normalization. n=3-5. (C) Expression levels of *ApoE*, determined by qRT-PCR, in systemic white blood cells from mice fed a control diet or a diet supplemented with GW3965 or T0901317 at 20 mg/kg for 10 days. n=3-6. (D) B16F10 control cells or B16F10 cells expressing shRNAs targeting mouse *LXRα* (sh_m*LXRα*) or mouse *LXRβ* (sh_m*LXRβ*) were subcutaneously injected into C57BL/6-WT, *LXRα*-/-, or *LXRβ*-/- mice. Once the tumors reached 5-10 mm^3 in volume, mice were fed a control diet or a diet supplemented with GW3965 (20 mg/kg) for 7 days, after which final tumor volume was measured. Representative tumor images extracted at the end point are shown in the right panel. n=6-18. (E) *ApoE* transcript levels, quantified by qRT-PCR, in systemic white blood cells extracted from *LXRα* -/- or *LXRβ* -/- mice fed a control diet or a GW3965-supplemented diet (20 mg/kg) for 10 days. n=3-5. (F) Subcutaneous tumor growth by 5×10^4 B16F10 control cells or B16F10 cells expressing an shRNA targeting mouse *ApoE* (sh_m*ApoE*) in C57BL/6-WT or *ApoE*-/- mice. Following the formation of tumors measuring 5-10 mm^3 in volume, mice were fed a control diet or a diet supplemented with GW3965 (20 mg/kg) for 7 days, and final tumor volume was quantified. Representative images of tumors extracted at the final day of measurement (d12) are shown on the right. n=8-18. (G) Lung colonization by 5×10^4 B16F10 cells transduced with a control shRNA or sh_mA*poE* and intravenously injected into C57BL/6-WT or *ApoE*-/- mice. Starting 10

days prior to cancer cell injection, mice were assigned to a control diet or a GW3965-supplemented diet (20 mg/kg) treatment. Lung metastasis was quantified on d22 by bioluminescence imaging. Representative lungs extracted at the end point (d22) are shown in the right panel. n=5-10. (H) ApoE protein expression, determined by blinded immunohistochemical analysis, in non-metastatic (n=39) and metastatic (n=34) primary melanoma skin lesion samples obtained from patients at MSKCC. The fraction of ApoE-positively staining cell area was quantified as a percentage of total tumor area. (I) Kaplan-Meier curves for the MSKCC cohort (n=71) depicting the metastasis-free survival of patients as a function of ApoE protein expression in patients' primary melanoma lesions. Melanomas that had ApoE levels above the median of the population were classified as ApoE-positive (pos), whereas tumors with ApoE expression below the median were classified as ApoE-negative (neg). All data are represented as mean ± SEM. Scale bar, 5 mm (D and F), 100 μm (H).

Figure 31. Activation of LXRβ Suppresses the *In Vivo* Growth of Melanoma Lines Resistant to Dacarbazine and Vemurafenib. (A) *In vitro* cell growth by 2.5×10^4 B16F10 parental cells and *in vitro*-derived B16F10 DTIC-resistant cells in response to varying doses of dacarbazine (DTIC) added to the cell media for 4 days. n=3. (B-D) Tumor growth by 5×10^4 DTIC-sensitive B16F10 parental cells (B) or 5×10^4 DTIC-resistant B16F10 cells (C) subcutaneously injected into C57BL/6-WT mice. Following tumor growth to 5-10 mm^3 in volume, mice were treated with dacarbazine (50 mg/kg, i.p., daily) or a control vehicle and randomly assigned to regular chow or a chow supplemented with GW3965 (100 mg/kg). Final day tumor volume measurements are shown in (D). n=8-16 (B), 7-8 (C). (E-F) Tumor growth by DTIC-sensitive MeWo parental cells and *in vivo*-derived DTIC-resistant MeWo human melanoma cells in response to DTIC or GW3965 treatments. 5×10^5 cells were subcutaneously injected into NOD Scid gamma mice. After formation of tumors measuring 5-10 mm^3 in volume, mice were blindedly assigned to a control treatment, a DTIC treatment (50 mg/kg, i.p., administered daily in 5-day cycles with 2-day off-treatment intervals), or a GW3965-supplemented diet treatment (100 mg/kg). Final day tumor measurements are show in (F). n=6-8. (G) Tumor growth by 2×10^6 SK-Mel-239 vemurafenib-resistant clone cells subcutaneously injected into NOD Scid gamma mice that were assigned to a control diet or a diet supplemented with GW3965 (100 mg/kg) subsequent to growth of tumors to 5-10 mm^3 in volume. n=7-8. (H) Overall mouse survival

post-grafting of 2 × 10^6 SK-Mel-239 vemurafenib-resistant cells. Upon the growth of tumors to 5-10 mm^3 in volume, mice were continuously fed a control diet or a diet supplemented with GW3965 (100 mg/kg). n=7. (I) Experimentally derived model depicting the engagement of systemic and melanoma-autonomous ApoE by LXRβ activation therapy in mediating the suppression of melanoma progression phenotypes. Extracellular ApoE suppresses melanoma metastasis by coordinately inhibiting melanoma cell invasion and non-cell-autonomous endothelial recruitment through targeting melanoma-cell LRP1 and endothelial-cell LRP8 receptors, respectively. All data are represented as mean ± SEM. Scale bar, 5 mm.

Figure 32. Dacarbazine-Induced Suppression of Tumor Growth by Human Melanoma Cells. (A) Tumor growth by 5 × 10^5 DTIC-sensitive MeWo parental cells subcutaneously injected into Nod SCID gamma mice. When tumors reached 5-10 mm^3 volume, mice were treated with a control vehicle or DTIC (50 mg/kg, i.p., administered daily in 5-day cycles with 2-day off-treatment intervals), and tumor volume was measured twice a week. n=6.

Figure 33. ApoE-mediated suppression of cell invasion across multiple cancer types. (A-B) 5 × 10^4 MUM2B and OCM1 human uveal melanoma cells, (C-E) 5 × 10^4 MDA-231, MDA-468, and BT 549 human triple-negative breast cancer cells, (F-G) 5 × 10^4 PANC1 and BXPC-3 human pancreatic cancer cells, and (H-I) 5 × 10^4 786-00 and RCC4 human renal cancer cells were tested for their ability to invade through matrigel-coated trans-well inserts *in vitro*. BSA or recombinant ApoE were added to the cell media at 100 μg/mL at the start of the assay. n=4. All data are represented as mean ± SEM; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 34. Effects of LXR agonists LXR-623, WO-2007-002563 Ex. 19, WO-2010-0138598 Ex. 9, and SB742881 on ApoE expression in human melanoma cells. (A-D) MeWo human melanoma cells were treated with DMSO or the LXR agonists LXR-623 (A), WO-2007-002563 (B), WO-2010-0138598 (C), or SB742881 (D) at 500 nM, 1 μM, or 2 μM for 48 hours. The expression levels of ApoE were subsequently quantified by qRT-PCR. n=3. All data are represented as mean ± SEM. *$p<0.05$, **$p<0.01$.

Figure 35. Treatment with the LXR agonist GW3965 inhibits *In Vitro* tumor cell invasion of renal cancer, pancreatic cancer, and lung cancer. (A-C) Trans-well matrigel invasion by 5 × 10^4 RCC human renal cancer cells (A), 5 × 10^4 PANC1 human pancreatic

cancer cells (B), and 5×10^4 H460 human lung cancer cells (C) that were treated with DMSO or GW3965 at 1 μM for 72 hours prior to the assay. n=4. All data are represented as mean ± SEM. *p<0.05, **p<0.01.

Figure 36. Treatment with the LXR agonist GW3965 inhibits breast cancer tumor growth *In Vivo*. Primary tumor growth by 2×10^6 MDA-468 human breast cancer cells injected into the mammary fat pads of NOD Scid gamma mice. Two days prior to cancer cell injection, the mice were assigned to a control diet treatment or a diet supplemented with GW3965 (75 mg/kg) and maintained on the corresponding diet throughout the experiment. n=8. All data are represented as mean ± SEM. ***p<0.001.

Figure 37. Effects of LXR agonists LXR-623, WO-2007-002563 Ex. 19, WO-2010-0138598 Ex. 9, and SB742881 on *in vitro* melanoma progression phenotypes. (A) Cell invasion by 1×10^5 MeWo human melanoma cells pre-treated with DMSO, LXR-623, WO-2007-002563 Ex. 19, WO-2010-0138598 Ex. 9, or SB742881 at 1 μM each for 72 hours. The number of cells invading into the basal side of matrigel-coated trans-well inserts was quantified. n=5. (B) Endothelial recruitment by 5×10^4 MeWo cells pre-treated with DMSO, LXR-623, WO-2007-002563 Ex. 19, WO-2010-0138598 Ex.9, or SB742881 at 1 μM each for 72 hours. Cancer cells were seeded at the bottom of a 24-well plate. Endothelial cells were seeded in a trans-well insert fitted into each well and allowed to migrate towards the cancer cells. The number of endothelial cells migrating to the basal side of each trans-well insert was quantified. n=4-5. All data are represented as mean ± SEM. *p<0.05, **p<0.01.

Figure 38. Effects of LXR agonists LXR-623, WO-2007-002563 Ex. 19, WO-2010-0138598 Ex. 9, and SB742881 on *in vivo* tumor growth. (A-D) Tumor growth by 5×10^4 B16F10 mouse melanoma cells subcutaneously injected into 7-week-old C57BL/6 mice. After tumors reached 5-10 mm^3 in volume, the mice were randomly assigned to a control diet treatment, an LXR-623-supplemented diet treatment at 20 mg/kg/day (A) a WO-2007-002563 Ex. 19-supplemented diet treatment at 100 mg/kg/day (B), a WO-2010-0138598 Ex. 19-supplemented diet treatment at 10 mg/kg/day or 100 mg/kg/day (C), or an SB742881-supplemented diet treatment at 100 mg/kg/day (D). n=8-10. All data are represented as mean ± SEM.

DETAILED DESCRIPTION OF THE INVENTION

The present invention features methods for preventing or reducing aberrant proliferation, differentiation, or survival of cells. For example, compounds of the invention may be useful in reducing the risk of, or preventing, tumors from increasing in size or from reaching a metastatic state. The subject compounds may be administered to halt the progression or advancement of cancer. In addition, the instant invention includes use of the subject compounds to reduce the risk of, or prevent, a recurrence of cancer.

Metastatic progression requires that sets of effector proteins involved in common cellular phenotypes be coherently expressed (Gupta and Massagué, 2006 Cell *127*, 679-695; Hanahan and Weinberg, 2011 Cell *144*, 646-674; Talmadge and Fidler, 2010 Cancer Res. *70*, 5649-5669; Hynes, 2003 Cell *113*, 821-823). Such concerted expression states are apparent in gene expression profiles of primary breast cancers that metastasize (Wang et al., 2005 Lancet *365*, 671-679), as well as profiles of human cancer cell clones that display enhanced metastatic activity (Kang et al., 2003 Cancer Cell *3*, 537-549; Minn et al., 2005 Nature *436*, 518-524). In recent years, post-transcriptional regulation has emerged as a pervasive and robust mode of concerted expression-state and phenotype-level control. The most studied class of post-transcriptional regulators with metastatic regulatory activity are small non-coding RNAs (miRNAs) (Bartel, 2009 Cell *136*, 215-233; Fabian et al., 2010 Annu. Rev. Biochem, *79*, 351-379; Filipowicz et al., 2008 Nat. Rev. Genet. *9*, 102-114). Metastasis promoter miRNAs (Ma et al., 2007 Nature *449*, 682-688; Huang et al., 2008 Nat. Cell Biol. *10*, 202-210) and suppressor miRNAs (Tavazoie et al., 2008 Nature *451*, 147-152) were originally discovered in breast cancer. Subsequent studies revealed many more miRNAs with regulatory roles in the tumorigenesis and metastasis of other cancer types (Hatziapostolou et al., 2011 Cell *147*, 1233-1247; Hurst et al., 2009 Cancer Res. *69*, 7495-7498; Olson et al., 2009 Genes Dev. *23*, 2152-2165; Zhang et al., 2010 Oncogene *29*, 937-948) In many cases, the expression levels of these miRNAs in human cancer samples have supported their experimental roles in metastasis. Thus, deregulated miRNA expression (Garzon et al., 2010 Nat. Rev. Drug Discov. *9*, 775-789; Lujambio and Lowe, 2012 Nature *482*, 347-355) and, more recently, deregulated expression of long non-coding RNAs (Calin et al., 2007 Nat. Rev. Cancer *6*, 857-866; Gupta et al., 2010 Nature *464*, 1071-1076; Guttman et al., 2009 Nature *458*, 223-227; Huarte et al., 2010 .Cell *142*, 409-419; Loewer et al., 2010 Nat. Genet. *42*, 1113-1117) as well as non-coding pseudogenes competing for endogenous miRNA binding (Poliseno et al., 2010 Nature *465*, 1033-1038) appear to be pervasive features of human cancer. Clues regarding the robust control exerted by specific miRNAs on metastatic progression came from early work showing that concerted targeting of multiple metastasis genes by a single metastasis suppressor miRNA was responsible for the dramatic metastasis suppression effects (Tavazoie et al., 2008 Nature *451*, 147-152). Such divergent gene targeting by miRNAs has appeared to be a defining feature of these regulators.

At a conceptual level, the need for divergent regulation of gene expression in cancer is readily understood. A miRNA could exert robust metastatic suppression by virtue of its ability to target multiple genes required for metastasis. The miRNA's silencing through genetic or epigenetic mechanisms would readily promote cancer progression by de-repressing multiple promoters of metastasis (Png et al., 2011 Nature *481*, 190-194). A role for convergent regulation of a single gene by multiple metastasis regulatory miRNAs is more nuanced. This scenario would emerge if there existed a key gene that acted as a robust suppressor of metastatic progression. Convergent and cooperative targeting of this gene by multiple miRNAs could achieve maximal silencing of such a key metastasis suppressor gene. This scenario, as opposed to genetic deletion, may be seen in cases where complete loss of a target gene could not be tolerated by the cell, and the gene would be required at low levels to mediate metabolic actions, for example. Given this possibility, a search for cooperative metastasis promoter miRNAs may uncover novel genes that are pivotal for metastasis suppression and may provide therapeutic insights into more effective treatments for metastasis prevention.

As disclosed herein, via a systematic, in vivo selection-based approach, a set of miRNAs were identified to be deregulated in multiple independent metastatic lines derived from multiple patients with melanoma—a highly prevalent cancer with increasing incidence (Garbe and Leiter, 2009 Clin. Dermatol. *27*, 3-9). As disclosed herein, miR-1908, miR-199a-3p, and miR-199a-5p act as robust endogenous promoters of melanoma metastasis through convergent targeting of the metabolic gene ApoE and the heat-shock protein DNAJA4. Through loss-of-function, gain-of-function, and epistatic analyses, a cooperative miRNA network that maximally silences ApoE signaling is delineated. Cancer cell-secreted ApoE inhibits metastatic invasion and endothelial recruitment, which is mediated through its actions on distinct receptors on melanoma and endothelial cells. These miRNAs display significant prognostic capacity in identifying patients that develop melanoma metastatic relapse, while therapeutic delivery of LNAs targeting these miRNAs significantly inhibits melanoma metastasis. The current lack of effective therapies for the prevention of melanoma metastasis after surgical resection (Garbe et al., 2011 Oncologist *16*, 5-24) requires an improved molecular and mechanistic understanding of melanoma metastatic progression. To this end, the findings disclosed herein reveal a number of key novel non-coding and coding genes involved in melanoma progression and offer a novel avenue for both identifying patients at high-risk for melanoma metastasis and treating them.

Listed below are the nucleic acid and amino acid sequences of the members of the above-mentioned network and a number of other sequences.

APOE – RNA sequence (SEQ ID NO: 1)
gggatccttgagtcctactcagccccagcggaggtgaaggacgtccttccccaggagccgactggccaatcacaggcaggaaga
tgaaggttctgtgggctgcgttgctggtcacattcctggcaggatgccaggccaaggtggagcaagcggtggagacagagccgg
agcccgagctgcgccagcagaccgagtggcagagcggccagcgctgggaactggcactgggtcgcttttgggattacctgcgct
gggtgcagacactgtctgagcaggtgcaggaggagctgctcagctcccaggtcacccaggaactgagggcgctgatggacgag
accatgaaggagttgaaggcctacaaatcggaactggaggaacaactgaccccggtggcggaggagacgcgggcacggctgtc
caaggagctgcaggcggcgcaggcccggctgggcgcggacatggaggacgtgtgcggccgcctggtgcagtaccgcggcga
ggtgcaggccatgctcggccagagcaccgaggagctgcgggtgcgcctcgcctcccacctgcgcaagctgcgtaagcggctcc
tccgcgatgccgatgacctgcagaagcgcctggcagtgtaccaggccggggcccgcgagggcgccgagcgcggcctcagcgc
catccgcgagcgcctggggcccctggtggaacagggccgcgtgcgggccgccactgtgggctccctggccggccagccgcta
caggagcgggcccaggcctggggcgagcggctgcgcgcgcggatggaggagatgggcagccggacccgcgaccgcctgga
cgaggtgaaggagcaggtggcggaggtgcgcgccaagctggaggagcaggcccagcagatacgcctgcaggccgaggcctt
ccaggcccgcctcaagagctggttcgagcccctggtggaagacatgcagcgccagtgggccgggctggtggagaaggtgcag
gctgccgtgggcaccagcgccgcccctgtgcccagcgacaatcactgaacgccgaagcctgcagccatgcgaccccacgccac
cccgtgcctcctgcctccgcgcagcctgcagcgggagaccctgtccccgccccagccgtcctcctggggtggaccctagtttaata
aagattcaccaagtttcacgcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

APOE – Amino acid sequence (SEQ ID NO: 2)
mkvlwaallv tflagcqakv eqavetepep elrqqtewqs gqrwelalgr fwdylrwvqt lseqvqeell ssqvtqelra
lmdetmkelk aykseleeql tpvaeetrar lskelqaaqa rlgadmedvc grlvqyrgev qamlgqstee lrvrlashlr
klrkrllrda ddlqkrlavy qagaregaer glsairerlg plveqgrvra atvgslagqp lqeraqawge rlrarmeemg
srtrdrldev keqvaevrak leeqaqqirl qaeafqarlk swfeplvedm qrqwaglvek vqaavgtsaa pvpsdnh
(Underlined residues 136-150 represent the LRP-binding domain of Apo E)

DNAJA4 isoform 1 – RNA sequence (SEQ ID NO: 3)
agucccacccuucggcgcagggcuccggccaacacagcccuccaggccgccuacucuccagccagccggcuccacggac
ccacggaagggcaagggggcggccucggggcggcgggacaguugucggagggcgcccuccaggcccaagccgccuuc
uccggcccccgccauggcccggggcggcagucagagcuggagcuccggggaaucagacgggcagccaaaggagcagac
gcccgagaagcccagacacaagauggugaaggagacccaguacuaugacauccugggcgugaagcccagcgcgucccc
ggaggagaucaagaaggccuaucggaagcuggcgcucaaguaccacccggacaagaacccggaugagggcgagaaguu
uaaacucauaucccaggcauaugaagugcuuucagauccaaagaaaagggauguuuaugaccaaggcggagagcaggc

aauuaaagaaggaggcucaggcagccccagcuucucuucacccauggacaucuuugacauguucuuugguggugug gacggauggcuagagagagaagaggcaagaauguuguacaccaguuaucuguaacucuugaagaucuauauaaugga gucacgaagaaauuggcccuccagaaaaauguaauuugugagaaaugugaagguguuggugggaagaagggaucggu ggagaagugcccgcugugcaaggggcgggggaugcagauccacauccagcagaucgggccgggcaugguacagcaga uccagaccgugugcaucgagugcaagggccagggugagcgcaucaaccccaaggaccgcugcgagagcugcagcgggg ccaaggugauccgugagaagaagauuaucgagguacauguugaaaagguaugaaagaugggcaaaagauacuauuuc auggagaaggagaucaggagccugagcuggagccuggugaugucauaauugugcuugaucagaaggaucauagugu cuuucagagacgaggccaugacuugaucaugaaaaugaaaauucagcuuucugaagcucuuuguggcuucaagaagacg auaaaaacauuggacaaucgaauucuuguuauuacauccaaagcaggugaggugauaaagcacggggaccugagaugc gugcgcgaugaaggaaugcccaucuacaaagcaccccuggaaaaagggauucugaucauacaguuuuuaguaaucuuu ccugaaaaacacuggcuuucucuggaaaagcuuccucagcuggaagcuuuacuccccuccucgacagaaagugaggauu acagaugacauggaucagguggagcugaaggaguuuugucccaaugagcagaacuggcgucagcacagggaggccuac gaggaggacgaagacgggccccaggcuggagugcagugccagacggcaugacgugguggcggggcagcguggccccac cggacuagcacaugaugaauguaaaguuggcacaaugaaaaugacaucgcuuuaauggccuuguguuugggauguccc ugugauauguguucagcauucuuaauugcugagugucuuuuggcuuuucuuuugguuguaacuuaaguuauagcuu aauuuauauuuaaauguuuuaaguauaaaucaccucuagucugcauauggaaucuguucauuucuauuuucaggaua uacuuuugagaugucagugauugcaccaauacuuugugcuucuaguggcuuugccauaauucagugucaccaauaag gcacagcccaguuagcagcuuagccccccuagcaaaccccaaggcacaaagugggcauccugacucaucucuaggucug ugguuucuccccucuucccuuggcagaguuauugagggcaugaucucagggcugcuaagauaacauuucugaggauu cuagaugauccucuuaaagaauaaaagcacauccguggaucggacauggcugcaugugccugcuuaacagggccaacu uaguuccuacuguucugugcccuucaguggauggaacgugagugucugaucaucucucuuggaaguuuucugaaccu uccaagcucugugguggaggacaaaccaguguuugaaucauaugcugauaacuguuugccugugacccucacaccuug uucuucaggguuuuaaugauuuucuguugacaacuuuugcaaugcuuucccaccaaagugcuuacuuguaaagaaaa cuaaauccuucuguguccccggcagccucagugcagcaacagaagccaagggagaaugcugcugguuuggcccauggc acagccagcuucucugaccaguaauccgggggugacuugagggucugcaaaggcauagaacucccccaguguuuuccacc ucauucucccagauugagcucccuuccaaaggaucguuccucucauugcacagccauauuacaaaggguuuccugcuc aagugauguuuugguaagaacuucgcugaguuccacuguggauuacaguuuguauggacuacuacuguaaauuauag cuuguuuggagggauauuagucauuauuuuauucaugacagguagacuacaauucgaacuuaggguuaccucagucu uuagccauuacugcuuauuucuuuuccccaagucacaaaaaacuuguaagcugcuggguuaaagcagaggccaccugu cagaucuacccuacccuuauuugguuacauggcaccugagaguuucacucagaccagggaucuuccuuaggaggguca aagugcagaucagaccaugcagguaaggugaaccagcugcacggaccagguucccgcaaaacauugccagcuagugag gcauaauuugcucaaaguauagaaacagcccaccugugcccacuuugaccauuggugaggauagauauaaaaucacuu cuuccaacgaagccuaggugaaaaucuauuuauaaauggaccacaacucugggggugucguuuuugugcugugacuuc cuaauuauugcuaaagaacuacuguuuaguugguaaugguguaaaauuacauucagcuccuucuugucauauaaaag gaauuuggagggugucgcuuaaaauuuuauuccaccuguacauuugucacuuuaaaauuaaaauugagcugguauga gagauaaaaaaaaaaaaaaaaaaa

DNAJA4 isoform 1 – Amino acid sequence (SEQ ID NO: 4)

marggsqsws sgesdgqpke qtpekprhkm vketqyydil gvkpsaspee ikkayrklal kyhpdknpde gekfklisqa yevlsdpkkr dvydqggeqa ikeggsgsps fsspmdifdm ffggggrmar errgknvvhq lsvtledlyn gvtkklalqk nvicekcegv ggkkgsvekc plckgrgmqi hiqqigpgmv qqiqtvciec kgqgerinpk drcescsgak virekkiiev hvekgmkdgq kilfhgegdq epelepgdvi ivldqkdhsv fqrrghdlim kmkiqlseal cgfkktiktl dnrilvitsk agevikhgdl rcvrdegmpi ykaplekgil iiqflvifpe khwlsleklp qleallpprq kvritddmdq velkefcpne qnwrqhreay eededgpqag vqcqta

DNAJA4 isoform 2 – RNA sequence (SEQ ID NO: 5)

gugaccgugacgcgcgagcgggcggcgggggcgcgggccaggggcgcgggccagggugccggcaggggcguccggg
gcgcucugaccggccucgcccgcccccccgcagacacaagauggugaaggagacccaguacuaugacauccugggcg
ugaagcccagcgcguccccggaggagaucaagaaggccuaucggaagcuggcgcucaaguaccacccggacaagaaccc
ggaugagggcgagaaguuuaaacucauaucccaggcauaugaagugcuuucagauccaaagaaaagggauguuuauga
ccaaggcggagagcaggcaauuaaagaaggaggcucaggcagccccagcuucucuucacccauggacaucuuugacau
guucuuugguggugguggacggauggcuagagagagaagaggcaagaauguuguacaccaguuaucuguaacucuug
aagaucuauauaauggagucacgaagaaauuggcccuccagaaaaauguaauuugugagaaaugugaagguguuggu
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cgacagaaagugaggauuacagaugacauggaucagguggagcugaaggaguuuugucccaaugagcagaacuggcg
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uuguguuugggauguccuguguauguguucagcauucuuaauugcugagugucuuuuggcuuucuuuugguug
uaacuuaaguuauagcuuaauuuauauuuaaauguuuuaaguauaaaucaccucuagucugcauauggaaucuguuc
auuucuauuuucaggauauacuuuugagaugucagugauugcaccaauacuuugugcuucuaguggcuuugccauaa
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gacucaucucuaggucugugguuucuccccucuucccuuggcagaguuauugagggcaugaucucagggcugcuaag
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ugcuuaacagggccaacuuaguuccuacuguucugugcccuucaguggauggaacgugagugucugaucaucucucu
uggaaguuuucugaaccuuccaagcucugguggugaggacaaaccaguguuugaaucauaugcugauaacuguuugcc
ugugacccucacaccuuguucuucaggguuuuaaugauuuucuguugacaacuuuugcaaugcuuucccaccaaagu
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gcugguuuggcccauggcacagccagcuucucugaccaguaauccggggugacuugagggucugcaaaggcauagaa
cuccccaguguuuccaccucauucucccagauugagcucccuuccaaaggaucguuccucucauugcacagccauau
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cuacuacuguaaauuauagcuuguuuggagggauauuagucauuauuuuauucaugacagguagacuacaauucgaa
cuuaggguuaccucagucuuuagccauuacugcuuauuucuuuuccccaagucacaaaaaacuuguaagcugcuggg
uuaaagcagaggccaccugucagaucuacccuacccuuauuugguuacauggcaccugagaguuucacucagaccagg
gaucuuccuuaggagggucaaagugcagaucagaccaugcagguaaggugaaccagcugcacggaccagguucccgca
aaacauugccagcuagugaggcauaauuugcucaaaguauagaaacagcccaccugugcccacuuugaccauuggug a
ggauagauauaaaaucacuucuuccaacgaagccuaggugaaaaucuauuuauaaauggaccacaacucugggguguc
guuuuugugcugugacuuccuaauuauugcuaaagaacuacuguuuaguugguaaugguguaaaauuacauucagcu
ccuucuugucauauaaaaggaauuuggagggugucgcuuaaaauuuuauuccaccuguacauuugucacuuuaaaau
uaaaauugagcugguaugagagauaaaaaaaaaaaaaaaaaa

DNAJA4 isoform 2 – Amino acid sequence (SEQ ID NO: 6)
mvketqyydi lgvkpsaspe eikkayrkla lkyhpdknpd egekfklisq ayevlsdpkk rdvydqggeq
aikeggsgsp sfsspmdifd mffggggrma rerrgknvvh qlsvtledly ngvtkklalq knvicekceg
vggkkgsvek cplckgrgmq ihiqqigpgm vqqiqtvcie ckgqgerinp kdrcescsga kvirekkiie
vhvekgmkdg qkilfhgegd qepelepgdv iivldqkdhs vfqrrghdli mkmkiqlsea lcgfkktikt ldnrilvits
kagevikhgd lrcvrdegmp iykaplekgi liiqflvifp ekhwlslekl pqleallppr qkvritddmd qvelkefcpn
eqnwrqhrea yeededgpqa gvqcqta

DNAJA4 isoform 3 – RNA sequence (SEQ ID NO: 7)
acauuucagcaagcuggcuaaagacaugugggaaagccugacccuggauucaggucaaaucucagcacucacaagauu
uaaacucauaucccaggcauaugaagugcuuucagauccaaagaaaagggauguuuaugaccaaggcggagagcaggc
aauuaaagaaggaggcucaggcagccccagcuucucuucacccauggacaucuuugacauguucuuuggugguggug
gacggauggcuagagagagaagaggcaagaauguuguacaccaguuaucuguaacucuugaagaucuauauaaugga
gucacgaagaaauuggcccuccagaaaaauguaauuugugagaaaugugaagguguuggugggaagaagggaucggu
ggagaagugcccgcugugcaaggggcgggggaugcagauccacauccagcagaucgggccgggcaugguacagcaga
uccagaccgugugcaucgagugcaagggccagggugagcgcaucaaccccaaggaccgcugcgagagcugcagcgggg
ccaaggugauccgugagaagaagauuaucgagguacauguugaaaaagguaugaaagaugggcaaaagauacuauuuc
auggagaaggagaucaggagccugagcuggagccuggugaugucauaauugugcuugaucagaaggaucauaguguc
uuucagagacgaggccaugacuugaucaugaaaaugaaaauucagcuuucugaagcucuuuguggcuucaagaagacg
auaaaaacauuggacaaucgaauucuuguuauuacauccaaagcaggugaggugauaaagcacggggaccugagaugc
gugcgcgaugaaggaaugcccaucuacaaagcaccccuggaaaaagggauucugaucauacaguuuuaguaaucuuu
ccugaaaaacacuggcuuucucuggaaaagcuuccucagcuggaagcuuuacucccuccucgacagaaagugaggauu
acagaugacauggaucagguggagcugaaggaguuuugucccaaugagcagaacuggcgucagcacagggaggccuac
gaggaggacgaagacgggccccaggcuggagugcagugccagacggcaugacguggugcggggcagcguggccccac
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uguguauguguucagcauucuuaauugcugagugucuuuuggcuuuucuuuugguuguaacuuaaguuauagcuu
aauuuauauuuaaauguuuuaaguauaaaucaccucuagucugcauauggaaucuguucauuucuauuuucaggaua
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gcacagcccaguuagcagcuuagccccccuagcaaaccccaaggcacaaagugggcauccugacucaucucuaggucug
ugguuucuccccucuucccuuggcagaguuauugagggcaugaucucagggcugcuaagauaacauuucugaggauu
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cuaaauccuucuguguccccggcagccucagugcagcaacagaagccaagggagaaugcugcugguuuggcccauggc
acagccagcuucucugaccaguaauccggggugacuugagggucugcaaaggcauagaacuccccaguguuuuccacc
ucauucucccagauugagcucccuuccaaaggaucguuccucucauugcacagccauauuacaaaggguuuccugcuc
aagugauguuuugguaagaacuucgcugaguuccacuguggauuacaguuuguauggacuacuacuguaaauuauag
cuuguuuggagggauauuagucauuauuuuauucaugacagguagacuacaauucgaacuuagggguuaccucagucu
uuagccauuacugcuuauuucuuuuccccaagucacaaaaaacuuguaagcugcugggguuaaagcagaggccaccugu
cagaucuacccuacccuuauuugguuacauggcaccugagaguuucacucagaccagggaucuuccuuaggagguca
aagugcagaucagaccaugcagguaaggugaaccagcugcacggaccagguucccgcaaaacauugccagcuagugag
gcauaauuugcucaaaguauagaaacagcccaccugugcccacuuugaccauuggugaggauagauauaaaaucacuu
cuuccaacgaagccuaggugaaaaucuauuuauaaauggaccacaacucuggggugucguuuuugugcugugacuuc
cuaauuauugcuaaagaacuacuguuuaguugguaaugguguaaaauuacauucagcuccuucuugucauauaaaag

gaauuuggagggugucgcuuaaaauuuuauuccaccuguacauuugucacuuuaaaauuaaaauugagcugguauga
gagauaaaaaaaaaaaaaaaaaa

DNAJA4 isoform 3 – Amino acid sequence (SEQ ID NO: 8)

mwesltldsg qisaltrfkl isqayevlsd pkkrdvydqg geqaikeggs gspsfsspmd ifdmffgggg
rmarerrgkn vvhqlsvtle dlyngvtkkl alqknvicek cegvggkkgs vekcplckgr gmqihiqqig
pgmvqqiqtv cieckgqger inpkdrcesc sgakvirekk iievhvekgm kdgqkilfhg egdqepelep
gdviivldqk dhsvfqrrgh dlimkmkiql sealcgfkkt iktldnrilv itskagevik hgdlrcvrde gmpiykaple
kgiliiqflv ifpekhwlsl eklpqleall pprqkvritd dmdqvelkef cpneqnwrqh reayeededg pqagvqcqta

LRP1 – RNA sequence (SEQ ID NO: 9)

cagcggugcgagcuccaggcccaugcacugaggaggcggaaacaaggggagcccccagagcuccaucaagcccccucca
aaggcuccccuacccgguccacgcccccacccccccuccccgccuccucccaauugugcauuuuugcagccggaggcg
gcuccgagauggggcugugagcuucgcccggggaggggaaagagcagcgaggagugaagcgggggggugggguga
aggguuuggauuucggggcagggggcgcacccccgucagcaggcccucccccaaggggcucggaacucuaccucuucac
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LRP1 – Amino acid sequence (SEQ ID NO: 10)

mltppllll pllsalvaaa idapktcspk qfacrdqitc iskgwrcdge rdcpdgsdea peicpqskaq rcqpnehncl gtelcvpmsr lcngvqdcmd gsdegphcrc lqgncsrlgc qhhcvptldg ptcycnssfq lqadgktckd fdecsvygtc sqlctntdgs ficgcvegyl lqpdnrscka knepvdrppv lliansqnil atylsgaqvs titptstrqt tamdfsyane tvcwvhvgds aaqtqlkcar mpglkgfvde htinislslh hveqmaidwl tgnfyfvddi ddrifvcnrn gdtcvtlldl elynpkgial dpamgkvfft dygqipkver cdmdgqnrtk lvdskivfph gitldlvsrl vywadayldy ievvdyegkg rqtiiqgili ehlygltvfe nylyatnsdn anaqqktsvi rvnrfnstey qvvtrvdkgg alhiyhqrrq prvrshacen dqygkpggcs dicllanshk artcrcrsgf slgsdgksck kpehelflvy gkgrpgiirg mdmgakvpde hmipienlmn praldfhaet gfiyfadtts yligrqkidg teretilkdg ihnvegvavd wmgdnlywtd dgpkktisva rlekaaqtrk tliegkmthp raivvdplng wmywtdweed pkdsrrgrle rawmdgshrd ifvtsktvlw pnglsldipa grlywvdafy drictillng tdrkivyegp elnhafglch hgnylfwtey rsgsvyrler gvggapptvt llrserppif eirmydaqqq qvgtnkcrvn nggcsslcla tpgsrqcaca edqvldadgv tclanpsyvp ppqcqpgefa cansrciqer wkcdgdndcl dnsdeapalc hqhtcpsdrf kcennrcipn rwlcdgdndc gnsedesnat csartcppnq fscasgrcip iswtcdlddd cgdrsdesas cayptcfplt qftcnngrci ninwrcdndn dcgdnsdeag cshscsstqf kcnsgrcipe hwtcdgdndc gdysdethan ctnqatrppg gchtdefqcr ldglciplrw rcdgdtdcmd ssdekscegv thvcdpsvkf gckdsarcis kawvcdgdnd cednsdeenc eslacrppsh pcanntsvcl ppdklcdgnd dcgdgsdege lcdqcslnng gcshncsvap gegivcscpl gmelgpdnht cqiqsycakh lkcsqkcdqn kfsvkcscye gwvlepdges crsldpfkpf iifsnrheir ridlhkgdys vlvpglrnti aldfhlsqsa lywtdvvedk iyrgklldng altsfevviq yglatpegla vdwiagniyw vesnldqiev akldgtlrtt llagdiehpr aialdprdgi lfwtdwdasl prieaasmsg agrrtvhret gsggwpnglt vdylekrilw idarsdaiys arydgsghme vlrgheflsh pfavtlygge vywtdwrtnt lakankwtgh nvtvvqrtnt qpfdlqvyhp srqpmapnpc eanggqgpcs hlclinynrt vscacphlmk lhkdnttcye fkkfllyarq meirgvdlda pyynyiisft vpdidnvtvl dydareqrvy wsdvrtqaik rafingtgve tvvsadlpna hglavdwvsr nlfwtsydtn kkqinvarld gsfknavvqg lcqphglvvh plrgklywtd gdnismanmd gsnrtllfsg qkgpvglaid fpesklywis sgnhtinrcn ldgsglevid amrsqlgkat alaimgdklw wadqvsekmg tcskadgsgs vvlrnsttlv mhmkvydesi qldhkgtnpc svnngdcsql clptsettrs cmctagyslr sgqqacegvg sfllysvheg irgipldpnd ksdalvpvsg tslavgidfh aendtiywvd mglstisrak rdqtwredvv tngigrvegi avdwiagniy wtdqgfdvie varlngsfry vvisqgldkp raitvhpekg ylfwtewgqy priersrldg tervvlvnvs iswpngisvd yqdgklywcd artdkierid letgenrevv lssnnmdmfs vsvfedfiyw sdrthangsi krgskdnatd svplrtgigv qlkdikvfnr drqkgtnvca vanggcqqlc lyrgrgqrac acahgmlaed gascreyagy llysertilk sihlsdernl napvqpfedp ehmknviala fdyragtspg tpnriffsdi hfgniqqind dgsrritive nvgsveglay hrgwdtlywt syttstitrh tvdqtrpgaf eretvitmsg ddhprafvld ecqnlmfwtn wneqhpsimr aalsganvlt liekdirtpn glaidhraek lyfsdatldk ierceydgsh ryvilksepv hpfglavyge hifwtdwvrr avqrankhvg snmkllrvdi pqqpmgiiav andtnscels pcrinnggcq dlcllthqgh vncscrggri lqddltcrav nsscraqdef ecangecinf sltcdgvphc kdksdekpsy cnsrrckktf rqcsngrcvs nmlwcngadd cgdgsdeipc nktacgvgef rcrdgtcign ssrcnqfvdc edasdemncs atdcssyfrl gvkgvlfqpc ertslcyaps wvcdgandcg dysderdcpg vkrprcplny facpsgrcip mswtcdkedd cehgedethc nkfcseaqfe cqnhrciskq wlcdgsddcg dgsdeaahce gktcgpssfs cpgthvcvpe rwlcdgdkdc adgadesiaa gclynstcdd refmcqnrqc ipkhfvcdhd rdcadgsdes peceyptcgp sefrcangrc lssrqwecdg endchdqsde apknphctsq ehkcnassqf lcssgrcvae allcngqddc gdssdergch ineclsrkls gcsqdcedlk igfkcrcrpg frlkddgrtc advdecsttf pcsqrcinth gsykclcveg yaprggdphs ckavtdeepf lifanryylr klnldgsnyt llkqglnnav aldfdyreqm iywtdvttqg smirrmhlng snvqvlhrtg lsnpdglavd wvggnlywcd kgrdtievsk lngayrtvlv ssglrepral vvdvqngyly wtdwgdhsli grigmdgssr svivdtkitw pngltldyvt eriywadare dyiefasldg snrhvvlsqd iphifaltlf edyvywtdwe tksinrahkt tgtnktllis tlhrpmdlhv fhalrqpdvp nhpckvnngg csnlcllspg gghkcacptn fylgsdgrtc vsnctasqfv ckndkcipfw wkcdteddcg dhsdeppdcp efkcrpgqfq cstgictnpa ficdgdndcq dnsdeancdi hvclpsqfkc tntnrcipgi frcngqdncg dgederdcpe vtcapnqfqc sitkrciprv wvcdrdndcv dgsdepanct qmtcgvdefr ckdsgrcipa rwkcdgeddc gdgsdepkee cdertcepyq frcknnrcvp grwqcdydnd cgdnsdeesc tprpcsesef scangrciag rwkcdgdhdc adgsdekdct prcdmdqfqc ksghciplrw rcdadadcmd gsdeeacgtg vrtcpldefq cnntlckpla wkcdgeddcg dnsdenpeec arfvcppnrp frckndrvcl wigrqcdgtd ncgdgtdeed cepptahtth ckdkkeflcr nqrclssslr cnmfddcgdg sdeedcsidp kltscatnas icgdearcvr tekaaycacr sgfhtvpgqp gcqdineclr fgtcsqlcnn tkgghlcsca rnfmkthntc kaegseyqvl yiaddneirs lfpghphsay eqafqgdesv ridamdvhvk agrvywtnwh tgtisyrslp paappttsnr hrrqidrgvt hlnisglkmp rgiaidwvag nvywtdsgrd vievaqmkge nrktlisgmi dephaivvdp lrgtmywsdw gnhpkietaa mdgtlretlv qdniqwptgl avdyhnerly wadaklsvig sirlngtdpi vaadskrgls hpfsidvfed yiygvtyinn rvfkihkfgh splvnltggl shasdvvlyh qhkqpevtnp cdrkkcewlc llspsgpvct cpngkrldng tcvpvpsptp ppdaprpgtc nlqcfnggsc flnarrqpkc rcqprytgdk celdqcwehc rnggtcaasp sgmptcrcpt gftgpkctqq vcagycanns tctvnqgnqp qcrclpgflg drcqyrqcsg ycenfgtcqm aadgsrqcrc tayfegsrce vnkcsrcleg acvvnkqsgd vtcnctdgrv apscltcvgh csnggsctmn skmmpecqcp phmtgprcee hvfsqqqpgh iasilipllll lllvlvagv vfwykrrvqg akgfqhqrmt ngamnveign ptykmyegge pddvgglIda dfaldpdkpt nftnpvyatl ymgghgsrhs lastdekrel lgrgpedeig dpla

LRP8 isoform 1 – RNA sequence (SEQ ID NO: 11)

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LRP8 isoform 1 – Amino acid sequence (SEQ ID NO: 12)
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ekdceggade agcatlcaph efqcgnrscl aavfvcdgdd dcgdgsderg cadpacgpre frcggdggga
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gdefqcgdgt cvlaikhcnq eqdcpdgsde agclqglnec lhnnggcshi ctdlkigfec tcpagfqlld qktcgdidec
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cmsgyliwrn wkrkntksmn fdnpvyrktt eeedelhi grtaqighvy paaissfdrp lwaepclget
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LRP8 isoform 2 – RNA sequence (SEQ ID NO: 13)
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LRP8 isoform 2 – Amino acid sequence (SEQ ID NO: 14)

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prapsispst lspatsnhsq hyanedskmg stvtaavigi ivpivviall cmsgyliwrn wkrkntksmn fdnpvyrktt
eeededelhi grtaqighvy paaissfdrp lwaepclget repedpapal kelfvlpgep rsqlhqlpkn plselpvvks
krvalsledd glp

LRP8 isoform 3 – RNA sequence (SEQ ID NO: 15)

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LRP8 isoform 3 – Amino acid sequence (SEQ ID NO: 16)
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LRP8 isoform 4 – RNA sequence (SEQ ID NO: 17)
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ucagcucacccauggcugcugugacuccuaccagggcuuucuuggcuuuggcgaaggucaguguacagacauuccau
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uccuuacagagcuaaguggagacguuuccagaucagcccaaguuuacuauagagacuggcccaggcacugaaugucua
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gagaaagaguuugaguuucuuaacucugcuguucugccacuuacucccacuagacaaccagggacaaggugcaacaug
gaaguguuugacuuaaguaggagcagaggagcugcaucuaaucucaucauaccuggaacuugacacacuuaagcaaau
gccuucccaucccuaccugccagaugcccccaacucaaugaaguuggaugucucaccagcuugauacccuuugaauuu
ucagucagacauucuggaguucuagcauccuguaccuaggaccuuccucugugucacucuuggccuccuaaacucuaa
gaaaauaacuauauucuggagcuugggcaguguguuuugcauaauccagcaaucuccucaugacaugcauguguuga
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uggccaacuccuauggagcuuacucacauauuacuauucuuuuaagaacggaaaguaaaauuauuuuugacugaagaa
aaaugaugacagugaaaaacauggaaauguacucaaaacaagugacuuuuucuguaaccuuccaaagaaacugaauuu
uccaaggaauuaaaugauaacaguggcuaaggcauaguuucuaaacuuucaguaagauccuggcauucacagaaaaaa
augaugaaugggucuggacauacagccugagaucucaaaaugacaaugaaauucacaacuuuuucucagagacauuc
auguuuccugcauaugcuacaacugcaguuugaaagaggcagcaaugggagcaacccuuuacaagaaacaaauuguga
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uucagccucauuuugagguacaguuacauaucuuuugccuuuugcccccgugcauagcuaucuacagccaaucacaga
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agaucggauuuaaauguauggcaguaaguccuauugaucccuccaguuaucucaguaugacugcaguauauucauuc
acuaaaaccacucacuagauaccaacuacacaccuggcacugcagauguaaaggucagucacacauguucugacuuuac
agaguucacaguagcaguggaggaugauauauguggaaacaaaaaaggcauugauucuauucagagcacuguuaggg
cucaaaggagagaggggucuuuccaccuaagaaaugaggaauagggucaucauagaagugaccuuaagucuuaaaaau
uaagaaggggauuccaagcugcuucagacagagacacaucgagcuaaaacacagagguaugaaagagcacagggacuu
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gcagugggauuuucacagaugcaugccauguugucgagagccucuuagauuuucucaacugugagaaagaaaaacgaa
aauguugaagacguugagucuggagaggggauacuaaucacuguccaguugggcacuggugggaauggggaaauggc
acaggaaugcaagccucuccacccuacccccccgaacuccagccauacacucaucguuucacaaaauauaaaugaguuag
cauuaaauguuucagaguaaauaauuccuuuucccgaaaugcaugaagauagaguaacagacuucucacacuguauuu
uuaggguauggagaauuuagaagguuaaagaauuacugcuucaauuuuucaguuaaaaaaaaaucaggaagcucugu
ucauucaggcuaugcaccaugugcacagucaagaauuagcagaaacccucugcauuuacaaacacuuugugcuauaaa
aaaguaauuuuuaaaaagccacgugugugugugugugauauauauauauauauauauauauuuaaagccaagguuuugau
acuuuuuuacaaaaacuacaagagaaaacaaauauaccuguccaaaccauauacuuuuaaaagagcauuuuuuuuucca
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cugauucugcucuuuuugucuugucauucaaguuccguuagcuucuguacgcggugcccuuugcagucuggugucu
cuuccagaggcgagggggcugaggaugggggugcugcaucucacuagcuauacuggcaucaucuugguaaacugaaaa
ccaaauguggacauuuguaaaaucagugcacuguuucuagagagagauuaaauucauuuaaaaaaaa

LRP8 isoform 4 – Amino acid sequence (SEQ ID NO: 18)

mglpepgplr llalllllll llllqlqhla aaaadpllgg qgpakdcekd qfqcrnerci psvwrcdedd dcldhsdedd
cpkktcadsd ftcdnghcih erwkcdgeee cpdgsdesea tctkqvcpae klscgptshk cvpaswrcdg
ekdceggade agcatlcaph efqcgnrscl aavfvcdgdd dcgdgsderg cadpacgpre frcggdggga
ciperwvcdr qfdcedrsde aaelcgrpgp gatsapaaca tasqfacrsg ecvhlgwrcd gdrdckdksd eadcplgtcr
gdefqcgdgt cvlaikhcnq eqdcpdgsde agclqglnec lhnnggcshi ctdlkigfec tcpagfqlld qktcgdidec
kdpdacsqic vnykgyfkce cypgyemdll tknckaaagk spsliftnrh evrridlvkr nysrlipmlk
nvvaldveva tnriywcdls yrkiysaymd kasdpkeqev lideqlhspe glavdwvhkh iywtdsgnkt
isvatvdggr rrtlfsrnls epraiavdpl rgfmywsdwg dqakieksgl ngvdrqtlvs dniewpngit ldllsqrlyw
vdsklhqlss idfsggnrkt lisstdflsh pfgiavfedk vfwtdlenea ifsanrlngl eisilaenln nphdivifhe
lkqprapdac elsvqpnggc eylclpapqi sshspkytca cpdtmwlgpd mkrcyrapqs tstttlastm trtvpattra
pgttvhrsty qnhstetpsl taavpssvsv prapsispst lspatsnhsq hyanedskmg stvtaavigi ivpivviall
cmsgyliwrn wkrkntksmn fdnpvyrktt eeededelhi grtaqighvy parvalsled dglp

CTGF – RNA sequence (SEQ ID NO: 19)

aaacucacacaacaacucuuccccgcugagaggagacagccagugcgacuccacccuccagcucgacggcagccgcccc
ggccgacagccccgagacgacagcccggcgcgucccgguccccaccuccgaccaccgccagcgcuccaggccccgccgc
uccccgcucgccgccaccgcgcccuccgcuccgcccgcagugccaaccaugaccgccgccaguaugggccccguccgcg
ucgccuucgugguccuccucgcccucugcagccggccggccgucggccagaacugcagcgggccgugccggugcccgg
acgagccggcgccgcgcugcccggcgggcgugagccucgugcuggacggcugcggcugcugccgcgucugcgccaag
cagcugggcgagcugugcaccgagcgcgaccccugcgacccgcacaagggccucuucugugacuucggcuccccggcc
aaccgcaagaucggcgugugcaccgccaaagauggugcucccugcaucuucgguggacgguguaccgcagcggagag
uccuuccagagcagcugcaaguaccagugcacgugccuggacggggcggugggcugcaugccccugugcagcaugga
cguucgucugcccagcccugacugccccuucccgaggagggucaagcugcccgggaaaugcugcgaggagugggugu
gugacgagcccaaggaccaaaccgugguugggccugcccucgcggcuuaccgacuggaagacacguuuggcccagacc
caacuaugauuagagccaacugccugguccagaccacagaguggagcgccuguuccaagaccugugggaugggcaucu
ccacccggguuaccaaugacaacgccuccugcaggcuagagaagcagagccgccugugcauggucaggccuugcgaag
cugaccuggaagagaacauuaagaagggcaaaaagugcauccguacucccaaaaucuccaagccuaucaaguuugagcu
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cugugccugccauuacaacugucccggagacaaugacaucuuugaaucgcuguacuacaggaagauguacggagacau
ggcaugaagccagagagugagagacauuaacucauuagacuggaacuugaacugauucacaucucauuuuuccguaaa
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ugccaugucaaacaaauagucuaucaaccccagacacugguuugaagaauguuaagacuugacaguggaacuacauua
guacacagcaccagaauguauauuaagguguggcuuuaggagcagugggaggguaccagcagaaagguuaguaucau
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agaacagcagacucagcucugacauucugauucgaaugacacuguucaggaaucggaauccugucgauuagacuggac
agcuuguggcaagugaauuugccuguaacaagccagauuuuuuaaaauuuauauuguaaauauugugugugugugu
gugugugauauauauauauauguacaguuaucuaaguuaauuuaaaguuguuugugccuuuuuauuuuuguuuuu
aaugcuuugauauuucaauguuagccucaauuucugaacaccauagguagaauguaaagcuugucugaucguucaaa
gcaugaaauggauacuuauauggaaauucugcucagauagaaugacaguccgucaaaacagauuguuugcaaaggga
ggcaucaguguccuuggcaggcugauuucuagguaggaaaugugguagccucacuuuuaaugaacaaauggccuuua
uuaaaaacugagugacucuauauagcugaucaguuuuucaccuggaagcauuuguuucuacuuugauaugacuguu
uuucggacaguuuauuuguugagagugugaccaaaguuacauguuugcaccuuucuaguugaaaauaaaguguaua
uuuuuucuauaaaaaaaaaaaaaaaa

CTGF – Amino Acid sequence (SEQ ID NO: 20)
mtaasmgpvr vafvvllalc srpavgqncs gpcrcpdepa prcpagvslv ldgcgccrvc akqlgelcte
rdpcdphkgl fcdfgspanr kigvctakdg apcifggtvy rsgesfqssc kyqctcldga vgcmplcsmd
vrlpspdcpf prrvklpgkc ceewvcdepk dqtvvgpala ayrledtfgp dptmirancl vqttewsacs
ktcgmgistr vtndnascrl ekqsrlcmvr pceadleeni kkgkkcirtp kiskpikfel sgctsmktyr akfcgvctdg
rcctphrttt lpvefkcpdg evmkknmmfi ktcachyncp gdndifesly yrkmygdma

LXR-a isoform 1: RNA sequence (SEQ ID NO: 21)
aggaaggaggggguggccugaccccucggcagucccucccccucagccuuuccccaaauugcuacuucucuggggcucca
gguccugcuugugcucagcuccagcucacuggcuggccaccgagacuucuggacaggaaacugcaccauccucuucuc
ccagcaagggggcuccagagacugcccacccaggaagucugguggccugggggauuuggacagugccuugguaaugac
cagggcuccaggaagagauguccuuguggcugggggccccugugccugacauuccuccugacucugcgguggagcug
uggaagccaggcgcacaggaugcaagcagccaggcccagggaggcagcagcugcauccucagagaggaagccaggaug
ccccacucugcugggggguacugcaggggugggggcuggaggcugcagagcccacagcccugcucaccagggcagagccc
ccuucagaacccacagagauccguccacaaaagcggaaaaagggggccagcccccaaaaugcuggggaacgagcuaugca
gcgugugugggggacaaggccucgggcuuccacuacaauguucugagcugcgagggcugcaagggauucuuccgccgc
agcgucaucaagggagcgcacuacaucugccacaguggcggccacugccccauggacaccuacaugcgucgcaagugc
caggagugucggcuucgcaaaugccgucaggcuggcaugcgggaggagugugugccugucagaagaacagauccgccu
gaagaaacugaagcggcaagaggaggaacaggcucaugccacauccuugcccccccagggcuuccucacccccccaaauc
cugccccagcucagcccggaacaacugggcaugaucgagaagcucgucgcugcccagcaacaguguaaccggcgcucc
uuuucugaccggcuucgagucacgccuuggcccauggcaccagauccccauagccgggaggcccgucagcagcgcuuu
gcccacuucacugagcuggccaucgucucugugcaggagauaguugacuuugcuaaacagcuacccggcuuccugcag
cucagccgggaggaccagauugcccugcugaagaccucugcgaucgaggugaugcuucuggagacaucucggaggua
caacccugggagugagaguaucaccuuccucaaggauuucaguuauaaccgggaagacuuugccaaagcagggcugca
aguggaauucaucaaccccaucuucgaguucuccagggccaugaaugagcugcaacucaaugaugccgaguuugccuu
gcucauugcuaucagcaucuucucugcagaccggcccaacgugcaggaccagcuccagguagagaggcugcagcacac
auauguggaagcccugcaugccuacgucuccauccaccaucccccaugaccgacugauguucccacggaugcuaaugaa
acuggugagccuccggacccugagcagcguccacucagagcaaguguuugcacugcgucugcaggacaaaaagcuccc
accgcugcucucugagaucugggaugugcacgaaugacuguucuguccccauauuuucuguuuucuuggccggaugg
cugaggccugguggcugccuccuagaaguggaacagacugagaagggcaaacauuccugggagcugggcaaggagau
ccucccguggcauuaaaagagagucaaaggguugcgaguuuuguggcuacugagcaguggagcccucgcuaacacug
ugcugugucugaagaucaugcugaccccacaaacggaugggccugggggccacuuugcacaggguucuccagagcccu
gcccauccugccuccaccacuuccuguuuuucccacagggccccaagaaaaauucuccacugucaaaaaaaaa

LXR-a (NR1H3) isoform 1: Amino acid sequence (SEQ ID NO: 22)
mslwlgapvp dippdsavel wkpgaqdass qaqggsscil reearmphsa ggtagvglea aeptalltra
eppsepteir pqkrkkgpap kmlgnelcsv cgdkasgfhy nvlscegckg ffrrsvikga hyichsgghc
pmdtymrrkc qecrlrkcrq agmreecvls eeqirlkklk rqeeeqahat slpprasspp qilpqlspeq lgmieklvaa
qqqcnrrsfs drlrvtpwpm apdphsrear qqrfahftel aivsvqeivd fakqlpgflq lsredqiall ktsaievmll
etsrrynpgs esitflkdfs ynredfakag lqvefinpif efsramnelq lndaefalli aisifsadrp nvqdqlqver
lqhtyvealh ayvsihhphd rlmfprmlmk lvslrtlssv hseqvfalrl qdkklpplls eiwdvhe

LXR-a (NR1H3) isoform 2: RNA sequence (SEQ ID NO: 23)
aggaaggaggggguggccugaccccucggcagucccucccccucagccuuuccccaaauugcuacuucucuggggcucca
gguccugcuugugcucagcuccagcucacuggcuggccaccgagacuucuggacaggaaacugcaccauccucuucuc
ccagcaagggggcuccagagacugcccacccaggaagucugguggccugggggauuuggacagugccuugguaaugac
cagggcuccaggaagagauguccuuguggcugggggccccugugccugacauuccuccugacucugcgguggagcug

uggaagccaggcgcacaggaugcaagcagccaggcccagggaggcagcagcugcauccucagagaggaagccaggaug
ccccacucugcugggggguacugcaggggugggggcuggaggcugcagagcccacagcccugcucaccagggcagagccc
ccuucagaacccacagagauccguccacaaaagcggaaaaaggggccagcccccaaaaugcuggggaacgagcuaugca
gcguguguggggacaaggccucgggcuuccacuacaauguucugagcugcgagggcugcaagggauucuuccgccgc
agcgucaucaagggagcgcacuacaucugccacaguggcggccacugccccauggacaccuacaugcgucgcaagugc
caggagugucggcuucgcaaaugccgucaggcuggcaugcgggaggagugugucccugucagaagaacagauccgccu
gaagaaacugaagcggcaagaggaggaacaggcucaugccacauccuugccccccagggcuuccucaccccccccaaauc
cugccccagcucagcccggaacaacugggcaugaucgagaagcucgucgcugcccagcaacaguguaaccggcgcucc
uuuucugaccggcuucgagucacggugaugcuucuggagacaucucggagguacaacccugggagugagaguaucac
cuuccucaaggauuucaguuauaaccgggaagacuuugccaaagcagggcugcaaguggaauucaucaaccccaucuu
cgaguucuccagggccaugaaugagcugcaacucaaugaugccgaguuugccuugcucauugcuaucagcaucuucuc
ugcagaccggcccaacgugcaggaccagcuccagguagagaggcugcagcacacauauguggaagcccugcaugccua
cgucuccauccaccauccccaugaccgacugauguucccacggaugcuaaugaaacuggugagccuccggacccugag
cagcguccacucagagcaaguguuugcacugcgucugcaggacaaaaagcucccaccgcugcucucugagaucuggga
ugugcacgaaugacuguucuguccccauauuuucuguuuucuuggccggauggcugaggccugguggcugccuccua
gaaguggaacagacugagaagggcaaacauuccugggagcugggcaaggagauccucccguggcauuaaaagagaguc
aaaggguugcgaguuuuguggcuacugagcaguggagcccucgcuaacacugugcugugucugaagaucaugcugac
cccacaaacggaugggccugggggccacuuugcacaggguucuccagagcccugcccauccugccuccaccacuuccu
guuuucccacagggccccaagaaaaauucuccacugucaaaaaaaaa

LXR-a (NR1H3) isoform 2 : Amino acid sequence (SEQ ID NO: 24)

mslwlgapvp dippdsavel wkpgaqdass qaqggsscil reearmphsa ggtagvglea aeptalltra
eppsepteir pqkrkkgpap kmlgnelcsv cgdkasgfhy nvlscegckg ffrrsvikga hyichsgghc
pmdtymrrkc qecrlrkcrq agmreecvls eeqirlkklk rqeeeqahat slpprasspp qilpqlspeq lgmieklvaa
qqqcnrrsfs drlrvtvmll etsrrynpgs esitflkdfs ynredfakag lqvefinpif efsramnelq lndaefalli
aisifsadrp nvqdqlqver lqhtyvealh ayvsihhphd rlmfprmlmk lvslrtlssv hseqvfalrl qdkklppls
eiwdvhe

LXR-a (NR1H3) isoform 3: RNA sequence (SEQ ID NO: 25)

aucuuacuuagggaccugcuggggugcggggaaaaggcgcagucucggugggauugcgugcaggagggucguggguc
uggcuguggcggaggagcauaagaagacucugcgguggagcuguggaagccaggcgcacaggaugcaagcagccagg
cccagggaggcagcagcugcauccucagagaggaagccaggaugccccacucugcugggggguacugcaggggugggg
cuggaggcugcagagcccacagcccugcucaccagggcagagcccccuucagaacccacagagauccguccacaaaagc
ggaaaaaggggccagcccccaaaaugcuggggaacgagcuaugcagcguguguggggacaaggccucgggcuuccacu
acaauguucugagcugcgagggcugcaagggauucuuccgccgcagcgucaucaagggagcgcacuacaucugccaca
guggcggccacugccccauggacaccuacaugcgucgcaagugccaggagugucggcuucgcaaaugccgucaggcug
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augccacauccuugccccccagggcuuccucaccccccccaaauccugccccagcucagcccggaacaacugggcaugau
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cucugcgaucgaggugaugcuucuggagacaucucggagguacaacccugggagugagaguaucaccuuccucaagg
auuucaguuauaaccgggaagacuuugccaaagcagggcugcaaguggaauucaucaaccccaucuucgaguucucca
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ccaacgugcaggaccagcuccagguagagaggcugcagcacacauauguggaagcccugcaugccuacgucuccaucc
accauccccaugaccgacugauguucccacggaugcuaaugaaacuggugagccuccggacccugagcagcguccacu
cagagcaaguguuugcacugcgucugcaggacaaaaagcucccaccgcugcucucugagaucugggaugugcacgaau

gacuguucuguccccauauuuucuguuuucuuggccggauggcugaggccugguggcugccuccuagaaguggaaca gacugagaagggcaaacauuccugggagcugggcaaggagauccuccccguggcauuaaaagagagucaaggguugcg aguuuuguggcuacugagcaguggagcccucgcuaacacugugcugugucugaagaucaugcugaccccacaaacgga ugggccugggggccacuuugcacaggguucuccagagcccugcccauccugccuccaccacuuccuguuuuucccaca gggccccaagaaaaauucuccacugucaaaaaaaaa

LXR-a (NR1H3) isoform 3: Amino acid sequence (SEQ ID NO: 26)

mphsaggtag vgleaaepta lltraeppse pteirpqkrk kgpapkmlgn elcsvcgdka sgfhynvlsc egckgffrrs vikgahyich sgghcpmdty mrrkcqecrl rkcrqagmre ecvlseeqir lkklkrqeee qahatslppr assppqilpq lspeqlgmie klvaaqqqcn rrsfsdrlrv tpwpmapdph srearqqrfa hftelaivsv qeivdfakql pgflqlsred qiallktsai evmlletsrr ynpgsesitf lkdfsynred fakaglqvef inpifefsra mnelqlndae falliaisif sadrpnvqdq lqverlqhty vealhayvsi hhphdrlmfp rmlmklvslr tlssvhseqv falrlqdkkl ppllseiwdv he

LXR-a (NR1H3) isoform 4: RNA sequence (SEQ ID NO: 27)

gauucuaacuuagcuaagcaaugcuacuggagaccauaggcaaagccaagguacagcuucagggaagucuuuggugag cccaucucucauuaccaagguaacgaagcgcagacuccgggcccggguggggcggcaucaccaccagguucacgccgag aaggagcuggaggagagccgccccggcuccagccggaccgcuugcccgccaucaccguuguaaucuaugcagcaaacaa gcuggaacccgcugggguggcaccugcaagcagccgccccggacgcacccacucugcgguggagcuguggaagccaggcg cacaggaugcaagcagccaggcccagggaggcagcagcugcauccucagagaggaagccaggaugccccacucugcug gggguacugcaggggugggggcuggaggcugcagagcccacagcccugcucaccagggcagagcccccuucagaaccca cagagauccguccacaaaagcggaaaaaggggccagcccccaaaaugcuggggaacgagcuaugcagcgugugugggg acaaggccucgggcuuccacuacaauguucugagcugcgagggcugcaagggauucuuccgccgcagcgucaucaagg gagcgcacuacaucugccacaguggcggccacugccccauggacaccuacaugcgucgcaagugccaggagugucggc uucgcaaaugccgucaggcuggcaugcgggaggagugugugccugucagaagaacagauccgccugaagaaacugaagc ggcaagaggaggaacaggcucaugccacauccuugcccccagggcuuccucacccccccaaauccugccccagcucag cccggaacaacugggcaugaucgagaagcucgucgcugcccagcaacaguguaaccggcgcuccuuuucugaccggcu ucgagucacgccuuggcccauggcaccagauccccauagccgggaggcccgucagcagcgcuuugcccacuucacuga gcuggccaucgucucugugcaggagauaguugacuuugcuaaacagcuacccggcuuccugcagcucagccgggagg accagauugcccugcugaagaccucugcgaucgaggugaugcuucuggagacaucucggagguacaacccugggagu gagaguaucaccuuccucaaggauuucaguuauaaccgggaagacuuugccaaagcagggcugcaaguggaauucauc aacccccaucuucgaguucuccagggccaugaaugagcugcaacucaaugaugccgaguuugccuugcucauugcuauc agcaucuucucugcagaccggcccaacgugcaggaccagcuccagguagagaggcugcagcacacauauguggaagcc cugcaugccuacgucuccauccaccauccccaugaccgacugauguuccccacggaugcuaaugaaacuggugagcccuc cggacccugagcagcguccacucagagcaaguguuugcacugcgucugcaggacaaaaagcucccaccgcugcucucu gagaucugggaugugcacgaaugacuguucuguccccauauuuucuguuuucuuggccggauggcugaggccuggu ggcugccuccuagaaguggaacagacugagaagggcaaacauuccugggagcugggcaaggagauccuccccguggcau uaaaagagagucaaggguugcgaguuuuguggcuacugagcaguggagcccucgcuaacacugugcugugucugaa gaucaugcugaccccacaaacggaugggccugggggccacuuugcacaggguucuccagagcccugcccauccugccu ccaccacuuccuguuuuucccacagggccccaagaaaaauucuccacugucaaaaaaaaa

LXR-a (NR1H3) isoform 4: Amino acid sequence (SEQ ID NO: 28)

mqqtswnplg gtckqppgrt hsavelwkpg aqdassqaqg gsscilreea rmphsaggta gvgleaaept alltraepps epteirpqkr kkgpapkmlg nelcsvcgdk asgfhynvls cegckgffrr svikgahyic hsgghcpmdt ymrrkcqecr lrkcrqagmr eecvlseeqi rlkklkrqee eqahatslpp rassppqilp qlspeqlgmi eklvaaqqqc nrrsfsdrlr vtpwpmapdp hsrearqqrf ahftelaivs vqeivdfakq lpgflqlsre dqiallktsa ievmlletsr rynpgsesit flkdfsynre dfakaglqve finpifefsr amnelqlnda efalliaisi fsadrpnvqd qlqverlqht yvealhayvs ihhphdrlmf prmlmklvsl rtlssvhseq vfalrlqdkk lppllseiwd vhe

LXR-b (NR1H2) isoform 1: RNA sequence (SEQ ID NO: 29)

ucgucaaguuucacgcuccgccccucuuccggacgugacgcaagggcgggguugccggaagaaguggcgaaguuacu uuugaggguauuugaguagcggcggugugucaggggcuaaagaggaggacgaagaaaagcagagcaagggaacccag ggcaacaggaguaguucacuccgcgagaggccguccacgagaccccgcgcgcagccaugagccccgcccccgcuguu gcuuggagaggggcgggaccuggagagaggcugcuccgugaccccaccauguccucuccuaccacgaguucccuggau accccccugccuggaaauggcccccucagccuggcgccccuucuucuucacccacuguaaaggaggaggguccggag ccguggcccgggggucggacccugaugucccaggcacugaugaggccagcucagccugcagcacagacugggucauc ccagaucccgaagaggaaccagagcgcaagcgaaagaagggcccagccccgaagaugcuggccacgagcuuugccgu gucuguggggacaaggccuccggcuuccacuacaacgugcucagcugcgaaggcugcaagggcuucuuccggcgcag uguggucccgugguggggccaggcgcuaugccugccgggguggcggaaccugccagauggacgcuuucaugcggcgca agugccagcagugccggcugcgcaagugcaaggaggcagggaugagggagcagugcguccuuucugaagaacagauc cggaagaagaagauucggaaacaacagcagcaggagucacagucacagucgcagucaccugugggggccgcagggcagc agcagcucagccucugggccugggggcuuccccugguggaucugaggcaggcagccagggcuccggggaaggcgaggg uguccagcuaacagcggcucaagaacuaaugauccagcaguugguggcggcccaacugcagugcaacaaacgcuccuu cuccgaccagcccaaagucacgcccuggccccugggcgcagacccccagucccgagaugcccgccagcaacgcuuugcc cacuucacggagcuggccaucaucucaguccaggagaucguggacuucgcuaagcaagugccugguuuccugcagcug ggccgggaggaccagaucgcccuccugaaggcauccacuaucgagaucaugcugcuagagacagccaggcgcuacaac cacgagacagagugu aucaccuucuugaaggacuucaccuacagcaaggacgacuuccaccgugcaggccugcaggug gaguucaucaaccccaucuucgaguucucgcgggccaugcggcggcugggccuggacgacgcugaguacgcccugcuc aucgccaucaacaucuucucggccgaccggcccaacgugcaggagccgggccgcguggaggcguugcagcagcccuac guggaggcgcugcuguccuacacgcgcaucaagaggccgcaggaccagcugcgcuucccgcgcaugcucaugaagcug gugagccugcgcacgcugagcucugugcacucggagcaggucuucgccuugcggcuccaggacaagaagcugccgccu cugcugucggagaucugggacguccacgagugaggggcuggccacccagccccacagccuugccugaccacccuccag cagauagacgccggcaccccuuccucuuccuaggguggaaggggcccugggccgagccuguagaccuaucggcucuca ucccuugggauaagccccaguccagguccaggaggcucccucccugcccagcgagucuuccagaaggggugaaagggu ugcaggucccgaccacugacccuucccggcugcccucccuccccagcuuacaccucaagcccagcacgcagugcaccuu gaacagagggaggggaggacccauggcucucccccuagcccgggagaccagggccuuccucuuccucugcuuuuauu uaauaaaaacuaaaaacagaaacaggaaaauaaaauaugaauacaauccagcccggagcuggagugca

LXR-b (NR1H2) isoform 1: Amino acid sequence (SEQ ID NO: 30)

msspttssld tplpgngppq pgapssspty keegpepwpg gpdpdvpgtd eassacstdw vipdpeeepe rkrkkgpapk mlghelcrvc gdkasgfhyn vlscegckgf frrsvvrgga rryacrgggt cqmdafmrrk cqqcrlrkck eagmreqcvl seeqirkkki rkqqqqesqs qsqspvgpqg ssssasgpga spggseagsq gsgegegvql taaqelmiqq lvaaqlqcnk rsfsdqpkvt pwplgadpqs rdarqqrfah ftelaiisvq eivdfakqvp gflqlgredq iallkastie imlletarry nhetecitfl kdftyskddf hraglqvefi npifefsram rrlglddaey alliainifs adrpnvqepg rvealqqpyv eallsytrik rpqdqlrfpr mlmklvslrt lssvhseqvf alrlqdkklp pllseiwdvh e

LXR-b (NR1H2) isoform 2: RNA sequence (SEQ ID NO: 31)

ucgucaaguuucacgcuccgccccucuuccggacgugacgcaagggcgggguugccggaagaaguggcgaaguuacu uuugaggguauuugaguagcggcggugugucaggggcuaaagaggaggacgaagaaaagcagagcaagggaacccag ggcaacaggaguaguucacuccgcgagaggccguccacgagaccccgcgcgcagccaugagccccgcccccgcuguu gcuuggagaggggcgggaccuggagagaggcugcuccgugaccccaccauguccucuccuaccacgaguucccuggau accccccugccuggaaauggcccccucagccuggcgccccuucuucuucacccacuguaaaggaggaggguccggag ccguggcccgggggguccggacccugaugucccaggcacugaugaggccagcucagccugcagcacagacuggggcguc cuuucugaagaacagauccggaagaagaagauucggaaacaacagcagcaggagucacagucacagucgcagucaccug uggggccgcagggcagcagcagcucagccucugggccuggggcuuccccugguggaucugaggcaggcagccagggc uccggggaaggcgaggguguccagcuaacagcggcucaagaacuaaugauccagcaguugguggcggcccaacugcag ugcaacaaacgcuccuucuccgaccagcccaaagucacgcccuggccccugggcgcagacccccagucccgagaugccc gccagcaacgcuuugcccacuucacggagcuggccaucaucucaguccaggagaucguggacuucgcuaagcaagugc cugguuuccugcagcugggccgggaggaccagaucgcccuccugaaggcauccacuaucgagaucaugcugcuagaga cagccaggcgcuacaaccacgagacagaguguaucaccuucuugaaggacuucaccuacagcaaggacgacuuccaccg ugcaggccugcagguggaguucaucaaccccaucuucgaguucucgcgggccaugcggcggcugggccuggacgacg cugaguacgcccugcucaucgccaucaacaucuucucggccgaccggcccaacgugcaggagccgggccgcguggagg cguugcagcagcccuacguggaggcgcugcuguccuacacgcgcaucaagaggccgcaggaccagcugcgcuucccgc gcaugcucaugaagcuggugagccugcgcacgcugagcucugugcacucggagcaggucuucgccuugcggcuccag gacaagaagcugccgccucugcugucggagaucugggacguccacgagugaggggcuggccacccagccccacagccu ugccugaccacccuccagcagauagacgccggcaccccuuccucuuccuaggguggaaggggcccugggccgagccug uagaccuaucggcucucaucccuugggauaagccccaguccagguccaggaggcucccucccugcccagcgagucuuc cagaaggggugaaaggguugcaggucccgaccacugacccuucccggcugcccucccucccccagcuuacaccucaagcc cagcacgcagugcaccuugaacagagggaggggaggacccauggcucucccccccuagcccgggagaccagggccuucc ucuuccucugcuuuuauuuaauaaaaacuaaaaacagaaacaggaaaauaaaauaugaauacaauccagcccggagcug gagugca

LXR-b (NR1H2) isoform 2 : Amino acid sequence (SEQ ID NO: 32)
msspttssld tplpgngppq pgapssssptv keegpepwpg gpdpdvpgtd eassacstdw gvlseeqirk kkirkqqqqe sqsqsqspvg pqgssssasg pgaspggsea gsqgsgegeg vqltaaqelm iqqlvaaqlq cnkrsfsdqp kvtpwplgad pqsrdarqqr fahftelaii svqeivdfak qvpgflqlgr edqiallkas tieimlleta rrynheteci tflkdftysk ddfhraglqv efinpifefs ramrrlgldd aeyalliain ifsadrpnvq epgrvealqq pyveallsyt rikrpqdqlr fprmlmklvs lrtlssvhse qvfalrlqdk klppllseiw dvhe

has-miR-199a-1 sequence (SEQ ID NO: 33)
GCCAACCCAGUGUUCAGACUACCUGUUCAGGAGGCUCUCAAUGUGUACAGUA GUCUGCACAUUGGUUAGGC

has-miR-199a-2 sequence (SEQ ID NO: 34)
AGGAAGCUUCUGGAGAUCCUGCUCCGUCGCCCCAGUGUUCAGACUACCUGUU CAGGACAAUGCCGUUGUACAGUAGUCUGCACAUUGGUUAGACUGGGCAAGG GAGAGCA

has-miR-1908 sequence (SEQ ID NO: 35)
CGGGAAUGCCGCGGCGGGGACGGCGAUUGGUCCGUAUGUGUGGUGCCACCGG CCGCCGGCUCCGCCCCGGCCCCCGCCCC

has-miR-7-1 sequence (SEQ ID NO: 36)
UUGGAUGUUGGCCUAGUUCUGUGUGGAAGACUAGUGAUUUUGUUGUUUUUA GAUAACUAAAUCGACAACAAAUCACAGUCUGCCAUAUGGCACAGGCCAUGCC UCUACAG

has-miR-7-2 sequence (SEQ ID NO: 37)
CUGGAUACAGAGUGGACCGGCUGGCCCCAUCUGGAAGACUAGUGAUUUUGU

UGUUGUCUUACUGCGCUCAACAACAAAUCCCAGUCUACCUAAUGGUGCCAGC
CAUCGCA

has-miR-7-3 sequence (SEQ ID NO: 38)
AGAUUAGAGUGGCUGUGGUCUAGUGCUGUGUGGAAGACUAGUGAUUUUGUU
GUUCUGAUGUACUACGACAACAAGUCACAGCCGGCCUCAUAGCGCAGACUCC
CUUCGAC

miR-Zip 199a-3p sequence (SEQ ID NO: 39)
GATCCGACAGTAGCCTGCACATTAGTCACTTCCTGTCAGTAACCAATGTGCAGA
CTACTGTTTTTGAATT

miR-Zip 199a-5p sequence (SEQ ID NO: 40)
GATCCGCCCAGTGCTCAGACTACCCGTGCCTTCCTGTCAGGAACAGGTAGTCTG
A
ACACTGGGTTTTTGAATT

miR-Zip 1908 sequence (SEQ ID NO: 41)
GATCCGCGGCGGGAACGGCGATCGGCCCTTCCTGTCAGGACCAATCGCCGTCC
CCGCCGTTTTTGAATT

miR-Zip 7 sequence (SEQ ID NO: 42)
GATCCGTGGAAGATTAGTGAGTTTATTATCTTCCTGTCAGACAACAAAATCACT
AGTCTTCCATTTTTGAATT

The members of this network can be used as targets for treating metastatic melanoma. In addition, the members can be used a biomarkers for determining whether a subject has, or is at risk of having, a metastatic melanoma or for determining a prognosis or surveillance of patient having the disorder. Accordingly, the present invention encompasses methods of treating metastatic melanoma by targeting one or more of the members, methods of determining the efficacy of therapeutic regimens for inhibiting the cancer, and methods of identifying anti-cancer agent. Also provided are methods of diagnosing whether a subject has, or is at risk for having, metastatic melanoma, and methods of screening subjects who are thought to be at risk for developing the disorder. The invention also encompasses various kits suitable for carrying out the above mentioned methods.

ApoE polypeptides

The term "polypeptide or peptide" as used herein includes recombinantly or synthetically produced fusion or chimeric versions of any of the aforementioned metastasis suppressors, having the particular domains or portions that are involved in the network. The term also encompasses an analog, fragment, elongation or derivative of the peptide (e.g. that have an added amino-terminal methionine, useful for expression in prokaryotic cells).

"Apolipoprotein polypeptide or ApoE polypeptide" as used herein means a peptide, drug, or compound that mimics a function of the native apolipoprotein either in vivo or in vitro including apolipoprotein analogs, fragments, elongations or derivatives that are a peptide of between 10 and 200 amino acid residues in length, such peptides can contain either natural, or non-natural amino acids containing amide bonds. Apolipoprotein peptide fragments may be modified to improve their stability or bioavailability in vivo as known in the art and may contain organic compounds bound to the amino acid side chains through a variety of bonds.

In one aspect, our invention is a method for using an isolated apoEp1.B peptide having the amino acid sequence TQQIRLQAEIFQAR (murine)(SEQ. ID. No.43) or AQQIRLQAEAFQAR (human)(SEQ. ID. No.44) or an analog, fragment, elongation or derivative of the peptide. The invention also includes a nucleic acid molecule encoding the apoEpI.B peptide, or an analog, fragment, elongation or derivative thereof.

The term "analog" includes any peptide having an amino acid residue sequence substantially identical to the native peptide in which one or more residues have been conservatively substituted with a functionally similar residue and which displays the ability to mimic the native peptide. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as alanine, isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

The phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a nonderivatized residue provided that such polypeptide displays the requisite activity. Analogs of the peptides include peptides having the following sequences: TAQIRLQAEIFQAR (SEQ.ID.NO.:45); TQAIRLQAEIFQAR

(SEQ.ID.NO.:46); TQQARLQAEIFQAR (SEQ.ID.NO.:47) and TQQIALQAEIFQAR (SEQ.ID.NO.:48).

"Derivative" refers to a peptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyprolinc may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. Polypeptides of the present invention also include any polypeptide having one or more additions and/or deletions or residues relative to the sequence of a polypeptide whose sequence is shown herein, so long as the requisite activity is maintained.

The term "fragment" refers to any subject peptide having an amino acid residue sequence shorter than that of a peptide whose amino acid residue sequence is shown herein.

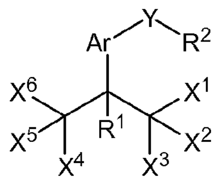
The term "elongation" refers to any subject peptide having an amino acid sequence longer by one or two amino acids (either at the carboxy or amino terminal end) than that of a peptide of the present invention. Preferably, the elongation occurs at the amino terminal end. Fragments and elongations of the peptides include peptides that have the following sequences: QTQQIRFQAEIFQAR (SEQ.ID.NO.:49) and QQIRFQAEIFQAR (SEQ.ID.NO.:50).

ApoE polypeptides and methods for their preparation are described in US patent No. 6,652,860.

LXR agonists

The methods of the invention can include administering a LXR agonist for the prevention and treatment of metastasis. The LXR agonist can be a compound according to the Formula I, II, III, or IV shown below.

Formula I is provided below:



Formula I

or a pharmaceutically acceptable salt thereof, wherein

Ar is an aryl group;

R^1 is a member selected from the group consisting of

-OH, $-CO_2H$, -O-(C_1-C_7)alkyl, -OC(O)- , -(C_1-C_7)alkyl, -O-(C_1-C_7)heteroalkyl, -OC (O)-(C_1-C_7)heteroalkyl, $-NH_2$, -NH(C_1-C_7) alkyl, -N((C_1-C_7)alkyl)$_2$ and -NH-S(O)$_2$(C_1-C_5)alkyl;

R^2 is a member selected from the group consisting of

(C_1-C_7)alkyl, (C_1-C_7)heteroalkyl, aryl and aryl (C_1-C_7)alkyl;

X^1, X^2, X^3, X^4, X^5 and X^6 are each independently a member selected from the group consisting of:

H, (C^1-C^5)alkyl, (C^1-C^5)heteroalkyl, F and CI, with the proviso that no more than three of X^1 through X^6 are H, (C^1-C^5)alkyl, (C^1-C^5)heteroalkyl; and

Y is a divalent linking group selected from the group consisting of:

-N(R^{12})S(O)$_m$-, -N(R^{12})S(O)$_m$N(R^{13})-, -N(R^{12})C(O)-, -N(R^{12})C(O)N(R^{13})-, -N(R^{12})C(S)- and -N(R^{12})C(O)O-;

wherein R^{12} and R^{13} are each independently selected from the group consisting of:

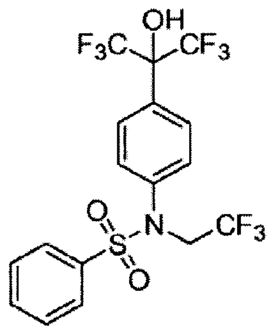
H, (C_1-C_7)alkyl, (C_1-C_7)heteroalkyl, aryl and aryl(C_1-C_7)alkyl, and optionally when Y is

-N(R^{12})S(O)$_m$- or -N(R^{12})S(O)$_m$N(R^{13})-, R^{12} forms a five- or six-membered ring fused to Ar or to R^2 through covalent attachment to Ar or to R^2, respectively; and the subscript m is an integer of from 1 to 2;

with the proviso that when R^1 is OH, and -Y-R^2 is -N(R^{12})S(O)m-R^2 or -N(R^{12})C(0)N(R^{13})-$R^2$and is attached to a position para to the quaternary carbon attached to Ar, and when R^2 is phenyl, benzyl, or benzoyl, then i) at least one of R^{12} or R^{13} is other than hydrogen and contains an electron-withdrawing substituent, or ii) R^2 is substituted with a moiety other than amino, acetamido, di(C_1-C_7)alkylamino, (C_1-C_7)alkylamino, halogen, hydroxy, nitro, or (C_1-C_7)alkyl, or iii) the benzene ring portion of R^2 is substituted with at least three independently selected groups in addition to the Y group or point of attachment to Y.

In some embodiments, Y is -N(R^{12})S$(O)^2$- and R^1 is OH.

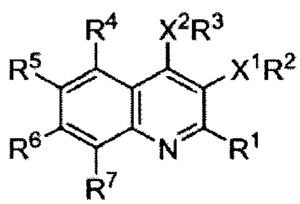
Accordingly, the compounds of Formula I include but are not limited the compound with the structure shown below:



I

Compounds of Formula I can be synthesized as described by US patent No. 6,316,503.

Formula II is provided below:



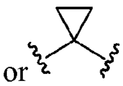
Formula II

wherein:

R^1 is-H;

X^1 is a bond, C_1 to C_5 alkyl, -C(O)-, -C(=CR^8R^9) - , -O-, -S$(O)_t$-, -NR^8-, -CR^8R^9- , -CHR^{23},

-CR^8(CR^9)-, -C(CR^8)$_2$-, -CR_8(OC(O)R^9)-, -C=NOR^9-, -C(O)NR^8-, -CH_2O-, -CH_2S-, -CH_2NR^8-, -OCH_2-,

$-SCH_2-$, $-NR^8CH_2-$, or  ;

R^2 is H, C_1 to C_6alkyl, $C_2$to C_6alkenyl, $C_2$to C_6alkynyl, C_3 to C_6 cycloalkyl, -CH_2OH, C7 to C_{11} arylalkyl, phenyl, naphthyl, C_1 to C_3 perfluoroalkyl, CN, $C(O)NH_2$, CO_2R^{12} or phenyl substituted independently by one or more of the groups independently selected from C_1 to C_3 alkyl, $C_2$to C_4 alkenyl, C_2 to C_4 alkynyl, C_1 to C_3 alkoxy, C_1 to C_3 perfluoroalkyl, halogen, $-NO_2$, $-NR^8R^9$, -CN, -OH, and C_1 to C_3alkyl substituted with 1 to 5 fluorines, or R^2 is a heterocycle selected from the group consisting of pyridine, thiophene, benzisoxazole, benzothiophene, oxadiazole, pyrrole, pyrazole, imidazole, and furan, each of which may be optionally substituted with one to three groups independently selected from C_1 to C_3alkyl, C_1 to C_3 alkoxy, C_1 to C_3 perfluoroalkyl, halogen, $-NO_2$, $-NR^8R^9$, -CN, and C_1 to C_3alkyl substituted with 1 to 5 fluorines;

X^2 is a bond or $-CH_2-$;

R^3 is phenyl, naphthyl, or phenyl or naphthyl substituted independently by one to four groups independently selected from C_1 to C_3 alkyl, hydroxy, phenyl, acyl, halogen, -NH_2,-CN, $-NO_2$, C_1 to C_3 alkoxy, C_1 to C_3perfluoroalkyl, C_1 to C_3 alkyl substituted with 1 to 5 fluorines, $NR^{14}R^{15}$, $-C(O)R^{10}$,
$-C(O)NR^{10}R^{11}$, $-C(O)NR^{11}A$, $-C{\equiv}CR^8$, $-CH{=}CHR^8$, -WA, $-C{\equiv}CA$, $-CH{=}CHA$, -WYA, -$WYNR^{11}$-A,
$-WYR^{10}$, $-WY(CH2)_jA$, $-WCHR^{11}(CH2)_jA$, $-W(CH2)_jA$, $-W(CH2)_jR^{10}$, -$CHR^{11}W(CH_2)_jR^{10}$,
$-CHR^{11}W(CH_2)_jA$, $-CHR^{11}NR^{12}YA$, $-CHR^{11}NR^{12}YR^{10}$, pyrrole, -$W(CH_2)_jA(CH_2)_kD(CH_2)_pZ$,
$-W(CR^{18}R^{19})A(CH_2)_kD(CH_2)_pZ$, $-(CH_2)_jWA(CH_2)_kD(CH_2)_pZ$, -$CH{=}CHA(CH2)_kD(CH2)pZ$,
$-C{\equiv}CA(CH_2)_kD(CH_2)_pZ$, $-W(CH_2)_jC{\equiv}CA(CH_2)_kD(CH2)_pZ$, and - $W(CH_2)_jZ$, or R^3 is a heterocycle selected from pyrimidine, thiophene, furan, benzothiophene, indole, benzofuran, benzimidazole, benzothiazole, benzoxazole, and quinoline, each of which may be optionally substituted with one to three groups independently selected from C_1 to C_3alkyl, C_1 to C_3 alkoxy, hydroxy, phenyl, acyl, halogen, $-NH_2$, -CN, $-NO_2$, C_1 to C_3 perfluoroalkyl, C_1 to C_3 alkyl substituted with 1 to 5 fluorines, $-C(O)R^{10}$, $-C(O)\ NR^{10}R^{11}$, -

C(O)NR^{11}A, -C≡CR^{8}, -CH=CHR^{8}, -WA, -C≡CA, -CH=CHA, -WYA, -WYR^{10}, -WY$(CH_2)_j$A,
-W$(CH_2)_j$A, -W$(CH_2)_j$$R^{10}$, -CHR^{11}W$(CH_2)_j$$R^{10}$, -CH$R^{11}W(CH_2)_j$A, -CH$R^{11}NR^{12}$YA, -CH$R^{11}NR^{12}YR^{10}$,
-WCH$R^{11}$$(CH_2)_j$A, -W$(CH_2)_jA(CH_2)_kD(CH_2)_p$Z, -W(C$R^{18}$$R^{19}$)A$(CH_2)_kD(CH_2)_p$Z,
-$(CH_2)_j$WA$(CH_2)_k$D$(CH_2)_p$Z, -CH=CHA$(CH_2)_k$D$(CH_2)_p$Z, -C≡CA$(CH_2)_k$D$(CH_2)_p$Z, -W$(CH_2)_j$C≡CA$(CH_2)_k$D$(CH_2)_p$Z, and -W$(CH_2)_j$Z;

W is a bond, -0-, -S-, -S(O)-, -$S(O)_2$-, -NR^{11}-, or -N(COR^{12})-;

Y is -CO-, -$S(O)^2$-, -CONR^{13},-CONR^{13}CO-,-CON$R^{13}$$SO_2$-, -C(NCN)-, -CSN$R^{13}$, -C(NH)N$R^{13}$, or -C(O)O-;

j is 0 to 3;

k is 0 to 3;

t is 0 to 2;

D is a bond, -CH=CH-, -C≡C-,-C=,-C(O)-, phenyl, -O-, -NH-, -S-, -CHR^{14}-, -C$R^{14}$$R^{15}$-,
-OCHR^{14}, -OC$R^{14}$$R^{15}$-, or -CH(OH)CH(OH)-;

p is 0 to 3;

Z is -CO_2R^{11}, -CON$R^{10}$$R^{11}$, -C(N$R^{10}$)N$R^{11}$$R^{12}$, -$CONH_2NH_2$,-CN, -$CH_2OH$, -N$R^{16}$$R^{17}$, phenyl, CONHCH($R^{20}$)CO$R^{12}$, phthalimide, pyrrolidine-2,5dione, thiazolidine-2,4-dione, tetrazolyl, pyrrole, indole, oxazole, 2-thioxo-l,3-thiazolinin-4-one, C_1 to C_7 amines, C_3 to C_7 cyclic amines, or C_1 to C_3 alkyl substituted with one to two OH groups; wherein said pyrrole is optionally substituted with one or two substituents independently selected from the group consisting of-CO_2CH_3,-CO_2H,-$COCH_3$,-$CONH_2$, and
-CN;

wherein said C_1 to C_7amines are optionally substituted with one to two substituents independently
selected from the group consisting of -OH, halogen, -OCH_3,and -C≡CH;

wherein said phenyl is optionally substituted with CO_2R^{11}, and wherein said C_3 to C_7 cyclic amines are optionally substituted with one or two substituents independently selected from the group consisting of -OH -CH_2OH, C_1 to C_3 alkyl, -CH_2OCH_3,-CO_2CH_3, and -$CONH_2$, and wherein said oxazole is optionally substituted with $CH_2CO_2R^{11}$;

A is phenyl, naphthyl, tetrahydronaphthyl, indan or biphenyl, each of which may be optionally substituted by one to four groups independently selected from halogen, C_1 to C_3 alkyl, C_2 to C_4 alkenyl, C_2 to C_4 alkynyl, acyl, hydroxy, halogen, -CN, $-NO_2$, $-CO_2R^{11}$, $-CH_2CO_2R^{11}$, phenyl, C_1 to C_3perfluoroalkoxy, C_1 to C_3 perfluoroalkyl, $-NR^{10}R^{11}$, $-CH_2NR^{10}R^{11}$, $-SR^{11}$, C_1 to C_6 alkyl substituted with 1 to 5 fluorines, C_1 to C_3alkyl substituted with 1 to 2-OH groups, C_1 to C_6 alkoxy optionally substituted with 1to 5 fluorines, or phenoxy optionally substituted with 1 to 2 CF_3 groups; or

A is a heterocycle selected from pyrrole, pyridine, pyridine-N-oxide, pyrimidine, pyrazole, thiophene, furan, quinoline, oxazole, thiazole, imidazole, isoxazole, indole, benzo[1,3]-dioxole, benzo[1,2,5]-oxadiazole, isochromen-l-one, benzothiophene, benzofuran,2,3-di- 5 hydrobenzo[1,4]-dioxine, bitheinyl, quinazolin-2,4-9[3H]dione, and 3-H-isobenzofuran-l-one, each of which may be optionally substituted by one to three groups independently selected from halogen, C_1 to C_3 alkyl, acyl, hydroxy, -CN,$-NO_2$,C_1 to C_3perfluoroalkyl, $-NR^{10}R^{11}$, $-CH_2NR^{10}R^{11}$, $-SR^{11}$, C_1 to C_3 alkyl substituted with 1 to 5 fluorines, and C_1 to C_3 alkoxy optionally substituted with 1 to 5 fluorines;

R^4, R^5, and R^6 are each, independently, -H or -F;

R^7 is C_1 to C_4 alkyl, C_1 to C_4 perfluoroalkyl, halogen, $-NO_2$, -CN, phenyl or phenyl substituted with one or two groups independently selected from halogen, C_1 to C_2alkyl and OH;

provided that if X_1R^2 forms hydrogen, then R^3 is selected from:

(a) phenyl substituted by $-W(CH_2)_jA(CH_2)_kD(CH_2)_pZ$, $-W(CR^{18}R^{19})A(CH_2)_kD(CH_2)_pZ$,
$-(CH_2)_jWA(CH_2)_kD(CH_2)_pZ$, $-CH{=}CHA(CH_2)_kD(CH_2)_pZ$, $-C{\equiv}CA(CH_2)_kD(CH_2)_pZ$, or $-W(CH_2)_jC{\equiv}CA(CH_2)_kD(CH_2)_pZ$, wherein the phenyl moiety is further optionally substituted with one or two groups independently selected from C_1 to C_2 alkyl, C_1 to C_2perfluoroalkyl, halogen, and CN; and

(b) a heterocycle selected from pyrimidine, thiophene, and furan, each of which is substituted by one of $-W(CH_2)_jA(CH_2)_kD(CH_2)_pZ$, $-W(CR^{18}R^{19})A(CH_2)_kD(CH_2)_pZ$, $-(CH2)_jWA(CH_2)_kD(CH_2)_pZ$,
$-CH{=}CHA(CH_2)_kD(CH_2)_pZ$, $-C{\equiv}CA(CH_2)_kD(CH_2)_pZ$, or $-W(CH_2)_jC{\equiv}CA(CH_2)_kD(CH_2)_pZ$;

each R^8 is independently-H, or C_1 to C_3alkyl;

each R^9 is independently-H, or C_1 to C_3alkyl;

each R^{10} is independently-H, -CH, C_1 to C_3alkoxy, C_1 to C_7 alkyl, C_3 to C_7 alkenyl, C_3 to C_7 alkynyl, C_3 to C_7 cycloalkyl, $-CH_2CH_2OCH_3$, 2-methyl-tetrahydro-furan, 2-methyl-tetrahydro-pyran, 4-methyl-piperidine, morpholine, pyrrolidine, or phenyl optionally substituted with one or two C_1 to C_3alkoxy groups, wherein said C_1 to C_7 alkyl is optionally substituted with 1, 2 or 3 groups independently selected from C_1 to C_3 alkoxy, C_1 to C_3thioalkoxy, and CN;

each R^{11} is independently-H, C_1 to C_3alkyl or R^{22}; or R^{10} and R^{11}, when attached to the same atom, together with said atom form:

a 5 to 7 membered saturated ring, optionally substituted by 1 to 2 groups independently selected from C_1 to C_3 alkyl, OH and C_1-C_3alkoxy; or a 5 to 7 membered ring containing 1 or 2 heteroatoms,
optionally substituted by 1 to 2 groups independently selected from C_1 to C_3alkyl, OH and C_1-C_3 alkoxy;

each R^{12} is independently-H, or C_1 to C_3alkyl;

each R^{13} is independently-H, or C_1 to C_3alkyl;

each R^{14} and R^{15} is, independently, C_1 toC_7 alkyl, C_3 to C_8 cycloalkyl, C_2 to C_7 alkenyl, C_2 to C_7 alkynyl,-CH, -F, C_7 to C_{14}arylalkyl, where said arylalkyl is optionally substituted with 1 to 3 groups independently selected from NO_2, C_1 to C_6 alkyl, C_1 toC_3perhaloalkyl, halogen, $CH_2CO_2R^{11}$, phenyl and C_1 to C_3 alkoxy, or R^{12} and R^{15} together with the atom to which they are attached can form a 3 to 7 membered saturated ring;

each R^{16} and R^{17} is, independently, hydrogen, C_1 to C_3 alkyl, C_1 to C_3alkenyl, C_1 to C_3 alkynyl, phenyl, benzyl or C_3 to C_8 cycloalkyl, wherein said C_1 to C_3 alkyl is optionally substituted with one OH group, and wherein said benzyl is optionally substituted with 1 to 3 groups selected from C_1 to C_3alkyl and C_1 to C_3alkoxy; or R^{16} and R^{17}, together with the atom to which they are attached, can form a 3 to 8 membered heterocycle which is optionally substituted with one or two substituents independently selected from the group consisting of C_1 to C_3alkyl,-OH, CH_2OH, $-CH_2OCH_3$,$-CO_2CH_3$, and$-CONH_2$;

each R^{18} and R^{19} is, independently, C_1 to C_3alkyl;

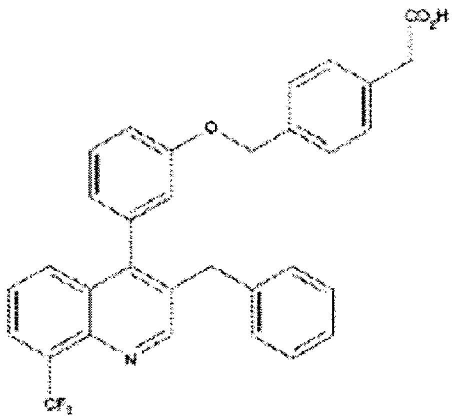
each R^{20} is independently H, phenyl, or the side chain of a naturally occurring alpha amino acid;

each R^{22} is independently arylalkyl optionally substituted with CH_2COOH; and

each R_{23} is phenyl;

or a pharmaceutically acceptable salt thereof.

Compounds of Formula II can be synthesized as described in US patent No. 7,576,215. The compound of formula II can be any of compounds 26-32, or a pharmaceutically acceptable salt thereof.



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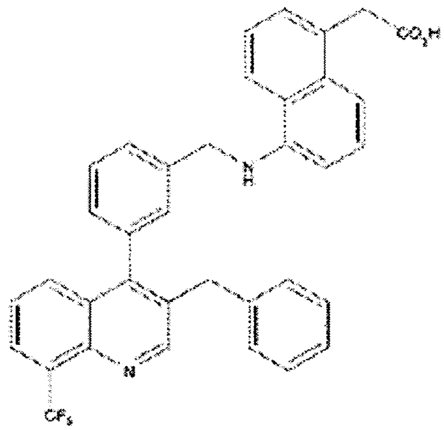

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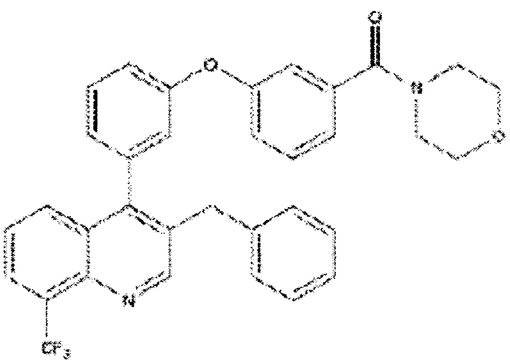

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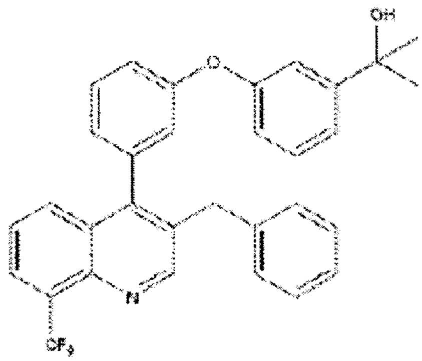

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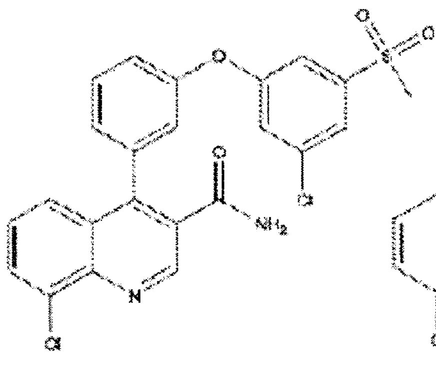

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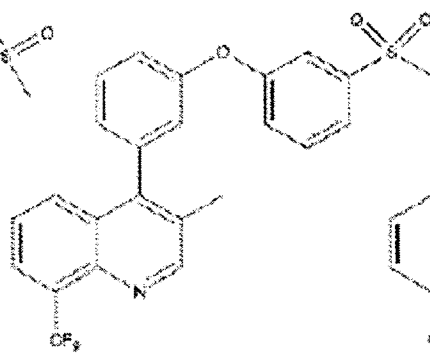

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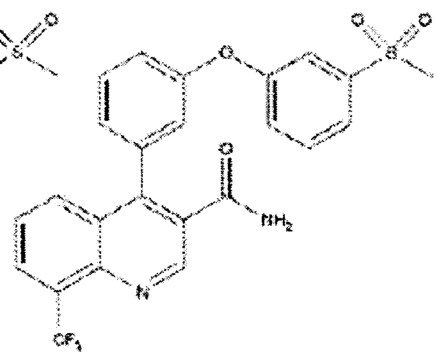

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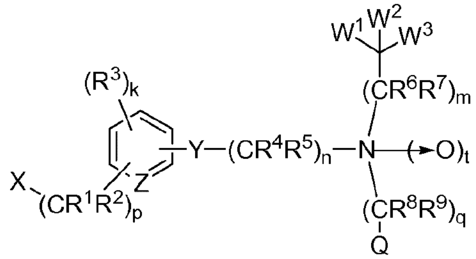
Formula III is provided below:



Formula III

wherein:

X is selected from hydrogen, C_1-C_8 alkyl, halo, -OR^{10}, -$NR^{10}R^{11}$, nitro, cyano, -$COOR^{10}$, or -COR^{10}.

Z is CH, CR^3 or N, wherein when Z is CH or CR^3, k is 0-4 and t is 0 or 1, and when Z is N, k is 0-3 and t is 0;

Y is selected from -O-, -S-, -N(R^{12})-, and -C(R^4)(R^5)- ;

W^1 is selected from C_1-C_6 alkyl, C_0-C_6 alkyl, C_3-C_6 cycloalkyl, aryl and Het, wherein said C_1-C_8 alkyl, C_3-C_8 cycloalkyl, Ar and Het are optionally unsubstituted or substituted with one or more groups independently selected from halo, cyano, nitro, C_1-C_6 alkyl, C_3-C_6 alkenyl, C_3-C_6 alkynyl,-C_0-C_6 alkyl-CO_2R^{12}, -C_0-C_6alkyl-C(O)SR^{12}, -C_0-C_6alkyl-$CONR^{13}R^{14}$, -C_0-C_6 alkyl-COR^{15}, -C_0-C_6 alkyl-$NR^{13}R^{14}$, -C_0-C_6 alkyl-SR^{12}, -C_0-C_6alkyl-OR^{12}, -C_0-C_6alkyl-SO_3H, -C_0-C_6alkyl-$SO_2NR^{13}R^{14}$, -C_0-C_6alkyl-SO_2R^{12}, -C_0-C_6alkyl-SOR^{15}, -C_0-C_6alkyl$OCOR^{15}$, -C_0-C_6alkyl-OC(O)$NR^{13}R^{14}$, -C_0-C_6alkyl-OC(O)OR^{15}, -C_0-C_6 alkyl-NR^{13}C(O)OR^{15}, -C_0-C_6 alkyl-NR^{13}C(O)$NR^{13}R^{14}$, and-C_0-C_6 alkyl-$NR^{13}COR^{15}$, where said C_1-C_6 alkyl, is optionally unsubstituted or substituted by one or more halo substituents;

W^2 is selected from H, halo, C_1-C_6alkyl, C_2-C_6alkenyl, C_2-C_6 alkynyl, -C_0-C_6 alkyl-$NR^{13}R^{14}$, -C_0--C_6alkyl-SR^{12}, -C_0-C_6 alkyl-OR^{12}, -C_0-C_6alkylCO_2R^{12}, -C_0-C_6alkyl-C(O)SR^{12}, -C_0-C_6 alkyl$CONR^{13}R^{14}$, -C_0-C_6alkyl-COR^{15}, -C_0-C_6 alkyl$OCOR^{15}$, -C_0-C_6alkyl-$OCONR^{13}R^{14}$, -C_0-C_6alkyl-$NR^{13}CONR^{13}R^{14}$, -C_0-C_6 alkyl-$NR^{13}COR^{15}$, -C_0-C_6alkyl-Het, -C_0-C_6alkyl-Ar and –C_0-C_6alkyl-C_3-C_7 cycloalkyl, wherein said C_1-C_6 alkyl is optionally unsubstituted or substituted by one or more halo substituents, and wherein the C_3-C_7cycloalkyl, Ar and Het moieties of said –C_0-C_6alkyl-Het, -C_0-C_6alkyl-Ar and –C_0-C_6alkyl-C_3-C_7cycloalkyl are optionally unsubstituted or substituted with one or more groups independently selected from halo, cyano, nitro, C_1-C_6 alkyl, C_3-C_6 alkenyl, C_3-C_6

alkynyl, -C_0-C_6alkyl-CO2R^{12}, -C_0-C_6 alkyl-C(O)SR^{12}, -C_0-C_6alkyl-CON$R^{13}R^{14}$, -C_0-C_6alkyl-COR^{15}, -C_0-C_6alkyl-N$R^{13}R^{14}$,-C_0-C_6alkyl-SR^{12}, -C_0-C_6alkyl-OR^{12}, -C_0-C_6 alkyl-SO_3H, -C_0-C_6alkyl-SO_2N$R^{13}R^{14}$, -C_0-C_6alkyl-SO_2R^{12}, -C_0-C_6alkyl-SOR^{15}, -C_0--C_6alkyl-OCOR^{15}, -C_0-C_6alkylOC(O)N$R^{13}R^{14}$,-C_0-C_6alkyl-OC(O)OR^{15}, -C_0-C_6alkyl-NR^{13}C(O)OR^{15}, -C_0-C_6alkyl-NR^{13}C(O)N$R^{13}R^{14}$, and –C_0-C_6alkyl-NR^{13}COR^{15}, where said C_1-C_6 alkyl, is optionally unsubstituted or substituted by one or more halo substituents;

W^3 is selected from the group consisting of: H, halo, C_1-C_6 alkyl, -C_0-C_6 alkyl-N$R^{13}R^{14}$,-C_0-C_6alkylSR^{12}, -C_0-C_6alkyl-OR^{12}, -C_0-C_6alkyl-CO_2R^{12}, -C_0-C_6alkyl-C(O)SR^{12}, -C_0-C_6alkyl-CON$R^{13}R^{14}$,

-C_0-C_6alkyl-COR^{15}, -C_0-C_6alkyl-OCOR^{15}, -C_0-C_6 alkyl-OCON$R^{13}R^{14}$, -C_0-C_6alkylNR^{13}CON$R^{13}R^{14}$,

-C_0-C_6alkyl-NR^{13}COR^{15}, -C_0-C_6alkyl-Het, -C_1-C_6alkyl-Ar and –C_1-C_6alkyl-C_3-C_7cycloalkyl, wherein said C_1-C_6 alkyl is optionally unsubstituted or substituted by one or more halo substituents;

Q is selected from C_3-C_8cycloalkyl, Ar and Het; wherein said C_3-C_8cycloalkyl, Ar and Het are optionally unsubstituted or substituted with one or more groups independently selected from halo, cyano, nitro, C_1-C_6alkyl, C_3-C_6alkenyl, C_3-C_6alkynyl,-C_0-C_6alkylCO_2R^{12}, -C_0-C_6 alkyl-C(O)SR^{12}, -C_0-C_6alkylCON$R^{13}R^{14}$, -C_0-C_6 alkyl-COR^{15}, -C_0-C_6alkylN$R^{13}R^{14}$, -C_0-C_6alkyl-SR^{12}, -C_0-C_6alkyl-OR^{12}, -C_0-C_6 alkyl-SO_3H, -C_0-C_6 alkyl-SO_2N$R^{13}R^{14}$, -C_0-C_6alkyl-SO_2R^{12}, -C_0-C_6alkyl-SOR^{15}, -C_0-C_6alkyl-OCOR^{15}, -C_0-C_6alkyl-OC(O)N$R^{13}R^{14}$, -C_0-C_6alkyl-OC(O)OR^{15},-C_0-C_6alkylNR^{13}C(O)OR^{15}, -C_0-C_6 alkyl-NR^{13}C(O)N$R^{13}R^{14}$, and –C_0-C_6alkyl-NR^{13}COR^{15}, where said C_1-C_6alkyl is optionally unsubstituted or substituted by one or more halo substituents;

p is 0-8;

n is 2-8;

m is 0 or 1;

q is 0 or 1;

t is 0 or 1;

each R^1 and R^2 are independently selected from H, halo, C_1-C_6alkyl, C_3-C_6alkenyl, C_3-C_6 alkynyl, -C_0-C_6alkyl-N$R^{13}R^{14}$, -C_0-C_6alkyl-OR^{12}, -C_0-C_6 alkyl-SR^{12}, -C_1-C_6alkyl-Het, -C_1-C_6alkyl-Ar and –C_1-C6alkyl-C_3-C_7cycloalkyl, or R^1 and R^2 together with the carbon to which they are attached form a 3-5 membered carbocyclic or heterocyclic ring, wherein said heterocyclic ring contains one, or more heteroatoms selected from N, O, and S, where any of said C_1-C_6 alkyl is optionally unsubstituted or substituted by one or more halo substituents;

each R^3 is the same or different and is independently selected from halo, cyano, nitro, C_1-C_6 alkyl, C_3-C_6alkenyl, C_3-C_6alkynyl, -C_0-C_6alkyl-Ar, -C_0-C_6alkyl-Het, -C_0-C_6alkyl-C_3-C_7cycloalkyl, -C_0-C_6alkyl-CO2R^{12}, -C_0-C_6alkyl-C(O)SR^{12}, -C_0-C_6alkyl-CON$R^{13}R^{14}$, -C_0-C_6alkyl-COR^{15}, -C_0-C_6alkyl-N$R^{13}R^{14}$, -C_0-C_6alkyl-SR^{12}, -C_0-C_6alkyl-OR^{12}, -C_0-C_6alkyl-SO_3H, -C_0-C_6alkyl$SO_2NR^{13}R^{14}$,-C_0-C_6 alkyl-SO_2R^{12}, -C_0-C_6alkylSOR^{15}, -C_0-C_6alkyl-OCOR^{15}, -C_0-C_6 alkyl-OC(O)N$R^{13}R^{14}$, -C_0-C_6alkyl-OC(O)OR^{15}, -C_0-C_6alkyl-NR^{13}C(O)OR^{15}, -C_0-C_6alkyl-NR^{13}C(O)N$R^{13}R^{14}$, and –C_0-C_6alkyl-NR^{13}COR^{15}, wherein said C_1-C_6alkyl is optionally unsubstituted or substituted by one or more halo substituents;

each R^4 and R^5 is independently selected from H, halo, C_1-C_6alkyl, -C_0-C_6alkyl-Het, -C_0-C_6alkyl-Ar and –C_0-C_6alkyl-C_3-C_7cycloalkyl;

R^6 and R^7 are each independently selected from H, halo, C_1-C_6 alkyl, -C_0-C_6alkyl-Het, -C_0-C_6 alkyl-Ar and –C_0-C_6alkyl-C_3-C_7cycloalkyl;

R^8 and R^9 are each independently selected from H, halo, C_1-C_6 alkyl, -C_0-C_6alkyl-Het, -C_0-C_6 alkyl-Ar and –C_0-C_6alkyl-C_3-C_7 cycloalkyl;

R^{10} and R^{11} are each independently selected from H, C_1-C_{12} alkyl, C_3-C_{12}alkenyl, C_3-C_{12}alkynyl,

-C_0-C_8alkyl-Ar, -C_0-C_8 alkyl-Het, -C_0-C_8 alkyl-C_3-C_7 cycloalkyl, -C_0-C_8 alkyl-O-Ar, -C_0-C_8alkyl-O-Het,

-C_0-C_8 alkyl-O-C_3-C_7cycloalkyl, -C_0-C_8alkyl-S$(O)_x$-C_0-C_6alkyl, -C_0-C_8alkyl-S$(O)_x$-Ar, -C_0-C_8 alkyl-S$(O)_x$-Het, -C_0-C_8 alkyl-S$(O)_x$-C_3-C_7cycloalkyl, -C_0-C_8alkyl-NH-Ar, -C_0-C_8alkyl-NH-Het, -C_0-C_8alkyl-NH-C_3-C_7cycloalkyl, -C_0-C_8alkyl-N(C_1-C_4 alkyl)-Ar, -C_0-C_8alkyl-N(C_1-C_4alkyl)-Het, -C_0-C_8alkyl-N(C_1-C_4alkyl-C_3-C_7cycloalkyl, -C_0-C_8alkyl-Ar, -C_0-C_8alkyl-Het and –C_0-C_8alkyl-C_3-C_7cycloalkyl, where x is 0, 1, or 2, or R^{10} and R^{11}, together with the nitrogen to which they are attached, form a 4-7 membered heterocyclic ring which optionally contains one or more additional heteroatoms selected from N, O, and S, wherein said C_1-C_{12}alkyl, C_3-C_{12} alkenyl, or C_3-C_{12}alkynyl is optionally substituted by one or more of the substituents independently selected from the group halo, ---OH, -SH, -NH_2, -

NH(unsubstituted C_1-C_6alkyl), -N(unsubstituted C_1-C_6 alkyl)(unsubstituted C_1-C_6alkyl), unsubstituted -OC_1-C_6 alkyl, -CO_2H,

-CO_2(unsubstituted C_1-C_6 alkyl), -$CONH_2$, -CONH(unsubstituted C_1-C_6 alkyl), -CON(unsubstituted C_1-C_6 alkyl)(unsubstituted C_1-C_6 alkyl), -SO_3H, -SO_2NH_2, -SO_2NH(unsubstituted C_1-C_6alkyl) and

-SO_2N(unsubstituted C_1-C_6alkyl)(unsubstituted C_1-C_6 alkyl);

R^{12} is selected from H, C_1-C_6 alkyl, C_3-C_6alkenyl, C_3-C_6alkynyl, -C_0-C_6alkyl-Ar, -C_0-C_6alkyl-Het and -C_0-C_6alkyl-C_3-C_7cycloalkyl;

each R^{13} and each R^{14} are independently selected from H, C_1-C_6alkyl, C_3-C_6alkenyl, C_3-C_6alkynyl, -C_0-C_6alkyl-Ar, -C_0-C_6alkyl-Het and-C_0-C_6alkyl-C_3-C_7cycloalkyl, or R^{13} and R^{14} together with the nitrogen to which they are attached form a 4-7 membered heterocyclic ring which optionally contains one or more additional heteroatoms selected from N, O, and S;

and R^{15} is selected from C_1-C_6alkyl, C_3-C_6 alkenyl, C_3-C_6alkynyl, -C_0-C_6alkyl-Ar, -C_0-C_6 alkyl-Het and -C_0-C_6 alkyl-C_3-C_7 cycloalkyl;

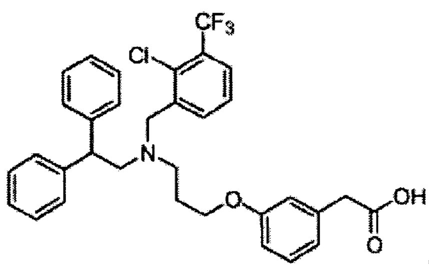
or a pharmaceutically acceptable salt thereof.

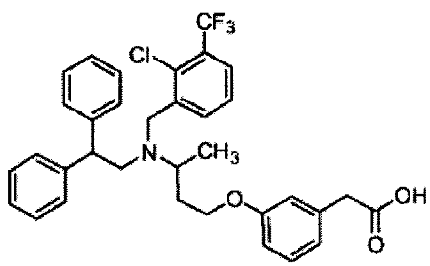
In some embodiments, X is hydrogen, p is 0, t is 0, Z is CH, and Y is -O-.

In further embodiments, X is hydrogen, p is 0, t is 0, Z is CH, and Y is -O-, W^1 and W^2 are phenyl, W^3 is hydrogen, q is 1, and R^8 and R^9 are hydrogen.

In other embodiments, X is hydrogen, p is 0, t is 0, Z is CH, and Y is -O-, W^1 and W^2 are phenyl, W^3 is hydrogen, q is 1, R^8 and R^9 are hydrogen, and Q is Ar.

Accordingly, the compounds of Formula III include but are not limited the compounds with structures shown below GW3965 **2** and SB742881 **25**:

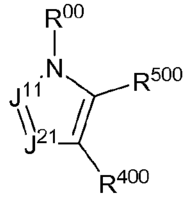




2, **25**

Compounds of Formula III can be synthesized as described in US patent No. 7,365,085 and 7,560,586.

Formula IV is shown below:



Formula IV

or a pharmaceutically acceptable salt thereof, wherein:

J^{11} is-N= and J^{21} is $-CR^{300}$-,or J^{11} is $-CR^{200}$- and J21 is =N-;

R^{00} is G^{1}, G^{21}, or R^{N};

R^{200} is G^{1}, G^{21}, or R^{C};

R^{300} and R^{400} are independently R^{C} or Q, provided one and only one of R^{300}, R^{400}, and R^{500} is Q;

Q is C_{3-6} cycloalkyl, heteroaryl or heterocyclyl, each optionally substituted with 1 to $4R^{Q}$, or Q is -X- Y-Z; wherein each R^{Q} is independently aryloxy, aralkyloxy, aryloxyalkyl, arylC_0-C_6alkylcarboxy, $C(R^{110})=C(R^{110})$- COOH, oxo, =S, -Z, - Y'-Z, or -X- Y-Z, wherein each R^{Q} is optionally substituted with 1 to 4 R^{80};

R^{500} is G^{1} G^{21}, Q, or R^{C}; provided that only one of R^{00}, R^{200}, and R^{500} is G^{1} and only one of R^{00}, N=, and R^{500} is G^{21};

G^{21} is $-J^{0}$-K^{o}, wherein J^{0} and K^{0} are independently aryl or heteroaryl, each optionally substituted with one to four R^{K} groups; each R^{K} is independently hydrogen, halogen, $CR^{110}=CR^{110}COOR^{110}$, nitro, -Z, -Y-Z, or -X-Y-Z;

G^{1} is $-L^{10}$-R, wherein L^{10} is a bond, L^{50}, L^{60}, $-L^{50}-L^{60}-L^{50}-$, or $-L^{60}-L^{50}-L^{50}-$, wherein each L^{50} is independently $-[C(R^{150})_2]_m-$;

each L^{60} is independently -CS-, -CO-, $-SO_2-$, -O-, $-CON(R^{110})-$, $-CONR^{110}N(R^{110})-$, $-C(=NR^{110})-$, $-C(NOR^{11})-$, $-C(=N-N(R^{110})_2)-$, $-C_3-C_8$cycloalkyl-, or -heterocyclyl-, wherein the cycloalkyl or heterocyclyl is optionally substituted with one to 4 R^{140} groups; or or each L^{60} is independently C_2-C_6 alidiyl, wherein the alidiyl chain is optionally interrupted by

$-C(R^{100})_2-$, $-C(R^{110})_2C(R^{110})_z-$, $-C(R^{11})C(R^{110})-$, $-C(R^{110})_2O-$, $-C(R^{110})_zNR^{110}-$, -C C-, -O-, -S-, -N(RO)CO-, $-N(R^{100})CO_2-$, $-CON(R^{110})-$, -CO-, $-CO_2-$, -OC(=O)-, $-OC(=O)N(R^{100})-$, -

SO_2-, -N(R^{100})SO_2-, or
-SO_2N(R^{100});

R is aryl, heterocyclyl, heteroaryl or -(C_3-C_6)cycloalkyl, wherein R is optionally substituted with 1 to 4 R^A, wherein each R^A is independently halogen, nitro, heterocyclyl, C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_8 cycloalkyl, (C_3-C_8 cycloalkyl)-C_1-C_6 alkyl-, (C_3-C_8 cycloalkenyl)-C_1-C_6 alkyl-, (C_3-C_8 cycloalkyl)-C_1C_6 alkenyl-, arylalkyl, aryloxy, arylC_{1-6} alkoxy, C_1-C_6 haloalkyl, SO_2R^{110}, OR^{110}, SR^{110}, N_3, SOR^{110}, COR^{110}, $SO_2N(R^{110})_2$, $SO_2NR^{110}COR^{110}$, C≡N, C(O)OR^{110}, CON$(R^{110})_2$, -CON(R^{110})OR^{110}, OCON$(R^{110})_2$, -$NR^{110}COR^{110}$, $NR^{110}CON(R^{110})_2$, $NR^{110}COOR^{110}$, -C(=N-OH)R^{110}, -C(=S)N$(R^{110})_2$,
-S(=O)N$(R^{110})_2$, -S(=O)OR^{110}, -N(R^{110})S(=O)$_2R^{110}$, -C(=O)N(R^{110})N$(R^{110})_2$, -OC(=O)-R^{110}, -OC(=O)-OR^{110} or N$(R^{11})_2$, wherein each R^A is optionally substituted with 1 to 4 groups which independently are -halogen, -C_1 -C_6 alkyl, aryloxy, C_{0-6} alkylSO_2R^{110}, C_{0-6} alkylCOOR ^{110}, C_{1-6} alkoxyaryl, C_1-C_6 haloalkyl,
-SO_2R^{110}, -OR^{110}, -SR^{110}, -N_3, -SO_2R^{110}, -COR^{110}, -$SO_2N(R^{110})_2$, -$SO_2NR^{110}COR^{110}$, -C≡N, -C(O)OR^{110},
-CON$(R^{110})_2$, -CON(R^{110})OR^{110}, -OCON$(R^{110})_2$, -$NR^{110}COR^{110}$, -$NR^{110}CON(R^{110})_2$, -$NR^{110}COOR^{110}$, or -N$(R^{110})_2$;

R^N is –L^{31}-R^{60}, wherein L^{31} is a bond, -X^3(CH_z)$_n$-X^3-, -(CH_2)$_m$-X3-(CH_2)$_n$- or -(CH_2)$_{1+w}$, -Y^3-(CH_2)$_w$ -, wherein each w is independently 0-5: and each X^3 is independently a bond, -C$(R^{110})_2$-, -C$(R^{110})_2$C$(R^{110})_2$-, -C(R^{110})=C(R^{110})-, -C≡C-, -CO-, -CS-, -CONR^{100}-, -C(=N)(R^{100})-, -C(=N-OR^{110})-, -C[=N-N$(R^{110})_2$], -CO_2-, -SO_2-, or –SO_2N(R^{110})-; and

Y^3 is -O-, -S-, -NR^{70}-, -N(R^{100})CO-, -N(R^{110})CO2-, -OCO-, -OC(=O)N (R^{100})-, -NR^{100}CONR^{100}-, -N(R^{110})SO_2-, or –NR^{100}CSNR^{100}-;

or L^{31} is C_{2-6} alidiyl chain wherein the alidiyl chain is optionally interrupted by -C$(R^{110})_2$-,
-C$(R^{110})_2$C$(R^{110})_2$-, -C(R^{110})=C(R^{110})-, -C$(R^{110})_2$O-, -C$(R^{110})_2$NR^{110}-, -C≡C-, -O-, -S-, -N(R^{100})CO-,
-N(R^{100})CO_2-, -CON(R^{100})-, -CO-, -CO_2-, -OC(=O)-,-OC(=O)N(R^{110})-, -SO_2-, -N(R^{100})SO_2-, or
-SO_2N(R^{100}); and

R^{60} is C_1-C_6 alkyl, C_1-C_6 halo alkyl, aryl, C_3-C_8 cycloalkyl, heteroaryl, heterocyclyl, -CN,
-C(=O)R^{110}, -C(=O)OR^{110}, -C(=O)N(R^{110})$_2$, -N(R^{110})$_2$, -SO_2R^{110}, -S(=O)$_2$N(R^{110})$_2$, -C(=O)N(R^{110})N(R^{110})$_2$, or -C(=O)N(R^{11})(OR^{110}), wherein the aryl, heteroaryl, cycloalkyl, or heterocyclyl is optionally substituted with 1 to 4 R^{60a}, wherein

each R^{60a} is independently -Z, -Y'-Z, or-X-Y-Z;

each R^{C} is independently –L^{30}-R^{70}, wherein

each L^{30} is independently a bond or-(CH_2)$_m$-V^{10}-(CH_2)$_n$-, wherein

V^{10} is -C(R^{110})$_2$-, -C(R^{110})$_2$C(R^{110})$_2$, -C(R^{110})=C(R^{110})-, -C(R^{110})$_2$O-, -C(R^{110})$_2$NR^{110}-, -C≡C-, -O-,
-S-, -NR^{10}-, -N(R^{100})CO-, -N(R^{100})CO2-, -OCO-, -CO-, -CS-, -CONR^{100}-, -C(=N-R^{110})-, -C(=N-OR^{110})-,
-C[=N-N(R^{110})$_2$], -CO_2-, -OC(=O)-, -OC(=O)N(R^{100})-, SO_2-, -N(R^{100})SO_2-, -SO_2N(R^{100})-, -NR^{100}CONR^{100}-, -NR^{100}CSNR^{100}-, C_3-C_6cyclo alkyl, or C_3-C_6 cyclohaloalkyl; or each L^{30} is independently C_2-C_6 alidiyl, wherein the alidiyl chain is optionally interrupted by –C(R^{110})$_2$-, -C(R^{110})$_2$C(R^{110})$_2$-, -C(R^{110})C(R^{110})-, -C(R^{110})$_2$O-, -C(R^{110})$_2$NR^{110}-, -C≡C-, -O-, -S-, -N(R^{100})CO-,
-N(R^{100})CO_2-, -NR^{110}-, -CON(R^{100})-, -CO-, -CO_2-, -O(C=O)-, -O(C=O)N(R^{100})-, -SO_2-, -N(R^{100})SO_2-, or –SO_2N(R^{100})-;

each R^{70} is independently hydrogen, halogen, nitro, aryl, heteroaryl, heterocyclyl, -Z, -Y-Z, or-X-YZ,

wherein the aryl, heteroaryl, and heterocyclyl, are each optionally substituted with 1 to 4 R^{70a}, wherein each R^{70a} is independently aryloxy, aralkyloxy, aryloxyalkyl, arylC_o-C_6alkylcarboxy, C(R^{110})=C(R^{110})COOH, oxo, -Z, - Y'-Z, or -X- Y-Z, wherein each R^{70a} is optionally substituted with 1 to 4 R^{80}, and wherein each R^{80} is independently halogen, C_1 -C_6 alkyl, C_1-C_6 alkoxy, C_1-C_8haloalkyl, C_1-C_8 haloalkyl(OR^{110}), C_0-C_6 alkylOR^{110}, C_0-C_6 alkylCON(R^{110})$_2$, C_0-C_6 alkylCOR^{110}, C_0-C_6 alkylCOOR^{110}, or C_0-C_6 alkylSO_2R^{110};

each R^{100} is independently –R^{110}, -C(=O)R^{110}, -CO_2R^{110}, or-SO_2R^{110};

each R^{110} is independently -hydrogen, -C_1-C_6 alkyl, C_2-C_6 alkenyl, C_1-C_6 alkynyl, -C_1-C_6 haloalkyl, or -N(R^{12})$_2$, wherein any of R^{110} is optionally substituted with 1 to 4 radicals of R^{120};

each R^{120} is independently halogen, cyano, nitro, oxo, -B(OR^{130}), C_0-C_6 alkylN(R^{13})$_2$, C_1-C_6haloalkyl, C_1-C_6 alkyl, C_1-C_6 alkoxy, (C_0-C_6 alkyl)C=O(OR^{130}), C_0-C_6 alkylOR^{130}, C_0-C_6 alkylCOR^{130},
C_0-C_6alkylSO_2R^{130}, C_o-C_6alkylCON(R^{13})$_2$, C_0-C_6alkylCONR^{130}OR^{130}, C_0-C_6alkylS0_2N(R^{130})$_2$, C_0-C_6alkylSR^{130}, C_0-C_6 haloalkylOR^{130}, C_0-C_6alkylCN, -C_0-C_6alkyN(R^{13})$_2$, -NR^{13}S$0_2$$R^{13}$, or-$OC_{O-6}$ alkylCOOR^{130};

each R^{130} is independently hydrogen, C_1-C_6 alkyl, C_2-C_6 alkenyl, or C_2-C_6 alkynyl;

each R^{140} is independently C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen, C_1-C_6 haloalkyl, C_0-C_6 alkylCON(R^{110})$_o$, C_0-C_6 alkylCON$R^{110}$$R^{10}$, C_0-C_6 alkylOR^{110}, or C_0-C_6 alkylCOOR^{110}; and

each R^{150} is independently hydrogen, halogen, OR^{130}, (C_1- C_6)alkyl or (C_1-C_6)haloalkyl, wherein
each alkyl is optionally substituted with at least one group which are each independently halogen, cyano, nitro, azido, OR^{130}, C(O)R^{130}, C(O)OR^{13}C(O)N(R^{130})$_2$, N(R^{130})$_2$, N(R^{130})C(O)R^{130}, N(R^{130})S(O)$_2$$R^{130}$,
-OC(O)OR^{130}, OC(O)N(R^{130})$_2$, N(R^{130})C(O)OR^{130}, N(R^{130})C(O)N(R^{130}), SR^{130}, S(O)R^{130}, S(O)$_2$R', or S(O)$_2$N(R^{130})$_2$; or two R^{150} (bonded to same or different atoms) can be taken together to form a C3-C6 cycloalkyl;

each X is independently -O-, -S-, or -N(R^{100})-;

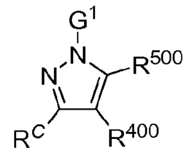
each Y is independently -[C(R^{150})$_2$]$_p$-, or-C_2-C_6 alkenyl, wherein p is 1, 2, 3, 4, 5, or 6;

each Y' is independently -[C(R^{150})$_2$]$_p$ -, -C_2-C_6 alkenyl C_3-C_8 cycloalkyl, or heterocyclyl, wherein the cycloalkyl or heterocyclyl is optionally substituted with 1 to 3 Z groups;

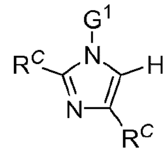
each Z is independently -H, halogen, -OR^{110}, -SR^{110}, -C(=O)R^{110}, -C(=O)OR^{110}, -C(=O)N(R^{110})$_2$,
-N(R^{100})$_2$, -N_3, -NO_2, -C(=N-OH)R^{110}, -C(=S)N(R^{110})$_2$, -CN, -S(=O)R^{110}, -S(=O)N(R^{110})$_2$, -S(=O)OR^{110},
-S(=O)$_2$$R^{110}$, S(=O)$_2$N($R^{110}$)$_2$, -N$R^{110}COR^{110}$, -N($R^{110}$)C(=O)N($R^{110}$)$_2$, -N($R^{110}$)COO$R^{110}$,
-N(R^{110})S(=O)$_2$$R^{110}$, -C(=O)N($R^{110}$)N($R^{110}$)$_2$, -C(=O)N($R^{110}$)(O$R^{110}$), -OC(=O)-$R^{110}$, -OC(=O)-O$R^{110}$, or
-OC(=O)-N(R^{110})$_2$; and

each m and n is independently 0, 1,2,3,4,5, or 6.

In some embodiments the compound of Formula IV has a structure of Formula V or VI:

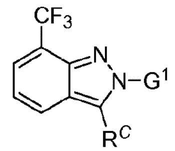


Formula V



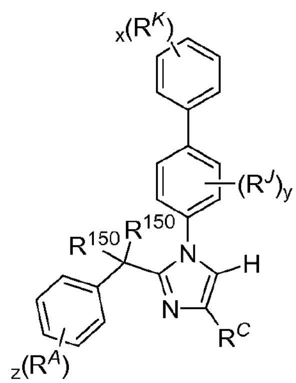
Formula VI

In other embodiments the compound of Formula VI has a structure of Formula VII:



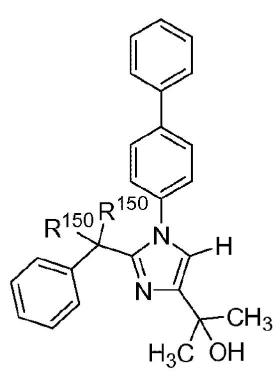
Formula VII

In yet other embodiments the compound of Formula VI has a structure of Formula VIII:



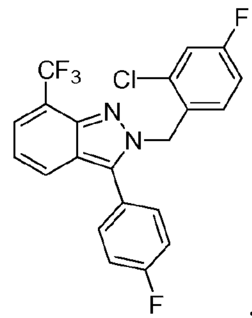
Formula VIII

In still further embodiments the compound of Formula VI has a structure of Formula IX:



Formula IX

Accordingly, the compounds of Formula IV which can be useful in the methods of the invention include, but are not limited to, compounds having the structures are shown below, and pharmaceutically acceptable salts thereof: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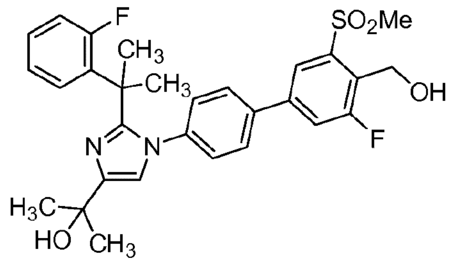
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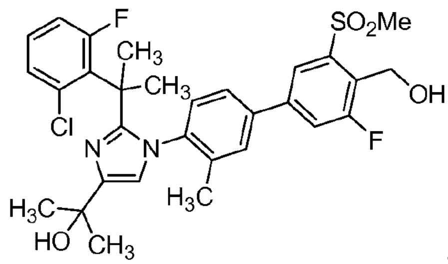
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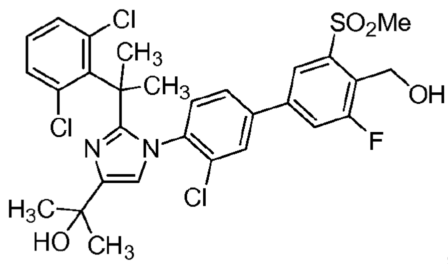
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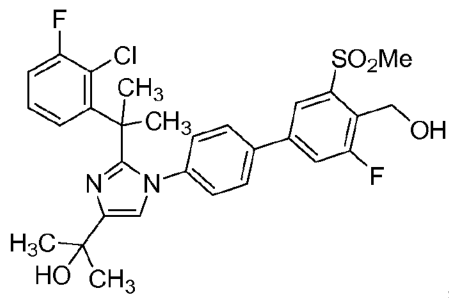
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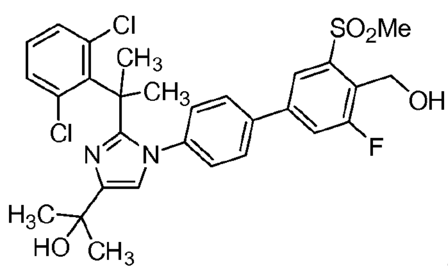
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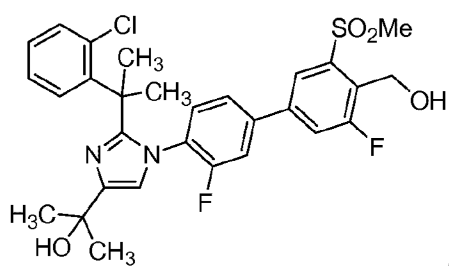
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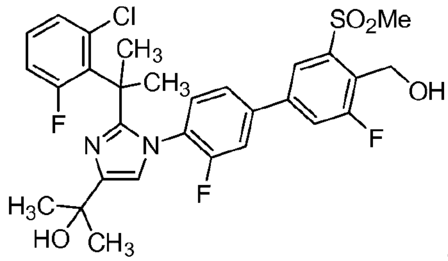
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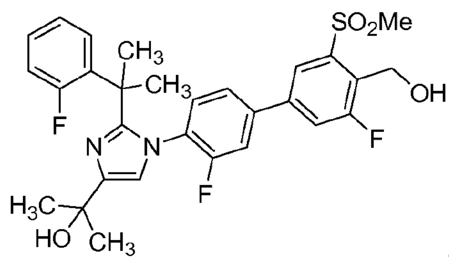
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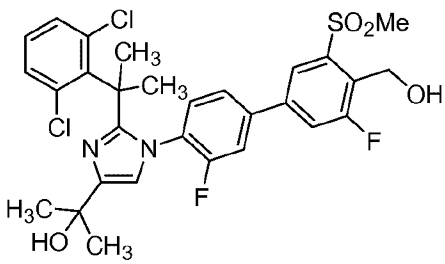
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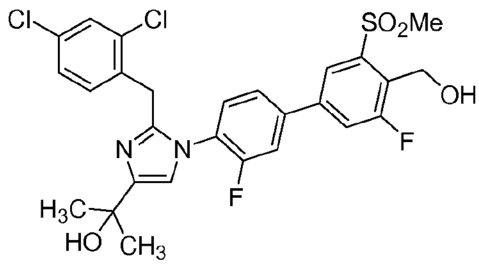
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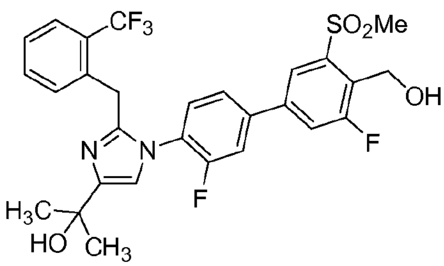
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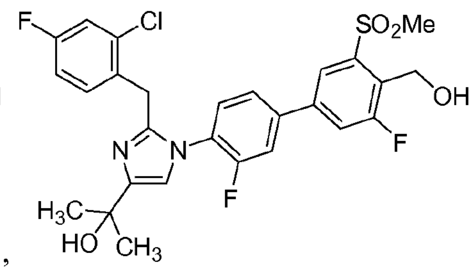
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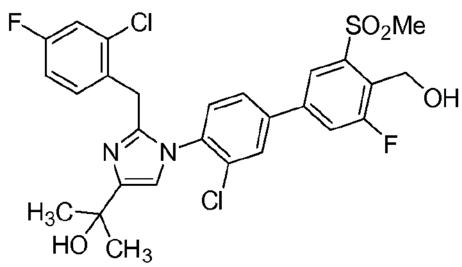
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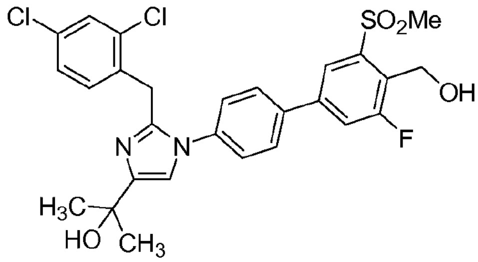,

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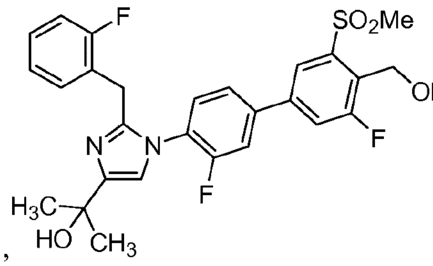,

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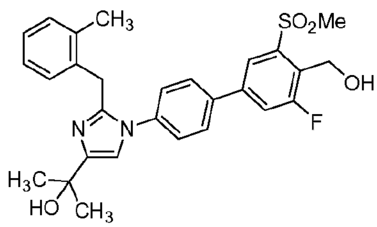,

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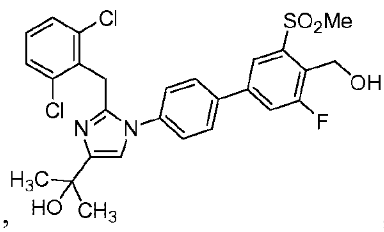,

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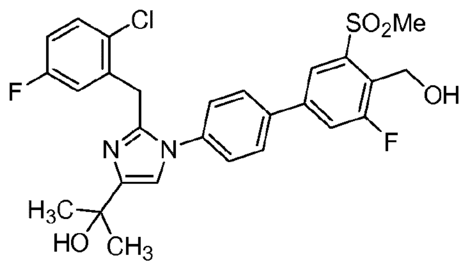,

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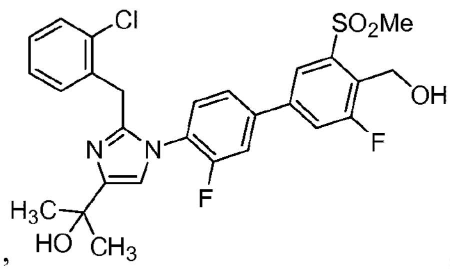,

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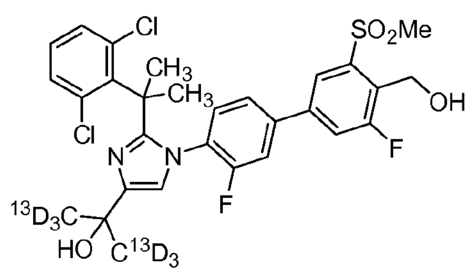,

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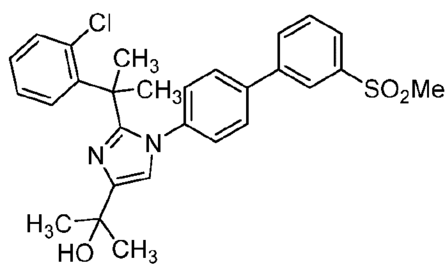,

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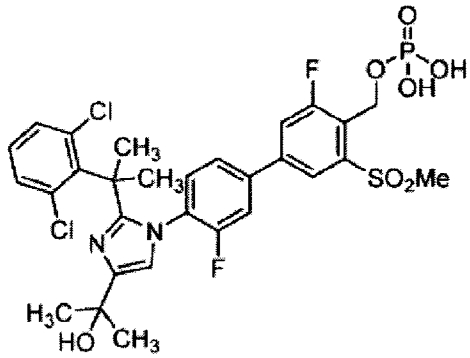

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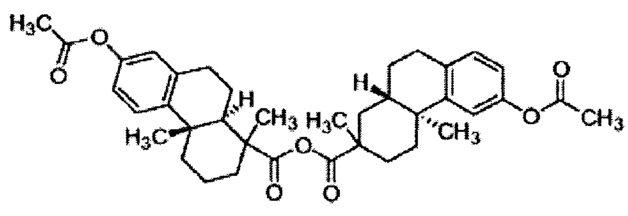
selected from the list comprising:

33 2-(l-(3chloro-3'-fluoro-4'-(hydroxymethyl)-5'-(methylsulfonyl)biphenyl-4-yl)-2-(2-(2,6dichlorophenyl) propan-2-yl)-lH-imidazol-4-yl)propan-2-ol; **34** 2-(2-(2(2-chloro-3-fluorophenyl)propan-2-yl)-l-(3'-fluoro-4'-(hydroxymethyl)-5'(methylsulfonyl)biphenyl-4-yl)-1H-imidazol-4-yl)propan-2-ol; **35** 2-(2-(2(2,6-dichlorophenyl)propan-2-yl)-1-(3'-fluoro-4'-(hydroxymcthyl)-5'(mcthylsulfonyl)biphcnyl-4-yl)-1H-imidazol-4-yl)propan-2-ol; **36** 2-(2-(2(2,6-dichlorophenyl)propan-2-yl)-l-(3,3'-difluoro-4'-(hydroxymethyl)-5'(methylsulfonyl)biphenyl-4-yl)-lH-imidazol-4-yl)propan-2-ol ;and **37** 2-(2-[1(2,6-dichlorophenyl)ethyl]-l-[3,3'-difluoro-4'-(hydroxymethyl)-5'(methylsulfonyl)biphenyl-4-yl]-lH-imidazol-4-yl)propan-2-ol. Compound **12** is also known as W02010 0138598 Ex. 9. Compound **38** is also known W02007 002563 Ex. 19. Compound **39** is also known as WO2012 0135082.

Compounds of Formula IV can be synthesized as described in PCT publication No. US2010/0069367 and WO2010/138598.

The LXR agonist that can be used for the treatment and/or prevention of metastasis can be compound 24, or a pharmaceutically acceptable salt thereof.



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In further embodiments compounds that can be used for the treatment and/or prevention of metastasis can be found in the PCT publications in the list consisting of:

W02006/094034, W02008/049047, W02009/020683, W02009/086138, W02009/086123, W02009/086130, W02009/086129, W02007/002559, W02007/002563, W02007/081335, W02006/017055, W02006/102067, W02009/024550, US2006/0074115, US2006/0135601, W02009/021868, W02009/040289, W02007/047991, W02007/050425, W02006/073363, W02006/073364, W02006/073365, W02006/073366, W02006/073367, US2009/0030082, W02008/065754, JP2008/179562, W02007/092065, US2010/0069367, US7998995, US7247748, WO2010/138598, US7365085, US75776215, US63136503, US2004/0072868, US2005/0107444, US2005/0113580, US2005/0131014, US2005/0282908, US2009/0286780.

LXRα and *LXRβ*, initially discovered by multiple groups at roughly the same time (Apfel et al., 1994; Willy et al., 1995; Song et al., 1994; Shinar et al., 1994; Teboul et al., 1995), belong to a family of nuclear hormone receptors that are endogenously activated by cholesterol and its oxidized derivatives to mediate transcription of genes involved in maintaining glucose, cholesterol, and fatty acid metabolism (Janowski et al., 1996; Calkin and Tontonoz, 2012). Given the intricate link between lipid metabolism and cancer cell growth (Cairns et al., 2011), the ubiquitous expression of *LXRβ* in melanoma is unlikely to be coincidental, allowing melanoma cells to synthesize lipids and lipoprotein particles to sustain their growth. At the same time, however, such stable basal expression levels make *LXRβ* an ideal therapeutic target, as exemplified by the broad-ranging responsiveness of melanoma cells to *LXRβ* activation therapy.

Compounds have been shown to have selectivity for *LXRβ* or *LXRα*. This selectivity may allow for increased activity and/or decreased off target effects. Examples of compounds with selectivity towards *LXRβ* or *LXRα* are shown in Table 1.

Table 1. EC5o values for selected compounds against *LXRα* and *LXRβ*

Compound	**EC_{50}- LXRα (nM)**	**EC_{50}- *LXRβ* (nM)**
GW3965 **2**	200	40
SB742881 **25**	74	25
TO901317 **1**	20	50
LXR-623 **3**	179	24

12	<100	11
38	101-1000	630

As used herein, reference to the activity of an LXR agonist at LXRα and LXRβ refer to the activity as measured using the ligand sensing assay (LiSA) described in Spencer et al. Journal of Medicinal Chemistry 2001, 44, 886-897. In some embodiments, the LXR agonist has an EC50 of less than 1μM in the ligand sensing assay (e.g., 0.5 nm to 500 nM, 10 nM to 100 nM). For example, the methods of the invention can be performed using an LXRβ agonist having activity for LXRβ that is at least 3-fold greater than the activity of the agonist for LXRα, or having activity for LXRβ that is at least 10-fold greater than the activity of the agonist for LXRα, or having activity for LXRβ that is at least 100-fold greater than the activity of said agonist for LXRα, or having activity for LXRβ that is at least within 3-fold of the activity of the agonist for LXRα. The term "greater activity" in the LiSA assay assay refers to a lower EC_{50}. For example, GW3965 **2** has approximately 6-fold greater activity for LXRβ (EC_{50}=30) compared to LXRa (EC_{50}=190).

As used herein, the term "increases the level of ApoE expression in vitro" refers to certain LXR agonists capable of increasing the level of ApoE expression 2.5-fold in the qPCR assay of Example 21 at a concentration of less than 5μM (e.g., at a concentration of 100 nM to 2μM, at a concentration of less than or equal to 1μM). The LXR agonists exhibiting this in vitro effect can be highly efficacious for use in the methods of the invention.

The term "alkyl" used is the present application relates a saturated branched or unbranched aliphatic univalent substituent. The alkyl substituent has 1 to 100 carbon atoms, (e.g., 1 to 22 carbon atoms, 1 to 10 carbon atoms 1 to 6 carbon atoms, 1 to 3 carbon atoms). Accordingly, examples of the alkyl substituent include methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl, *tert*-butyl, *n*-pentyl and *n*-hexyl.

The term "alkoxy" represents a chemical substituent of formula -OR, where R is an optionally substituted C1-C6 alkyl group, unless otherwise specified. In some embodiments, the alkyl group can be substituted, e.g., the alkoxy group can have 1, 2, 3, 4, 5 or 6 substituent groups as defined herein.

The term “alkoxyalkyl” represents a heteroalkyl group, as defined herein, that is described as an alkyl group that is substituted with an alkoxy group. Exemplary unsubstituted alkoxyalkyl groups include between 2 to 12 carbons. In some embodiments, the alkyl and the alkoxy each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective group.

As used herein, the term “cycloalkyl” refers to a monocyclic, bicyclic, or tricyclic substituent, which may be saturated or partially saturated, *i.e.* possesses one or more double bonds. Monocyclic substituents are exemplified by a saturated cyclic hydrocarbon group containing from 3 to 8 carbon atoms. Examples of monocyclic cycloalkyl substituents include cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cycloheptyl and cyclooctyl. Bicyclic fused cycloalkyl substituents are exemplified by a cycloalkyl ring fused to another cycloalkyl ring. Examples of bicyclic cycloalkyl substituents include, but are not limited to decalin, 1,2,3,7,8,8a-hexahydro-naphthalene, and the like. Tricyclic cycloalkyl substituents are exemplified by a cycloalkyl bicyclic fused ring fused to an additional cycloalkyl substituent.

The term “alkylene” used is the present application relates a saturated branched or unbranched aliphatic bivalent substituent (e.g. the alkylene substituent has 1 to 6 carbon atoms, 1 to 3 carbon atoms). Accordingly, examples of the alkylene substituent include methylene, ethylene, trimethylene, propylene, tetramethylene, isopropylidene, pentamethylene and hexamethylene.

The term “alkenylene or alkenyl” as used in the present application is an unsaturated branched or unbranched aliphatic bivalent substituent having a double bond between two adjacent carbon atoms (e.g. the alkenylene substituent has 2 to 6 carbon atoms, 2 to 4 carbon atoms). Accordingly, examples of the alkenylene substituent include but are not limited to vinylene, 1-propenylene, 2-propenylene, methylvinylene, 1-butenylene, 2-butenylene, 3-butenylene, 2-methyl-1-propenylene, 2-methyl-2-propenylene, 2-pentenylene, 2-hexenylene.

The term “alkynylene or alkynyl” as used is the present application is an unsaturated branched or unbranched aliphatic bivalent substituent having a tripple bond between two adjacent carbon atoms(e.g. the alkynylene substituent has 2 to 6 carbon atoms 2 to 4 carbon atoms). Examples of the alkynylene substituent include but are not limited to ethynylene, 1-

propynylene, 1-butynylene, 2-butynylene, 1-pentynylene, 2-pentynylene, 3-pentynylene and 2-hexynylene.

The term "alkadienylene" as used is the present application is an unsaturated branched or unbranched aliphatic bivalent substituent having two double bonds between two adjacent carbon atoms(e.g. the alkadienylene substituent has 4 to 10 carbon atoms). Accordingly, examples of the alkadienylene substituent include but are not limited to 2,4-pentadienylene, 2,4-hexadienylene, 4-methyl-2,4-pentadienylene, 2,4-heptadienylene, 2,6-heptadienylene, 3-methyl-2,4-hexadienylene, 2,6-octadienylene, 3-methyl-2,6-heptadienylene, 2-methyl-2,4-heptadienylene, 2,8-nonadienylene, 3-methyl-2,6-octadienylene, 2,6-decadienylene, 2,9-decadienylene and 3,7-dimethyl-2,6-octadienylene substituents.

The term "heteroaliphatic substituent or heteroalkyl", as used herein, refers to a monovalent or a bivalent substituent, in which one or more carbon atoms have been substituted with a heteroatom, for instance, with an oxygen, sulfur, nitrogen, phosphorus or silicon atom, wherein the nitrogen and sulfur atoms may optionally be oxidized, and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and S may be placed at any interior position of the heteroaliphatic substituent. Examples include $-CH_2-CH_2-O-CH_3$, $-CH_2-CH_2-NH-CH_3$, $-CH_2-CH_2-N(CH_3)-CH_3$, $-CH_2-S-CH_2-CH_3$, $-S(O)-CH_3$, $-CH_2-CH_2-S(O)_2-CH_3$, $-CH=CH-O-CH_3$, $-CH_2-CH=N-OCH_3$, and $-CH=CH-N(CH_3)-CH_3$. A heteroaliphatic substituent may be linear or branched, and saturated or unsaturated.

In one embodiment, the heteroaliphatic substituent has 1 to 100, (e.g 1 to 42 carbon atoms). In yet another embodiment, the heteroaliphatic substituent is a polyethylene glycol residue.

As used herein, "aromatic substituent or aryl" is intended to mean any stable monocyclic, bicyclic or polycyclic carbon ring of up to 10 atoms in each ring, wherein at least one ring is aromatic, and may be unsubstituted or substituted. Examples of such aromatic substituents include phenyl, *p*-toluenyl (4-methylphenyl), naphthyl, tetrahydronaphthyl, indanyl, biphenyl, phenanthryl, anthryl or acenaphthyl. In cases where the aromatic substituent is bicyclic and one ring is non-aromatic, it is understood that attachment is via the aromatic ring.

The term "alkylaryl substituents or arylalkyl" refers to alkyl substituents as described above wherein one or more bonds to hydrogen contained therein are replaced by a bond to an aryl substituent as described above. It is understood that an arylalkyl substituents is connected to the carbonyl group if the compound of the invention through a bond from the alkyl substituent. Examples of arylalkyl substituents include, but are not limited to, benzyl (phenylmethyl), *p*-trifluoromethylbenzyl (4-trifluoromethylphenylmethyl), 1-phenylethyl, 2-phenylethyl, 3-phenylpropyl, 2-phenylpropyl and the like.

The term "heteroaromatic substituent or heteroaryl" as used herein, represents a stable monocyclic, bicyclic or polycyclic ring of up to 10 atoms in each ring, wherein at least one ring is aromatic and contains from 1 to 4 heteroatoms selected from the group consisting of O, N and S. Bicyclic heteroaromatic substituents include phenyl, pyridine, pyrimidine or pyridizine rings that are

a) fused to a 6-membered aromatic (unsaturated) heterocyclic ring having one nitrogen atom;
b) fused to a 5- or 6-membered aromatic (unsaturated) heterocyclic ring having two nitrogen atoms;
c) fused to a 5-membered aromatic (unsaturated) heterocyclic ring having one nitrogen atom together with either one oxygen or one sulfur atom; or
d) fused to a 5-membered aromatic (unsaturated) heterocyclic ring having one heteroatom selected from O, N or S.

Heteroaryl groups within the scope of this definition include but are not limited to: benzoimidazolyl, benzofuranyl, benzofurazanyl, benzopyrazolyl, benzotriazolyl, benzothiophenyl, benzoxazolyl, carbazolyl, carbolinyl, cinnolinyl, furanyl, indolinyl, indolyl, indolazinyl, indazolyl, isobenzofuranyl, isoindolyl, isoquinolyl, isothiazolyl, isoxazolyl, naphthpyridinyl, oxadiazolyl, oxazolyl, oxazoline, isoxazoline, oxetanyl, pyranyl, pyrazinyl, pyrazolyl, pyridazinyl, pyridopyridinyl, pyridazinyl, pyridyl, pyrimidyl, pyrrolyl, quinazolinyl, quinolyl, quinoxalinyl, tetrazolyl, tetrazolopyridyl, thiadiazolyl, thiazolyl, thienyl, triazolyl, azetidinyl, aziridinyl, 1,4-dioxanyl, hexahydroazepinyl, dihydrobenzoimidazolyl, dihydrobenzofuranyl, dihydrobenzothiophenyl, dihydrobenzoxazolyl, dihydrofuranyl, dihydroimidazolyl, dihydroindolyl, dihydroisooxazolyl, dihydroisothiazolyl, dihydrooxadiazolyl, dihydrooxazolyl, dihydropyrazinyl, dihydropyrazolyl, dihydropyridinyl, dihydropyrimidinyl, dihydropyrrolyl, dihydroquinolinyl, dihydrotetrazolyl, dihydrothiadiazolyl, dihydrothiazolyl, dihydrothienyl, dihydrotriazolyl, dihydroazetidinyl, methylenedioxybenzoyl, tetrahydrofuranyl, tetrahydrothienyl, acridinyl, carbazolyl, cinnolinyl, quinoxalinyl, pyrrazolyl, indolyl, benzotriazolyl, benzothiazolyl, benzoxazolyl, isoxazolyl, isothiazolyl, furanyl, thienyl, benzothienyl, benzofuranyl, quinolinyl, isoquinolinyl, oxazolyl, isoxazolyl, indolyl, pyrazinyl, pyridazinyl, pyridinyl, pyrimidinyl, pyrrolyl, tetrahydroquinoline. In cases where the heteroaryl substituent is bicyclic and one ring is non-aromatic or contains no heteroatoms, it is understood that attachment is via the aromatic ring or via the heteroatom containing ring, respectively. If the heteroaryl contains nitrogen atoms, it is understood that the corresponding *N*-oxides thereof are also encompassed by this definition.

The aliphatic, heteroaliphatic, aromatic and heteroaromatic substituents can be optionally substituted one or more times, the same way or differently with any one or more of the following substituents including, but not limited to: aliphatic, heteroaliphatic, aromatic and heteroaromatic substituents, aryl, heteroaryl; alkylaryl; heteroalkylaryl; alkylheteroaryl; heteroalkylheteroaryl; alkoxy; aryloxy; heteroalkoxy; heteroaryloxy; alkylthio; arylthio; heteroalkylthio; heteroarylthio; F; Cl; Br; I; -OH; $-NO_2$; -CN; $-CF_3$; $-CH_2CF_3$; $-CHCl_2$; $-CH_2OH$; $-CH_2CH_2OH$; $-CH_2NH_2$; $-CH_2SO_2CH_3$; $-C(O)R_x$; $-CO_2(R_x)$; $-CON(R_x)_2$; $-OC(O)R_x$; $-OCO_2R_x$; $-OCON(R_x)_2$; $-N(R_X)_2$; $-S(O)R_x$; $-S(O)_2R_x$; $-NR_x(CO)R_x$ wherein each occurrence of R_x independently includes, but is not limited to, aliphatic, alicyclic, heteroaliphatic, heterocyclic, aromatic, heteroaromatic, aryl, heteroaryl, alkylaryl, alkylheteroaryl, heteroalkylaryl or heteroalkylheteroaryl, wherein any of the aliphatic, alicyclic, heteroaliphatic, heterocyclic, alkylaryl, or alkylheteroaryl substituents described above and herein may be substituted or unsubstituted, branched or unbranched, saturated or unsaturated, and wherein any of the aromatic, heteroaromatic, aryl, heteroaryl, (alkyl)aryl or (alkyl)heteroaryl substituents described above and herein may be substituted or unsubstituted. Additionally, it will be appreciated, that any two adjacent substituents taken together may represent a 4, 5, 6, or 7-membered substituted or unsubstituted alicyclic or heterocyclic substituents. Additional examples of generally applicable substituents are illustrated by the specific embodiments shown below.

The terms "halo" and "halogen" refer to a halogen atom selected from the group consisting of F, Cl, Br and I.

The term "halogenated alkyl substituent, haloalkyl" refers to an alkyl substituents as defined above which is substituted with at least one halogen atom. In an embodiment, the halogenated alkyl substituent is perhalogenated. In another embodiment, perfluoroalkyl refers to the halogenated alkyl substituent is a univalent perfluorated substituent of formula C_nF_{2n+1}. For example, the halogenated alkyl substituent may have 1 to 6 carbon atoms, (e.g. 1 to 3 carbon atoms). Accordingly, examples of the alkyl group include trifluoromethyl, 2,2,2-trifluoroethyl, *n*-perfluoropropyl, *n*-perfluorobutyl and *n*-perfluoropentyl.

The term "amino," as used herein, represents $–N(R^{N1})_2$, wherein each R^{N1} is, independently, H, OH, NO_2, $N(R^{N2})_2$, SO_2OR^{N2}, SO_2R^{N2}, SOR^{N2}, an *N*-protecting group, alkyl, alkenyl, alkynyl, alkoxy, aryl, alkaryl, cycloalkyl, alkcycloalkyl, heterocyclyl (e.g., heteroaryl), alkheterocyclyl (e.g., alkheteroaryl), or two R^{N1} combine to form a heterocyclyl or an *N*-protecting group, and wherein each R^{N2} is, independently, H, alkyl, or aryl. In a preferred embodiment, amino is $–NH_2$, or $–NHR^{N1}$, wherein R^{N1} is, independently, OH, NO_2, NH_2, $NR^{N2}{}_2$, SO_2OR^{N2}, SO_2R^{N2}, SOR^{N2}, alkyl, or aryl, and each R^{N2} can be H, alkyl, or aryl. The term "aminoalkyl," as used herein, represents a heteroalkyl group, as defined herein, that is described as an alkyl group, as defined herein, substituted by an amino group, as defined herein. The alkyl and amino each can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the respective group. For example, the alkyl moiety may comprise an oxo (=O) substituent.

As used herein, the term "aryloxy" refers to aromatic or heteroaromatic systems which are coupled to another residue through an oxygen atom. A typical example of an O-aryl is phenoxy. Similarly, "arylalkyl" refers to aromatic and heteroaromatic systems which are coupled to another residue through a carbon chain, saturated or unsaturated, typically of C1-C8, C1-C6, or more particularly C1-C4 or C1-C3 when saturated or C2-C8, C2-C6, C2-C4, or C2-C3 when unsaturated, including the heteroforms thereof. For greater certainty, arylalkyl thus includes an aryl or heteroaryl group as defined above connected to an alkyl, heteroalkyl, alkenyl, heteroalkenyl, alkynyl or heteroalkynyl moiety also as defined above. Typical arylalkyls would be an aryl(C6-C12)alkyl(C1-C8), aryl(C6-C12)alkenyl(C2-C8), or aryl(C6-C12)alkynyl(C2-C8), plus the heteroforms. A typical example is phenylmethyl, commonly referred to as benzyl.

Typical optional substituents on aromatic or heteroaromatic groups include independently halo, CN, NO_2, CF_3, OCF_3, COOR', $CONR'_2$, OR', SR', SOR', SO_2R',

NR'_2, NR'(CO)R',NR'C(O)OR', NR'C(O)NR'_2, $NR'SO_2NR'_2$, or $NR'SO_2R'$, wherein each R' is independently H or an optionally substituted group selected from alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, heteroalkynyl, heteroaryl, and aryl (all as defined above); or the substituent may be an optionally substituted group selected from alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, heteroalkynyl, aryl, heteroaryl, O-aryl, O-heteroaryl and arylalkyl.

Optional substituents on a non-aromatic group (e.g., alkyl, alkenyl, and alkynyl groups), are typically selected from the same list of substituents suitable for aromatic or heteroaromatic groups, except as noted otherwise herein. A non-aromatic group may also include a substituent selected from =O and =NOR' where R' is H or an optionally substituted group selected from alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, heteralkynyl, heteroaryl, and aryl (all as defined above).

In general, a substituent group (e.g., alkyl, alkenyl, alkynyl, or aryl (including all heteroforms defined above) may itself optionally be substituted by additional substituents. The nature of these substituents is similar to those recited with regard to the substituents on the basic structures above. Thus, where an embodiment of a substituent is alkyl, this alkyl may optionally be substituted by the remaining substituents listed as substituents where this makes chemical sense, and where this does not undermine the size limit of alkyl per se; e.g., alkyl substituted by alkyl or by alkenyl would simply extend the upper limit of carbon atoms for these embodiments, and is not included. However, alkyl substituted by aryl, amino, halo and the like would be included. For example, where a group is substituted, the group may be substituted with 1, 2, 3, 4, 5, or 6 substituents. Optional substituents include, but are not limited to: C1-C6 alkyl or heteroaryl, C2-C6 alkenyl or heteroalkenyl, C2-C6 alkynyl or heteroalkynyl, halogen; aryl, heteroaryl, azido(-N_3), nitro (-NO_2), cyano (-CN), acyloxy(-OC(=O)R'), acyl (-C(=O)R'), alkoxy (-OR'), amido (-NR'C(=O)R" or –C(=O)NRR'), amino (-NRR'), carboxylic acid (-CO_2H), carboxylic ester (-CO_2R'), carbamoyl (-OC(=O)NR'R" or -NRC(=O)OR'), hydroxy (-OH), isocyano (-NC), sulfonate (-S(=O)$_2$OR), sulfonamide (-S(=O)$_2$NRR' or –NRS(=O)$_2$R'), or sulfonyl (-S(=O)$_2$R), where each R or R' is selected, independently, from H, C1-C6 alkyl or heteroaryl, C2-C6 alkenyl or heteroalkenyl, 2C-6C alkynyl or heteroalkynyl, aryl, or heteroaryl. A substituted group may have, for example, 1, 2, 3, 4, 5, 6, 7, 8, or 9 substituents.

The term "heterocyclyl, heterocyclic, or Het" as used herein represents cyclic heteroalkyl or heteroalkenyl that is, e.g., a 3-, 4-, 5-, 6- or 7-membered ring, unless otherwise specified, containing one, two, three, or four heteroatoms independently selected from the group consisting of nitrogen, oxygen, and sulfur. The 5-membered ring has zero to two double bonds, and the 6- and 7-membered rings have zero to three double bonds. The term "heterocyclyl" also represents a heterocyclic compound having a bridged multicyclic structure in which one or more carbons and/or heteroatoms bridges two non-adjacent members of a monocyclic ring, e.g., a quinuclidinyl group. The term "heterocyclyl" includes bicyclic, tricyclic, and tetracyclic groups in which any of the above heterocyclic rings is fused to one, two, or three carbocyclic rings, e.g., an aryl ring, a cyclohexane ring, a cyclohexene ring, a cyclopentane ring, a cyclopentene ring, or another monocyclic heterocyclic ring, such as indolyl, quinolyl, isoquinolyl, tetrahydroquinolyl, benzofuryl, benzothienyl and the like.

Some of the compounds of the present invention can comprise one or more stereogenic centers, and thus can exist in various isomeric forms, *e.g.* stereoisomers and/or diastereomers. Thus, the compounds of the invention and pharmaceutical compositions thereof may be in the form of an individual enantiomer, diastereomer or geometric isomer, or may be in the form of a mixture of stereoisomers. In certain embodiments, the compounds of the invention are enantiopure compounds. In certain other embodiments, mixtures of stereoisomers or diastereomers are provided. Moreover, when compounds of the invention exist in tautomeric forms, each tautomer is embraced herein.

Furthermore, certain compounds, as described herein may have one or more double bonds that can exist as either the *Z* or *E* isomer, unless otherwise indicated. The invention additionally encompasses the compounds as individual isomers substantially free of other isomers and alternatively, as mixtures of various isomers, *e.g.,* racemic mixtures of stereoisomers. In addition to the above-mentioned compounds *per se,* this invention also encompasses pharmaceutically acceptable derivatives of these compounds and compositions comprising one or more compounds of the invention and one or more pharmaceutically acceptable excipients or additives.

Treatment Methods

As disclosed herein, miR-1908, miR-199a-3p, miR-199a-5p, and CTGF were identified as endogenous metastasis promoters of metastatic invasion, endothelial recruitment, and colonization in melanoma while DNAJA4, ApoE, LRP1, LRP8, LXR, and miR7 function as metastasis suppressors or inhibitors of the same process. In addition, it was found that these miRNAs convergently target ApoE and the heat-shock factor DNAJA4. Cancer-secreted ApoE suppresses invasion and endothelial recruitment by activating melanoma cell LRP1 and endothelial LRP8 receptors, respectively. DNAJA4, in turn, induces ApoE expression. These miRNAs strongly predict human metastatic outcomes. Pre-treatment with locked nucleic acids (LNAs) targeting miR-199a-3p, miR-199a-5p, and miR-1908 inhibits metastasis to multiple organs, while therapeutic delivery of these LNAs significantly suppresses human melanoma cell metastasis in a mouse model.

Accordingly, this invention provides methods for treating melanoma via increasing in the subject the expression level or activity level of one of the metastasis suppressors. This increasing can be achieved by, among others, forced expression of one or more of the metastasis suppressors DNAJA4, ApoE, LRP1, and LRP8, or decreasing the expression level or activity level of one or more miR-199a-3p, miR-199a-5p, and miR-1908. In addition, the treatment can be achieved by decreasing the expression level or activity level of one or more of the metastasis promoters.

The invention also provides methods for treating in a subject an angiogenic disorder or a disorder of angiogenesis. The terms "angiogenic disorder," "disorder of angiogenesis," and "angiogenesis disorder" are used interchangeably herein, and refer to a disorder characterized by pathological angiogenesis. A disorder characterized by pathological angiogenesis refers to a disorder where abnormal or aberrant angiogenesis, alone or in combination with others, contributes to causation, origination, or symptom of the disorder. Examples of this disorder include various cancers (e.g., vascularized tumors), eye disorders, inflammatory disorders, and others.

Typical vascularized tumors that can be treated with the method include solid tumors, particularly carcinomas, which require a vascular component for the provision of oxygen and nutrients. Exemplary solid tumors include, but are not limited to, carcinomas of the lung, breast, bone, ovary, stomach, pancreas, larynx, esophagus, testes, liver, parotid, biliary tract, colon, rectum, cervix, uterus, endometrium, kidney, bladder, prostate, thyroid, squamous cell carcinomas, adenocarcinomas, small cell carcinomas, melanomas, gliomas, glioblastomas, neuroblastomas, Kaposi's sarcoma, and sarcomas.

A number of disorders or conditions, other than cancer, also can be treated with the above-described method. Examples include arthritis, rheumatoid arthritis, psoriasis, atherosclerosis, diabetic retinopathy, age-related macular degeneration, Grave's disease, vascular restenosis (including restenosis following angioplasty), arteriovenous malformations (AVM), meningioma, hemangioma, neovascular glaucoma, chronic kidney disease, diabetic nephropathy, polycystic kidney disease, interstitial lung disease, pulmonary hypertension, chronic obstructive pulmonary disease (COPD), emphysema, autoimmune hepatitis, chronic inflammatory liver disease, hepatic cirrhosis, cutaneous T-cell lymphoma, rosacea, and basal cell carcinoma.

Other treatment targets include those described in, e.g., US Applications 2009004297, 20090175791, and 20070161553, such as angiofibroma, atherosclerotic plaques, corneal graft neovascularization, hemophilic joints, hypertrophic scars, Osler-Weber syndrome, pyogenic granuloma retrolental fibroplasia, scleroderma, trachoma, vascular adhesions, synovitis, dermatitis, various other inflammatory diseases and disorders, and endometriosis.

Forced Expression of Metastasis Suppressors

Both polypeptides of the aforementioned metastasis suppressors (e.g., DNAJA4, ApoE, LRP1, LRP8, and LXR) and nucleic acid encoding the polypeptides can be used to practice the invention. While many polypeptide preparations can be used, a highly purified or isolated polypeptide is preferred. The terms "peptide," "polypeptide," and "protein" are used herein interchangeably to describe the arrangement of amino acid residues in a polymer. A peptide, polypeptide, or protein can be composed of the standard 20 naturally occurring amino acid, in addition to rare amino acids and synthetic amino acid analogs. They can be any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

The polypeptide "of this invention" includes recombinantly or synthetically produced fusion or chimeric versions of any of the aforementioned metastasis suppressors, having the particular domains or portions that are involved in the network. The term also encompasses polypeptides that have an added amino-terminal methionine (useful for expression in prokaryotic cells).

Within the scope of this invention are fusion proteins containing one or more of the afore-mentioned sequences and a heterologous sequence. A "chimeric" or "fusion" refers to the combination of amino acid sequences of different origin in one polypeptide chain by in-frame combination of their coding nucleotide sequences. The term explicitly encompasses internal fusions, i.e., insertion of sequences of different origin within a polypeptide chain, in addition to fusion to one of its termini. A heterologous polypeptide, nucleic acid, or gene is one that originates from a foreign species, or, if from the same species, is substantially modified from its original form. Two fused domains or sequences are heterologous to each other if they are not adjacent to each other in a naturally occurring protein or nucleic acid.

An "isolated" or "purified" polypeptide refers to a polypeptide that has been separated from other proteins, lipids, and nucleic acids with which it is naturally associated. The polypeptide can constitute at least 10% (i.e., any percentage between 10% and 100%, e.g., 20%, 30%, 40%, 50%, 60%, 70 %, 80%, 85%, 90%, 95%, and 99%) by dry weight of the purified preparation. Purity can be measured by any appropriate standard method, for example, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. An isolated polypeptide described in the invention can be purified from a natural source, produced by recombinant DNA techniques, or by chemical methods.

A "recombinant" polypeptide refers to a polypeptide produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. A "synthetic" polypeptide refers to a polypeptide prepared by chemical synthesis. The term "recombinant" when used with reference, e.g., to a cell, nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified.

"Overexpression" refers to the expression of a RNA or polypeptide encoded by a nucleic acid introduced into a host cell, wherein the RNA or polypeptide or protein is either not normally present in the host cell, or wherein the RNA or polypeptide is present in said host cell at a higher level than that normally expressed from the endogenous gene encoding the RNA or polypeptide.

The amino acid composition of each of the above-mentioned polypeptides may vary without disrupting their functions - the ability to up-regulate the above-mentioned network (e.g., increase the activation level of the ApoE/LRP signaling pathway), thereby inhibiting metastasis to multiple organs. For example, it can contain one or more conservative amino acid substitutions. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), β-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in one of the above-described polypeptides (e.g., SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, and 18) is preferably replaced with another amino acid residue from the same side chain family. Alternatively, mutations can be introduced randomly along all or part of the sequences, such as by saturation mutagenesis, and the resultant mutants can be screened for the ability to up-regulate the above-mentioned network or ApoE/LRP signaling pathway, and trigger the respective cellular response to identify mutants that retain the activity as descried below in the examples.

A functional equivalent of a polypeptide of this invention refers to a derivative of the polypeptide, e.g., a protein having one or more point mutations, insertions, deletions, truncations, a fusion protein, or a combination thereof. It retains substantially the activity to of the above-mentioned polypeptide. The isolated polypeptide of this invention can contain the sequence of one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, and 18, or a functional equivalent or fragment thereof. In general, the functional equivalent is at least 75% (e.g., any number between 75% and 100%, inclusive, e.g., 70 %, 80%, 85%, 90%, 95%, and 99%) identical to one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, and 18.

A polypeptide described in this invention can be obtained as a recombinant polypeptide. To prepare a recombinant polypeptide, a nucleic acid encoding it can be linked to another nucleic acid encoding a fusion partner, e.g., glutathione-s-transferase (GST), 6x-His epitope tag, or M13 Gene 3 protein. The resultant fusion nucleic acid expresses in suitable host cells a fusion protein that can be isolated by methods known in the art. The isolated fusion protein can be further treated, e.g., by enzymatic digestion, to remove the fusion partner and obtain the recombinant polypeptide of this invention. Alternatively, the polypeptide of the invention can be chemically synthesized (see e.g., Creighton, "Proteins: Structures and Molecular Principles," W.H. Freeman & Co., NY, 1983). For additional guidance, skilled artisans may consult Ausubel *et al.* (Current Protocols in Molecular Biology and Short Protocols in Molecular Biology, 3rd Ed. 1987 & 1995), Sambrook *et al.* (Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989), and chemical synthesis Gait, M.J. Ed. (Oligonucleotide Synthesis, IRL Press, Oxford, 1984).

Due to their functions as cellular protein or membrane protein, DNAJA4, LRP1, LRP8, and LXR can be associated with, e.g., conjugated or fused to, one or more of an amino acid sequence comprising a cell-penetrating peptide (CPP) sequence, and the like. In this manner, a composition of the invention as discussed below can include a transport enhancer. A cell-penetrating peptide (CPP) generally consists of less than 30 amino acids and has a net positive charge. CPPs internalize in living animal cells in an endocytotic or receptor/energy-independent manner. There are several classes of CPPs with various origins, from totally protein-derived CPPs via chimeric CPPs to completely synthetic CPPs. Examples of CPPs are known in the art. See, e.g., U.S. Application Nos. 20090099066 and 20100279918. It is know that CPPs can delivery an exogenous protein into various cells.

All of naturally occurring versions, genetic engineered versions, and chemically synthesized versions of the above-mentioned polypeptides can be used to practice the invention disclosed therein. Polypeptides obtained by recombinant DNA technology may have the same amino acid sequence as a naturally occurring version (e.g., one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, and 18) or a functionally equivalent thereof. They also include chemically modified versions. Examples of chemically modified polypeptides include polypeptides subjected to conformational change, addition or deletion of a side chain, and those to which a compound such as polyethylene glycol has been bound. Once purified and tested by standard methods or according to the method described in the examples below or other methods known in the art, the polypeptides can be included in suitable composition.

For expressing the above-mentioned factors, the invention provides a nucleic acid that encodes any of the polypeptides mentioned above. Preferably, the nucleotide sequences are isolated and/or purified. A nucleic acid refers to a DNA molecule (e.g., but not limited to, a cDNA or genomic DNA), an RNA molecule (e.g., but not limited to, an mRNA), or a DNA or RNA analog. A DNA or RNA analog can be synthesized from nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded. An "isolated nucleic acid" is a nucleic acid the structure of which is not identical to that of any naturally occurring nucleic acid or to that of any fragment of a naturally occurring genomic nucleic acid. The term therefore covers, for example, (a) a DNA which has the sequence of part of a naturally occurring genomic DNA molecule but is not flanked by both of the coding sequences that flank that part of the molecule in the genome of the organism in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein.

The terms "RNA," "RNA molecule," and "ribonucleic acid molecule" are used interchangeably herein, and refer to a polymer of ribonucleotides. The term "DNA" or "DNA molecule" or deoxyribonucleic acid molecule" refers to a polymer of deoxyribonucleotides. DNA and RNA can be synthesized naturally (e.g., by DNA replication or transcription of DNA, respectively). RNA can be post-transcriptionally modified. DNA and RNA also can be chemically synthesized. DNA and RNA can be single-stranded (i.e., ssRNA and ssDNA, respectively) or multi-stranded (e.g., double-stranded, i.e., dsRNA and dsDNA, respectively).

The present invention also provides recombinant constructs having one or more of the nucleotide sequences described herein. Example of the constructs include a vector, such as a plasmid or viral vector, into which a nucleic acid sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred embodiment, the construct further includes regulatory sequences, including a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are also described in Sambrook *et al.* (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press).

Examples of expression vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of or Simian virus 40 (SV40), bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, a nucleic acid sequence encoding one of the polypeptides described above can be inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and related sub-cloning procedures are within the scope of those skilled in the art.

The nucleic acid sequence in the aforementioned expression vector is preferably operatively linked to an appropriate transcription control sequence (promoter) to direct mRNA synthesis. Examples of such promoters include: the retroviral long terminal (LTR) or SV40 promoter, the *E. coli* lac or trp promoter, the phage lambda PL promoter, and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or viruses. The expression vector can also contain a ribosome binding site for translation initiation, and a transcription terminator. The vector may include appropriate sequences for amplifying expression. In addition, the expression vector preferably contains one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell cultures, or such as tetracycline or ampicillin resistance in *E. coli*.

The vector containing the appropriate nucleic acid sequences as described above, as well as an appropriate promoter or control sequence, can be employed to transform an appropriate host to permit the host to express the polypeptides described above. Such vectors can be used in gene therapy. Examples of suitable expression hosts include bacterial cells (e.g., *E. coli*, *Streptomyces*, *Salmonella typhimurium*), fungal cells (yeast), insect cells (e.g., *Drosophila* and *Spodoptera frugiperda* (Sf9)), animal cells (e.g., CHO, COS, and HEK 293), adenoviruses, and plant cells. The selection of an appropriate host is within the scope of those skilled in the art. In some embodiments, the present invention provides methods for producing the above mentioned polypeptides by transfecting a host cell with an expression vector having a nucleotide sequence that encodes one of the polypeptides. The host cells are then cultured under a suitable condition, which allows for the expression of the polypeptide.

Decreasing Expression or Activity Level of Metastasis Promoters

As mentioned above, one can use an inhibitory agent that decreases the expression or activity level of miR-199a-3p, miR-199a-5p, miR-1908, or CTGF in treating melanoma. An inhibitory agent (i.e., inhibitor) can be a nucleic acid, a polypeptide, an antibody, or a small molecule compound. In one example, the inhibitor functions at a level of transcription, mRNA stability, translation, protein stability/degradation, protein modification, and protein binding.

A nucleic acid inhibitor can encode a small interference RNA (e.g., an RNAi agent) that targets one or more of the above-mentioned genes, e.g., CTGF, and inhibits its expression or activity. The term “RNAi agent” refers to an RNA, or analog thereof, having sufficient sequence complementarity to a target RNA to direct RNA interference. Examples also include a DNA that can be used to make the RNA. RNA interference (RNAi) refers to a sequence-specific or selective process by which a target molecule (e.g., a target gene, protein or RNA) is down-regulated. Generally, an interfering RNA (“iRNA”) is a double stranded short-interfering RNA (siRNA), short hairpin RNA (shRNA), or single-stranded micro-RNA (miRNA) that results in catalytic degradation of specific mRNAs, and also can be used to lower or inhibit gene expression.

The term “short interfering RNA” or “siRNA” (also known as “small interfering RNAs”) refers to an RNA agent, preferably a double-stranded agent, of about 10-50 nucleotides in length, preferably between about 15-25 nucleotides in length, more preferably about 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length, the strands optionally having overhanging ends comprising, for example 1, 2 or 3 overhanging nucleotides (or nucleotide analogs), which is capable of directing or mediating RNA interference. Naturally-occurring siRNAs are generated from longer dsRNA molecules (e.g., >25 nucleotides in length) by a cell's RNAi machinery (e.g., Dicer or a homolog thereof).

The term "miRNA" or "microRNA" refers to an RNA agent, preferably a single-stranded agent, of about 10-50 nucleotides in length, preferably between about 15-25 nucleotides in length, more preferably about 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length, which is capable of directing or mediating RNA interference. Naturally-occurring miRNAs are generated from stem-loop precursor RNAs (i.e., pre-miRNAs) by Dicer. The term "Dicer" as used herein, includes Dicer as well as any Dicer orthologue or homologue capable of processing dsRNA structures into siRNAs, miRNAs, siRNA-like or miRNA-like molecules. The term microRNA (or "miRNA") is used interchangeably with the term "small temporal RNA" (or "stRNA") based on the fact that naturally-occurring microRNAs (or "miRNAs") have been found to be expressed in a temporal fashion (e.g., during development).

The term "shRNA", as used herein, refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region.

Within the scope of this invention is utilization of RNAi featuring degradation of RNA molecules (e.g., within a cell). Degradation is catalyzed by an enzymatic, RNA-induced silencing complex (RISC). A RNA agent having a sequence sufficiently complementary to a target RNA sequence (e.g., the above-mentioned CTGF gene) to direct RNAi means that the RNA agent has a homology of at least 50%, (e.g., 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99%, or 100% homology) to the target RNA sequence so that the two are sufficiently complementary to each other to hybridize and trigger the destruction of the target RNA by the RNAi machinery (e.g., the RISC complex) or process. A RNA agent having a "sequence sufficiently complementary to a target RNA sequence to direct RNAi" also means that the RNA agent has a sequence sufficient to trigger the translational inhibition of the target RNA by the RNAi machinery or process. A RNA agent also can have a sequence sufficiently complementary to a target RNA encoded by the target DNA sequence such that the target DNA sequence is chromatically silenced. In other words, the RNA agent has a sequence sufficient to induce transcriptional gene silencing, *e.g.*, to down-modulate gene expression at or near the target DNA sequence, *e.g.*, by inducing chromatin structural changes at or near the target DNA sequence.

The above-mentioned polynucleotides can be delivered using polymeric, biodegradable microparticle or microcapsule delivery devices known in the art. Another way to achieve uptake of the polynucleotides is using liposomes, prepared by standard methods. The polynucleotide can be incorporated alone into these delivery vehicles or co-incorporated with tissue-specific antibodies. Alternatively, one can prepare a molecular conjugate composed of a plasmid or other vector attached to poly-L-lysine by electrostatic or covalent forces. Poly-L-lysine binds to a ligand that can bind to a receptor on target cells (Cristiano, *et al*., 1995, J. Mol. Med. 73:479). Alternatively, tissue specific targeting can be achieved by the use of tissue-specific transcriptional regulatory elements that are known in the art. Delivery of naked DNA (i.e., without a delivery vehicle) to an intramuscular, intradermal, or subcutaneous site is another means to achieve *in vivo* expression.

siRNA, miRNA, and asRNA (antisense RNA) molecules can be designed by methods well known in the art. siRNA, miRNA, and asRNA molecules with homology sufficient to provide sequence specificity required to uniquely degrade any RNA can be designed using programs known in the art, including, but not limited to, those maintained on websites for AMBION, Inc. and DHARMACON, Inc. Systematic testing of several designed species for optimization of the siRNA, miRNA, and asRNA sequence can be routinely performed by those skilled in the art. Considerations when designing short interfering nucleic acid molecules include, but are not limited to, biophysical, thermodynamic, and structural considerations, base preferences at specific positions in the sense strand, and homology. These considerations are well known in the art and provide guidelines for designing the above-mentioned RNA molecules.

An antisense polynucleotide (preferably DNA) of the present invention can be any antisense polynucleotide so long as it possesses a base sequence complementary or substantially complementary to that of the gene encoding a component of the aforementioned network. The base sequence can be at least about 70%, 80%, 90%, or 95% homology to the complement of the gene encoding the polypeptide. These antisense DNAs can be synthesized using a DNA synthesizer.

The antisense DNA of the present invention may contain changed or modified sugars, bases or linkages. The antisense DNA, as well as the RNAi agent mentioned above, may also be provided in a specialized form such as liposomes, microspheres, or may be applied to gene therapy, or may be provided in combination with attached moieties. Such attached moieties include polycations such as polylysine that act as charge neutralizers of the phosphate backbone, or hydrophobic moieties such as lipids (e.g., phospholipids, cholesterols, etc.) that enhance the interaction with cell membranes or increase uptake of the nucleic acid. Preferred examples of the lipids to be attached are cholesterols or derivatives thereof (e.g., cholesteryl chloroformate, cholic acid, etc.). These moieties may be attached to the nucleic acid at the 3' or 5' ends thereof and may also be attached thereto through a base, sugar, or intramolecular nucleoside linkage. Other moieties may be capping groups specifically placed at the 3' or 5' ends of the nucleic acid to prevent degradation by nucleases such as exonuclease, RNase, etc. Such capping groups include, but are not limited to, hydroxyl protecting groups known in the art, including glycols such as polyethylene glycol, tetraethylene glycol and the like. The inhibitory action of the antisense DNA can be examined using a cell-line or animal based gene expression system of the present invention *in vivo* and *in vitro*.

The above-discussed nucleic acids encoding one or more of the polypeptides mentioned above or RNAi agents can be cloned in a vector for delivering to cells *in vitro* or *in vivo*. For *in vivo* uses, the delivery can target a specific tissue or organ (e.g., skin). Targeted delivery involves the use of vectors (e.g., organ-homing peptides) that are targeted to specific organs or tissues after systemic administration. For example, the vector can have a covalent conjugate of avidin and a monoclonal antibody to a liver specific protein.

In certain embodiments, the present invention provides methods for *in vivo* expression the above-mentioned metastsis suppressors. Such method would achieve its therapeutic effect by introduction of nucleic acid sequences encoding any of the factors into cells or tissues of a human or a non-human animal in need of inhibiting endothelial recruitment, cancer cell invasion, or metastatic angiogenesis. Delivery of the nucleic acid sequences can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Preferred for therapeutic delivery of the nucleic acid sequences is the use of targeted liposomes.

Various viral vectors which can be utilized for gene therapy disclosed herein include, adenovirus, adeno-associated virus (AAV), herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus and a lentivirus. Preferably, the retroviral vector is a lentivirus or a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes.

All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. Retroviral vectors can be made target-specific by attaching, for example, a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using a target-specific antibody or hormone that has a receptor in the target. Those of skill in the art will recognize that specific polynucleotide sequences can be inserted into the retroviral genome or attached to a viral envelope to allow target specific delivery of the retroviral vector.

Another targeted system for delivery of nucleic acids is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. RNA, DNA, and intact virions can be encapsulated within the aqueous interior and delivered to cells in a biologically active form. Methods for efficient gene transfer using a liposome vehicle are known in the art. The composition of the liposome is usually a combination of phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidyl-ethanolamine, sphingolipids, cerebrosides, and gangliosides. Exemplary phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine, and distearoyl-phosphatidylcholine. The targeting of liposomes is also possible based on, for example, organ-specificity, cell-specificity, and organelle-specificity and is known in the art.

When used *in vivo*, it is desirable to use a reversible delivery-expression system. To that end, the Cre-loxP or FLP/FRT system and other similar systems can be used for reversible delivery-expression of one or more of the above-described nucleic acids. See WO2005/112620, WO2005/039643, U.S. Applications 20050130919, 20030022375, 20020022018, 20030027335, and 20040216178. In particular, the reversible delivery-expression system described in US Application NO 20100284990 can be used to provide a selective or emergency shut-off.

In another example, the above-mentioned inhibitory agent can be a polypeptide or a protein complex, such as an antibody. The term "antibody" refers to an immunoglobulin molecule or immunologically active portion thereof, i.e., an antigen-binding portion. Examples include, but are not limited to, a protein having at least one or two, heavy (H) chain variable regions (V_H), and at least one or two light (L) chain variable regions (V_L). The V_H and V_L regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, termed "framework regions" (FR). As used herein, the term "immunoglobulin" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized human immunoglobulin genes include the kappa, lambda, alpha (IgA1 and IgA2), gamma (IgG1, IgG2, IgG3, and IgG4), delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes.

The term "antigen-binding portion" of an antibody (or "antibody portion") refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., LRP1, LRP8, and CTGF). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a V_H domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242:423-

426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

Antibodies that specifically bind to one of the above-mentioned target proteins (e.g., CTGF) can be made using methods known in the art. This antibody can be a polyclonal or a monoclonal antibody. In one embodiment, the antibody can be recombinantly produced, e.g., produced by phage display or by combinatorial methods. In another embodiment, the antibody is a fully human antibody (e.g., an antibody made in a mouse which has been genetically engineered to produce an antibody from a human immunoglobulin sequence), a humanized antibody, or a non-human antibody, for example, but not limited to, a rodent (mouse or rat), goat, primate (for example, but not limited to, monkey), rabbit, or camel antibody. Examples of methods to generate humanized version of antibodies include, but are not limited to, CDR grafting (Queen *et al.,* U.S. Pat. No. 5,585,089; Riechmann *et al.,* Nature 332:323 (1988)), chain shuffling (U.S. Pat. No. 5,565,332); and veneering or resurfacing (EP 592,106; EP 519,596); Padlan, Molecular Immunology 28(415):489-498 (1991); Studnicka *et al.,* Protein Engineering 7(6):805-814 (1994); Roguska. *et al.*, PNAS 91:969-973 (1994)). Examples of methods to generate fully human antibodies include, but are not limited to, generation of antibodies from mice that can express human immunoglobulin genes and use of phage-display technology to generate and screen human immunoglobulin gene libraries.

An "isolated antibody" is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (e.g., an isolated antibody that specifically binds CTGF is substantially free of antibodies that specifically bind antigens other than such an antigen). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

The term "human antibody", as used herein, is intended to include antibodies having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. Furthermore, if the antibody contains a constant region, the constant region also is derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. In one embodiment, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic nonhuman animal, e.g., a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

The term "recombinant human antibody," as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (e.g., a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom (described further below), (b) antibodies isolated from a host cell transformed to express the human antibody, e.g., from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable regions in which the framework and CDR regions are derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to in vitro mutagenesis (or, when an animal transgenic for human Ig sequences is used, in vivo somatic mutagenesis) and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire in vivo.

As used herein, "isotype" refers to the antibody class (e.g., IgM or IgG1) that is encoded by the heavy chain constant region genes. The phrases "an antibody recognizing an antigen" and "an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen." As used herein, the term "high affinity" for an IgG antibody refers to an antibody having a K_D of 10^{-7} M or less, preferably 10^{-8} M or less, more preferably 10^{-9} M or less and even more preferably 10^{-10} M or less for a target antigen. However, "high affinity" binding can vary for other antibody isotypes. For example, "high affinity" binding for an IgM isotype refers to an antibody having a K_D of 10^{-7} M or less, more preferably 10^{-8} M or less.

In one example, a composition contains a monoclonal antibody that neutralizes CTGF. In one embodiment, this antibody can be a fully human antibody, a humanized antibody, or a non-human antibody, for example, but not limited to, a rodent (mouse or rat), goat, primate (for example, but not limited to, monkey), rabbit, or camel antibody. In one embodiment, one or more amino-acids of this monoclonal monoclonal antibody may be substituted in order to alter its physical properties. These properties include, but are not limited to, binding specificity, binding affinity, immunogenicity, and antibody isotype. Pharmaceutical compositions containing fully human or humanized versions of the above described antibodies can be used for treating melanoma or for inhibiting endothelial recruitment, cancer cell invasion, or metastatic angiogenesis.

As used herein, a "subject" refers to a human and a non-human animal. Examples of a non-human animal include all vertebrates, e.g., mammals, such as non-human mammals, non-human primates (particularly higher primates), dog, rodent (e.g., mouse or rat), guinea pig, cat, and rabbit, and non-mammals, such as birds, amphibians, reptiles, etc. In one embodiment, the subject is a human. In another embodiment, the subject is an experimental animal or animal suitable as a disease model. A subject to be treated for a disorder can be identified by standard diagnosing techniques for the disorder. Optionally, the subject can be examined for mutation, expression level, or activity level of one or more of the miR-199a-3p, miR-199a-5p, miR-1908, and CTGF mentioned above by methods known in the art or described above before treatment. If the subject has a particular mutation in the gene, or if the gene expression or activity level is, for example, greater in a sample from the subject than that in a sample from a normal person, the subject is a candidate for treatment of this invention.

To confirm the inhibition or treatment, one can evaluate and/or verify the inhibition of endothelial recruitment or resulting angiogenesis using technology known in the art before and/or after the administering step. Exemplary technologies include angiography or arteriography, a medical imaging technique used to visualize the inside, or lumen, of blood vessels and organs of the body, can generally be done by injecting a radio-opaque contrast agent into the blood vessel and imaging using X-ray based techniques such as fluoroscopy.

"Treating" or "treatment" as used herein refers to administration of a compound or agent to a subject who has a disorder with the purpose to cure, alleviate, relieve, remedy, delay the onset of, prevent, or ameliorate the disorder, the symptom of a disorder, the disease state secondary to the disorder, or the predisposition toward the disorder. An "effective amount" or "therapeutically effective amount" refers to an amount of the compound or agent that is capable of producing a medically desirable result in a treated subject. The treatment method can be performed *in vivo* or *ex vivo*, alone or in conjunction with other drugs or therapy. A therapeutically effective amount can be administered in one or more administrations, applications or dosages and is not intended to be limited to a particular formulation or administration route.

The expression "effective amount" as used herein, refers to a sufficient amount of the compound of the invention to exhibit the desired therapeutic effect. The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the particular therapeutic agent and the like. The compounds of the invention are preferably formulated in dosage unit form for ease of administration and uniformity of dosage. The expression "dosage unit form" as used herein refers to a physically discrete unit of therapeutic agent appropriate for the patient to be treated. It will be understood, however, that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient or organism will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the anticancer activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts.

A therapeutic agent can be administered *in vivo* or *ex vivo*, alone or co-administered in conjunction with other drugs or therapy, i.e., a cocktail therapy. As used herein, the term "co-administration" or "co-administered" refers to the administration of at least two agent(s) or therapies to a subject. For example, in the treatment of tumors, particularly vascularized, malignant tumors, the agents can be used alone or in combination with, e.g., chemotherapeutic, radiotherapeutic, apoptopic, anti-angiogenic agents and/or immunotoxins or coaguligands. In some embodiments, the co-administration of two or more agents/therapies is concurrent. In other embodiments, a first agent/therapy is administered prior to a second agent/therapy. Those of skill in the art understand that the formulations and/or routes of administration of the various agents/therapies used may vary.

In an *in vivo* approach, a compound or agent is administered to a subject. Generally, the compound is suspended in a pharmaceutically-acceptable carrier (such as, for example, but not limited to, physiological saline) and administered orally or by intravenous infusion, or injected or implanted subcutaneously, intramuscularly, intrathecally, intraperitoneally, intrarectally, intravaginally, intranasally, intragastrically, intratracheally, or intrapulmonarily.

The dosage required depends on the choice of the route of administration; the nature of the formulation; the nature of the patient's illness; the subject's size, weight, surface area, age, and sex; other drugs being administered; and the judgment of the attending physician. Suitable dosages are in the range of 0.01-100 mg/kg. Variations in the needed dosage are to be expected in view of the variety of compounds available and the different efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by i.v. injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization as is well understood in the art. Encapsulation of the compound in a suitable delivery vehicle (e.g., polymeric microparticles or implantable devices) can increase the efficiency of delivery, particularly for oral delivery.

Compositions

Within the scope of this invention is a composition that contains a suitable carrier and one or more of the therapeutic agents described above. The composition can be a pharmaceutical composition that contains a pharmaceutically acceptable carrier, a dietary composition that contains a dietarily acceptable suitable carrier, or a cosmetic composition that contains a cosmetically acceptable carrier.

The term "pharmaceutical composition" refers to the combination of an active agent with a carrier, inert or active, making the composition especially suitable for diagnostic or therapeutic use *in vivo* or *ex vivo*. A "pharmaceutically acceptable carrier," after administered to or upon a subject, does not cause undesirable physiological effects. The carrier in the pharmaceutical composition must be "acceptable" also in the sense that it is compatible with the active ingredient and can be capable of stabilizing it. One or more solubilizing agents can be utilized as pharmaceutical carriers for delivery of an active compound. Examples of a pharmaceutically acceptable carrier include, but are not limited to, biocompatible vehicles, adjuvants, additives, and diluents to achieve a composition usable as a dosage form. Examples of other carriers include colloidal silicon oxide, magnesium stearate, cellulose, sodium lauryl sulfate, and D&C Yellow # 10.

As used herein, the term "pharmaceutically acceptable salt" refers to those salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like, and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts of amines, carboxylic acids, and other types of compounds, are well known in the art. For example, S.M. Berge, *et al.* describe pharmaceutically acceptable salts in detail in *J. Pharmaceutical Sciences*, 66: 1-19 (1977). The salts can be prepared *in situ* during the final isolation and purification of the compounds of the invention, or separately by reacting a free base or free acid function with a suitable reagent, as described generally below. For example, a free base function can be reacted with a suitable acid. Furthermore, where the compounds of the invention carry an acidic moiety, suitable pharmaceutically acceptable salts thereof may, include metal salts such as alkali metal salts, *e.g.* sodium or potassium salts; and alkaline earth metal salts, *e.g.* calcium or magnesium salts. Examples of pharmaceutically acceptable, nontoxic acid addition salts are salts of an amino group formed with inorganic acids such as hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid and perchloric acid or with organic acids such as acetic acid, oxalic acid, maleic acid, tartaric acid, citric acid, succinic acid or malonic acid or by using other methods used in the art such as ion exchange. Other pharmaceutically acceptable salts, include adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, formate, fumarate, glucoheptonate, glycerophosphate, gluconate, hemisulfate, heptanoate, hexanoate, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, p-toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like. Further pharmaceutically acceptable salts include, when appropriate, nontoxic ammonium, quaternary ammonium, and amine cations formed using counterions such as halide, hydroxide, carboxylate, sulfate, phosphate, nitrate, loweralkyl sulfonate and aryl sulfonate.

As described above, the pharmaceutical compositions of the present invention additionally comprise a pharmaceutically acceptable carrier, which, as used herein, includes any and all solvents, diluents, or other liquid vehicle, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's Pharmaceutical Sciences, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980) discloses various carriers used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Except insofar as any conventional carrier medium is incompatible with the compounds of the invention, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition, its use is contemplated to be within the scope of this invention. Some examples of materials which can serve as pharmaceutically acceptable carriers include, but are not limited to, sugars such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatine; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil, sesame oil; olive oil; corn oil and soybean oil; glycols; such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; natural and synthetic phospholipids, such as soybean and egg yolk phosphatides, lecithin, hydrogenated soy lecithin, dimyristoyl lecithin, dipalmitoyl lecithin, distearoyl lecithin, dioleoyl lecithin, hydroxylated lecithin, lysophosphatidylcholine, cardiolipin, sphingomyelin, phosphatidylcholine, phosphatidyl ethanolamine, diastearoyl phosphatidylethanolamine (DSPE) and its pegylated esters, such as DSPE-PEG750 and, DSPE-PEG2000, phosphatidic acid, phosphatidyl glycerol and phosphatidyl serine. Commercial grades of lecithin which are preferred include those which are available under the trade name Phosal® or Phospholipon® and include Phosal 53 MCT, Phosal 50 PG, Phosal 75 SA, Phospholipon 90H, Phospholipon 90G and Phospholipon 90 NG; soy-phosphatidylcholine (SoyPC) and DSPE-PEG2000 are particularly preferred; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol, and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator.

The above-described composition, in any of the forms described above, can be used for treating melanoma, or any other disease or condition described herein. An effective amount refers to the amount of an active compound/agent that is required to confer a therapeutic effect on a treated subject. Effective doses will vary, as recognized by those skilled in the art, depending on the types of diseases treated, route of administration, excipient usage, and the possibility of co-usage with other therapeutic treatment.

A pharmaceutical composition of this invention can be administered parenterally, orally, nasally, rectally, topically, or buccally. The term "parenteral" as used herein refers to subcutaneous, intracutaneous, intravenous, intrmuscular, intraarticular, intraarterial, intrasynovial, intrasternal, intrathecal, intralesional, or intracranial injection, as well as any suitable infusion technique.

A sterile injectable composition can be a solution or suspension in a non-toxic parenterally acceptable diluent or solvent. Such solutions include, but are not limited to, 1,3-butanediol, mannitol, water, Ringer's solution, and isotonic sodium chloride solution.

In addition, fixed oils are conventionally employed as a solvent or suspending medium (e.g., synthetic mono- or diglycerides). Fatty acid, such as, but not limited to, oleic acid and its glyceride derivatives, are useful in the preparation of injectables, as are natural pharmaceutically acceptable oils, such as, but not limited to, olive oil or castor oil, polyoxyethylated versions thereof. These oil solutions or suspensions also can contain a long chain alcohol diluent or dispersant such as, but not limited to, carboxymethyl cellulose, or similar dispersing agents. Other commonly used surfactants, such as, but not limited to, Tweens or Spans or other similar emulsifying agents or bioavailability enhancers, which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms also can be used for the purpose of formulation.

A composition for oral administration can be any orally acceptable dosage form including capsules, tablets, emulsions and aqueous suspensions, dispersions, and solutions. In the case of tablets, commonly used carriers include, but are not limited to, lactose and corn starch. Lubricating agents, such as, but not limited to, magnesium stearate, also are typically added. For oral administration in a capsule form, useful diluents include, but are not limited to, lactose and dried corn starch. When aqueous suspensions or emulsions are administered orally, the active ingredient can be suspended or dissolved in an oily phase combined with emulsifying or suspending agents. If desired, certain sweetening, flavoring, or coloring agents can be added.

Pharmaceutical compositions for topical administration according to the described invention can be formulated as solutions, ointments, creams, suspensions, lotions, powders, pastes, gels, sprays, aerosols, or oils. Alternatively, topical formulations can be in the form of patches or dressings impregnated with active ingredient(s), which can optionally comprise one or more excipients or diluents. In some preferred embodiments, the topical formulations include a material that would enhance absorption or penetration of the active agent(s) through the skin or other affected areas.

A topical composition contains a safe and effective amount of a dermatologically acceptable carrier suitable for application to the skin. A "cosmetically acceptable" or "dermatologically-acceptable" composition or component refers a composition or component that is suitable for use in contact with human skin without undue toxicity, incompatibility, instability, allergic response, and the like. The carrier enables an active agent and optional component to be delivered to the skin at an appropriate concentration(s).

The carrier thus can act as a diluent, dispersant, solvent, or the like to ensure that the active materials are applied to and distributed evenly over the selected target at an appropriate concentration. The carrier can be solid, semi-solid, or liquid. The carrier can be in the form of a lotion, a cream, or a gel, in particular one that has a sufficient thickness or yield point to prevent the active materials from sedimenting. The carrier can be inert or possess dermatological benefits. It also should be physically and chemically compatible with the active components described herein, and should not unduly impair stability, efficacy, or other use benefits associated with the composition.

Combination Therapies

In some embodiments, the pharmaceutical composition may further comprise an additional compound having antiproliferative activity. The additional compound having antiproliferative activity can be selected from a group of antiproliferative agents including those shown in Table 2.

It will also be appreciated that the compounds and pharmaceutical compositions of the present invention can be formulated and employed in combination therapies, that is, the compounds and pharmaceutical compositions can be formulated with or administered concurrently with, prior to, or subsequent to, one or more other desired therapeutics or medical procedures. The particular combination of therapies (therapeutics or procedures) to employ in a combination regimen will take into account compatibility of the desired therapeutics and/or procedures and the desired therapeutic effect to be achieved. It will also be appreciated that the therapies employed may achieve a desired effect for the same disorder, or they may achieve different effects (e.g., control of any adverse effects).

By "antiproliferative agent" is meant any antiproliferative agent, including those antiproliferative agents listed in Table 2, any of which can be used in combination with a LXR agonist to treat the medical conditions recited herein. Antiproliferative agents also include organo-platine derivatives, naphtoquinone and benzoquinone derivatives, chrysophanic acid and anthroquinone derivatives thereof.

Table 2		
Alkylating agents	Busulfan dacarbazine ifosfamide hexamethylmelamine thiotepa dacarbazine lomustine cyclophosphamide	Chlorambucil procarbazine altretamine estramustine phosphate mechlorethamine streptozocin temozolomide Semustine
Platinum agents	spiroplatin tetraplatin ormaplatin iproplatin ZD-0473 (AnorMED) oxaliplatin carboplatin	lobaplatin (Aeterna) satraplatin (Johnson Matthey) BBR-3464 (Hoffmann-La Roche) SM-11355 (Sumitomo) AP-5280 (Access) cisplatin
Antimetabolites	azacytidine Floxuridine 2-chlorodeoxyadenosine 6-mercaptopurine 6-thioguanine cytarabine 2-fluorodeoxy cytidine methotrexate tomudex fludarabine raltitrexed	trimetrexate deoxycoformycin pentostatin hydroxyurea decitabine (SuperGen) clofarabine (Bioenvision) irofulven (MGI Pharma) DMDC (Hoffmann-La Roche) ethynylcytidine (Taiho) gemcitabine capecitabine
Topoisomerase inhibitors	amsacrine epirubicin etoposide teniposide or mitoxantrone 7-ethyl-10-hydroxy-camptothecin dexrazoxanet (TopoTarget) pixantrone (Novuspharma) rebeccamycin analogue (Exelixis) BBR-3576 (Novuspharma) rubitecan (SuperGen) irinotecan (CPT-11) topotecan	exatecan mesylate (Daiichi) quinamed (ChemGenex) gimatecan (Sigma-Tau) diflomotecan (Beaufour-Ipsen) TAS-103 (Taiho) elsamitrucin (Spectrum) J-107088 (Merck & Co) BNP-1350 (BioNumerik) CKD-602 (Chong Kun Dang) KW-2170 (Kyowa Hakko) hydroxycamptothecin (SN-38)

Table 2		
Antitumor antibiotics	valrubicin therarubicin idarubicin rubidazone plicamycin porfiromycin mitoxantrone (novantrone) amonafide	azonafide anthrapyrazole oxantrazole losoxantrone MEN-10755 (Menarini) GPX-100 (Gem Pharmaceuticals) Epirubicin mitoxantrone doxorubicin
Antimitotic agents	colchicine vinblastine vindesine dolastatin 10 (NCI) rhizoxin (Fujisawa) mivobulin (Warner-Lambert) cemadotin (BASF) RPR 109881A (Aventis) TXD 258 (Aventis) epothilone B (Novartis) T 900607 (Tularik) T 138067 (Tularik) cryptophycin 52 (Eli Lilly) vinflunine (Fabre) auristatin PE (Teikoku Hormone) BMS 247550 (BMS) BMS 184476 (BMS) BMS 188797 (BMS) taxoprexin (Protarga) SB 408075 (GlaxoSmithKline) Vinorelbine Trichostatin A	E7010 (Abbott) PG-TXL (Cell Therapeutics) IDN 5109 (Bayer) A 105972 (Abbott) A 204197 (Abbott) LU 223651 (BASF) D 24851 (ASTAMedica) ER-86526 (Eisai) combretastatin A4 (BMS) isohomohalichondrin-B (PharmaMar) ZD 6126 (AstraZeneca) AZ10992 (Asahi) IDN-5109 (Indena) AVLB (Prescient NeuroPharma) azaepothilone B (BMS) BNP-7787 (BioNumerik) CA-4 prodrug (OXiGENE) dolastatin-10 (NIH) CA-4 (OXiGENE) docetaxel vincristine paclitaxel
Aromatase inhibitors	aminoglutethimide atamestane (BioMedicines) letrozole anastrazole	YM-511 (Yamanouchi) formestane exemestane
Thymidylate synthase inhibitors	pemetrexed (Eli Lilly) ZD-9331 (BTG)	nolatrexed (Eximias) CoFactor™ (BioKeys)

Table 2		
DNA antagonists	trabectedin (PharmaMar) glufosfamide (Baxter International) albumin + 32P (Isotope Solutions) thymectacin (NewBiotics)	edotreotide (Novartis) mafosfamide (Baxter International) apaziquone (Spectrum Pharmaceuticals) O6 benzyl guanine (Paligent)
Farnesyltransferase inhibitors	arglabin (NuOncology Labs) lonafarnib (Schering-Plough) BAY-43-9006 (Bayer)	tipifarnib (Johnson & Johnson) perillyl alcohol (DOR BioPharma)
Pump inhibitors	CBT-1 (CBA Pharma) tariquidar (Xenova) MS-209 (Schering AG)	zosuquidar trihydrochloride (Eli Lilly) biricodar dicitrate (Vertex)
Histone acetyltransferase inhibitors	tacedinaline (Pfizer) SAHA (Aton Pharma) MS-275 (Schering AG)	pivaloyloxymethyl butyrate (Titan) depsipeptide (Fujisawa)
Metalloproteinase inhibitors	Neovastat (Aeterna Laboratories) marimastat (British Biotech)	CMT-3 (CollaGenex) BMS-275291 (Celltech)
Ribonucleoside reductase inhibitors	gallium maltolate (Titan) triapine (Vion)	tezacitabine (Aventis) didox (Molecules for Health)
TNF alpha agonists/antagonists	virulizin (Lorus Therapeutics) CDC-394 (Celgene)	revimid (Celgene)
Endothelin A receptor antagonist	atrasentan (Abbott) ZD-4054 (AstraZeneca)	YM-598 (Yamanouchi)
Retinoic acid receptor agonists	fenretinide (Johnson & Johnson) LGD-1550 (Ligand)	alitretinoin (Ligand)

Table 2		
Immuno-modulators	interferon oncophage (Antigenics) GMK (Progenics) adenocarcinoma vaccine (Biomira) CTP-37 (AVI BioPharma) IRX-2 (Immuno-Rx) PEP-005 (Peplin Biotech) synchrovax vaccines (CTL Immuno) melanoma vaccine (CTL Immuno) p21 RAS vaccine (GemVax) MAGE-A3 (GSK) nivolumab (BMS) abatacept (BMS)	dexosome therapy (Anosys) pentrix (Australian Cancer Technology) ISF-154 (Tragen) cancer vaccine (Intercell) norelin (Biostar) BLP-25 (Biomira) MGV (Progenics) ß-alethine (Dovetail) CLL therapy (Vasogen) Ipilimumab (BMS), CM-10 (cCam Biotherapeutics) MPDL3280A (Genentech)
Hormonal and antihormonal agents	estrogens conjugated estrogens ethinyl estradiol chlortrianisen idenestrol hydroxyprogesterone caproate medroxyprogesterone testosterone testosterone propionate; fluoxymesterone methyltestosterone diethylstilbestrol megestrol bicalutamide flutamide nilutamide	dexamethasone prednisone methylprednisolone prednisolone aminoglutethimide leuprolide octreotide mitotane P-04 (Novogen) 2-methoxyestradiol (EntreMed) arzoxifene (Eli Lilly) tamoxifen toremofine goserelin Leuporelin bicalutamide
Photodynamic agents	talaporfin (Light Sciences) Theralux (Theratechnologies) motexafin gadolinium (Pharmacyclics)	Pd-bacteriopheophorbide (Yeda) lutetium texaphyrin (Pharmacyclics) hypericin

Table 2		
Kinase Inhibitors	imatinib (Novartis) leflunomide (Sugen/Pharmacia) ZD1839 (AstraZeneca) erlotinib (Oncogene Science) canertinib (Pfizer) squalamine (Genaera) SU5416 (Pharmacia) SU6668 (Pharmacia) ZD4190 (AstraZeneca) ZD6474 (AstraZeneca) vatalanib (Novartis) PKI166 (Novartis) GW2016 (GlaxoSmithKline) EKB-509 (Wyeth) trastuzumab (Genentech) OSI-774 (Tarceva^{TM}) CI-1033 (Pfizer) SU11248 (Pharmacia) RH3 (York Medical) Genistein Radicinol Met-MAb (Roche)	EKB-569 (Wyeth) kahalide F (PharmaMar) CEP-701 (Cephalon) CEP-751 (Cephalon) MLN518 (Millenium) PKC412 (Novartis) Phenoxodiol (Novogen) C225 (ImClone) rhu-Mab (Genentech) MDX-H210 (Medarex) 2C4 (Genentech) MDX-447 (Medarex) ABX-EGF (Abgenix) IMC-1C11 (ImClone) Tyrphostins Gefitinib (Iressa) PTK787 (Novartis) EMD 72000 (Merck) Emodin Radicinol Vemurafenib (B-Raf enzyme inhibitor, Daiichi Sankyo)

Table 2	
SR-27897 (CCK A inhibitor, Sanofi-Synthelabo)	ceflatonin (apoptosis promotor, ChemGenex)
tocladesine (cyclic AMP agonist, Ribapharm)	BCX-1777 (PNP inhibitor, BioCryst)
alvocidib (CDK inhibitor, Aventis)	ranpirnase (ribonuclease stimulant, Alfacell)
CV-247 (COX-2 inhibitor, Ivy Medical)	galarubicin (RNA synthesis inhibitor, Dong-A)
P54 (COX-2 inhibitor, Phytopharm)	tirapazamine (reducing agent, SRI International)
CapCell™ (CYP450 stimulant, Bavarian Nordic)	N-acetylcysteine (reducing agent, Zambon)
GCS-100 (gal3 antagonist, GlycoGenesys)	R-flurbiprofen (NF-kappaB inhibitor, Encore)
G17DT immunogen (gastrin inhibitor, Aphton)	3CPA (NF-kappaB inhibitor, Active Biotech)
efaproxiral (oxygenator, Allos Therapeutics)	seocalcitol (vitamin D receptor agonist, Leo)
PI-88 (heparanase inhibitor, Progen)	131-I-TM-601 (DNA antagonist, TransMolecular)
tesmilifene (histamine antagonist, YM BioSciences)	eflornithine (ODC inhibitor , ILEX Oncology)
histamine (histamine H2 receptor agonist, Maxim)	minodronic acid (osteoclast inhibitor, Yamanouchi)
tiazofurin (IMPDH inhibitor, Ribapharm)	indisulam (p53 stimulant, Eisai)
cilengitide (integrin antagonist, Merck KGaA)	aplidine (PPT inhibitor, PharmaMar)
SR-31747 (IL-1 antagonist, Sanofi-Synthelabo)	gemtuzumab (CD33 antibody, Wyeth Ayerst)
CCI-779 (mTOR kinase inhibitor, Wyeth)	PG2 (hematopoiesis enhancer, Pharmagenesis)
exisulind (PDE V inhibitor, Cell Pathways)	Immunol™ (triclosan oral rinse, Endo)
CP-461 (PDE V inhibitor, Cell Pathways)	triacetyluridine (uridine prodrug , Wellstat)
AG-2037 (GART inhibitor, Pfizer)	SN-4071 (sarcoma agent, Signature BioScience)
WX-UK1 (plasminogen activator inhibitor, Wilex)	TransMID-107™ (immunotoxin, KS Biomedix)
PBI-1402 (PMN stimulant, ProMetic LifeSciences)	PCK-3145 (apoptosis promotor, Procyon)
bortezomib (proteasome inhibitor, Millennium)	doranidazole (apoptosis promotor, Pola)
SRL-172 (T cell stimulant, SR Pharma)	CHS-828 (cytotoxic agent, Leo)
TLK-286 (glutathione S transferase inhibitor, Telik)	trans-retinoic acid (differentiator, NIH)
PT-100 (growth factor agonist, Point Therapeutics)	MX6 (apoptosis promotor, MAXIA)
midostaurin (PKC inhibitor, Novartis)	apomine (apoptosis promotor, ILEX Oncology)
bryostatin-1 (PKC stimulant, GPC Biotech)	urocidin (apoptosis promotor, Bioniche)
CDA-II (apoptosis promotor, Everlife)	Ro-31-7453 (apoptosis promotor, La Roche)
SDX-101 (apoptosis promotor, Salmedix)	brostallicin (apoptosis promotor, Pharmacia)
rituximab (CD20 antibody, Genentech	β-lapachone
carmustine	
Mitoxantrone	
Bleomycin	
Absinthin	
Chrysophanic acid	
Cesium oxides	
BRAF inhibitors,	
PDL1 inhibitors	

Diagnosis and Prognosis Methods

The above-describe genes can be used in determining whether a subject has, or is at risk of having, metastatic melanoma. Alternatively, they can be used for determining a prognosis of such a disorder in a subject.

Diagnosis Methods

In one aspect, the invention provides qualitative and quantitative information to determine whether a subject has or is predisposed to metastatic melanoma or other disease characterized by endothelial recruitment, cancer cell invasion, or metastatic angiogenesis. A subject having such a disorder or prone to it can be determined based on the expression levels, patterns, or profiles of the above-described genes or their products (mRNAs, microRNAs, or polypeptides) in a test sample from the subject. In other words, the products can be used as markers to indicate the presence or absence of the disorder. Diagnostic and prognostic assays of the invention include methods for assessing the expression level of the products. The methods allow one to detect the disorder. For example, a relative increase in the expression level of one or more promoters (i.e., miR-199a-3p, miR-199a-5p, miR-1908, and CTGF) is indicative of presence the disorder. Conversely, a lower expression level or a lack of the expression is indicative lack of the disorder.

The presence, level, or absence of, an mRNA, microRNA, or polypeptide product in a test sample can be evaluated by obtaining a test sample from a test subject and contacting the test sample with a compound or an agent capable of detecting the nucleic acid (e.g., RNA or DNA probe) or polypeptide. The "test sample" includes tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. The level of expression of a gene(s) of interest can be measured in a number of ways, including measuring the RNA encoded by the gene.

Expressed RNA samples can be isolated from biological samples using any of a number of well-known procedures. For example, biological samples can be lysed in a guanidinium-based lysis buffer, optionally containing additional components to stabilize the RNA. In some embodiments, the lysis buffer can contain purified RNAs as controls to monitor recovery and stability of RNA from cell cultures. Examples of such purified RNA templates include the Kanamycin Positive Control RNA from PROMEGA (Madison, WI), and 7.5 kb Poly(A)-Tailed RNA from LIFE TECHNOLOGIES (Rockville, MD). Lysates may be used immediately or stored frozen at, e.g., -80°C.

Optionally, total RNA can be purified from cell lysates (or other types of samples) using silica-based isolation in an automation-compatible, 96-well format, such as the RNEASY purification platform (QIAGEN, Inc., Valencia, CA). Other RNA isolation methods are contemplated, such as extraction with silica-coated beads or guanidinium. Further methods for RNA isolation and preparation can be devised by one skilled in the art.

The methods of the present invention can be performed using crude samples (e.g., blood, serum, plasma, or cell lysates), eliminating the need to isolate RNA. RNAse inhibitors are optionally added to the crude samples. When using crude cellular lysates, it should be noted that genomic DNA can contribute one or more copies of a target sequence, e.g., a gene, depending on the sample. In situations in which the target sequence is derived from one or more highly expressed genes, the signal arising from genomic DNA may not be significant. But for genes expressed at low levels, the background can be eliminated by treating the samples with DNAse, or by using primers that target splice junctions for subsequent priming of cDNA or amplification products.

The level of RNA corresponding to a gene in a cell can be determined both *in situ* and *in vitro*. RNA isolated from a test sample can be used in hybridization or amplification assays that include, Southern or Northern analyses, PCR analyses, and probe arrays. A preferred diagnostic method for the detection of RNA levels involves contacting the isolated RNA with a nucleic acid probe that can hybridize to the RNA encoded by the gene. The probe can be a full-length nucleic acid or a portion thereof, such as an oligonucleotide of at least 10 nucleotides in length and sufficient to specifically hybridize under stringent conditions to the RNA.

In one format, RNA (or cDNA prepared from it) is immobilized on a surface and contacted with the probes, for example, by running the isolated RNA on an agarose gel and transferring the RNA from the gel to a membrane, such as nitrocellulose. In another format, the probes are immobilized on a surface and the RNA (or cDNA) is contacted with the probes, for example, in a gene chip array. A skilled artisan can adapt known RNA detection methods for detecting the level of RNA.

The level of RNA (or cDNA prepared from it) in a sample encoded by a gene to be examined can be evaluated with nucleic acid amplification, e.g., by standard PCR (U.S.

Patent No. 4,683,202), RT-PCR (Bustin S. J Mol Endocrinol. 25:169-93, 2000), quantitative PCR (Ong Y. *et al.*, Hematology. 7:59-67, 2002), real time PCR (Ginzinger D. Exp Hematol. 30:503-12, 2002), and *in situ* PCR (Thaker V. Methods Mol Biol. 115:379-402, 1999), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques known in the art. In another embodiment, the methods of the invention further include contacting a control sample with a compound or agent capable of detecting the RNA of a gene and comparing the presence of the RNA in the control sample with the presence of the RNA in the test sample.

The above-described methods and markers can be used to assess the risk of a subject for developing melanoma. In particular, the invention can be applied to those in high risk cohort who already have certain risks so as to gain critical insight into early detection. A change in levels of miR gene products associated with melanoma can be detected prior to, or in the early stages of, the development of transformed or neoplastic phenotypes in cells of a subject. The invention therefore also provides a method for screening a subject who is at risk of developing melanoma, comprising evaluating the level of at least one gene product, or a combination of gene products, associated with melanoma in a biological sample obtained form the subject's skin. Accordingly, an alteration in the level of the gene product, or combination of gene products, in the biological sample as compared to the level of a corresponding gene product in a control sample, is indicative of the subject being at risk for developing melanoma. The biological sample used for such screening can include skin tissue that is either normal or suspected to be cancerous. Subjects with a change in the level of one or more gene products associated with melanoma are candidates for further monitoring and testing. Such further testing can comprise histological examination of tissue samples, or other techniques within the skill in the art.

As used herein, the term “diagnosis” means detecting a disease or disorder or determining the stage or degree of a disease or disorder. Usually, a diagnosis of a disease or disorder is based on the evaluation of one or more factors and/or symptoms that are indicative of the disease. That is, a diagnosis can be made based on the presence, absence or amount of a factor which is indicative of presence or absence of the disease or condition. Each factor or symptom that is considered to be indicative for the diagnosis of a particular disease does not need be exclusively related to the particular disease; i.e. there may be differential diagnoses that can be inferred from a diagnostic factor or symptom. Likewise, there may be instances where a factor or symptom that is indicative of a particular disease is present in an individual that does not have the particular disease. The diagnostic methods may be used independently, or in combination with other diagnosing and/or staging methods known in the medical art for a particular disease or disorder, e.g., melanoma.

Prognosis Methods

The diagnostic methods described above can identify subjects having, or at risk of developing, a melanoma. In addition, changes in expression levels and/or trends of the above-mentioned genes in a biological sample, e.g., peripheral blood samples, can provide an early indication of recovery or lack thereof. For example, a further increase (or decline) or persistently-altered gene expression levels of the promoter genes (or inhibitor genes) indicate a poor prognosis, i.e., lack of improvement or health decline. Accordingly, these genes allow one to assess post-treatment recovery of melanoma. The analysis of this select group of genes or a subset thereof indicates outcomes of the conditions.

The prognostic assays described herein can be used to determine whether a subject is suitable to be administered with an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat melanoma or other disorders associated with endothelial recruitment, cancer cell invasion, or metastatic angiogenesis. For example, such assays can be used to determine whether a subject can be administered with a chemotherapeutic agent.

Thus, also provided by this invention is a method of monitoring a treatment for a cellular proliferative disorder in a subject. For this purpose, gene expression levels of the genes disclosed herein can be determined for test samples from a subject before, during, or after undergoing a treatment. The magnitudes of the changes in the levels as compared to a baseline level are then assessed. A decrease in the expression of the above-mentioned promoter genes (miR-199a-3p, miR-199a-5p, miR-1908, and CTGF) after the treatment indicates that the subject can be further treated by the same treatment. Similarly, an increase in the inhibitors (DNAJA4, ApoE, LRP1, and LRP8) also indicates that the subject can be further treated by the same treatment. Conversely, further increase or persistent high expression levels of one or more of the promoter genes is indicate lack of improvement or health decline.

Information obtained from practice of the above assays is useful in prognostication, identifying progression of, and clinical management of diseases and other deleterious conditions affecting an individual subject's health status. In preferred embodiments, the foregoing diagnostic assays provide information useful in prognostication, identifying progression of and management of melanoma and other conditions characterized by endothelial recruitment, cancer cell invasion, or metastatic angiogenesis. The information more specifically assists the clinician in designing chemotherapeutic or other treatment regimes to eradicate such conditions from the body of an afflicted subject, a human.

The term "prognosis" as used herein refers to a prediction of the probable course and outcome of a clinical condition or disease. A prognosis is usually made by evaluating factors or symptoms of a disease that are indicative of a favorable or unfavorable course or outcome of the disease. The phrase "determining the prognosis" as used herein refers to the process by which the skilled artisan can predict the course or outcome of a condition in a patient. The term "prognosis" does not refer to the ability to predict the course or outcome of a condition with 100% accuracy instead, the skilled artisan will understand that the term "prognosis" refers to an increased probability that a certain course or outcome will occur; that is, that a course or outcome is more likely to occur in a patient exhibiting a given condition, when compared to those individuals not exhibiting the condition.

The terms "favorable prognosis" and "positive prognosis," or "unfavorable prognosis" and "negative prognosis" as used herein are relative terms for the prediction of the probable course and/or likely outcome of a condition or a disease. A favorable or positive prognosis predicts a better outcome for a condition than an unfavorable or negative prognosis. In a general sense, a "favorable prognosis" is an outcome that is relatively better than many other possible prognoses that could be associated with a particular condition, whereas an unfavorable prognosis predicts an outcome that is relatively worse than many other possible prognoses that could be associated with a particular condition. Typical examples of a favorable or positive prognosis include a better than average cure rate, a lower propensity for metastasis, a longer than expected life expectancy, differentiation of a benign process from a cancerous process, and the like. For example, a positive prognosis is one where a patient has a 50% probability of being cured of a particular cancer after treatment, while the average patient with the same cancer has only a 25% probability of being cured.

The terms "determining," "measuring," "assessing," and "assaying" are used interchangeably and include both quantitative and qualitative measurement, and include determining if a characteristic, trait, or feature is present or not. Assessing may be relative or absolute. "Assessing the presence of" a target includes determining the amount of the target present, as well as determining whether it is present or absent.

Arrays

Also provided in the invention is a biochip or array. The biochip/array may contain a solid or semi-solid substrate having an attached probe or plurality of probes described herein. The probes may be capable of hybridizing to a target sequence under stringent hybridization conditions. The probes may be attached at spatially defined address on the substrate. More than one probe per target sequence may be used, with either overlapping probes or probes to different sections of a particular target sequence. The probes may be capable of hybridizing to target sequences associated with a single disorder appreciated by those in the art. The probes may either be synthesized first, with subsequent attachment to the biochip, or may be directly synthesized on the biochip.

"Attached" or "immobilized" as used herein to refer to a nucleic acid (e.g., a probe) and a solid support may mean that the binding between the probe and the solid support is sufficient to be stable under conditions of binding, washing, analysis, and removal. The binding may be covalent or non-covalent. Covalent bonds may be formed directly between the probe and the solid support or may be formed by a cross linker or by inclusion of a specific reactive group on either the solid support or the probe or both molecules. Non-covalent binding may be one or more of electrostatic, hydrophilic, and hydrophobic interactions. Included in non-covalent binding is the covalent attachment of a molecule, such as streptavidin, to the support and the non-covalent binding of a biotinylated probe to the streptavidin. Immobilization may also involve a combination of covalent and non-covalent interactions.

The solid substrate can be a material that may be modified to contain discrete individual sites appropriate for the attachment or association of the probes and is amenable to at least one detection method. Examples of such substrates include glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, TeflonJ, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses and plastics. The substrates may allow optical detection without appreciably fluorescing.

The substrate can be planar, although other configurations of substrates may be used as well. For example, probes may be placed on the inside surface of a tube, for flow-through sample analysis to minimize sample volume. Similarly, the substrate may be flexible, such as flexible foam, including closed cell foams made of particular plastics.

The array/biochip and the probe may be derivatized with chemical functional groups for subsequent attachment of the two. For example, the biochip may be derivatized with a chemical functional group including, but not limited to, amino groups, carboxyl groups, oxo groups or thiol groups. Using these functional groups, the probes may be attached using functional groups on the probes either directly or indirectly using a linker. The probes may be attached to the solid support by either the 5' terminus, 3' terminus, or via an internal nucleotide. The probe may also be attached to the solid support non-covalently. For example, biotinylated oligonucleotides can be made, which may bind to surfaces covalently coated with streptavidin, resulting in attachment. Alternatively, probes may be synthesized on the surface using techniques such as photopolymerization and photolithography. Detailed discussion of methods for linking nucleic acids to a support substrate can be found in, e.g., U.S. Patent Nos. 5837832, 6087112, 5215882, 5707807, 5807522, 5958342, 5994076, 6004755, 6048695, 6060240, 6090556, and 6040138.

In some embodiments, an expressed transcript (e.g., a transcript of a microRNA gene described herein) is represented in the nucleic acid arrays. In such embodiments, a set of binding sites can include probes with different nucleic acids that are complementary to different sequence segments of the expressed transcript. Examples of such nucleic acids can be of length of 15 to 200 bases, 20 to 100 bases, 25 to 50 bases, 40 to 60 bases. Each probe sequence can also include one or more linker sequences in addition to the sequence that is complementary to its target sequence. A linker sequence is a sequence between the sequence that is complementary to its target sequence and the surface of support. For example, the nucleic acid arrays of the invention can have one probe specific to each target microRNA gene. However, if desired, the nucleic acid arrays can contain at least 2, 5, 10, 100, 200, 300, 400, 500 or more probes specific to some expressed transcript (e.g., a transcript of a microRNA gene described herein).

Kits

In another aspect, the present invention provides kits embodying the methods, compositions, and systems for analysis of the polypeptides and microRNA expression as described herein. Such a kit may contain a nucleic acid described herein together with any or all of the following: assay reagents, buffers, probes and/or primers, and sterile saline or another pharmaceutically acceptable emulsion and suspension base. In addition, the kit may include instructional materials containing directions (e.g., protocols) for the practice of the methods described herein. For example, the kit may be a kit for the amplification, detection, identification or quantification of a target mRNA or microRNA sequence. To that end, the kit may contain a suitable primer (e.g., hairpin primers), a forward primer, a reverse primer, and a probe.

In one example, a kit of the invention includes one or more microarray slides (or alternative microarray format) onto which a plurality of different nucleic acids (each corresponding to one of the above-mentioned genes) have been deposited. The kit can also include a plurality of labeled probes. Alternatively, the kit can include a plurality of polynucleotide sequences suitable as probes and a selection of labels suitable for customizing the included polynucleotide sequences, or other polynucleotide sequences at the discretion of the practitioner. Commonly, at least one included polynucleotide sequence corresponds to a control sequence, e.g., a normalization gene or the like. Exemplary labels include, but are not limited to, a fluorophore, a dye, a radiolabel, an enzyme tag, that is linked to a nucleic acid primer.

In one embodiment, kits that are suitable for amplifying nucleic acid corresponding to the expressed RNA samples are provided. Such a kit includes reagents and primers suitable for use in any of the amplification methods described above. Alternatively, or additionally, the kits are suitable for amplifying a signal corresponding to hybridization between a probe and a target nucleic acid sample (e.g., deposited on a microarray).

In addition, one or more materials and/or reagents required for preparing a biological sample for gene expression analysis are optionally included in the kit. Furthermore, optionally included in the kits are one or more enzymes suitable for amplifying nucleic acids, including various polymerases (RT, Taq, etc.), one or more deoxynucleotides, and buffers to provide the necessary reaction mixture for amplification.

Typically, the kits are employed for analyzing gene expression patterns using mRNA or microRNA as the starting template. The RNA template may be presented as either total cellular RNA or isolated RNA; both types of sample yield comparable results. In other embodiments, the methods and kits described in the present invention allow quantitation of other products of gene expression, including tRNA, rRNA, or other transcription products.

Optionally, the kits of the invention further include software to expedite the generation, analysis and/or storage of data, and to facilitate access to databases. The software includes logical instructions, instructions sets, or suitable computer programs that can be used in the collection, storage and/or analysis of the data. Comparative and relational analysis of the data is possible using the software provided.

The kits optionally contain distinct containers for each individual reagent and/or enzyme component. Each component will generally be suitable as aliquoted in its respective container. The container of the kits optionally includes at least one vial, ampule, or test tube. Flasks, bottles and other container mechanisms into which the reagents can be placed and/or aliquoted are also possible. The individual containers of the kit are preferably maintained in close confinement for commercial sale. Suitable larger containers may include injection or blow-molded plastic containers into which the desired vials are retained. Instructions, such as written directions or videotaped demonstrations detailing the use of the kits of the present invention, are optionally provided with the kit.

In a further aspect, the present invention provides for the use of any composition or kit herein, for the practice of any method or assay herein, and/or for the use of any apparatus or kit to practice any assay or method herein.

A "test sample" or a "biological sample" as used herein may mean a sample of biological tissue or fluid that comprises nucleic acids. Such samples include, but are not limited to, tissue or body fluid isolated from animals. Biological samples may also include sections of tissues such as biopsy and autopsy samples, frozen sections taken for histological purposes, blood, plasma, serum, sputum, stool, tears, mucus, urine, effusions, amniotic fluid, ascitic fluid, hair, and skin. Biological samples also include explants and primary and/or transformed cell cultures derived from patient tissues. A biological sample may be provided by removing a sample of cells from an animal, but can also be accomplished by using previously isolated cells (e.g., isolated by another person, at another time, and/or for another purpose), or by performing the methods described herein in vivo. Archival tissues, such as those having treatment or outcome history, may also be used.

The term "body fluid" or "bodily fluid" refers to any fluid from the body of an animal. Examples of body fluids include, but are not limited to, plasma, serum, blood, lymphatic fluid, cerebrospinal fluid, synovial fluid, urine, saliva, mucous, phlegm and sputum. A body fluid sample may be collected by any suitable method. The body fluid sample may be used immediately or may be stored for later use. Any suitable storage method known in the art may be used to store the body fluid sample: for example, the sample may be frozen at about -20°C to about -70°C. Suitable body fluids are acellular fluids. "Acellular" fluids include body fluid samples in which cells are absent or are present in such low amounts that the miRNA level determined reflects its level in the liquid portion of the sample, rather than in the cellular portion. Such acellular body fluids are generally produced by processing a cell-containing body fluid by, for example, centrifugation or filtration, to remove the cells. Typically, an acellular body fluid contains no intact cells however, some may contain cell fragments or cellular debris. Examples of acellular fluids include plasma or serum, or body fluids from which cells have been removed.

The term "gene" used herein refers to a natural (e.g., genomic) or synthetic gene comprising transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (e.g., introns, 5'- and 3'-untranslated sequences). The coding region of a gene may be a nucleotide sequence coding for an amino acid sequence or a functional RNA, such as tRNA, rRNA, catalytic RNA, siRNA, miRNA or antisense RNA. A gene may also be an mRNA or cDNA corresponding to the coding regions (e.g., exons and miRNA) optionally comprising 5'- or 3'-untranslated sequences linked thereto. A gene may also be an amplified nucleic acid molecule produced in vitro comprising all or a part of the coding region and/or 5'- or 3'-untranslated sequences linked thereto. The term also includes pseudogenes, which are dysfunctional relatives of known genes that have lost their protein-coding ability or are otherwise no longer expressed in a cell.

"Expression profile" as used herein refers to a genomic expression profile, e.g., an expression profile of microRNAs. Profiles may be generated by any convenient means for determining a level of a nucleic acid sequence e.g., quantitative hybridization of microRNA, cRNA, etc., quantitative PCR, ELISA for quantification, and the like, and allow the analysis of differential gene expression between two samples. A subject or patient sample, e.g., cells or a collection thereof, e.g., tissues, is assayed. Samples are collected by any convenient method, as known in the art. Nucleic acid sequences of interest are nucleic acid sequences that are found to be predictive, including the nucleic acid sequences of those described herein, where the expression profile may include expression data for 5, 10, 20, 25, 50, 100 or more of, including all of the listed nucleic acid sequences. The term “expression profile” may also mean measuring the abundance of the nucleic acid sequences in the measured samples.

“Differential expression” refers to qualitative or quantitative differences in the temporal and/or cellular gene expression patterns within and among cells and tissue. Thus, a differentially expressed gene can qualitatively have its expression altered, including an activation or inactivation, in, e.g., normal versus disease tissue. Genes may be turned on or turned off in a particular state, relative to another state thus permitting comparison of two or more states. A qualitatively regulated gene will exhibit an expression pattern within a state or cell type that may be detectable by standard techniques. Some genes will be expressed in one state or cell type, but not in both. Alternatively, the difference in expression may be quantitative, e.g., in that expression is modulated, up-regulated, resulting in an increased amount of transcript, or down-regulated, resulting in a decreased amount of transcript. The degree to which expression differs need only be large enough to quantify via standard characterization techniques such as expression arrays, quantitative reverse transcriptase PCR, Northern analysis, and RNase protection.

“Nucleic acid” or “oligonucleotide” or “polynucleotide” as used herein refers to at least two nucleotides covalently linked together. The depiction of a single strand also defines the sequence of the complementary strand. Thus, a nucleic acid also encompasses the complementary strand of a depicted single strand. Many variants of a nucleic acid may be used for the same purpose as a given nucleic acid. Thus, a nucleic acid also encompasses substantially identical nucleic acids and complements thereof. A single strand provides a probe that may hybridize to a target sequence under stringent hybridization conditions. Thus, a nucleic acid also encompasses a probe that hybridizes under stringent hybridization conditions.

Nucleic acids may be single stranded or double stranded, or may contain portions of both double stranded and single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA, or a hybrid, where the nucleic acid may contain combinations of deoxyribo- and ribo-nucleotides, and combinations of bases including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine and isoguanine. Nucleic acids may be obtained by chemical synthesis methods or by recombinant methods.

The term "primer" refers to any nucleic acid that is capable of hybridizing at its 3' end to a complementary nucleic acid molecule, and that provides a free 3' hydroxyl terminus which can be extended by a nucleic acid polymerase. As used herein, amplification primers are a pair of nucleic acid molecules that can anneal to 5' or 3' regions of a gene (plus and minus strands, respectively, or vice-versa) and contain a short region in between. Under appropriate conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule having the nucleotide sequence flanked by the primers. For in situ methods, a cell or tissue sample can be prepared and immobilized on a support, such as a glass slide, and then contacted with a probe that can hybridize to RNA. Alternative methods for amplifying nucleic acids corresponding to expressed RNA samples include those described in, e.g., U.S. Patent No. 7,897,750.

The term "probe" as used herein refers to an oligonucleotide capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. Probes may bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. There may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids described herein. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. A probe may be single stranded or partially single and partially double stranded. The strandedness of the probe is dictated by the structure, composition, and properties of the target sequence. Probes may be directly labeled or indirectly labeled such as with biotin to which a streptavidin complex may later bind.

"Complement" or "complementary" as used herein to refer to a nucleic acid may mean Watson-Crick (e.g., A-T/U and C-G) or Hoogsteen base pairing between nucleotides or nucleotide analogs of nucleic acid molecules. A full complement or fully complementary may mean 100% complementary base pairing between nucleotides or nucleotide analogs of nucleic acid molecules.

"Stringent hybridization conditions" as used herein refers to conditions under which a first nucleic acid sequence (e.g., probe) hybridizes to a second nucleic acid sequence (e.g., target), such as in a complex mixture of nucleic acids. Stringent conditions are sequence-dependent and be different in different circumstances, and can be suitably selected by one skilled in the art. Stringent conditions may be selected to be about 5-10°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength pH. The Tm may be the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at Tm, 50% of the probes are occupied at equilibrium). Stringent conditions may be those in which the salt concentration is less than about 1.0 M sodium ion, such as about 0.01-1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., about 10-50 nucleotides) and at least about 60°C for long probes (e.g., greater than about 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal may be at least 2 to 10 times background hybridization. Exemplary stringent hybridization conditions include the following: 50% formamide, 5xSSC, and 1% SDS, incubating at 42°C, or, 5xSSC, 1% SDS, incubating at 65°C., with wash in 0.2xSSC, and 0.1% SDS at 65°C. However, several factors other than temperature, such as salt concentration, can influence the stringency of hybridization and one skilled in the art can suitably select the factors to accomplish a similar stringency.

As used herein the term "reference value" refers to a value that statistically correlates to a particular outcome when compared to an assay result. In preferred embodiments, the reference value is determined from statistical analysis of studies that compare microRNA expression with known clinical outcomes. The reference value may be a threshold score value or a cutoff score value. Typically a reference value will be a threshold above (or below) which one outcome is more probable and below which an alternative threshold is more probable.

In one embodiment, a reference level may be one or more circulating miRNA levels expressed as an average of the level of the circulating miRNA from samples taken from a control population of healthy (disease-free) subjects. In another embodiment, the reference level may be the level in the same subject at a different time, e.g., before the present assay, such as the level determined prior to the subject developing the disease or prior to initiating therapy. In general, samples are normalized by a common factor. For example, acellular body fluid samples are normalized by volume body fluid and cell-containing samples are normalized by protein content or cell count. Nucleic acid samples may also be normalized relative to an internal control nucleic acid.

As disclosed herein, the difference of the level of one or more polypeptides or RNAs (mRNAs or microRNAs) is indicative of a disease or a stage thereof. The phrase "difference of the level" refers to differences in the quantity of a particular marker, such as a nucleic acid, in a sample as compared to a control or reference level. For example, the quantity of a particular biomarker may be present at an elevated amount or at a decreased amount in samples of patients with a neoplastic disease compared to a reference level. In one embodiment, a "difference of a level" may be a difference between the quantity of a particular biomarker present in a sample as compared to a control (e.g., reference value) of at least about 1%, 2%, 3%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 75%, 80% 100%, 150%, 200%, or more. In one embodiment, a "difference of a level" may be a statistically significant difference between the quantities of a biomarker present in a sample as compared to a control. For example, a difference may be statistically significant if the measured level of the biomarker falls outside of about 1.0 standard deviation, about 1.5 standard deviations, about 2.0 standard deviations, or about 2.5 stand deviations of the mean of any control or reference group. With respect to miRNA measurement, the level may be measured from real-time PCR as the Ct value, which may be normalized to a ΔCt value as described in the Examples below.

Drug Screening

The invention provides a method for identifying a compound that are useful for treating melanoma or for inhibiting endothelial recruitment, cell invasion, or metastatic angiogenesis.

Candidate compounds to be screened (e.g., proteins, peptides, peptidomimetics, peptoids, antibodies, small molecules, or other drugs) can be obtained using any of the numerous approaches in combinatorial library methods known in the art. Such libraries include: peptide libraries, peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone that is resistant to enzymatic degradation); spatially addressable parallel solid phase or solution phase libraries; synthetic libraries obtained by deconvolution or affinity chromatography selection; and the "one-bead one-compound" libraries. See, e.g., Zuckermann *et al*. 1994, J. Med. Chem. 37:2678-2685; and Lam, 1997, Anticancer Drug Des. 12:145. Examples of methods for the synthesis of molecular libraries can be found in, e.g., DeWitt *et al*., 1993, PNAS USA 90:6909; Erb *et al*., 1994, PNAS USA 91:11422; Zuckermann *et al*., 1994, J. Med. Chem. 37:2678; Cho *et al*., 1993, Science 261:1303; Carrell *et al*., 1994, Angew. Chem. Int. Ed. Engl. 33:2059; Carell *et al*., 1994, Angew. Chem. Int. Ed. Engl. 33:2061; and Gallop *et al*., 1994 J. Med. Chem. 37:1233. Libraries of compounds may be presented in solution (e.g., Houghten, 1992, Biotechniques 13:412-421), or on beads (Lam, 1991, Nature 354:82-84), chips (Fodor, 1993, Nature 364:555-556), bacteria (U.S. Patent No. 5,223,409), spores (U.S. Patent No. 5,223,409), plasmids (Cull *et al*., 1992, PNAS USA 89:1865-1869), or phages (Scott and Smith 1990, Science 249:386-390; Devlin, 1990, Science 249:404-406; Cwirla *et al*., 1990, PNAS USA 87:6378-6382; Felici 1991, J. Mol. Biol. 222:301-310; and U.S. Patent No. 5,223,409).

To identify a useful compound, one can contact a test compound with a system containing test cells expressing a reporter gene encoded by a nucleic acid operatively liked to a promoter of a marker gene selected from the above-mentined metastasis promoters or suppressors. The system can be an *in vitro* cell line model or an *in vivo* animal model. The cells can naturally express the gene, or can be modified to express a recombinant nucleic acid. The recombinant nucleic acid can contain a nucleic acid coding a reporter polypeptide to a heterologous promoter. One then measures the expression level of the miRNA, polypeptide, or reporter polypeptide.

For the polypeptide, the expression level can be determined at either the mRNA level or at the protein level. Methods of measuring mRNA levels in a cell, a tissue sample, or a body fluid are well known in the art. To measure mRNA levels, cells can be lysed and the levels of mRNA in the lysates or in RNA purified or semi-purified from the lysates can be determined by, e.g., hybridization assays (using detectably labeled gene-specific DNA or RNA probes) and quantitative or semi-quantitative RT-PCR (using appropriate gene-specific primers). Alternatively, quantitative or semi-quantitative in situ hybridization assays can be carried out using tissue sections or unlysed cell suspensions, and detectably (e.g., fluorescent or enzyme) labeled DNA or RNA probes. Additional mRNA-quantifying methods include RNA protection assay (RPA) and SAGE. Methods of measuring protein levels in a cell or a tissue sample are also known in the art.

To determine the effectiveness of a candidate compound to treat melanoma or inhibiting endothelial recruitment, cell invasion, or metastatic angiogenesis, one can compare the level obtained in the manner described above with a control level (e.g., one obtained in the absence of the candidate compound). The compound is identified as being effective if (i) a metastasis suppressor's level is lower than a control or reference value or (ii) a metastasis promoter's level is higher than the control or reference value. One can further verify the efficacy of a compound thus-identified using the in vitro cell culture model or an in vivo animal model as disclosed in the example below.

EXAMPLES

EXAMPLE 1 Materials And Methos

This example descibes materials and methos used in EXAMPLES 2-11 below.

Compounds

Table 3 Compound Names

Compound #	Compound Name
1	T0901317
2	GW3965
3	LXR-623
12	WO-2010-0138598 Ex. 9 or WO-201000138598
25	SB742881
38	WO-2007-002563 Ex. 19 or WO-2007-002563

Animal Studies

All mouse experiments were conducted in agreement with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at The Rockefeller University. 6-8-week old age-matched and sex-matched mice were used for primary tumor growth and metastasis assays as previously described (Minn et al., 2005; Tavazoie et al., 2008). See Extended Experimental Procedures.

Cell Culture

All cancer cell lines were cultured as previously described (Tavazoie et al., 2008). 293T and human umbilical vein endothelial cells (HUVEC's) were maintained in standard conditions. miRNA and gene knock-down/over-expression studies in cell lines and in vitro functional assays are detailed in Extended Experimental Procedures.

Microarray Hybridization

In order to identify miRNAs deregulated across highly metastatic derivatives, small RNAs were enriched from total RNA derived from MeWo and A375 cell lines and profiled by LC sciences. In order to identify potential gene targets of miR-199a-3p, miR-199a-5p, and miR-1908, total RNA from MeWo cell lines was labeled and hybridized onto Illumina HT-12 v3 Expression BeadChip arrays by The Rockefeller University genomics core facility. See Extended Experimental Procedures for thresholds and criteria used to arrive at miRNA and mRNA targets.

Analysis of miRNA Expression in Human Melanoma Skin Lesions

All human clinical samples used in this study were obtained, processed, and analyzed in accordance with IRB guidelines. Total RNA was extracted from paraffin-embedded cross-sections of primary melanoma skin lesions previously resected from patients at MSKCC, and specific miRNA expression levels were analyzed in a blinded fashion using TaqMan miRNA Assays (Applied Biosystems). Kaplan-Meier curves representing each patient's metastasis-free-survival data as a function of primary tumor miRNA expression values were generated using the GraphPad Prism software package.

In Vivo LNA Therapy

Following tail-vein injection of 4×10^4 MeWo-LM2 cells, NOD-SCID mice were treated intravenously twice a week for four weeks with in vivo-optimized LNAs (Exiqon) antisense to miR-199a-3p, miR-199a-5p, and miR-1908 at a combinatorial dose of 12.5 mg/kg delivered in 0.1 mL of PBS.

Histology

For gross macroscopic metastatic nodule visualization, 5-μm-thick lung tissue sections were H&E stained. For in vivo endothelial content analyses, lung sections were double-stained with antibodies against MECA-32 (Developmental Studies Hybridoma Bank, The University of Iowa, IA), which labels mouse endothelial cells, and human vimentin (Vector Laboratories), which labels human melanoma cells. See Extended Experimental Procedures.

Data Analysis

All data are represented as mean ± SEM. The Kolmogorov-Smirnov test was used to determine significance of differences in metastatic blood vessel density cumulative distributions. The prognostic power of the miRNAs to predict metastatic outcomes was tested for significance using the Mantel-Cox log-rank test. The one-way Mann-Whitney t-test was used to determine significance values for non-Gaussian bioluminescence measurements. For all other comparisons, the one-sided student's t-test was used. P values < 0.05 were deemed to be statistically significant.

In Vivo Selection, Experimental Metastasis, and Primary Tumor Growth Assays

All mouse experiments were conducted in agreement with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at The Rockefeller University. To generate multiple metastatic derivatives from two independent human melanoma cell lines, in vivo selection was performed as previously described (Minn et al., 2005 Nature *436*, 518-524; Pollack and Fidler, 1982 J. Natl. Cancer Inst. *69*, 137-141). In brief, 1×10^6 pigmented MeWo or non-pigmented A375 melanoma parental cells were resuspended in 0.1 mL of PBS and intravenously injected into 6-8-week old immunocompromised NOD-SCID mice. Following lung metastases formation, nodules were dissociated and cells were propagated in vitro, giving rise to first generation of lung metastatic derivatives (LM1). The LM1 cells were then subjected to another round of in vivo selection by injecting 2×10^5 cells via the tail-vein into NOD-SCID mice, giving rise to metastatic nodules, whose subsequent dissociation yielded second generation of lung metastatic derivatives (LM2). For the A375 cell line, a third round of in vivo selection was performed, yielding the highly metastatic A375-LM3 derivatives.

In order to monitor metastasis in vivo through bioluminescence imaging, A375 and MeWo parental cells and their metastatic derivatives were transduced with a retroviral construct expressing a luciferase reporter (Ponomarev et al., 2004 Eur J Nucl Med Mol Imaging *31*, 740-751). For all metastasis experiments, lung or systemic colonization was monitored over time and quantified through non-invasive bioluminescence imaging as previously described (Minn et al., 2005). To determine whether in vivo selection had been achieved, 4×10^4 MeWo parental or MeWo-LM2 cells and 1×10^5 A375 parental or A375-LM3 cells were resuspended in 0.1 mL of PBS and injected via the lateral tail vein into 6-8-week old NOD-SCID mice. For experimental metastasis assays testing the effects of putative promoter miRNAs on lung colonization, 4×10^4 MeWo parental cells over-expressing miR-199a, miR-1908, miR-214, or a control hairpin, 4×10^4 MeWo-LM2 cells with silenced expression of miR-199a-3p, miR-199a-5p, miR-1908, or a control sequence, and 2×10^5 A375-LM3 cells inhibited for miR-199a-3p, miR-199a-5p, miR-1908, or a control sequence were resuspended in 0.1 mL of PBS and tail-vein injected into 6-8-week old NOD-SCID mice. For epistasis experiments, 1×10^5 MeWo-LM2 cells expressing an shRNA targeting ApoE, DNAJA4, or a control sequence or siRNA inhibiting LRP1 or a control sequence in the setting of miRNA inhibition were intravenously injected into 6-8-week old NOD-SCID mice. For ApoE pre-treatment experiments, MeWo-LM2 cells were incubated in the presence of ApoE or BSA at 100 μg/mL at 37 °C. After 24 hours, 4×10^4 cells were injected via the tail-vein into 7-week old NOD-SCID mice. To determine the effect of pre-treating highly metastatic melanoma cells with LNAs targeting miR-199a-3p, miR-199a-5p, and miR-1908 on metastasis, MeWo-LM2 cells were transfected with each LNA individually, a cocktail of LNAs targeting all three miRNAs, or a control LNA. After 48 hours, 1×10^5 cells, resuspended in 0.1 mL of PBS, were administered intravenously into 7-week old NOD-SCID mice for lung metastatic colonization studies or through intracardiac injection into 7-week old athymic nude mice for systemic metastasis assays. To determine the effect of genetic deletion of ApoE on metastasis, 8-week old C57BL/6-WT or C57BL/6-ApoE-/- mice were intravenously injected with 5×10^4 B16F10 mouse melanoma cells. For primary tumor growth studies, 1×10^6 parental MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin were mixed 1:1 with matrigel and subcutaneously injected into the lower right flank of 6-week old immunodeficient NOD-SCID mice. Animals were palpated weekly for tumor formation, after which sizeable tumors were measured twice a week. Tumor volume was calculated as (small diameter)^2 × (large diameter)/2.

Lentiviral miRNA Inhibition and Gene Knock-Down

293T cells were seeded in a 10-cm plate and allowed to reach 60% confluency. Prior to transfection, the cell media was replaced with fresh antibiotic-free DMEM media supplemented with 10% FBS. 6 μg of vector A, 12 μg of vector K, and 12 μg of the appropriate miR-Zip (System Biosciences, Mountain View, CA) or shRNA plasmid construct (MSKCC HTS Core Facility, New York, NY) were co-transfected using 60 μL of TransIT-293 transfection reagent (MIR 2700, Mirus Bio LLC, Madison, WI). The cells were incubated at 37 °C for 48 hours, and the virus was harvested by spinning the cell media for 10 minutes at 2000g followed by virus filtration through a 0.45 μm filter. 1×10^5 cancer cells were transduced with 2 mL of the appropriate virus in the presence of 10 μg/mL of polybrene (TR-1003-G, Millipore, Billerica, MA) for 6 hrs. After 48 hours, 2 μg/mL of puromycin (P8833, Sigma-Aldrich, St Louis, MO) was added to the cell media for lentiviral selection. The cells were kept in puromycin selection for 72 hours. The following miR-Zip sequences were used:

miR-Zip-199a-3p: 5'-
GATCCGACAGTAGCCTGCACATTAGTCACTTCCTGTCAGTAACCAATG
TGCAGACTACTGTTTTTGAATT-3'

miR-Zip-199a-5p: 5'-
GATCCGCCCAGTGCTCAGACTACCCGTGCCTTCCTGTCAGGAACAGGTAG
TCTGAACACTGGGTTTTTGAATT-3'

miR-Zip-1908 5'-
GATCCGCGGCGGGAACGGCGATCGGCCCTTCCTGTCAGGACCAATCGCCGTCC
CC GCCGTTTTTGAATT-3'

The following shRNA sequences were used:

shAPOE[1]:

5'CCGGGAAGGAGTTGAAGGCCTACAACTCGAGTTGTAGGCCTTCAACTCCTTCTTTTT3'

shAPOE[2]:

5'CCGGGCAGACACTGTCTGAGCAGGTCTCGAGACCTGCTCAGACAGTGTCTGCTTTTT3'

shDNAJA4[1]:

5'CCGGGCGAGAAGTTTAAACTCATATCTCGAGATATGAGTTTAAACTTCTCGCTTTTT3'

shDNAJA4[2]:

5'CCGGCCTCGACAGAAAGTGAGGATTCTCGAGAATCCTCACTTTCTGTCGAGGTTTTT3'

Retroviral miRNA and Gene Over-Expression

6 μg of vector VSVG, 12 μg of vector Gag-Pol, and 12 μg of pBabe plasmid containing the coding sequences of human ApoE, DNAJA4, or an empty vector or miR-Vec containing the precursor sequence of miR-199a, miR-214, miR-1908, or a control hairpin were co-transfected into 60%-confluent 293T cells using 60 μL of TransIT-293 transfection reagent. The cells were incubated at 37 °C for 48 hours, after which the virus was harvested and transduced into cancer cells in the presence of 10 μg/mL of polybrene for 6 hours. After 48 hours, 2 μg/mL of puromycin or 10 μg/mL of blasticidin (15205, Sigma-Aldrich, St Louis, MO) were added to the cell media for retroviral selection. The cells were kept in puromycin selection for 72 hours or in blasticidin selection for 7 days. The following cloning primers were used for over-expression of the coding sequences of ApoE and DNAJA4:

ApoE_CDS_Fwd: 5'-TCATGAGGATCCATGAAGGTTCTGTGGGCT-3'

ApoE_CDS_Rev: 5'-TAGCAGAATTCTCAGTGATTGTCGCTGGG-3'

DNAJA4_CDS_Fwd: 5'-ATCCCTGGATCCATGTGGGAAAGCCTGACCC-3'

DNAJA4_CDS_Rev: 5'-TACCATGTCGACTCATGCCGTCTGGCACTGC-3'

LNA-Based miRNA Knock-Down

LNAs complimentary to mature miR-199a-3p, miR-199a-5p, miR-1908, or a control sequence (426917-00, 426918-00, 426878-00, and 1990050, respectively; Exiqon, Vedbaek, Denmark) were transfected at a final concentration of 50 nM into 50% confluent MeWo-LM2 cancer cells cultured in antibiotics-free media using lipofectamine™ 2000 transfection reagent (11668-09, Invitrogen, Carlsbad, CA). After 8 hours, the transfection media was replaced with fresh media. After 48 hours, 1×10^5 cells were injected intravenously into NOD-SCID mice to assess lung metastatic colonization or through intracardiac injection into athymic nude mice to assess systemic metastasis. For cell invasion and endothelial recruitment in vitro assays, the cells were used 96 hours post-transfection.

siRNA-Based mRNA Knock-Down

siRNAs targeting LRP1, LRP8, VLDLR, LDLR, or a control seqeuence were transfected into cancer cells or HUVEC's at a final concentration of 100 nM using lipofectamine™ 2000 transfection reagent. After 5 hours, the transfection media was replaced with fresh media. The cells were subjected to matrigel invasion and endothelial recruitment assays 96 hours post-transfection. Cells transduced with siRNAs targeting LRP1 or a control sequence in the setting of miRNA inhibition were tail-vein injected for lung colonization assays 72 hours post-transfecton. Control non-targeting siRNAs were obtained from Dharmacon. The following LRP1 and LRP8 target sequences were used:

siLRP1[1]: 5'-CGAGGACGAUGACUGCUUA-3';

siLRP1[2]: 5'-GCUAUGAGUUUAAGAAGUU-3';

siLRP8[1]: 5'-CGAGGACGAUGACUGCUUA-3';

siLRP8[2]: 5'-GAACUAUUCACGCCUCAUC-3'.

Cell Proliferation Assay

To determine the effects of miR-199a or miR-1908 over-expression and combinatorial LNA-induced miRNA inhibition on cell proliferation, 2.5×10^4 cells were seeded in triplicate in 6-well plates, and viable cells were counted after 5 days. To assess the effects of recombinant ApoE addition on melanoma cell or endothelial cell proliferation, 3×10^4 melanoma MeWo-LM2 cells or endothelial cells were incubated in the presence of ApoE (100 μM) or BSA (100 μM). Viable cells were counted after 8, 24, 48, 72, and 120 hours.

Matrigel Invasion Assay

Cancer cells were serum-starved in 0.2% FBS DMEM-based media for 12 hours. Trans-well invasion chambers (354480, BD Biosciences, Bedford, MA) were pre-equilibrated prior to beginning the assay by adding 0.5 mL of starvation media to the top and bottom chambers. After 30 minutes, the media in the top chamber was removed, and 0.5 mL of media containing 1×10^5 cancer cells was added into each matrigel-coated trans-well insert and incubated at 37°C for 24 hours. For neutralization antibody and/or recombinant protein experiments, antibody/recombinant protein was added to each well at the start of the assay at the following concentrations as indicated in the figures: 5-40 μg/mL anti-ApoE 1D7 (Heart Institute, University of Ottawa), 5-40 μg/mL anti-IgG (AB-108-C, R&D Systems, Minneapolis, MN), 100 μM recombinant human ApoE3 (4696, BioVision, Mountain View, CA), and 100 μM BSA (A2153, Sigma-Aldrich). Upon completion of the assay, matrigel-coated inserts were washed with PBS, the cells at the top side of each insert were scraped off, and the inserts were fixed in 4% paraformaldehyde for 15 minutes. The inserts were then cut out and mounted onto slides using VectaShield mounting medium containing DAPI (H-1000, Vector Laboratories, Burlingame, CA). The basal side of each insert was imaged using an inverted fluorescence microscope (Zeiss Axiovert 40 CFL) at 5X magnification, taking three representative images for each insert. The number of invaded cells was quantified using ImageJ (NIH).

Endothelial Recruitment Assay

5×10^4 cancer cells were seeded into 24-well plates approximately 24 hours prior to the start of the assay. HUVEC's were grown to 80% confluency and serum starved in EGM-2 media supplemented with 0.2% FBS for 16 hours. HUVEC's were then pulsed with Cell Tracker Red CMTPX dye (C34552, Invitrogen) for 45 minutes. Meanwhile, cancer cells were washed with PBS, 0.5 mL of 0.2 % FBS EGM-2 media was added to each well, and a 3.0 μm HTS Fluoroblock insert (351151, BD Falcon, San Jose, CA) was placed into each well. 1×10^5 HUVEC's, resuspended in 0.5 mL of starvation media, were seeded into each trans-well insert, and the recruitment assay was allowed to proceed for 16-18 hours at

37 °C. For neutralization antibody and/or recombinant protein experiments, antibody/protein was then added to each well at the appropriate concentration as indicated in the figures: 40 μg/mL anti-ApoE 1D7, 40 μg/mL anti-IgG, 100 μM rhApoE3, and 100 μM BSA. Upon completion of the assay, the inserts were processed and analyzed as described for the matrigel invasion assay above (See Matrigel Invasion Assay).

Endothelial Migration Assay

Serum-starved HUVEC's were pulsed with Cell Tracker Red CMTPX dye for 45 minutes and seeded into HTS Fluoroblock trans-well inserts at a concentration of 1×10^5 HUVEC's in 0.5 mL starvation media per each insert. The assay was allowed to proceed for 16-18 hours at 37 °C, and the inserts were processed and analyzed as described above (See Matrigel Invasion Assay).

Chemotaxis Assay

HUVEC's were serum-starved in 0.2% FBS EGM-2 media for 16 hours and labeled with Cell Tracker Red CMTPX dye for 45 minutes. Meanwhile, the indicated amounts (1-5 μg) of recombinant human ApoE3 or BSA were mixed with 250 μL of matrigel (356231, BD Biosciences) and allowed to solidify at the bottom of a 24-well plate for 30 min. 250 μL of HUVEC EGM-2 media containing 0.2% FBS was then added to each matrigel-coated well, and 3.0 μM HTS Fluoroblock inserts were fitted into each well. 1×10^5 HUVEC's, resuspended in 0.5 mL of starvation media, were seeded into each insert and allowed to migrate along the matrigel gradient for 16-18 hours at 37 °C. Upon completion of the assay, the inserts were mounted on slides and analyzed as described above (See Matrigel Invasion Assay).

Endothelial Adhesion Assay

HUVEC's were seeded in 6-well plates and allowed to form monolayers. Cancer cells were serum starved in 0.2% FBS DMEM-based media for 30 minutes and pulsed with Cell Tracker Green CMFDA dye (C7025, Invitrogen) for 45 minutes. 2×10^5 cancer cells, resuspended in 0.5 mL starvation media, were seeded onto each endothelial monolayer. The cancer cells were allowed to adhere to the HUVEC monolayers for 30 minutes at 37 °C. The endothelial monolayers were then washed gently with PBS and fixed with 4% paraformaldehyde for 15 minutes. Each well was then coated with PBS, and 8 images were taken for each endothelial monolayer using an inverted Fluorescence microscope (Zeiss Axiovert 40 CFL) at 10X magnification. The number of cancer cells adhering to HUVEC's was quantified using ImageJ.

Anoikis Assay

1×10^6 MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin were seeded in low adherent plates containing cell media supplemented with 0.2 % methylcellulose. Following 48 hours in suspension, the numbers of dead and viable cells were counted using trypan blue.

Serum Starvation Assay

To determine the effects of miR-199a and miR-1908 on melanoma cell serum starvation capacity, 1×10^5 MeWo parental cells over-expressing miR-199a, miR-1908, or a control hairpin were seeded in quadruplicate into 6-well plates and incubated in 0.2% FBS starvation DMEM-based media for 48 hours, after which the number of viable cells was counted using trypan blue. To determine the effect of recombinant ApoE3 addition on the survival of melanoma cells or endothelial cells in serum starvation conditions, 3×10^4 MeWo-LM2 cells or endothelial cells were incubated in the presence of ApoE3 (100 μM) or BSA (100 μM) in low serum conditions (0.2% FBS). The number of viable cells was counter after 8, 16, and 24 hours.

Colony Formation Assay

Fifty MeWo parental cells over-expressing miR-199a, miR-1908, or a control hairpin were seeded in quadruplicate into 6-cm plates. After two weeks, the cells were washed with PBS, fixed with 6% glutaraldehyde, and stained with 0.5 % crystal violet. The number of positive-staining colonies was counted.

miRNA Microarray Hybridization

For identification of miRNAs showing deregulated expression across highly metastatic melanoma cell line derivatives, total RNA from multiple independent metastatic derivatives and their respective parental MeWo and A375 cell populations was used to enrich for small RNAs which were then labelled and hybridized onto microfluidic custom microarray platforms by LC sciences. The arrays were designed to detect 894 mature miRNAs corresponding to the miRNA transcripts listed in Sanger miRBase Release 13.0. Out of all the probes analyzed, those corresponding to 169 miRNAs yielded signal above a background threshold across the multiple cell lines analyzed. The raw signal intensities, corresponding to probe hybridization, were median-normalized for each cell line. A threshold of 2-fold or higher up-regulation of median-normalized expression values were used in order to identify miRNAs commonly induced in multiple metastatic derivatives for two independent human melanoma cell lines.

Microarray-Based Gene Target Prediction for miR-199a and miR-1908

In order to identify potential genes targeted by miR-199a-3p, miR-199a-5p, and miR-1908, total RNA was extracted from MeWo cell lines with loss- or gain-of-function of each miRNA and submitted to the genomics core facility at The Rockefeller University for hybridization onto Illumina HT-12 v3 Expression BeadChip microarrays. The raw signal intensities, corresponding to probe hybridization, were then median-normalized for each cell line sample. Three sets of microarray profile comparisons were generated: (1) MeWo control cells relative to MeWo cells over-expressing miR-199a or miR-1908, (2) MeWo-LM2 control cells relative to MeWo-LM2 cells expressing a short hairpin (miR-Zip) targeting miR-199a-3p, miR-199a-5p, or miR-1908, and (3) MeWo parental cells relative to MeWo-LM2 cells. Based on the median-normalized expression values from these arrays, the following criteria were used to arrive at possible target genes common to miR-199a and miR-1908: (1) Genes down-regulated by more than 1.5 fold upon individual over-expression of each miR-199a and miR-1908, (2) Genes up-regulated by more than 1.5 fold upon inhibition of either both miR-199a-3p and miR-1908 or both miR-199a-5p and miR-1908, and (3) genes down-regulated by more than 1.5 fold in LM2 cells, which express physiologically higher levels of the three miRNAs, relative to MeWo parental cells.

Analysis of miRNA and mRNA Expression in Cell Lines

Total RNA was extracted from various cell lines using the miRvana kit (AM1560, Applied Biosystems, Austin, TX). The expression levels of mature miRNAs were quantified using the Taqman miRNA expression assay (4427975-0002228, Applied Biosystems). RNU44 was used as an endogenous control for normalization. For mRNA expression analyses, 600 ng of total RNA was reverse transcribed using the cDNA First-

Strand Synthesis Kit (18080-051, Invitrogen), and roughly 200 ng of the resulting cDNA was then mixed with SYBR green PCR Master Mix (4309155, Applied Biosystems) and the appropriate primers. Each reaction was performed in quadruplicate, and mRNA expression was quantified by performing real-time PCR amplification using an ABI Prism 7900HT Real-Time PCR System (Applied Biosystems). GAPDH was used as an endogenous control for normalization. The following primers were used:

ApoE_Fwd: 5'-TGGGTCGCTTTTGGGATTAC-3'
ApoE_Rev: 5'-TTCAACTCCTTCATGGTCTCG-3'
DNAJA4_Fwd: 5'-CCAGCTTCTCTTCACCCATG-3'
DNAJA4_Rev:5'-GCCAATTTCTTCGTGACTCC-3'
GAPDH_Fwd: 5'-AGCCACATCGCTCAGACAC-3'
GAPDH_Rev: 5'-GCCCAATACGACCAAATCC-3'
LRP1_Fwd: 5'-TTTAACAGCACCGAGTACCAG-3'
LRP1_Rev: 5'CAGGCAGATGTCAGAGCAG-3'
LRP8_Fwd: 5'-GCTACCCTGGCTACGAGATG-3'
LRP8_Rev: 5'-GATTAGGGATGGGCTCTTGC-3'

ELISA

Conditioned cancer cell media was prepared by incubating cells in 0.2% FBS serum starvation DMEM-based media for 24 hours. ApoE levels in conditioned media were determined using the APOE ELISA kit (IRAPKT031, Innovative Research, Novi, Michigan).

Luciferase Reporter Assays

Heterologous luciferase reporter assays were performed as previously described (Tavazoie et al., 2008). In brief, full-length 3'UTRs and CDS's of ApoE and DNAJA4 were cloned downstream of a renilla luciferase reporter into the psiCheck2 dual luciferase reporter vector (C8021, Promega, Madison, WI). 5×10^4 parental MeWo cells, MeWo-LM2 cells, MeWo cells over-expressing miR-199a, miR-1908, or a control hairpin, and MeWo-LM2 cells expressing a miR-Zip hairpin targeting miR-199a-3p, miR-199a-5p, miR-1908, or a control sequence were transfected with 100 ng of the respective specific reporter constructs using TransiT-293 transfection reagent. Twenty-four hours post-transfection, the cells were lysed, and the ratio of renilla to firefly luciferase expression was determined using the dual luciferase assay (E1910, Promega). Putative miRNA binding sites in each target construct were identified by alignment to the complimentary miRNA seed sequences (miR-199a-3p: 5'-CAGUAGUC-3'; miR-199a-5p: 5'-CCAGUGUU-3'; miR-1908: 5'-GGCGGGGA-3'). The miRNA complimentary sites on each target construct were mutated using the QuickChange Multi Site-Directed Mutagenesis Kit (200514, Agilent Technologies, Santa Clara, CA). Based on miRNA seed sequence complimentarity analysis, the CDS of ApoE was mutated at position 141 (CTG to ACT), the 3'UTR of ApoE was mutated at positions 83 (GCC to ATA) and 98 (CTG to ACA), the CDS of DNAJA4 was mutated at positions 373 (CGC to TAT) and 917 (CTG to AGA), and the 3'UTR of DNAJA4 was mutated at positions 576 (CTG to ACA), 1096 (CTG to TCT), 1396 (CGC to TGT), and 1596 (CTG to TGT). The following primers were used to clone the 3'UTR's and CDS's of ApoE and DNAJA4:

ApoE_CDS_Fwd: 5'-AGTACCTCGAGGGGATCCTTGAGTCCTACTC-3'
APOE_CDS_Rev: 5'-TAATTGCGGCCGCTCAGACAGTGTCTGCACCCAG-3'
DNAJA4_CDS_Fwd: 5'-TAATATCTCGAGATGTGGGAAAGCCTGACCC-3'
DNAJA4_CDS_Rev: 5'-CAATTGCGGCCGCTCATGCCGTCTGGCACTGC-3'
APOE_3'UTR_Fwd: 5'-TTAGCCTCGAGACGCCGAAGCCTGCAGCCA-3'
APOE_3'UTR_Rev: 5'-TTACTGCGGCCGCTGCGTGAAACTTGGTGAATCTT-3'
DNAJA4_3'UTR_Fwd: 5'-TAATATCTCGAGCGTGGTGCGGGGCAGCGT-3'
DNAJA4_3'UTR_Rev: 5'-CAATTGCGGCCGCTTATCTCTCATACCAGCTCAAT-3'

The following primers were used to mutagenize the miRNA binding sites on each target:

APOE_CDS_mut: 5'-GCCAGCGCTGGGAACTGGCAACTGGTCGCTTTTGGGATTACCT-3'
APOE_3'UTR_mut1: 5'-CAGCGGGAGACCCTGTCCCCATACCAGCCGTCCTCCTGGGGTG-3'
APOE_3'UTR_mut2: 5'-TCCCCGCCCCAGCCGTCCTCACAGGGTGGACCCTAGTTTAATA-3'
DNAJA4_CDS_mut1: 5'-GGGATCGGTGGAGAAGTGCCTATTGTGCAAGGGGCGGGGGATG-3'

DNAJA4_CDS_mut2: 5'-
GTAGGGGGCGGGGAACGTGTTATCCGTGAAGAGGTGGCTAGGG-3'
DNAJA4_3'UTR_mut1: 5'-
CAGGGCCAACTTAGTTCCTAACATTCTGTGCCCTTCAGTGGAT-3'
DNAJA4_3'UTR_mut2: 5'-
ACAGTTTGTATGGACTACTATCTTAAATTATAGCTTGTTTGGA-3'
DNAJA4_3'UTR_mut3: 5'-
TAATTATTGCTAAAGAACTATGTTTTAGTTGGTAATGGTGTAA-3'
DNAJA4_3'UTR_mut4: 5'-
CAGCTGCACGGACCAGGTTCCATAAAAACATTGCCAGCTAGTGAG-3'

Analysis of miRNA Expression in Human Melanoma Skin Lesions

All human clinical samples used in this study were obtained, processed, and analyzed in accordance with institutional IRB guidelines. Paraffin-embedded cross-sections of primary melanoma skin lesions from 71 human patients were obtained from MSKCC. The samples were de-paraffinized by five consecutive xylene washes (5 minutes each). Following de-paraffinization, the malignancy-containing region was identified by H&E staining, dissected, and total RNA was extracted from it using the RecoverAll Total Nucleic Acid Isolation Kit (AM1975, Applied Biosystems). The expression levels of mature miR-199a-3p, miR-199a-5p, and miR-1908 in each sample were quantified in a blinded fashion using the Taqman miRNA assay. RNU44 was used as an endogenous control for normalization. The expression levels of each miRNA were compared between primary melanomas with propensity to metastasize and primary melanomas that did not metastasize. Kaplan-Meier curves were plotted using metastasis-free survival data of patients as a function of the expression levels for each miRNA in each patient's tumor. Metastatic recurrence to such sites as lung, brain, bone, and soft tissue were previously documented and allowed for a retrospective analysis of the relationship between the expression levels of identified miRNAs and metastatic recurrence.

Histology

Animals were perfused with PBS followed by fixation with 4% paraformaldehyde infused via intracardiac and subsequently intratracheal injection. The lungs were sectioned out, incubated in 4% paraformaldehyde at 4°C overnight, embedded in paraffin, and sliced into 5-μm-thick increments. For gross macroscopic metastatic nodule visualization, lung sections were H&E stained. For endothelial content analysis in metastatic nodules formed by human melanoma MeWo cells in mice, representative lung sections were double-stained with primary antibodies against MECA-32 (Developmental Studies Hybridoma Bank, The University of Iowa, IA), which labels mouse endothelial cells, and human vimentin (VP-V684, Vector Laboratories), which labels human melanoma cells. Various Alexa Flour dye-conjugated secondary antibodies were used to detect primary antibodies. To determine the blood vessel density within metastatic nodules, fluorescence was measured using a Zeiss laser scanning confocal microscope (LSM 510), and the MECA-32 signal within each metastatic nodule, outlined based on co-staining with human vimentin, was quantified in a blinded fashion using ImageJ (NIH). For endothelial content analysis in metastatic nodules formed by mouse B16F10 mouse melanoma cells in wild type and ApoE genetically null mice, representative lung sections were stained for MECA-32, and the MECA-32 signal within each nodule, demarcated based on cell pigmentation, was quantified in a blinded fashion. The collective vessel area, given as the percentage area covered by blood vessels relative to the total area of each metastatic nodule, was obtained by background subtraction (rolling ball radius of 1 pixel) and use of a pre-determined threshold as a cut-off. A metastatic nodule was defined as any region of greater than 2000 μm^2 total area. For large nodules, minimum of four representative images were obtained, and their average blood vessel density was calculated.

In Vivo Matrigel Plug Assay

10 μg/mL recombinant human ApoE3 (4696, BioVision), 10 μg/mL BSA (A2153, Sigma Aldrich), or 400 ng/ml VEGF were mixed with matrigel (356231, BD Biosciences) as indicated. 400 μL of matrigel containing the indicated recombinant proteins were injected subcutaneously just above the ventral flank of immunocompromised NOD-SCID mice. Plugs were extracted on day 3 post-injection and fixed in 4% paraformaldehyde for 48 hours. Plugs were then paraffin-embedded and sectioned at 5-μm-thick increments. Plug cross-sectional sections were immunohistochemically stained using a primary antibody against the mouse endothelial antigen MECA-32 (Developmental Studies Hybridoma Bank, The University of Iowa, IA), detected by peroxidase-conjugated secondary antibody, and

subsequently visualized by DAB oxidization. To quantify the extent of endothelial cell invasion into each matrigel plug, the number of endothelial cells was counted in 4-5 random fields for each plug, and the average number of endothelial cells per given plug area was calculated.

Tissue Culture

The SK-Mel-334 primary human melanoma line was established from a soft tissue metastasis of a *Braf*-mutant melanoma of a patient at the MSKCC. Following minimum expansion *in vitro*, the cells were *in vivo* selected (Pollack and Fidler, 1982) to generate the lung-metastatic derivatives SK-Mel-334.2. The SK-Mel-239 vemurafenib-resistant clone (C1) was a gift from Poulikos Poulikakos (Mount Sinai Medical School) and the $B\text{-}Raf^{V600E/+}$; $Pten^{-/-}$; $CDKN2A^{-/-}$ primary murine melanoma cell line was generously provided by Marcus Rosenberg (Yale University). All other cell lines used were purchased from ATCC.

ApoE Elisa

Extracellular ApoE levels in serum-free conditioned media from melanoma cells treated with DMSO, GW3965, or T0901317 (1 μM each) were quantified using the ApoE ELISA kit (Innovative Research) at 72 hours following treatment.

Western Blotting

Mouse lung and brain tissue samples were homogenized on ice in RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitors (Roche). Mouse adipose tissue was homogenized on ice in TNET buffer (1.5 mM Tris pH 7.5, 150 mM NaCl, 2mM EDTA 1% triton, protease inhibitors). Total protein lysate (2 μg) was separated by SDS-PAGE, transferred to PVDF membrane, and blotted with an anti-mouse ApoE (ab20874, Abcam) and anti-tubulin α/β (2148, Cell Signaling) antibodies.

ApoE Expression Analysis in Melanoma Clinical Samples

All clinical sample procurement, processing, and analyses were performed in strict agreement with IRB guidelines. Primary melanoma skin lesions were previously resected from patients at the MSKCC, formalin-fixed, paraffin-embedded, and sectioned into 5-μm-thick slides. ApoE protein expression was assessed by double-blinded immunohistochemical analysis using the D6E10 anti-ApoE antibody (ab1906, Abcam).

Histochemistry

Animals were intracardially perfused with PBS followed by 4% paraformaldehyde (PFA). Fixed lungs were embedded in paraffin and sectioned into 5-μm-thick increments. Macroscopic lung metastatic nodules were visualized by H&E staining. For analysis of tumor endothelial cell content, proliferation, and apoptosis, primary tumor paraffin-embedded sections were stained with antibodies against MECA-32 (Developmental Studies Hybridoma Bank, University of Iowa), KI-67 (ab15580, Abcam), and cleaved caspase-3 (9661, Cell Signaling), respectively.

Tail-Vein Metastasis Assays

Melanoma cells used for *in vivo* metastasis assays were transduced with a stably expressed retroviral construct encoding a luciferase reporter gene (Ponomarev et al., 2004), allowing us to monitor the *in vivo* progression of melanoma cells by bioluminescence imaging. The following numbers of melanoma cells, resuspended in 100 μL of PBS, were injected intravenously via the tail-vein: 4×10^4 MeWo cells, 2.5×10^5 HT-144 cells, 2×10^5 SK-Mel-334.2 cells, 5×10^4 B16F10 cells, and 1×10^5 YUMM cells. The MeWo, HT-144, and SK-Mel-334.2 cells were injected into 6-8 week-old sex-matched NOD scid mice, while the B16F10 and YUMM cells were injected into 6-8 week-old sex-matched C57BL/6 mice. In all experiments assessing at the effects of GW3965 on metastasis formation, mice were pre-treated on a control diet or a GW3965-supplemented diet (20 mg/kg) for 10 days. To assess the effect of GW3965 treatment on brain metastasis, 1×10^5 MeWo brain-metastatic derivatives were injected intracardially into athymic nude mice. Immediately following injection, mice were randomly assigned to a control diet or GW3965-supplemented diet (100 mg/kg). To determine whether oral delivery of GW3965 can inhibit the progression of incipient metastasis, NOD Scid mice were intravenously injected with 4×10^4 MeWo cells and the cells were allowed to colonize the lungs for 42 days, after which mice were blindedly assigned to a control diet or a GW3965-supplemented diet (100 mg/kg) treatment.

Orthotopic Metastasis Assays

To determine the effect of GW3965 treatment on lung colonization by melanoma cells dissociated from an orthotopic site, 1×10^6 MeWo cells expressing a luciferase reporter were subcutaneously injected into both lower flanks of NOD Scid mice. Upon the formation of tumors measuring ~300 mm^3 in volume, the tumors were excised and the mice were randomly assigned to a control diet or a GW3965-supplemented diet (100 mg/kg) treatment. One month after tumor excision, the lungs were extracted and lung colonization was measured by *ex vivo* bioluminescence imaging. To histologically confirm the extent of melanoma lung colonization, lungs were then fixed in 4% PFA overnight, paraffin-embedded, section into 5-μM increments and stained for human vimentin (VP-V684, Vector Laboratories).

Generation of Dacarbazine-Resistant Melanoma Cells

Dacarbazine-resistant B16F10 mouse melanoma cells were generated by continuously culturing the cells in the presence of DTIC (D2390, Sigma-Aldrich, St. Louis, MO). First, the cells were treated with 500 μg/mL DTIC for one week. Following this initial DTIC treatment, the remaining (~10%) viable cells were allowed to recover for one week, after which 750 μg/mL of DTIC was added to the cell media for 5 days. Subsequent to this high-dose treatment, the cells were allowed to recover in the presence of low-dose DTIC (100 μg/mL) for one week. The cells were then continuously cultured in cell media containing 200 μg/mL DTIC for at least one month prior to grafting the cells into mice. DTIC was added to fresh cancer cell media every 3 days. For tumor growth experiments, 5×10^4 B16F10 parental and DTIC-resistant cells were subcutaneously injected into the lower flank of 7-week-old C57BL/6 mice. Following formation of small tumors measuring 5-10 mm^3 in volume, the mice were randomly assigned to the following treatment groups: (1) control diet + vehicle, i.p.; (2) control diet + DTIC i.p. (50 mg/kg); (3) GW3965-supplemented diet (100 mg/kg) + vehicle i.p.. DTIC was dissolved in the presence of citric acid (1:1 by weight) in water and administered daily by intraperitoneal injection.
The DTIC-resistant MeWo human melanoma cell line clone was generated following DTIC treatment of mice bearing MeWo tumors measuring 600-800 mm^3 in volume. After initial tumor shrinkage in response to daily DTIC dosing (50 mg/kg, i.p.) during the first two weeks, the tumors eventually developed resistance and resumed growth, at which point

tumor cells were dissociated and the DTIC-resistant MeWo cell line was established. The cells were expanded *in vitro* in the presence of DTIC (200 μg/mL) for one week, after which 5×10^5 DTIC-resistant MeWo cells were re-injected into 8-week old Nod SCID gamma mice. Following growth of tumors to 5-10 mm^3 in volume, mice were blindedly assigned to the following treatment groups: (1) control diet; (2) control diet + DTIC (50 mg/kg); (3) GW3965-supplemented diet (100 mg/kg). To determine the effect of DTIC on tumor growth by parental unselected MeWo cells, 5×10^5 MeWo cells were subcutaneously injected into Nod SCID gamma mice, and the mice were treated with a control vehicle or DTIC (50 mg/kg) subsequent to formation of tumors measuring 5-10 mm^3 in volume. DTIC was administered daily, as described above, in cycles consisting of 5 consecutive daily treatments interspersed by 2-day off-treatment intervals. Tumor growth was measured twice a week.

Genetically-Initiated Model of Melanoma Progression

The *Tyr::CreER; B-Raf*$^{V600E/+;}$ *Pten*$^{lox/+}$ / *Tyr::CreER; B-Raf*$^{V600E/+;}$ *Pten*$^{lox/lox}$ conditional model of melanoma progression was previously established and characterized by Dankort et al. (2009). Briefly, melanoma in these mice was induced at 6 weeks of age by intraperitoneally injecting 4-HT (H6278, 70% isomer, Sigma-Aldrich, St Louis, MO) at 25 mg/kg administered in peanut oil on three consecutive days. The 4-HT stock solution was prepared by dissolving it in 100% EtOH at 50 mg/mL by heating at 45 °C for 5 min and mixing. Once dissolved, the stock 4-HT solution was then diluted by 10-fold in peanut oil, yielding a 5 mg/mL 4-HT working solution that was then injected into mice. After the first 4-HT injection, mice were blindedly assigned to receive either a control diet or a diet supplemented with GW3965 (100 mg/kg). Mice were examined three times a week for the presence and progression of melanoma lesions. At day 35, dorsal skin samples were harvested from control-treated and GW3965-treated mice, fixed in 4% PFA and photographed at 10X. The percentage of pigmented melanoma lesion area out of the total skin area was quantified using ImageJ. For survival analyses, mice were monitored daily for melanoma progression and euthanized according to a standard body condition score, taking into account initial signs of moribund state and discomfort associated with the progression of melanoma burden. Post-mortem, the lungs, brains, and salivary glands were harvested and examined for the presence of macroscopic melanoma lesions.

Mouse Genotyping

All mouse genotyping was performed using standard PCR conditions, as recommended by Jackson Labs. The following genotyping primers were used for the respective PCR reactions:

Tyr::CreER; B-Raf$^{V600E/+;}$ *Pten*$^{lox/+}$ and *Tyr::CreER; B-Raf*$^{V600E/+;}$ *Pten*$^{lox/lox}$ mice:
B-Raf Forward: 5'-TGA GTA TTT TTG TGG CAA CTG C-3'
B-Raf Reverse: 5'-CTC TGC TGG GAA AGC GGC-3'
Pten Forward: 5'-CAA GCA CTC TGC GAA CTG AG-3'
Pten Reverse: 5'-AAG TTT TTG AAG GCA AGA TGC-3'
Cre Transgene Forward: 5'-GCG GTC TGG CAG TAA AAA CTA TC-3'
Cre Transgene Reverse: 5'-GTG AAA CAG CAT TGC TGT CAC TT-3'
Internal Positive Control Forward: 5'-CTA GGC CAC AGA ATT GAA AGA TCT-3'
Internal Positive Control Reverse: 5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3'

ApoE-/- mice:
Common Forward: 5'-GCC TAG CCG AGG GAG AGC CG-3'
Wild-type Reverse: 5'-TGT GAC TTG GGA GCT CTG CAG C-3'
Mutant Reverse: 5'-GCC GCC CCG ACT GCA TCT-3'

LXRα-/- mice:
Common Forward: 5'-TCA GTG GAG GGA AGG AAA TG-3'
Wild-type Reverse: 5'-TTC CTG CCC TGG ACA CTT AC-3'
Mutant Reverse: 5'-TTG TGC CCA GTC ATA GCC GAA T-3'

LXRβ-/- mice:
Common Forward: 5'-CCT TTT CTC CCT GAC ACC G-3'
Wild-type Reverse: 5'-GCA TCC ATC TGG CAG GTT C-3'
Mutant Reverse: 5'-AGG TGA GAT GAC AGG AGA TC-3'

Cell Proliferation and Viability Assay:

To determine the effects of GW3965, T0901317, and Bexarotene on *in vitro* cell growth, 2.5×10^4 melanoma cells were seeded in triplicate in 6-well plates and cultured in the presence of DMSO, GW3965, T0901317, or Bexarotene at 1 μM each. After 5 days, the number of viable and dead cells was counted using the trypan blue dye (72-57-1, Sigma-Aldrich), which selectively labels dead cells.

Cell Invasion Assay

The cell invasion assay was performed as previously described in detail (Pencheva et al., 2012) using a trans-well matrigel invasion chamber system (354480, BD Biosciences). In brief, various melanoma cells were cultured in the presence of DMSO, GW3965, T0901317, or Bexarotene at 1 μM for 56 hours, after which melanoma cells were switched to starvation media (0.2 % FBS) for 16 hours in the presence of each drug. Following starvation, cells were seeded into matrigel-coated trans-well inserts, and the invasion assay was allowed to proceed for 24 hours at 37°C. For ApoE antibody neutralization experiments, 40 μg/mL 1D7 anti-ApoE blocking antibody (Heart Institute, University of Ottawa, Ottawa, Canada) or 40μ g/mL anti-IgG control antibody (AB-108-C, R&D Systems, Minneapolis, MN) was added to each trans-well insert at the start of the assay.

Endothelial Recruitment Assay

The endothelial recruitment assay was carried out as previously described (Pencheva et al., 2012; Png et al., 2012). Melanoma cells were treated with DMSO, GW3965, T0901317, or Bexarotene at 1 μM for 56 hours, after which 5×10^4 cells were seeded in a 24-well plate in the presence of each drug and allowed to attach for 16 hours prior to starting the assay. HUVEC cells were serum-starved overnight in EGM-2 media containing 0.2% FBS. The following day, 1×10^5 HUVEC cells were seeded into a 3.0 μm HTS Fluoroblock trans-well migration insert (351151, BD Falcon, San Jose, CA) fitted into each well containing cancer cells at the bottom. The HUVEC cells were allowed to migrate towards the cancer cells for 20 hours at 37°C, after which the inserts were processed as previously described (Pencheva et al., 2012). For ApoE antibody neutralization experiments, 40 μg/mL 1D7 anti-ApoE blocking antibody (Heart Institute, University of Ottawa, Ottawa, Canada) or 40 μg/mL anti-IgG control antibody (AB-108-C, R&D Systems, Minneapolis, MN) was added to each trans-well insert at the start of the assay.

Lentiviral shRNA-Based Gene Knockdown

shRNAs were integrated into lentiviral particles that were prepared by transfection of 6 μg of vector A, 12 μg of vector K, and 12 μg of shRNA plasmid into HEK-293T packaging cells, as previously described (Pencheva et al., 2012; Png et al., 2012). Lentiviral shRNA transduction was performed in the presence of 10 μg/mL of polybrene (TR-1003-G, Millipore, Billerica, MA) for 6 hours, as described previously (Pencheva et al., 2012). The cells were expanded for 72 hours after transduction and lentiviral selection was performed by culturing the cells in the presence of 2 μg/mL of puromycin (P8833, Sigma-Aldrich) for 72 hours.

The following shRNA sequences were used:

Human:

sh_1*LXRα*: 5'-
CCGGCCGACTGATGTTCCCACGGATCTCGAGATCCGTGGGAACATCAGTCGGTTTTT-3'

sh_2*LXRα*: 5'-
CCGGGCAACTCAATGATGCCGAGTTCTCGAGAACTCGGCATCATTGAGTTGCTTTTT-3'

sh_1*LXRβ*: 5'-
CCGGAGAGTGTATCACCTTCTTGAACTCGAGTTCAAGAAGGTGATACACTCTTTTTT-3'

sh_2*LXRβ*: 5'-
CCGGGAAGGCATCCACTATCGAGATCTCGAGATCTCGATAGTGGATGCCTTCTTTTT-3'

sh*ApoE*: 5'-
CCGGGCAGACACTGTCTGAGCAGGTCTCGAGACCTGCTCAGACAGTGTCTGCTTTTT-3'

Mouse:

sh_m*LXRα*: 5'-
CCGGGCAACTCAATGATGCTGAGTTCTCGAGAACTCAGCATCATTGAGTTGCTTTTT-3'

sh_m*LXRβ*: 5'-

CCGGTGAGATCATGTTGCTAGAAACCTCGAGGTTTCTAGCAACATGATCTCATT TTTG-3'

sh_m*ApoE*: 5'-
CCGGGAGGACACTATGACGGAAGTACTCGAGTACTTCCGTCATAGTGTCCTCTT TTT-3'

Gene Expression Analysis by qRT-PCR:

RNA was extracted from whole cell lysates using the Total RNA Purification Kit (17200, Norgen, Thorold, Canada). 600 ng of total RNA was then reverse transcribed into cDNA using the cDNA First-Strand Synthesis Kit (18080-051, Invitrogen), and quantitative real-time PCR amplification was performed as previously described (Pencheva et al., 2012) using an ABI Prism 7900HT Real-Time PCR System (Applied Biosystems, Austin, TX). Each PCR reaction was carried out in quadruplicates. Gene expression was normalized to GAPDH, which was used as an endogenous control.

The following primers were used:

Human:

ApoE Forward: 5'-TGGGTCGCTTTTGGGATTAC-3'
ApoE Reverse: 5'-TTCAACTCCTTCATGGTCTCG-3'
GAPDH Forward: 5'-AGCCACATCGCTCAGACAC-3'
GAPDH Reverse: 5'-GCCCAATACGACCAAATCC-3'
LXRα_Fwd: 5'- GTTATAACCGGGAAGACTTTGC-3'
LXRα_Rev: 5'- AAACTCGGCATCATTGAGTTG-3'
LXRβ_Fwd: 5'- TTTGAGGGTATTTGAGTAGCGG-3'
LXRβ_Rev: 5'- CTCTCGCGGAGTGAACTAC-3'

Mouse:

ApoE Forward: 5'-GACCCTGGAGGCTAAGGACT-3'
ApoE Reverse: 5'-AGAGCCTTCATCTTCGCAAT-3'
GAPDH Forward: 5'-GCACAGTCAAGGCCGAGAAT-3'
GAPDH Reverse: 5'-GCCTTCTCCATGGTGGTGAA-3'
LXRα Forward: 5'-GCGCTCAGCTCTTGTCACT-3'
LXRα Reverse: 5'-CTCCAGCCACAAGGACATCT-3'

LXRβ Forward: 5'-GCTCTGCCTACATCGTGGTC-3'
LXRβ Reverse: 5'-CTCATGGCCCAGCATCTT-3'
ABCA1 Forward: 5'- ATGGAGCAGGGAAGACCAC-3'
ABCA1 Reverse: 5'- GTAGGCCGTGCCAGAAGTT-3'

ApoE Promoter Activity Assay

The *ApoE* promoter, consisting of a sequence spanning 980 base pairs upstream and 93 base pairs downstream of the *ApoE* gene, was cloned into a pGL3-Basic vector (E1751, Promega Corporation, Madison, WI) upstream of the firefly luciferase gene using NheI and SacI restriction enzymes. Then, multi-enhancer elements 1 (ME.1) and 2 (ME.2) were cloned directly upstream of the *ApoE* promoter using MluI and SacI restriction enzymes. To assess *ApoE* promoter- and ME.1/ME.2-driven transcriptional activation by LXR agonists, 5×10^4 MeWo cells were seeded into a 24-well plate. The following day, 100 ng of pGL3-ME.1/ME.2-*ApoE* promoter construct and 2 ng of pRL-CMV renilla luciferase construct (E2261, Promega) were co-transfected into cells in the presence of DMSO, GW3965, or T0901317 at 1μM, each condition in quadruplicate. To assess transcriptional activation by LXRα or LXRβ, 5×10^4 MeWo cells expressing a control shRNA or shRNA targeting *LXRα* or *LXRβ* were seeded into a 24-well plate. The following day, 200 ng of pGL3-ME.1/ME.2-*ApoE* promoter construct and 2 ng of pRL-CMV renilla luciferase were co-transfected into cells in the presence of DMSO, GW3965, or T0901317 at 1μM, each condition in quadruplicate. After 24 hours, cells were lysed, and cell lysate was analyzed for firefly and renilla luciferase activity using the Dual Luciferase Assay System (E1960, Promega) and a Bio-Tek Synergy NEO Microplate Reader. Firefly luciferase signal was normalized to renilla luciferase signal and all data are expressed relative to the luciferase activity ratio measured in the DMSO-treated control cells.

The following cloning primers were used:

ApoE-promoter Forward: 5'-TCA TAG CTA GCG CAG AGC CAG GAT TCA CGC CCT G-3'

ApoE-promoter Reverse: 5'-TGG TCC TCG AGG AAC CTT CAT CTT CCT GCC TGT GA-3'

ME.1 Forward: 5'-TAG TTA CGC GTA GTA GCC CCC ATC TTT GCC-3'
ME.1 Reverse: 5'-AAT CAG CTA GCC CCT CAG CTG CAA AGC TC-3'

ME.2 Forward: 5'-TAG TTA CGC GTA GTA GCC CCC TCT TTG CC-3'
ME.2 Reverse: 5'-AAT CAG CTA GCC CTT CAG CTG CAA AGC TCT G-3'

Tumor Histochemistry

Tumors were excised from mice and fixed in 4% paraformaldehyde at 4 °C for 48 hours. Then, tumors were paraffin-embedded and sectioned into 5-µm-thick increments. For endothelial cell content analysis in tumors, tumor sections were stained with a primary antibody against the mouse endothelial cell marker MECA-32 (Developmental Studies Hybridoma Bank, The University of Iowa, IA) and counterstained with DAPI nuclear stain. To determine tumor cell proliferation and apoptosis, tumor sections were stained with antibodies against the proliferative marker Ki-67 (Abcam, ab15580, Cambridge, MA) and the apoptotic marker cleaved caspase-3 (9661, Cell Signaling, Danvers, MA), respectively. Various Alexa Flour dye-conjugated secondary antibodies were used to detect primary antibodies. Fluorescence was measured using inverted fluorescence microscope (Zeiss Axiovert 40 CFL) at 5X magnification for MECA-32 and Ki-67 staining and 10X magnification for cleaved caspase-3 staining. Endothelial cell content density and tumor proliferation rate were quantified by calculating the average percentage of MECA-32 or Ki-67 positively-staining area out of the total tumor area. Tumor apoptosis was measured by counting the number of cleaved caspase-3 expressing cells per given tumor area.

Analysis of ApoE Expression in Primary Melanoma Lesions

Human primary melanoma skin samples were resected from melanoma patients at MSKCC, formalin-fixed, embedded in paraffin, and sectioned into 5-µm-thick increments. To determine ApoE protein expression, the samples were first de-paraffinized by two consecutive xylene washes (5 minutes each), and rehydrated in a series of ethanol washes (100%, 95%, 80%, and 70% EtOH). ApoE antigen was retrieved by incubating the samples in the presence of proteinase K (5 µg/mL) for 20 minutes at room temperature. To quench endogenous peroxidase activity, the slides were incubated in 3% H_2O_2 solution. The slides were then blocked in three consecutive Avidin, Biotin, and horse serum block solutions for 15 min each at room temperature (SP-2001, Vector Laboratories, Burlingame, CA). ApoE was detected by staining with D6E10 anti-ApoE antibody (ab1908, Abcam), which was used at a 1:100 dilution in PBS at 4 °C overnight. The primary antibody was then recognized by incubating the slides in a peroxidase-conjugated secondary antibody (PK-4002, Vector Laboratories) and exposed by DAB (SK-4105, Vector Laboratories) oxidation reaction. The slides were imaged at 10X magnification and analysed in a double-blinded manner. ApoE expression was quantified by counting the number of DAB-positive cells and measuring the area of extracellular ApoE staining. Total ApoE staining signal was expressed as the percentage staining area per given tumor area, determined based on matched H&E-stained slides for each sample. Kaplan-Meier curves depicting patients' metastasis-free survival times were generated by plotting each patient's relapse-free survival data as a function of ApoE expression in that patient's primary melanoma lesion. Patients whose tumors had ApoE levels lower than the median ApoE expression of the population were classified as ApoE-negative, whereas patients whose melanomas expressed ApoE above the median were classified as ApoE-positive. Previously documented patients' history of metastatic recurrence to sites such as lung, brain, bone, soft and subcutaneous tissues, and skin enabled us to retrospectively determine the relationship between ApoE expression at a primary melanoma site and metastatic relapse.

EXAMPLE 2 *Endogenous Mir-1908, Mir-199a-3p, And Mir-199a-5p Promote Human Melanoma Metastasis*

In order to identify miRNA regulators of melanoma metastasis, *in vivo* selection (Pollack and Fidler, 1982) was utilized with the pigmented MeWo and non-pigmented A375 human melanoma cell lines to generate multiple second (LM2) and third generation (LM3) lung metastatic derivatives. Comparison of the metastatic potential of the MeWo-LM2 and A375-LM3 lines showed these derivatives to metastasize significantly more efficiently than their respective parental populations in lung colonization assays (Figures 12A-B). Hybridization-based small RNA profiling of 894 mature miRNAs followed by quantitative stem-loop PCR (qRT-PCR) revealed four miRNAs (miR-1908, miR-199a-3p, miR-199a-5p, and miR-214) to be upregulated greater than two-fold in multiple A375 and MeWo metastatic derivatives relative to their respective parental cells (Figures 1A-B, 12C). The significant induction of miR-199a-3p, miR-199a-5p, miR-214, and miR-1908 across multiple metastatic derivatives suggested a metastasis-promoting role for these miRNAs. Retrovirally mediated transduction and over-expression of the precursors for miR-199a-3p and miR-199a-5p (over-expressed concomitantly as the miR-199a hairpin) and miR-1908 lead to a robust increase in lung metastatic colonization based on both bioluminescence signal quantification and gross lung histology (Figure 1C, 12D; 9.64-fold increase, P = 0.016 for miR-1908; 8.62-fold increase, P = 0.028 for miR-199a), while miR-214 over-expression did not significantly affect metastasis. Importantly, over-expression of each miR-199a and miR-1908 increased the number of metastatic nodules formed (Figure 12E), consistent with a role for these miRNAs in metastatic initiation. These findings also revealed miR-199a and miR-1908 to be sufficient for enhanced metastatic colonization.

Next, assays were carried out to examine if endogenous levels of these miRNAs promote metastasis. To this end, miR-1908 and each of the two miRNAs arising from the miR-199a hairpin (miR-199a-3p and miR-199a-5p) were inhibited in the highly metastatic cells through miR-Zip technology. Individual inhibition of each of these miRNAs suppressed metastatic colonization by more than 7-fold (Figure 1D; P = 0.047 for miR-1908 inhibition; P = 0.010 for miR-199a-3p inhibition; P = 0.015 for miR-199a-5p inhibition) and dramatically decreased the number of metastatic nodules formed (Figure 12F).

To determine whether these miRNAs also promote metastasis in an independent cell line, their expression was silenced in the A375 metastatic derivative cell line. Indeed, inhibition of miR-1908, miR-199a-3p, or miR-199a-5p significantly reduced the lung colonization capacity of metastatic A375-LM3 cells (Figure 1E), establishing these three miRNAs as endogenous promoters of metastasis by human melanoma cells.

Given the robust functional roles of miR-1908, miR-199a-3p, and miR-199a-5p in promoting melanoma metastasis in a mouse model of human cell metastasis, further assays were carried out to examine whether expression of these miRNAs correlates with the capacity of human primary melanoma lesions to metastasize. To this end, 71 primary melanoma skin lesions obtained from Memorial Sloan-Kettering Cancer Center (MSKCC) patients were analyzed in a blinded fashion for the expression levels of miR-1908, miR-199a-3p, and miR-199a-5p through qRT-PCR. Consistent with the above functional studies, all three miRNAs were significantly induced in primary melanomas that had metastasized relative to those that had not (Figure 1F; P = 0.037 for miR-1908; P = 0.0025 for miR-199a-3p; P = 0.0068 for miR-199a-5p), suggesting that upregulated expression of these miRNAs in primary lesions is an early event predictive of melanoma cancer progression.

EXAMPLE 3 *Mir-1908, Mir-199a-3p, and Mir-199a-5p Promote Cell Invasion and Endothelial Recruitment*

In this Examiner, assays were carried out to determine the cellular mechanisms by which miR-1908, miR-199a-3p, and miR-199a-5p regulate metastasis.

First, it was examined if these miRNAs promote metastasis by enhancing proliferation or tumor growth. Contrary to this, over-expression of each miRNA reduced cell proliferation (Figure 13A). More importantly, miR-1908 over-expression did not increase primary tumor growth, while miR-199a over-expression actually lead to a significant decrease (35%; $P < 0.001$) in tumor volume (Figure 2A), indicating that the pro-metastatic effects of miR-1908 and miR-199a are not secondary to tumor growth promotion or enhanced cell proliferation.

Next, it was examined whether these miRNAs regulate cell invasion, a key metastatic phenotype. Metastatic LM2 cells, which express higher levels of these miRNAs, displayed significantly increased matrigel invasion capacity relative to their less metastatic parental population (Figure 13B). Accordingly, over-expression of miR-199a and miR-1908 individually enhanced the ability of parental MeWo cells to invade through matrigel (Figure 2B; three-fold increase for miR-199; two-fold increase for miR-1908). Conversely, individual inhibition of miR-199a-3p, miR-199a-5p, and miR-1908 significantly decreased the invasive capacity of MeWo-LM2 (Figure 2C) as well as A375-LM3 (Figure 2D) metastatic melanoma cell derivatives.

Given the robust effects of these miRNAs on metastatic progression, further analyses were conducted to examiner whether they may regulate any additional pro-metastatic phenotypes. While over-expression of miR-199a or miR-1908 did not modulate melanoma cell adhesion to endothelial cells (Figure 13C), resistance to anoikis (Figures 13D), survival in the setting of serum starvation (Figure 13E), or colony formation (Figure 13F), each miRNA dramatically enhanced (more than three-fold increase) the ability of parental MeWo cells to recruit endothelial cells in trans-well endothelial recruitment assays (Figure 2E). Consistent with this, metastatic Mewo-LM2 cells, which physiologically over-express miR-199a and miR-1908, were more efficient at recruiting endothelial cells relative to their parental cells (Figure 13G). Conversely, inhibition of miR-199a-3p, miR-199a-5p, or miR-1908 in the metastatic MeWo-LM2 (Figure 2F) as well as A375-LM3 cells (Figures 2G) suppressed endothelial recruitment, consistent with the requirement and sufficiency of these miRNAs for enhanced endothelial recruitment capacity of metastatic melanoma cells.

To determine whether endogenous miR-199a-3p, miR-199a-5p, and miR-1908 regulate endothelial recruitment by metastatic cells in vivo, assays were carried out to examine metastatic blood vessel density by performing co-immunostaining for human vimentin, which labels human MeWo melanoma cells, and mouse endothelial cell antigen (MECA-32), which labels mouse endothelial cells. Strikingly, inhibition of miR-199a-3p, miR-199a-5p, or miR-1908 individually led to pronounced decreases (an average of 3-fold for miR-199a-3p and miR-199a-5p and 4.7-fold for miR-1908) in blood vessel density within metastatic nodules (Figure 2H; $P < 0.001$ for miR-199a-3p; $P < 0.001$ for miR-199a-5p; and $P < 0.001$ for miR-1908), revealing a role for these miRNAs in promoting metastatic endothelial content and metastatic angiogenesis. Conversely, over-expression of each miRNA in poorly metastatic melanoma cells dramatically increased metastatic blood vessel density (Figure 13H). These findings reveal miR-199a-3p, miR-199a-5p, and miR-1908 as necessary and sufficient for enhanced invasion and endothelial recruitment during melanoma progression.

EXAMPLE 4 *Mir-1908, Mir-199a-3p, and Mir-199a-5p Convergently and Cooperatively Target Apoe and DNAJA4*

In this example, a systematic and unbiased approach was employed to identify the direct molecular targets of these miRNAs.

Since miR-1908, miR-199a-3p, and miR-199a-5p mediate the same sets of in vitro and in vivo phenotypes and miR-199a-5p and miR-199a-3p arise from the same precursor hairpin, it was hypothesized that the pro-metastatic phenotypes of these miRNAs may arise through silencing of common target genes. Given that mammalian miRNAs act predominantly by destabilizing target mRNA transcripts (Guo et al., 2010 Nature *466*, 835-840), transcriptomic profiling of melanoma cells was performed in the context of both loss- and gain-of-function for each miRNA. This revealed a small set of genes that were repressed by both miR-199a and miR-1908 and that also displayed lower levels in the metastatic LM2 derivatives, which express higher endogenous levels of these miRNAs (Figure 14A). Quantitative RT-PCR validated two genes, the metabolic gene

Apolipoprotein E (ApoE) and the heat-shock protein DNAJA4, as significantly modulated by miR-199a and miR-1908 and dramatically silenced in the highly metastatic LM2 cells (Figures 3A and 14B-D).

To determine whether ApoE and DNAJA4 are directly targeted by miR-1908, miR-199a-3p, and miR-199a-5p, the effects of each miRNA on the stability of its putative targets were examined through heterologous luciferase reporter assays. Interestingly, over-expression of miR-199a repressed the stability of the 3' untranslated region (UTR) and coding sequence (CDS) of both ApoE and DNAJA4, while over-expression of miR-1908 destabilized the 3'UTR of ApoE and the 3'UTR and CDS of DNAJA4. Consistent with direct targeting, mutating the miRNA complementary sequences on each target abrogated miRNA-mediated regulation (Figure 3B). In a direct test of endogenous targeting, individual miRNA inhibition in metastatic LM2 cells resulted in increased target stability (Figures 3C) that was abrogated upon mutating the miRNA target sites (Figure 14E), revealing ApoE to be directly targeted by miR-1908 and miR-199a-5p and DNAJA4 to be directly targeted by all three miRNAs (Figure 3D). Importantly, the CDS's and 3'UTR's of both of these genes were less stable in the highly metastatic LM2 cells, which express physiologically higher levels of the three regulatory miRNAs, indicating that endogenous targeting of ApoE and DNAJA4 by these miRNAs is relevant to melanoma metastasis (Figure 3E).

Given the molecular convergence of miR-199a-3p, miR-199a-5p, and miR-1908 onto common target genes, it was next examined whether these targets, ApoE and DNAJA4, could mediate the metastatic phenotypes conferred by these miRNAs. Over-expression of each gene in the metastatic LM2 cells led to pronounced reductions in cell invasion and endothelial recruitment phenotypes (Figures 3F-G, 14F). Conversely, knock-down of ApoE or DNAJA4 in the poorly metastatic cells using independent hairpins significantly enhanced cell invasion and endothelial recruitment (Figures 3H-I, 14G), revealing ApoE and DNAJA4 to act as endogenous suppressors of these pro-metastatic phenotypes—consistent with their targeting by the above mentioned metastasis-promoting miRNAs.

EXAMPLE 5 *ApoE and DNAJA4 Mediate miR-199a- and miR-1908-Dependent Metastatic Invasion, Endothelial Recruitment, and Colonization*

To determine whether ApoE and DNAJA4 are the direct biological effectors downstream of miR-199a and miR-1908, assays were carried out to examine whether these two target genes epistatically interact with each miRNA. As expected, miRNA silencing reduced the invasion and endothelial recruitment capacity of highly metastatic melanoma cells. Importantly, knock-down of ApoE or DNAJA4 in the setting of miRNA inhibition significantly occluded the suppression of invasion (Figures 4A and 4C) and endothelial recruitment (Figures 4B and 4D) upon silencing of each miRNA. Strikingly, knock-down of either of these genes in cells depleted for miR-1908 or miR-199a-5p fully rescued the dramatic suppression of metastatic colonization resulting from miRNA inhibition (Figure 4E-F, 15E). Conversely, over-expression of ApoE or DNAJA4 in cells over-expressing miR-1908 (Figure 4G-H, 15F) or miR-199a (Figure 15G-I) was sufficient to suppress cell invasion and endothelial recruitment. Additionally, ApoE or DNAJA4 over-expression was sufficient to inhibit miRNA-mediated metastatic colonization (Figure 15J). Importantly, ApoE and DNAJA4 were also required for miRNA-dependent enhanced cell invasion and endothelial recruitment by the highly metastatic A375-LM3 cells (Figures 4 I-J, 15K).

To determine whether ApoE and DNAJA4 also regulate miRNA-dependent metastatic endothelial recruitment in vivo, co-immunostaining of melanoma metastases (human vimentin) and endothelial cells (MECA-32) was performed in lung metastatic nodules formed by cells knocked-down for each of these genes in the context of miRNA inhibition. Notably, knock-down of ApoE or DNAJA4 resulted in a significant (>3.5-fold) increase in metastatic blood vessel density in metastases arising from cells with miRNA silencing (Figure 4K, $P < 0.01$ for both ApoE and DNAJA4 knock-down cells). These findings reveal ApoE and DNAJA4 as direct downstream effectors of miRNA-dependent metastatic invasion, colonization, and endothelial recruitment phenotypes induced by these pro-metastatic miRNAs in melanoma.

EXAMPLE 6 *Melanoma Cell-Secreted Apoe Is Both a Necessary and Sufficient Mediator of Invasion and Endothelial Recruitment, While Genetic Deletion of Apoe Promotes Metastasis*

ApoE is a secreted factor. As such, it was examined whether melanoma-cell secreted ApoE could suppress invasion and endothelial recruitment. Accordingly, extracellular ApoE levels, detected by ELISA, were 3.5-fold lower in metastatic LM2 cells—which express higher levels of miR-199a and miR-1908—than their less metastatic parental cells (Figure 5A). Secreted ApoE levels were also significantly suppressed by endogenous miR-199a and miR-1908 (Figures 5B and 16A).

Next, inhibiting ApoE through use of a neutralizing antibody (1D7) that recognizes the receptor-binding domain of ApoE enhanced both cell invasion (Figure 5C; 1.68-fold increase) and endothelial recruitment (Figure 5D; 1.84-fold increase) by parental MeWo cells, which express high endogenous levels of ApoE (Figure 14C). Conversely, addition of recombinant human ApoE significantly suppressed invasion and endothelial recruitment by metastatic LM2 cells (Figure 5E), which exhibit low endogenous ApoE levels (Figure 14C). Importantly, recombinant ApoE addition did not affect melanoma cell or endothelial cell in vitro proliferation (Figure 16B-C) or survival in serum starvation conditions (Figure 16D-E), indicating that suppression of these phenotypes by recombinant ApoE is not secondary to a decrease in proliferation or impaired survival. Consistent with ApoE being epistatically downstream of miR-199a and miR-1908, neutralization of ApoE with the ApoE neutralizing antibody 1D7 significantly abrogated the suppressed invasion and endothelial recruitment phenotypes seen with inhibition of each miRNA (Figures 5F-G). The above findings reveal melanoma cell-secreted ApoE as a necessary and sufficient suppressor of miRNA-dependent invasion and endothelial recruitment phenotypes in melanoma.

Further assays were carried out to investigate the mechanism by which DNAJA4, a poorly characterized heat-shock protein, mediates endothelial recruitment and invasion. Given the phenotypic commonalities displayed by ApoE and DNAJA4, it was hypothesized that DNAJA4 may play a regulatory role and enhance ApoE levels. Indeed, knock-down of DNAJA4 reduced both ApoE transcript levels (Figure 16F) as well as secreted ApoE levels (Figure 5H), while DNAJA4 over-expression substantially elevated ApoE expression (Figure 16G). Consistent with DNAJA4 acting upstream of ApoE, addition of recombinant ApoE abrogated the enhanced cell invasion and endothelial recruitment phenotypes seen with DNAJA4 knock-down (Figure 5I-J). Conversely, the suppression of invasion and endothelial recruitment seen with DNAJA4 over-expression phenotypes were significantly occluded by antibody neutralization of ApoE (Figures 16H-I). These findings reveal DNAJA4 to suppress melanoma invasion and endothelial recruitment through positive regulation of ApoE expression and resulting secretion.

In view of the regulatory convergence of three metastasis-promoting miRNAs and the DNAJA4 gene on ApoE, assays were carried out to determine whether ApoE expression correlates with human melanoma progression. To this end, published array-based expression data for ApoE (Haqq et al., 2005 Proc. Natl. Acad. Sci. U S A *102*, 6092-6097) was analyzed in nevi, primary, and metastatic lesions. Consistent with a metastasis-suppressive role, ApoE levels were significantly lower in distal organ metastases relative to primary ($P < 0.025$) and nevi lesions ($P < 0.0003$) (Figure 5K).

Given its significant correlation with human melanoma progression, it was next examined whether increasing ApoE signaling in melanoma cells could have therapeutic efficacy in suppressing melanoma metastasis. More specifically, metastatic MeWo-LM2 cells were pre-incubated with recombinant ApoE or BSA for 24 hours prior to injection into mice. Strikingly, pre-treatment of cancer cells with ApoE robustly suppressed metastatic colonization by over 300-fold (Figure 5L). This dramatic suppression of metastasis by ApoE pre-incubation of melanoma cells reflects that the effects of ApoE on melanoma cells are pivotal for metastatic initiation, as cells pre-treated with ApoE exhibit reduced invasive ability, which is needed to initiate metastatic events leading to lung colonization.

Given the robust influence exerted by ApoE on metastasis and metastatic phenotypes, as well as its strong association with human melanoma progression, further assays were carried out to investigate the impact of genetic deletion of systemic ApoE on melanoma progression in an immunocompetent mouse model of melanoma metastasis. Consistent with a major suppressive role for extracellular ApoE in metastasis, B16F10 mouse melanoma cells injected into the circulation exhibited a greater than 7-fold increase in metastatic colonization in ApoE genetically null mice compared to their wild-type littermates (Figure 5M). These findings establish systemic and cancer-secreted ApoE as a robust suppressor of human and mouse melanoma metastasis.

EXAMPLE 7 *Extracellular ApoE Divergently Targets Melanoma Cell LRP1 and Endothelial Cell LRP8 Receptors*

In this example, assays were carried out to investigate the molecular mechanisms by which ApoE suppresses metastasis.

In order to identify the ApoE receptor(s) that mediate(s) invasion, down all four known ApoE receptors, VLDLR, LRP1, LRP8, and LDLR (Hatters et al., 2006 Trends

Biochem. Sci. *31*, 445-454; Hauser et al., 2011 Prog. Lipid Res. *50*, 62-74) were knocked in melanoma cells. Interestingly, knock-down of LRP1, but not the other ApoE receptors, abolished the cell invasion suppression effect induced by recombinant ApoE (Figure 6A). Importantly, knock-down of LRP1 in metastatic LM2 cells, which display low levels of ApoE, only modestly increased cell invasion (Figure 17A), suggesting the effects of LRP1 to be mediated by endogenous ApoE.

To determine if LRP1 also mediates the miRNA-dependent effects on invasion and metastatic colonization, LRP1 was knocked down in the context of miRNA inhibition. LRP1 knock-down in the setting of miRNA silencing rescued the suppressed invasion phenotype arising from miRNA inhibition (Figures 6B, 17B). Consistent with these in vitro results, LRP1 knock-down significantly enhanced in vivo metastatic colonization by LM2 cells silenced for miR-1908 (Figure 6C, 17C). These findings reveal LRP1 to be epistatically downstream of miRNA/ApoE-dependent melanoma invasion and metastatic colonization.

While the invasion phenotype reflects the cell-autonomous effects of ApoE on melanoma cells, the endothelial recruitment phenotype suggests a non-cell-autonomous role of cancer-expressed ApoE directly on endothelial cells. Consistent with this, pre-treatment of endothelial cells with ApoE significantly reduced their ability to migrate towards highly metastatic cancer cells (Figure 6D). In order to identify the ApoE receptor(s) on endothelial cells that mediate(s) the endothelial recruitment phenotype, all four known ApoE receptors were knocked down on endothelial cells. Interestingly, unlike for cancer cell invasion, knock-down of endothelial LRP8, but not any of the other receptors, selectively and significantly abrogated the inhibition of endothelial recruitment caused by miRNA silencing (Figures 6E, 17D-E). These findings are consistent with the LRP8 receptor being the downstream endothelial mediator of miRNA/ApoE-dependent effects on endothelial recruitment.

Next, assays were carried out to examine whether ApoE/LRP8 signaling might also regulate general endothelial migration in a cancer cell-free system. Accordingly, antibody neutralization of ApoE, which is present in endothelial cell media, significantly enhanced endothelial migration (Figure 6F), while recombinant ApoE was sufficient to inhibit endothelial migration in a trans-well assay (Figure 6G) and a gradient-based chemotactic assay (Figure 6H) in an endothelial cell LRP8 receptor-dependent manner. Importantly, addition of ApoE lead to a dramatic (greater than 40-fold) suppression of VEGF-induced endothelial recruitment in vivo into subcutaneous matrigel plugs (Figure 6I).

Given the requirement and sufficiency of ApoE in mediating endothelial recruitment, further assays were carried out to examine whether systemic ApoE might regulate metastatic angiogenesis. Consistent with the robust suppression of metastatic endothelial content by melanoma cell-secreted ApoE (Figure 4K), genetically null ApoE mice displayed higher blood vessel densities within their lung metastatic nodules formed by B16F10 mouse melanoma cells compared to their wild-type littermates (Figure 6J; 2.41-fold increase, $P = 0.0055$). Taken together, the above findings reveal dual cell-autonomous/non-cell-autonomous roles for ApoE in metastasis suppression through divergent signaling mediated by melanoma cell LRP1 and endothelial cell LRP8 receptors.

EXAMPLE 8 *MiR-199a-3p, miR-199a-5p, and miR-1908 as Robust Prognostic and Therapeutic Targets in Melanoma Metastasis*

To examine whether the metastasis promoter miRNAs described herein could serve as clinical predictors of metastatic outcomes, the expression levels of miR-199a-3p, miR-199a-5p, and miR-1908 were quantified in a blinded fashion by qRT-PCR in a cohort of human melanoma samples obtained from patients at MSKCC. The relationships between the levels of these miRNAs in primary melanoma lesions and metastatic relapse outcomes were then determined.

Importantly, patients whose primary melanoma lesions expressed higher (greater than the median for the population) levels of miR-199a-3p, miR-199a-5p, or miR-1908 were more likely to develop distal metastases and exhibited significantly shorter metastasis-free survival times than patients whose primary melanomas expressed lower levels of each of these miRNAs (Figures 7A-C, $P = 0.0032$ for miR-199a-3p, $P = 0.0034$ for miR-199a-5p, and $P = 0.027$ for miR-1908). Strikingly, the aggregate expression levels of the three miRNAs displayed the strongest prognostic capacity in stratifying patients at high risk from those with very low risk for metastatic relapse (Figure 7D, $P < 0.0001$). These clinical findings are consistent with functional cooperativity between these miRNAs in the regulation of cancer progression and suggest utility for these molecules as clinical prognostic biomarkers of melanoma metastasis.

In light of the current lack of effective treatment options for the prevention of melanoma metastasis and the strong prognostic value of the three regulatory miRNAs in melanoma metastasis, these miRNAs therapeutically targeted using antisense LNA therapy (Elmer et al., 2008(a); Elmer et al., 2008(b)). Highly metastatic MeWo-LM2 cells pre-treated with LNA oligonucleotides antisense to mature miR-199a-3p, miR-199a-5p, or miR-1908 exhibited roughly a four-fold decrease in metastatic activity. Given clinical evidence for cooperativity among these miRNAs, the impact of silencing all three miRNAs on metastatic progression was examined. Remarkably, co-transfection of LNAs against all three miRNAs suppressed metastatic colonization by over seventy-fold, revealing dramatic synergy and cooperativity between endogenous miR-199a-3p, miR-199a-5p, and miR-1908 (Figure 7E, $P = 0.004$). Importantly, inhibition of these miRNAs with triple LNA pre-treatment did not result in decreased in vitro proliferation (Figure 18A), indicating that the dramatic metastasis suppression phenotype is not secondary to impaired proliferation. Combinatorial LNA-mediated miRNA targeting in the independent A375 metastatic derivative line also significantly inhibited lung colonization (Figure 18B).

Next, it was examined whether combinatorial LNA-induced miRNA inhibition could suppress systemic melanoma metastasis to multiple distant organs. Indeed, intracardiac injection of highly metastatic melanoma cells pre-treated with a cocktail of LNAs targeting the three regulatory miRNAs revealed endogenous miR-199a-3p, miR-199a-5p, and miR-1908 to promote systemic melanoma metastasis (Figure 7F). Combinatorial LNA-mediated inhibition of the three miRNAs lead to a reduction in the number of systemic metastatic foci (Figure 7G) in distal sites such as the brain and bone (Figures 7H-I).

Further assays were carried out to examine the therapeutic efficacy of systemically administered in vivo-optimized LNAs in melanoma metastasis prevention. To this end, highly metastatic MeWo-LM2 cells were injected into mice. The following day, mice were intravenously treated with LNAs targeting miR-199a-3p, miR-199a-5p, and miR-1908 at a low total dose (12.5 mg/kg) on a bi-weekly basis for four weeks. Notably, combinatorial LNA treatment reduced lung colonization by 9-fold (Figure 7J, $P = 0.031$) without any apparent signs of toxicity (Figure 18C). Taken together, the above findings reveal a novel miRNA-dependent regulatory network that converges on ApoE signaling to control cell-autonomous and non-cell-autonomous features of melanoma metastatic progression (Figure

7K). The above basic studies have identified a set of miRNAs with powerful prognostic and therapeutic potential in the clinical management of melanoma.

EXAMPLE 9 *miRNA-Dependent Targeting Of Apoe/LRP1 Signaling Promotes Cancer Cell Invasion and Endothelial Recruitment through CTGF Induction*

In this example, Connective Tissue Growth Factor (CTGF) was identified as a down-stream mediator of ApoE/LRP1 signaling in cancer cell invasion and endothelial recruitment. CTGF expression level, as determined by qRT-PCR analysis and ELISA, is mediated by ApoE/LRP1 signaling (Figure 8A, 8B, and 8C). Additionally, ApoE/LRP1 regulated cancer cell invasion and endothelial recruitment are mediated by CTGF (Figure 8D, 8E).

EXAMPLE 10 *CTGF Mediates miRNA-Dependent Metastatic Invasion, Endothelial Recruitment, and Colonization*

In this Examiner, assays were carried out to investigate whether CTGF mediates miRNA-dependent invasion and endothelial recruitment. Briefly, trans-well cell invasion and endothelial recruitment assays were performed on parental MeWo cells over-expressing miR-199a or miR-1908 in the presence of a blocking antibody targeting CTGF. Indeed, it was found that mir-199a and mir-1908 dependent metastatic invasion and endothelial recruitment are mediated by CTGF (Figure 9A and 9B). In order to investigate whether *in vivo* melanoma metastasis (metastatic colonization) is mediated by CTGF, bioluminescence imaging was performed on lung metastasis by 5×10^4 parental MeWo cells knocked down for CTGF in the setting of miR-199a or miR-1908 over-expression. Knock-down of CTGF in this setting resulted in significant reduction of *in vivo* melanoma metastasis (Figure 9C).

EXAMPLE 11 *Treatment with LXR Agonist GW3965 Elevates Melanoma Cell ApoE and DNAJA4 Levels and Suppresses Cancer Cell Invasion, Endothelial Recruitment, and Metastatic Colonization*

Small molecule agonists of the Liver X Receptor (LXR) have previously been shown to increase Apo E levels. To investigate whether increasing Apo-E levels via LXR activation resulted in therapeutic benefit, assays were carried out to assess the effect of the LXR agonist GW3965 [chemical name: 3-[3-[N-(2-Chloro-3-trifluoromethylbenzyl)-(2,2-

diphenylethyl)amino]propyloxy] phenylacetic acid hydrochloride) on Apo-E levels, tumor cell invasion, endothelial recruitment, and *in vivo* melanoma metastasis (Figure 10). Incubation of parental MeWo cells in the presence of therapeutic concentrations of GW3965 increased expression of ApoE and DNAJA4 (Figure 10A and 10B). Pre-treatment of MeWO cells with GW3965 decreased tumor cell invasion (Figure 10C) and endothelial recruitment (Figure 10D). To test whether GW3965 could inhibit metastasis *in vivo*, mice were administered a grain-based chow diet containing GW3965 (20mg/kg) or a control diet, and lung metastasis was assayed using bioluminescence after tail-vein injection of 4×10^4 parental MeWo cells into the mice (Figure 10E). Oral administration of GW3965 to the mice in this fashion resulted in a significant reduction in *in vivo* melanoma metastasis (Figure 10E).

EXAMPLE 12 *Identification of Mir-7 as an Endogenous Suppressor of Melanoma Metastasis*

In this example, miR-7 was identified as an endogenous suppressor of melanoma metastasis (Figure 11). To test whether miR-7 suppresses melanoma metastasis *in vivo*, its expression was knocked down in parental MeWo cells using miR-Zip technology (Figure 11A). Bioluminescence imaging plot of lung metastatic colonization following intravenous injection of 4×10^4 parental MeWo cells expressing a short hairpin (miR-Zip) inhibitor of miR-7 (miR-7 KD) significantly increased lung metastasis in vivo (Figure 11A). Conversely, overexpression of miR-7 in LM2 cells significantly reduced lung metastasis *in vivo* (Figure 11B).

The complexity of cancer requires the application of systematic analyses (Pe'er and Hacohen, 2011). Via a systematic global approach, a cooperative network of miRNAs was uncovered. The miRNAs are i) upregulated in highly metastatic human melanoma cells, ii) required and sufficient for metastatic colonization and angiogenesis in melanoma, and iii) robust pathologic predictors of human melanoma metastatic relapse. Through a transcriptomic-based and biologically guided target identification approach, miR-1908, miR-199a-3p, and miR-199a-5p were found to convergently target the heat shock factor DNAJA4 and the metabolic gene ApoE. The requirement of each individual miRNA for metastasis indicates that these three convergent miRNAs are non-redundant in promoting melanoma metastasis, while the robust synergistic metastasis suppression achieved by combinatorial miRNA inhibition reveals functional cooperativity between these miRNAs, presumably achieved through maximal silencing of ApoE and DNAJA4. The identification of ApoE as a gene negatively regulated by three metastasis promoter miRNAs, positively regulated by a metastasis suppressor gene (DNAJA4), and silenced in clinical metastasis samples highlights the significance of this gene as a suppressor of melanoma progression.

EXAMPLE 13 *Identification of LXRβ Signaling as a Novel Therapeutic Target in Melanoma*

To identify nuclear hormone receptors that show broad expression in melanoma, we examined the expression levels of all nuclear hormone receptor family members across the NCI-60 collection of human melanoma cell lines. Several receptors exhibited stable expression across multiple melanoma lines, suggesting that they could represent novel potential targets in melanoma (Figures 19A and 20A). Notably, out of these, liver-X receptors (LXRs) were previously shown to enhance *ApoE* transcription in adipocytes and macrophages (Laffitte et al., 2001), while pharmacologic activation of RXRs was found to drive *ApoE* expression in pre-clinical Alzheimer's models (Cramer et al., 2012).

Given the recently uncovered metastasis-suppressive role of ApoE in melanoma (Pencheva et al., 2012), the ubiquitous basal expression of LXRβ and RXRα in melanoma, and the availability of pharmacologic agents to therapeutically activate LXRs and RXRs, we investigated whether activation of LXRs or RXRs in melanoma cells might inhibit melanoma progression phenotypes. In light of the established roles of nuclear hormone receptors such as ER and AR in regulating breast and prostate cancer cell proliferation, we first examined whether pharmacologic agonism of LXRs or RXRs in melanoma cells affects *in vitro* cell growth.

Treatment of melanoma cells with two structurally-distinct LXR agonists, GW3965 **2** or T0901317 **1**, or the RXR agonist bexarotene did not affect cell proliferation or cell viability rates (Figure 20 B-C). We next assessed the effects of LXR or RXR activation on cell invasion and endothelial recruitment—phenotypes displayed by metastatic melanoma and metastatic breast cancer populations (Pencheva et al., 2012; Png et al., 2012). Treatment of the mutationally diverse MeWo (*B-Raf/N-Ras* wild-type), HT-144 (*B-Raf* mutant), and SK-Mel-2 (*N-Ras* mutant) human melanoma lines as well as the SK-Mel-334.2 (*B-Raf* mutant) primary human melanoma line with GW3965 **2** or T0901317 **1** consistently suppressed the ability of melanoma cells to invade through matrigel and to recruit endothelial cells in trans-well assays (Figure 19B-C). In comparison, treatment with bexarotene suppressed invasion only in half of the melanoma lines tested and it did not significantly affect the endothelial recruitment phenotype (Figures 19B-C).

Given the superiority of LXR over RXR agonism in broadly inhibiting both cell invasion and endothelial recruitment across multiple melanoma lines, we investigated the requirement for LXR signaling in mediating the suppressive effects of LXR agonists. Knockdown of melanoma *LXRβ*, but not *LXRα*, abrogated the ability of GW3965 **2** and T0901317 **1** to suppress invasion and endothelial recruitment (Figure 19D-G and Figures 20D-G), revealing melanoma-cell LXRβ to be the functional target of LXR agonists in eliciting the suppression of these *in vitro* phenotypes. Our molecular findings are consistent with *LXRβ* being the predominant LXR isoform expressed by melanoma cells (Figure 19A, $P < 0.0001$).

The ubiquitous basal expression of *LXRβ* in melanoma is likely reflective of the general role that LXRs play in controlling lipid transport, synthesis, and catabolism (Calkin and Tontonoz, 2013). While such stable *LXRβ* expression would be key to maintaining melanoma cell metabolism and growth, it also makes LXR signaling an attractive candidate for broad-spectrum therapeutic targeting in melanoma.

EXAMPLE 14 *Therapeutic Delivery of LXR Agonists Suppresses Melanoma Tumor Growth*

LXR agonists were originally developed as oral drug candidates for the purpose of cholesterol lowering in patients with dyslipidemia and atherosclerosis (Collins et al., 2002; Joseph and Tontonoz, 2003). These compounds were abandoned clinically secondary to their inability to reduce lipid levels in large-animal pre-clinical models (Groot et al., 2005).

Given the robust ability of GW3965 **2** and T0901317 **1** to suppress *in vitro* melanoma progression phenotypes (Figure 19B-C), we investigated whether therapeutic LXR activation could be utilized for the treatment of melanoma. Indeed, oral administration of GW3965 **2** or T0901317 **1** at low doses (20 mg/kg), subsequent to formation of subcutaneous tumors measuring 5-10 mm^3 in volume, suppressed tumor growth by the aggressive B16F10 mouse melanoma cells in an immunocompetent model by 67% and 61%, respectively (Figure 21A-B). Administration of a higher LXR agonist dose

(100 mg/kg) led to an 80% reduction in tumor growth (Figure 21A), consistent with dose-dependent suppressive effects.

Oral administration of GW3965 **2** also robustly suppressed tumor growth by the MeWo (70% inhibition) and SK-Mel-2 (49% inhibition) human melanoma cell lines, as well as the SK-Mel-334.2 primary human melanoma line (73% inhibition) (Figure 21C-E and Figure 22A).

Encouraged by the robust tumor-suppressive impact of LXR agonists on small tumors (5-10 mm^3) (Figure 21A-E), we next investigated whether LXR activation therapy could inhibit the growth of large (~150 mm^3) tumors.

We found that treatment with GW3965 **2** led to a roughly 50% reduction in the growth of established large B16F10 tumors (Figure 21F). Importantly, therapeutic delivery of GW3965 **2** subsequent to tumor establishment substantially prolonged the overall survival time of immunocompetent mice injected with mouse B16F10 cells, immunocompromised mice bearing tumor xeongrafts derived from the human MeWo established melanoma line, as well as the SK-Mel.334-2 primary human melanoma line (Figure 21G-I). These findings are consistent with broad-spectrum responsiveness to LXR activation therapy across melanotic and amelanotic established melanoma tumors of diverse mutational subtypes: *B-Raf* and *N-Ras* wild-type (B16F10 and MeWo; Figure 21A-C), *B-Raf* mutant (SK-Mel-334.2; Figure 21D), and *N-Ras* mutant (SK-Mel-2; Figure 21E).

We next sought to determine the cell biological phenotypes regulated by LXR agonists in suppressing tumor growth. Consistent with the inhibitory effects of GW3965 **2** on endothelial recruitment by melanoma cells *in vitro*, GW3965 **2** administration led to a roughly 2-fold reduction in the endothelial cell content of tumors (Figure 21J). This effect was accompanied by a modest decrease (23%) in the number of actively proliferating tumor cells *in vivo* (Figure 21K) without a change in the number of apoptotic cells (Figure 21L). These results suggest that, in addition to reducing local tumor invasion, LXR activation suppresses melanoma tumor growth primarily through inhibition of tumor angiogenesis with a resulting reduction in *in vivo* proliferation.

EXAMPLE 15 *LXR Agonism Suppresses Melanoma Metastasis to the Lung and Brain and Inhibits the Progression of Incipient Metastases*

The strong suppressive effects of LXR agonists on melanoma tumor growth motivated us to examine whether LXR activation could also suppress metastatic colonization by melanoma cells. To this end, pre-treatment of human MeWo melanoma cells with GW3965 **2** led to a more than 50-fold reduction in their metastatic colonization capacity (Figure 23A). In light of this dramatic inhibitory effect, we next assessed the ability of orally administered LXR agonists to suppress metastasis. Immunocompromised mice that were orally administered GW3965 **2** or T0901317 **1** experienced 31-fold and 23-fold respective reductions in lung metastatic colonization by human MeWo cells (Figure 23B-C). Treatment with GW3965 2 also suppressed metastatic colonization by the HT-144 melanoma line (Figure 23D) as well as the SK-Mel-334.2 primary melanoma line (Figure 23E).

GW3965 **2** is a lipophilic molecule that can efficiently cross the blood brain barrier and potently activate LXR signaling in the brain. Consistent with this, oral delivery of GW3965 **2** was previously shown to improve amyloid plaque pathology and memory deficits in pre-clinical models of Alzheimer's disease (Jiang et al., 2008). We thus wondered whether LXR agonism could exhibit therapeutic activity in the suppression of melanoma brain metastasis—a dreaded melanoma outcome in dire need of effective therapies (Fonkem et al., 2012). Notably, oral administration of GW3965 **2** inhibited both systemic dissemination and brain colonization following intracardiac injection of brain-metastatic melanoma cells derived from the MeWo parental line (Figure 23F). These results reveal robust metastasis suppression by LXR activation therapy across multiple melanoma lines and in multiple distal organ metastatic sites.

Encouraged by the robust effects observed in suppressing metastasis formation (Figure 23A-F), we next sought to determine whether LXR activation therapy could halt the progression of melanoma cells that had already metastatically disseminated. We first tested the ability of GW3965 **2** to reduce lung colonization by melanoma cells disseminating from an orthotopic site following removal of the primary tumor (Figure 23G). Importantly, oral administration of GW3965 **2** post-tumor excision inhibited lung colonization by disseminated melanoma cells by 17-fold (Figure 23H). Remarkably, treatment of mice with GW3965 **2** also dramatically suppressed (28-fold) colonization by incipient lung metastases that had progressed 8-fold from the baseline at seeding (Figure 23I). Consistent with LXR activation inhibiting metastatic initiation, GW3965 **2** treatment decreased the number of macroscopic metastatic nodules formed (Figure 23J). Finally, treatment of mice with GW3965 **2** in this 'adjuvant' pre-clinical context significantly prolonged their survival times following metastatic colonization (Figure 23K).

EXAMPLE 16 *LXR Activation Reduces Melanoma Progression and Metastasis in a Genetically-Driven Mouse Model of Melanoma*

Roughly 60% of human melanoma tumors are marked by activating mutations in the *Braf* oncogene, with one single amino acid variant, *B-Raf*^{V600E}, being the predominant mutation found (Davies et al., 2002). Nearly 20% of melanomas exhibit activating mutations in *B-Raf* with concurrent silencing of the *Pten* tumor-suppressor, which drives progression to a malignant melanoma state (Tsao et al., 2004; Chin et al., 2006). Recently, *Tyrosinase* (*Tyr*)-driven conditional *B-Raf* activation and *Pten* loss were shown to genetically cooperate in driving mouse melanoma progression (Dankort et al., 2009).

To determine whether LXR activation could suppress melanoma progression in this genetically-initiated model, we induced melanomas in *Tyr::CreER; B-Raf*$^{V600E/+}$*; Pten*$^{lox/+}$ and *Tyr::CreER; B-Raf*$^{V600E/+}$*; Pten*$^{lox/lox}$ mice by intraperitoneal administration of 4-hydroxytamoxifen (4-HT). Notably, oral administration of GW3965 **2** following melanoma initiation attenuated tumor progression and significantly extended the overall survival times of both *PTEN* heterozygous *Tyr::CreER; B-Raf*$^{V600E/+;}$ *Pten*$^{lox/+}$ and *PTEN* homozygous *Tyr::CreER; B-Raf*$^{V600E/+;}$ *Pten*$^{lox/lox}$ mice (Figure 24A-B and Figure 25A-B). Next, we examined the ability of GW3965 **2** to suppress melanoma metastasis in this genetic context. While we did not detect macroscopic metastases in the lungs or brains of 4-HT-treated *Tyr::CreER; B-Raf*$^{V600E/+;}$ *Pten*$^{lox/lox}$ control mice, we consistently observed melanoma metastases to the salivary gland lymph nodes. Importantly, *Tyr::CreER; B-Raf*$^{V600E/+;}$ *Pten*$^{lox/lox}$ mice treated with GW3965 **2** exhibited a decrease in the number of lymphatic metastases detected post-mortem (Figure 24C). These findings indicate that LXR activation inhibits orthotopic metastasis in a genetically-driven melanoma model, in addition to its suppressive effects on primary melanoma tumor progression.

The cooperativity between *B-Raf* activation and *Pten* loss in driving melanoma progression can be further enhanced by inactivation of *CDKN2A*, a cell cycle regulator frequently mutated in familial melanomas (Hussussian et al., 1994; Kamb et al., 1994). We thus examined the effect of LXR activation on *B-Raf*$^{V600E/+}$*; Pten*$^{-/-}$*; CDKN2A*$^{-/-}$ melanomas, allowing us to test the therapeutic efficacy of LXR agonism in a more aggressive genetically-driven melanoma progression model. Importantly, therapeutic delivery of GW3965 **2** robustly inhibited tumor growth and lung metastasis by *B-Raf*$^{V600E/+}$*; Pten*$^{-/-}$*; CDKN2A*$^{-/-}$ primary mouse melanoma cells injected into syngeneic immunocompetent mice and extended the overall survival of mice bearing *B-Raf*$^{V600E/+}$*; Pten*$^{-/-}$*; CDKN2A*$^{-/-}$ melanoma burden (Figure 24D-F). Taken together, the robust suppression of melanoma progression across independent xenograft and genetically-induced immunocompetent melanoma mouse models that exhibit the diverse mutational profiles of human melanomas motivates the clinical testing of LXR activation therapy.

EXAMPLE 17 *Pharmacologic Activation of LXRβ Suppresses Melanoma Phenotypes by Transcriptionally Inducing Melanoma-Cell ApoE expression*

We next sought to determine the downstream molecular target of LXRβ that mediates suppression of melanoma progression. To this end, we transcriptomically profiled human MeWo melanoma cells treated with the LXR agonist GW3965 **2**.

Out of the 365 genes that were significantly induced in response to LXR activation, we identified *ApoE*, a previously validated transcriptional target of LXRs in macrophages and adipocytes (Laffitte et al., 2001), as the top upregulated secreted factor in melanoma cells (Figure 26). Quantitative real-time PCR (qRT-PCR) validation revealed robust upregulation of *ApoE* transcript expression following treatment with independent LXR agonists across multiple human melanoma lines (Figure 27A-C).

In light of the previously reported metastasis-suppressive function of ApoE in melanoma (Pencheva et al., 2012), we investigated whether LXRβ activation suppresses melanoma progression through transcriptional induction of *ApoE*. Indeed, GW3965 **2** and T0901317 **1** were found to enhance the melanoma cell-driven activity of a luciferase reporter construct containing the *ApoE* promoter fused to either of two previously characterized LXR-binding multi-enhancer elements (ME.1 or ME.2) (Laffitte et al., 2001) (Figure 28A). Importantly, this transcriptional induction resulted in elevated levels of secreted ApoE protein (Figure 28B). Consistent with direct LXRβ targeting of *ApoE* in melanoma cells, neutralization of extracellular ApoE with an antibody fully blocked the

LXRβ-mediated suppression of cell invasion and endothelial recruitment and further enhanced these phenotypes relative to the control IgG treatment (Figure 28C-G and Figure 27D-F), revealing the effects of LXR agonism to be modulated by extracellular ApoE.

Additionally, molecular knockdown of *ApoE* in melanoma cells also blocked the GW3965 **2**-mediated suppression of cell invasion and endothelial recruitment phenotypes (Figure 27G-H). In agreement with this, melanoma cell depletion of *LXRβ*, but not *LXRα*, abrogated the ability of GW3965 **2** and T0901317 **1** to upregulate *ApoE* transcription and ultimately protein expression (Figure 28H-I and Figure 27I-K). Collectively, these findings indicate that pharmacologic activation of LXRβ, the predominant LXR isoform expressed by melanoma cells, suppresses cell-intrinsic invasion and endothelial recruitment by melanoma cells through transcriptionally activating *ApoE* expression in melanoma cells.

EXAMPLE 18 *Engagement of Melanoma-Derived and Systemic ApoE by LXRβ Activation Therapy*

The LXRβ-induced suppression of key melanoma phenotypes by extracellular ApoE *in vitro* suggested that the suppressive effects of LXR agonists *in vivo* might be further augmented by the activation of LXRs in peripheral tissues, which could serve as robust sources of extracellular ApoE.

Importantly, such non-transformed tissues would be less vulnerable to developing resistance to LXR activation therapy, allowing for chronic *ApoE* induction in patients. We thus investigated whether therapeutic LXR agonism suppresses melanoma progression by inducing *ApoE* derived from melanoma cells or systemic tissues. Consistent with LXRβ agonism increasing *ApoE* expression in melanoma cells *in vivo*, *ApoE* transcript levels were upregulated in melanoma primary tumors as well as in melanoma lung and brain metastases dissociated from mice that were fed an LXR agonist-supplemented diet (Figure 29A-E). Importantly, treatment of mice with either GW3965 **2** or T0901317 **1** significantly elevated ApoE protein expression in systemic adipose, lung, and brain tissues of mice (Figures 30A-B) and also upregulated *ApoE* transcript levels in circulating white blood cells (Figure 30C). These results indicate that LXR activation therapy induces both melanoma-cell and systemic tissue *ApoE* expression *in vivo*.

To determine the *in vivo* requirement of melanoma-derived and systemic LXR activation for the tumor-suppressive effects of orally administered LXR agonists, we first tested the ability of GW3965 **2** to suppress tumor growth by B16F10 mouse melanoma cells depleted of LXRβ.

Consistent with our findings in human melanoma cells, knockdown of mouse melanoma-cell *LXRβ* abrogated the GW3965-mediated induction of *ApoE* expression (Figure 29F-H). Despite this, melanoma-cell *LXRβ* knockdown was unable to prevent the suppression of tumor growth by GW3965 **2** (Figure 29D), implicating a role for systemic LXR activation in tumor growth inhibition by GW3965 **2**. To identify the LXR isoform that mediates this non-tumor autonomous suppression of melanoma growth by LXR agonists, we examined the effects of GW3965 **2** on tumors implanted onto *LXRα* or *LXRβ* genetically null mice. Interestingly, genetic ablation of systemic *LXRβ* blocked the ability of GW3965 to suppress melanoma tumor growth, while *LXRα* inactivation had no effect on tumor growth inhibition by GW3965 (Figure 6D). Importantly, the upregulation of systemic *ApoE* expression by GW3965 **2**, an agonist with 6-fold greater activity towards LXRβ than LXRα, was abrogated in *LXRβ* -/-, but not in *LXRα* -/- mice (Figure 30E and Figure 29I). These results indicate that *ApoE* induction by GW3965 **2** in peripheral tissues is predominantly driven by systemic LXRβ activation. In agreement with this, we find systemic LXRβ to be the primary molecular target and effector of GW3965 **2** in mediating melanoma tumor growth suppression.

We next examined whether ApoE is required for the *in vivo* melanoma-suppressive effects of LXR agonists. Consistent with the lack of an impact for melanoma-cell *LXRβ* knockdown on the tumor-suppressive activity of GW3965 **2**, depletion of melanoma-cell *ApoE* did not prevent tumor growth inhibition by GW3965 **2** neither (Figure 29F-H and Figure 30F). These findings suggest that the tumor suppressive effects of GW3965 **2** might be primarily mediated through *ApoE* induction in systemic tissues.

Indeed, GW3965 **2** was completely ineffective in suppressing tumor growth in mice genetically inactivated for *ApoE* (Figure 30F), revealing systemic *ApoE* as the downstream effector of systemic LXRβ in driving melanoma tumor growth suppression. Interestingly, in contrast to primary tumor growth regulation, knockdown of melanoma-cell *ApoE* partially prevented the metastasis-suppressive effect of GW3965 **2** (Figure 30G). Similarly, genetic inactivation of *ApoE* only partially prevented the metastasis suppression elicited by GW3965 **2** as well (Figure 30G). The GW3965-driven inhibition of metastasis was completely blocked only in the context of both melanoma-cell *ApoE* knockdown and genetic inactivation of systemic *ApoE* (Figure 30G), indicative of a requirement for both melanoma-derived and systemic *ApoE* engagement by LXRβ in suppressing metastasis. We thus conclude that the effects of LXRβ activation on primary tumor growth are elicited primarily through systemic *ApoE* induction, while the effects of LXRβ agonism on metastasis are mediated through *ApoE* transcriptional induction in both melanoma cells and systemic tissues.

The identification of ApoE as the sole downstream mediator of the LXRβ-induced suppression of melanoma phenotypes further highlights the importance of this gene as a suppressor of melanoma progression. To determine whether ApoE expression is clinically prognostic of melanoma metastatic outcomes, we assessed ApoE protein levels by performing blinded immunohistochemical analysis on 71 surgically resected human primary melanoma lesions.

We found that patients whose melanomas had metastasized exhibited roughly 3-fold lower ApoE expression in their primary tumors relative to patients whose melanomas did not metastasize (Figure 30H, $P = 0.002$). Remarkably, ApoE expression levels in patients' primary melanoma lesions robustly stratified patients at high risk from those at low risk for metastatic relapse (Figure 30I, $P = 0.002$). These observations are consistent with previous findings that revealed significantly lower levels of ApoE in distant melanoma metastases relative to primary lesions (Pencheva et al., 2012). Collectively, this work indicates that *ApoE*, as a single gene, could likely act as a prognostic and predictive biomarker in primary melanomas to identify patients that i.) are at risk for melanoma metastatic relapse and as such ii.) could obtain clinical benefit from LXRβ agonist-mediated *ApoE* induction.

EXAMPLE 19 *LXRβ Activation Therapy Suppresses the Growth of Melanomas Resistant to Dacarbazine and Vemurafenib*

Encouraged by the robust ability of LXRβ activation therapy to suppress melanoma tumor growth and metastasis across a wide range of melanoma lines of diverse mutational backgrounds, we next sought to determine whether melanomas that are resistant to two of the mainstay clinical agents used in the management of metastatic melanoma—dacarbazine and vemurafenib—could respond to LXRβ-activation therapy.

To this end, we generated B16F10 clones resistant to dacarbazine (DTIC) by continuously culturing melanoma cells in the presence of DTIC for two months. This yielded a population of cells that exhibited a 7-fold increase in viability in response to high-dose DTIC treatment compared to the parental B16F10 cell line (Figure 31A). To confirm that this *in vitro*-derived DTIC clone was also resistant to DTIC *in vivo, we* assessed the effects of dacarbazine treatment on tumor growth.

While dacarbazine significantly suppressed the growth of the DTIC-sensitive parental line (Figure 31B), it did not affect tumor growth by B16F10 DTIC-resistant cells (Figure 31C). GW3965 **2** robustly suppressed tumor growth by the DTIC-resistant B16F10 melanoma clone by more than 70% (Figures 31C-D). Importantly, oral delivery of GW3965 **2** also strongly inhibited the growth of *in vivo*-derived DTIC-resistant human melanoma tumors formed by the independent MeWo cell line (Figure 31E-F and Figure 32A).

These results reveal that LXRβ agonism is effective in suppressing multiple melanoma cell populations that are resistant to dacarbazine—the only FDA-approved cytotoxic chemotherapeutic in metastatic melanoma. Our findings have important clinical implications for melanoma treatment since all stage IV patients who are treated with dacarbazine ultimately progress by developing tumors that are resistant to this agent.

We tested the impact of LXRβ activation therapy on melanoma cells resistant to the recently approved B-Raf kinase inhibitor, vemurafenib—a regimen that shows activity against *B-Raf*-mutant melanomas (Bollag et al., 2010; Sosman et al., 2012). Numerous investigators have derived melanoma lines resistant to vemurafenib (Poulikakos et al., 2011; Shi et al., 2012, Das Thakur et al., 2013). GW3965 **2** treatment suppressed the growth of the previously derived SK-Mel-239 vemurafenib-resistant line by 72% (Figure 31G) and significantly prolonged the survival of mice bearing vemurafenib-resistant melanoma burden (Figure 31H). Our findings from combined pharmacologic, molecular and genetic studies in diverse pre-clinical models of melanoma establish LXRβ targeting as a novel therapeutic approach that robustly suppresses melanoma tumor growth and metastasis through the transcriptional induction of *ApoE*—a key suppressor of melanoma invasion and metastatic angiogenesis (Pencheva et al., 2012; Figure 31I).

EXAMPLE 20 *Treatment with ApoE inhibits tumor cell invasion and endothelial recruitment across multiple cancer types, including breast cancer, renal cell cancer and pancreatic cancer*

In order to determine if ApoE treatment could be effective for treating cancer types in addition to melanoma, in vitro assays were performed to assess the effect of ApoE treatment on several different cancer cell lines, including breast cancer, renal cell cancer, and pancreatic cancer cell lines (Figure 33).

The ability of cancer cells to invade through matrigel *in vitro* was tested by using a trans-well matrigel invasion chamber system (354480, BD Biosciences). Various cancer cell lines were serum-starved overnight in media containing 0.2% FBS. The following day, invasion chambers were pre-equilibrated prior to the assay by adding 0.5 mL of starvation media to the top and bottom wells. Meanwhile, cancer cells were trypsinized and viable cells were counted using the trypan blue dead cell exclusion dye. Cancer cells were then resuspended at a concentration of 1×10^5 cells/1 mL starvation media, and 0.5 mL of cell suspension, containing 5×10^4 cells, was seeded into each trans-well. To determine the effect of recombinant ApoE on cancer cell invasion, human recombinant ApoE3 (4696, Biovision) or BSA were added to each trans-well at 100 μg/mL at the start of the assay. The invasion assay was allowed to proceed for 24 hours at 37°C. Upon completion of the assay, the inserts were washed in PBS, the cells that did not invade were gently scraped off from the top side of each insert using q-tips, and the cells that invaded into the basal insert side were fixed in 4% PFA for 15 minutes at room temperature. Following fixation, the inserts were washed in PBS and then cut out and mounted onto slides using VectaShield mounting medium containing DAPI nuclear stain (H-1000, Vector Laboratories). The basal side of each insert was imaged using an inverted fluorescence microscope (Zeiss Axiovert 40 CFL) at 5X magnification, and the number of DAPI-positive cells was quantified using ImageJ.

Indeed, treatment with ApoE inhibited both tumor cell invasion and endothelial recruitment across all three of these cancer types (Figure 33A-I).

EXAMPLE 21 *LXR agonists LXR-623, WO-2007-002563 Ex. 19, WO-2010-0138598 Ex. 9, and SB742881, induce ApoE expression in human melanoma cells*

Given that ApoE activation by treatment with LXR agonists GW3965 **2** and T0901317 **1** resulted in therapeutic benefit for inhibiting tumor growth and metastasis, we next examined the ability of other LXR agonists to induce ApoE expression in human melanoma cell lines (Figure 34).

To determine the effect of the various LXR agonists (LXR-623, WO-2007-002563 Ex. 19, WO-2010-0138598 Ex. 9, and SB742881 on ApoE expression in melanoma cells, 1 × 10^5 human MeWo melanoma cells were seeded in a 6-well plate. The following day, DMSO or the respective LXR agonist was added to the cell media at a concentration of 500 nM, 1 μM, or 2 μM, as indicated, and the cells were incubated in the presence of DMSO or the drug for 48 hours at 37°C. The total amount of DMSO added to the cell media was kept below 0.2%. RNA was extracted from whole cell lysates using the Total RNA Purification Kit (17200, Norgen). For every sample, 600 ng of RNA was reverse transcribed into cDNA using the cDNA First-Strand Synthesis kit (Invitrogen). Approximately 200 ng of cDNA was mixed with SYBR® green PCR Master Mix and the corresponding forward and reverse primers specific for detection of human ApoE. Each reaction was carried out in quadruplicates, and ApoE mRNA expression levels were measured by quantitative real-time PCR amplification using an ABI Prism 7900HT Real-Time PCR System (Applied Biosystems). The relative ApoE expression was determined using the ΔΔCt method. GAPDH was used as an endogenous control for normalization purposes.

Indeed, treatment with the LXR agonists LXR-623, WO-2007-002563 Ex. 19, WO-2010-0138598 Ex. 9, and SB742881 all led to varied degrees of ApoE expression induction. (Figure 34A-C).

EXAMPLE 22 *Treatment with the LXR agonist GW3965 inhibits in vitro tumor cell invasion of renal cancer, pancreatic cancer, and lung cancer*

We have demonstrated that treatment with LXR agonists resulted in inhibition of melanoma tumor cell invasion. Given that this effect is mediated by activation of ApoE expression, we hypothesized that treatment with LXR agonists would result in inhibition of in vitro tumor cell invasion in breast cancer, pancreatic cancer, and renal cancer, since these cancer types were responsive to ApoE treatment. In order to test this hypothesis, we performed in vitro tumor cell invasion assays by treating breast cancer, pancreatic cancer, and renal cell cancer cell lines with the LXR agonist GW3965 **2** (Figure 35).

Various cell lines (5 × 10^4 RCC human renal cancer cells, 5 × 10^4 PANC1 human pancreatic cancer cells, and 5 × 10^4 H460 human lung cancer cells) were treated with DMSO or GW3965 at 1 μM for 56 hours. The cells were serum starved for 16 hours in 0.2% FBS media in the presence of DMSO or GW3965. Following serum starvation, the cells were subjected to the trans-well invasion assay using a matrigel invasion chamber system (354480, BD Biosciences). Invasion chambers were pre-equilibrated prior to the assay by adding 0.5 mL of starvation media to the top and bottom wells. Meanwhile, cancer cells were trypsinized and viable cells were counted using trypan blue. Cancer cells were then resuspended at a concentration of 1×10^5 cells/1 mL starvation media, and 0.5 mL of cell suspension, containing 5×10^4 cells, was seeded into each trans-well. The invasion assay was allowed to proceed for 24 hours at 37°C. Upon completion of the assay, the inserts were washed in PBS, the cells that did not invade were gently scraped off from the top side of each insert using q-tips, and the cells that invaded into the basal insert side were fixed in 4% PFA for 15 minutes at room temperature. Following fixation, the inserts were washed in PBS and then cut out and mounted onto slides using VectaShield mounting medium containing DAPI nuclear stain (H-1000, Vector Laboratories). The basal side of each insert was imaged using an inverted fluorescence microscope (Zeiss Axiovert 40 CFL) at 5X magnification, and the number of DAPI-positive cells was quantified using ImageJ.

Indeed, treatment with GW3965 **2** resulted in inhibition of tumor cell invasion in all three cancer types tested (Figure 35A-C). This further demonstrated the broad therapeutic potential of LXR agonists for treating various cancer types.

EXAMPLE 23 *Treatment with the LXR agonist GW3965 inhibits breast cancer tumor growth In Vivo*

We have demonstrated that LXR agonists inhibit in vitro cancer progression phenotypes in breast cancer, pancreatic cancer, and renal cancer. To investigate if LXR agonist treatment inhibits breast cancer primary tumor growth in vivo, mice injected with MDA-468 human breast cancer cells were treated with either a control diet or a diet supplemented with LXR agonist GW3965 **2** (Figure 36).

To determine the effect of orally delivered GW3965 **2** on breast cancer tumor growth, 2×10^6 MDA-468 human breast cancer cells were resuspended in 50 μL PBS and 50 μL matrigel and the cell suspension was injected into both lower memory fat pads of 7-week-old Nod Scid gamma female mice. The mice were assigned to a control diet treatment or a GW3965-supplemented diet treatment (75 mg/kg/day) two days prior to injection of the cancer cells. The GW3965 **2** drug compound was formulated in the mouse chow by Research Diets, Inc. Tumor dimensions were measured using digital calipers, and tumor volume was calculated as (small diameter)^2 × (large diameter)/2.

Treatment with GW3965 resulted in significant reduction in breast cancer tumor size in vivo (Figure 36).

EXAMPLE 24 *Effects of treatment with LXR agonists LXR-623, WO-2007-002563 Ex. 19, WO-2010-0138598 Ex. 9, and SB742881 on in vitro melanoma progression phenotypes*

We have demonstrated the ability of various LXR agonists to induce ApoE expression with varying potency in melanoma cells (Figure 34). Since the therapeutic effect of LXR agonists on cancer is via activation of ApoE expression, we hypothesized that the therapeutic potency of any given LXR agonist is directly correlated with its ability to induce ApoE expression. To confirm this, we quantified the effect of treatment with various LXR agonists on in vitro endothelial recruitment and tumor cell invasion of melanoma cells. As shown in Figure 37, the degree to which LXR agonists inhibit in vitro cancer progression phenotypes is related to the LXR agonist's ApoE induction potency.

Cell Invasion: MeWo human melanoma cells were treated with DMSO, LXR-623, WO-2007-002563 Ex. 19, WO-2010-0138598 Ex. 9, or SB742881 at 1 μM each for 56 hours. The cells were then serum starved for 16 hours in 0.2% FBS media in the presence of each corresponding drug or DMSO. Following serum starvation, the cells were subjected to the trans-well invasion assay using a matrigel invasion chamber system (354480, BD Biosciences). Invasion chambers were pre-equilibrated prior to the assay by adding 0.5 mL of starvation media to the top and bottom wells. Meanwhile, cancer cells were trypsinized and viable cells were counted using trypan blue. Cancer cells were then resuspended at a concentration of 2 × 10^5 cells/1 mL starvation media, and 0.5 mL of cell suspension, containing 1 × 10^5 cells, was seeded into each trans-well. The invasion assay was allowed to proceed for 24 hours at 37°C. Upon completion of the assay, the inserts were washed in PBS, the cells that did not invade were gently scraped off from the top side of each insert using q-tips, and the cells that invaded into the basal insert side were fixed in 4% PFA for 15 minutes at room temperature. Following fixation, the inserts were washed in PBS, cut out, and mounted onto slides using VectaShield mounting medium containing DAPI nuclear stain (H-1000, Vector Laboratories). The basal side of each insert was imaged using an inverted fluorescence microscope (Zeiss Axiovert 40 CFL) at 5X magnification, and the number of DAPI-positive cells was quantified using ImageJ.

Endothelial Recruitment: MeWo human melanoma cells were treated with DMSO, LXR-623, WO-2007-002563 Ex. 19, WO-2010-0138598 Ex. 9, or SB742881 at 1 μM each for 56 hours. Subsequently, 5×10^4 cancer cells were seeded into 24-well plates in the presence of each drug or DMSO and allowed to attach for 16 hours prior to starting the assay. Human umbilical vein endothelial cells (HUVEC cells) were serum-starved in 0.2 % FBS-containing media overnight. The following day, 1×10^5 HUVEC cells were seeded into a 3.0 μm HTS Fluoroblock insert (351151, BD Falcon) fitted into each well containing the cancer cells at the bottom. The HUVEC cells were allowed to migrate towards the cancer cells for 20 hours, after which the inserts were washed in PBS, fixed in 4% PFA, labeled with DAPI, and mounted on slides. The basal side of each insert was imaged using an inverted fluorescence microscope (Zeiss Axiovert 40 CFL) at 5X magnification, and the number of DAPI-positive cells was quantified using ImageJ.

LXR agonists that potently induce ApoE expression (e.g. WO-2010-0138598 Ex. 9 and SB742881) are more effective at inhibiting cancer progression phenotypes (Figure 37) than lower potency LXR agonists. This further demonstrates that the therapeutic benefit of LXR agonist treatment for cancer is a result of ApoE induction.

EXAMPLE 25 *Treatment with LXR agonists inhibit melanoma tumor growth in vivo*

We have demonstrated that LXR agonists that induce ApoE expression inhibit in vitro tumor activity. To confirm if these agonists inhibit melanoma tumor growth in vivo, mice that were injected with B16F10 melanoma cells were treated with either *LXR-623, WO-2007-002563 Ex. 19, WO-2010-0138598 Ex. 9, or SB742881.*

To assess the effect of orally administered LXR-623, WO-2007-002563 Ex. 19, WO-2010-0138598 Ex. 9, or SB742881 on melanoma tumor growth, 5×10^4 B16F10 mouse melanoma cells were resuspended in 50 μL PBS and 50 μL matrigel and the cell suspension was subcutaneously injected into both lower dorsal flanks of 7-week-old C57BL/6 mice. The mice were palpated daily for tumor formation and after detection of tumors measuring 5-10 m^3 in volume, the mice were assigned to a control chow or a chow containing each respective LXR agonist: LXR-623 (20 mg/kg/day), WO-2007-002563 Ex. 19 (100 mg/kg/day), WO-2010-0138598 Ex. 9 (10 mg/kg/day or 100 mg/kg/day), or

SB742881 (100 mg/kg/day). The LXR drug compounds were formulated in the mouse chow by Research Diets, Inc. Tumor dimensions were measured using digital calipers, and tumor volume was calculated as (small diameter)^2 × (large diameter)/2.

Consistent with our in vitro data, LXR agonists that potently induce ApoE expression in vitro (WO-2010-0138598 Ex. 9, and SB742881) significantly inhibited melanoma primary tumor growth in vivo (Figure 38). This is also consistent with our results demonstrating that other LXR agonists which potently induce ApoE expression (GW3965 **2**, T0901317 **1**) also inhibit primary tumor growth in vivo (Figure 21).

Accordingly, the above examples focused on characterizing the molecular and cellular mechanisms by which it exerts its effects. To this end, it was found that ApoE targets two distinct, yet homologous, receptors on two diverse cell types. ApoE acting on melanoma cell LRP1 receptors inhibits melanoma invasion, while its action on endothelial cell LRP8 receptors suppresses endothelial migration. The results from loss-of-function, gain-of-function, epistasis, clinical correlation, and in vivo selection derivative expression analyses give rise to a model wherein three miRNAs convergently target a metastasis suppressor network to limit ApoE secretion, thus suppressing ApoE/LRP1 signaling on melanoma cells and ApoE/LRP8 signaling on endothelial cells (Figure 7K). Although the above systematic analysis has identified ApoE and DNAJA4 as key targets and direct mediators of the metastatic phenotypes regulated by these miRNAs, it cannot be excluded that the three miRNAs may individually retain additional target genes whose silencing may contribute to metastatic progression. The ability of ApoE or DNAJA4 knock-down to fully rescue the metastasis suppression phenotypes seen with individual miRNA silencing, however, strongly suggests that these genes are the key mediators of the miRNA-dependent effects on metastasis.

The above results reveal combined molecular, genetic, and in vivo evidence for a required and sufficient role for ApoE in the suppression of melanoma metastatic progression. ApoE can distribute in the circulatory system both in a lipoprotein-bound and a lipid-free state (Hatters et al., 2006). While it has been shown that lipid-free recombinant ApoE is sufficient to suppress melanoma invasion and endothelial migration, it is possible that ApoE contained in lipoprotein particles could also suppress melanoma invasion and endothelial recruitment. The ability of recombinant ApoE to inhibit these pro-metastatic phenotypes, as well as the increased melanoma invasion and endothelial recruitment phenotypes seen with antibody-mediated ApoE neutralization suggests that the ApoE molecule itself is the key mediator of these phenotypes. Consistent with the findings disclosed herein, a synthetic peptide fragment of ApoE was previously found to inhibit endothelial migration through unknown mechanisms (Bhattacharjee et al., 2011). The findings disclosed herein are consistent with a role for melanoma cell-secreted and systemic endogenous ApoE in inhibiting endothelial recruitment, which is not secondary to impaired endothelial cell growth.

The above-described molecular, genetic, and in vivo studies reveal a role for endogenous cancer-derived ApoE in the modulation of endothelial migration and cancer angiogenesis through endothelial LRP8 receptor signaling. This robust non-cell-autonomous endothelial recruitment phenotype mediated by ApoE/LRP8 signaling suggests that ApoE may also modulate metastatic angiogenesis in other cancer types, and such a general role for ApoE in cancer angiogenesis biology remains to be explored. ApoE is a polymorphic molecule with well-established roles in lipid, cardiovascular, and neurodegenerative disorders. Its three major variants, ApoE2, ApoE3, and ApoE4, display varying representations in the human population, with ApoE3 being the most common variant (Hatters et al., 2006). The three isoforms differ at residues 112 and 158 in the N-terminal domain, which contains the ApoE receptor-binding domain. These structural variations are thought to give rise to distinct functional attributes among the variants. Consistent with this, the three ApoE isoforms differ in their binding affinity for members of the LDL receptor family, lipoprotein-binding preferences, and N-terminus stability. Namely, ApoE2 has 50- to 100-fold attenuated LDL receptor binding ability compared to ApoE3 and ApoE4 (Weisgraber et al., 1982), while ApoE4, unlike the other two variants, preferentially binds to large lower-density lipoproteins (Weisgraber et al., 1990) and exhibits the lowest N-terminus stability (Morrow et al., 2000). These functional differences confer pathophysiological properties to select ApoE isoforms. While ApoE3, found in 78% of the population, is considered a neutral allele, ApoE2 is associated with type III hyperlipoprotenemia (Hatters et al., 2006) and ApoE4 represents the major known genetic risk factor for Alzheimer's disease (Corder et al., 1993) and also correlates with a modest increase in the risk of developing cardiovascular disease (Luc et al., 1994). Given that the multiple human melanoma cell lines analyzed in the above study are homozygous for the ApoE3 allele, as well as the ability of recombinant ApoE3 to inhibit melanoma invasion and endothelial recruitment, the above findings are consistent with ApoE3 being sufficient and required for the suppression of melanoma metastatic progression. However, it will be of interest in the future to determine whether ApoE2 and ApoE4 can modulate these pro-metastatic phenotypes to a similar extent as ApoE3 and whether specific ApoE genotypes confer enhanced risk of melanoma metastatic progression.

Besides surgical resection of primary melanoma lesions, there are currently no effective therapies for the prevention of melanoma metastasis with interferon therapy increasing overall survival rates at 5 years by a meager 3% based on meta-analyses, while phase III trial data demonstration of significant survival benefits is still outstanding (Garbe et al., 2011). The dramatic enhancement of melanoma metastatic progression in the context of genetic ablation of systemic ApoE suggests that modulating ApoE levels may have significant therapeutic implications for melanoma—a disease that claims approximately 48,000 lives a year globally (Lucas et al., 2006). Given the robust ability of ApoE to suppress melanoma invasion, endothelial migration, metastatic angiogenesis, and metastatic colonization, therapeutic approaches aimed at pharmacological induction of endogenous ApoE levels may significantly reduce melanoma mortality rates by decreasing metastatic incidence.

The above-described unbiased in vivo selection based approach led to discovery of deregulated miRNAs that synergistically and dramatically promote metastasis by cancer cells from independent patients' melanoma cell lines representing both melanotic and amelanotic melanomas. While miR-1908 has not been previously characterized, miR-199a has been implicated in hepatocellular carcinoma (Hou et al., 2011; Shen et al., 2010) and osteosarcoma (Duan et al., 2011) that, contrary to melanoma, display down-regulation of miR-199a expression levels. These differences are consistent with the established tissue-specific expression profiles of miRNAs in various cancer types. The identification of miR-199a as a promoter of melanoma metastasis is supported by a previous clinical association study revealing that increased miR-199a levels correlate with uveal melanoma progression (Worley et al., 2008), suggesting that induced miR-199a expression may be a defining feature of metastatic melanoma regardless of site of origin. Previous studies have implicated additional miRNAs in promoting melanoma metastatic progression such as miR-182 (Segura et al., 2009), miR-214 (which was upregulated in metastatic melanoma cells, but it did not functionally perform in the above studies; Penna et al., 2011), and miR-

30b/miR-30d (Gaziel-Sovran et al., 2011). Each of these miRNAs have been reported to only modestly modulate melanoma metastasis, leading to 1.5- to 2-fold increased or decreased metastasis modulation upon miRNA over-expression or knock-down, respectively. In contrast, over-expression of either miR-199a or miR-1908 enhanced metastasis by 9-fold (Figure 1C), while combinatorial miRNA knock-down synergistically suppressed melanoma metastasis by over 70-fold (Figure 7E). Therefore, the study disclosed herein represents the first systematic discovery of multiple miRNAs that convergently and robustly promote human melanoma metastasis, as well as the first to assign dual cell-autonomous/non-cell-autonomous roles to endogenous metastasis-regulatory miRNAs in cancer.

Previous systematic analysis of miRNAs in breast cancer revealed primarily a decrease in the expression levels of multiple microRNAs in in vivo selected metastatic breast cancer cells (Tavazoie et al., 2008). Those findings were consistent with the subsequent discovery of many additional metastasis suppressor miRNAs in breast cancer (Shi et al., 2010; Wang and Wang, 2011), the identification of a number of miRNAs as direct transcriptional targets of the p53 tumour suppressor (He et al., 2007), the downregulation of miRNAs in breast cancer relative to normal tissues (Calin and Groce, 2006; Iorio et al., 2005), the downregulation of drosha and dicer in breast cancer (Yan et al., 2011) and metastatic breast cancer (Grelier et al., 2011), as well as the pro-tumorigenic and pro-metastatic effects of global miRNA silencing through dicer knock-down (Kumar et al., 2007; Kumar et al., 2009; Martello et al., 2010; Noh et al., 2011). In contrast to breast cancer, the above findings in melanoma reveal a set of miRNAs upregulated in metastatic human melanoma, raising the intriguing possibility that miRNA processing may actually act in a pro-tumorigenic or pro-metastatic manner in melanoma. Consistent with this, dicer is required for melanocytic development (Levy et al., 2010), and dicer expression was recently found to positively correlate with human melanoma progression in a clinico-pathological study (Ma et al., 2011). These findings, when integrated with the findings disclosed here, motivate future studies to investigate the functional requirement for dicer (Bernstein et al., 2001) in melanoma metastasis.

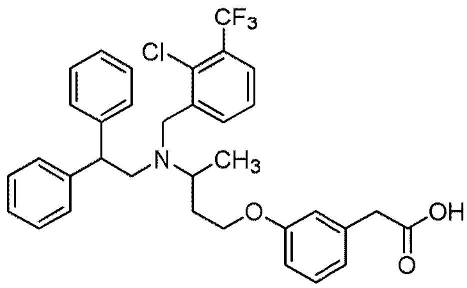
The establishment of in vivo selection models of melanotic and amelanotic melanoma metastasis has allowed one to identify the cellular phenotypes displayed by highly metastatic melanoma cells. The work reveals that, in addition to enhanced invasiveness, the capacity of melanoma cells to recruit endothelial cells is significantly enhanced in highly metastatic melanoma cells relative to poorly metastatic melanoma cells. Additionally, it was found that three major post-transcriptional regulators of metastasis strongly mediate endothelial recruitment. It was further found that the downstream signaling pathway modulated by these miRNAs also regulates endothelial recruitment. These findings reveal endothelial recruitment to be a defining feature of metastatic melanoma cells. Enhanced endothelial recruitment capacity was also recently found to be a defining feature of metastatic breast cancer, wherein suppression of metastasis by miR-126 was mediated through miRNA targeting of two distinct signaling pathways that promote endothelial recruitment (Png et al., 2012). In breast cancer, endothelial recruitment increased the likelihood of metastatic initiation rather than tumor growth. Similarly, the melanoma metastasis promoter miRNAs studied here dramatically enhanced metastatic colonization, without enhancing primary tumor growth, and increased the number of metastatic nodules—consistent with a role for these miRNAs and their regulatory network in metastatic initiation rather than tumor growth promotion. Taken together, these findings are consistent with endothelial recruitment into the metastatic niche acting as a promoter of metastatic initiation and colonization in these distinct epithelial cancer types. Such a non-canonical role for endothelial cells in cancer progression would contrast with the established role of endothelial cells in angiogenic enhancement of blood flow spurring enhanced tumor growth. Endothelial cells are known to play such non-canonical roles in development by supplying cues to neighboring cells during organogenesis (Lammert et al., 2001). Such cues have also been recently shown to promote organ regeneration (Ding et al., 2011; Ding et al., 2010; Kobayashi et al., 2010). Future work is needed to determine the metastasis stimulatory factors provided by endothelial cells that catalyze metastatic initiation.

The ability of miR-199a-3p, miR-199a-5p, and miR-1908 to individually predict metastasis-free survival in a cohort of melanoma patients indicates the significance of each miRNA as a clinical predictor of melanoma cancer progression. Importantly, the dramatic and highly significant capacity of the three miRNA aggregate signature (Figure 7D) to stratify patients at high risk from those at essentially no risk for metastatic relapse reveals both the cooperativity of these miRNAs, as well as their clinical potential as melanoma biomarkers (Sawyers, 2008) for identifying the subset of patients that might benefit from miRNA inhibition therapy. Therapeutic miRNA targeting has gained momentum through the use of in vivo LNAs that have been shown to antagonize miRNAs in mice (Elmer et al., 2008(b); Krützfeldt et al., 2005; Obad et al., 2011) and primates (Elmer et al., 2008(a)) and are currently being tested in human clinical trials. The powerful prognostic capacity of the three miRNAs, proof-of-principle demonstration of robust synergistic metastasis prevention achieved by treating highly metastatic melanoma cells with a cocktail of LNAs targeting miR-199a-3p, miR-199a-5p, and miR-1908 (Figure 7E), as well as the metastasis suppression effect of therapeutically delivered in vivo-optimized LNAs targeting these miRNAs (Figure 7J) motivate future clinical studies aimed at determining the therapeutic potential of combinatorially targeting these pro-metastatic and pro-angiogenic miRNAs in patients at high risk for melanoma metastasis—an outcome currently lacking effective chemotherapeutic options.

The foregoing examples and description of the preferred embodiments should be taken as illustrating, rather than as limiting the present invention. As will be readily appreciated, numerous variations and combinations of the features set forth above can be utilized without departing from the present invention. Such variations are not regarded as a departure from the scope of the invention, and all such variations are intended to be included within the scope of the present invention.

Claims:

1. Use of an LXRβ agonist to treat metastatic melanoma in a subject in need thereof, wherein the LXRβ agonist is:



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or a pharmaceutically acceptable salt thereof in an amount sufficient to suppress metastatic progression of said melanoma.

2. The use of claim 1, wherein said metastatic melanoma is resistant to dacarbazine, a BRAF inhibitor, a MEK inhibitor, a CTLA-4 inhibitor, a PD-1 inhibitor and/or a PD-L1 inhibitor.

3. The use of claim 2, wherein said metastatic melanoma is resistant to vemurafenib and/or dacarbazine.

4. Use of an LXRβ agonist or a pharmaceutically acceptable salt thereof, and a PD-1 inhibitor or PD-L1 inhibitor to treat drug resistant cancer in a subject in need thereof, wherein said LXRβ agonist is:

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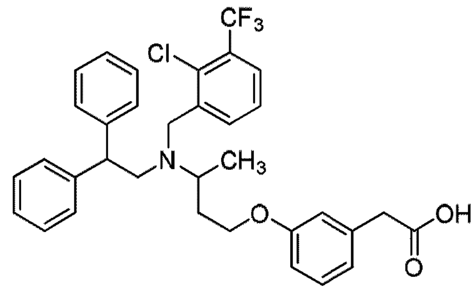

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5. The use of claim 4, wherein said drug resistant cancer is metastatic.

6. The use of claim 4, wherein said LXRβ agonist is in an amount sufficient to slow the spread of said drug resistant cancer.

7. The use of claim 4, wherein said drug resistant cancer is ovarian cancer.

8. The use of claim 4, wherein said drug resistant cancer is breast cancer.

9. The use of claim 8, wherein said breast cancer is triple negative breast cancer.

10. The use of claim 4, wherein said drug resistant cancer is non-small cell lung cancer.

11. The use of claim 4, comprising the LXRβ agonist, or the pharmaceutically acceptable salt thereof, and the PD-1 inhibitor, wherein the PD-1 inhibitor is nivolumab.

12. The use of claim 4, comprising the LXRβ agonist, or the pharmaceutically acceptable salt thereof, and the PD-L1 inhibitor, wherein the PD-L1 inhibitor is Atezolizumab (MPDL3280A).

13. The use of claim 4, comprising the use of the LXRβ agonist, or a pharmaceutically acceptable salt thereof and the PD-1 inhibitor within 28 days of each other.

14. The use of claim 4, comprising the use of the LXRβ agonist, or a pharmaceutically acceptable salt thereof and the PD-1 inhibitor within 21 days of each other.

15. The use of claim 4, comprising the use of the LXRβ agonist, or a pharmaceutically acceptable salt thereof and the PD-1 inhibitor within 14 days of each other.

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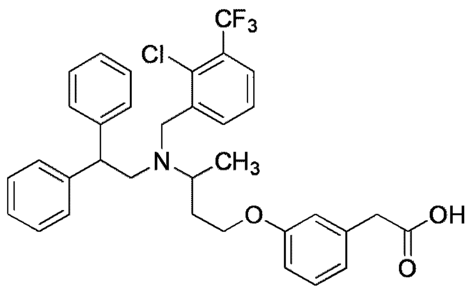
16. The use of claim 4, comprising the use of the LXRβ agonist, or a pharmaceutically acceptable salt thereof and the PD-1 inhibitor within 7 days of each other.

17. The use of claim 4, comprising the use of the LXRβ agonist, or a pharmaceutically acceptable salt thereof and the PD-1 inhibitor.

18. Use of an effective amount of LXRβ agonist, or a pharmaceutically acceptable salt thereof, and a PD-1 inhibitor, one or more of a PD-L1 inhibitor, and a CTLA-4 inhibitor to treat cancer in a subject in need thereof, wherein the cancer is resistant to the PD-1 inhibitor, the PD-L1 inhibitor, and/or the CTLA-4 inhibitor, wherein the LXRβ agonist is:



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or a pharmaceutically acceptable salt thereof,

wherein the cancer is ovarian cancer, breast cancer, lung cancer, glioblastoma, melanoma, bladder cancer, head and neck cancer, renal cell cancer, colorectal cancer, biliary tract cancer, bladder cancer, brain cancer, cervical cancer, choriocarcinoma, endometrial cancer, esophageal cancer, gastric cancer, hematological neoplasms, intraepithelial neoplasms, liver cancer, lymphomas, neuroblastomas, oral cancer, pancreatic cancer, prostate cancer, sarcoma, basocellular cancer, squamous cell cancer, testicular cancer, stromal tumors, germ cell tumors, thyroid cancer, renal cancer, endometrial cancer, lymphoma, leukemia, multiple myeloma, or hepatocellular carcinoma.

19. The use of claim 18, wherein the cancer is breast cancer, ovarian cancer, lung cancer, glioblastoma, endometrial cancer, or melanoma.

20. The use of claim 19, wherein the cancer is ovarian cancer.

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21. The use of claim 19, wherein the cancer is breast cancer.

22. The use of claim 21, wherein the breast cancer is triple negative breast cancer.

23. The use of claim 19, wherein the cancer is lung cancer.

24. The use of claim 23, wherein the lung cancer is non-small cell lung cancer.

25. The use of claim 19, wherein the cancer is glioblastoma.

26. The use of claim 19, wherein the cancer is melanoma.

27. The use of claim 26, wherein the melanoma is also resistant to dacarbazine, a BRAF inhibitor, and/or a MEK inhibitor.

28. The use of claim 18, wherein the cancer is bladder cancer.

29. The use of claim 18, wherein the cancer is head and neck cancer.

30. The use of claim 18, wherein the cancer is renal cell cancer.

31. The use of claim 18, wherein the cancer is colorectal cancer.

32. The use of claim 18, wherein the cancer is lymphoma.

33. The use of claim 18, wherein the cancer is leukemia.

34. The use of claim 18, wherein the cancer is multiple myeloma.

35. The use of claim 18, wherein the cancer is hepatocellular carcinoma.

36. The use of claim 18, wherein the cancer is metastatic.

37. The use of claim 18, comprising the use of the LXRβ agonist, or a pharmaceutically acceptable salt thereof, and the PD-1 inhibitor or PD-L1 inhibitor or CTLA-4 inhibitor within 28 days of each other.

38. Use of an LXRβ agonist to treat metastatic cancer in a subject in need thereof, wherein the LXRβ agonist is:

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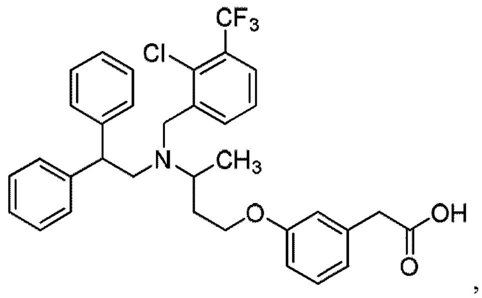

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or a pharmaceutically acceptable salt thereof, in an amount sufficient to suppress progression of the metastatic cancer.

39. The use of claim 38, wherein the metastatic cancer is breast cancer, lung cancer, bladder cancer, head and neck cancer, renal cell cancer, colorectal cancer, lymphoma, hepatocellular carcinoma, biliary tract cancer, bladder cancer, brain cancer, cervical cancer, choriocarcinoma, endometrial cancer, esophageal cancer, gastric cancer, hematological neoplasms, intraepithelial neoplasms, liver cancer, lymphomas, neuroblastomas, oral cancer, pancreatic cancer, prostate cancer, sarcoma, basocellular cancer, squamous cell cancer, testicular cancer, stromal tumors, germ cell tumors, thyroid cancer, renal cancer, endometrial cancer, or prostate cancer.

40. The use of claim 39, wherein the metastatic cancer is breast cancer.

41. The use of claim 40, wherein the breast cancer is triple negative breast cancer.

42. The use of claim 39, wherein the metastatic cancer is lung cancer.

43. The use of claim 42, wherein the lung cancer is non-small cell lung cancer.

44. The use of claim 42, wherein the lung cancer is small cell lung cancer.

45. The use of claim 39, wherein the metastatic cancer is bladder cancer.

46. The use of claim 39, wherein the metastatic cancer is head and neck cancer.

47. The use of claim 39, wherein the metastatic cancer is renal cell cancer.

48. The use of claim 39, wherein the metastatic cancer is colorectal cancer.

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49. The use of claim 39, wherein the metastatic cancer is lymphoma.

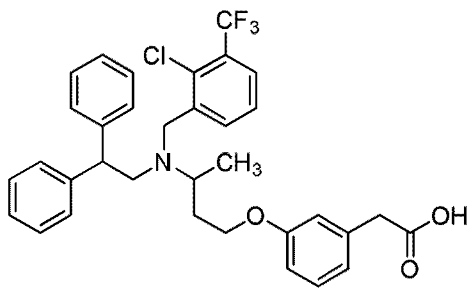
50. The use of claim 39, wherein the metastatic cancer is hepatocellular carcinoma.

51. The use of claim 39, wherein the metastatic cancer is prostate cancer.

52. The use of claim 39, wherein the metastatic cancer is drug resistant.

53. The use of claim 39, wherein the amount of compound 25 or pharmaceutically acceptable salt thereof is sufficient for reducing or stopping the formation of new metastatic tumors and/or reducing, stopping, or reversing the load of a metastatic tumor.

54. Use of an LXRβ agonist to treat ovarian cancer in a subject in need thereof, wherein the LXRβ agonist is:



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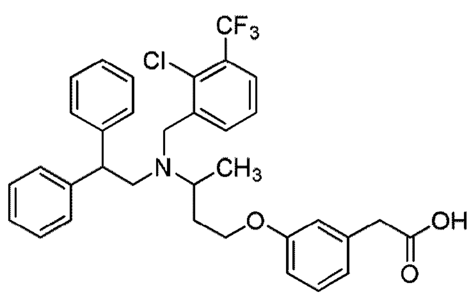
or a pharmaceutically acceptable salt thereof in an amount sufficient to suppress the invasion of surrounding tissue by the ovarian cancer and/or suppress metastatic colonization of the ovarian cancer.

55. Use of an LXRβ agonist to treat small cell carcinoma in a subject in need thereof, wherein the LXRβ agonist is:



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or a pharmaceutically acceptable salt thereof in an amount sufficient to suppress the invasion of surrounding tissue by the small cell carcinoma and/or suppress metastatic colonization of the small cell carcinoma, wherein the small cell carcinoma is lung cancer or prostate cancer.

56. The use of claim 55, wherein the small cell carcinoma is lung cancer.

57. The use of any one of claims 1-3, wherein said LXRβ agonist is in an amount sufficient to increase the expression level or activity level of ApoE to a level sufficient to slow the spread of metastasis of said metastatic melanoma.

58. The use any one of claims 1-57, wherein the use comprises suppression of metastatic colonization by compound 25 or its pharmaceutically acceptable salt.

59. The use of claim 58, wherein the suppression comprises suppression of metastatic colonization to the lung and/or the brain.

60. The use of any one of claims 1-59, wherein the use comprises suppression of invasion of surrounding tissue.

61. The use of any one of claims 1-3, wherein the metastatic melanoma progressed on or after treatment with platinum-containing chemotherapy, a PD-1 inhibitor, a PD-L1 inhibitor, a CTLA-4 inhibitor, an IDO inhibitor, an anti-hormonal therapy, an antimitotic agent, an angiogenesis inhibitor, an IDO inhibitor, an alkylating agent, a topoisomerase inhibitor, an antimetabolite, and/or a kinase inhibitor.

62. The use of any one of claims 1-3, and 38 to 56, further comprising an additional anticancer therapy.

63. The use of claim 62, wherein the additional anticancer therapy is selected from the group consisting of a platinum-containing chemotherapy, an immunomodulator, an anti-hormonal therapy, a topoisomerase inhibitor, an antimetabolite, an alkylating agent, an antimitotic agent, an angiogenesis inhibitor, an antiproliferative and a kinase inhibitor.

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64. The use of claim 63, wherein said antiproliferative is a PD1 inhibitor, a VEGF inhibitor, a VEGFR2 inhibitor, a CTLA4 inhibitor, and/or a PDL1 inhibitor.

65. The use of claim 64, wherein said antiproliferative is nivolumab, ipilmumab, dacarbazine, or vemurafenib.

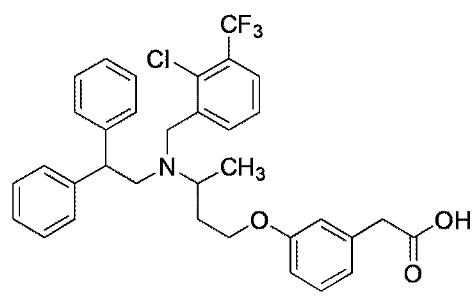
66. The use of claim 63, wherein the antimetabolite is gemcitabine.

67. The use of claim 63, wherein the platinum-containing chemotherapy comprises oxaliplatin, carboplatin, or cisplatin, the immunomodulator is a PD-1 inhibitor, a CTLA4 inhibitor, an IDO inhibitor, and/or a PDL1 inhibitor, the kinase inhibitor is erlotinib or trastuzumab, the angiogenesis inhibitor is bevacizumab, and/or the antimitotic agent is paclitaxel or docetaxel.

68. The use of claim 63, wherein the immunomodulator is a PD-1 inhibitor, a CTLA4 inhibitor, and/or a PDL1 inhibitor.

69. The use of any one of claims 62-68, wherein compound 25 or its pharmaceutically acceptable salt is not formulated for concurrent administration with the additional anticancer therapy.

70. Use of an LXRβ agonist to treat cancer in a subject in need thereof, wherein the LXRβ agonist is:



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or a pharmaceutically acceptable salt thereof, wherein the subject or the cancer has decreased expression of ApoE compared to a reference.

71. The use of claim 42, wherein the lung cancer is resistant to PD-1 inhibitor.

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72. The use of claim 42, further comprising use of a PD-1 inhibitor, a PD-L1 inhibitor, or a CTLA-4 inhibitor.

73. The use of claim 72, wherein the lung cancer is NSCLC.

74. The use of claim 56, wherein the small cell carcinoma is metastatic.

75. The use of claim 56, wherein the small cell carcinoma is resistant to PD-1 inhibitor.

76. The use of claim 56, further comprising use of a PD-1 inhibitor, a PD-L1 inhibitor, or a CTLA-4 inhibitor.

77. The use of claim 4, wherein said drug resistant cancer is selected from the group consisting of lung cancer, breast cancer, ovarian cancer, colorectal cancer, biliary tract cancer, bladder cancer, brain cancer, cervical cancer, choriocarcinoma, endometrial cancer, esophageal cancer, gastric cancer, hematological neoplasms, multiple myeloma, leukemia, intraepithelial neoplasms, liver cancer, lymphomas, neuroblastomas, oral cancer, pancreatic cancer, prostate cancer, sarcoma, melanoma, basocellular cancer, squamous cell cancer, testicular cancer, stromal tumors, germ cell tumors, thyroid cancer, and renal cancer.

78. The use of claim 4, wherein said drug resistant cancer is endometrial cancer.

79. The use of claim 19, wherein the cancer is endometrial cancer.

80. The use of claim 39, wherein the metastatic cancer is endometrial cancer.

81. The use of claim 70, wherein the cancer is selected from the group consisting of lung cancer, breast cancer, ovarian cancer, colorectal cancer, biliary tract cancer, bladder cancer, brain cancer, cervical cancer, choriocarcinoma, endometrial cancer, esophageal cancer, gastric cancer, hematological neoplasms, multiple myeloma, leukemia, intraepithelial neoplasms, liver cancer, lymphomas, neuroblastomas, oral cancer, pancreatic cancer, prostate cancer, sarcoma, melanoma, basocellular cancer, squamous cell cancer, testicular cancer, stromal tumors, germ cell tumors, thyroid cancer, and renal cancer.

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82. The use of claim 18, comprising the LXRβ agonist, or a pharmaceutically acceptable salt thereof, and the CTLA-4 inhibitor, wherein the cancer is endometrial cancer.

83. The use of any one of claims 18-36 and 82, wherein the CTLA-4 inhibitor is ipilimumab.

84. The use of any one of claims 18-36 and 82-83, wherein the cancer is resistant to a PD-1 inhibitor and/or a PD-L1 inhibitor.

85. The use of any one of claims 4 to 17, wherein said LXRβ agonist is in an amount sufficient to increase the expression level or activity level of ApoE to a level sufficient to slow the spread of the metastasis of said drug resistant cancer.

86. The use of any one of claims 18 to 37, wherein said LXRβ agonist is in an amount sufficient to increase the expression level or activity level of ApoE to a level sufficient to slow the spread of the metastasis of said cancer or said endometrial cancer.

87. The use of any one of claims 38 to 40, 42, and 45 to 53, wherein said LXRβ agonist is in an amount sufficient to increase the expression level or activity level of ApoE to a level sufficient to slow the spread of the metastasis of said metastatic cancer.

88. The use of claim 41, wherein said LXRβ agonist is in an amount sufficient to increase the expression level or activity level of ApoE to a level sufficient to slow the spread of the metastasis of said breast cancer.

89. The use of any one of claims 43 to 44, wherein said LXRβ agonist is in an amount sufficient to increase the expression level or activity level of ApoE to a level sufficient to slow the spread of the metastasis of said lung cancer.

90. The use of claim 54, wherein said LXRβ agonist is in an amount sufficient to increase the expression level or activity level of ApoE to a level sufficient to slow the spread of the metastasis of said ovarian cancer.

91. The use of any one of claims 55 to 56, wherein said LXRβ agonist is in an amount sufficient to increase the expression level or activity level of ApoE to a level sufficient to slow the spread of the metastasis of said small cell carcinoma.

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92. The use of any one of claims 4-17, wherein the drug resistant cancer progressed on or after treatment with platinum-containing chemotherapy, a PD-1 inhibitor, a PD-L1 inhibitor, a CTLA-4 inhibitor, an IDO inhibitor, an anti-hormonal therapy, an antimitotic agent, an angiogenesis inhibitor, an IDO inhibitor, an alkylating agent, a topoisomerase inhibitor, an antimetabolite, and/or a kinase inhibitor.

93. The use of any one of claims 18-37, wherein the cancer or the endometrial cancer progressed on or after treatment with platinum-containing chemotherapy, a PD-1 inhibitor, a PD-L1 inhibitor, a CTLA-4 inhibitor, an IDO inhibitor, an anti-hormonal therapy, an antimitotic agent, an angiogenesis inhibitor, an IDO inhibitor, an alkylating agent, a topoisomerase inhibitor, an antimetabolite, and/or a kinase inhibitor.

94. The use of any one of claims 38 to 40, 42 and 45 to 53, wherein the metastatic cancer progressed on or after treatment with platinum-containing chemotherapy, a PD-1 inhibitor, a PD-L1 inhibitor, a CTLA-4 inhibitor, an IDO inhibitor, an anti-hormonal therapy, an antimitotic agent, an angiogenesis inhibitor, an IDO inhibitor, an alkylating agent, a topoisomerase inhibitor, an antimetabolite, and/or a kinase inhibitor.

95. The use of claim 41, wherein the breast cancer progressed on or after treatment with platinum-containing chemotherapy, a PD-1 inhibitor, a PD-L1 inhibitor, a CTLA-4 inhibitor, an IDO inhibitor, an anti-hormonal therapy, an antimitotic agent, an angiogenesis inhibitor, an IDO inhibitor, an alkylating agent, a topoisomerase inhibitor, an antimetabolite, and/or a kinase inhibitor.

96. The use of any one of claims 43 to 44, wherein the lung cancer progressed on or after treatment with platinum-containing chemotherapy, a PD-1 inhibitor, a PD-L1 inhibitor, a CTLA-4 inhibitor, an IDO inhibitor, an anti-hormonal therapy, an antimitotic agent, an angiogenesis inhibitor, an IDO inhibitor, an alkylating agent, a topoisomerase inhibitor, an antimetabolite, and/or a kinase inhibitor.

97. The use of claim 54, wherein the ovarian cancer progressed on or after treatment with platinum-containing chemotherapy, a PD-1 inhibitor, a PD-L1 inhibitor, a CTLA-4 inhibitor, an IDO inhibitor, an anti-hormonal therapy, an antimitotic agent, an angiogenesis inhibitor, an IDO inhibitor, an alkylating agent, a topoisomerase inhibitor, an antimetabolite, and/or a kinase inhibitor.

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98. The use of any one of claims 55 to 56, wherein the small cell carcinoma progressed on or after treatment with platinum-containing chemotherapy, a PD-1 inhibitor, a PD-L1 inhibitor, a CTLA-4 inhibitor, an IDO inhibitor, an anti-hormonal therapy, an antimitotic agent, an angiogenesis inhibitor, an IDO inhibitor, an alkylating agent, a topoisomerase inhibitor, an antimetabolite, and/or a kinase inhibitor.

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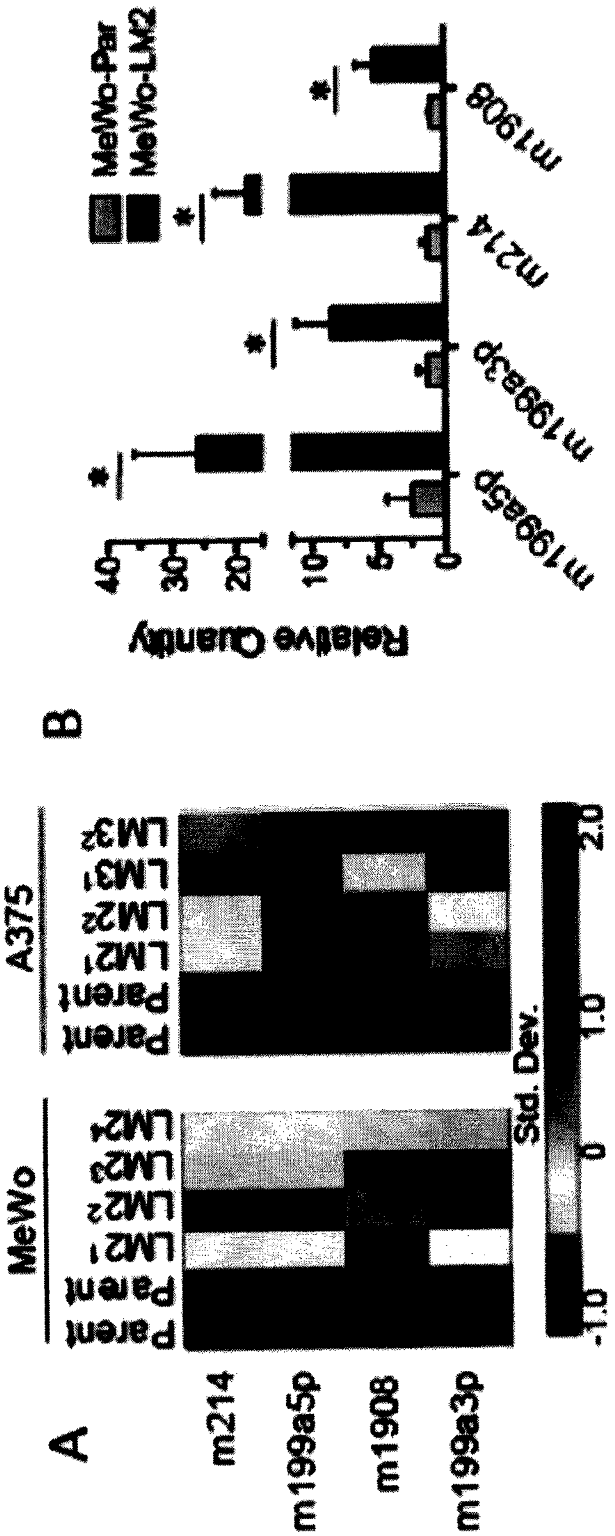
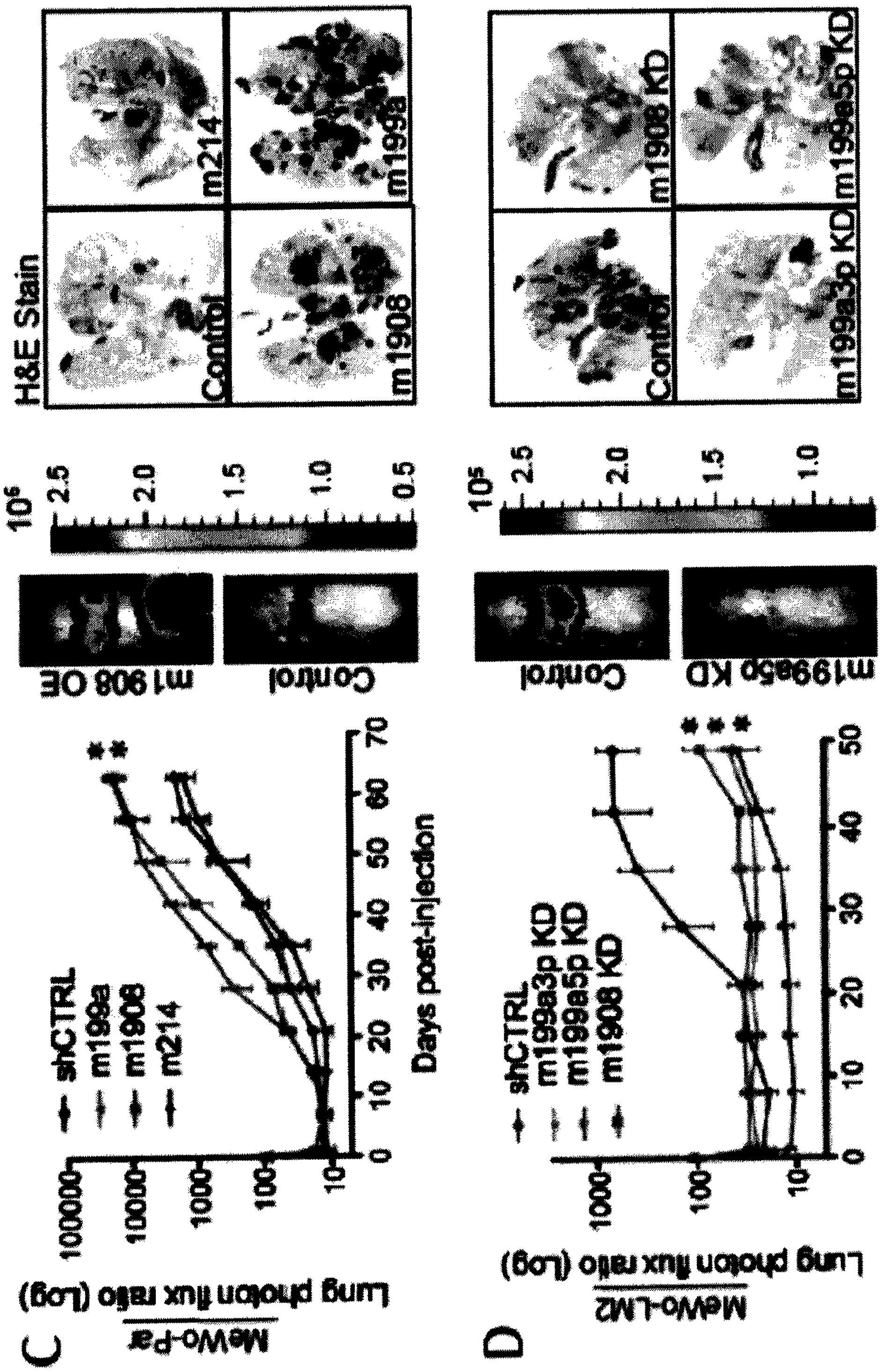
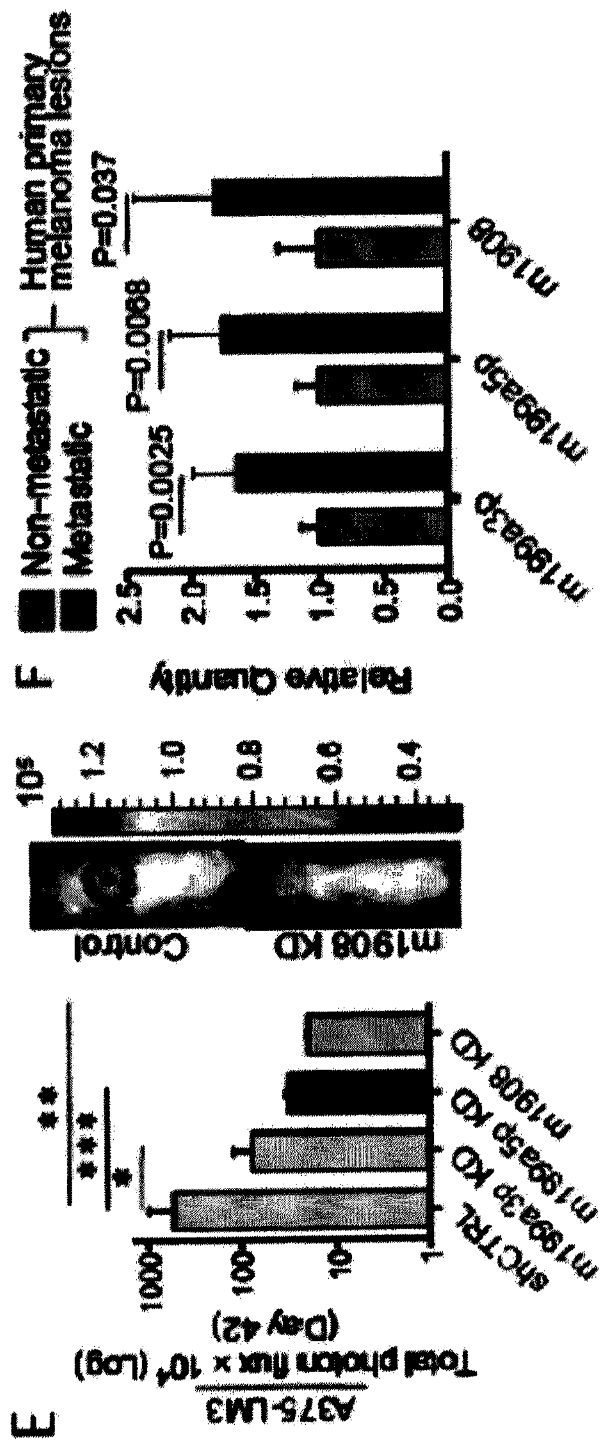

A
MeWo
A375
Parent
Parent
LM2¹
LM2²
LM2³
LM2⁴
Parent
Parent
LM2¹
LM2²
LM3¹
LM3²
m214
m199a5p
m1908
m199a3p
Std. Dev.
-1.0
0
1.0
2.0
B
Relative Quantity
MeWo-Par
MeWo-LM2
m199a5p
m199a3p
m214
m1908
40
30
20
10
5
0
*

FIGURES 1A-B

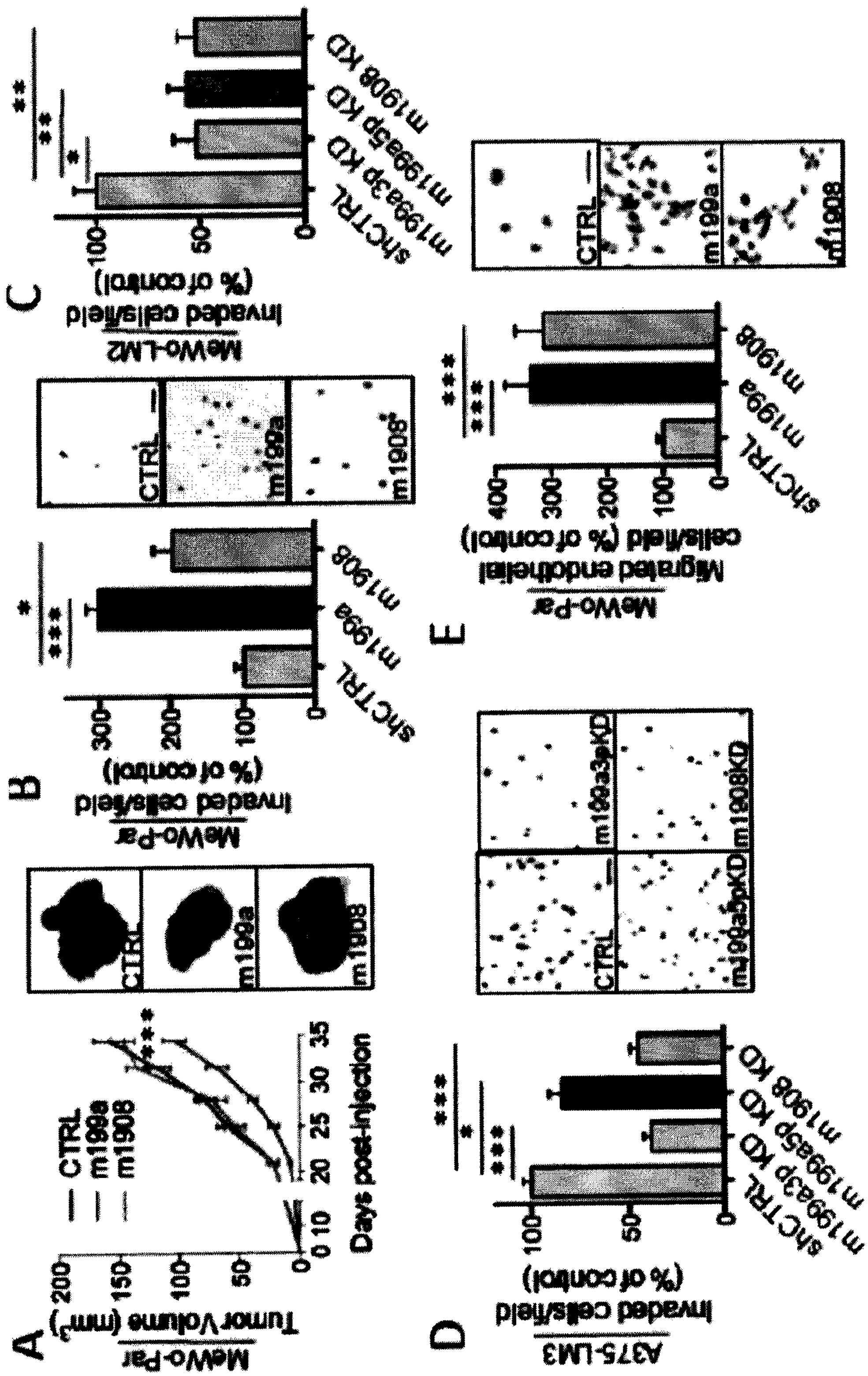

C
MeWo-Par
Lung photon flux ratio (Log)
100000
10000
1000
100
10
shCTRL
m199a
m1908
m214
Days post-injection
0
10
20
30
40
50
60
70
m1908 OE
Control
10⁶
2.5
2.0
1.5
1.0
0.5
H&E Stain
Control
m214
m1908
m199a
D
MeWo-LM2
Lung photon flux ratio (Log)
1000
100
10
shCTRL
m199a3p KD
m199a5p KD
m1908 KD
Days post-injection
0
10
20
30
40
50
Control
m199a5p KD
10⁵
2.5
2.0
1.5
1.0
Control
m1908 KD
m199a3p KD
m199a5p KD

FIGURES 1C-D

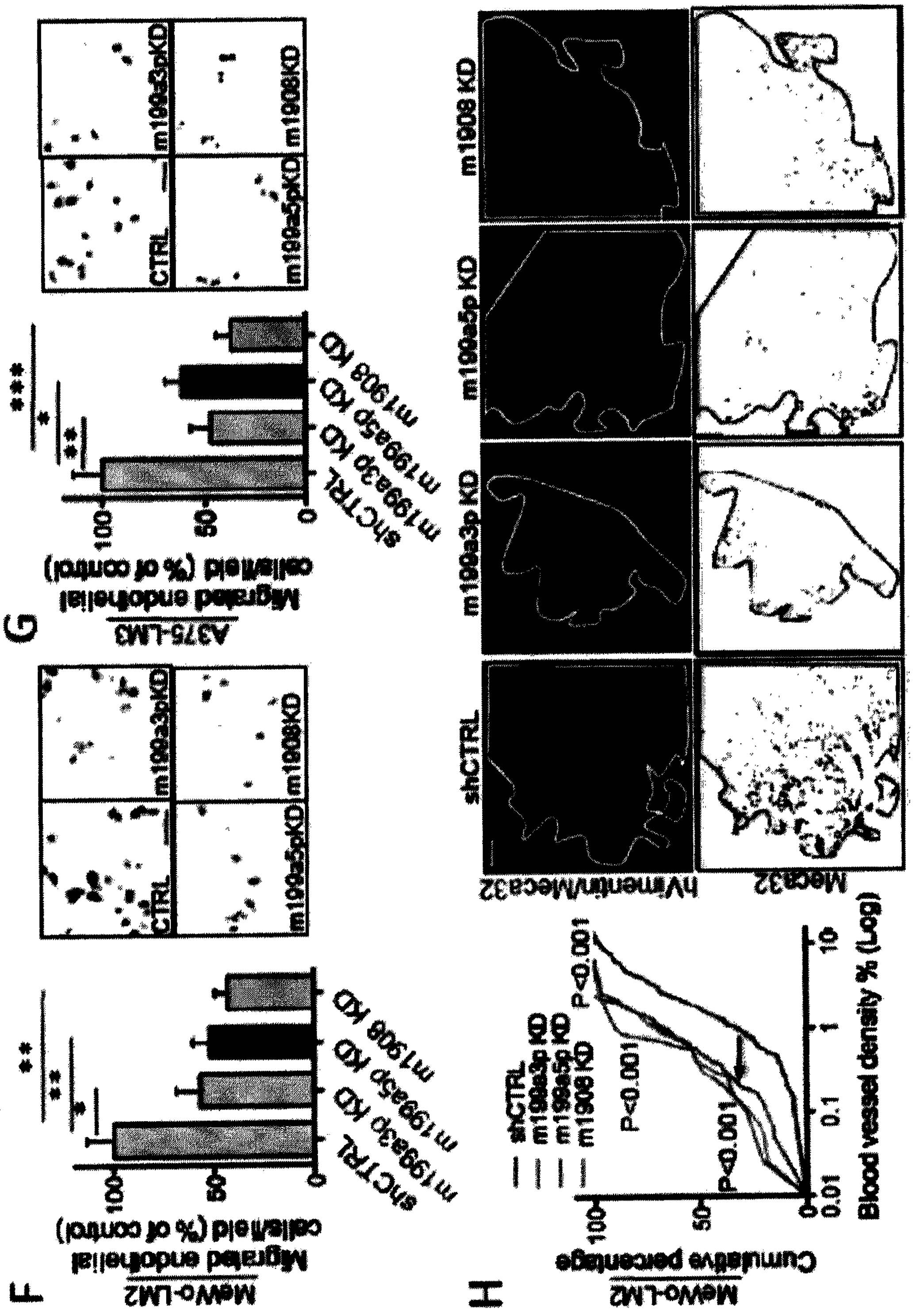


E
A375-LM3
Total photon flux x 10⁴ (Log)
(Day 42)
1000
100
10
1
shCTRL
m1998a3p KD
m1998a5p KD
m1908 KD
*
***
**
Control
m1908 KD
10⁵
1.2
1.0
0.8
0.6
0.4
F
Non-metastatic
Metastatic
Human primary melanoma lesions
Relative Quantity
2.5
2.0
1.5
1.0
0.5
0.0
P=0.0025
P=0.0068
P=0.037
m1998a3p
m1998a5p
m1908

FIGURES 1E-F

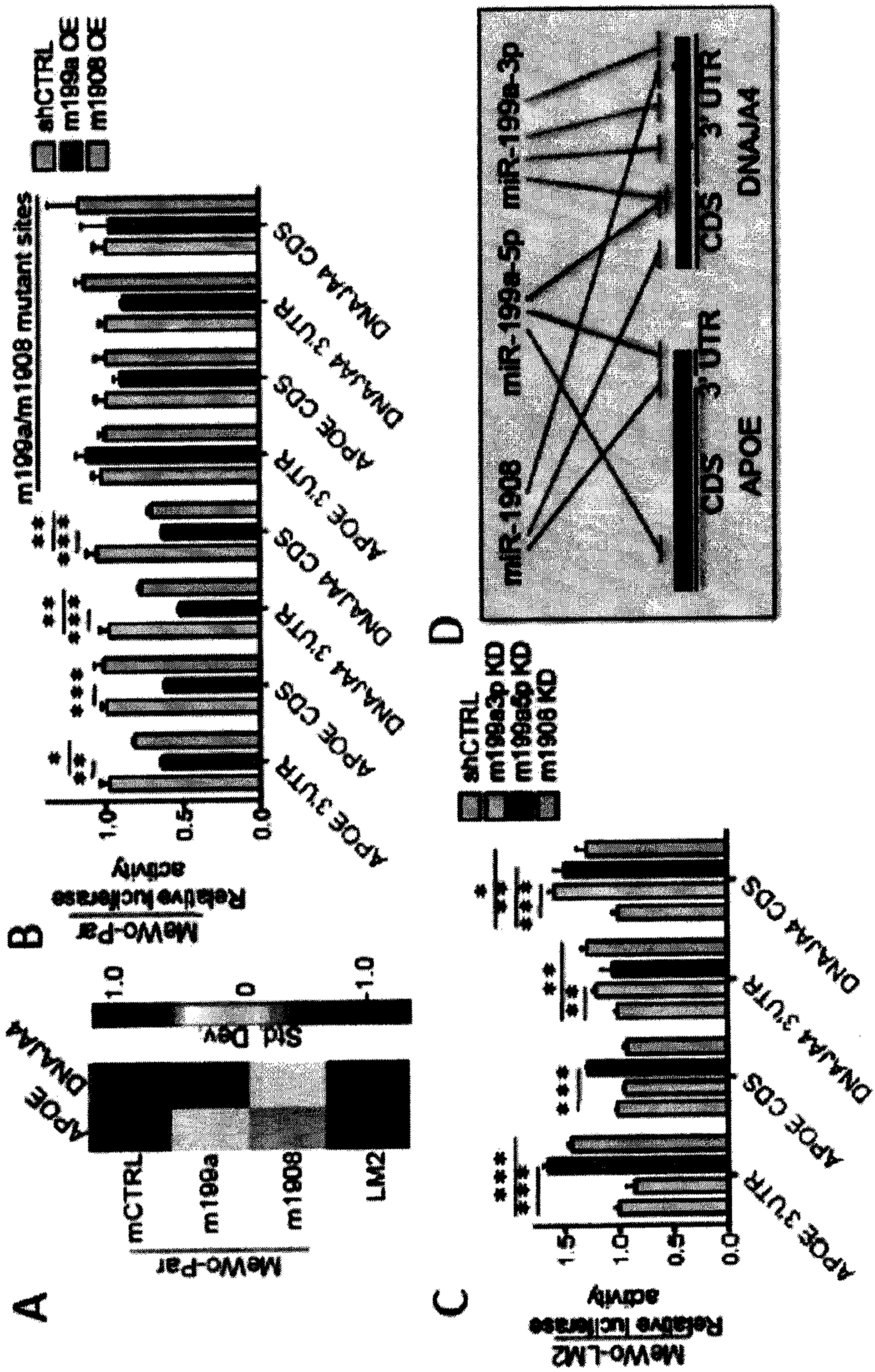

A
MeWo-Par
Tumor Volume (mm3)
Days post-injection
CTRL
m199a
m1908
B
MeWo-Par
Invaded cells/field (% of control)
shCTRL
m199a
m1908
C
MeWo-LM2
Invaded cells/field (% of control)
shCTRL
m199a3p KD
m199a5p KD
m1908 KD
D
A375-LM3
Invaded cells/field (% of control)
shCTRL
m199a3p KD
m199a5p KD
m1908 KD
E
MeWo-Par
Migrated endothelial cells/field (% of control)
shCTRL
m199a
m1908

FIGURES 2A-E

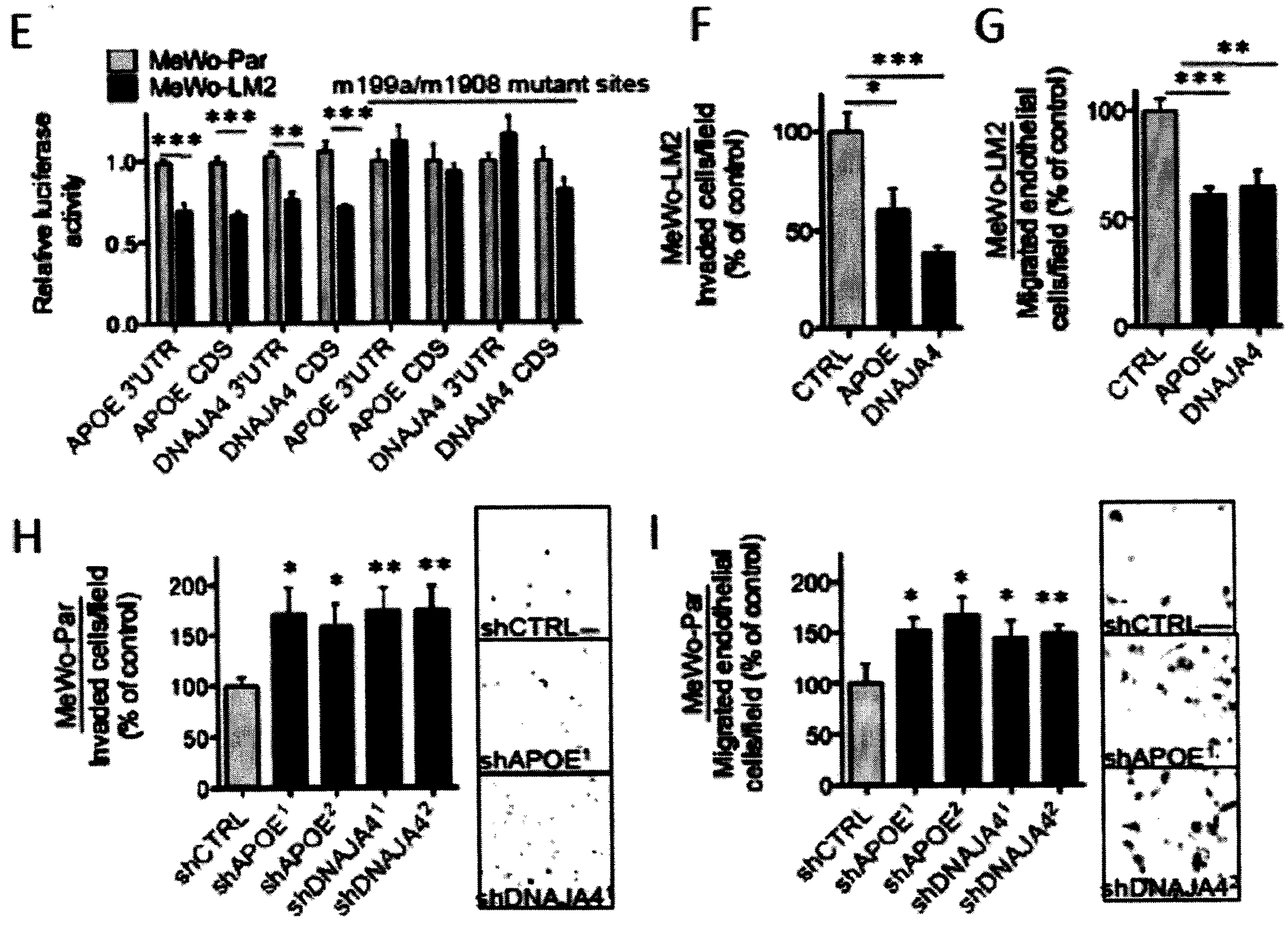

F
MeWo-LM2
Migrated endothelial cells/field (% of control)
shCTRL
m199a3p KD
m199a5p KD
m1908 KD
CTRL
m199a3pKD
m199a5pKD
m1908KD
G
A375-LM3
H
Cumulative percentage
Blood vessel density % (Log)
P<0.001
hVimentin/Meca32
Meca32

FIGURES 2F - H

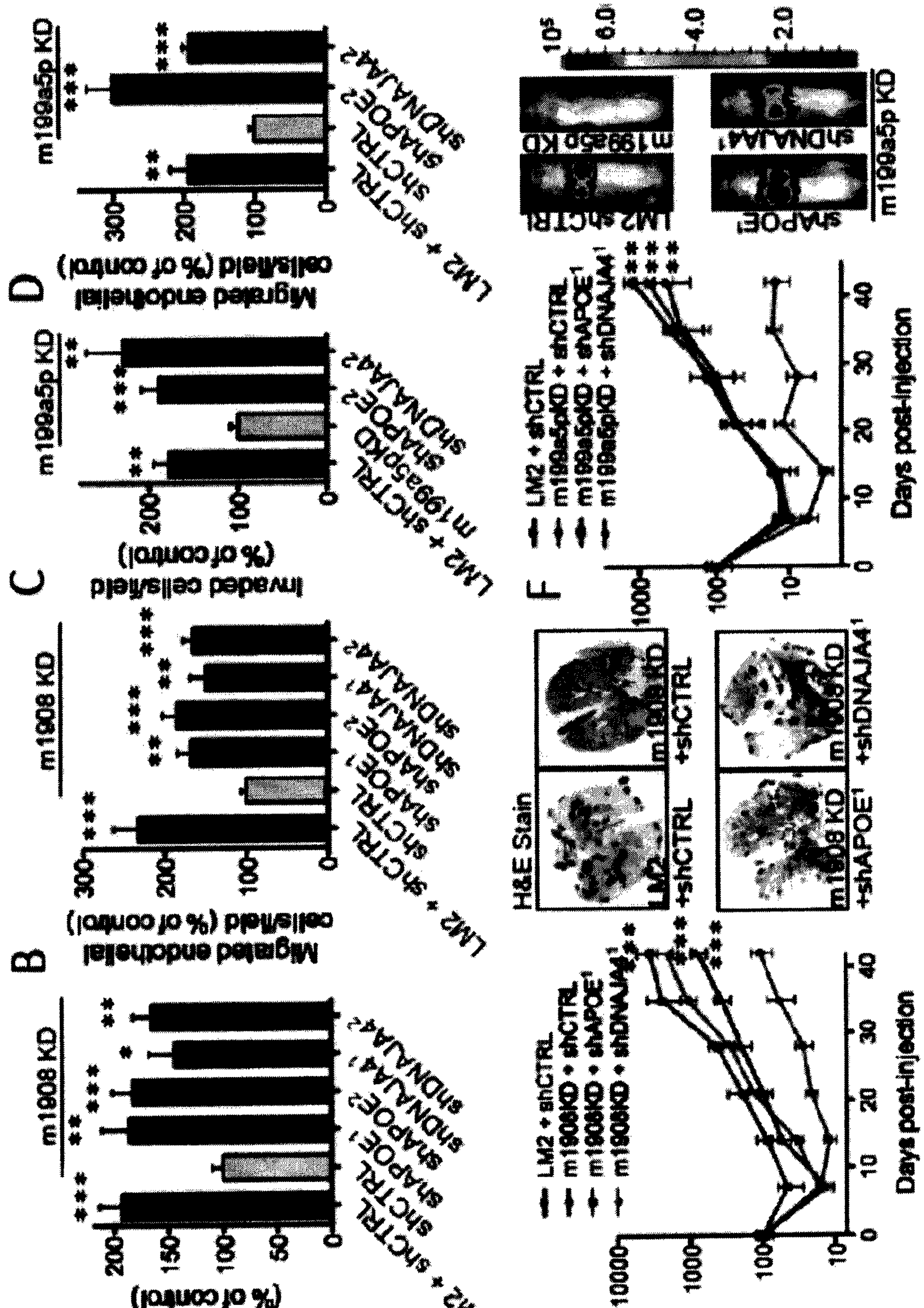
Figure 3



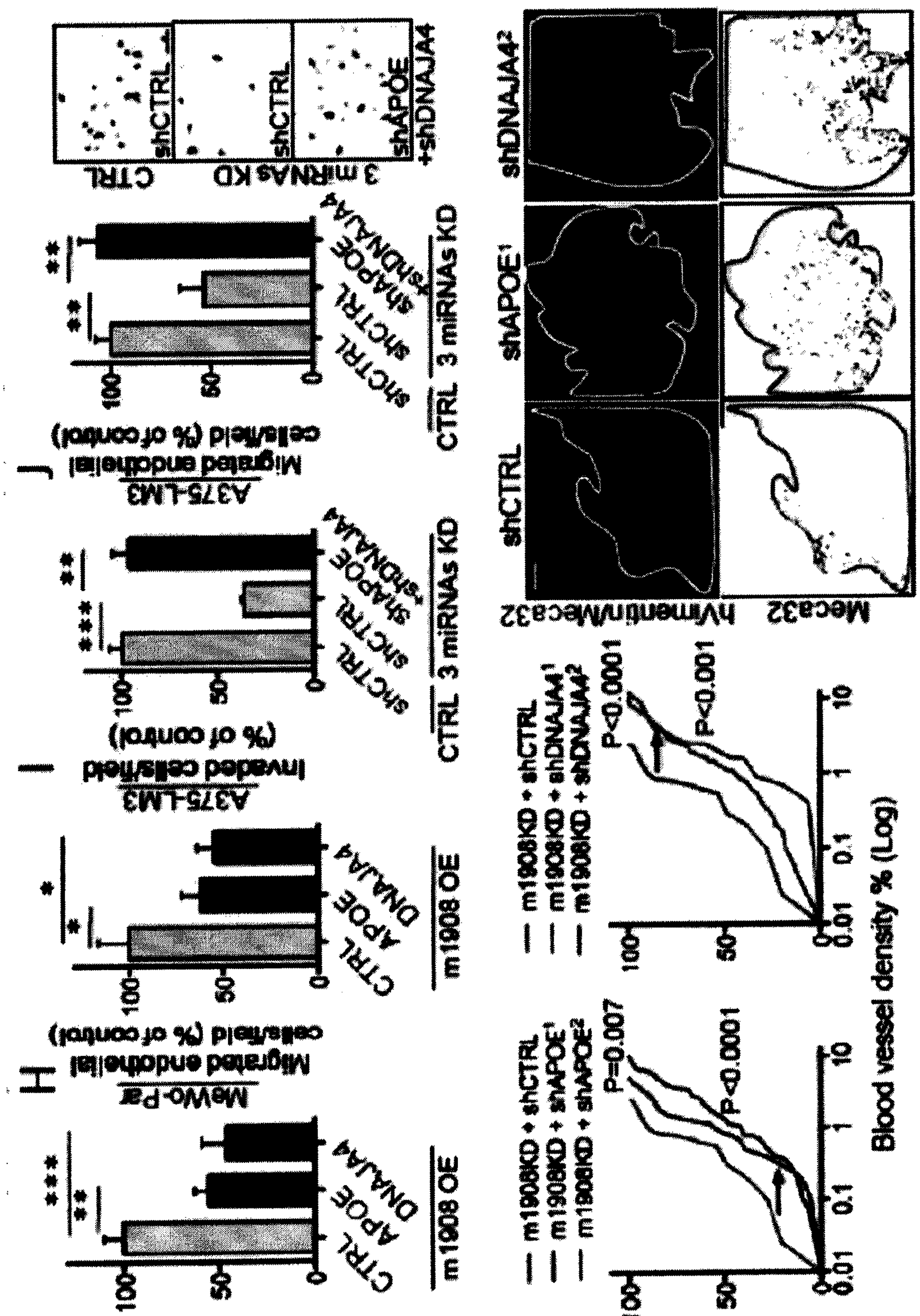
FIGURES 3A-D


E
MeWo-Par
MeWo-LM2
m199a/m1908 mutant sites
Relative luciferase activity
1.0
0.5
0.0
APOE 3'UTR
APOE CDS
DNAJA4 3'UTR
DNAJA4 CDS
F
MeWo-LM2
Invaded cells/field (% of control)
100
50
0
CTRL
APOE
DNAJA4
G
MeWo-LM2
Migrated endothelial cells/field (% of control)
H
MeWo-Par
Invaded cells/field (% of control)
200
150
shCTRL
shAPOE1
shAPOE2
shDNAJA41
shDNAJA42
shDNAJA41
I
MeWo-Par
Migrated endothelial cells/field (% of control)
shDNAJA42

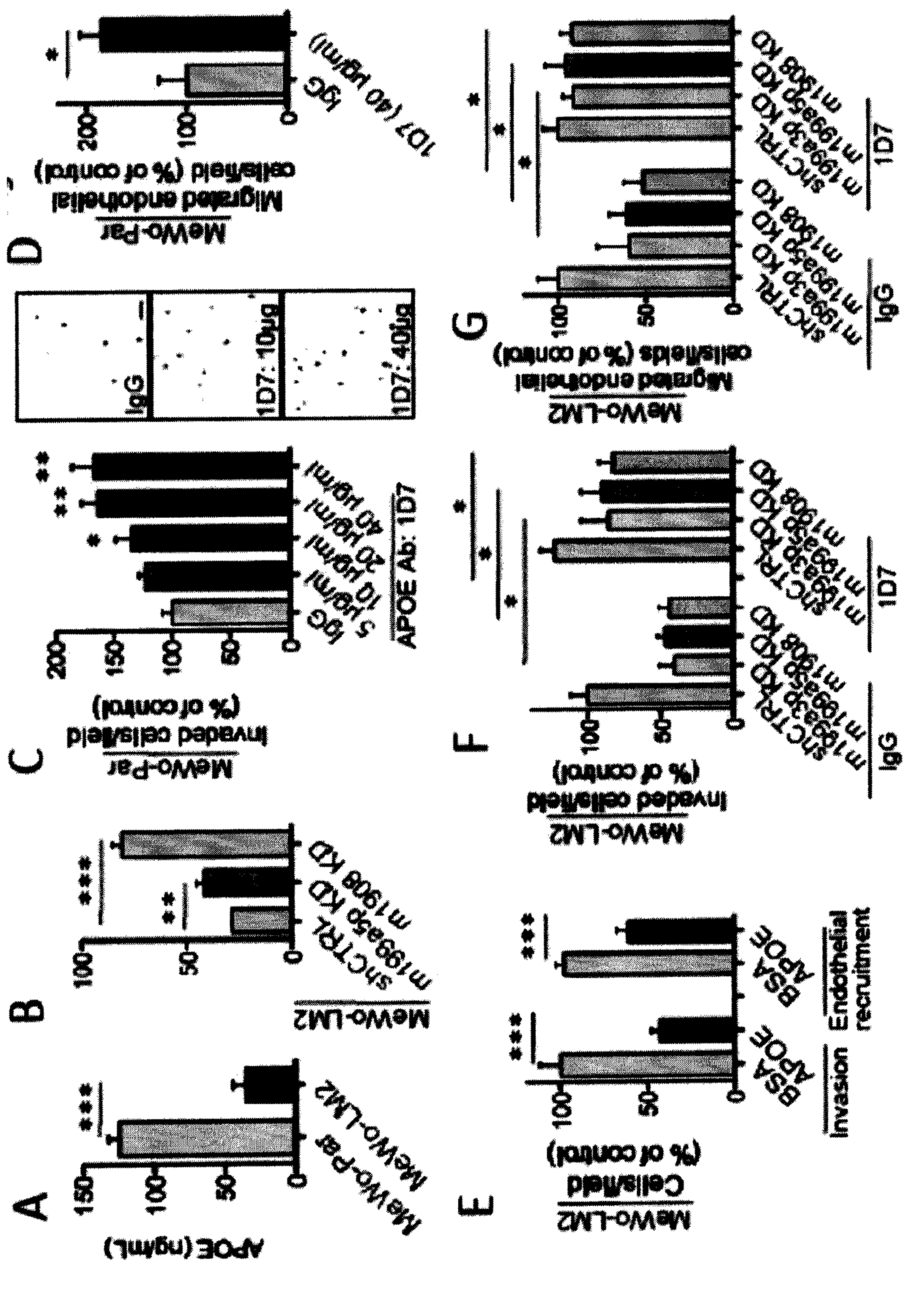
FIGURES 3E-I


A
B
C
D
E
F
MeWo-LM2
Invaded cells/field (% of control)
Migrated endothelial cells/field (% of control)
m1908 KD
m199a5p KD
LM2 + shCTRL
shCTRL
shAPOE1
shAPOE2
shDNAJA41
shDNAJA42
m199a5pKD
Lung photon flux ratio (Log)
Days post-injection
LM2 + shCTRL
m1908KD + shCTRL
m1908KD + shAPOE1
m1908KD + shDNAJA41
m199a5pKD + shCTRL
m199a5pKD + shAPOE1
m199a5pKD + shDNAJA41
H&E Stain
LM2 +shCTRL
m1908 KD +shCTRL
m1908 KD +shAPOE1
m1908 KD +shDNAJA41
LM2 shCTRL
m199a5p KD
shAPOE1
shDNAJA41

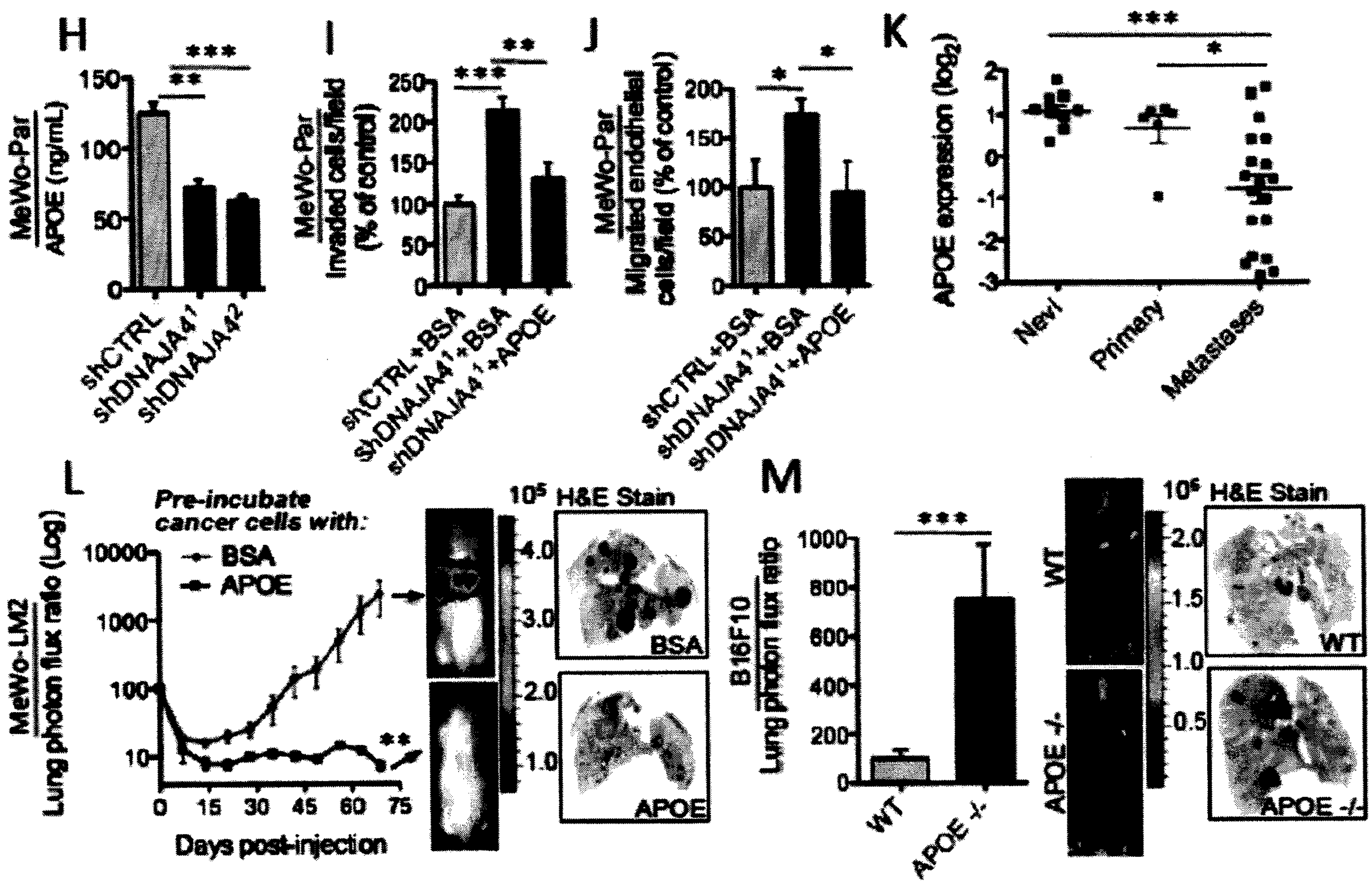
FIGURES 4A-F


G
MeWo-Par
Invaded cells/field (% of control)
CTRL
APOE
DNAJA4
m1908 OE
H
MeWo-Par
Migrated endothelial cells/field (% of control)
I
A375-LM3
Invaded cells/field (% of control)
shCTRL
shAPOE
+shDNAJA4
CTRL
3 miRNAs KD
J
A375-LM3
Migrated endothelial cells/field (% of control)
K
MeWo-LM2
Cumulative percentage
m1908KD + shCTRL
m1908KD + shAPOE¹
m1908KD + shAPOE²
P=0.007
P<0.0001
m1908KD + shDNAJA4¹
m1908KD + shDNAJA4²
P<0.0001
P<0.001
Blood vessel density % (Log)
hVimentin/Meca32
Meca32
shDNAJA4²

FIGURES 4G-K


A
APOE (ng/mL)
MeWo-Par
MeWo-LM2
B
MeWo-LM2
shCTRL
m199a5p KD
m1908 KD
C
MeWo-Par
Invaded cells/field (% of control)
IgG
5 µg/ml
10 µg/ml
20 µg/ml
40 µg/ml
APOE Ab: 1D7
IgG
1D7: 10µg
1D7: 40µg
D
MeWo-Par
Migrated endothelial cells/field (% of control)
IgG
1D7 (40 µg/ml)
E
MeWo-LM2
Cells/field (% of control)
BSA
APOE
Invasion
Endothelial recruitment
F
MeWo-LM2
Invaded cells/field (% of control)
shCTRL
m199a3p KD
m199a5p KD
m1908 KD
IgG
1D7
G
MeWo-LM2
Migrated endothelial cells/fields (% of control)
IgG
1D7

FIGURES 5A-G



H
MeWo-Par
APOE (ng/mL)
shCTRL
shDNAJA4[1]
shDNAJA4[2]
I
MeWo-Par
Invaded cells/field (% of control)
shCTRL+BSA
shDNAJA4[1]+BSA
shDNAJA4[1]+APOE
J
MeWo-Par
Migrated endothelial cells/field (% of control)
shCTRL+BSA
shDNAJA4[1]+BSA
shDNAJA4[1]+APOE
K
APOE expression (log_2)
Nevi
Primary
Metastases
L
MeWo-LM2
Lung photon flux ratio (Log)
Pre-incubate cancer cells with:
BSA
APOE
Days post-injection
10^5 H&E Stain
BSA
APOE
M
B16F10
Lung photon flux ratio
WT
APOE -/-
10^6 H&E Stain

FIGURES 5H-M

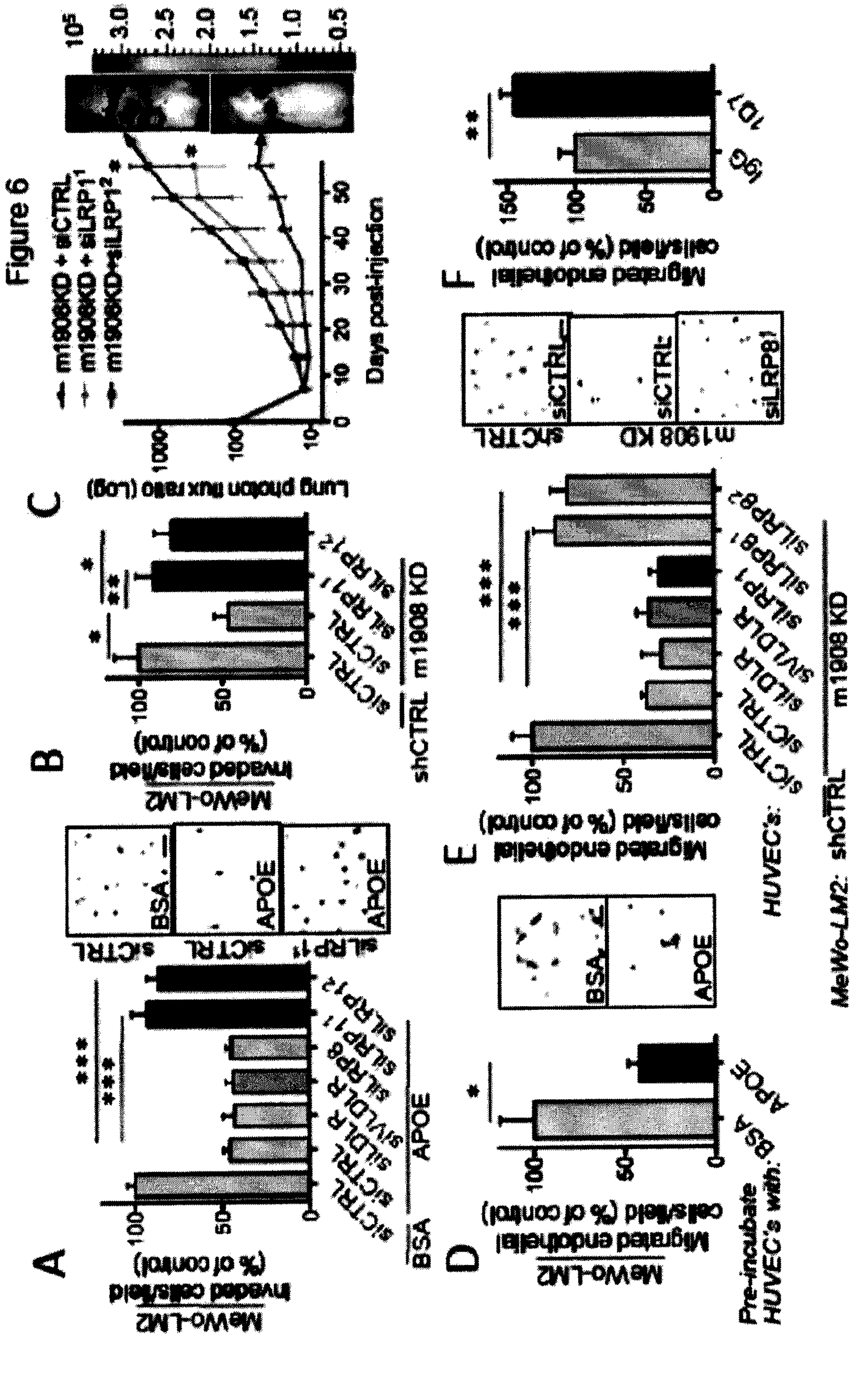
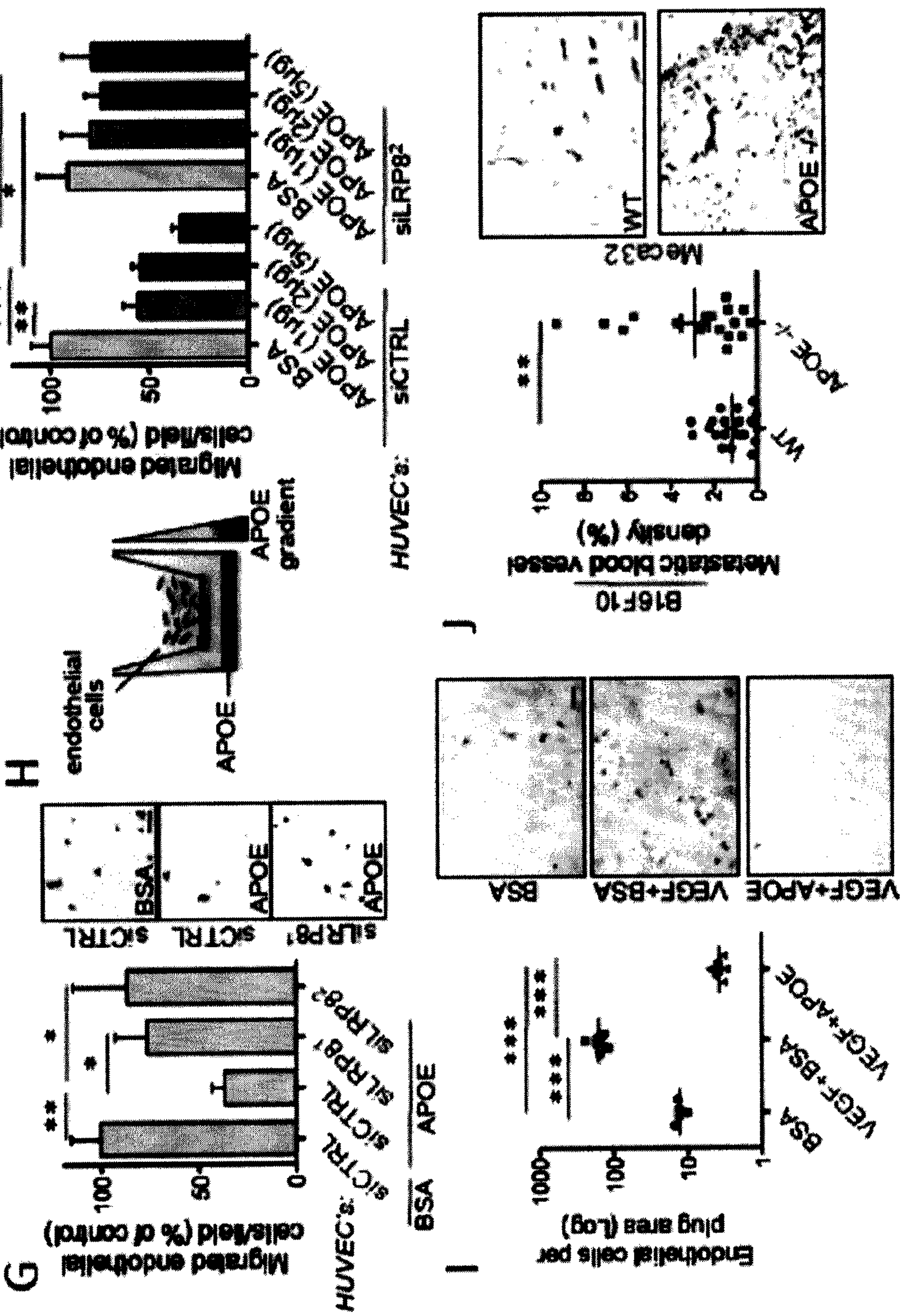
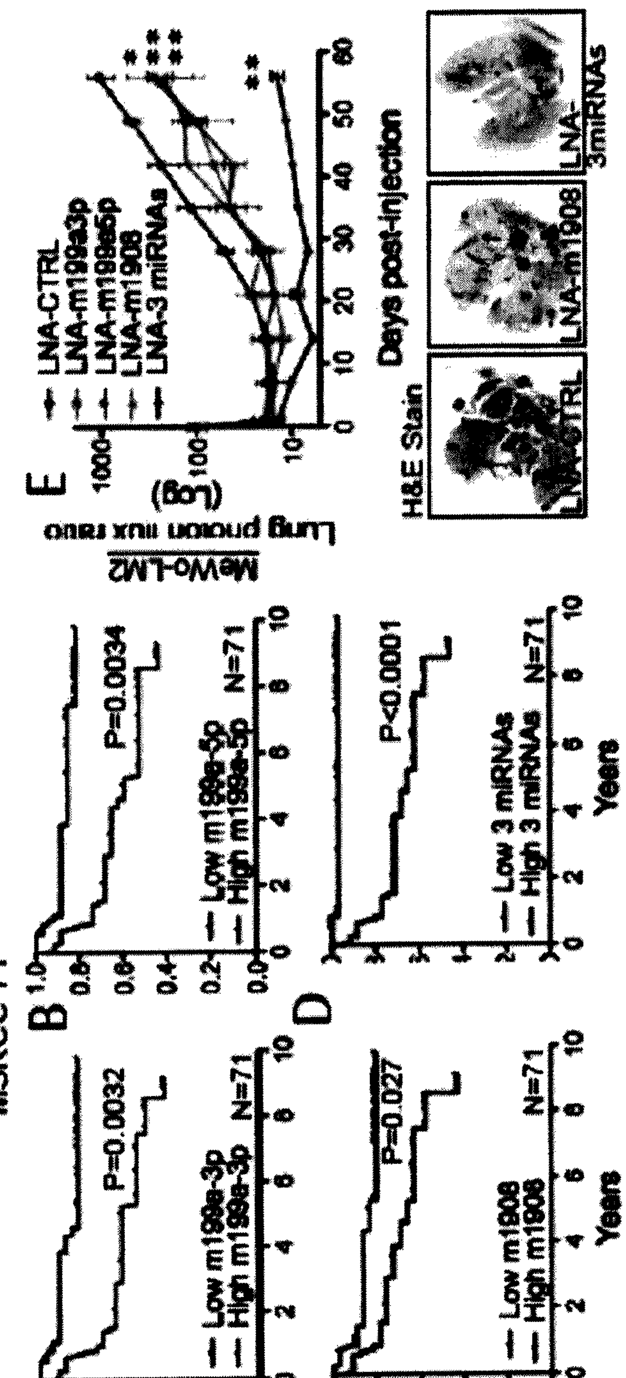


Figure 6
A
MeWo-LM2
Invaded cells/field (% of control)
BSA
APOE
siCTRL
siLDLR
siVLDLR
siLRP8
siLRP1¹
siLRP1²
B
C
m1908KD + siCTRL
m1908KD + siLRP1¹
m1908KD+siLRP1²
Lung photon flux ratio (Log)
Days post-injection
shCTRL
m1908 KD
D
Migrated endothelial cells/field (% of control)
Pre-incubate HUVEC's with:
E
HUVEC's:
MeWo-LM2:
siLRP8¹
siLRP8²
F
IgG
1D7

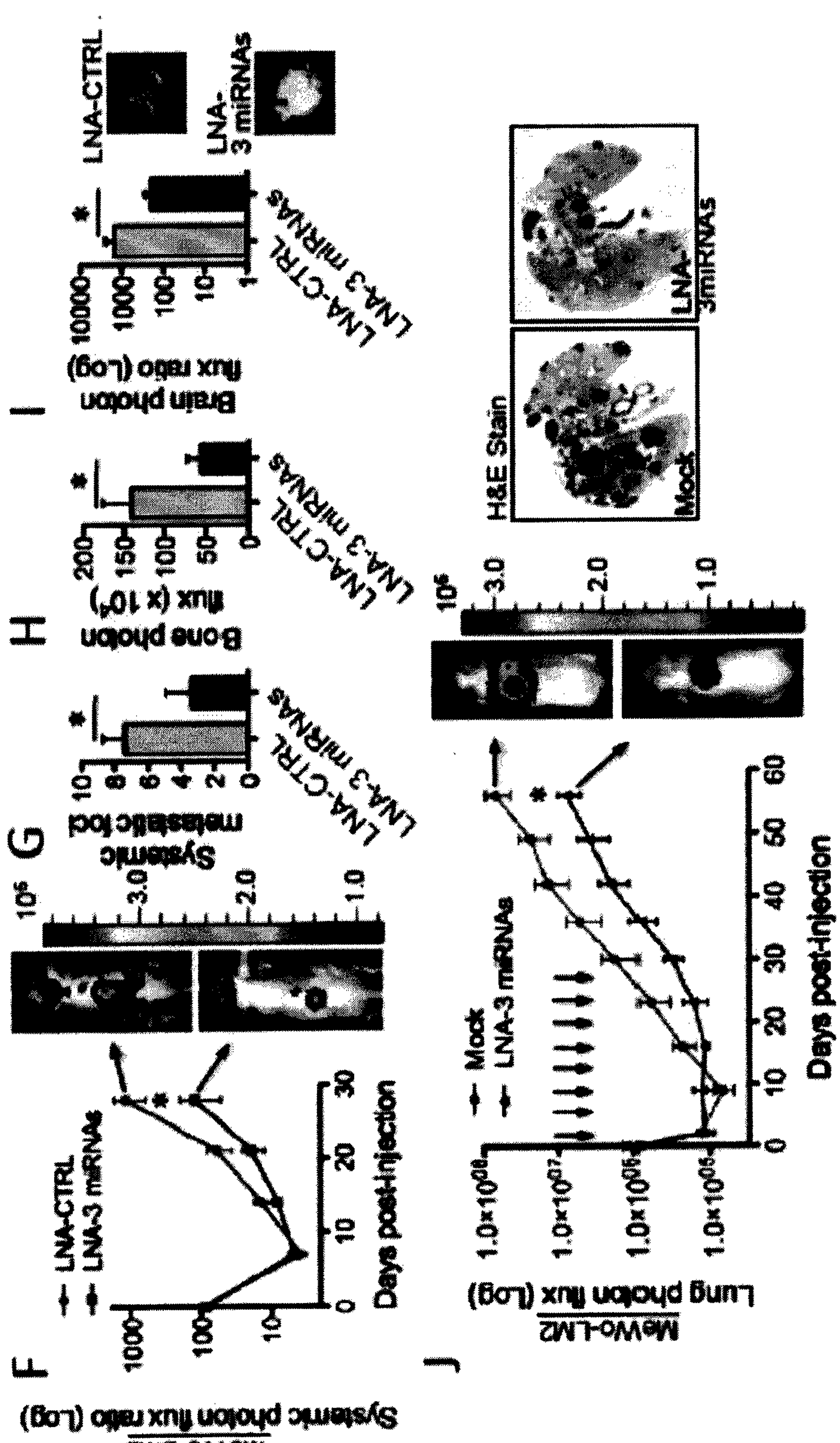
FIGURES 6A-F


G
Migrated endothelial cells/field (% of control)
HUVEC's:
BSA
APOE
siCTRL
siLRP8¹
siLRP8²
H
endothelial cells
APOE
APOE gradient
siCTRL
siLRP8²
I
Endothelial cells per plug area (Log)
BSA
VEGF+BSA
VEGF+APOE
J
B16F10
Metastatic blood vessel density (%)
WT
APOE -/-
Meca32

FIGURES 6G-J


MSKCC 71
A
B
C
D
E
Metastasis-free survival
P=0.0032
Low m199a-3p
High m199a-3p
N=71
Years
P=0.0034
Low m199a-5p
High m199a-5p
P=0.027
Low m1908
High m1908
P<0.0001
Low 3 miRNAs
High 3 miRNAs
MeWo-LM2
Lung photon flux ratio (Log)
LNA-CTRL
LNA-m199a3p
LNA-m199a5p
LNA-m1908
LNA-3 miRNAs
Days post-injection
H&E Stain
LNA-CTRL
LNA-m1908
LNA-3miRNAs

FIGURES 7A-E


F
MeWo-LM2
Systemic photon flux ratio (Log)
LNA-CTRL
LNA-3 miRNAs
Days post-injection
G
Systemic metastatic foci
LNA-CTRL
LNA-3 miRNAs
H
Bone photon flux (x 10⁴)
LNA-CTRL
LNA-3 miRNAs
I
Brain photon flux ratio (Log)
LNA-CTRL
LNA-3 miRNAs
LNA-CTRL
LNA-3 miRNAs
J
MeWo-LM2
Lung photon flux (Log)
Mock
LNA-3 miRNAs
Days post-injection
H&E Stain
Mock
LNA-3miRNAs

FIGURES 7F-J

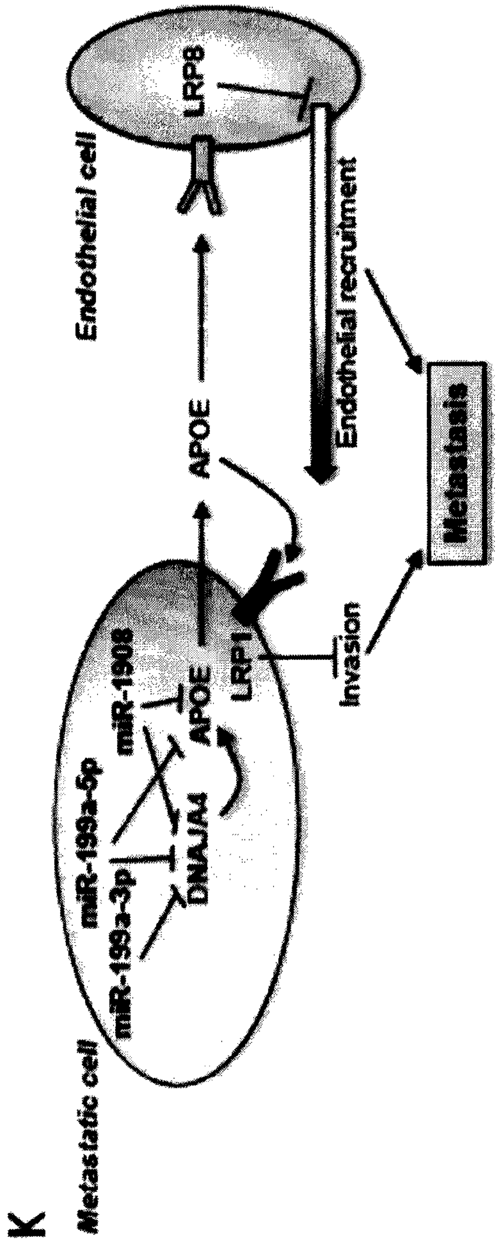
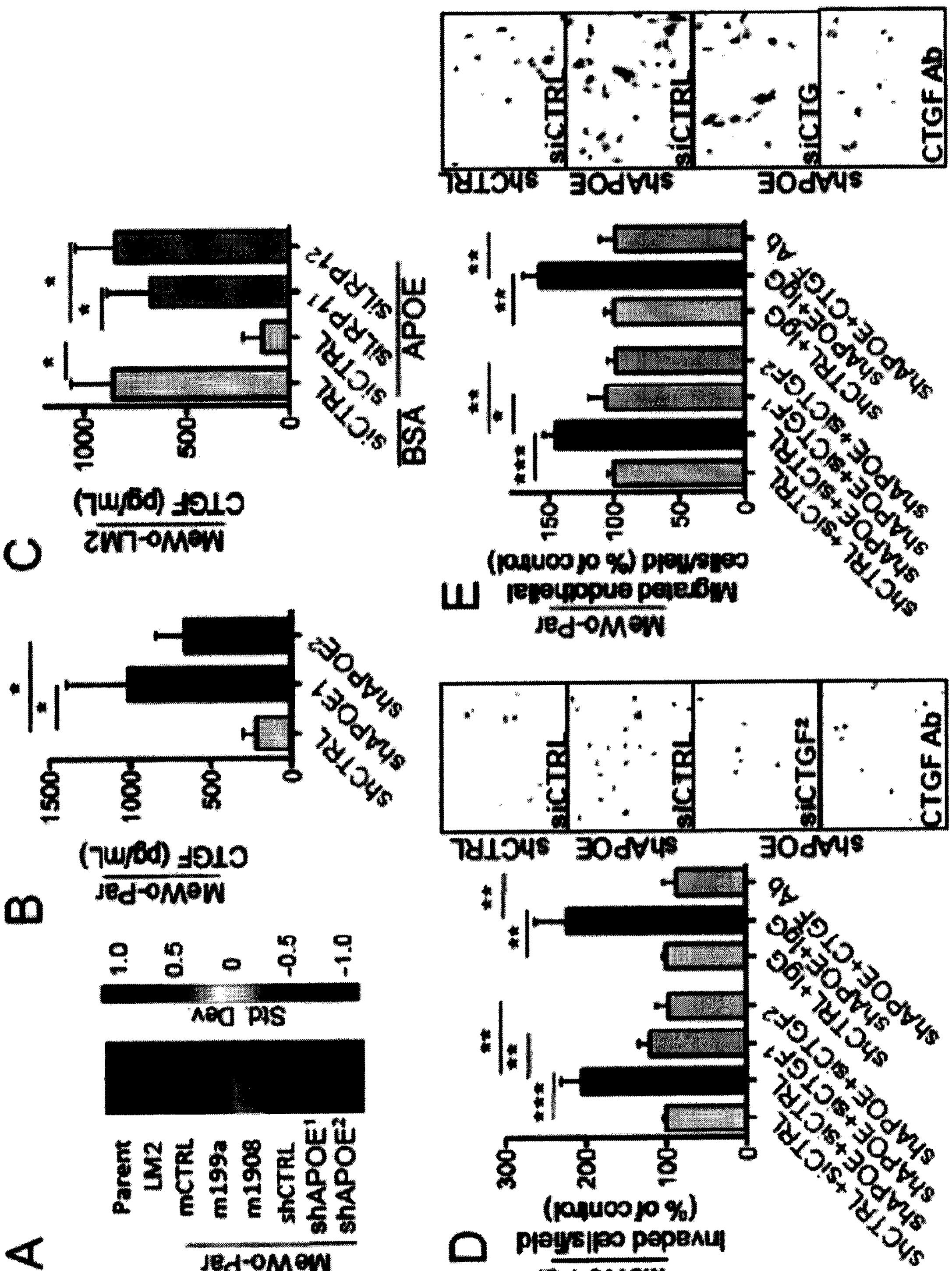
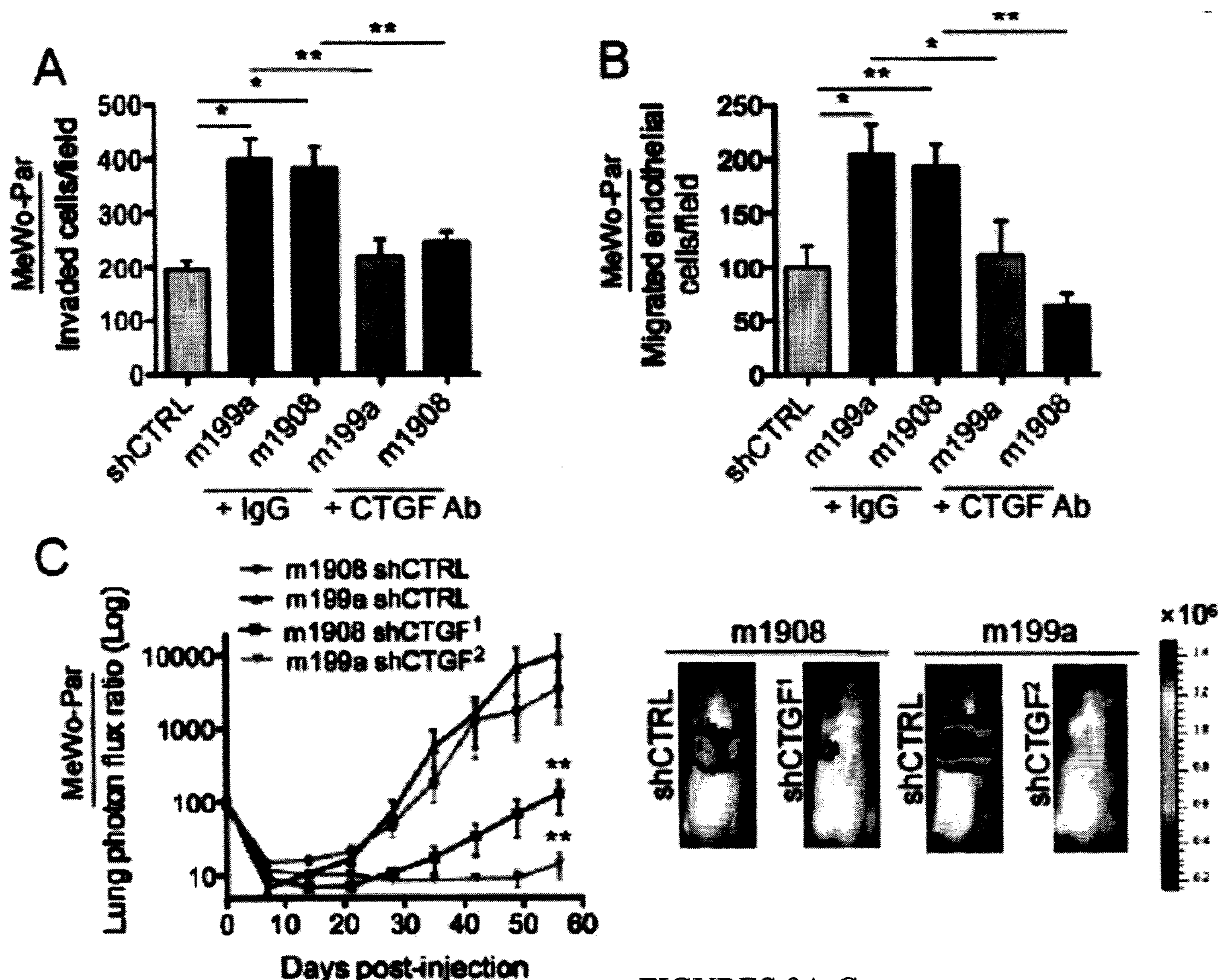

K
Metastatic cell
miR-199a-3p
miR-199a-5p
miR-1908
DNAJA4
APOE
LRP1
Invasion
APOE
Endothelial cell
LRP8
Endothelial recruitment
Metastasis

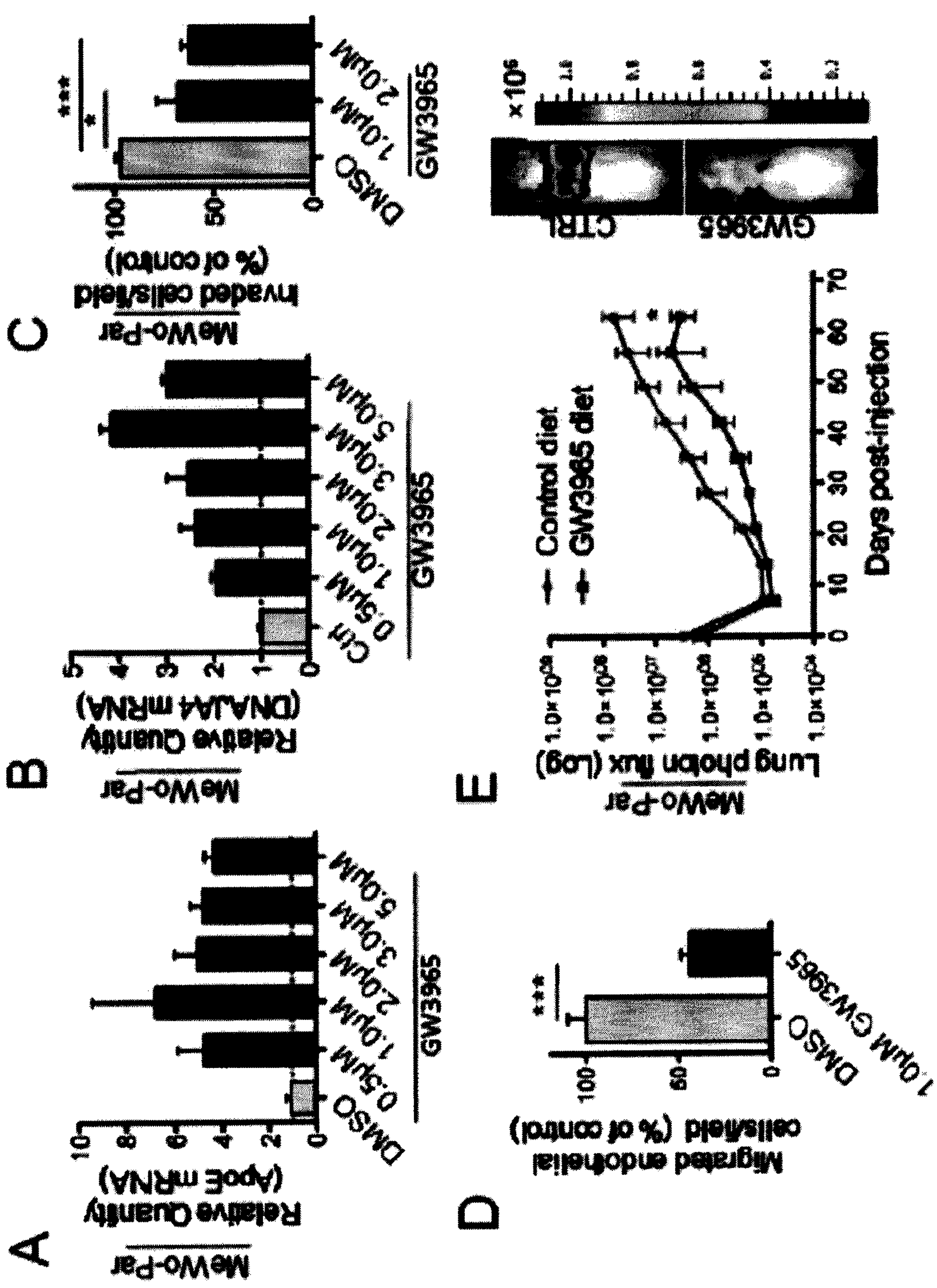
FIGURE 7K


A
MeWo-Par
Parent
LM2
mCTRL
m199a
m1908
shCTRL
shAPOE1
shAPOE2
Std. Dev.
1.0
0.5
0
-0.5
-1.0
B
MeWo-Par
CTGF (pg/mL)
shCTRL
shAPOE1
shAPOE2
C
MeWo-LM2
CTGF (pg/mL)
siCTRL
siCTRL
siLRP11
siLRP12
BSA
APOE
D
MeWo-Par
Invaded cells/field (% of control)
shCTRL+siCTRL
shAPOE+siCTRL
shAPOE+siCTGF1
shAPOE+siCTGF2
shCTRL+IgG
shAPOE+IgG
shAPOE+CTGF Ab
shCTRL
shAPOE
siCTRL
siCTGF2
CTGF Ab
E
MeWo-Par
Migrated endothelial cells/field (% of control)
siCTG

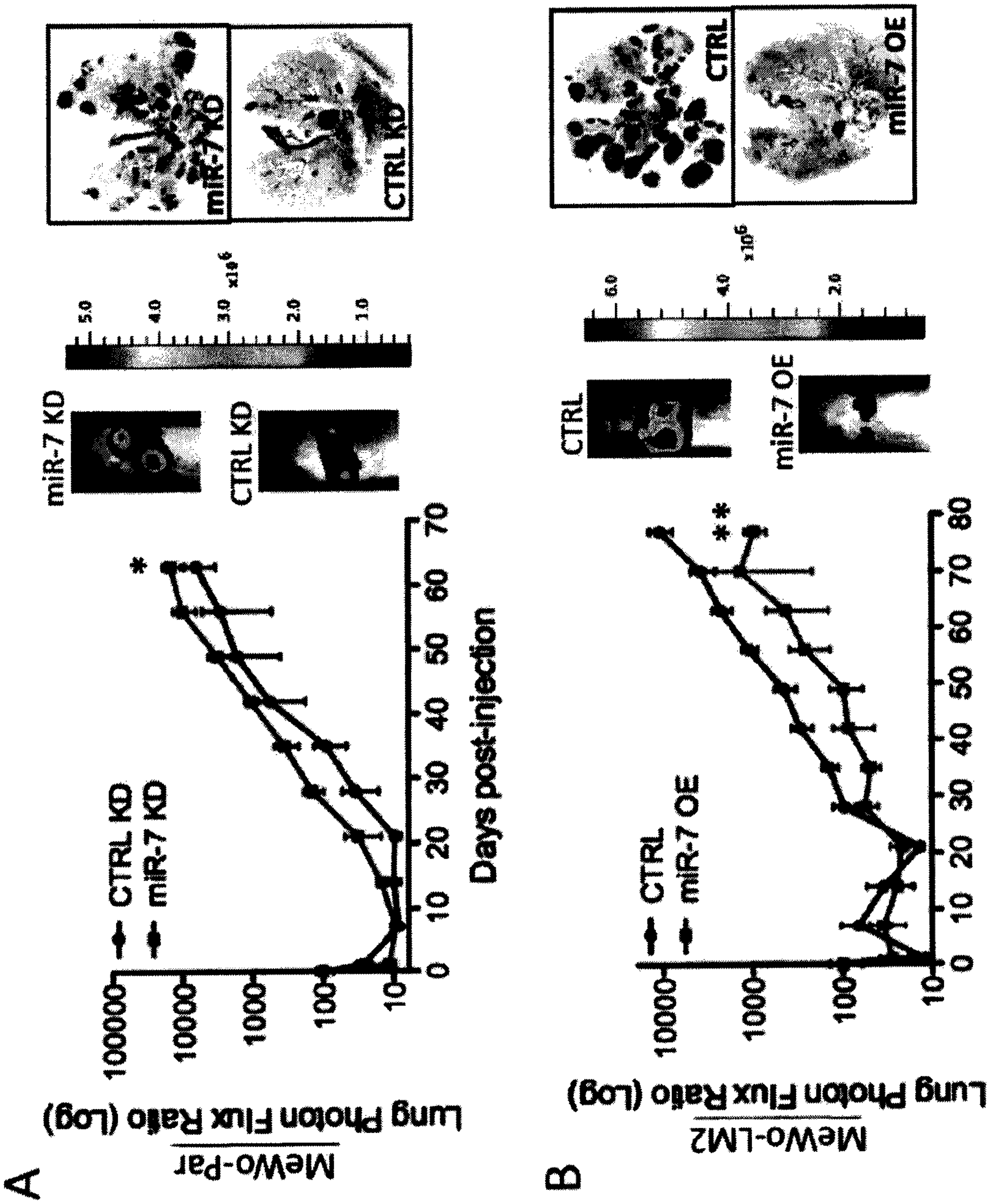
FIGURES 8A-E


A
B
C
MeWo-Par
Invaded cells/field
Migrated endothelial cells/field
Lung photon flux ratio (Log)
shCTRL
m199a
m1908
+ IgG
+ CTGF Ab
m1908 shCTRL
m199a shCTRL
m1908 shCTGF1
m199a shCTGF2
Days post-injection
m1908
m199a
shCTRL
shCTGF1
shCTGF2
x10⁶

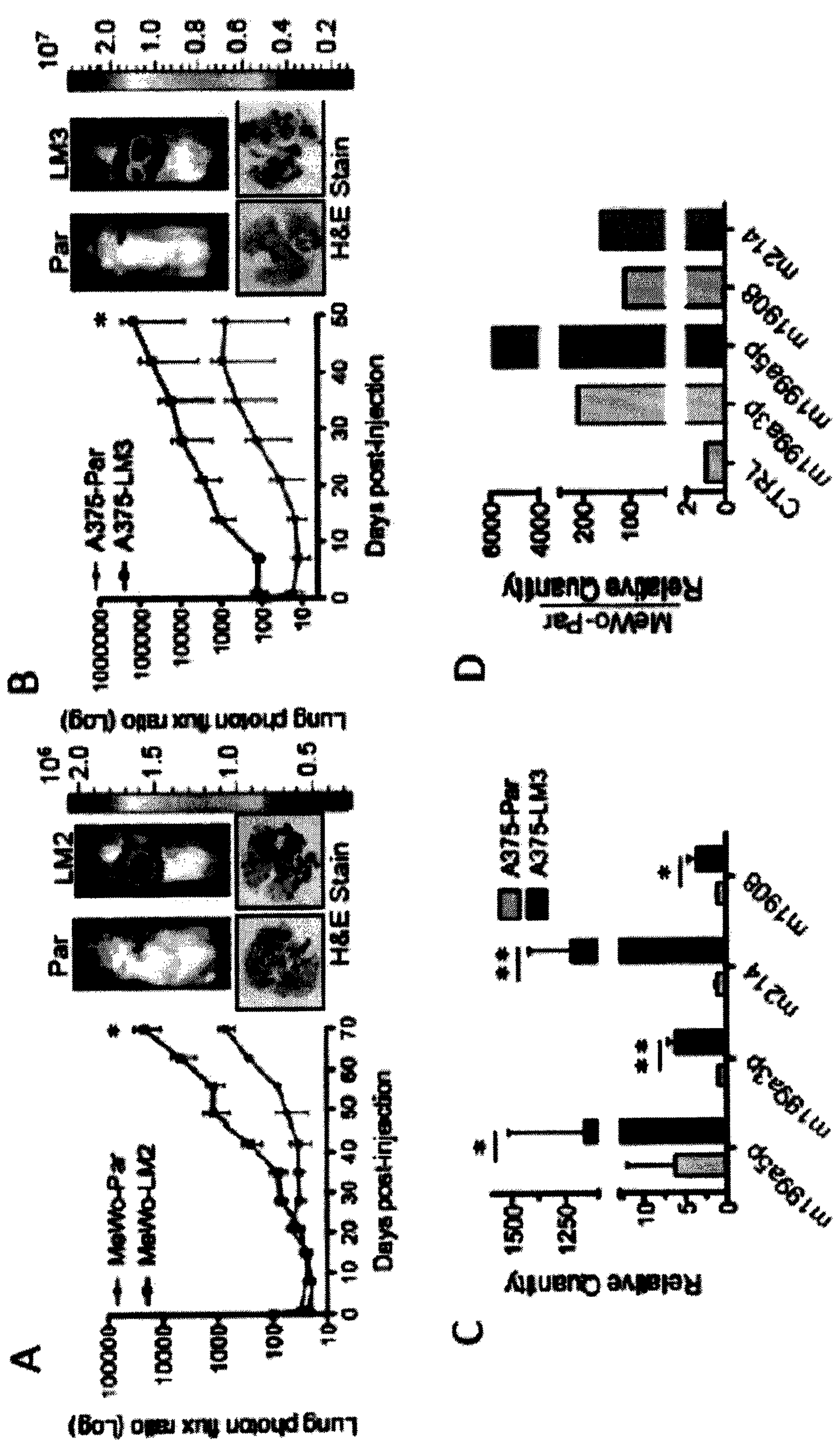
FIGURES 9A-C



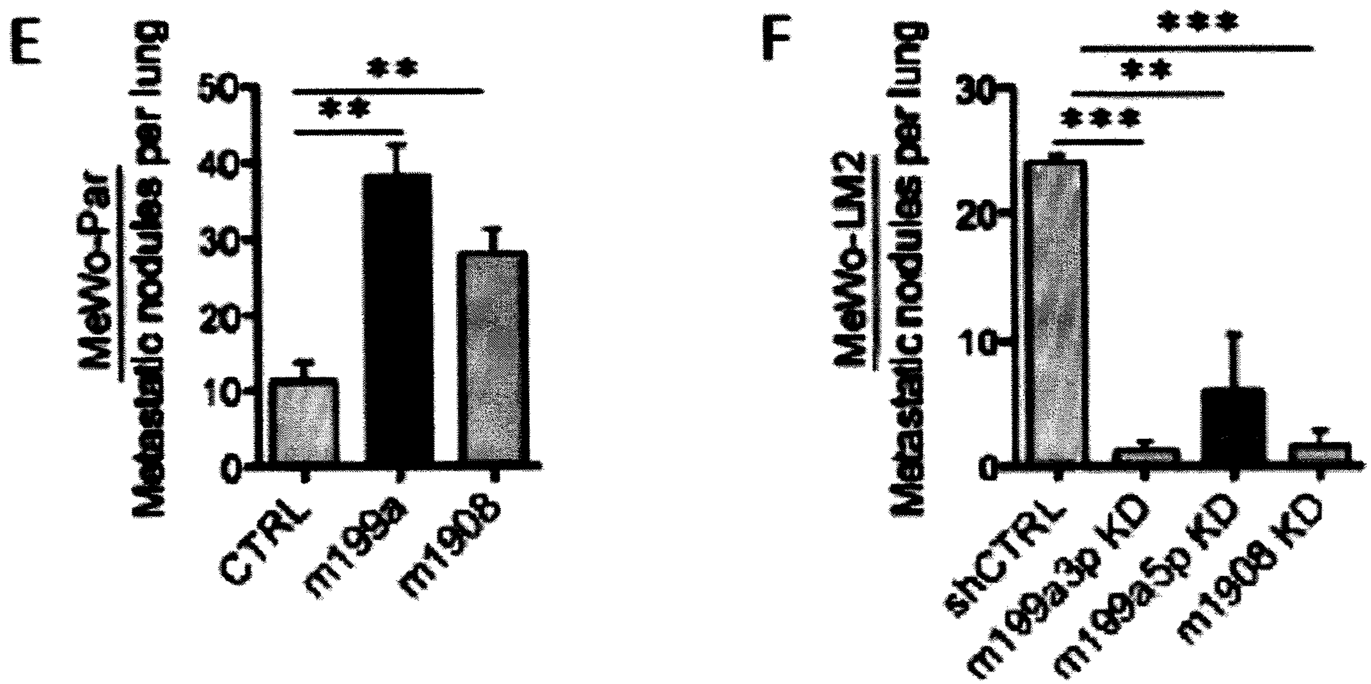
FIGURES 10A-E



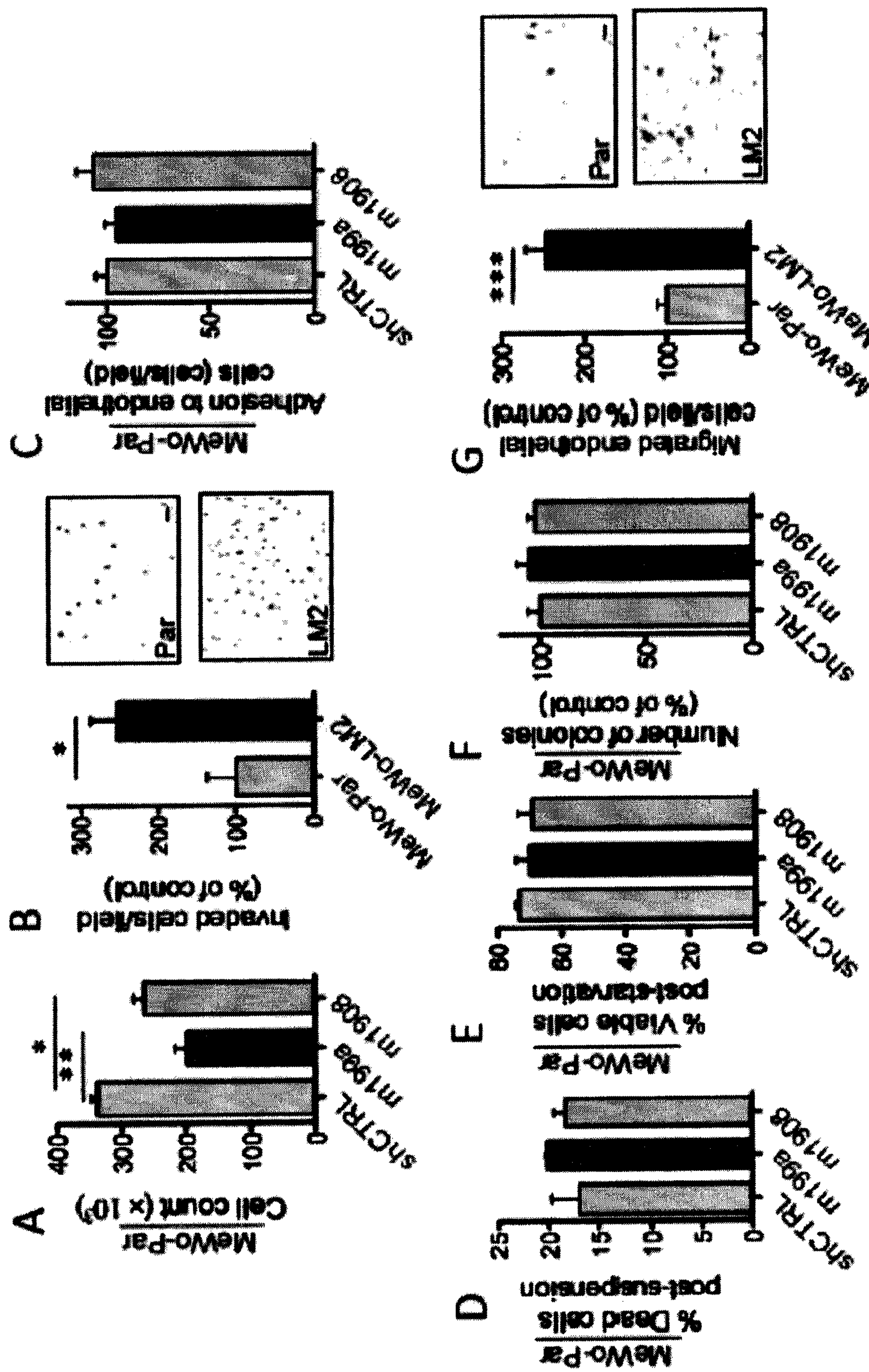
FIGURES 11A-B



FIGURES 12A-D



FIGURES 12E-F



FIGURES 13A-G

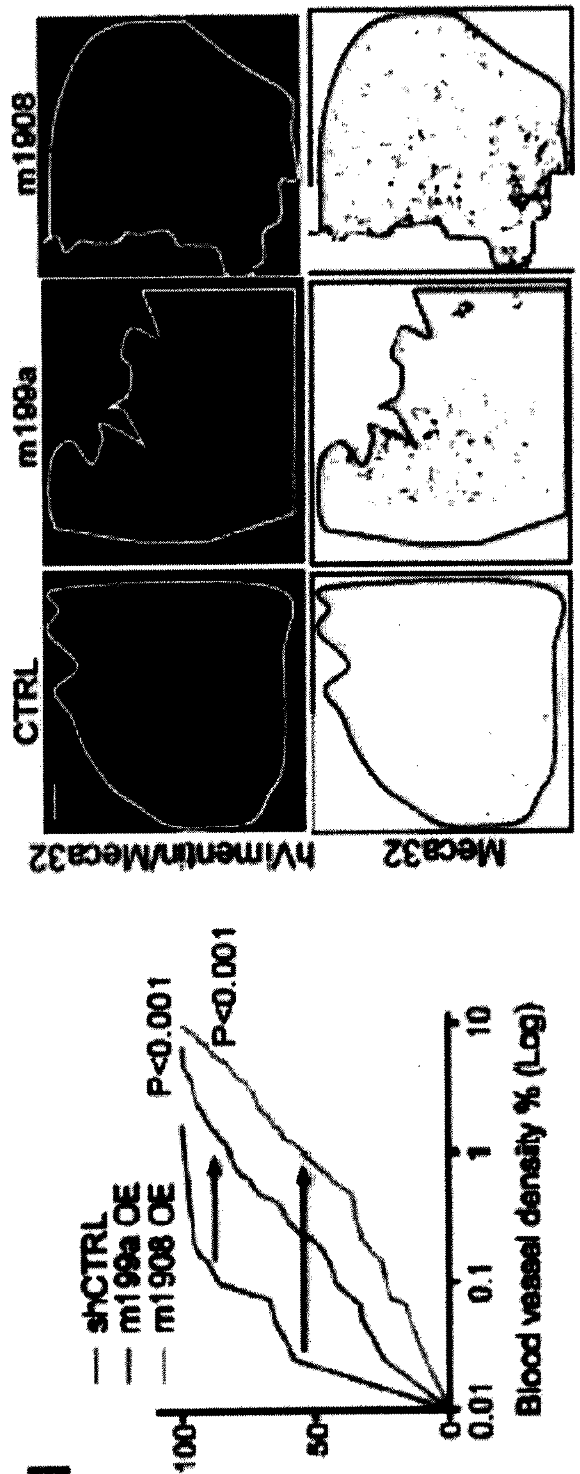
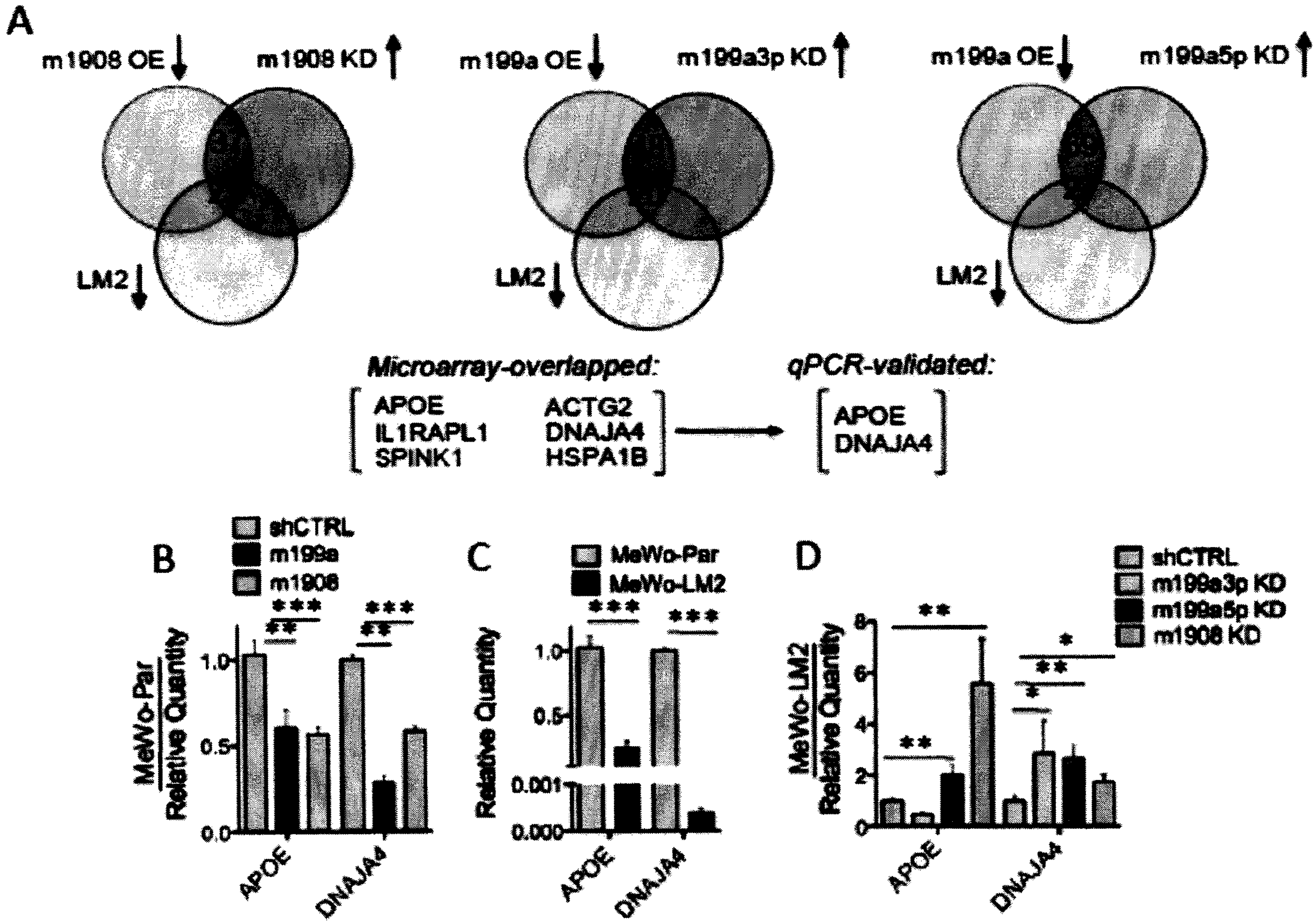
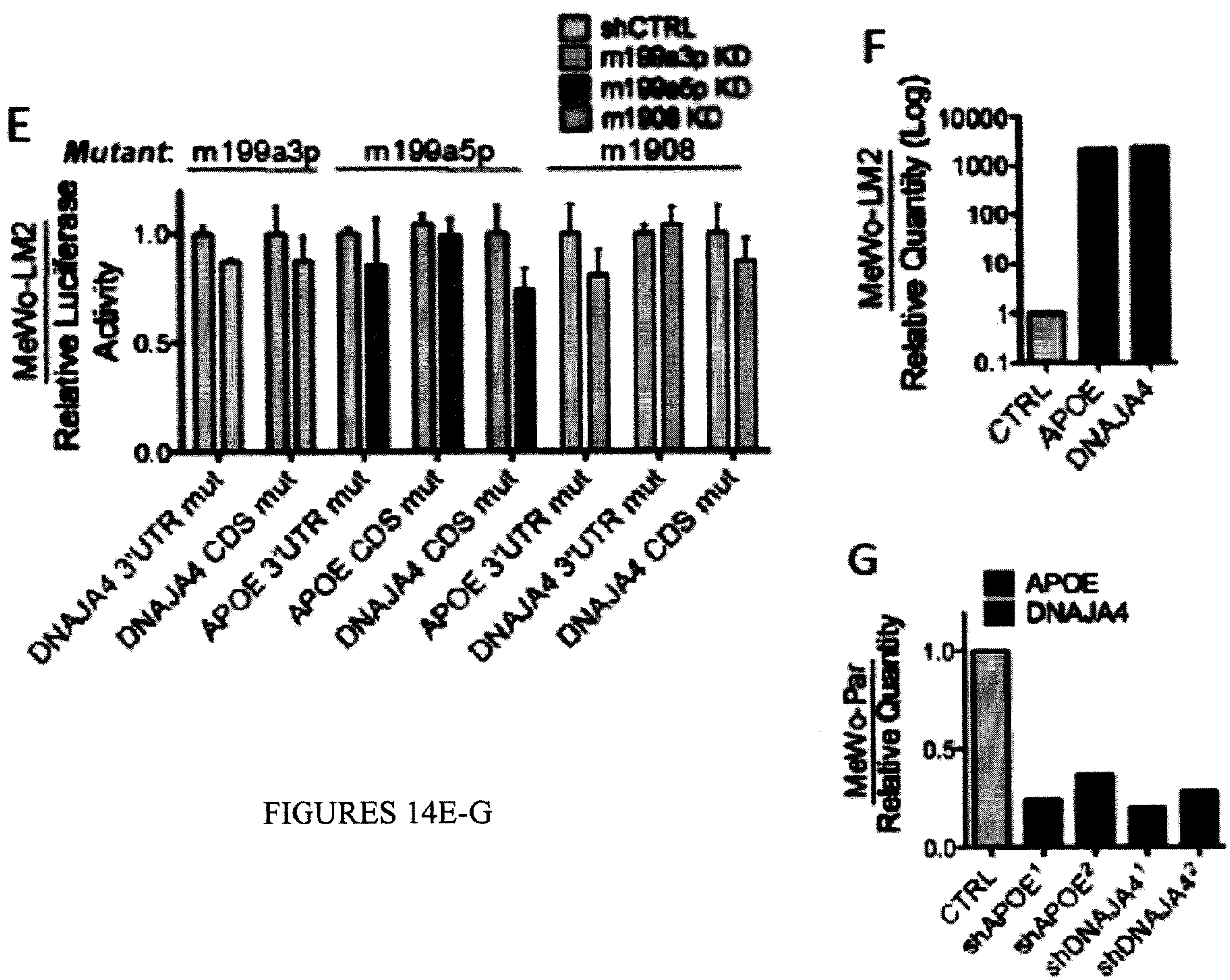

H
CTRL
m199a
m1908
hVimentin/Meca32
Meca32
MeWo-Par
Cumulative percentage
shCTRL
m199a OE
m1908 OE
P<0.001
P<0.001
100
50
0
0.01
0.1
1
10
Blood vessel density % (Log)

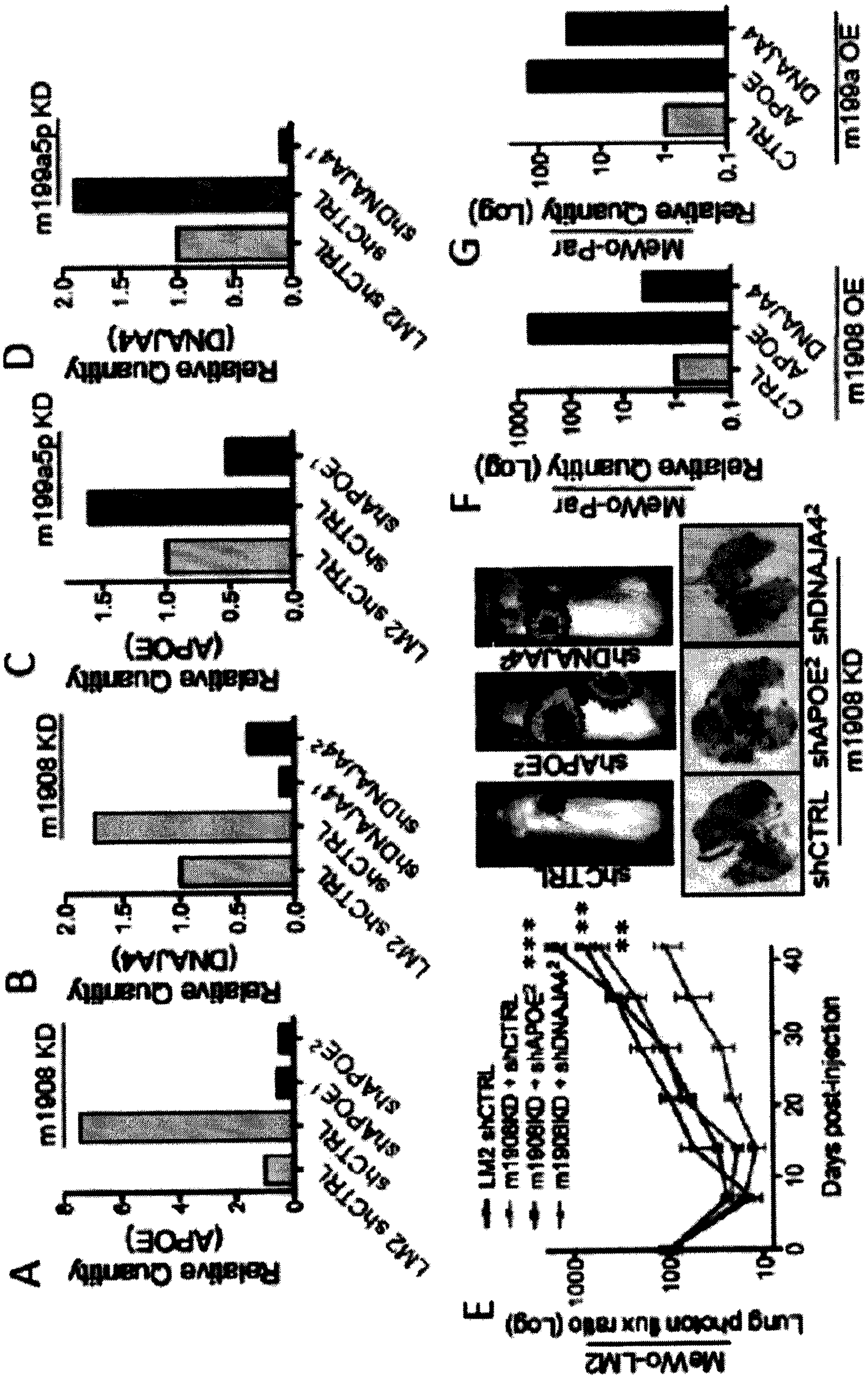
FIGURE 13H



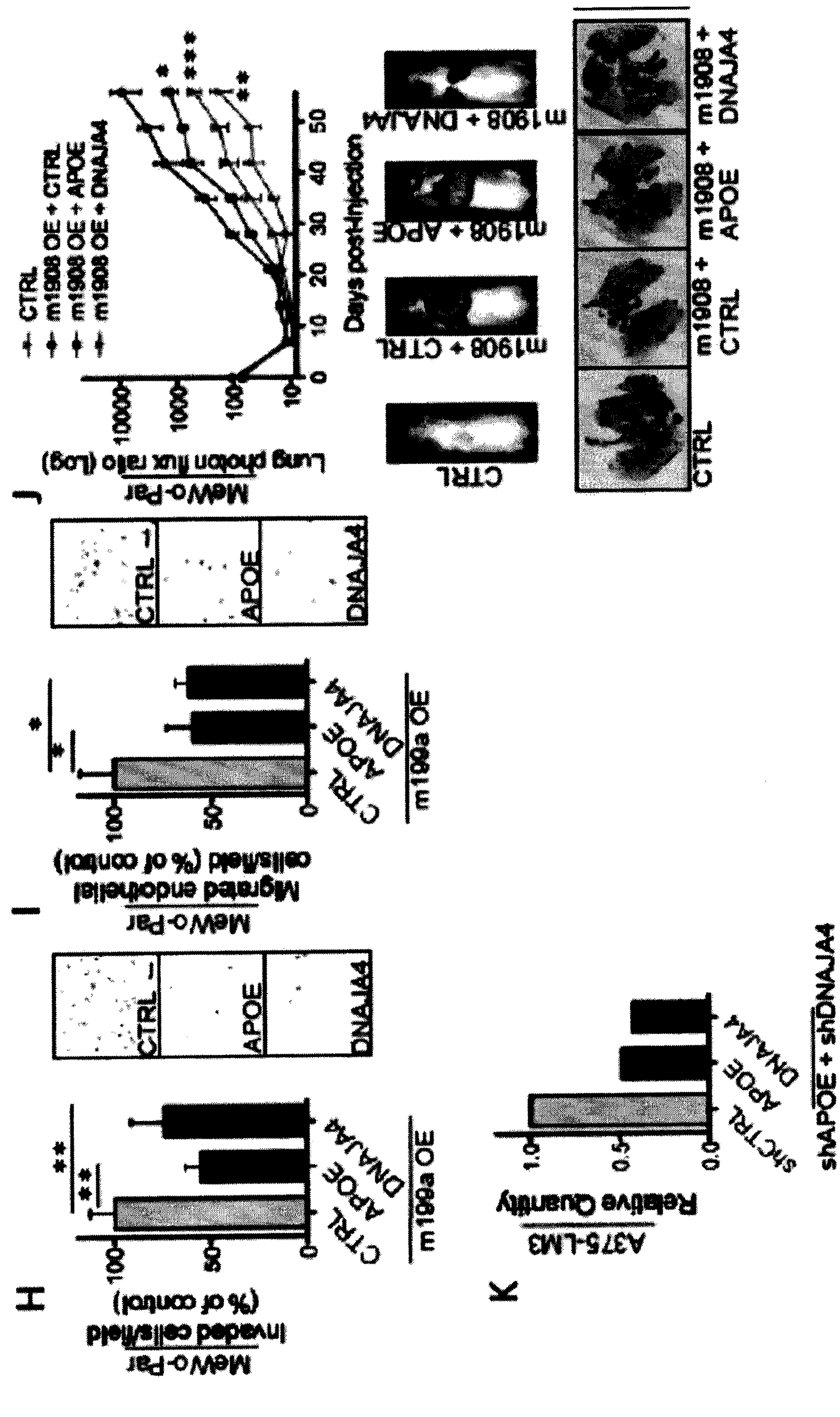
FIGURES 14A-D



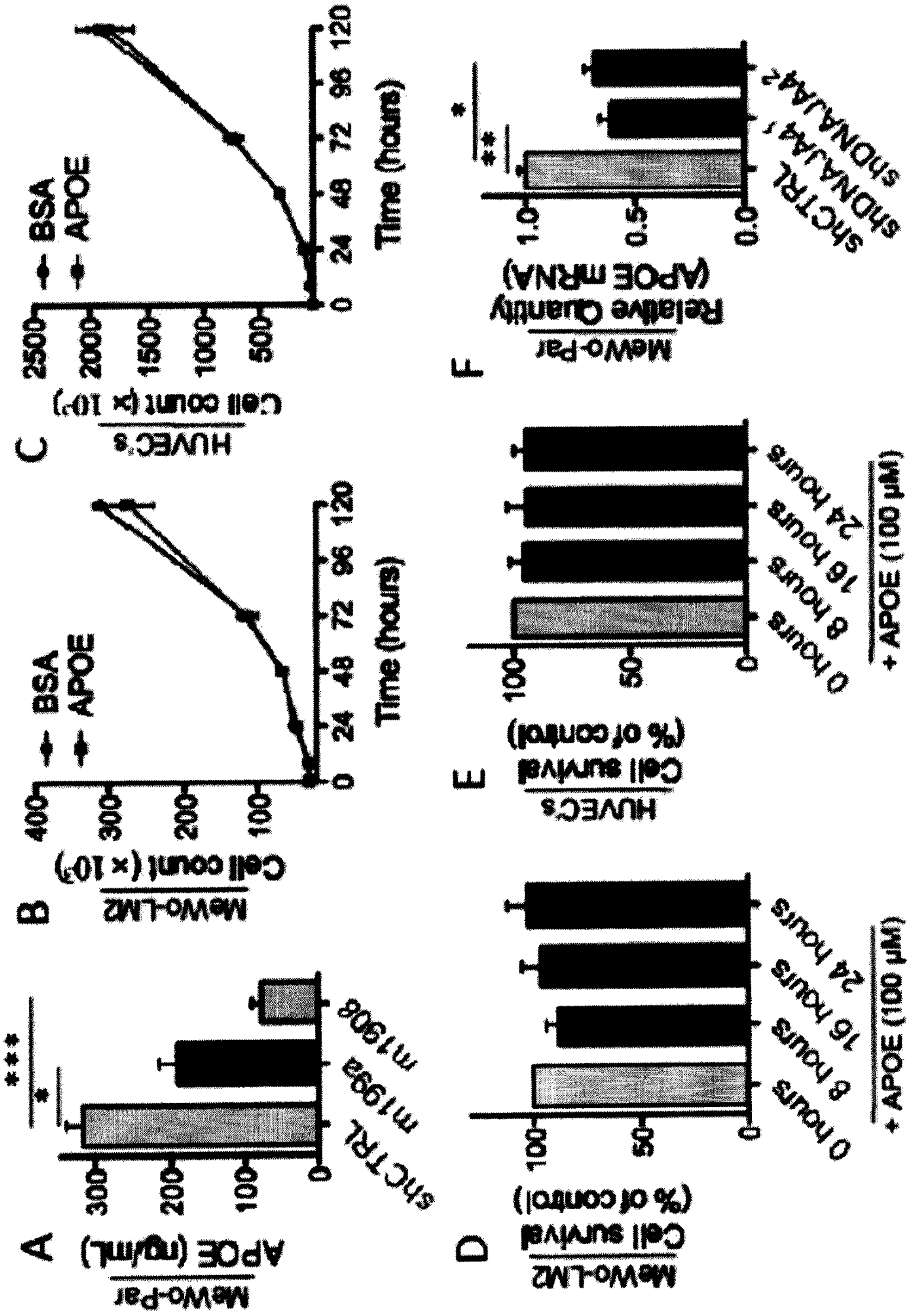
FIGURES 14E-G



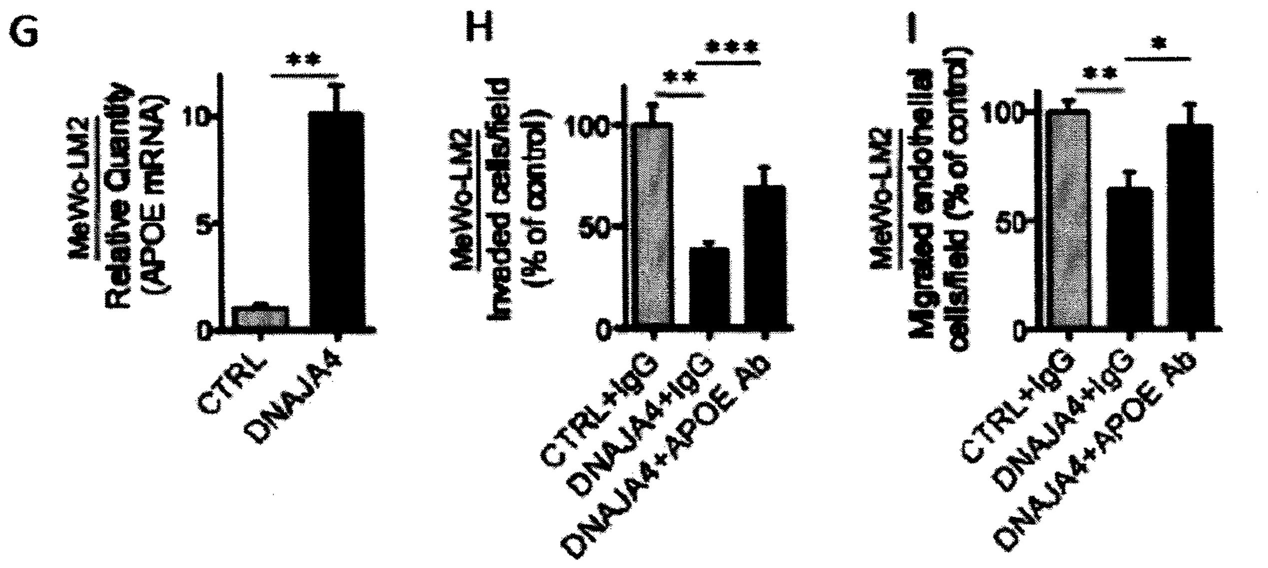
FIGURES 15A-G



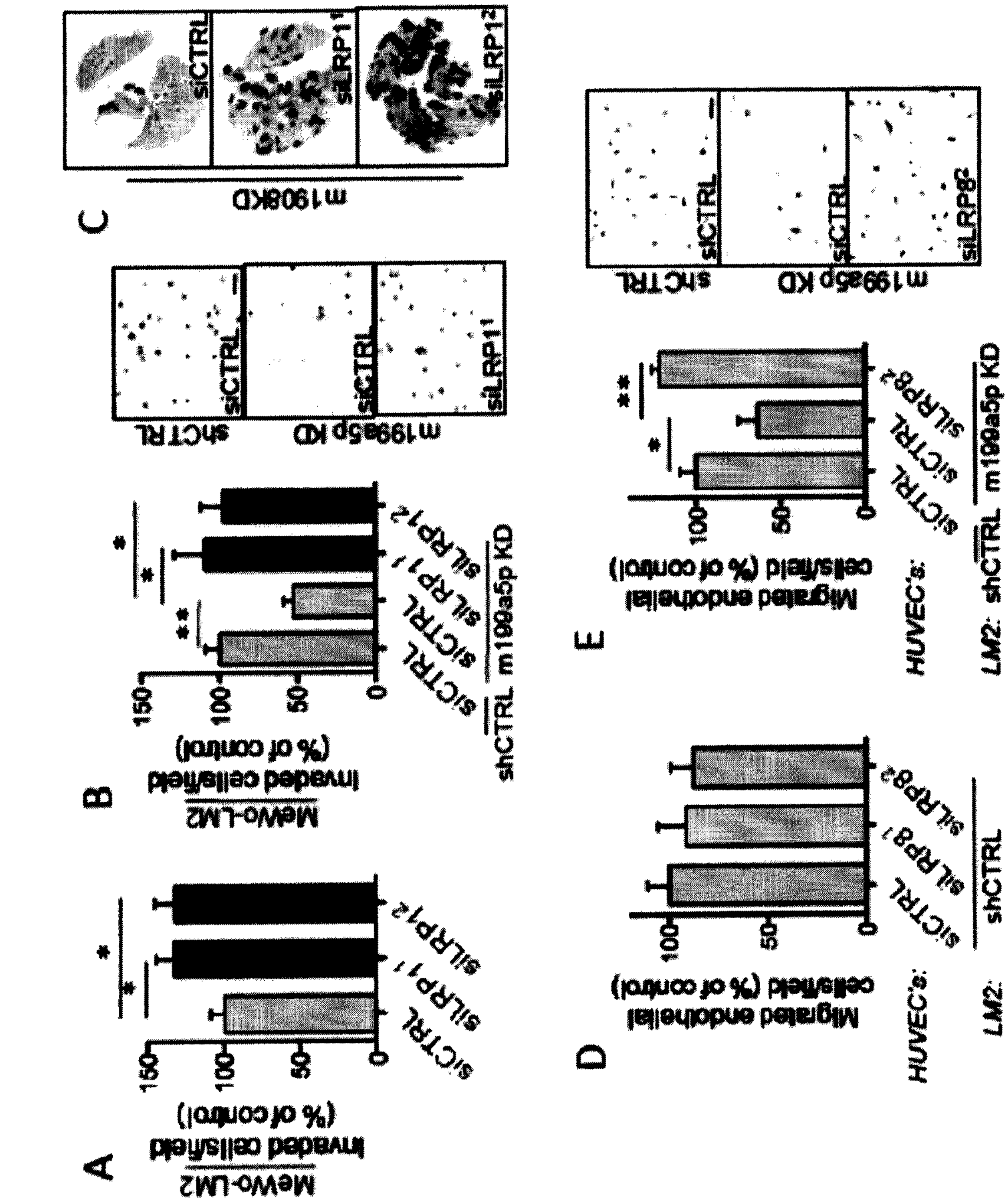
FIGURES 15H-K



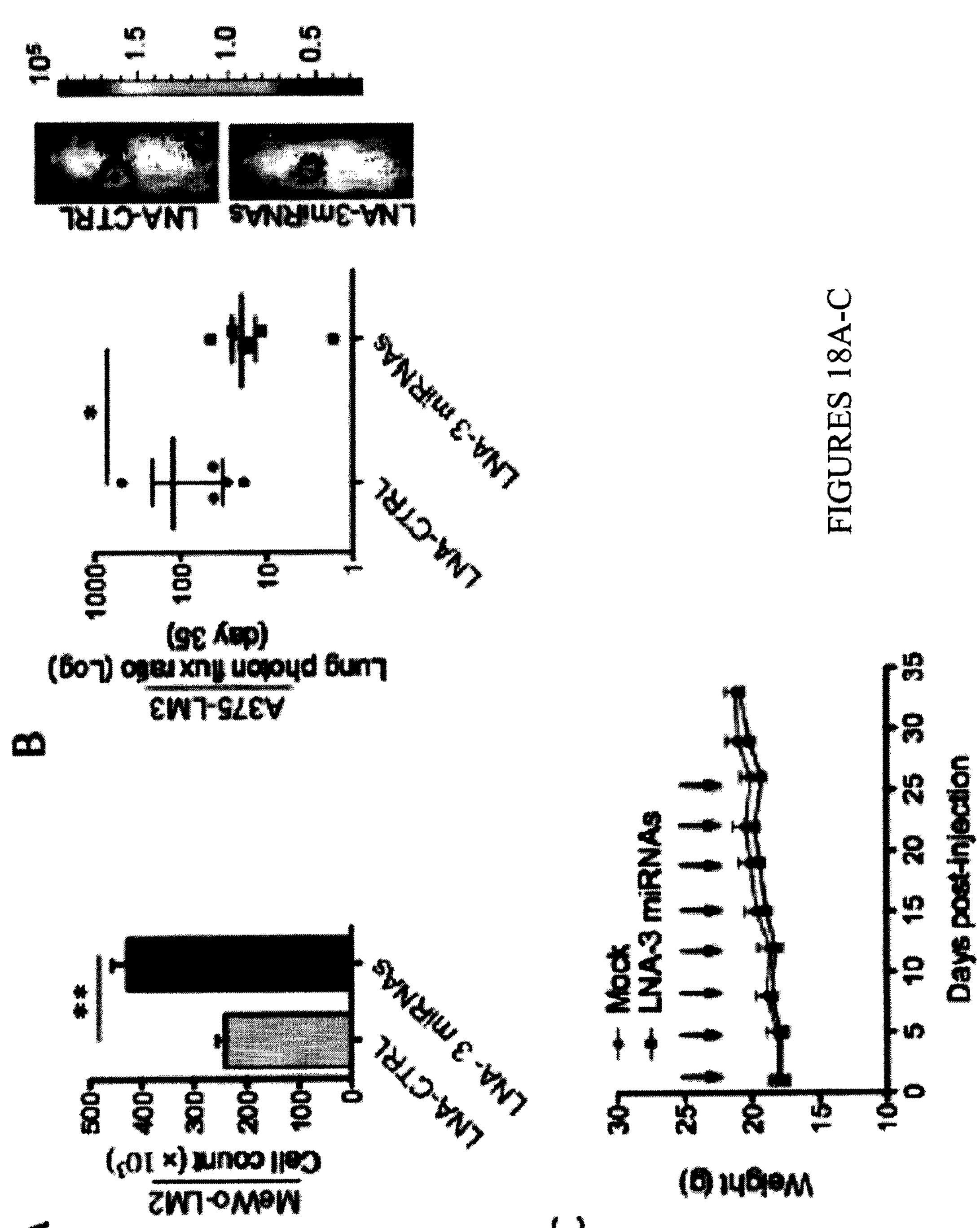
FIGURES 16A-F



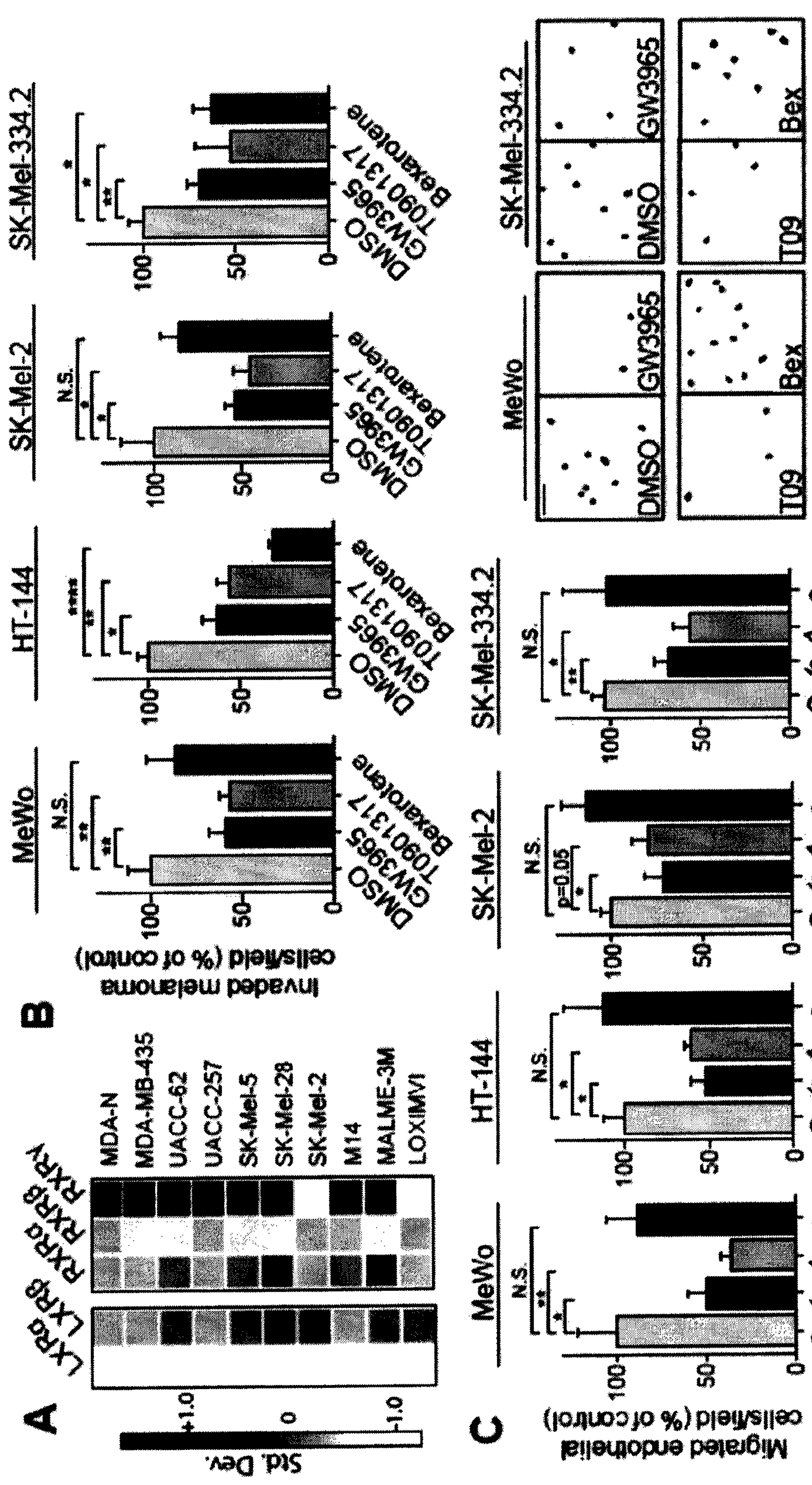
FIGURES 16G-I



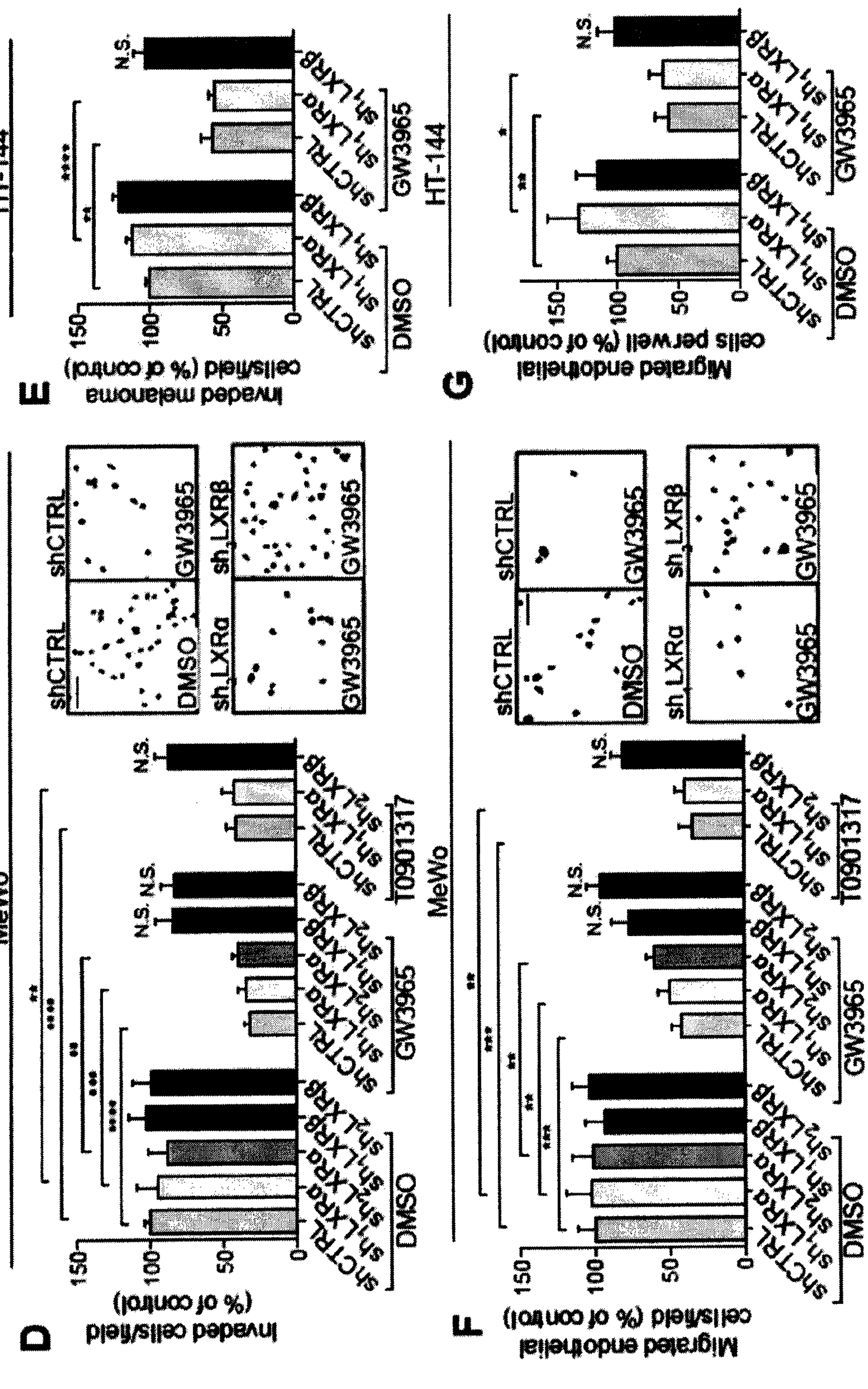
FIGURES 17A-E



FIGURES 18A-C



FIGURES 19A-C



FIGURES 19D-F

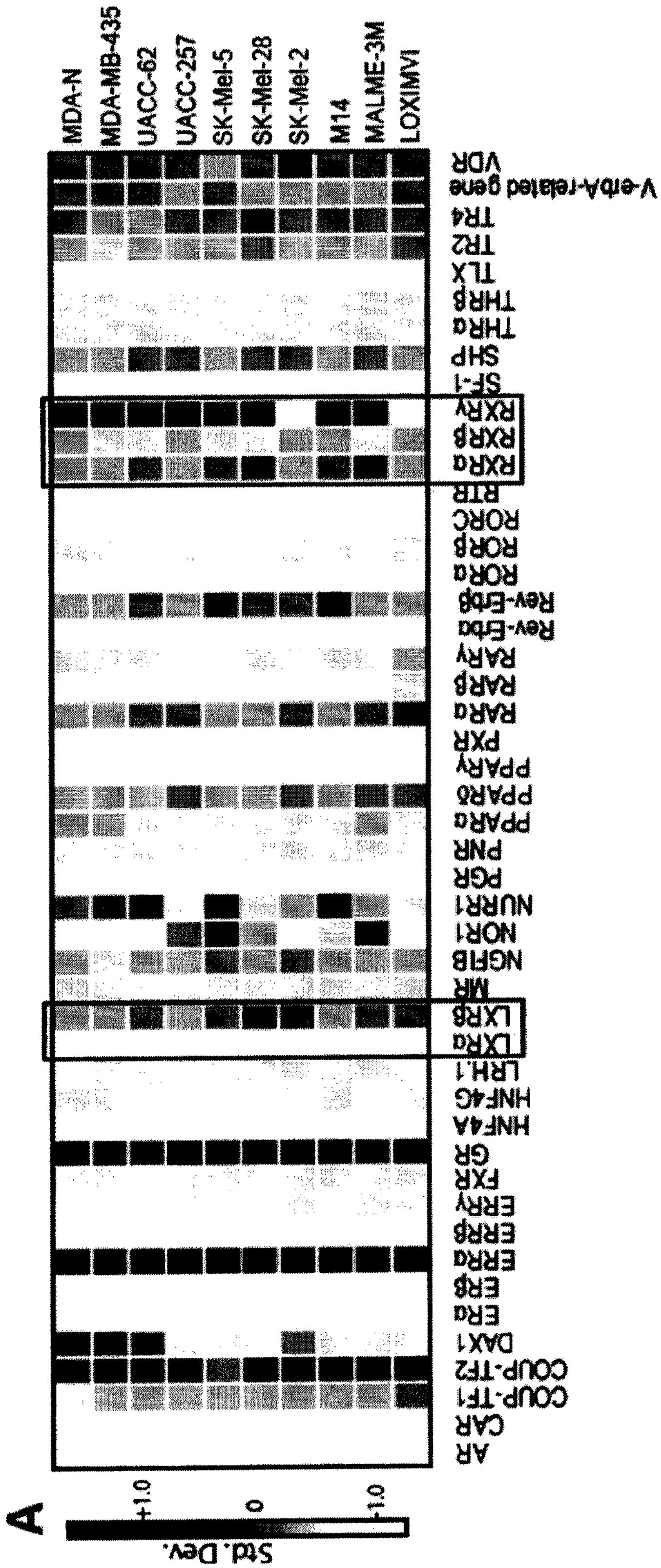
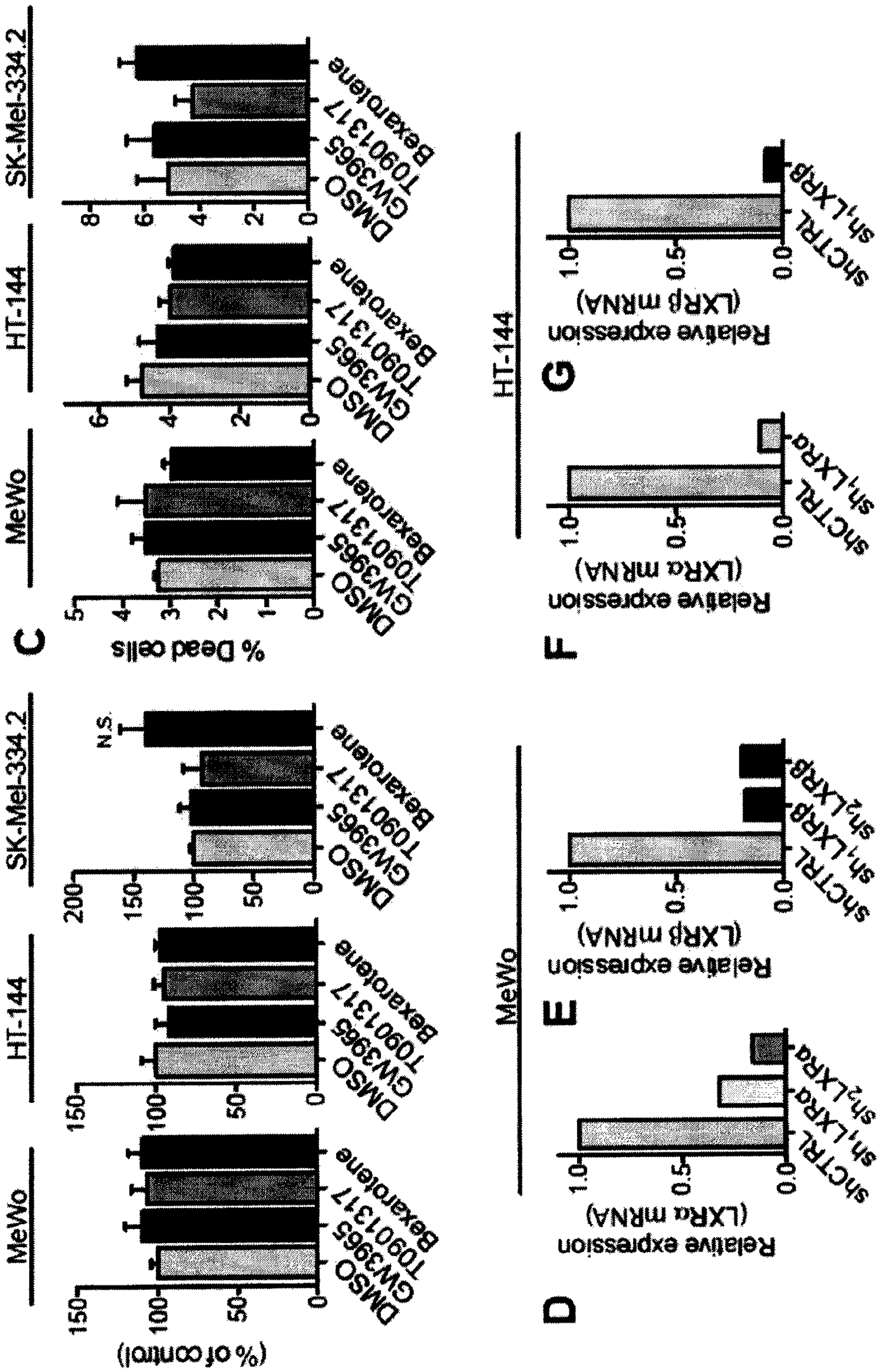
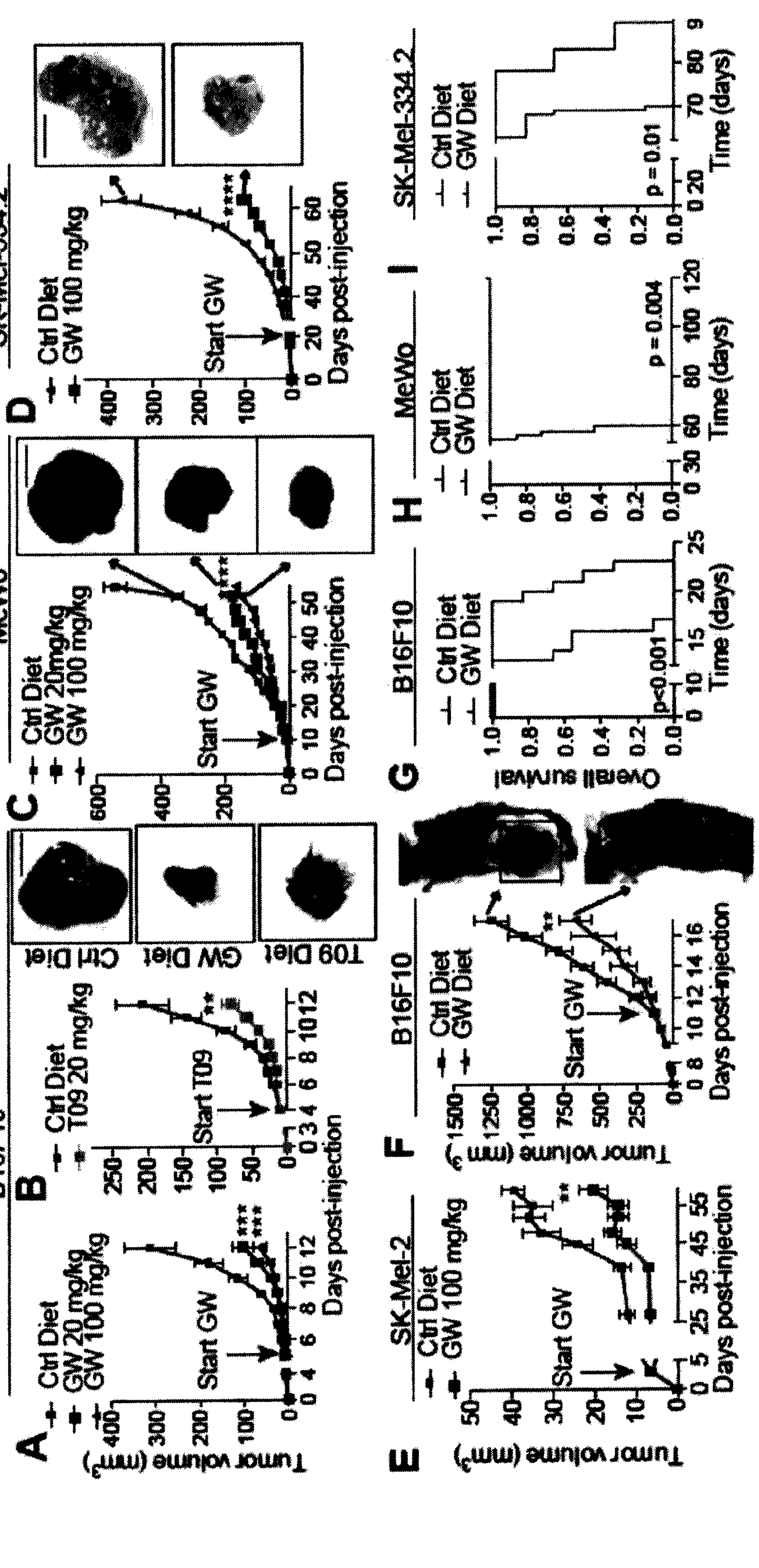

A
Std. Dev.
+1.0
0
-1.0
MDA-N
MDA-MB-435
UACC-62
UACC-257
SK-Mel-5
SK-Mel-28
SK-Mel-2
M14
MALME-3M
LOXIMVI
VDR
V-erbA-related gene
TR4
TR2
TLX
THRβ
THRα
SHP
SF-1
RXRγ
RXRβ
RXRα
RTR
RORC
RORβ
RORα
Rev-Erbβ
Rev-Erbα
RARγ
RARβ
RARα
PXR
PPARγ
PPARδ
PPARα
PNR
PGR
NURR1
NOR1
NGFIB
MR
LXRβ
LXRα
LRH.1
HNF4G
HNF4A
GR
FXR
ERRγ
ERRβ
ERRα
ERβ
ERα
DAX1
COUP-TF2
COUP-TF1
CAR
AR

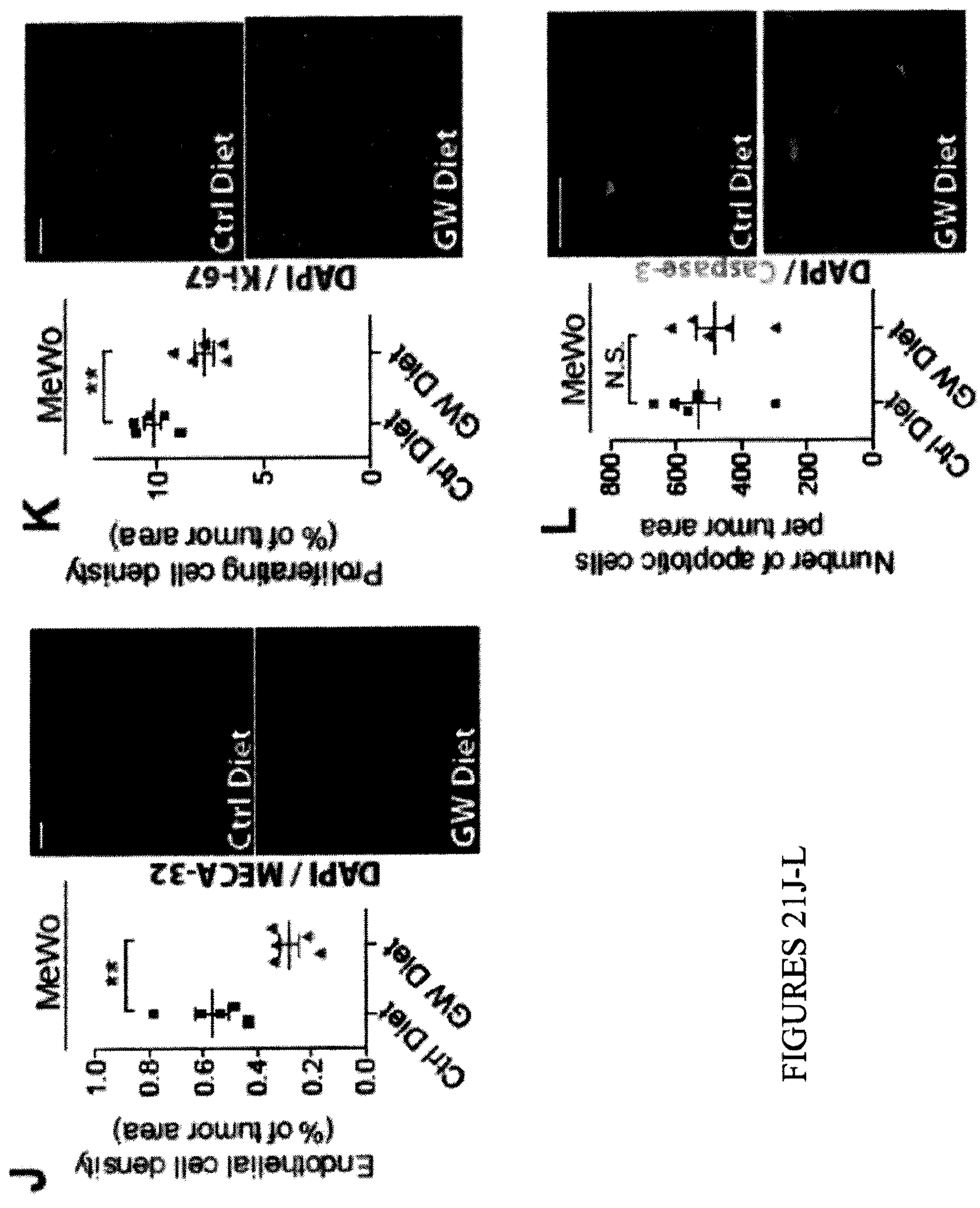
FIGURE 20A



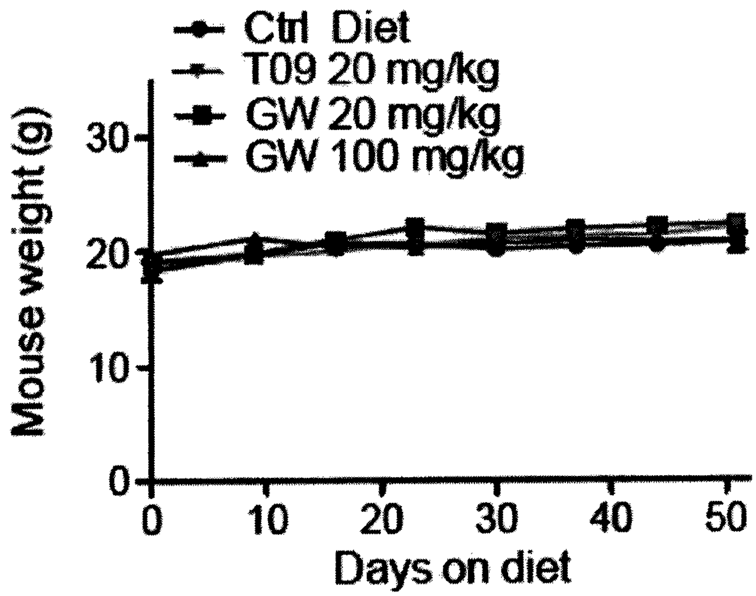
FIGURES 20B-G



FIGURES 21A-I

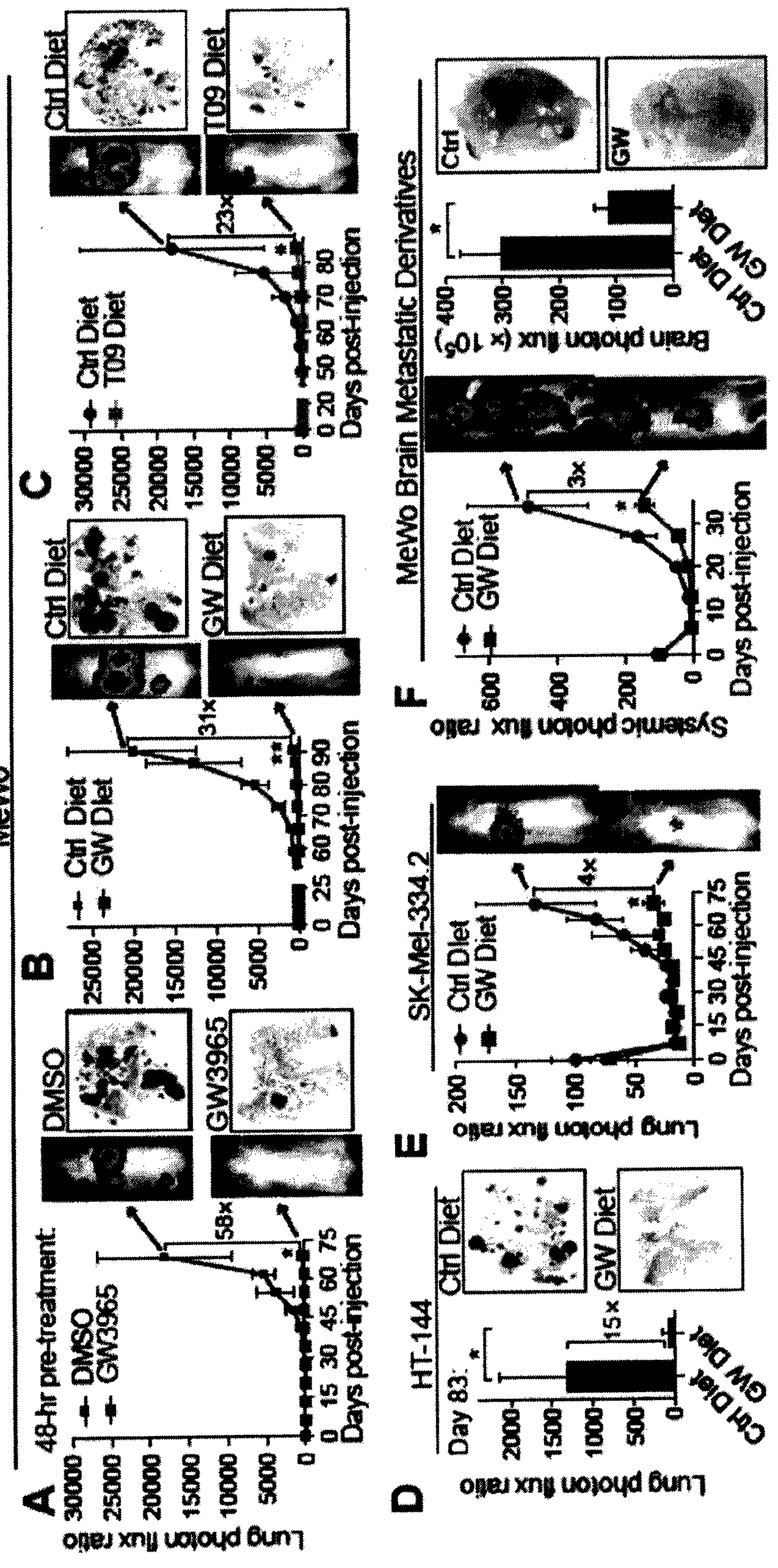


FIGURES 21J-L

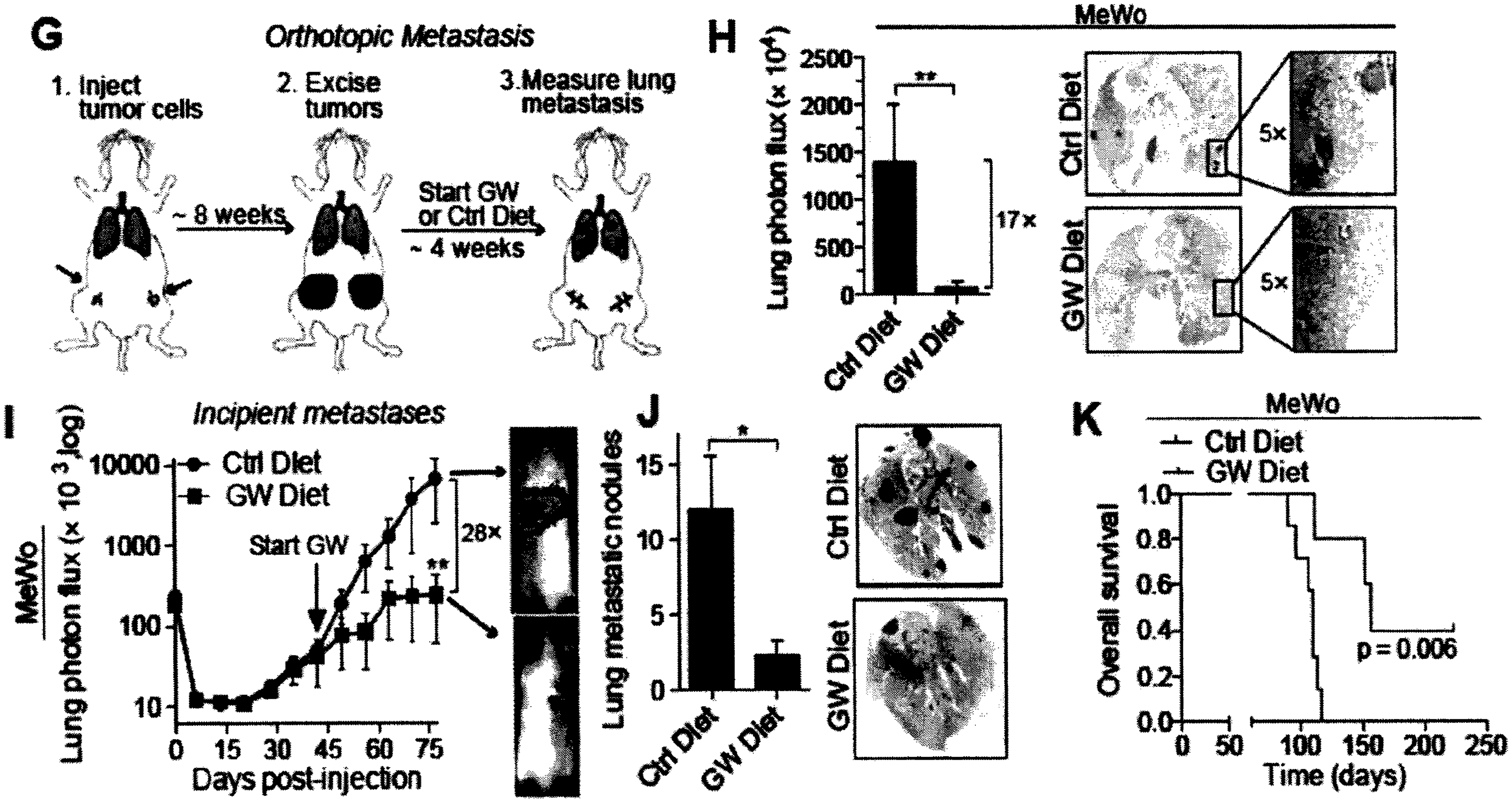


Ctrl Diet
T09 20 mg/kg
GW 20 mg/kg
GW 100 mg/kg
Mouse weight (g)
30
20
10
0
0
10
20
30
40
50
Days on diet

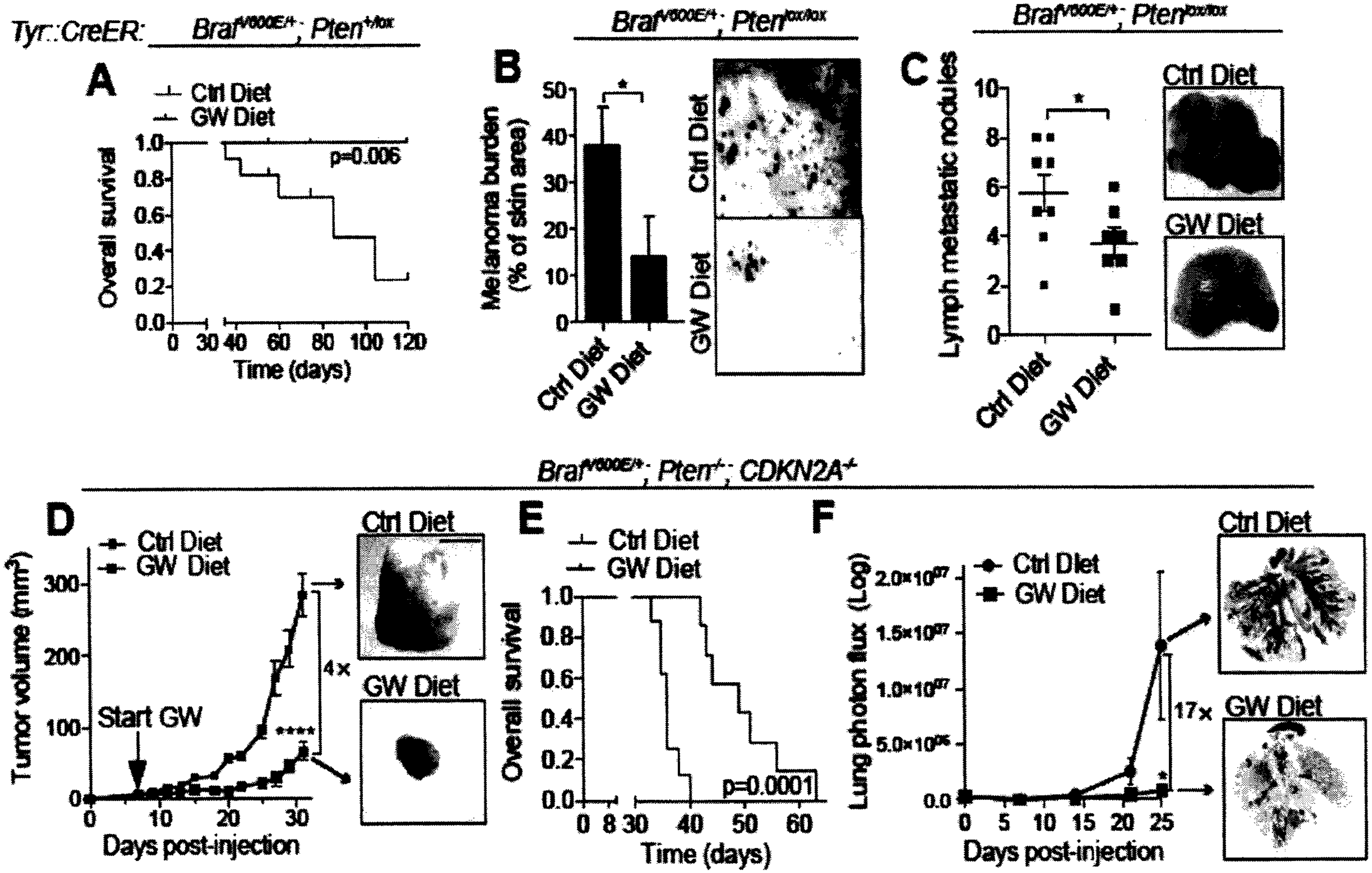
FIGURE 22



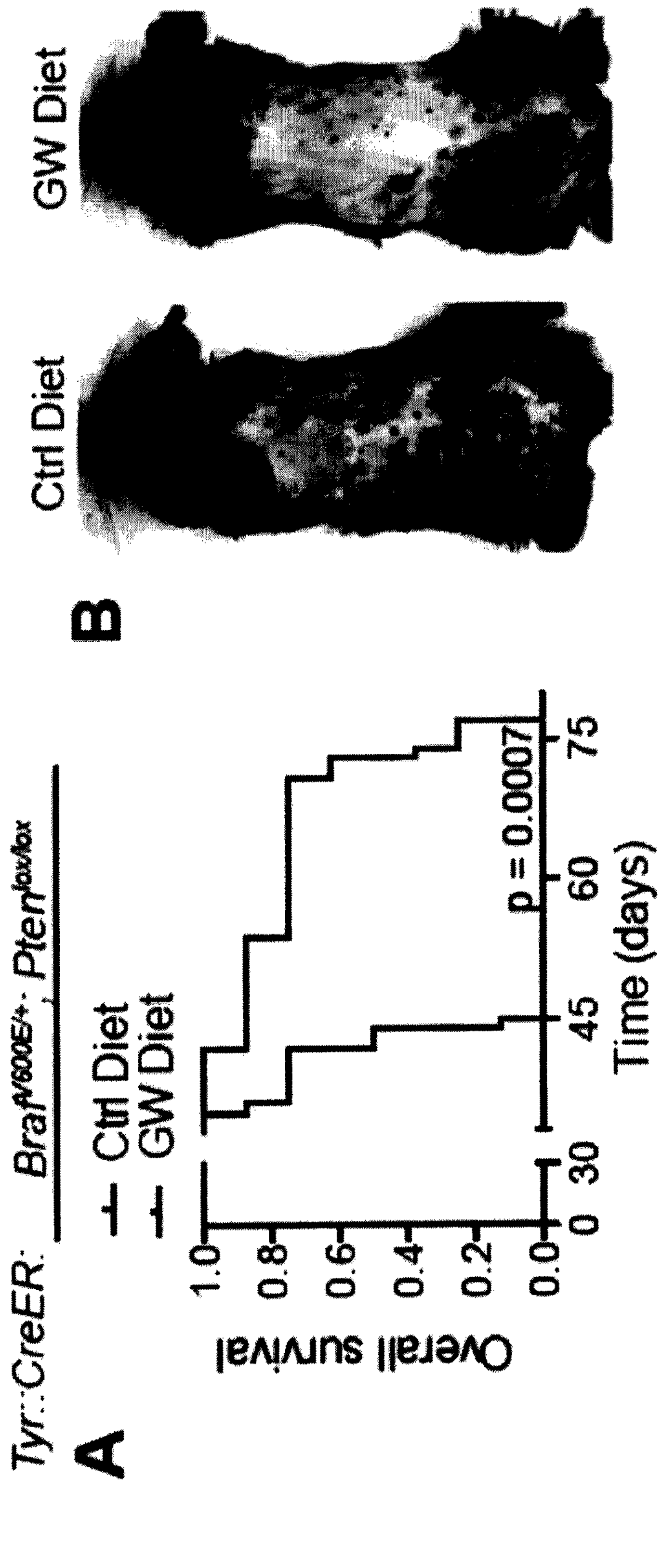
FIGURES 23A-F



FIGURES 23G-K



FIGURES 24A-F



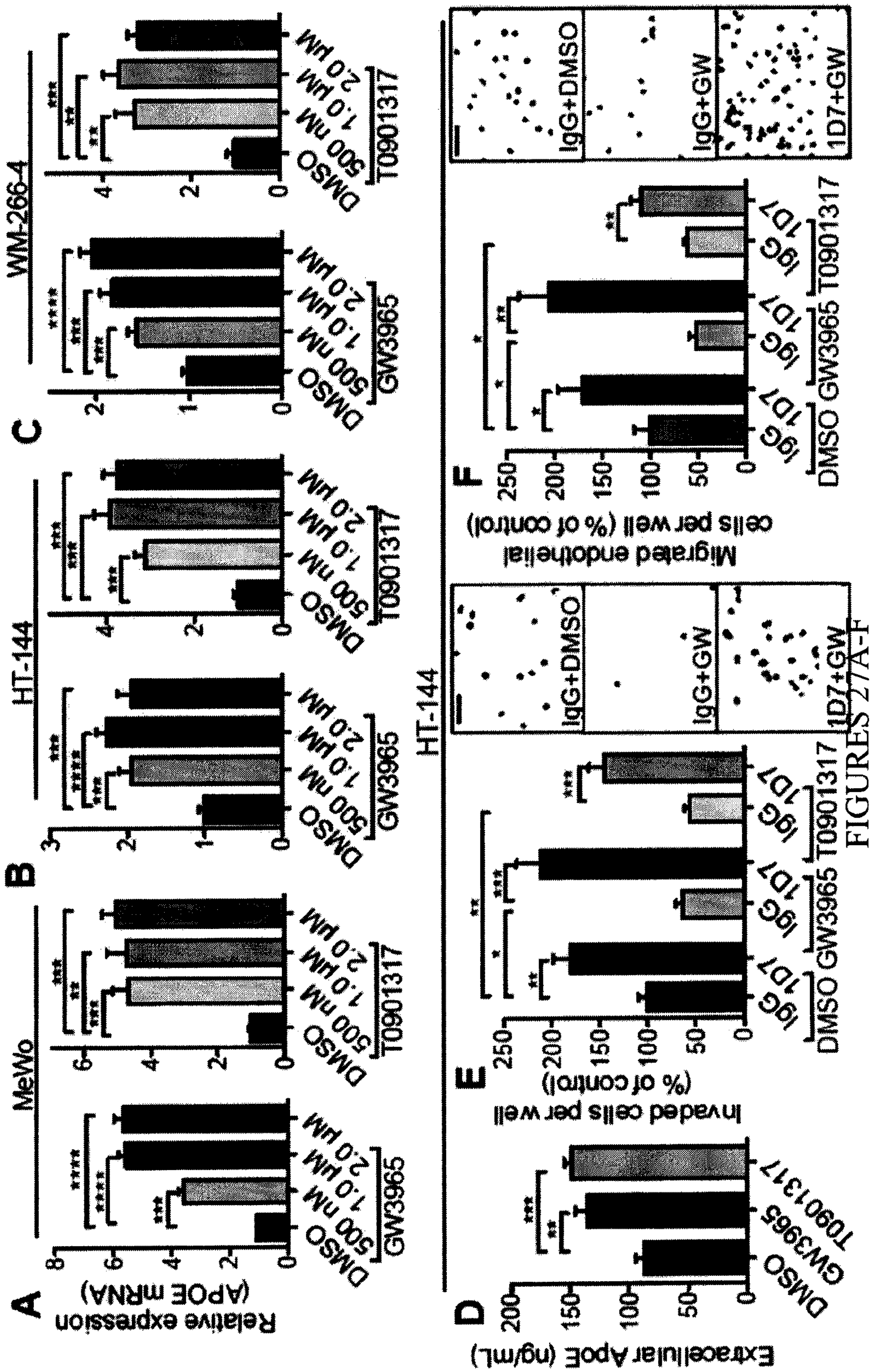
FIGURES 25A-B

Table S1 List of the 50 most upregulated genes in MeWo human melanoma cells in response to GW3965 treatment

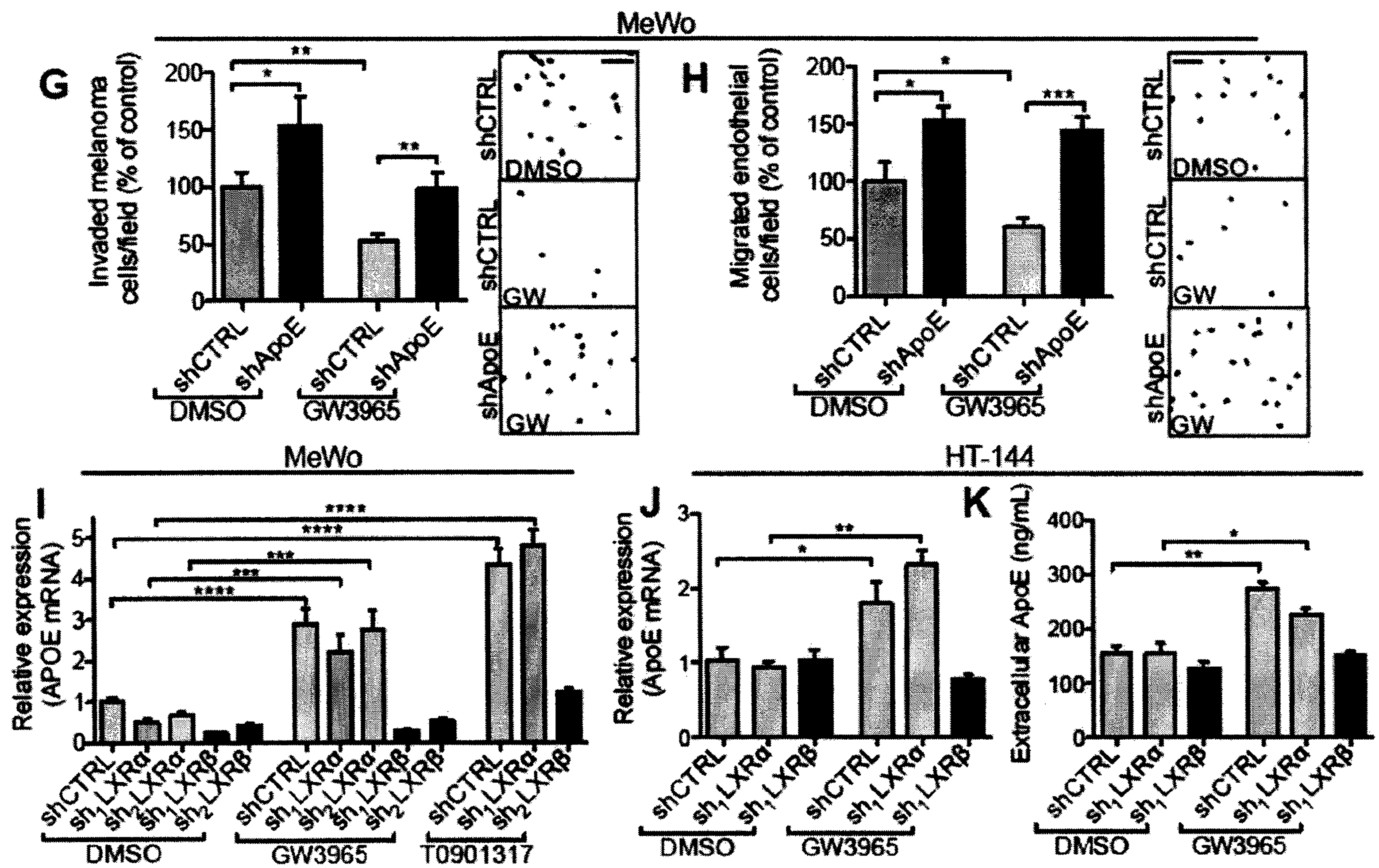
Gene ID	Fold-change*	q-value	Rank	Gene ID	Fold-change*	q-value	Rank
ABCA1	19.34	1.98E-11	1	*CEACAM1*	1.83	5.58E-06	26
SREBF1	8.08	9.12E-15	2	*LDLR*	1.79	1.29E-05	27
FABP7	7.43	1.85E-11	3	*IL1RAPL1*	1.78	1.58E-09	28
LOC645313	4.34	2.25E-10	4	*PPP1R3C*	1.78	1.64E-07	29
APOE	4.11	1.04E-07	5	*ABCD1*	1.76	4.24E-07	30
KRT34	4.09	5.99E-18	6	*C10ORF75*	1.67	2.91E-06	31
FASN	3.72	2.48E-07	7	*LSS*	1.66	1.86E-05	32
IGFBP5	2.91	1.15E-13	8	*DHCR7*	1.64	2.58E-06	33
TF	2.88	2.37E-12	9	*CAPS*	1.63	8.35E-06	34
INSIG1	2.62	3.15E-16	10	*PCYT2*	1.63	3.08E-06	35
MYLIP	2.56	9.21E-07	11	*C5ORF28*	1.62	3.71E-02	36
LPCAT3	2.54	2.80E-15	12	*TMEM119*	1.60	1.15E-04	37
ACACA	2.43	1.56E-12	13	*LPXN*	1.60	1.15E-07	38
SCD	2.42	3.56E-08	14	*SMPDL3A*	1.59	2.39E-04	39
FCRLA	2.19	9.13E-09	15	*SPOCD1*	1.58	7.18E-07	40
FADS1	2.12	2.01E-09	16	*ACLY*	1.58	6.56E-11	41
ACSS2	2.02	1.88E-08	17	*VGLL3*	1.57	1.32E-04	42
SLC2A6	2.02	5.99E-18	18	*MVD*	1.57	1.13E-03	43
ACSL3	1.94	5.35E-10	19	*NAV3*	1.55	2.32E-04	44
TMEM135	1.91	9.06E-07	20	*HS.538962*	1.55	1.40E-07	45
ADM	1.88	3.78E-05	21	*TUBB2B*	1.53	3.46E-08	46
LPIN1	1.88	5.94E-08	22	*LOC728285*	1.53	2.19E-06	47
MID1IP1	1.85	7.48E-09	23	*PHLDA2*	1.52	2.77E-07	48
FDPS	1.84	5.49E-05	24	*APOLD1*	1.51	9.50E-05	49
CCL2	1.83	1.00E-08	25	*BEX1*	1.51	1.08E-09	50

* Fold-upregulation in array-based gene expression for GW3965 treatment (1 μM, 48 hrs) versus DMSO control treatment

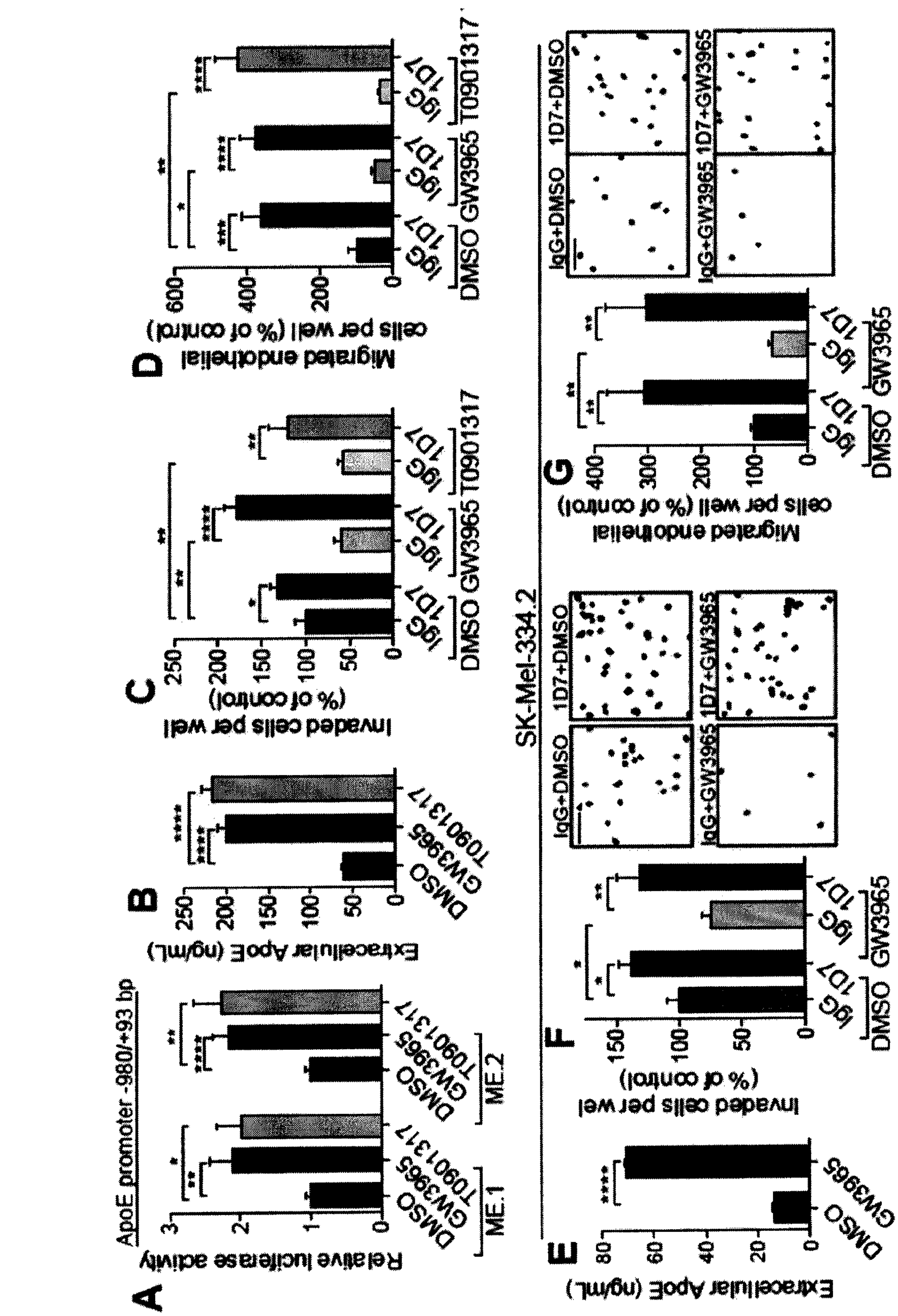
FIGURE 26



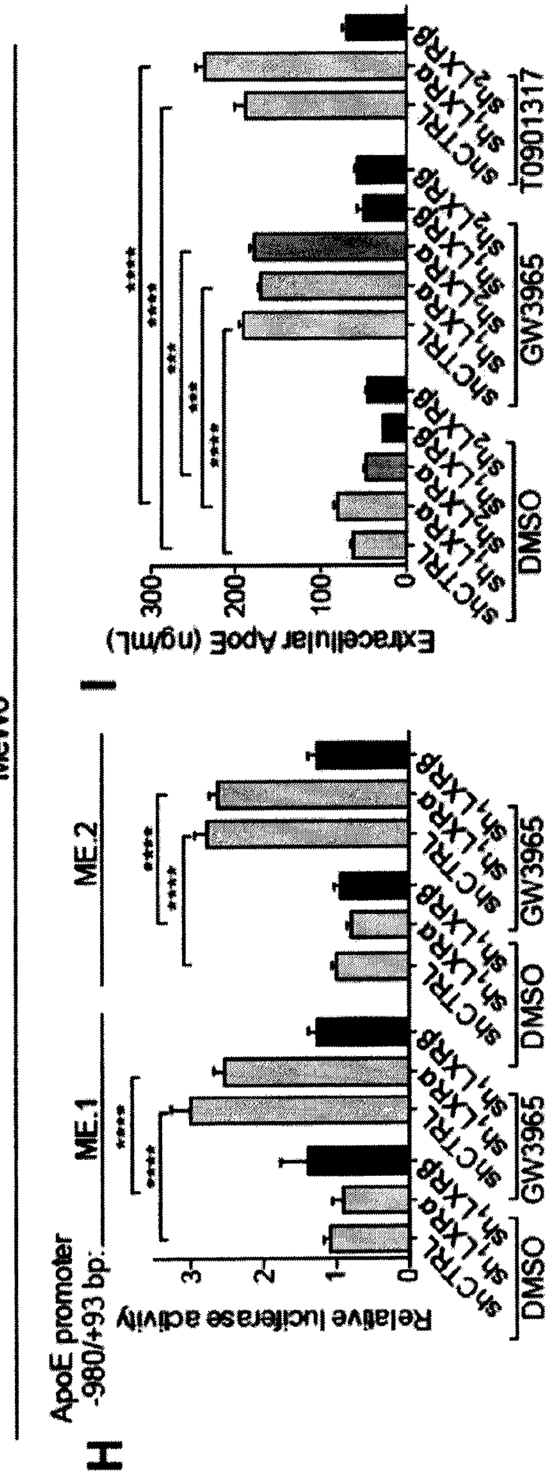
FIGURES 27A-F



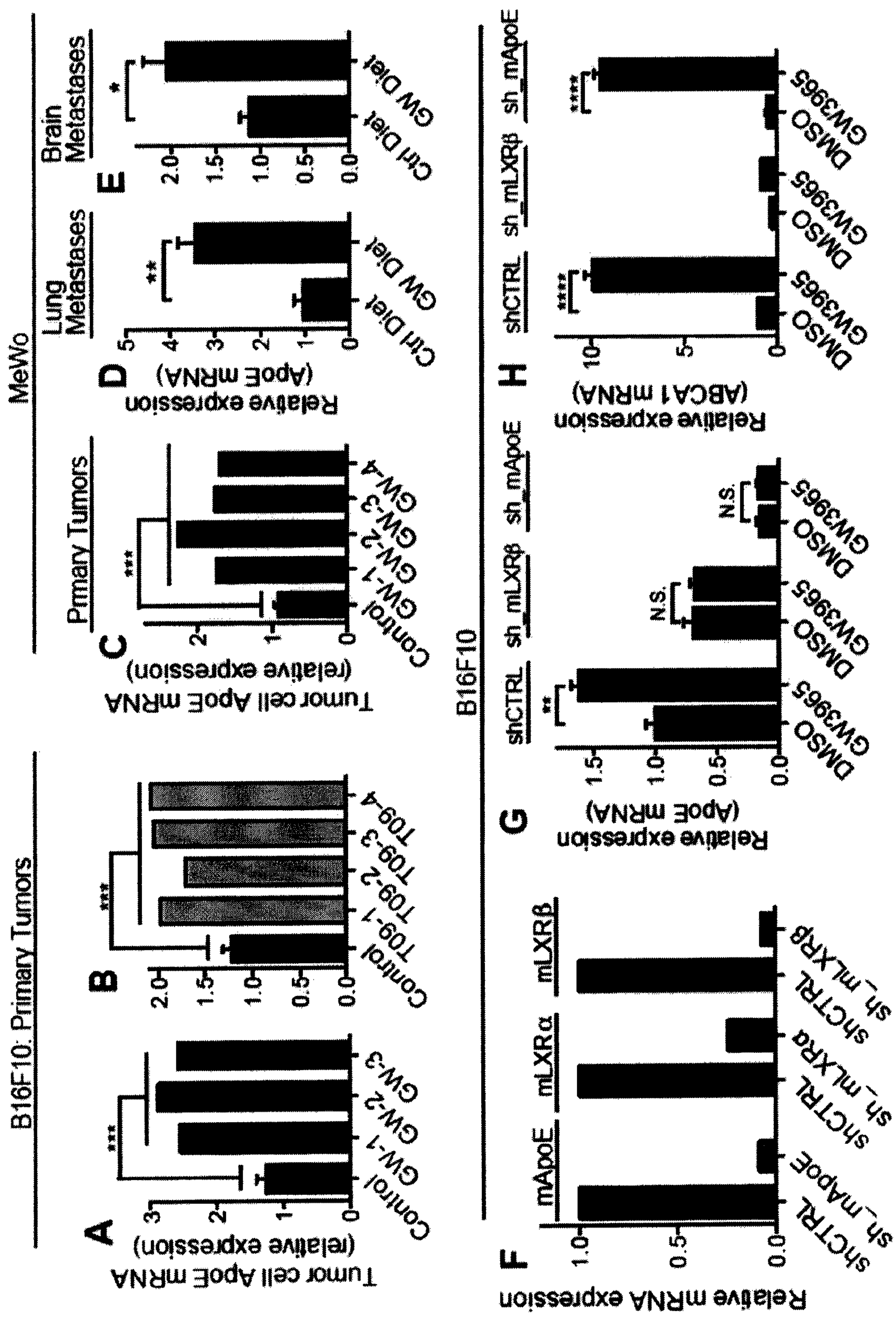
FIGURES 27G-K



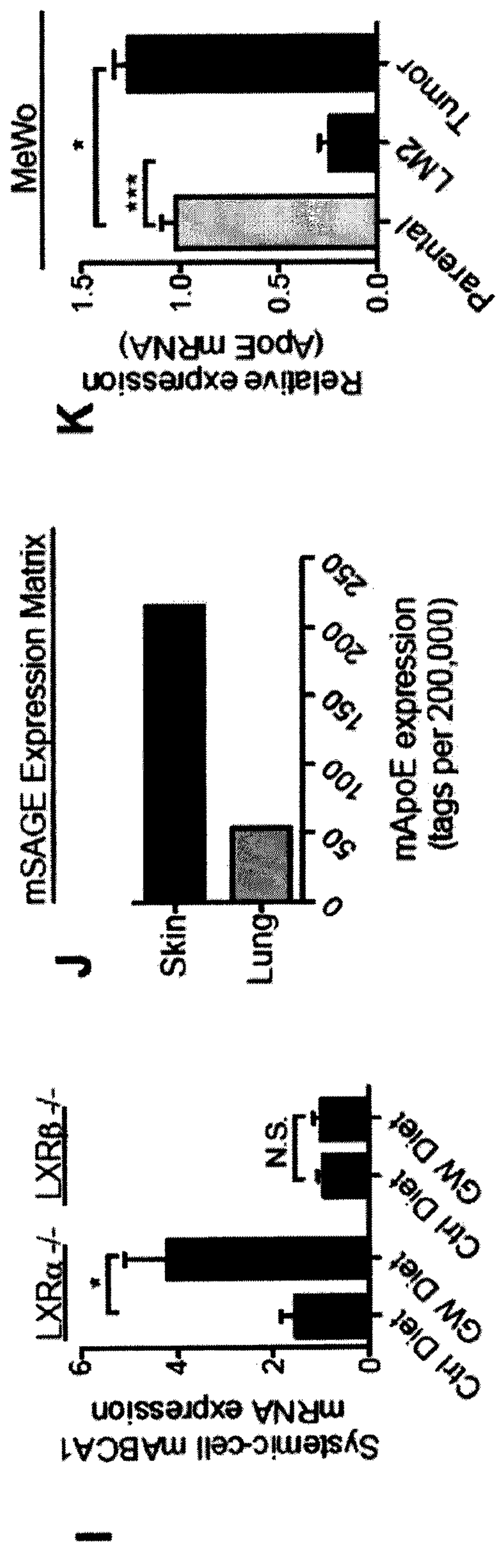
FIGURES 28A-G



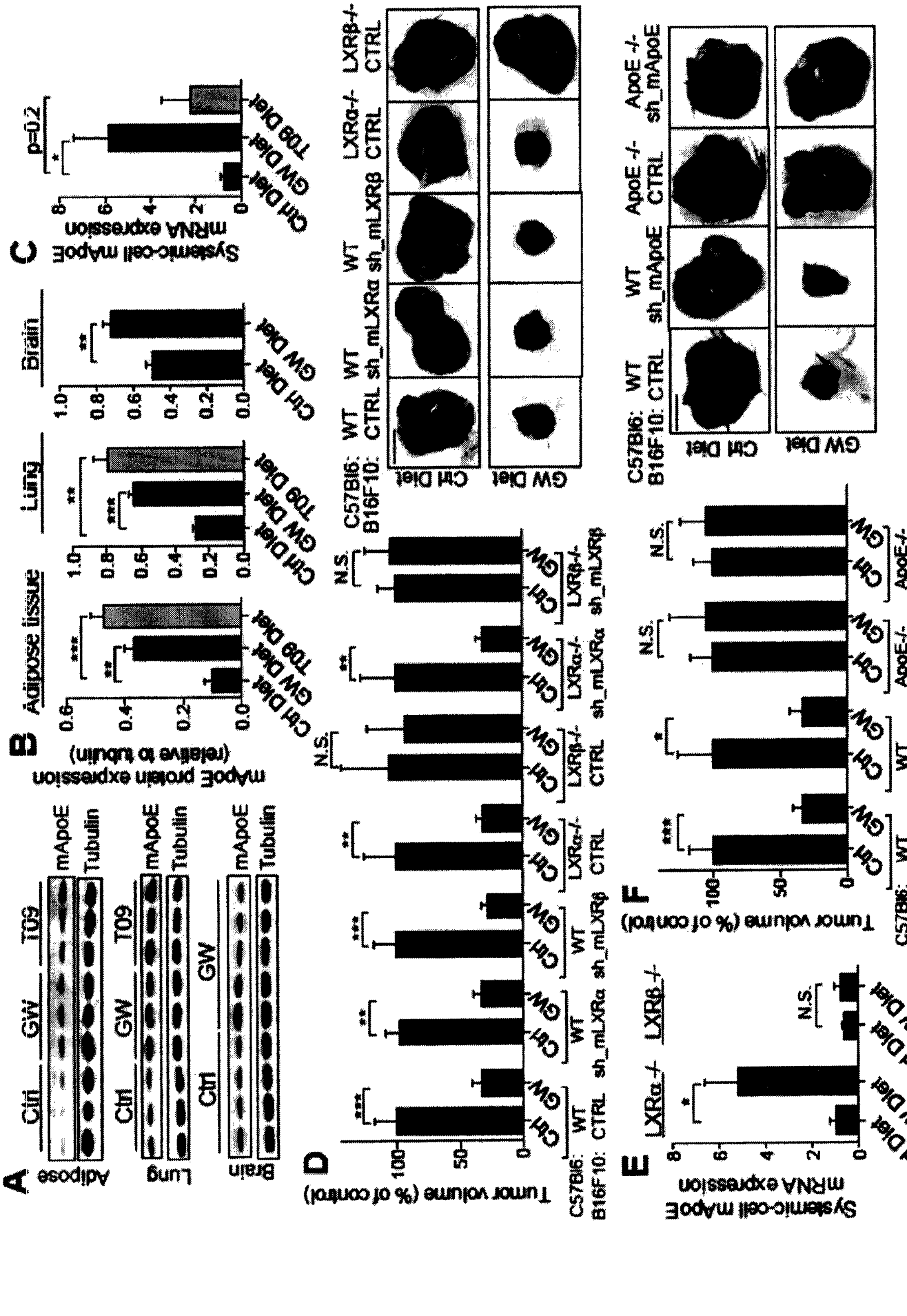
FIGURES 28H-I



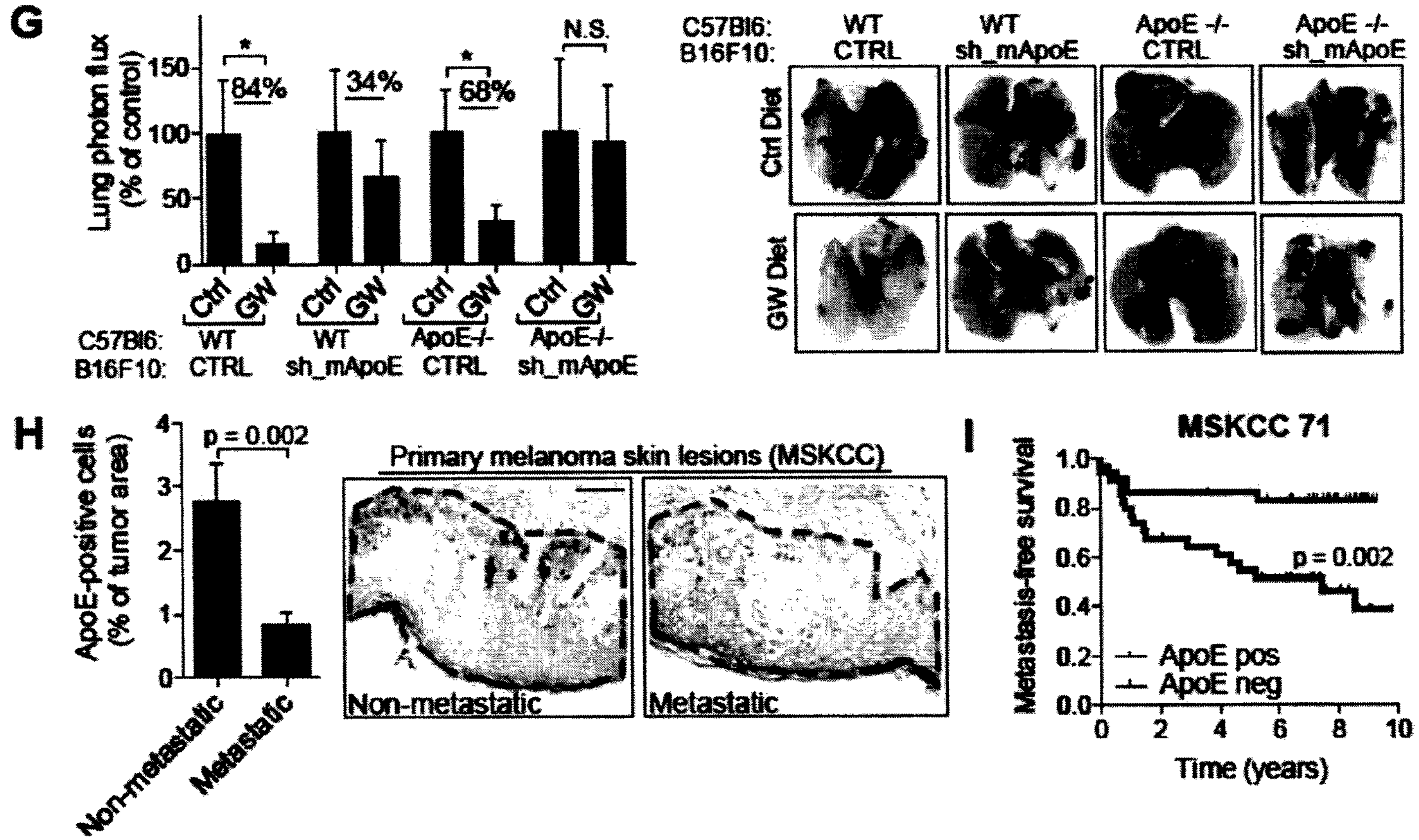
FIGURES 29A-H



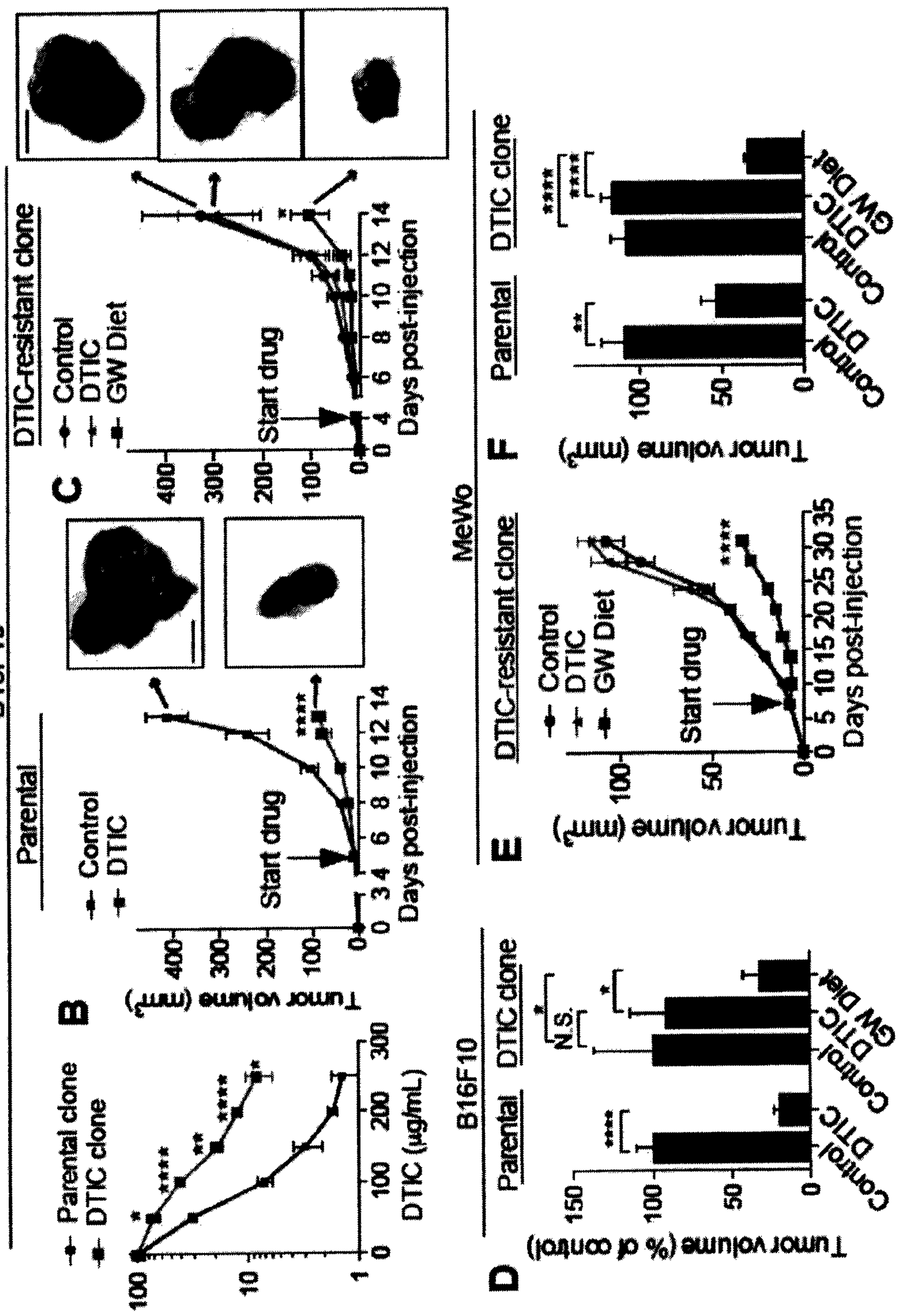
FIGURES 29I-K



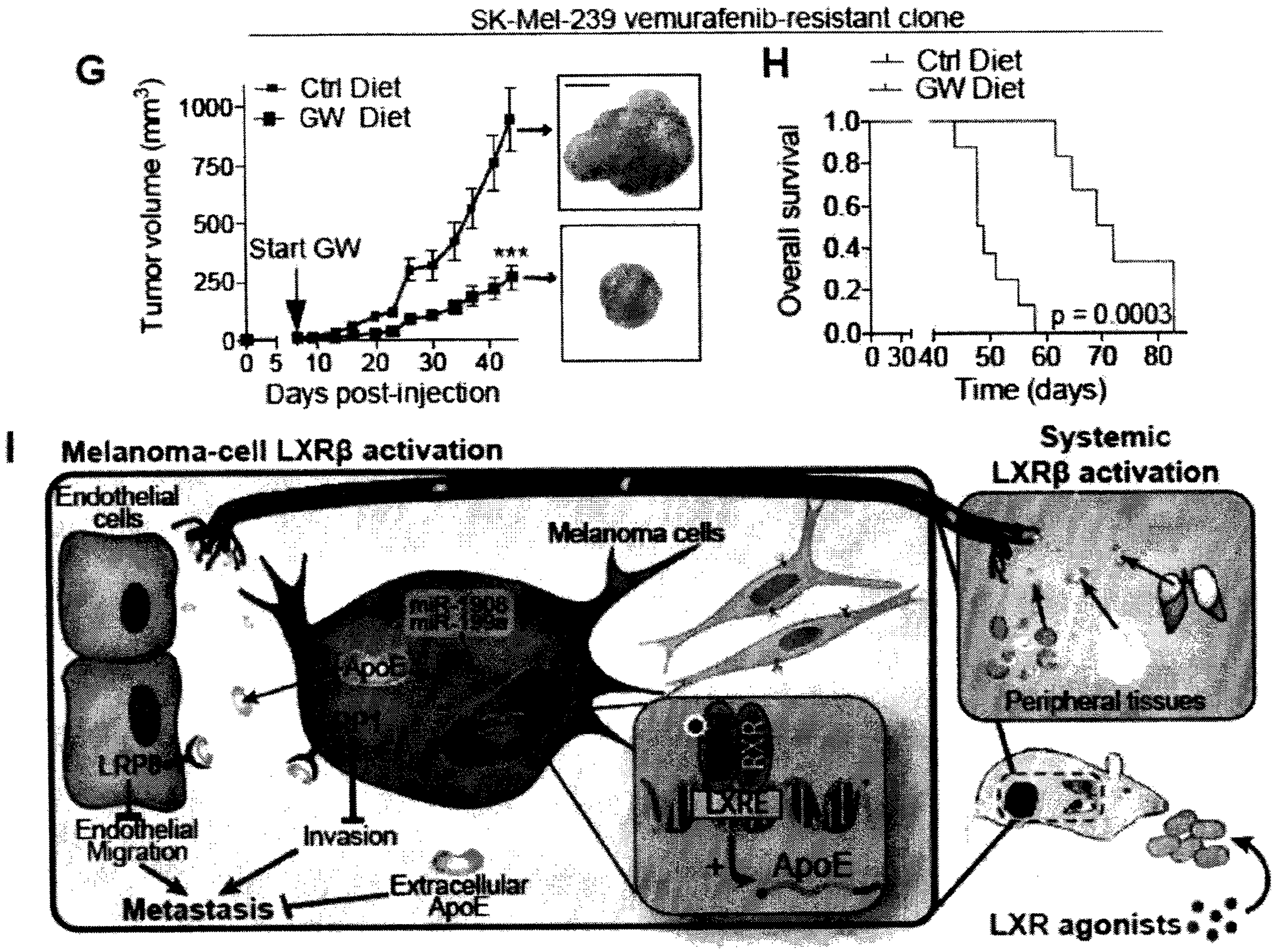
FIGURES 30A-F



FIGURES 30G-I



FIGURES 31A-F



FIGURES 31G-I

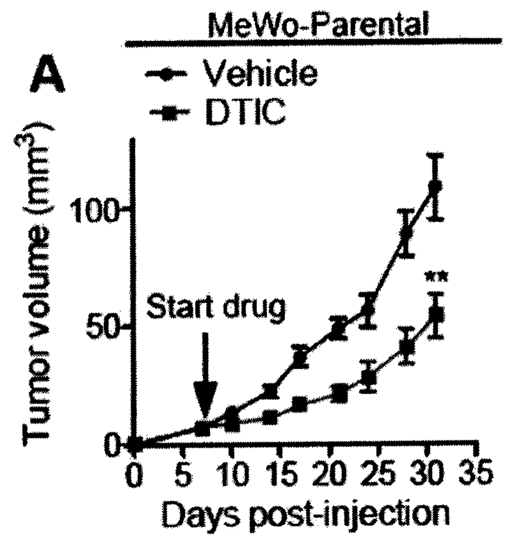
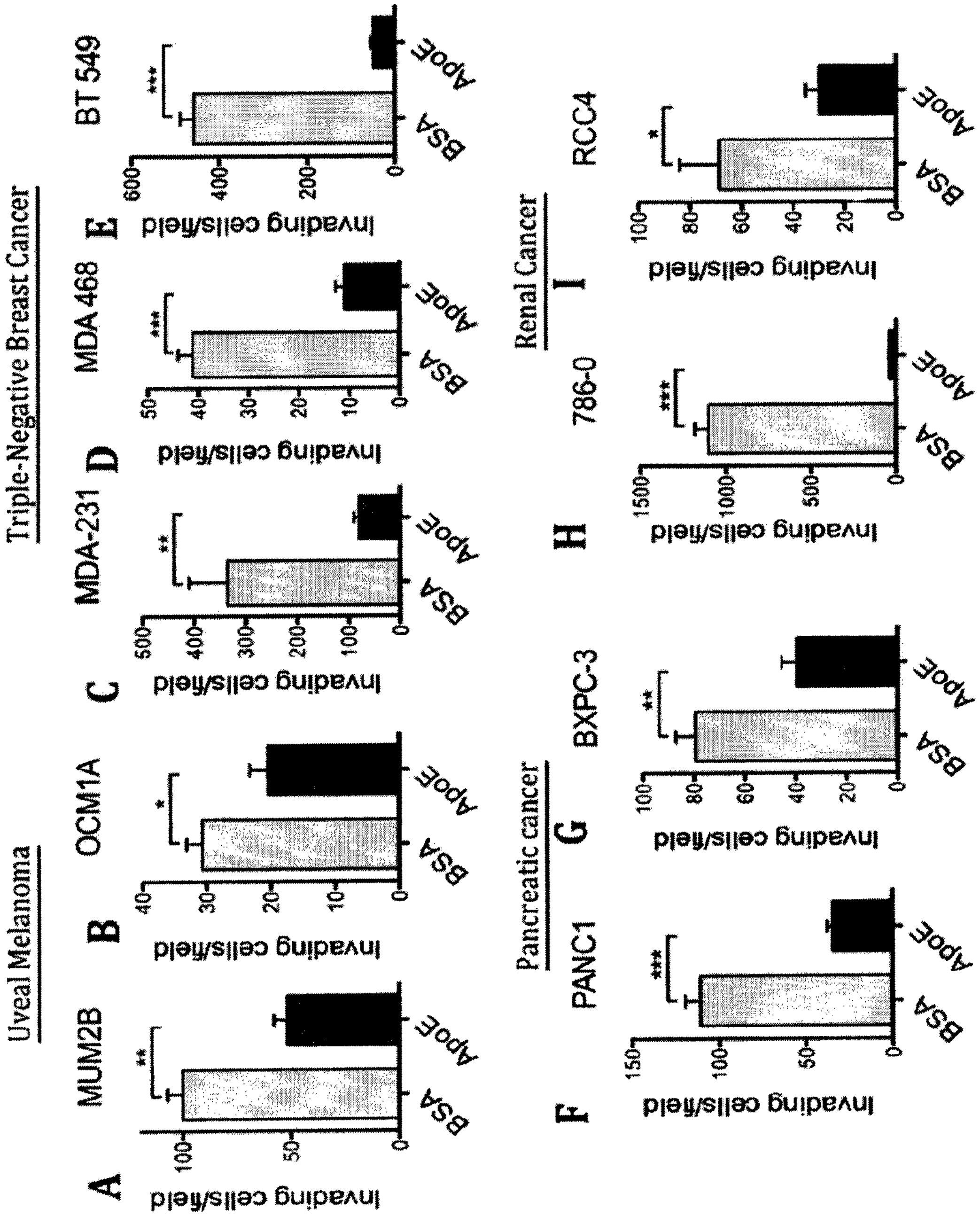
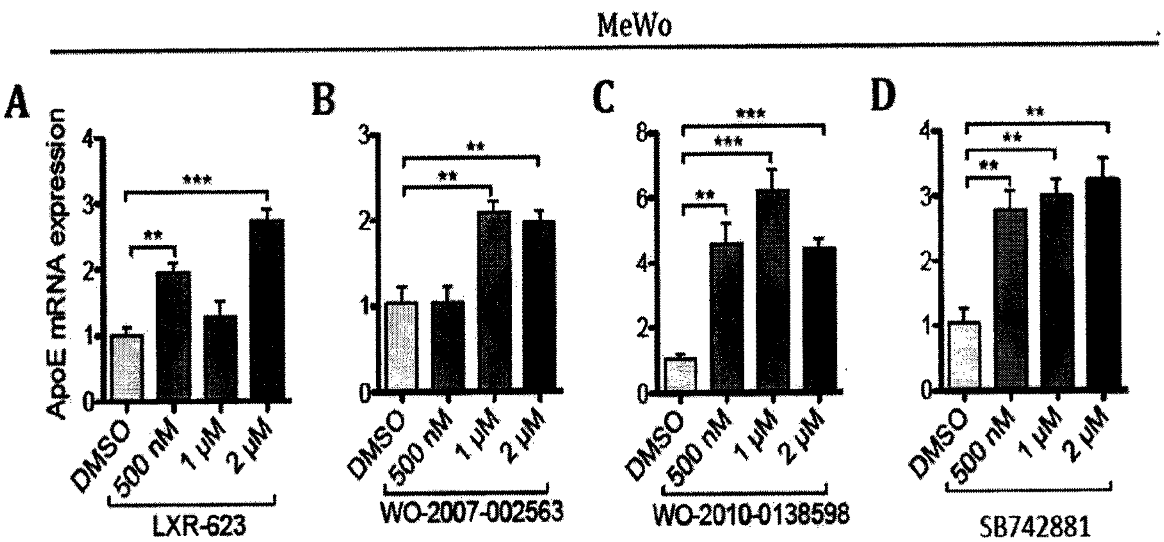

MeWo-Parental
A
Vehicle
DTIC
Tumor volume (mm3)
100
50
0
Start drug
**
0 5 10 15 20 25 30 35
Days post-injection

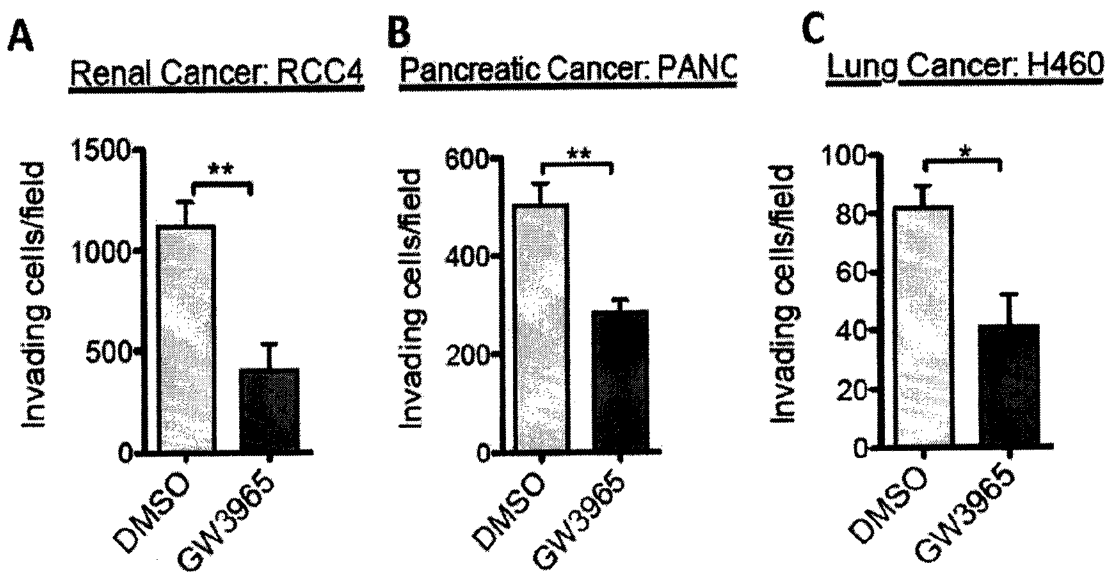
FIGURE 32



FIGURES 33A-I



FIGURES 34A-D



FIGURES 35A-C

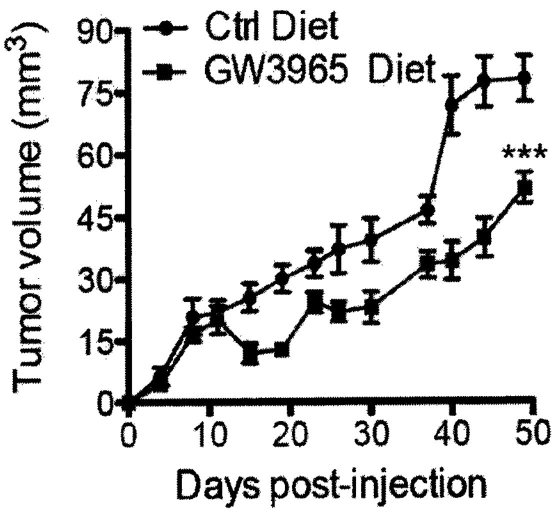
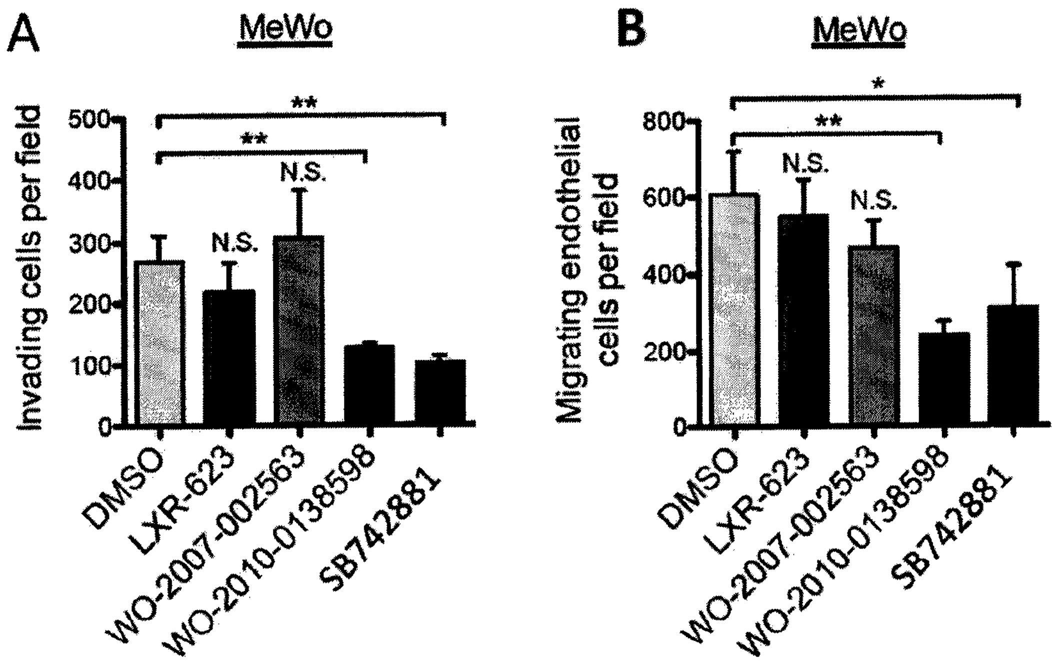
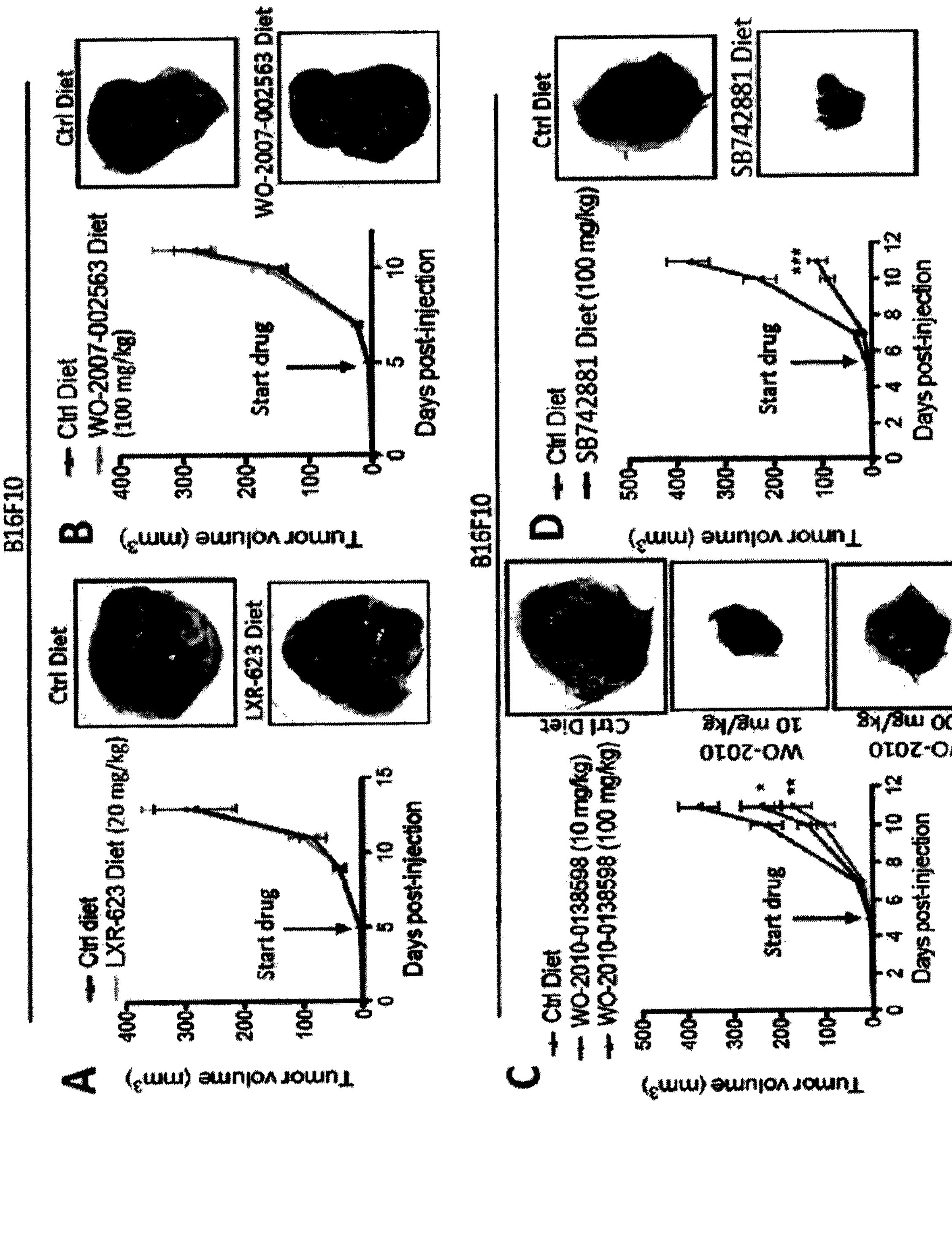


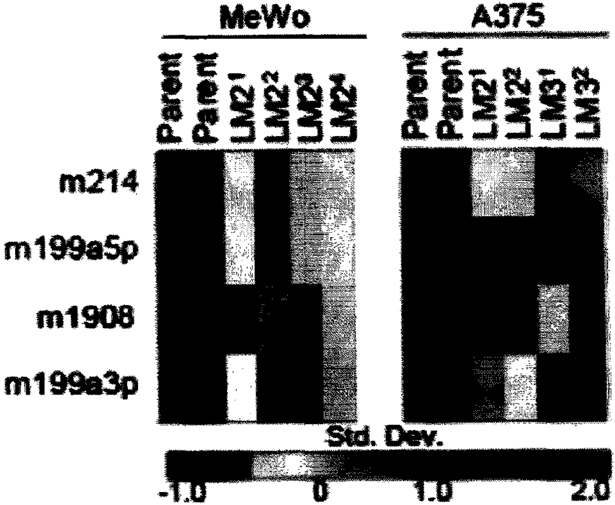
FIGURE 36



FIGURES 37A-B



FIGURES 38A - D


MeWo
A375
Parent
Parent
LM2¹
LM2²
LM2³
LM2⁴
Parent
Parent
LM2¹
LM2²
LM3¹
LM3²
m214
m199a5p
m1908
m199a3p
Std. Dev.
-1.0
0
1.0
2.0