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(54) **Title:** MATERIALS AND METHODS RELATED TO MIR-221 AND HEPATOCELLULAR CARCINOMA

(57) **Abstract:** The present invention provides, inter alia, anti-miR-221 oligonucleotides controlling cancer growth in vivo, methods of treating hepatocellular carcinoma, a miR-221 transgenic mouse model which exhibits increased liver cancer susceptibility, and methods to use the miR-221 mouse model to study the features of human hepatocellular carcinoma.



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TITLE

Materials and Methods Related to miR-221 and Hepatocellular Carcinoma

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CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Provisional Application No. 61/553,399 filed October 31, 2011, the disclosure of which is incorporated herein by reference for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with U.S. Government support under grant number RO1CA135030 awarded by the National Cancer Institute at the National Institutes for Health. The U.S. government has certain rights in the invention.

STATEMENT REGARDING THE SEQUENCE LISTING

[0003] This application is being filed electronically via the United States Receiving Office USPTO EFS-WEB server, as authorized and set forth in MPEP§1730 II.B.2(a)(A), and this electronic filing includes an electronically submitted sequence (SEQ ID) listing. The entire content of this sequence listing is herein incorporated by reference for all purposes. The sequence listing is identified on the electronically filed .txt file as follows: 604_53322_SEQ_LIST_2012-026.txt, created on October 31, 2012 and is 5,004 bytes in size.

FIELD OF THE INVENTION

[0004] This invention is in the field of medicine and drug discovery, particularly in molecular biology related to microRNAs and cancer.

BACKGROUND OF THE INVENTION

[0005] Hepatocellular carcinoma (HCC) is the most common type of liver cancer. Many cases of HCC in humans follow cirrhosis or a viral hepatitis infection. Detection of HCC is variable and may occur at a late stage. Currently available treatment options are limited and are, in general, not specific to the underlying cellular processes.

[0006] There is need for additional tools and animal models for the study of hepatocellular dysplasia, cellular processes of oncogenesis, microRNA involvement in liver cancer, and the effect of toxins, hormones, and viral infection on the liver.

SUMMARY OF THE INVENTION

[0007] In a first aspect, described herein is the use of anti-miR-221 oligonucleotide as an anti-cancer drug.

[0008] In another aspect, described herein is the use of anti-miR-221 oligonucleotides to inhibit tumor growth in a transgenic model. Also described herein is a useful animal model for studying liver pathology and tumorigenesis. The transgenic mouse whose genome comprises a nucleic acid construct capable of overexpressing at least one miR-221 gene in liver cells. The transgenic mouse model exhibits an inappropriate over-expression of miR-221 in liver. This transgenic model is characterized by the appearance of spontaneous liver tumors in a fraction of male mice and a strong acceleration of tumor development in 100% of mice treated with diethylnitrosamine (DEN). This transgenic model represents a tool to perform pre-clinical drug investigations and analysis of toxicity in other industrial applications.

[0009] Also provided is such a transgenic mouse, wherein when the at least one miR-221 gene is overexpressed in liver cells of the transgenic mouse, the mouse develops malignant liver cancer cells.

[0010] Also provided is such a transgenic mouse, wherein a construct comprises a nucleotide sequence having at least 90% sequence identity to **SEQ ID NO: 1** (precursor miR-221) or **SEQ ID NO: 2** (mature miR-221).

[0011] Also provided is such a transgenic mouse, wherein the construct comprises a vector. In certain embodiments, the vector comprises a promoter. Also, in certain embodiments, the promoter comprises a α 1-antitrypsin promoter operably linked to a hepatitis B enhancer.

[0012] Also provided is such a transgenic mouse, wherein the construct comprises pWhere-Ell- α 1AT vector.

[0013] Also provided is such a transgenic mouse, wherein the construct comprises a pWhere-Ell- α 1AT vector comprising at least one operably linked miR-221 sequence.

[0014] Also provided is such a transgenic mouse, wherein the construct comprises a pWhere-Ell- α 1AT vector comprising at least one operably linked nucleic acid of **SEQ ID NO: 1** (precursor miR-221) and/or **SEQ ID NO: 2** (mature miR-221).

[0015] Also provided is such a transgenic mouse, wherein the mouse develops hepatocellular carcinoma. In certain embodiments, the transgenic mouse is male.

[0016] Also provided is such a transgenic mouse, which further exhibits high levels of steatosis.

[0017] Also provided is such a transgenic mouse, which further comprises an attribute selected from the group consisting of: repression of Cdkn1b/p27; repression of Cdkn1c/p57; repression of Bmf proteins; upregulation of miR-221; downregulation of miR-122; downregulation of miR-199; and upregulation of miR-21.

[0018] In another aspect, described herein are methods of determining whether a test agent affects a liver condition in a subject, comprising: a) administering a test agent to a transgenic mouse; and b) after the agent has been administered to the transgenic mouse, comparing one or more

symptoms and/or indications of a liver condition in the transgenic animal to those of a control animal of the same genotype, wherein the control animal has not been administered the agent, wherein a difference in the detectability and/or rate of appearance of the one or more symptoms and/or indications of the liver condition in the transgenic animal, relative to the control animal, is indicative of the test agent affecting the liver condition.

[0019] In certain embodiments, a liver tumor-inducing treatment is administered concurrent with, or after, step a.

[0020] In certain embodiments, the liver tumor-inducing treatment is diethylnitrosamine.

[0021] In certain embodiments, the liver condition hepatocellular carcinoma. In certain embodiments, the liver condition is visible tumor, pseudoglandular, or trabecular cell growth.

[0022] In certain embodiments, the liver condition basophilic cell invasion.

[0023] In certain embodiments, the one or more symptoms and/or indications of the liver condition are selected from the group consisting of: enlarged abdomen; externally-visible lumps; body weight loss; and a combination thereof.

[0024] In certain embodiments, one of more symptoms and/or indications of the liver condition comprises an attribute selected from the group consisting of: repression of Cdkn1b/p27; repression of Cdkn1cp57; repression of Bmf proteins; up-regulation of miR-221; down-regulation of miR-122; down-regulation of miR-199; and up-regulation of miR-21.

[0025] In certain embodiments, the transgenic mouse is further compared or evaluated with that of a wild type mouse.

[0026] In another aspect, described herein are methods for screening an inducer/promoter or an inhibitor of a liver condition, wherein an alteration of liver morphology, molecular biology or function is determined after administering a test substance to the transgenic mouse herein or contacting a tissue, an organ or cells comprising cells derived from the transgenic mouse herein with the test substance.

[0027] In another aspect, described herein are methods of testing the therapeutic efficacy of a test agent in treating or preventing a liver condition in a subject comprising: (a) administering a test agent to a transgenic mouse; and (b) after the agent has been administered to the transgenic animal, comparing one or more symptoms and/or indications of the liver condition in the transgenic animal with those of a control animal of the same genotype, wherein the agent has not been administered to the control animal; wherein if the agent inhibits, prevents and/or reduces the one or more symptoms and/or indications of the liver condition in the transgenic animal, relative to the control animal, then the agent is considered to have therapeutic efficacy in treating or preventing a liver condition in a subject .

[0028] In certain embodiments, at least one anti-miR-221 gene is administered to the transgenic mouse.

[0029] In another aspect, described herein are methods for suppressing a liver cancer,

comprising: contacting a liver cell with at least one antisense miR-221 oligonucleotide, thereby suppressing a liver cancer oncogene.

[0030] In another aspect, described herein are at least one anti-miR-221 gene comprises a nucleotide sequence having at least 90% sequence identity to **SEQ ID NO: 5** (anti-precursor miR-221) and/or **SEQ ID NO: 6** (anti-mature miR-221).

[0031] In certain embodiments, the transgenic mouse that is treated is compared/evaluated to a transgenic mouse that has not been treated.

[0032] In certain embodiments, the alteration of liver morphology, molecular biology or function comprises an attribute selected from the group consisting of: hepatocellular carcinoma; visible tumor, pseudoglandular, or trabecular cell growth; basophilic cell invasion; upregulation of miR-221; repression of Cdkn1b/p27; repression of Cdkn1c/p57; repression of Bmf proteins; downregulation of miR-122; downregulation of miR-199; and upregulation of miR-21; enlarged abdomen; externally-visible lumps; body weight loss; and a combination thereof.

[0033] In another aspect, described herein are methods to ameliorate the effects of miR-221 on liver cells, comprising administering at least one anti-miR-221 oligonucleotide to liver cells.

[0034] In certain embodiments, the anti-miR-221 oligonucleotide is modified to further comprise a stabilizing group.

[0035] In certain embodiments, the anti-miR-221 oligonucleotide is modified to further comprise 2'-O-methyl RNA bases with a phosphothioate bond.

[0036] In another aspect, described herein are methods of ameliorating the risk of liver cancer in a subject, comprising: administering a pharmacologically effective amount of **SEQ ID NO: 5** (anti-precursor miR-221) and/or **SEQ ID NO: 6** (anti-mature miR-221) and/or **SEQ ID NO: 7** (modified anti-miR-221 with 2-O-methyl RNA bases and phosphorothioate bonds) to a subject with elevated miR-221, and ameliorating the risk of liver cancer in the subject.

[0037] In certain embodiments, the suppressed liver cancer oncogene is a miR-221 gene product.

[0038] In certain embodiments, the methods further suppress liver cancer cell proliferation, liver cancer cell growth, or liver cell tumor development.

[0039] Also described herein are methods of ameliorating the symptoms of liver cancer in a subject, comprising: administering a pharmacologically effective amount of **SEQ ID NO: 5** (anti-precursor miR-221) and/or **SEQ ID NO: 6** (anti-mature miR-221); and/or **SEQ ID NO: 7** (modified anti-miR-221 with 2-O-methyl RNA bases and phosphorothioate bonds) to a subject with liver cancer symptoms, and ameliorating the liver cancer symptoms in the subject.

[0040] Also described herein are methods of reducing liver tumor growth in a subject, comprising: administering a pharmacologically effective amount of **SEQ ID NO: 5** (anti-precursor miR-221) and/or **SEQ ID NO: 6** (anti-mature miR-221); and/or **SEQ ID NO: 7** (modified anti-miR-221 with 2-O-methyl RNA bases and phosphorothioate bonds) to a subject with liver tumors, and

reducing liver tumor growth in the subject.

[0041] In certain embodiments, the methods further comprise administering a chemotherapeutic agent or conducting cancer or tumor resection surgery.

[0042] In certain embodiments, the subject is selected from the group consisting of: mouse; rat; guinea pig; cat; dog; horse; cow; pig; and human.

[0043] In certain embodiments, the cancer or tumor is hepatocellular carcinoma.

[0044] Other systems, methods, features, and advantages of the present invention will be or will become apparent to one with skill in the art upon examination of the following drawings and detailed description. It is intended that all such additional systems, methods, features, and advantages be included within this description, be within the scope of the present invention, and be protected by the accompanying claims

BRIEF DESCRIPTION OF THE FIGURES

[0045] The patent or application file may contain one or more drawings executed in color and/or one or more photographs. Copies of this patent or patent application publication with color drawing(s) and/or photograph(s) will be provided by the U.S. Patent and Trademark Office upon request and payment of the necessary fees.

[0046] **Figures 1A-1B. Diagram and expression of the vector pWhere-EII- α 1AT-miR-221.**

[0047] **Figure 1A** shows schematic map of a vector showing the two H19 insulators (H19 ins); the hybrid α 1Anti-trypsin promoter coupled with the enhancer II sequence of human hepatitis B virus (EII- α 1AT); the mmu-mir-221 miRNA locus (miR-221); gene reporter β -galactosidase without CpG dinucleotides (LacZ Δ CpG NLS); poly-adenylation site (EF1 pAn).

[0048] **Figure 1B** shows the expression of miR-221 in the livers of transgenic and wild type mice at the age of 9 or 12 months. Significance of the difference is shown as p-values.

[0049] **Figures 2A-2D. Histology liver characterization.** Liver histology of wild type (**Figure 2A**) and transgenic mice (**Figure 2B**) is shown by hematoxylin and eosin staining. Transgenic livers were characterized by variable extents of steatohepatic changes, with hepatocyte degeneration characterized by lipidic vacuoli. Oil Red staining for lipid and fat was performed on frozen sections of wild type (**Figure 2C**) and transgenic livers (**Figure 2D**). Red dots are lipids, while nuclei appear in a pale blue.

[0050] **Figures 3A-3C. miR-221 gene targets exhibit a reduced expression in the livers of transgenic mice.**

[0051] **Figure 3A** shows that miR-221 “seed” matches with 3’UTR sequences present in mouse Cdkn1b/p27, Cdkn1c/p57 and Bmf genes. **Figure 3A** discloses SEQ ID NOS: 18, 4, 19, 4, 20 and 4, respectively, in order of appearance.

[0052] **Figure 3B** shows Western blot analyses of p27, p57 and Bmf target proteins and β -actin in the normal livers of transgenic (TG NL) and wild type (WT NL) mice.

[0053] **Figure 3C** shows protein expression was quantified and normalized versus the levels of β -actin. The p-values of the comparisons are shown in each panel.

[0054] **Figures 4A-4D. miR-221 over-expression in mouse liver is correlated with development of cancer.** At 6 months of age, following DENA treatment, transgenic mice exhibited an increased number and size of tumors than control wild type mice.

[0055] **Figure 4A** shows the distribution of the number of nodules shown in the Table on the left side of the figure.

[0056] **Figure 4B** shows the tumor burden, deduced by the weight of the livers, is significantly increased in transgenic mice.

[0057] **Figure 4C** shows that body weight is significantly reduced in transgenic mice bearing tumors.

[0058] **Figure 4D** shows examples of liver tumors in a transgenic or wild type male mouse at 6 months of age exposed to DENA (**Figure 4D**, left panel and center panel, respectively), in comparison with a normal liver (**Figure 4D**, right panel).

[0059] **Figures 5A-5D. miRNA and gene expression analysis in HCCs versus normal liver of miR-221 transgenic mice.** (**Figures 5A-5D**) MicroRNA expression analysis in liver cancer versus normal livers revealed a statistically significant difference of expression levels: miR-221 and miR-21 exhibited an increased expression, while miR-122 and miR-199 exhibited a down-regulation.

[0060] **Figures 6A-6D. Cdkn1b/p27, Cdkn1c/p57 and Bmf inhibition by miR-221 in HCCs versus normal livers.**

[0061] **Figure 6A** shows the proteins levels in tumor and normal tissues of transgenic mice were evaluated by Western Blot analysis, revealing a strong reduction of all the analyzed miR-221 target genes in HCC tissues.

[0062] **Figures 6B-6D** show the protein expression data were normalized versus the levels of β -actin and the p-values of the comparisons are shown in each panel.

[0063] **Figures 7A-7F. Short and long-term expression of miR-221 in livers after *in vivo* delivery of anti-miR-221 oligonucleotides and effect on tumorigenicity.** A group of transgenic mice were injected intraperitoneally with DENA at 10 days of age and after 2 months they received three consecutive every 15 days intravenous injection into their tail vein of an anti-miR-221 synthetic oligonucleotide.

[0064] **Figure 7A** shows that forty eight hours after first injection, liver and blood of three mice were harvested to evaluate miR-221 expression levels as a result of the treatment. Quantitative PCR analysis revealed a significant decrease in miR-221 amounts in the liver of treated mice in comparison with untreated ones. **Figure 21** indicates a similar reduction in serum.

[0065] **Figures 7B-7C** shows the reduced level of miR-221 correlated with an increase in Cdkn1b/p27 target protein levels. Protein expression data were normalized versus the levels of β -tubulin and the p-value of the comparisons is shown.

[0066] **Figure 7D** shows that, at 4 months (120 days of age), AMOs treated and untreated mice were sacrificed. Even macroscopically, number and size of tumors appeared to be smaller in treated mice (**Figure 7D**, three columns on the right side). An experimental timeline shows the time of DENA (10 days) and anti-miR-221 (60, 75 and 90 days) treatments and the time of final sacrifices (120 days). (**Figure 7D**, bottom row) Hematoxylin and eosin–stained histological sections confirmed the results (magnification, x20; visible nodules are indicated by arrows).

[0067] **Figures 7E-7F** shows histological analyses which confirm that the number of liver lesions of treated mice were significantly fewer than in control animals (**Figure 7E**), and tumor size of the nodules was smaller (**Figure 7F**).

[0068] **Figure 7G** shows that, in tumors of AMOs treated mice, the expression of miR-221 revealed a significant long-term reduction in comparison with tumors developed in control animals.

[0069] **Figure 8. Progressive development of HCC in transgenic female mice.** Transgenic and wild type female mice were treated intraperitoneally with DENA at 10 days of age and sacrificed at different time-points (6, 9 and 12 months of age). The number of visible tumors was clearly higher in transgenic animals (TG) than in wild type control animals (WT).

[0070] **Figures 9A-9B. Analysis of HCC tissues of female transgenic mice.** HCC tissues of female transgenic mice analyzed by Western Blot analysis exhibited a significant reduction of Cdkn1b/p27 protein in comparison with normal livers.

[0071] **Figure 9C** shows the quantitative PCR analysis revealed a higher expression of miR-221 in tumor tissues than in matched normal livers. Protein expression data were normalized versus β -tubulin levels.

[0072] **Figure 10. Western Blot of Bmf inhibition by miR-221 in HCCs versus normal livers.** Bmf protein levels were evaluated in tumor and normal tissues of transgenic mice by Western Blot. A reduction of Bmf was generally detected in tumor tissues.

[0073] **Figure 11. Bmf inhibition by miR-221 in HCCs versus normal livers.** The relative density represents the Bmf density values normalized on β -actin. The difference of expression was significant.

[0074] **Figures 12A-12C. Long-term effect of anti-miR-221 oligonucleotides on tumorigenicity.** A group of transgenic mice injected intraperitoneally with DENA at 10 days of age received after two months three consecutive intravenous injection into the tail vein of an anti-miR-221 synthetic oligonucleotide.

[0075] **Figure 12A** shows where the animals were sacrificed at 5 months (150 days of age) and analyzed in comparison with DENA-only treated mice (**Figure 12A**, left panel). Similar to what observed at 4 months, the number of liver lesions and the size of the nodules were significantly reduced in treated mice; see **FIGS. 12B-12C**).

[0076] **Figures 13A-13B. Immunohistochemistry of Cdkn1b/p27 in the liver of transgenic and wild type mice.**

[0077] **Figure 13A** shows that, in transgenic mice, no or poor p27 accumulation in hepatocytes nuclei was detectable and a vacuolar degeneration of cytoplasm was evident.

[0078] **Figure 13B** shows livers of wild type mice displayed a well defined nuclear p27 immunostaining and did not show the vacuolar degeneration observed in transgenic animals. 20x magnification.

[0079] **Figures 14A-14B. Transgenic and wild type normal livers differ in gene expression.** A different molecular background between the livers of transgenic versus wt mice was confirmed by differences in gene expression profiles.

[0080] **Figure 14A** shows hierarchical clustering of gene expression in normal livers of transgenic mice versus wild type mice. The list of 473 differentially expressed genes is shown in **Figure 22**. The colors of the genes represented on the heat map correspond to the expression values normalized on mean expression across all samples: green means down-regulated, red means up-regulated in the sample.

[0081] **Figure 14B** shows functional analysis revealed that many differentially expressed genes were involved in the regulation of the interferon gamma (Ifng) gene, which is expressed at lower level in the livers of transgenic animals, showing an important role of this pathway in the different liver phenotypes.

[0082] **Figures 15A-15D. Spontaneous liver tumors in miR-221 transgenic mice.**

[0083] **Figure 15A** shows that some transgenic male mice developed spontaneous tumors at 9-12 months of age.

[0084] **Figure 15B** shows the expression of miR-221 analyzed by quantitative PCR analysis, showed higher levels in HCC tissues than in matched normal livers.

[0085] **Figures 15C-15D** show Western Blot analysis of miR-221 target gene protein CDKN1B/p27, revealed a simultaneous reduction of protein levels in tumor tissues. Protein expression data were normalized versus the levels of β -tubulin and the p-value of the comparison is shown in the figure.

[0086] **Figure 16. Progressive development of HCC in transgenic mice.** Several groups of male animals, transgenic and wild type, were treated intraperitoneally with DENA and sacrificed at different time-points (3, 4 and 6 months of age). The images demonstrate that the number of visible tumors developed in transgenic mice (TG) is higher than in wild type control animals (WT). The difference was already apparent by the third month of age.

[0087] **Figures 17A-17E. Histopathology of liver nodules and hepatocellular carcinomas (HCCs) in miR-221 transgenic mice.**

[0088] **Figure 17A** shows many liver nodules of transgenic mice displayed pleomorphic cells, and anaplastic features with loss of trabecular arrangement.

[0089] **Figure 17B** shows other nodules of transgenic mice exhibited a front of an invasive trabecular pattern of growth with no demarcation from the surrounding liver parenchyma.

[0090] **Figure 17C** shows the presence of necrotic areas, marked angiogenesis with slit-like sinusoids lined by endothelium (black arrows) were common.

[0091] **Figure 17D** shows intravasation of tumor cells was also detected.

[0092] **Figure 17E** shows control WT mice displayed tumor nodules characterized by a better defined tumor margin without a fibrous capsule, together with less evident angiogenesis with single cell plates and adenoma-like features in the smallest nodules.

[0093] **Figure 18. miR-221 levels in serum of transgenic mice treated with anti-miR-221.**

[0094] **Figures 19A-19B. Analyses of miR-221 expression vector *in vitro* and *in vivo*.**

[0095] **Figure 19A** shows the pWhere-EII- α 1AT-miR-221 plasmid vector was transfected into the SNU398 hepatocarcinoma derived cells, which express miR-221 at low level. The relative expression of miR-221 in transfected cells (pWhere/miR-221) versus untransfected control (NT) was assessed by quantitative PCR analysis of RNA extracted at 48 hours.

[0096] **Figure 19B** shows the quantitative PCR analysis carried out on different tissues revealed a significant increase in miR-221 expression levels in liver and kidney of transgenic animals in comparison with wild type controls. No additional significant differences were found in other tissues. The shown data represent mean $\pm$ SD.

[0097] **Figures 20A-20B. Macroscopic features of livers from transgenic versus wild type mice.**

[0098] **Figure 20A** shows the differences in macroscopic appearance of transgenic livers, which show a pale exterior with increased evidence of the microlobular structure in comparison with wild type. (**Figure 20A** top panel – wild type; bottom panel – transgenic). Both the wild type and transgenic are shown at 6 months.

[0099] **Figure 20B** shows the distribution of liver weights of transgenic and wild type mice. Transgenic livers show a slight but significant increased in weight.

[0100] **Figure 21. Table showing the number of tumor nodules developed in transgenic and wild type male mice.**

[0101] **Figure 22. Table showing differentially expressed RNA transcripts in the livers of miR-221 transgenic mice versus wild type mice.**

DETAILED DESCRIPTION

[0102] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not intended to limit the scope of the current teachings. In this application, the use of the singular includes the plural unless specifically stated otherwise.

[0103] Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew et al. (eds.), *The*

Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

[00104] *Definitions*

[00105] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

[00106] Also, the use of "comprise", "contain", and "include", or modifications of those root words, for example but not limited to, "comprises", "contained", and "including", are not intended to be limiting. The term "and/or" means that the terms before and after can be taken together or separately. For illustration purposes, but not as a limitation, "X and/or Y" can mean "X" or "Y" or "X and Y".

[00107] The term "combinations thereof" as used herein refers to all permutations and combinations of the listed items preceding the term. For example, "A, B, C, or combinations thereof" is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, ACB, CBA, BCA, BAC, or CAB.

[00108] The terms "agent" and "drug" generally refer to any therapeutic agents (e.g., chemotherapeutic compounds and/or molecular therapeutic compounds), antisense therapies, radiation therapies, or surgical interventions, used in the treatment of a particular disease or disorder.

[00109] The term "clinical outcome" generally refers to the health status of a subject following treatment for a disease or disorder, or in the absence of treatment. Clinical outcomes include, but are not limited to, an increase in the length of time until death, a decrease in the length of time until death, an increase in the chance of survival, an increase in the risk of death, survival, disease-free survival, chronic disease, metastasis, advanced or aggressive disease, disease recurrence, death, and favorable or poor response to therapy.

[00110] The term "decrease in survival" generally refers to a decrease in the length of time before death of a subject, or an increase in the risk of death for the subject.

[00111] The term "control" generally refers to a sample or standard used for comparison with an experimental sample, such as a sample obtained from a subject. In some embodiments, the control is a sample obtained from a healthy subject. In some embodiments, the control is cell/tissue sample obtained from the same subject. In some embodiments, the control is a historical control or standard value (i.e., a previously tested control sample or group of samples that represent baseline or normal values, such as the level in a control sample). In other embodiments, the control is a sample obtained from a healthy subject, such as a donor. Test samples and control samples can be obtained according to any method known in the art.

[00112] The term "cytokines" generally refers to proteins produced by a wide variety of hematopoietic and non-hematopoietic cells that affect the behavior of other cells. Cytokines are

important for both the innate and adaptive immune responses.

[00113] The terms “prevent,” “preventing” and “prevention” generally refer to a decrease in the occurrence of disease or disorder in a subject. The prevention may be complete, e.g., the total absence of the disease or disorder in the subject. The prevention may also be partial, such that the occurrence of the disease or disorder in the subject is less than that which would have occurred without the present invention. “Preventing” a disease generally refers to inhibiting the full development of a disease.

[00114] The terms “treating” and/or “ameliorating a disease” generally refer to a therapeutic intervention that ameliorates a sign or symptom of a disease or disorder after it has begun to develop. “Ameliorating” generally refers to the reduction in the number or severity of signs or symptoms of a disease or disorder.

[00115] The terms “treat,” “treating” and “treatment”, as used herein, refer to ameliorating symptoms associated with a disease or condition, for example, cancer, including preventing or delaying the onset of the disease symptoms, and/or lessening the severity or frequency of symptoms of the disease or condition. The terms “subject” and “individual” are defined herein to include animals, such as mammals, including, but not limited to, primates, cows, sheep, goats, horses, dogs, cats, rabbits, guinea pigs, rats, mice or other bovine, ovine, equine, canine, feline, rodent, or murine species. In a preferred embodiment, the animal is a human.

[00116] In other embodiments of the treatment methods, an effective amount of at least one compound that inhibits miR expression can be administered to the subject. As used herein, “inhibiting miR expression” means that the production of the precursor and/or active, mature form of miR gene product after treatment is less than the amount produced prior to treatment. One skilled in the art can readily determine whether miR expression has been inhibited in a cancer cell, using, for example, the techniques for determining miR transcript level discussed herein. Inhibition can occur at the level of gene expression (i.e., by inhibiting transcription of a miR gene encoding the miR gene product) or at the level of processing (e.g., by inhibiting processing of a miR precursor into a mature, active miR).

[00117] The term “subject” includes human and non-human animals. The preferred subject for treatment is a human. “Subject” and “subject” are used interchangeably herein.

[00118] The term “therapeutic” generally is a generic term that includes both diagnosis and treatment. The term “therapeutic agent” generally refers to a chemical compound, small molecule, or other composition, such as an antisense compound, protein, peptide, small molecule, nucleic acid, antibody, protease inhibitor, hormone, chemokine or cytokine, capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject. As used herein, a “candidate agent” is a compound selected for screening to determine if it can function as a therapeutic agent. “Incubating” includes a sufficient amount of time for an agent to interact with a cell or tissue. “Contacting” includes incubating an agent in solid or in liquid form with a cell or tissue. “Treating” a

cell or tissue with an agent includes contacting or incubating the agent with the cell or tissue.

[00119] The term “therapeutically effective amount” generally refers to that amount of the therapeutic agent sufficient to result in amelioration of one or more symptoms of a disorder, or prevent advancement of a disorder, or cause regression of the disease or disorder. For example, in one embodiment, a therapeutically effective amount will refer to the amount of a therapeutic agent that decreases the rate of rejection, or increases survival time by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 100%.

[00120] A “therapeutically effective amount” can be a quantity of a specified pharmaceutical or therapeutic agent sufficient to achieve a desired effect in a subject, or in a cell, being treated with the agent. For example, this can be the amount of a therapeutic agent that alters the expression of miR/s, and thereby prevents, treats or ameliorates the disease or disorder in a subject. The effective amount of the agent will be dependent on several factors, including, but not limited to the subject or cells being treated, and the manner of administration of the therapeutic composition.

[00121] The term “pharmaceutically acceptable vehicles” generally refers to such pharmaceutically acceptable carriers (vehicles) as would be generally used. Remington’s Pharmaceutical Sciences, by E. W. Martin, Mack Publishing Co., Easton, PA, 20 Edition, describes compositions and formulations suitable for pharmaceutical delivery of one or more therapeutic compounds, molecules or agents. In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

[00122] The term “pharmaceutically acceptable salt” generally refers to any salt (e.g., obtained by reaction with an acid or a base) of a compound of the present invention that is physiologically tolerated in the target animal (e.g., a mammal). Salts of the compounds of the present invention may be derived from inorganic or organic acids and bases. Examples of acids include, but are not limited to, hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycolic, lactic, salicylic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, sulfonic, naphthalene-2-sulfonic, benzenesulfonic acid, and the like. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, may be employed in the

preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid addition salts. Examples of bases include, but are not limited to, alkali metal (e.g., sodium) hydroxides, alkaline earth metal (e.g., magnesium) hydroxides, ammonia, and the like. Examples of salts include, but are not limited to: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, flucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, chloride, bromide, iodide, 2-hydroxyethanesulfonate, lactate, maleate, mesylate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, palmoate, pectinate, persulfate, phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, undecanoate, and the like. Other examples of salts include anions of the compounds of the present invention compounded with a suitable cation such as Na⁺, NH₄⁺, and NW₄⁺ (wherein W is a C1-4 alkyl group), and the like. For therapeutic use, salts of the compounds of the present invention are contemplated as being pharmaceutically acceptable. However, salts of acids and bases that are non-pharmaceutically acceptable may also find use, for example, in the preparation or purification of a pharmaceutically acceptable compound.

[00123] MicroRNAs

[00124] MicroRNAs are generally 21-23 nucleotides in length. MicroRNAs are processed from primary transcripts known as pri-miRNA to short stem-loop structures called precursor (pre)-miRNA and finally to functional, mature microRNA. Mature microRNA molecules are partially complementary to one or more messenger RNA molecules, and their primary function is to down-regulate gene expression. MicroRNAs regulate gene expression through the RNAi pathway.

[00125] As used herein interchangeably, a "miR gene product," "microRNA," "miR," or "miRNA" refers to the unprocessed or processed RNA transcript from a miR gene. As the miR gene products are not translated into protein, the term "miR gene products" does not include proteins. The unprocessed miR gene transcript is also called a "miR precursor," and typically comprises an RNA transcript of about 70-100 nucleotides in length. The miR precursor can be processed by digestion with an RNase (for example, Dicer, Argonaut, RNase III (e.g., E. coli RNase III)) into an active 19-25 nucleotide RNA molecule. This active 19-25 nucleotide RNA molecule is also called the "processed" miR gene transcript or "mature" miRNA.

[00126] The active nucleotide RNA molecule can be obtained from the miR precursor through natural processing routes (e.g., using intact cells or cell lysates) or by synthetic processing routes (e.g., using isolated processing enzymes, such as isolated Dicer, Argonaut, or RNase III). It is understood that the active 19-25 nucleotide RNA molecule can also be produced directly by biological or chemical synthesis, without having to be processed from the miR precursor. When a microRNA is referred to herein by name, the name corresponds to both the precursor and mature forms, unless otherwise indicated.

[00127] "miRNA nucleic acid" generally refers to RNA or DNA that encodes a miR as defined

above, or is complementary to a nucleic acid sequence encoding a miR, or hybridizes to such RNA or DNA and remains stably bound to it under appropriate stringency conditions. Particularly included are genomic DNA, cDNA, mRNA, miRNA and antisense molecules, pri-miRNA, pre-miRNA, mature miRNA and miRNA seed sequences. Also included are nucleic acids based on alternative backbones or including alternative bases. MiRNA nucleic acids can be derived from natural sources or synthesized.

[00128] It is to be understood that a miRNAs or pre-miRNAs can be 18-100 nucleotides in length, and more preferably from 18-80 nucleotides in length. For example, mature miRNAs can have a length of 19-30 nucleotides, preferably 21-25 nucleotides, particularly 21, 22, 23, 24, or 25 nucleotides. MicroRNA precursors typically have a length of about 70-100 nucleotides and have a hairpin conformation. Thus, once a sequence of a miRNA or a pre-miRNA is known, a miRNA antagonist that is sufficiently complementary to a portion of the miRNA or the pre-miRNA can be designed according to the rules of Watson and Crick base pairing. As used herein, the term "sufficiently complementary" generally means that two sequences are sufficiently complementary such that a duplex can be formed between them under physiologic conditions. A miRNA antagonist sequence that is sufficiently complementary to a miRNA or pre-miRNA target sequence can be 70%, 80%, 90%, or more identical to the miRNA or pre-miRNA sequence. In one embodiment, the miRNA antagonist contains no more than 1, 2 or 3 nucleotides that are not complementary to the miRNA or pre-miRNA target sequence. In another embodiment, the miRNA antagonist is 100% complementary to a miRNA or pre-miRNA target sequence. Sequences for miRNAs are available publicly through the miRBase registry (Griffiths-Jones, et al., *Nucleic Acids Res.*, 36(Database Issue):D154-D158 (2008); Griffiths-Jones, et al., *Nucleic Acids Res.*, 36(Database Issue):D140-D144 (2008); Griffiths-Jones, et al., *Nucleic Acids Res.*, 36(Database Issue):D109-D111 (2008)).

[00129] The term "miRNA" generally refers to a single-stranded molecule, but in specific embodiments, molecules can encompass a region or an additional strand that is partially (between 10 and 50% complementary across length of strand), substantially (greater than 50% but less than 100% complementary across length of strand) or fully complementary to another region of the same single-stranded molecule or to another nucleic acid. Thus, nucleic acids may encompass a molecule that comprises one or more complementary or self-complementary strand(s) or "complement(s)" of a particular sequence comprising a molecule. For example, precursor miRNA may have a self-complementary region, which is up to 100% complementary miRNA probes of the invention can be or be at least 60, 65, 70, 75, 80, 85, 90, 95, or 100% (and all ranges in-between) complementary to their target.

[00130] "MicroRNA seed sequence," "miRNA seed sequence," "seed region" and "seed portion" generally refer to nucleotides 2-7 or 2-8 of the mature miRNA sequence. The miRNA seed sequence is typically located at the 5' end of the miRNA.

[00131] A "miR-specific inhibitor" may be an anti-miRNA (anti-miR) oligonucleotide. Anti-

miRNAs may be single stranded molecules.

[00132] Anti-miRs may comprise RNA or DNA or have non-nucleotide components. Anti-miRs anneal with and block mature microRNAs through extensive sequence complementarity. In some embodiments, an anti-miR may comprise a nucleotide sequence that is a perfect complement of the entire miRNA. In some embodiments, an anti-miR comprises a nucleotide sequence of at least 6 consecutive nucleotides that are complementary to the seed region of a microRNA molecule at positions 2-8 and has at least 50%, 60%, 70%, 80%, or 90% complementarity to the rest of the miRNA. In other embodiments, the anti-miR may comprise additional flanking sequence, complementary to adjacent primary (pri-miRNA) sequences. Chemical modifications, such as 2'-O-methyl; locked nucleic acids (LNA); and 2'-O-methyl, phosphorothioate, cholesterol (antagomir); 2'-O-methoxyethyl can be used. Chemically modified anti-miRs are commercially available from a variety of sources, including but not limited to Sigma-Prologo, Ambion, Exiqon, and Dharmacon.

[00133] The miRNA antagonists can be oligomers or polymers of RNA or DNA, and can contain modifications to their nucleobases, sugar groups, phosphate groups, or covalent internucleoside linkages. In certain embodiment, modifications include those that increase the stability of the miRNA antagonists or enhance cellular uptake of the miRNA antagonists. In one embodiment, the miRNA antagonists are antagomirs, which have 2'-O-methylation of the sugars, a phosphorothioate backbone and a terminal cholesterol moiety.

[00134] In some embodiments, miR-specific inhibitors possess at least one microRNA binding site, mimicking the microRNA target (target mimics). These target mimics may possess at least one nucleotide sequence comprising 6 consecutive nucleotides complementary to positions 2-8 of the miRNA seed region. Alternatively, these target mimics may comprise a nucleotide sequence with complementarity to the entire miRNA. These target mimics may be vector encoded. Vector encoded target mimics may have one or more microRNA binding sites in the 5' or 3' UTR of a reporter gene. The target mimics may possess microRNA binding sites for more than one microRNA family. The microRNA binding site of the target mimic may be designed to mismatch positions 9-12 of the microRNA to prevent miRNA-guided cleavage of the target mimic.

[00135] In an alternative embodiment, a miR-specific inhibitor may interact with the miRNA binding site in a target transcript, preventing its interaction with a miRNA.

[00136] The terms "miRNA specific inhibitor" and "miRNA antagonist," generally refer to an agent that reduces or inhibits the expression, stability, or activity of a miRNA (e.g., miR-155). A miRNA antagonist may function, for example, by blocking the activity of a miRNA (e.g., blocking the ability of a miRNA to function as a translational repressor and/or activator of one or more miRNA targets), or by mediating miRNA degradation. Exemplary miRNA antagonists include nucleic acids, for example, antisense locked nucleic acid molecules (LNAs), antagomirs, or 2'-O-methyl antisense RNAs targeting a miRNA.

[00137] The phrase "inhibiting expression of a target gene" generally refers to the ability of an

RNAi agent, such as a siRNA, to silence, reduce, or inhibit expression of a target gene. The another way, to "inhibit", "down-regulate", or "reduce", it is meant that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits, is reduced below that observed in the absence of the RNAi agent.

[00138] For example, in one embodiment, inhibition, down-regulation, or reduction contemplates inhibition of the target mRNA below the level observed in the presence of, for example, a siRNA molecule with scrambled sequence or with mismatches.

[00139] To examine the extent of gene silencing, a test sample (e.g., a biological sample from organism of interest expressing the target gene(s) or a sample of cells in culture expressing the target gene(s)) is contacted with a siRNA that silences, reduces, or inhibits expression of the target gene(s). Expression of the target gene in the test sample is compared to expression of the target gene in a control sample (e.g., a biological sample from organism of interest expressing the target gene or a sample of cells in culture expressing the target gene) that is not contacted with the siRNA. Control samples (i.e., samples expressing the target gene) are assigned a value of 100%. Silencing, inhibition, or reduction of expression of a target gene is achieved when the value of the test sample relative to the control sample is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 10% or 0%. Suitable assays include, e.g., examination of protein or mRNA levels using techniques known to those of skill in the art such as dot blots, northern blots, in situ hybridization, ELISA, microarray hybridization, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

[00140] An "effective amount" or "therapeutically effective amount" of a miR-specific inhibitor is an amount sufficient to produce the desired effect, e.g., inhibition of expression of a target sequence in comparison to the normal expression level detected in the absence of the miR-specific inhibitor. Inhibition of expression of a target gene or target sequence by a miR-specific inhibitor is achieved when the expression level of the target gene mRNA or protein is about 90%, 80%, 70%, 60%, 50%, 40%, 30%, 25%, 20%, 15%, 10%, 5%, or 0% relative to the expression level of the target gene mRNA or protein of a control sample. The desired effect of a miR-specific inhibitor may also be measured by detecting an increase in the expression of genes down-regulated by the miRNA targeted by the miR-specific inhibitor.

[00141] As used herein, an "effective amount" of a compound that inhibits miR expression is an amount sufficient to inhibit proliferation of a cancer cell in a subject suffering from a cancer (e.g., cancer). One skilled in the art can readily determine an effective amount of a miR expression-inhibiting compound to be administered to a given subject, by taking into account factors, such as the size and weight of the subject; the extent of disease penetration; the age, health and gender of the subject; the route of administration; and whether the administration is regional or systemic.

[00142] For example, an effective amount of the expression-inhibiting compound can be based on

the approximate weight of a tumor mass to be treated, as described herein. An effective amount of a compound that inhibits miR expression can also be based on the approximate or estimated body weight of a subject to be treated, as described herein.

[00143] One skilled in the art can also readily determine an appropriate dosage regimen for administering a compound that inhibits miR expression to a given subject, as described herein.

[00144] By "modulate" is meant that the expression of the gene, or level of RNA molecule or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits is up-regulated or down-regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the modulator. For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

[00145] Non-limiting examples of suitable sequence variants of miRNA can include: substitutional, insertional or deletional variants. Insertions include 5' and/or 3' terminal fusions as well as intrasequence insertions of single or multiple residues. Insertions can also be introduced within the mature sequence. These, however, can be smaller insertions than those at the 5' or 3' terminus, on the order of 1 to 4 residues, preferably 2 residues, most preferably 1 residue.

[00146] Insertional sequence variants of miRNA are those in which one or more residues are introduced into a predetermined site in the target miRNA. Most commonly insertional variants are fusions of nucleic acids at the 5' or 3' terminus of the miRNA.

[00147] Deletion variants are characterized by the removal of one or more residues from the miRNA sequence. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding miRNA, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture. However, variant miRNA fragments may be conveniently prepared by *in vitro* synthesis. The variants typically exhibit the same qualitative biological activity as the naturally-occurring analogue, although variants also are selected in order to modify the characteristics of miRNA.

[00148] Substitutional variants are those in which at least one residue sequence has been removed and a different residue inserted in its place. While the site for introducing a sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target region and the expressed miRNA variants screened for the optimal combination of desired activity. Various suitable techniques for making substitution mutations at predetermined sites in DNA having a known sequence can be used.

[00149] Nucleotide substitutions are typically of single residues; insertions usually will be on the order of about from 1 to 10 residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs; i.e., a deletion of 2 residues or insertion of 2 residues. Substitutions, deletion, insertions or any combination thereof may be combined to arrive at

a final construct.

[00150] Changes may be made to decrease the activity of the miRNA, and all such modifications to the nucleotide sequences encoding such miRNA are encompassed.

[00151] An “isolated nucleic acid or DNA” is generally understood to mean chemically synthesized DNA, cDNA or genomic DNA with or without the 3' and/or 5' flanking regions. DNA encoding miRNA can be obtained from other sources by, for example” a) obtaining a cDNA library from cells containing mRNA; b) conducting hybridization analysis with labeled DNA encoding miRNA or fragments thereof in order to detect clones in the cDNA library containing homologous sequences; and, c) analyzing the clones by restriction enzyme analysis and nucleic acid sequencing to identify full-length clones.

[00152] As used herein nucleic acids and/or nucleic acid sequences are “homologous” when they are derived, naturally or artificially, from a common ancestral nucleic acid or nucleic acid sequence. Homology is generally inferred from sequence identity between two or more nucleic acids or proteins (or sequences thereof). The precise percentage of identity between sequences that is useful in establishing homology varies with the nucleic acid and protein at issue, but as little as 25% sequence identity is routinely used to establish homology. Higher levels of sequence identity, e.g., 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more can also be used to establish homology. Methods for determining sequence similarity percentages (e.g., BLASTN using default parameters) are generally available. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information.

[00153] The term “detecting the level of miR expression” generally refers to quantifying the amount of such miR present in a sample. Detecting expression of a miR, or any microRNA, can be achieved using any method known in the art or described herein, such as by qRT-PCR. Detecting expression of a miR includes detecting expression of either a mature form of the miR or a precursor form that is correlated with the miR expression. For example, miRNA detection methods involve sequence specific detection, such as by RT-PCR. miR-specific primers and probes can be designed using the precursor and mature miR nucleic acid sequences, which are known in the art and include modifications which do not change the function of the sequences.

[00154] The terms “low miR- expression” and “high miR- expression” are relative terms that refer to the level of miR/s found in a sample. In some embodiments, low miR- and high miR- expression are determined by comparison of miR/s levels in a group of test samples and control samples. Low and high expression can then be assigned to each sample based on whether the expression of a miR in a sample is above (high) or below (low) the average or median miR expression level. For individual samples, high or low miR expression can be determined by comparison of the sample to a control or reference sample known to have high or low expression, or by comparison to a standard value. Low and high miR expression can include expression of either the precursor or mature forms of miR, or both.

[00155] The term "expression vector" generally refers to a nucleic acid construct that can be generated recombinantly or synthetically. An expression vector generally includes a series of specified nucleic acid elements that enable transcription of a particular gene in a host cell. Generally, the gene expression is placed under the control of certain regulatory elements, such as constitutive or inducible promoters.

[00156] The term "operably linked" is used to describe the connection between regulatory elements and a gene or its coding region. That is, gene expression is typically placed under the control of certain regulatory elements, for example, without limitation, constitutive or inducible promoters, tissue-specific regulatory elements, and enhancers. A gene or coding region is to be "operably linked to" or "operatively linked to" or "operably associated with" the regulatory elements, meaning that the gene or coding region is controlled or influenced by the regulatory element.

[00157] MicroRNA detection

[00158] In some methods herein, it is desirable to identify miRNAs present in a sample.

[00159] The sequences of precursor microRNAs (pre-miRNAs) and mature miRNAs are publicly available, such as through the miRBase database, available online by the Sanger Institute (see Griffiths-Jones et al., Nucleic Acids Res. 36:D154-D158, 2008; Griffiths-Jones et al., Nucleic Acids Res. 34:D140-D144, 2006; and Griffiths-Jones, Nucleic Acids Res. 32:D109-D111, 2004). The sequences of the precursor and mature forms of the presently disclosed preferred family members are provided herein.

[00160] Detection and quantification of RNA expression can be achieved by any one of a number of methods well known in the art. Using the known sequences for RNA family members, specific probes and primers can be designed for use in the detection methods described below as appropriate.

[00161] In some cases, the RNA detection method requires isolation of nucleic acid from a sample, such as a cell or tissue sample. Nucleic acids, including RNA and specifically miRNA, can be isolated using any suitable technique known in the art. For example, phenol-based extraction is a common method for isolation of RNA. Phenol-based reagents contain a combination of denaturants and RNase inhibitors for cell and tissue disruption and subsequent separation of RNA from contaminants. Phenol-based isolation procedures can recover RNA species in the 10-200-nucleotide range (e.g., precursor and mature miRNAs, 5S and 5.8S ribosomal RNA (rRNA), and U1 small nuclear RNA (snRNA)). In addition, extraction procedures such as those using TRIZOL™ or TRI REAGENT™, will purify all RNAs, large and small, and are efficient methods for isolating total RNA from biological samples that contain miRNAs and small interfering RNAs (siRNAs).

[00162] In some embodiments, use of a microarray is desirable. A microarray is a microscopic, ordered array of nucleic acids, proteins, small molecules, cells or other substances that enables parallel analysis of complex biochemical samples. A DNA microarray consists of different nucleic acid probes, known as capture probes that are chemically attached to a solid substrate, which can be a microchip, a glass slide or a microsphere-sized bead. Microarrays can be used, for example, to

measure the expression levels of large numbers of messenger RNAs (mRNAs) and/or miRNAs simultaneously.

[00163] Microarrays can be fabricated using a variety of technologies, including printing with fine-pointed pins onto glass slides, photolithography using pre-made masks, photolithography using dynamic micromirror devices, ink-jet printing, or electrochemistry on microelectrode arrays.

[00164] Microarray analysis of miRNAs, for example (although these procedures can be used in modified form for any RNA analysis) can be accomplished according to any method known in the art. In one example, RNA is extracted from a cell or tissue sample, the small RNAs (18-26-nucleotide RNAs) are size-selected from total RNA using denaturing polyacrylamide gel electrophoresis. Oligonucleotide linkers are attached to the 5' and 3' ends of the small RNAs and the resulting ligation products are used as templates for an RT-PCR reaction with 10 cycles of amplification. The sense strand PCR primer has a fluorophore attached to its 5' end, thereby fluorescently labeling the sense strand of the PCR product. The PCR product is denatured and then hybridized to the microarray. A PCR product, referred to as the target nucleic acid that is complementary to the corresponding miRNA capture probe sequence on the array will hybridize, via base pairing, to the spot at which the capture probes are affixed. The spot will then fluoresce when excited using a microarray laser scanner. The fluorescence intensity of each spot is then evaluated in terms of the number of copies of a particular miRNA, using a number of positive and negative controls and array data normalization methods, which will result in assessment of the level of expression of a particular miRNA.

[00165] In an alternative method, total RNA containing the small RNA fraction (including the miRNA) extracted from a cell or tissue sample is used directly without size-selection of small RNAs, and 3' end labeled using T4 RNA ligase and either a fluorescently-labeled short RNA linker. The RNA samples are labeled by incubation at 30°C for 2 hours followed by heat inactivation of the T4 RNA ligase at 80°C for 5 minutes. The fluorophore-labeled miRNAs complementary to the corresponding miRNA capture probe sequences on the array will hybridize, via base pairing, to the spot at which the capture probes are affixed. The microarray scanning and data processing is carried out as described above.

[00166] There are several types of microarrays than be employed, including spotted oligonucleotide microarrays, pre-fabricated oligonucleotide microarrays and spotted long oligonucleotide arrays. In spotted oligonucleotide microarrays, the capture probes are oligonucleotides complementary to miRNA sequences. This type of array is typically hybridized with amplified PCR products of size-selected small RNAs from two samples to be compared (such as non-cancerous tissue and cancerous or sample tissue) that are labeled with two different fluorophores. Alternatively, total RNA containing the small RNA fraction (including the miRNAs) is extracted from the two samples and used directly without size-selection of small RNAs, and 3' end labeled using T4 RNA ligase and short RNA linkers labeled with two different fluorophores. The samples can be mixed and hybridized to one single microarray that is then scanned, allowing the visualization of up-

regulated and down-regulated miRNA genes in one assay.

[00167] In pre-fabricated oligonucleotide microarrays or single-channel microarrays, the probes are designed to match the sequences of known or predicted miRNAs. There are commercially available designs that cover complete genomes (for example, from Affymetrix or Agilent). These microarrays give estimations of the absolute value of gene expression and therefore the comparison of two conditions requires the use of two separate microarrays.

[00168] In some embodiments, use of quantitative RT-PCR is desirable. Quantitative RT-PCR (qRT-PCR) is a modification of polymerase chain reaction used to rapidly measure the quantity of a product of polymerase chain reaction. qRT-PCR is commonly used for the purpose of determining whether a genetic sequence, such as a miR, is present in a sample, and if it is present, the number of copies in the sample. Any method of PCR that can determine the expression of a nucleic acid molecule, including a miRNA, falls within the scope of the present disclosure. There are several variations of the qRT-PCR method known in the art. Methods for quantitative polymerase chain reaction include, but are not limited to, via agarose gel electrophoresis, the use of SYBR Green (a double stranded DNA dye), and the use of a fluorescent reporter probe. The latter two can be analyzed in real-time.

[00169] Screening

[00170] As used herein, “screening” refers to the process used to evaluate and identify candidate agents that affect such disease. Expression of a microRNA can be quantified using any one of a number of techniques known in the art and described herein, such as by microarray analysis or by qRT-PCR.

[00171] In some embodiments, screening comprises contacting the candidate agents with cells. The cells can be primary cells obtained from a patient, or the cells can be immortalized or transformed cells.

[00172] The candidate agents can be any type of agent, such as a protein, peptide, small molecule, antibody or nucleic acid. In some embodiments, the candidate agent is a cytokine. In some embodiments, the candidate agent is a small molecule. Screening includes both high-throughout screening and screening individual or small groups of candidate agents.

[00173] Methods of screening candidate agents to identify therapeutic agents for the treatment of disease are well known in the art. Methods of detecting expression levels of RNA and proteins are known in the art and are described herein, such as, but not limited to, microarray analysis, RT-PCR (including qRT-PCR), in situ hybridization, in situ PCR, and Northern blot analysis. In one embodiment, screening comprises a high-throughput screen. In another embodiment, candidate agents are screened individually.

[00174] The candidate agents can be any type of molecule, such as, but not limited to nucleic acid molecules, proteins, peptides, antibodies, lipids, small molecules, chemicals, cytokines, chemokines, hormones, or any other type of molecule that may alter cancer disease state(s) either directly or

indirectly.

[00175] Typically, an endogenous gene, miRNA or mRNA is modulated in the cell. In particular embodiments, the nucleic acid sequence comprises at least one segment that is at least 70, 75, 80, 85, 90, 95, or 100% identical in nucleic acid sequence to one or more miRNA sequence listed in Table 1. Modulation of the expression or processing of an endogenous gene, miRNA, or mRNA can be through modulation of the processing of an mRNA, such processing including transcription, transportation and/or translation within a cell. Modulation may also be effected by the inhibition or enhancement of miRNA activity within a cell, tissue, or organ. Such processing may effect the expression of an encoded product or the stability of the mRNA. In still other embodiments, a nucleic acid sequence can comprise a modified nucleic acid sequence. In certain aspects, one or more miRNA sequence may include or comprise a modified nucleobase or nucleic acid sequence.

[00176] It will be understood in methods of the invention that a cell or other biological matter such as an organism (including patients) can be provided a miRNA or miRNA molecule corresponding to a particular miRNA by administering to the cell or organism a nucleic acid molecule that functions as the corresponding miRNA once inside the cell. The form of the molecule provided to the cell may not be the form that acts as a miRNA once inside the cell. Thus, it is contemplated that in some embodiments, biological matter is provided a synthetic miRNA or a nonsynthetic miRNA, such as one that becomes processed into a mature and active miRNA once it has access to the cell's miRNA processing machinery. In certain embodiments, it is specifically contemplated that the miRNA molecule provided to the biological matter is not a mature miRNA molecule but a nucleic acid molecule that can be processed into the mature miRNA once it is accessible to miRNA processing machinery. The term "nonsynthetic" in the context of miRNA means that the miRNA is not "synthetic," as defined herein. Furthermore, it is contemplated that in embodiments of the invention that concern the use of synthetic miRNAs, the use of corresponding nonsynthetic miRNAs is also considered an aspect of the invention, and vice versa. It will be understood that the term "providing" an agent is used to include "administering" the agent to a patient.

[00177] In certain embodiments, methods also include targeting a miRNA to modulate in a cell or organism. The term "targeting a miRNA to modulate" means a nucleic acid of the invention will be employed so as to modulate the selected miRNA. In some embodiments the modulation is achieved with a synthetic or non-synthetic miRNA that corresponds to the targeted miRNA, which effectively provides the targeted miRNA to the cell or organism (positive modulation). In other embodiments, the modulation is achieved with a miRNA inhibitor, which effectively inhibits the targeted miRNA in the cell or organism (negative modulation).

[00178] In some embodiments, the miRNA targeted to be modulated is a miRNA that affects a disease, condition, or pathway. In certain embodiments, the miRNA is targeted because a treatment can be provided by negative modulation of the targeted miRNA. In other embodiments, the miRNA is targeted because a treatment can be provided by positive modulation of the targeted miRNA.

[00179] In certain methods of the invention, there is a further step of administering the selected miRNA modulator to a cell, tissue, organ, or organism (collectively “biological matter”) in need of treatment related to modulation of the targeted miRNA or in need of the physiological or biological results discussed herein (such as with respect to a particular cellular pathway or result like decrease in cell viability).

[00180] Consequently, in some methods there is a step of identifying a patient in need of treatment that can be provided by the miRNA modulator(s). It is contemplated that an effective amount of a miRNA modulator can be administered in some embodiments. In particular embodiments, there is a therapeutic benefit conferred on the biological matter, where a “therapeutic benefit” refers to an improvement in the one or more conditions or symptoms associated with a disease or condition or an improvement in the prognosis, duration, or status with respect to the disease. It is contemplated that a therapeutic benefit includes, but is not limited to, a decrease in pain, a decrease in morbidity, a decrease in a symptom.

[00181] For example, with respect to cancer, it is contemplated that a therapeutic benefit can be inhibition of tumor growth, prevention of metastasis, reduction in number of metastases, inhibition of cancer cell proliferation, induction of cell death in cancer cells, inhibition of angiogenesis near cancer cells, induction of apoptosis of cancer cells, reduction in pain, reduction in risk of recurrence, induction of chemo- or radiosensitivity in cancer cells, prolongation of life, and/or delay of death directly or indirectly related to cancer.

[00182] Furthermore, it is contemplated that the miRNA compositions may be provided as part of a therapy to a patient, in conjunction with traditional therapies or preventative agents. Moreover, it is contemplated that any method discussed in the context of therapy may be applied as preventatively, particularly in a patient identified to be potentially in need of the therapy or at risk of the condition or disease for which a therapy is needed.

[00183] In addition, methods of the invention concern employing one or more nucleic acids corresponding to a miRNA and a therapeutic drug. The nucleic acid can enhance the effect or efficacy of the drug, reduce any side effects or toxicity, modify its bioavailability, and/or decrease the dosage or frequency needed. In certain embodiments, the therapeutic drug is a cancer therapeutic. Consequently, in some embodiments, there is a method of treating cancer in a patient comprising administering to the patient the cancer therapeutic and an effective amount of at least one miRNA molecule that improves the efficacy of the cancer therapeutic or protects non-cancer cells. Cancer therapies also include a variety of combination therapies with both chemical and radiation based treatments.

[00184] Generally, inhibitors of miRNAs can be given to achieve the opposite effect as compared to when nucleic acid molecules corresponding to the mature miRNA are given. Similarly, nucleic acid molecules corresponding to the mature miRNA can be given to achieve the opposite effect as compared to when inhibitors of the miRNA are given. For example, miRNA molecules that increase

cell proliferation can be provided to cells to increase proliferation or inhibitors of such molecules can be provided to cells to decrease cell proliferation.

[00185] The present invention contemplates these embodiments in the context of the different physiological effects observed with the different miRNA molecules and miRNA inhibitors disclosed herein. These include, but are not limited to, the following physiological effects: increase and decreasing cell proliferation, increasing or decreasing apoptosis, increasing transformation, increasing or decreasing cell viability, reduce or increase viable cell number, and increase or decrease number of cells at a particular phase of the cell cycle. Methods of the invention are generally contemplated to include providing or introducing one or more different nucleic acid molecules corresponding to one or more different miRNA molecules. It is contemplated that the following, at least the following, or at most the following number of different nucleic acid molecules may be provided or introduced: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, or any range derivable therein. This also applies to the number of different miRNA molecules that can be provided or introduced into a cell.

[00186] General Description

[00187] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not intended to limit the scope of the current teachings. In this application, the use of the singular includes the plural unless specifically stated otherwise. In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided.

[00188] Mouse Model

[00189] The presently described mouse model develops spontaneous liver cancers and is highly susceptible to the carcinogen diethylnitrosamine. In this model, the cell cycle inhibitors p27 and p57 and the pro-apoptotic protein Bmf are modulated by the up-regulation of miR-221. The miR-221 transgenic mouse model exhibits an increased liver cancer susceptibility. The liver tumors in the miR-221 mouse model exhibit molecular features of human hepatocellular carcinoma.

[00190] Transgenic mouse model carrying a liver-deregulated miR-221.

[00191] The transgenic mouse model exhibits an inappropriate over-expression of miR-221 in liver. The transgenic model is characterized by the appearance of spontaneous liver tumors in a fraction of male mice and a strong acceleration of tumor development in 100% of mice treated with diethylnitrosamine (DEN). The mouse model is useful as a tool to perform pre-clinical investigations on the use of miRNA or anti-miRNA approaches for liver cancer therapy.

[00192] A miR-221 expression vector was developed by employing the pWhere plasmid (Invivogen, San Diego, CA) as vector backbone. The pWhere vector is characterized by the presence of two murine H19 insulators, which protect the integrated transcriptional unit from negative as well

as positive influences from neighboring sequences.

[00193] To specifically drive a liver-specific expression, the pWhere vector was modified by inserting a regulatory element that included a liver specific $\alpha 1$ Anti-trypsin promoter coupled with the enhancer II (EII) sequence of human hepatitis B virus (HBV). This chimeric DNA element acts as a potent and steady promoter, and is able to ensure a constant and high level of gene expression in the liver. The tissue-specificity of this EII- $\alpha 1$ AT chimeric promoter, cloned upstream a luciferase reporter gene into a pGL3 plasmid, was tested in different types of hepatic and non-hepatic cell lines. It was confirmed that the highest level of luciferase expression was detectable in hepatic cells, thereby confirming the liver-specificity of the promoter (data not shown).

[00194] A DNA segment of 890 bp, which included the mmu-mir-221 locus, was amplified from mouse genomic DNA and cloned into the pWhere-EII $\alpha 1$ AT vector downstream of the EII- $\alpha 1$ AT promoter (**Figure 1A**). Expression of miR-221 from this vector was shown to be functional in a liver cancer derived cell line (**Figure 19A**).

[00195] To generate a line of transgenic mice, the pWhere-EII- $\alpha 1$ AT-miR-221 plasmid was linearized using the *PacI* restriction enzyme. The purified 9 kb fragment containing the transgene was used to microinject fertilized oocytes of a B6D2F2 mouse strain to complete their development. Following several crosses, a homozygous line of transgenic mice over-expressing the miR-221 in the liver was produced and used in all subsequent experiments.

[00196] *Transgenic mouse strain over-expressing miR-221 in the liver develops malignant HCCs.*

[00197] The miR-221 transgenic mouse is based on a genetically wild type animal.

[00198] The *in vivo* oncogenic activity of miR-221 was shown in the transgenic mice since the transgenic mice present a stable increase of miR-221 in the liver. The miR-221 transgenic mice exhibited a strong predisposition to the development of liver tumors. The transgenic mice spontaneously developed visible neoplastic lesions starting at 9 months of age, which were undetectable in wild type mice. If treated with DENA, the transgenic mice developed a significantly higher number and larger tumor lesions that became evident much earlier than in wild type animals treated with the same carcinogen. Histologically, tumors of transgenic mice were typical HCCs, characterized by an invasive trabecular growth and high level of angiogenesis. In comparison, tumors in wild type DENA-treated control mice displayed tumor nodules characterized by a better defined tumor margin without a fibrous capsule, together with less evident angiogenesis. The predisposition was stronger in males, a result supporting a protective effect of estrogens and a stimulating effect of androgen hormones in the development of HCC.

[00199] These tumors did not arise on a cirrhotic background (which is typical of most human HCCs). However, the livers of transgenic mice exhibited high levels of steatosis, a condition that, in humans, is frequently observed in the context of metabolic dysfunctions that predispose to HCC. The existence of a different molecular background driven by the aberrantly expressed miR-221 was shown by gene expression analyses of non-neoplastic livers of transgenic versus wild type mice. This

different molecular background is now believed to be responsible for the differences in liver phenotypes, including the predisposition to liver cancer. Intriguingly, many of the differentially expressed protein coding genes were connected to the modulation of interferon gamma, which was itself expressed at lower levels in the livers of transgenic mice.

[00200] At the molecular level, these tumors revealed a further increase of miR-221, accompanied by a strong repression of the cell cycle inhibitors Cdkn1b/p27, Cdkn1c/p57 and the pro-apoptotic Bmf proteins. In addition to miR-221, other miRNAs that play a key role in human HCC were found to be dysregulated in the tumors arising in this model. Among them, the down-regulated miR-122 and miR-199 or the up-regulated miR-21 were dysregulated in the same direction observed in human HCC, thus indicating that the pattern of miRNA expression in HCCs arising in this mouse model faithfully overlaps with that of human HCC.

[00201] The development of tumors in the miR-221 transgenic mouse required either about 1 year for the appearance of spontaneous tumors or the treatment with a carcinogen to accelerate tumorigenesis.

[00202] Anti-miR-221 can be effectively delivered to the liver, block miR-221 and induce a significant inhibition of tumor growth.

[00203] Anti-miR-221 as a potential anti-cancer molecule was investigated through the use of intra-tumor injections of anti-microRNA oligonucleotide (AMOs) targeting miR-221 in PC-3 derived tumors and in melanoma cells xenotransplants.

[00204] The intravenous injection of synthetic 2'-O-methyl modified oligonucleotides targeting miR-221 in transgenic mice showed that these molecules specifically silenced miRNA expression in the liver, as well as in the circulatory system. Furthermore, in DENA-treated transgenic mice, systemic administration of AMOs led to a significant containment of liver tumor growth in comparison with control animals.

[00205] This demonstrates that miR-221 is an oncogenic driving force *in vivo* for liver cancer and demonstrates that miR-221 can be effectively targeted to reduce tumor growth. Significantly, this effect was achieved without any appreciable toxicity. For HCC, this quality is particularly important. In fact, HCC conveys a very poor prognosis not only because a small fraction of tumors can be surgically resected, but also because systemic chemotherapy in advanced HCC is often only marginally effective or too toxic to be tolerated.

[00206] Constructs/Transgenes

[00207] The term "transgene" refers to a nucleic acid sequence introduced into one or more cells of a non-human animal by way of human intervention, such as by way of the methods described herein. The introduced genetic information may be foreign to the species of animal to which the recipient belongs, foreign only to the particular individual recipient, or genetic information already possessed by the recipient. In the latter case, the introduced genetic information may be differentially-expressed, as compared to the native endogenous gene.

[00208] The transgenic non-human animals have a genome that comprises a nucleic acid construct/transgene that is capable of expressing a miR-221 gene product. As used herein, “miR-221 gene” refers to any DNA (including artificially-modified DNA) which comprises DNA which encodes the unprocessed (e.g., precursor) or processed (e.g., mature) miR-221, such as, but not limited to, a miR-221 gene from mouse (*Mus musculus*). “miR-221 gene” does not require, but does not exclude, naturally-occurring or artificially-constructed noncoding sequences, such as, for example, promoters, enhancers and other regulatory elements.

[00209] For example a “miR-221 gene” includes a DNA sequence which encodes a stem-loop precursor miR-221 sequence for mouse miR-221. a “miR-221 gene” for mouse miR-221 is 5’-ATCCAGGTCTGGGGCATGAACCTGGCATAACAATGTAGATTTCTGTGTTTGGTTAGGCAACA GCTACATTGTCTGCTGGGTTTCAGGCTACCTGGAA-3’ (**SEQ ID NO: 1**). For example a “miR-221 gene” includes a DNA sequence which encodes a mature mouse miR-221 gene. Mature mouse miR-221 gene sequence is 5’ AGCTACATTGTCTGCTGGGTTTC -3’ (**SEQ ID NO: 2**).

[00210] Therefore, as used herein, “miR-221 gene product” refers to RNA which comprises RNA of the unprocessed (e.g., precursor) or processed (e.g., mature) RNA transcript from a miR-221 DNA sequence, such as, but not limited to, a miR-221 gene from mouse (*Mus musculus*). A precursor miR-221 gene product from mouse is represented by the nucleotide sequence: 5’-AUCCAGGUCUGGGGCAUGAACCGUGCAUACAAUGUAGAUUUCUGUGUUUGUUAGGCAACAGCUACAUGUCUGCUGGGUUUCAGGCUACCUGGAA-3’ (mirBase accession number MI0000709). (**SEQ ID NO:3**), while the processed, or mature, mouse miR-221 gene product is represented by the nucleotide sequence: 5’-AGCUACAUGUCUGCUGGGUUUC-3’ (mirBase accession number MIMAT0000669) (**SEQ ID NO:4**).

[00211] In certain embodiments, the miR-221 gene product comprises a nucleotide sequence having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or 100%, sequence identity to the nucleotide sequences of **SEQ ID NO:3** and/or **SEQ ID NO:4**.

[00212] In a particular embodiment, the miR-221 gene comprises a nucleotide sequence having 100% identity to the nucleotide sequence of **SEQ ID NO: 1**. In another embodiment, the miR-221 gene comprises a nucleotide sequence having 100% identity to the nucleotide sequence of **SEQ ID NO: 2**.

[00213] The actual comparison of two sequences can be accomplished by well-known methods, for example, using a mathematical algorithm. A preferred, non-limiting example of such a mathematical algorithm is described in Karlin *et al.* (*Proc. Natl. Acad. Sci. USA*, 90:5873-5877 (1993)). Such an algorithm is incorporated into the BLASTN and BLASTX programs (version 2.2) as described in Schaffer *et al.* (*Nucleic Acids Res.*, 29:2994-3005 (2001)). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., BLASTN; available at the Internet site for the National Center for Biotechnology Information) can be used. In one embodiment, the database searched is a non-redundant (NR) database, and parameters for

sequence comparison can be set at: no filters; Expect value of 10; Word Size of 3; the Matrix is BLOSUM62; and Gap Costs have an Existence of and an Extension of 1.

[00214] Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0), which is part of the GCG (Accelrys, San Diego, California) sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Additional algorithms for sequence analysis are known in the art and include ADVANCE and ADAM as described in Torellis and Robotti (Comput. Appl. Biosci., 10: 3-5, 1994); and FASTA described in Pearson and Lipman (Proc. Natl. Acad. Sci USA, 85: 2444-2448, 1988).

[00215] In another embodiment, the percent identity between two amino acid sequences can be accomplished using the GAP program in the GCG software package (Accelrys, San Diego, California) using either a Blossom 63 matrix or a PAM250 matrix, and a gap weight of 12, 10, 8, 6, or 4, and a length weight of 2, 3, or 4. In yet another embodiment, the percent identity between two nucleic acid sequences can be accomplished using the GAP program in the GCG software package (Accelrys, San Diego, California), using a gap weight of 50 and a length weight of 3.

[00216] Expression

[00217] According to one aspect, the transgenic non-human animal possesses a genome that comprises a nucleic acid construct in which a nucleic acid sequence encoding a miR-221 gene product is operably linked to at least one transcriptional regulatory sequence capable of directing expression in liver cells of the animal. The term "transcriptional regulatory sequence" is used according to its art-recognized meaning. It is intended to mean any DNA sequence that can, by virtue of its sequence, cause the linked gene to be either up- or down-regulated in a particular cell. In the case of a promoter, the promoter will generally be adjacent to the coding region. In the case of an enhancer, however, the enhancer may function at some distance from the coding region, such that there is an intervening DNA sequence between the enhancer and the coding region. To direct expression of the genetic information, which may include a DNA sequence encoding a particular protein (or "coding region"), the coding region of interest may be coupled to at least one transcriptional regulatory sequence in a functional manner. Transcriptional regulatory sequences may be used to increase, decrease, regulate or designate to certain tissues or to certain stages of development, the expression of a gene. The transcriptional regulatory sequences need not be naturally occurring sequences.

[00218] A nucleic acid molecule is "capable of expressing" or "capable of directing expression of" a microRNA if it contains nucleotide sequence(s) that contain transcriptional regulatory information, and such sequence(s) are "operably linked" to nucleotide sequence(s) that encode the microRNA. An operable linkage is a linkage in which regulatory nucleic acid sequence(s) and the nucleic acid sequence(s) sought to be expressed are connected in such a way as to permit gene expression.

[00219] In general, the regulatory regions needed for gene expression include, but are not limited

to, transcriptional regulatory sequences (e.g., a promoter region, an enhancer region), as well as DNA sequence(s) that, when transcribed into RNA, contribute to the stability of the gene transcript.

[00220] The term "promoter" is used according to its art-recognized meaning. It is intended to mean the DNA region, usually upstream to the coding sequence of a gene or operon, which binds RNA polymerase and directs the enzyme to the correct transcriptional start site. A promoter region is operably linked to a DNA sequence if the promoter is capable of effecting transcription of that DNA sequence.

[00221] The term "enhancer" is used according to its art-recognized meaning. It is intended to mean a sequence found in eukaryotes and certain eukaryotic viruses, which can increase transcription from a gene when located (in either orientation) up to several kilobases from the gene being studied. These sequences usually act as enhancers when on the 5' side (upstream) of the gene in question. However, some enhancers are active when placed on the 3' side (downstream) of the gene. In some cases, enhancer elements can activate transcription from a gene with no (known) promoter.

[00222] The nucleic acid construct may also include sequences that promote expression and/or stability of the construct and/or a gene product expressed from the construct. In a particular embodiment, the nucleic acid construct comprises the 3' UTR and poly(A) sequence of a β -globin gene (e.g., a mouse β -globin gene). Other sequences that promote expression and/or stability of the construct and/or a gene product expressed from the construct are known in the art and are encompassed herein.

[00223] Transgenic Animals

[00224] The term "transgenic non-human animal" is used herein to include all vertebrate animals, except humans. In one embodiment, the transgenic non-human animal is a mammal. Such transgenic non-human animals include, for example, transgenic pigs, transgenic rats, transgenic rabbits, transgenic cattle, transgenic goats, and other transgenic animal species, particularly mammalian species. Additionally, other members of the rodent family, e.g., rats, and guinea pigs, and nonhuman primates, such as chimpanzees, may be used to practice the embodiments described herein. In a particular embodiment, the transgenic non-human animal is a mouse. The transgenic non-human animals described herein include individual animals in all stages of development, including embryonic and fetal stages.

[00225] A "transgenic animal" is an animal containing one or more cells bearing genetic information received, directly or indirectly, by deliberate genetic manipulation at a subcellular level, such as by microinjection or infection with a recombinant virus. The introduced nucleic acid molecule may be integrated within a chromosome, or it may be extra-chromosomally replicating DNA. Suitable transgenic animals described herein include, but are not limited to, those animals in which the genetic information was introduced into a germ line cell, thereby conferring the ability to transfer the information to offspring. If such offspring in fact possess some or all of that information, then they, too, are transgenic animals.

[00226] To produce transgenic animals, any method known in the art for introducing a recombinant construct or transgene into an embryo, such as, for example, microinjection, use of a cell gun, transfection, liposome fusion, electroporation, and the like, may be used. In a particular embodiment, the method for producing a transgenic animal is microinjection, which involves injecting a DNA molecule into the male pronucleus of a fertilized egg (see, e.g., U.S. Pat. Nos. 4,870,009; 5,550,316; 4,736,866; and 4,873,191). Methods for introducing a recombinant construct/transgene into mammals and their germ cells were originally developed in the mouse. Such methods were subsequently adopted for use with larger animals, including livestock species (see, e.g., PCT Publications Nos. WO 88/00239, WO 90/05188 and WO 92/11757). Microinjection of DNA into the cytoplasm of a zygote can also be used to produce transgenic animals.

[00227] The methods for evaluating the presence of the introduced transgene as well as its expression are readily available and well-known in the art. Such methods include, but are not limited to, DNA (Southern) hybridization to detect the exogenous DNA, polymerase chain reaction (PCR), polyacrylamide gel electrophoresis (PAGE) and blots to detect DNA, RNA or protein.

[00228] The present embodiments are not limited to any one species of animal, but provides for any appropriate non-human vertebrate species. For example, as described and exemplified herein, transgenic mice can be produced. Other non-limiting examples include, e.g., other non-human mammals described herein, such as guinea pigs, rabbits, pigs, sheep, etc. The success rate for producing transgenic animals by microinjection is highest in mice, where approximately 25% of fertilized mouse eggs into which the DNA has been injected, and which have been implanted in a female, will develop into transgenic mice.

[00229] After the agent has been administered to the transgenic animal, one or more symptoms and/or indications of the condition in the transgenic animal are compared with those of a control animal of the same genotype, which has not been administered the agent. If the agent inhibits, prevents and/or reduces one or more symptoms and/or indications of the condition in the transgenic animal to which it has been administered, relative to the control animal, then the agent is considered to have therapeutic efficacy in treating or preventing a condition.

[00230] According to another embodiment, potential therapeutic modalities or agents for preventing and/or treating disorders may be tested by measuring the anti-disorder activity of such modalities in animals produced according to one or more aspects as described herein. Such activity may be assessed by measuring the capacity of a potential therapeutic modality to inhibit, prevent, and/or destroy one or more of the symptoms or indications of disorder exhibited by transgenic animals produced according to one embodiment and/or in "recipient" animals produced according to another embodiment.

[00231] A variety of therapeutic modalities or agents, such as proteins (e.g., antibodies), peptides, peptidomimetics, small organic molecules, nucleic acids and the like, can be tested for preventing and/or treating disorders. According to the methods described herein, agents can be individually

screened or one or more agents can be tested simultaneously. Where a mixture of compounds is tested, the compounds selected by the processes described can be separated (as appropriate) and identified using suitable methods (e.g., sequencing, chromatography). The presence of one or more compounds in a test sample can also be determined according to these methods.

[00232] Agents that prevent and/or treat disorders can be identified, for example, by screening libraries or collections of molecules, such as, the Chemical Repository of the National Cancer Institute, in assays that measure inhibition and/or prevention of one or more of the symptoms or indications of disorder exhibited by the transgenic animals described herein. Libraries, such as combinatorial libraries, of compounds (e.g., organic compounds, recombinant or synthetic peptides, "peptoids", nucleic acids) produced by combinatorial chemical synthesis or other methods can be tested. Where compounds selected from a library carry unique tags, identification of individual compounds by chromatographic methods is possible.

[00233] Identified therapeutic modalities can further be formulated in accordance with known methods to produce pharmaceutically-acceptable compositions. Therapeutic modalities or compositions comprising such therapeutic modalities may be administered to subjects (e.g., transgenic animals) in a variety of standard ways. For example, the agent can be administered using a variety of routes, including, for example, oral, dietary, topical, transdermal, rectal, parenteral (e.g., intravenous, intraarterial, intramuscular, subcutaneous, intradermal injection), and inhalation (e.g., intrabronchial, intranasal, oral inhalation, intranasal drops). Administration can be local or systemic as indicated. The preferred mode of administration can vary depending upon the antibody or antigen-binding fragment to be administered and the particular condition (e.g., disease) being treated, however, oral or parenteral administration is generally preferred.

[00234] Agents can be administered parenterally such as, for example, by intravenous, intramuscular, intrathecal or subcutaneous injection. Parenteral administration can be accomplished by incorporating the agent(s) into a solution or suspension. Such solutions or suspensions may also include sterile diluents, such as water for injection, saline solution, bacteriostatic saline (saline containing about 0.9% mg/ml benzyl alcohol), phosphate-buffered saline (referred to herein as PBS), Hank's solution, Ringer's-lactate, fixed oils, polyethylene glycols, glycerine, propylene glycol, and other synthetic solvents. Parenteral formulations may also include antibacterial agents (e.g., benzyl alcohol, methyl parabens), antioxidants (e.g., ascorbic acid, sodium bisulfite), and chelating agents (e.g., EDTA). Buffers, such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride and dextrose, may also be added. The parenteral preparation can be enclosed in ampules, disposable syringes, or multiple dose vials made of glass or plastic.

[00235] The level of at least one miR gene product can be measured in cells of a biological sample obtained from the subject. For example, a tissue sample can be removed from a subject suspected of having hepatocellular carcinoma cancer, by conventional biopsy techniques. In another embodiment, a blood sample can be removed from the subject, and white blood cells can be isolated

for DNA extraction by standard techniques. The blood or tissue sample is preferably obtained from the subject prior to initiation of radiotherapy, chemotherapy or other therapeutic treatment. A corresponding control tissue or blood sample, or a control reference sample, can be obtained from unaffected tissues of the subject, from a normal human individual or population of normal individuals, or from cultured cells corresponding to the majority of cells in the subject's sample. The control tissue or blood sample is then processed along with the sample from the subject, so that the levels of miR gene product produced from a given miR gene in cells from the subject's sample can be compared to the corresponding miR gene product levels from cells of the control sample.

Alternatively, a reference sample can be obtained and processed separately (e.g., at a different time) from the test sample and the level of a miR gene product produced from a given miR gene in cells from the test sample can be compared to the corresponding miR gene product level from the reference sample.

[00236] In one embodiment, the level of the at least one miR gene product in the test sample is greater than the level of the corresponding miR gene product in the control sample (i.e., expression of the miR gene product is "up-regulated" or "increased"). As used herein, expression of a miR gene product is increased when the amount of miR gene product in a cell or tissue sample from a subject is greater than the amount of the same gene product in a control cell or tissue sample. In another embodiment, the level of the at least one miR gene product in the test sample is less than the level of the corresponding miR gene product in the control sample (i.e., expression of the miR gene product is "down-regulated" or "decreased"). As used herein, expression of a miR gene is decreased when the amount of miR gene product produced from that gene in a cell or tissue sample from a subject is less than the amount produced from the same gene in a control cell or tissue sample. The relative miR gene expression in the control and normal samples can be determined with respect to one or more RNA expression standards. The standards can comprise, for example, a zero miR gene expression level, the miR gene expression level in a standard cell line, the miR gene expression level in unaffected tissues of the subject, or the average level of miR gene expression previously obtained for a population of normal human controls.

[00237] An alteration (i.e., an increase or decrease) in the level of a miR gene product in the sample obtained from the subject, relative to the level of a corresponding miR gene product in a control sample, is indicative of the presence of liver pathology in the subject. In one embodiment, the level of at least one miR gene product in the test sample is greater than the level of the corresponding miR gene product in the control sample. In another embodiment, the level of at least one miR gene product in the test sample is less than the level of the corresponding miR gene product in the control sample.

[00238] The level of a miR gene product in a sample can be measured using any technique that is suitable for detecting RNA expression levels in a biological sample. Suitable techniques (e.g., Northern blot analysis, RT-PCR, in situ hybridization) for determining RNA expression levels in a

biological sample (e.g., cells, tissues) are well known to those of skill in the art. In a particular embodiment, the level of at least one miR gene product is detected using Northern blot analysis. For example, total cellular RNA can be purified from cells by homogenization in the presence of nucleic acid extraction buffer, followed by centrifugation. Nucleic acids are precipitated, and DNA is removed by treatment with DNase and precipitation. The RNA molecules are then separated by gel electrophoresis on agarose gels according to standard techniques, and transferred to nitrocellulose filters. The RNA is then immobilized on the filters by heating. Detection and quantification of specific RNA is accomplished using appropriately labeled DNA or RNA probes complementary to the RNA in question. See, for example, *Molecular Cloning: A Laboratory Manual*, J. Sambrook et al., eds., 2nd edition, Cold Spring Harbor Laboratory Press, 1989, Chapter 7.

[00239] Suitable probes (e.g., DNA probes, RNA probes) for Northern blot hybridization of a given miR gene product can be produced from the nucleic acid sequences provided herein and include, but are not limited to, probes having at least about 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% complementarity to a miR gene product of interest, as well as probes that have complete complementarity to a miR gene product of interest. Methods for preparation of labeled DNA and RNA probes, and the conditions for hybridization thereof to target nucleotide sequences, are described in *Molecular Cloning: A Laboratory Manual*, J. Sambrook et al., eds., 2nd edition, Cold Spring Harbor Laboratory Press, 1989, Chapters 10 and 11, the disclosures of which are incorporated herein by reference.

[00240] For example, the nucleic acid probe can be labeled with, e.g., a radionuclide, such as ^3H , ^{32}P , ^{33}P , ^{14}C , or ^{35}S ; a heavy metal; a ligand capable of functioning as a specific binding pair member for a labeled ligand (e.g., biotin, avidin or an antibody); a fluorescent molecule; a chemiluminescent molecule; an enzyme or the like.

[00241] Probes can be labeled to high specific activity by either the nick translation method of Rigby et al. (1977), *J. Mol. Biol.* 113:237-251 or by the random priming method of Fienberg et al. (1983), *Anal. Biochem.* 132:6-13, the entire disclosures of which are incorporated herein by reference. The latter is the method of choice for synthesizing ^{32}P -labeled probes of high specific activity from single-stranded DNA or from RNA templates. For example, by replacing preexisting nucleotides with highly radioactive nucleotides according to the nick translation method, it is possible to prepare ^{32}P -labeled nucleic acid probes with a specific activity well in excess of 10^8 cpm/microgram. Autoradiographic detection of hybridization can then be performed by exposing hybridized filters to photographic film. Densitometric scanning of the photographic films exposed by the hybridized filters provides an accurate measurement of miR gene transcript levels. Using another approach, miR gene transcript levels can be quantified by computerized imaging systems, such as the Molecular Dynamics 400-B 2D Phosphorimager available from Amersham Biosciences, Piscataway, NJ.

[00242] Where radionuclide labeling of DNA or RNA probes is not practical, the random-primer

method can be used to incorporate an analogue, for example, the dTTP analogue 5-(N-(N-biotinyl-epsilon-aminocaproyl)-3-aminoallyl)deoxyuridine triphosphate, into the probe molecule. The biotinylated probe oligonucleotide can be detected by reaction with biotin-binding proteins, such as avidin, streptavidin and antibodies (e.g., anti-biotin antibodies) coupled to fluorescent dyes or enzymes that produce color reactions.

[00243] In addition to Northern and other RNA hybridization techniques, determining the levels of RNA transcripts can be accomplished using the technique of in situ hybridization. This technique requires fewer cells than the Northern blotting technique and involves depositing whole cells onto a microscope cover slip and probing the nucleic acid content of the cell with a solution containing radioactive or otherwise labeled nucleic acid (e.g., cDNA or RNA) probes. This technique is particularly well-suited for analyzing tissue biopsy samples from subjects. The practice of the in situ hybridization technique is described in more detail in U.S. Patent No. 5,427,916, the entire disclosure of which is incorporated herein by reference. Suitable probes for in situ hybridization of a given miR gene product can be produced from the nucleic acid sequences provided herein, and include, but are not limited to, probes having at least about 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% complementarity to a miR gene product of interest, as well as probes that have complete complementarity to a miR gene product of interest, as described above.

[00244] The relative number of miR gene transcripts in cells can also be determined by reverse transcription of miR gene transcripts, followed by amplification of the reverse-transcribed transcripts by polymerase chain reaction (RT-PCR). The levels of miR gene transcripts can be quantified in comparison with an internal standard, for example, the level of mRNA from a “housekeeping” gene present in the same sample. A suitable “housekeeping” gene for use as an internal standard includes, e.g., myosin or glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Methods for performing quantitative and semi-quantitative RT-PCR, and variations thereof, are well known to those of skill in the art.

[00245] In some instances, it may be desirable to simultaneously determine the expression level of a plurality of different miR gene products in a sample. In other instances, it may be desirable to determine the expression level of the transcripts of all known miR genes correlated with a cancer. Assessing cancer-specific expression levels for hundreds of miR genes or gene products is time consuming and requires a large amount of total RNA (e.g., at least 20 µg for each Northern blot) and autoradiographic techniques that require radioactive isotopes.

[00246] To overcome these limitations, an oligolibrary, in microchip format (i.e., a microarray), may be constructed containing a set of oligonucleotide (e.g., oligodeoxynucleotide) probes that are specific for a set of miR genes. Using such a microarray, the expression level of multiple microRNAs in a biological sample can be determined by reverse transcribing the RNAs to generate a set of target oligodeoxynucleotides, and hybridizing them to probe the oligonucleotides on the microarray to generate a hybridization, or expression, profile. The hybridization profile of the test sample can then

be compared to that of a control sample to determine which microRNAs have an altered expression level in cancer cells. As used herein, "probe oligonucleotide" or "probe oligodeoxynucleotide" refers to an oligonucleotide that is capable of hybridizing to a target oligonucleotide. "Target oligonucleotide" or "target oligodeoxynucleotide" refers to a molecule to be detected (e.g., via hybridization). By "miR-specific probe oligonucleotide" or "probe oligonucleotide specific for a miR" is meant a probe oligonucleotide that has a sequence selected to hybridize to a specific miR gene product, or to a reverse transcript of the specific miR gene product.

[00247] An "expression profile" or "hybridization profile" of a particular sample is essentially a fingerprint of the state of the sample; while two states may have any particular gene similarly expressed, the evaluation of a number of genes simultaneously allows the generation of a gene expression profile that is unique to the state of the cell. That is, normal tissue may be distinguished from cancer cells, and within cancer cell types, different prognosis states (for example, good or poor long term survival prospects) may be determined. By comparing expression profiles of liver cells in different states, information regarding which genes are important (including both up- and down-regulation of genes) in each of these states is obtained. The identification of sequences that are differentially expressed in liver cancer cells or normal cells, as well as differential expression resulting in different prognostic outcomes, allows the use of this information in a number of ways. For example, a particular treatment regime may be evaluated (e.g., to determine whether a chemotherapeutic drug acts to improve the long-term prognosis in a particular patient). Similarly, diagnosis may be done or confirmed by comparing patient samples with known expression profiles. Furthermore, these gene expression profiles (or individual genes) allow screening of drug candidates that suppress the miR or disease expression profile or convert a poor prognosis profile to a better prognosis profile.

[00248] Accordingly, described herein are methods of determining the effectiveness of intervention therapy, comprising reverse transcribing RNA from a test sample obtained from the subject to provide a set of target oligodeoxynucleotides, hybridizing the target oligodeoxynucleotides to a microarray comprising miRNA-specific probe oligonucleotides to provide a hybridization profile for the test sample, and comparing the test sample hybridization profile to a hybridization profile generated from a control sample, wherein an alteration in the signal of at least one miRNA is indicative of the subject either having, or being at risk for developing, liver cancer. In one embodiment, the microarray comprises miRNA-specific probe oligonucleotides for human miRNAs.

[00249] The microarray can be prepared from gene-specific oligonucleotide probes generated from known miRNA sequences. The array may contain two different oligonucleotide probes for each miRNA, one containing the active, mature sequence and the other being specific for the precursor of the miRNA. The array may also contain controls, such as one or more mouse sequences differing from human orthologs by only a few bases, which can serve as controls for hybridization stringency conditions. tRNAs and other RNAs (e.g., rRNAs, mRNAs) from both species may also be printed on

the microchip, providing an internal, relatively stable, positive control for specific hybridization. One or more appropriate controls for non-specific hybridization may also be included on the microchip. For this purpose, sequences are selected based upon the absence of any homology with any known miRNAs.

[00250] The microarray may be fabricated using techniques known in the art. For example, probe oligonucleotides of an appropriate length, e.g., 40 nucleotides, are 5'-amine modified at position C6 and printed using commercially available microarray systems, e.g., the GeneMachine OmniGrid™ 100 Microarrayer and Amersham CodeLink™ activated slides. Labeled cDNA oligomer corresponding to the target RNAs is prepared by reverse transcribing the target RNA with labeled primer. Following first strand synthesis, the RNA/DNA hybrids are denatured to degrade the RNA templates. The labeled target cDNAs thus prepared are then hybridized to the microarray chip under hybridizing conditions, e.g., 6X SSPE/30% formamide at 25°C for 18 hours, followed by washing in 0.75X TNT at 37°C for 40 minutes. At positions on the array where the immobilized probe DNA recognizes a complementary target cDNA in the sample, hybridization occurs. The labeled target cDNA marks the exact position on the array where binding occurs, allowing automatic detection and quantification. The output consists of a list of hybridization events, indicating the relative abundance of specific cDNA sequences, and therefore the relative abundance of the corresponding complementary miRs, in the patient sample. According to one embodiment, the labeled cDNA oligomer is a biotin-labeled cDNA, prepared from a biotin-labeled primer. The microarray is then processed by direct detection of the biotin-containing transcripts using, e.g., Streptavidin-Alexa647 conjugate, and scanned utilizing conventional scanning methods. Image intensities of each spot on the array are proportional to the abundance of the corresponding miR in the patient sample.

[00251] The use of the array has several advantages for miRNA expression detection. First, the global expression of several hundred genes can be identified in the same sample at one time point. Second, through careful design of the oligonucleotide probes, expression of both mature and precursor molecules can be identified. Third, in comparison with Northern blot analysis, the chip requires a small amount of RNA, and provides reproducible results using 2.5 µg of total RNA. The relatively limited number of miRNAs (a few hundred per species) allows the construction of a common microarray for several species, with distinct oligonucleotide probes for each. Such a tool would allow for analysis of trans-species expression for each known miR under various conditions.

[00252] In addition to use for quantitative expression level assays of specific miRs, a microchip containing miRNA-specific probe oligonucleotides corresponding to a substantial portion of the miRNome, preferably the entire miRNome, may be employed to carry out miR gene expression profiling, for analysis of miR expression patterns. Distinct miR signatures can be associated with established disease markers, or directly with a disease state.

[00253] According to the expression profiling methods described herein, total RNA from a sample from a subject is quantitatively reverse transcribed to provide a set of labeled target

oligodeoxynucleotides complementary to the RNA in the sample. The target oligodeoxynucleotides are then hybridized to a microarray comprising miRNA-specific probe oligonucleotides to provide a hybridization profile for the sample. The result is a hybridization profile for the sample representing the expression pattern of miRNA in the sample. The hybridization profile comprises the signal from the binding of the target oligodeoxynucleotides from the sample to the miRNA-specific probe oligonucleotides in the microarray. The profile may be recorded as the presence or absence of binding (signal vs. zero signal). More preferably, the profile recorded includes the intensity of the signal from each hybridization. The profile is compared to the hybridization profile generated from a normal, e.g., noncancerous, control sample. An alteration in the signal is indicative of the presence of, or propensity to develop, cancer in the subject.

[00254] Other techniques for measuring miR gene expression are also within the skill in the art, and include various techniques for measuring rates of RNA transcription and degradation.

[00255] The invention also provides methods of determining the prognosis of a subject with cancer, comprising measuring the level of at least one miR gene product, which is associated with a particular prognosis in liver cancer (e.g., a good or positive prognosis, a poor or adverse prognosis), in a test sample from the subject. According to these methods, an alteration in the level of a miR gene product that is associated with a particular prognosis, in the test sample, as compared to the level of a corresponding miR gene product in a control sample, is indicative of the subject having liver cancer with a particular prognosis. In one embodiment, the miR gene product is associated with an adverse (i.e., poor) prognosis. Examples of an adverse prognosis include, but are not limited to, low survival rate and rapid disease progression.

[00256] In certain embodiments, the level of the at least one miR gene product is measured by reverse transcribing RNA from a test sample obtained from the subject to provide a set of target oligodeoxynucleotides, hybridizing the target oligodeoxynucleotides to a microarray that comprises miRNA-specific probe oligonucleotides to provide a hybridization profile for the test sample, and comparing the test sample hybridization profile to a hybridization profile generated from a control sample.

[00257] As demonstrated herein, alterations in the level of one or more miR-221 gene products in cells can result in the deregulation of one or more intended targets for these miRs, which can lead to the formation of cancer, pathology, or toxicity. Therefore, altering the level of the miR-221 gene product (e.g., by decreasing the level of a miR-221 that is up-regulated in cancer, pathology, or toxicity), successfully treats the cancer, pathology, or toxicity.

[00258] Accordingly, also described herein are methods of treating miR-221-associated liver cancer, pathology, or toxicity in a subject. In one embodiment, the level of at least one miR gene product in a test sample is greater than the level of the corresponding miR gene product in a control sample. When the at least one isolated miR gene product is down-regulated in the cancer, pathology, or toxicity, the method comprises administering an effective amount of the at least one isolated miR

gene product, or an isolated variant or biologically-active fragment thereof, such that proliferation of cancer cells in the subject is inhibited.

[00259] As defined herein, a "variant" of a miR gene product refers to a miRNA that has less than 100% identity to a corresponding wild-type miR gene product and possesses one or more biological activities of the corresponding wild-type miR gene product. Examples of such biological activities include, but are not limited to, inhibition of expression of a target RNA molecule (e.g., inhibiting translation of a target RNA molecule, modulating the stability of a target RNA molecule, inhibiting processing of a target RNA molecule) and inhibition of a cellular process associated with cancer (e.g., cell differentiation, cell growth, cell death). These variants include species variants and variants that are the consequence of one or more mutations (e.g., a substitution, a deletion, an insertion) in a miR gene. In certain embodiments, the variant is at least about 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% identical to a corresponding wild-type miR gene product.

[00260] As defined herein, a "biologically-active fragment" of a miR gene product refers to an RNA fragment of a miR gene product that possesses one or more biological activities of a corresponding wild-type miR gene product. As described above, examples of such biological activities include, but are not limited to, inhibition of expression of a target RNA molecule and inhibition of a cellular process associated with cancer, pathology, or toxicity. In certain embodiments, the biologically-active fragment is at least about 5, 7, 10, 12, 15, or 17 nucleotides in length. In a particular embodiment, an isolated miR gene product can be administered to a subject in combination with one or more additional anti-cancer treatments. Suitable anti-cancer treatments include, but are not limited to, chemotherapy, radiation therapy and combinations thereof (e.g., chemoradiation).

[00261] When the at least one isolated miR gene product is up-regulated in the cancer cells, the method comprises administering to the subject an effective amount of a compound that inhibits expression of the at least one miR gene product, such that proliferation of cancer cells is inhibited. Such compounds are referred to herein as miR gene expression-inhibition compounds. Examples of suitable miR gene expression-inhibition compounds include, but are not limited to, those described herein (e.g., double-stranded RNA, antisense nucleic acids and enzymatic RNA molecules). In a particular embodiment, a miR gene expression-inhibiting compound can be administered to a subject in combination with one or more additional anti-cancer treatments. Suitable anti-cancer treatments include, but are not limited to, chemotherapy, radiation therapy and combinations thereof (e.g., chemoradiation).

[00262] Suitable compounds for inhibiting miR gene expression include double-stranded RNA (such as short- or small-interfering RNA or "siRNA"), antisense nucleic acids, and enzymatic RNA molecules, such as ribozymes. Each of these compounds can be targeted to a given miR gene product and interfere with the expression (e.g., by inhibiting translation, by inducing cleavage and/or degradation) of the target miR gene product.

[00263] For example, expression of a given miR gene can be inhibited by inducing RNA

interference of the miR gene with an isolated double-stranded RNA (“dsRNA”) molecule which has at least 90%, for example at least 95%, at least 98%, at least 99%, or 100%, sequence homology with at least a portion of the miR gene product. In a particular embodiment, the dsRNA molecule is a “short or small interfering RNA” or “siRNA.”

[00264] siRNA useful in the present methods comprise short double-stranded RNA from about 17 nucleotides to about 29 nucleotides in length, preferably from about 19 to about 25 nucleotides in length. The siRNA comprise a sense RNA strand and a complementary antisense RNA strand annealed together by standard Watson-Crick base-pairing interactions (hereinafter “base-paired”). The sense strand comprises a nucleic acid sequence that is substantially identical to a nucleic acid sequence contained within the target miR gene product.

[00265] As used herein, a nucleic acid sequence in a siRNA that is “substantially identical” to a target sequence contained within the target mRNA is a nucleic acid sequence that is identical to the target sequence, or that differs from the target sequence by one or two nucleotides. The sense and antisense strands of the siRNA can comprise two complementary, single-stranded RNA molecules, or can comprise a single molecule in which two complementary portions are base-paired and are covalently linked by a single-stranded “hairpin” area.

[00266] The siRNA can also be altered RNA that differs from naturally-occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siRNA or to one or more internal nucleotides of the siRNA, or modifications that make the siRNA resistant to nuclease digestion, or the substitution of one or more nucleotides in the siRNA with deoxyribonucleotides.

[00267] One or both strands of the siRNA can also comprise a 3' overhang. As used herein, a “3' overhang” refers to at least one unpaired nucleotide extending from the 3'-end of a duplexed RNA strand. Thus, in certain embodiments, the siRNA comprises at least one 3' overhang of from 1 to about 6 nucleotides (which includes ribonucleotides or deoxyribonucleotides) in length, from 1 to about 5 nucleotides in length, from 1 to about 4 nucleotides in length, or from about 2 to about 4 nucleotides in length. In a particular embodiment, the 3' overhang is present on both strands of the siRNA, and is 2 nucleotides in length. For example, each strand of the siRNA can comprise 3' overhangs of dithymidylic acid (“TT”) or diuridylic acid (“uu”).

[00268] The siRNA can be produced chemically or biologically, or can be expressed from a recombinant plasmid or viral vector, as described above for the isolated miR gene products.

Exemplary methods for producing and testing dsRNA or siRNA molecules are described in U.S. Published Patent Application No. 2002/0173478 to Gewirtz and in U.S. Published Patent Application No. 2004/0018176 to Reich et al., the entire disclosures of both of which are incorporated herein by reference.

[00269] Expression of a given miR gene can also be inhibited by an antisense nucleic acid. As used herein, an “antisense nucleic acid” refers to a nucleic acid molecule that binds to target RNA by

means of RNA-RNA, RNA-DNA or RNA-peptide nucleic acid interactions, which alters the activity of the target RNA. Antisense nucleic acids suitable for use in the present methods are single-stranded nucleic acids (e.g., RNA, DNA, RNA-DNA chimeras, peptide nucleic acids (PNA)) that generally comprise a nucleic acid sequence complementary to a contiguous nucleic acid sequence in a miR gene product. The antisense nucleic acid can comprise a nucleic acid sequence that is 50-100% complementary, 75-100% complementary, 90-100% complementary, or 95-100% complementary to a contiguous nucleic acid sequence in a miR gene product. Nucleic acid sequences of particular human miR gene products are provided in the Tables herein. Without wishing to be bound by any theory, it is believed that the antisense nucleic acids activate RNase H or another cellular nuclease that digests the miR gene product/antisense nucleic acid duplex.

[00270] Antisense nucleic acids can also contain modifications to the nucleic acid backbone or to the sugar and base moieties (or their equivalent) to enhance target specificity, nuclease resistance, delivery or other properties related to efficacy of the molecule. Such modifications include cholesterol moieties, duplex intercalators, such as acridine, or one or more nuclease-resistant groups.

[00271] Antisense nucleic acids can be produced chemically or biologically, or can be expressed from a recombinant plasmid or viral vector, using a number of standard techniques. For example, the antisense nucleic acids can be chemically synthesized or recombinantly produced using methods known in the art. In one embodiment, antisense nucleic acids are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. Commercial suppliers of synthetic RNA molecules or synthesis reagents include, e.g., Prologo (Hamburg, Germany), Dharmacon Research (Lafayette, CO, U.S.A.), Pierce Chemical (part of Perbio Science, Rockford, IL, U.S.A.), Glen Research (Sterling, VA, U.S.A.), ChemGenes (Ashland, MA, U.S.A.) and Cruachem (Glasgow, UK). Exemplary methods for producing and testing are within the skill in the art; see, e.g., Stein and Cheng (1993), Science 261:1004 and U.S. Patent No. 5,849,902 to Woolf et al., the entire disclosures of which are incorporated herein by reference.

[00272] Alternatively, the antisense nucleic acids can be expressed from recombinant circular or linear DNA plasmids using any suitable promoter. Suitable promoters for expressing RNA from a plasmid include, e.g., the U6 or H1 RNA pol III promoter sequences, or the cytomegalovirus promoters. Selection of other suitable promoters is within the skill in the art. The recombinant plasmids of the invention can also comprise inducible or regulatable promoters for expression of the antisense nucleic acids in cancer cells.

[00273] The antisense nucleic acids that are expressed from recombinant plasmids can be isolated from cultured cell expression systems by standard techniques. The antisense nucleic acids that are expressed from recombinant plasmids can also be delivered to, and expressed directly in, the cancer cells.

[00274] The antisense nucleic acids can also be expressed from recombinant viral vectors. The RNA expressed from the recombinant viral vectors can either be isolated from cultured cell

expression systems by standard techniques, or can be expressed directly in cancer cells. The use of recombinant viral vectors to deliver the antisense nucleic acids to cancer cells is discussed in more detail below.

[00275] The recombinant viral vectors of the invention comprise sequences encoding the antisense nucleic acids and any suitable promoter for expressing the RNA sequences. Suitable promoters include, but are not limited to, the U6 or H1 RNA pol III promoter sequences, or the cytomegalovirus promoters. Selection of other suitable promoters is within the skill in the art. The recombinant viral vectors of the invention can also comprise inducible or regulatable promoters for expression of the miR gene products in a cancer cell.

[00276] Any viral vector capable of accepting the coding sequences for the antisense nucleic acids can be used; for example, vectors derived from adenovirus (AV); adeno-associated virus (AAV); retroviruses (e.g., lentiviruses (LV), Rhabdoviruses, murine leukemia virus); herpes virus, and the like. The tropism of the viral vectors can be modified by pseudotyping the vectors with envelope proteins or other surface antigens from other viruses, or by substituting different viral capsid proteins, as appropriate.

[00277] For example, lentiviral vectors of the invention can be pseudotyped with surface proteins from vesicular stomatitis virus (VSV), rabies, Ebola, Mokola, and the like. AAV vectors of the invention can be made to target different cells by engineering the vectors to express different capsid protein serotypes. For example, an AAV vector expressing a serotype 2 capsid on a serotype 2 genome is called AAV 2/2. This serotype 2 capsid gene in the AAV 2/2 vector can be replaced by a serotype 5 capsid gene to produce an AAV 2/5 vector. Techniques for constructing AAV vectors that express different capsid protein serotypes are within the skill in the art; see, e.g., Rabinowitz, J.E., et al. (2002), *J. Virol.* 76:791-801, the entire disclosure of which is incorporated herein by reference.

[00278] Selection of recombinant viral vectors suitable for use in the invention, methods for inserting nucleic acid sequences for expressing RNA into the vector, methods of delivering the viral vector to the cells of interest, and recovery of the expressed RNA products are within the skill in the art. See, for example, Dornburg (1995), *Gene Therap.* 2:301-310; Eglitis (1988), *Biotechniques* 6:608-614; Miller (1990), *Hum. Gene Therap.* 1:5-14; and Anderson (1998), *Nature* 392:25-30, the entire disclosures of which are incorporated herein by reference.

[00279] Particularly suitable viral vectors are those derived from AV and AAV. A suitable AV vector for expressing the antisense nucleic acids, a method for constructing the recombinant AV vector, and a method for delivering the vector into target cells, are described in Xia et al. (2002), *Nat. Biotech.* 20:1006-1010, the entire disclosure of which is incorporated herein by reference. Suitable AAV vectors for expressing the antisense nucleic acids, methods for constructing the recombinant AAV vector, and methods for delivering the vectors into target cells are described in Samulski et al. (1987), *J. Virol.* 61:3096-3101; Fisher et al. (1996), *J. Virol.*, 70:520-532; Samulski et al. (1989), *J. Virol.* 63:3822-3826; U.S. Patent No. 5,252,479; U.S. Patent No. 5,139,941; International Patent

Application No. WO 94/13788; and International Patent Application No. WO 93/24641, the entire disclosures of which are incorporated herein by reference. In one embodiment, the antisense nucleic acids are expressed from a single recombinant AAV vector comprising the CMV intermediate early promoter.

[00280] Expression of a given miR gene can also be inhibited by an enzymatic nucleic acid. As used herein, an “enzymatic nucleic acid” refers to a nucleic acid comprising a substrate binding region that has complementarity to a contiguous nucleic acid sequence of a miR gene product, and which is able to specifically cleave the miR gene product. The enzymatic nucleic acid substrate binding region can be, for example, 50-100% complementary, 75-100% complementary, 90-100% complementary, or 95-100% complementary to a contiguous nucleic acid sequence in a miR gene product. The enzymatic nucleic acids can also comprise modifications at the base, sugar, and/or phosphate groups. An exemplary enzymatic nucleic acid for use in the present methods is a ribozyme.

[00281] The enzymatic nucleic acids can be produced chemically or biologically, or can be expressed from a recombinant plasmid or viral vector, as described above. Exemplary methods for producing and testing dsRNA or siRNA molecules are described in Werner and Uhlenbeck (1995), *Nucl. Acids Res.* 23:2092-96; Hammann et al. (1999), *Antisense and Nucleic Acid Drug Dev.* 9:25-31; and U.S. Patent No. 4,987,071 to Cech et al, the entire disclosures of which are incorporated herein by reference.

[00282] Administration of at least one compound for inhibiting miR expression, will inhibit the proliferation of cancer cells in a subject who has a cancer. As used herein, to “inhibit the proliferation of a cancer cell” means to kill the cell, or permanently or temporarily arrest or slow the growth of the cell. Inhibition of cancer cell proliferation can be inferred if the number of such cells in the subject remains constant or decreases after administration of the miR gene expression-inhibiting compounds. An inhibition of cancer cell proliferation can also be inferred if the absolute number of such cells increases, but the rate of tumor growth decreases.

[00283] The number of cancer cells in the body of a subject can be determined by direct measurement, or by estimation from the size of primary or metastatic tumor masses. For example, the number of cancer cells in a subject can be measured by immunohistological methods, flow cytometry, or other techniques designed to detect characteristic surface markers of cancer cells.

[00284] The miR gene expression-inhibiting compounds can be administered to a subject by any means suitable for delivering these compounds to cancer cells of the subject. For example, the miR expression-inhibiting compounds can be administered by methods suitable to transfect cells of the subject with these compounds, or with nucleic acids comprising sequences encoding these compounds. In one embodiment, the cells are transfected with a plasmid or viral vector comprising sequences encoding at least one miR gene expression-inhibiting compound.

[00285] Transfection methods for eukaryotic cells are well known in the art, and include, e.g., direct injection of the nucleic acid into the nucleus or pronucleus of a cell; electroporation; liposome

transfer or transfer mediated by lipophilic materials; receptor-mediated nucleic acid delivery, bioballistic or particle acceleration; calcium phosphate precipitation, and transfection mediated by viral vectors.

[00286] For example, cells can be transfected with a liposomal transfer compound, e.g., DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium methylsulfate, Boehringer-Mannheim) or an equivalent, such as LIPOFECTIN. The amount of nucleic acid used is not critical to the practice of the invention; acceptable results may be achieved with 0.1-100 micrograms of nucleic acid/ 10^5 cells. For example, a ratio of about 0.5 micrograms of plasmid vector in 3 micrograms of DOTAP per 10^5 cells can be used.

[00287] A miR gene expression-inhibiting compound can also be administered to a subject by any suitable enteral or parenteral administration route. Suitable enteral administration routes for the present methods include, e.g., oral, rectal, or intranasal delivery. Suitable parenteral administration routes include, e.g., intravascular administration (e.g., intravenous bolus injection, intravenous infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature); peri- and intra-tissue injection (e.g., peri-tumoral and intra-tumoral injection, intra-retinal injection, or subretinal injection); subcutaneous injection or deposition, including subcutaneous infusion (such as by osmotic pumps); direct application to the tissue of interest, for example by a catheter or other placement device (e.g., a retinal pellet or a suppository or an implant comprising a porous, non-porous, or gelatinous material); and inhalation. Particularly suitable administration routes are injection, infusion and direct injection into the tumor.

[00288] In the present methods, a miR gene product expression-inhibiting compound can be administered to the subject either as naked RNA, in combination with a delivery reagent, or as a nucleic acid (e.g., a recombinant plasmid or viral vector) comprising sequences that express the miR gene expression-inhibiting compound. Suitable delivery reagents include, e.g., the Mirus Transit TKO lipophilic reagent; LIPOFECTIN; lipofectamine; cellfectin; polycations (e.g., polylysine) and liposomes.

[00289] Recombinant plasmids and viral vectors comprising sequences that express the miR gene expression-inhibiting compounds, and techniques for delivering such plasmids and vectors to cancer cells, are discussed herein and/or are well known in the art.

[00290] In a particular embodiment, liposomes are used to deliver a miR gene expression-inhibiting compound (or nucleic acids comprising sequences encoding them) to a subject. Liposomes can also increase the blood half-life of the gene products or nucleic acids. Suitable liposomes for use in the invention can be formed from standard vesicle-forming lipids, which generally include neutral or negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of factors, such as the desired liposome size and half-life of the liposomes in the blood stream. A variety of methods are known for preparing liposomes.

[00291] The liposomes for use in the present methods can comprise a ligand molecule that targets

the liposome to cancer cells. Ligands that bind to receptors prevalent in cancer cells, such as monoclonal antibodies that bind to tumor cell antigens, are preferred.

[00292] The liposomes for use in the present methods can also be modified so as to avoid clearance by the mononuclear macrophage system (“MMS”) and reticuloendothelial system (“RES”). Such modified liposomes have opsonization-inhibition moieties on the surface or incorporated into the liposome structure. In a particularly preferred embodiment, a liposome of the invention can comprise both an opsonization-inhibition moiety and a ligand.

[00293] Opsonization-inhibiting moieties for use in preparing the liposomes of the invention are typically large hydrophilic polymers that are bound to the liposome membrane. As used herein, an opsonization-inhibiting moiety is “bound” to a liposome membrane when it is chemically or physically attached to the membrane, e.g., by the intercalation of a lipid-soluble anchor into the membrane itself, or by binding directly to active groups of membrane lipids. These opsonization-inhibiting hydrophilic polymers form a protective surface layer that significantly decreases the uptake of the liposomes by the MMS and RES; e.g., as described in U.S. Patent No. 4,920,016, the entire disclosure of which is incorporated herein by reference.

[00294] Opsonization-inhibiting moieties suitable for modifying liposomes are preferably water-soluble polymers with a number-average molecular weight from about 500 to about 40,000 daltons, and more preferably from about 2,000 to about 20,000 daltons. Such polymers include polyethylene glycol (PEG) or polypropylene glycol (PPG) or derivatives thereof; e.g., methoxy PEG or PPG, and PEG or PPG stearate; synthetic polymers, such as polyacrylamide or poly N-vinyl pyrrolidone; linear, branched, or dendrimeric polyamidoamines; polyacrylic acids; polyalcohols, e.g., polyvinylalcohol and polyxylitol to which carboxylic or amino groups are chemically linked, as well as gangliosides, such as ganglioside GM1. Copolymers of PEG, methoxy PEG, or methoxy PPG, or derivatives thereof, are also suitable. In addition, the opsonization-inhibiting polymer can be a block copolymer of PEG and either a polyamino acid, polysaccharide, polyamidoamine, polyethyleneamine, or polynucleotide. The opsonization-inhibiting polymers can also be natural polysaccharides containing amino acids or carboxylic acids, e.g., galacturonic acid, glucuronic acid, mannuronic acid, hyaluronic acid, pectic acid, neuraminic acid, alginic acid, carrageenan; aminated polysaccharides or oligosaccharides (linear or branched); or carboxylated polysaccharides or oligosaccharides, e.g., reacted with derivatives of carbonic acids with resultant linking of carboxylic groups. Preferably, the opsonization-inhibiting moiety is a PEG, PPG, or a derivative thereof. Liposomes modified with PEG or PEG-derivatives are sometimes called “PEGylated liposomes.”

[00295] The opsonization-inhibiting moiety can be bound to the liposome membrane by any one of numerous well-known techniques. For example, an N-hydroxysuccinimide ester of PEG can be bound to a phosphatidyl-ethanolamine lipid-soluble anchor, and then bound to a membrane. Similarly, a dextran polymer can be derivatized with a stearylamine lipid-soluble anchor via reductive amination using $\text{Na}(\text{CN})\text{BH}_3$ and a solvent mixture, such as tetrahydrofuran and water in a 30:12 ratio

at 60°C.

[00296] Liposomes modified with opsonization-inhibition moieties remain in the circulation much longer than unmodified liposomes. For this reason, such liposomes are sometimes called “stealth” liposomes. Stealth liposomes are known to accumulate in tissues fed by porous or “leaky” microvasculature. Thus, tissue characterized by such microvasculature defects, for example, solid tumors (e.g., liver cancers), will efficiently accumulate these liposomes; see Gabizon, et al. (1988), Proc. Natl. Acad. Sci., U.S.A., 18:6949-53. In addition, the reduced uptake by the RES lowers the toxicity of stealth liposomes by preventing significant accumulation of the liposomes in the liver and spleen. Thus, liposomes that are modified with opsonization-inhibition moieties are particularly suited to deliver the miR gene expression-inhibition compounds (or nucleic acids comprising sequences encoding them) to tumor cells.

[00297] The miR gene expression-inhibition compounds can be formulated as pharmaceutical compositions, sometimes called “medicaments,” prior to administering them to a subject, according to techniques known in the art. Accordingly, the invention encompasses pharmaceutical compositions for treating liver cancer. In one embodiment, the pharmaceutical composition comprises at least one isolated anti-miR-221 gene product, or an isolated variant or biologically-active fragment thereof, and a pharmaceutically-acceptable carrier. The pharmaceutical compositions of the invention comprise at least one miR-221 expression-inhibition compound. In a particular embodiment, the at least one miR gene expression-inhibition compound is specific for a miR-221 gene whose expression is greater in liver cancer cells than control cells.

[00298] Certain embodiments of the present invention are defined in the Examples herein. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

[00299] **EXAMPLES**

[00300] Example 1.

[00301] miR-221 vector construction.

[00302] The hybrid promoter EII- α 1AT includes a α 1-antitrypsin promoter linked to a hepatitis B enhancer. This chimeric promoter was amplified by PCR from the pGL3-EII- α 1AT vector using the primers EII33_XhoI_Fwd (5'-CTC GAG CCC TAT ATA TGG ATC CGC-3' **SEQ ID NO:8**) and EII512_SmaI_Rev (5'-CCC GGG TTC ACT GTC CCA GGT CA-3' **SEQ ID NO:9**). The 497 bp PCR product was directionally cloned into the XhoI and SmaI restriction sites of the pWhere vector (Invivogen, San Diego, CA).

[00303] The miR-221 DNA segment was amplified by PCR from mouse genomic DNA using the following primers: miR-221_18SmaI Fwd (5'-CCC GGG CCA GAG TTT GAT GAA GGA TGA

AG-3' **SEQ ID NO:10**) miR-221_836NcoI_Rev (5'CCA TGG GGA GGG ACA GAA ACA GAC CA-3' **SEQ ID NO:11**). The 615 bp miR-221 sequence was cloned downstream the EII- α 1AT promoter into the SmaI and NcoI restriction sites, present in the multiple cloning sites of the pWhere vector.

[00304] The accuracy of the sequence of the final vector pWhere-EII- α 1AT-miR-221 was verified by nucleotide sequencing using the following primers: pWhere-2237-Rev (5'-GCA AAC CTC CAC TCT CCA TT-3' **SEQ ID NO:12**), EII512_SmaI_Rev (5' - CCC GGG TTC ACT GTC CCA GGT CA-3' **SEQ ID NO:13**) and miR-221-375-Rev (5'-GTT CAG CCT GCA AAT TAT_CCA TA-3' **SEQ ID NO:14**).

[00305] Production of miR-221 transgenic mice.

[00306] To generate a line of transgenic mice, the pWhere-EII- α 1AT-miR-221 plasmid was linearized using the *PacI* restriction enzyme. The purified 9 kb fragment containing the transgene was used to microinject fertilized oocytes of a B6D2F2 mouse strain. The mouse strain B6D2F2 is an intercross between B6D2F1 (C57Bl6/J x DBA/2J) animals. 10 μ g of pWhere-EII α 1AT-miR-221 vector were linearized with the *PacI* restriction enzyme, purified and injected into fertilized oocytes, which were reimplanted into a surrogate mother to complete their development. To identify potential founders, PCR analyses were performed on genomic DNAs isolated from tail clips. DNAs from tail biopsies of newborn mice were used to identify the transgenics by using the specific primers α 1AT_Fwd (5'-AAT ACG GAC GAG GAC AGG G-3' **SEQ ID NO:15**) and miR-221_836Rev (5'-GGA GGG ACA GAA ACA GAC CA -3' **SEQ ID NO:16**), able to detect the DNA transgene. Template DNA for mouse genotyping was from tail biopsy samples with a diameter of 2 mm. Samples are incubated over night at 56°C in lysis buffer (50mM Tris-HCl, 100mM EDTA pH8, 100mM NaCl, 1% SDS) with Proteinase K (20 μ g/ml, Invitrogen). The genomic DNA was extracted from tissues lysates following a phenol-chloroform standard protocol, precipitated with Sodium Acetate 0,3M pH4.8 and Ethanol 100% and finally resuspended in TE buffer.

[00307] Mice were screened for the presence of the transgene by PCR analysis on genomic DNA. Several transgenics were identified. An intercross with wild type B6D2 mice demonstrated the germline transmission and established 9 heterozygous F1 lines. To generate homozygous transgenic mice over-expressing miR-221 in the liver, the miR-221 expression levels in liver were assessed by quantifying mature miR-221 in RNAs isolated from the livers of F1 transgenic animals by Real Time PCR using miR-221 TaqMan labelled probes (Applied Biosystems). The analysis of miR-221 expression in liver revealed that mice derived from 2 "founders" exhibited a 10-fold higher expression than control non-transgenic mice, while the others showed inconsistent differences of expression in comparison with wild type B6D2 mice (data not shown). F1 animals over-expressing the miR-221 were intercrossed and a F2 line was obtained. To identify potential homozygous mice, the transgene copy number was first assessed by quantitative PCR in DNAs isolated from tails. Then, animals with the highest transgene copy number were intercrossed with *wild-type* mice to confirm their true

homozygous status. Finally, a pure homozygous F3 line of transgenic animals was produced by crossing the identified homozygous mice.

[00308] RT and Real Time TaqMan PCR analysis.

[00309] Total RNA was extracted from frozen liver tissues after homogenization, with Trizol reagent (Invitrogen) according to the manufacturer's instructions. For quantitative PCR analysis, 5ng of purified RNA were retro-transcribed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystem) and mature miR-221 expression was evaluated by TaqMan miRNA Assay, with specific labeled probes for mmu-miR-221 (Applied Biosystem, assay ID001134) on a Biorad-Chromo4 thermal cycler. The reaction was carried out in a 96-well PCR plate at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was analyzed in triplicate. The level of miRNA was measured using Ct (threshold cycle) and the amount of target was calculated using 2- $\Delta\Delta$ Ct method.

[00310] To normalize the expression levels of miR-221, TaqMan murine endogenous control snoRNA412 (Applied Biosystem, assay ID001243) was used. To quantify total fg of circulating miR-221 in animals blood, all plasma samples were frozen and conserved at -80°C until RNA extraction. As a normalization control, 1.25 μ l of *C. elegans* synthetic miR-39 5nM were added every 100 μ l of plasma. RNA isolation was carried out using the TRIZOL reagent protocol (Invitrogen). For quantitative PCR analysis, a standard curve with several dilutions of a DNA oligo (5'- AGC TAC ATT GTC TGC TGG GTT TC-3' **SEQ ID NO:17**, IDT) corresponding to the miR-221 mature sequence was done. The levels of Cel miR-39 and mmu-miR-221 in each sample were assessed by TaqMan MicroRNA Assay as described above (Cel miR-39 Assay, Applied Biosystem, assay ID000200; mmu-miR-221 Assay, Applied Biosystem, assay ID001134).

[00311] Real Time EvaGreen PCR analysis:

[00312] Genomic DNA extracted from mice tails (as described in the examples above) were analyzed by quantitative PCR analysis. 25ng of DNA were amplified using the specific primers α 1AT_Fwd (5'-AAT ACG GAC GAG GAC AGG G-3' **SEQ ID NO:15**) and miR-221_836Rev (5'- GGA GGG ACA GAA ACA GAC CA -3' **SEQ ID NO:16**), with Qiagen Taq DNA Polymerase (QIAGEN, 201203) for EvaGreen detection (Biotium Inc, Hayward, CA, USA). The reactions were incubated in a 96-well PCR plate at 95°C for 15 min followed by 40 cycles of 95°C for 30 s and 58°C for 1 min. Each sample was analyzed in triplicate. Fluorescence measurements were completed using a Biorad-Chromo4 thermal cycler real-time PCR instrument.

[00313] Microarray gene expression analysis.

[00314] RNAs were hybridized on Agilent Whole Mouse Gene Expression Microarray (#G4122F, Agilent Technologies, Palo Alto, CA). One-color gene expression was performed according to the manufacturer's procedure. Briefly, total RNA fraction is obtained from samples by using the Trizol Reagent (Invitrogen). RNA quality is assessed by the use of Agilent 2100 Bioanalyzer (Agilent Technologies). Low quality RNAs (RNA integrity number below 7) were excluded from microarray

analyses. Labeled cRNA is synthesized from 500ng of total RNA using the Low RNA Input Linear Amplification Kit (Agilent Technologies) in the presence of cyanine 3-CTP (Perkin-Elmer Life Sciences, Boston, MA). Hybridizations were performed at 65°C for 17 hours in a rotating oven. Images at 5 um resolution were generated by an Agilent scanner and the Feature Extraction 10.5 software (Agilent Technologies) was used to obtain the microarray raw-data.

[00315] Microarray results were analyzed by using the GeneSpring GX 11 software (Agilent Technologies). Data transformation was applied to set all the negative raw values at 1.0, followed by a quantile normalization. A filter on low gene expression was used to keep only the probes expressed in at least one sample (flagged as Marginal or Present). Differentially expressed genes were selected as having a 2- fold expression difference between the groups of interest and a p-value < 0.05 at unpaired t test; Benjamin-Hoechberg multiple testing correction was applied to obtain the false discovery rates. Hierarchical clustering was performed using Manhattan correlation as a measure of similarity. All microarray data were submitted to ArrayExpress, accession number: E-TABM-1203.

[00316] *In vivo studies.*

[00317] All animal experimentation was performed in accordance with institutional ethical committee. The mice were maintained in a sterile room at 25°C with 12-hour light-dark cycle and provided food and water ad libitum. 10-day newborn mice received one intraperitoneal injection of DENA (7,5mg/kg body weight) and then sacrificed after six or nine months. All the mice were subjected to autopsy and all the tissues were partly fixed in 10% formalin and partly frozen in liquid nitrogen. Mice and livers were both weighed. The anti-miR oligonucleotide against miR-221 used is: 5'-mG*mA*mA mAmCmC mCmAmG mCmAmG mAmCmA mAmUmG mU*mA*mG* mC*mU-3' (where "m" represents 2'-O-Methyl RNA bases and "*" represents phosphorothioate bond) (**SEQ ID NO:7**).

[00318] For *in vivo* evaluation of miR-221 targeting, several mice received a single intravenous dose of 350 µg (10 mg/kg) of anti-miR-221 diluted in saline solution. All the animals were sacrificed after 48 hours. Blood and livers were analyzed as described herein.

[00319] For assessing anti-tumor activity of *in vivo* anti-miR treatments, 10-day newborn mice received one intraperitoneal injection of DENA (7,5mg/kg body weight) and after 2 months each mouse received intravenously a single dose of anti-miR-221 (10mg/kg) every 15 days for a total of three injections (about 1 mg total dose for each mouse). The mice were sacrificed at 4 months of age and all the tissues were treated as described above.

[00320] *Western Blot analysis.*

[00321] Tissue samples were collected, immediately frozen in liquid nitrogen and stored at -80°C until protein extraction. Samples were subjected to mechanical pulverization in dry ice and then dissolved by repeated syringing in lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 2.5 mmol/L MgCl₂, 0.5% Triton X-100, 1 mmol/L DTT, and protease inhibitors]. Homogenates were then centrifuged at 13000 rpm for fifteen minutes at 4°C and supernatants were collected and analyzed by Western blot to

assess Bmf, Cdkn1c/p57 and Cdkn1b/p27 expression. Rabbit polyclonal antibodies against Bmf (Novus Biologicals, Littleton, CO), p57/Kip2 (clone- EP2515Y, Epitomics) and p27/Kip1 (clone- EPFHCR16, Epitomics, Burlingame, CA) were diluted in 1:500, 1:1000 and 1:2000 in T-PBS, respectively, and incubated for 16 h at 4°C. A horseradish-conjugated secondary antibody (labeled polymer horseradish peroxidase anti-rabbit, Envision system; DAKO Cytomation, Glostrup, Denmark) was used. After autoradiography acquisition, the membranes were reprobed for 1 h at room temperature with anti-β-actin monoclonal antibody (Clone C4, sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-β-tubulin polyclonal antibody (H-235, sc-9104, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 as housekeeping genes. Digital images of autoradiographies were acquired with Fluor-S MultiImager and band signals were acquired in the linear range of the scanner using a specific densitometric software (Quantity One).

[00322] *Histological Procedures and Morphological Criteria Used for the Classification of Liver Lesions.*

[00323] Tissue samples from at least two representative fragments of each lobe of the liver were taken at autopsy and fixed in 10% phosphate-buffered formalin for 12 to 24 hours, then were embedded in paraffin for histological examination. Three- to five-micron serial sections were stained with hematoxylin and eosin for the histological examination. The criteria of Koen and Becker (Becker, 1982; Koen et al., 1983b) slightly modified, were used for the classification of liver lesions. Hepatocyte proliferative lesions: 1 mm were defined as liver tumors (Becker, 1982) and classified as adenomas or carcinomas based on cytological features and architectural patterns.

[00324] The LCD and basophilic liver cell nodules (BLCN) were identified according to Solt et al. (Solt et al., 1977). Similar cytological modifications associated with other pathological conditions (inflammation, amyloidosis) of the liver were not considered as dysplastic-preneoplastic lesions and were not included in the analysis. All of the proliferative liver lesions were grouped together as: liver cell proliferative nodules.

[00325] *Immunohistochemistry.*

[00326] The presence and localization of Cdkn1b/p27 protein in liver tissues from transgenic mice and controls were immunohistochemically assessed on formalin-fixed, paraffin embedded sections. Serial 4 μm thick sections were processed for hematoxylin and eosin staining and for immunohistochemistry. After re-hydration, inhibition of endogenous peroxidases was obtained by incubation of slides in 1% H₂O₂-methanol for 20 minutes at 4°C. Pressure-cooker pre-treatment was performed with a citrate buffer solution (pH 6.0). 10% goat serum solution was used for 15 minutes at room temperature for non-specific epitopes masking. Primary antibody was incubated overnight at 4.0 °C for all primary antibodies. The following primary antibody and dilutions were used: anti-p27/Kip1 (clone- EPFHCR16, Epitomics, Burlingame, CA) 1:100/1:50. Sections were then washed twice in 0.1M PBS (0.1M Phosphate Buffered NaCl solution, pH 7.20). Secondary marking was performed with a Horse-Radish-Peroxidase-rabbit EnVision system (DAKO, Denmark) for 30

minutes at room temperature. After repeated washes in PBS the immunohistochemical staining was visualized using the diaminobenzidine color substrate (Sigma Chemical Company, St. Louis, MO, USA). After rinsing in distilled water, slides were counterstained with Mayer's hematoxylin and mounted with DPX mountant (Sigma Chemical Company, St. Louis, MO, USA). Negative controls were obtained by omitting the primary antibody.

[00327] Cell cultures, Transfection and Luciferase Activity Assay.

[00328] Transfections were performed by Lipofectamine 2000 reagent (Invitrogen) diluting the DNA plasmids in Opti-MEM reduced serum medium (Invitrogen-Gibco), in a final volume of 250 μ l. SNU398 cells were cultured in a 24 wells plate and transfected in triplicate with 400ng of pWhere-EII α /AT-miR-221 vector. Cells were collected 48h after transfection and the RNA was extracted following TRIZOL protocol. HepG2, H460 and NIH3T3 cell lines were cultured in 24 wells plates and transfected with 400ng of either pGL3 Control or pGL3+ EII- α /AT plasmids together with 40ng pRL-TK vector (Promega), which contains the Renilla luciferase gene. Each transfection was repeated in triplicate. At 24h after transfection, Firefly and Renilla luciferase activity were measured using the Dual-Luciferase Reporter Assay (Promega). Lung carcinoma derived H460 (ATCC, HTB-177), hepatocellular carcinomas derived HepG2 (ATCC, HB-8065) and SNU398 (ATCC, CRL-2233), mouse fibroblast NIH3T3 (ATCC, CRL-1658) cell lines were maintained in IMDM medium, supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and 0,1% Gentamicin (Sigma-Aldrich).

[00329] Example 2.

[00330] Characterization of the livers of miR-221 transgenic mice.

[00331] To assess miR-221 expression levels in the transgenic model, livers taken from homozygous mice at different ages were analyzed by Real Time PCR. In comparison with wild type mice, the analysis revealed a stable and increased expression of miR-221 in the livers of transgenic animals, thereby confirming the development of a homozygous transgenic mice over-expressing miR-221 in hepatic cells (**Figure 1B**).

[00332] From the analysis of 12 different mouse tissues (liver, intestine, stomach, lung, skeletal muscle, heart, kidney, bladder, spleen, brain, thymus and fat tissue), only liver and kidney exhibited a significant increase of miR-221 (**Figure 19B**).

[00333] Macroscopically, the livers of transgenic mice exhibited an increase in volume and weight in comparison with controls (**Figure 20**). Histologically, while both groups displayed a conserved liver architecture, transgenic livers were characterized by variable extents of steatohepatic changes, with hepatocyte degeneration characterized by enlarged cells with large dysplastic nuclei, lipidic vacuoli and focal coagulative necrosis (**Figure 2**). These changes were more evident in older transgenic animals and absent among wild type controls.

[00334] To assess whether miR-221 up-regulation could affect the expression of its targets, an immunoblotting analysis was performed in order to verify the expression of the miR-221 target

proteins Cdkn1b/p27, Cdkn1c/p57 and Bmf. In normal liver, it was demonstrated that Bmf and Cdkn1b/p27 were both significantly down-regulated in transgenic mice. Cdkn1c/p57 was also generally down-regulated although it did not reach statistical significance (**Figure 3**).

[00335] Immunostaining for Cdkn1b/p27 demonstrated the strong reduction of the protein in transgenic animals (**Figure 13**).

[00336] In addition to the above characteristics, a gene expression profiling proved that the livers of transgenics differed from wild type also at a deeper molecular level (**Figure 14A** and **Figure 22**). Interaction analysis revealed that many of the identified protein coding genes were connected to the modulation of interferon gamma, which was itself expressed at lower levels in the livers of transgenic mice (**Figure 14B**).

[00337] *Example 3.*

[00338] *miR-221 promotes liver tumorigenesis.*

[00339] The miR-221 transgenic mouse model analyzed to confirm predisposition to the development of liver cancer. By monitoring mice at different ages (3, 6, 9, 12 months), it emerged that females did not develop tumors, while a fraction of males developed spontaneous tumors that became visible not earlier than 9 months of age. Four of 8 observed male mice (50%), aged at least 9 months (range 9-12 months) showed evidence of small but visible liver tumors. The observations were supported by a significant up-regulation of miR-221 in the spontaneous tumors analyzed (**Figure 15**).

[00340] Transgenic mice also exhibited an increased susceptibility to treatment with the carcinogen diethylnitrosamine (DEN). Transgenic mice as well as wild type mice were injected intra-peritoneally with 7.5 mg/kg DEN at 10 days of age. All the mice were daily monitored and periodically sacrificed at 3, 4 and 6 months of age. An increasing development of tumors was observed at the different time points in all the mice, which was stronger in the transgenic mice than in wild type control mice (**Figure 16**). At 6 months, all male mice treated with DEN showed evidence of multiple large tumors. Transgenic mice exhibited a larger number of foci, which were also larger in size than in wild type control mice. Tumor burden caused a significant increase in liver weight.

[00341] Possibly because of the presence of debilitating liver tumors, transgenic mice exhibited a significant decrease in body weight than controls (**Figure 21**). In females treated with DEN, liver tumors were not visible at 6 months. However, starting at 9 months of age, tumors began to become visible also in transgenic females.

[00342] Liver nodules in miR-221 transgenic mice displayed variable degrees of cell differentiation and architecture, ranging from adenoma-like lesions to overt carcinomas (**Figure 17**). Neoplastic cells show a stronger basophilic reaction. In both miR-221 transgenic mice and control mice, multifocal HCCs were detectable and displayed a pseudoglandular or, more often, a trabecular pattern of growth. Their size varied in diameter from 1 mm to 1 cm. At six months of age, in DEN-treated transgenic males, tumors almost completely substituted the entire liver by confluent neoplastic

nodules, which were characterized by an infiltrative invasive front with no demarcation from the surrounding liver parenchyma, presence of necrotic areas, marked angiogenesis with slit-like sinusoids lined by endothelium, intravasation of tumor cells (**Figure 17D**).

[00343] Conversely, DENA-treated control mice displayed tumor nodules smaller in size and lower in number, characterized by a better defined tumor margin without a fibrous capsule, together with less evident angiogenesis with single cell plates and adenoma-like features in the smallest nodules (**Figure 17E**). All tumors were composed almost entirely of basophilic cells and those cells were more evident in zones of trabeculation of large tumors. They were irregularly branched and composed of cells with basophilic cytoplasm and central oval nucleus with small nucleoli. Mitoses were rare.

[00344] At the molecular level, tumors were characterized by a further increase in miR-221 expression (**Figure 5**). Other miRNAs typically deregulated in human HCC were analyzed: miR-21 was up-regulated, while miR-122 and miR-199 were downregulated, results that mimic the human HCC condition. The further increase in miR-221 expression was likely responsible for the strong inhibition detected on its targets Cdkn1b/p27, Cdkn1c/p57 and Bmf (**Figure 6, Figure 10, and Figure 11**).

[00345] Example 4.

[00346] Anti-miR-221 controls in vivo tumorigenicity

[00347] To confirm that the up-regulation of miR-221 is oncogenic and important in the maintenance of liver tumors and also investigate the potential anti-tumor activity of anti-miR-221 AMOs in this transgenic mouse model, the endogenous miR-221 was inhibited through the *in vivo* delivery of anti-miR-221 oligonucleotides.

[00348] To assess the effects on miR-221 levels, first, a group of three transgenic mice were intravenously injected through the tail vein with a single dose of an antisense 2' O methyl oligoribonucleotide targeting miR-221 (10 mg/kg). Forty-eight hours after injection, molecular analysis revealed a significant down-regulation of miR-221 levels both in liver and plasma of anti-miR treated mice in comparison with untreated controls, thus revealing a functional antisense inhibition of the miR-221 *in vivo* (**Figure 7 and Figure 21**).

[00349] These effects were also accompanied and supported by a concurrent increase in Cdkn1b/p27 protein expression in liver (**Figure 6**).

[00350] Then, to assess the effect of anti-miR-221 oligonucleotides on liver tumorigenicity in this transgenic mouse model and establish if miR-221 could represent an anti-tumor therapeutic target, a group of five mice were treated with anti-miR-221 AMOs (10mg/kg at 60, 75 and 90 days) after intraperitoneal injection with DENA (at 10-days). Three mice were sacrificed at 120 days of age and two at 150 days of age. Significantly, a reduction in the number and size of tumors was observed in anti-miR-221 treated mice in comparison with same age (4 or 5 months) mice treated with DENA only (**Figure 7 and Figure 12**).

[00351] These anti-tumor effects were accompanied by a persistent significant decrease of miR-221 expression in tumors arising in the group of AMOs treated mice (**Figure 7**).

[00352] Example 5

[00353] Therapeutic/Prophylactic Methods and Compositions

[00354] Also provided herein are methods of treatment and prophylaxis by administration to a subject an effective amount of a therapeutic, i.e., a monoclonal (or polyclonal) antibody, viral vector, mimic and/or antagonist. In a preferred aspect, the therapeutic is substantially purified. The subject is preferably an animal, including but not limited to, animals such as cows, pigs, chickens, etc., and is preferably a mammal, and most preferably human.

[00355] Various delivery systems are known and are used to administer a therapeutic of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, expression by recombinant cells, receptor-mediated endocytosis, construction of a therapeutic nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, and oral routes. The compounds are administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local.

[00356] In a specific embodiment, it may be desirable to administer the pharmaceutical compositions locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, the implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration is by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

[00357] In a specific embodiment where the therapeutic is a nucleic acid encoding a protein therapeutic the nucleic acid is administered *in vivo* to modulate expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus. Alternatively, a nucleic acid therapeutic can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

[00358] Also provided herein are pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a therapeutic, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes, but is not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The carrier and composition can be sterile. The formulation will suit the mode of administration.

[00359] The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

[00360] In one embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. For example, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition also includes a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it is be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline is provided so that the ingredients are mixed prior to administration.

[00361] The therapeutic formulation can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

[00362] The amount of the therapeutic formulation which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and is determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and is decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

[00363] Also provided herein is a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions. Optionally associated with such container(s) is a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[00364] Methods of treating cancer patients

[00365] This example describes a method of selecting and treating patients that are likely to have a favorable response to treatments with compositions herein.

[00366] In certain circumstances, a patient diagnosed with cancer ordinarily first undergoes tissue resection with an intent to cure. Tumor samples are obtained from the portion of the tissue removed from the patient. RNA is then isolated from the tissue samples using any appropriate method for extraction of small RNAs that are well known in the art, such as by using TRIZOLTM. Purified RNA is then subjected to RT-PCR using primers specific miR21 or other differentially expressed miRNAs disclosed, optionally in conjunction with genetic analysis. These assays are run to determine the expression level of the pertinent RNA in the tumor. If differentially expressed miR expression pattern is determined, especially if mutant status is ascertained, the patient is a candidate for treatment with the compositions herein.

[00367] Accordingly, the patient is treated with a therapeutically effective amount of the compositions according to methods known in the art. The dose and dosing regimen of the compositions will vary depending on a variety of factors, such as health status of the patient and the stage of the cancer. Typically, treatment is administered in many doses over time.

[00368] Methods of Diagnosing Cancer Patients

[00369] In one particular aspect, there is provided herein a method of diagnosing whether a subject has, or is at risk for developing, a particular type of cancer. The method generally includes measuring the differential miR expression pattern of the miR compared to control. If a differential miR expression pattern is ascertained, the results are indicative of the subject either having, or being at risk for developing, cancer. In certain embodiments, the level of the at least one gene product is measured using Northern blot analysis. Also, in certain embodiments, the level of the at least one gene product in the test sample is less than the level of the corresponding miR gene product in the control sample, and/or the level of the at least one miR gene product in the test sample is greater than the level of the corresponding miR gene product in the control sample.

[00370] Measuring miR Gene Products

[00371] The level of the at least one miR gene product can be measured by reverse transcribing RNA from a test sample obtained from the subject to provide a set of target oligodeoxynucleotides; hybridizing the target oligodeoxynucleotides to a microarray comprising miRNA-specific probe oligonucleotides to provide a hybridization profile for the test sample; and, comparing the test sample hybridization profile to a hybridization profile generated from a control sample. An alteration in the signal of at least one miRNA is indicative of the subject either having, or being at risk for developing, lung cancer, particularly EGFR mutant lung cancer.

[00372] Diagnostic and Therapeutic Applications

[00373] In another aspect, there is provided herein are methods of treating a cancer in a subject, where the signal of at least one miRNA, relative to the signal generated from the control sample, is

de-regulated (e.g., down-regulated and/or up-regulated).

[00374] Also provided herein are methods of diagnosing whether a subject has, or is at risk for developing, a cancer associated with one or more adverse prognostic markers in a subject, by reverse transcribing RNA from a test sample obtained from the subject to provide a set of target oligodeoxynucleotides; hybridizing the target oligodeoxynucleotides to a microarray comprising miRNA-specific probe oligonucleotides to provide a hybridization profile for the test sample; and, comparing the test sample hybridization profile to a hybridization profile generated from a control sample. An alteration in the signal is indicative of the subject either having, or being at risk for developing, the cancer.

[00375] Kits

[00376] Any of the compositions described herein may be comprised in a kit. In a non-limiting example, reagents for isolating miRNA, labeling miRNA, and/or evaluating a miRNA population using an array are included in a kit. The kit may further include reagents for creating or synthesizing miRNA probes. The kits will thus comprise, in suitable container means, an enzyme for labeling the miRNA by incorporating labeled nucleotide or unlabeled nucleotides that are subsequently labeled. It may also include one or more buffers, such as reaction buffer, labeling buffer, washing buffer, or a hybridization buffer, compounds for preparing the miRNA probes, and components for isolating miRNA. Other kits may include components for making a nucleic acid array comprising oligonucleotides complementary to miRNAs, and thus, may include, for example, a solid support.

[00377] For any kit embodiment, including an array, there can be nucleic acid molecules that contain a sequence that is identical or complementary to all or part of any of the sequences herein.

[00378] The components of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit (labeling reagent and label may be packaged together), the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The kits of the present invention also will typically include a means for containing the nucleic acids, and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

[00379] When the components of the kit are provided in one and/or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being one preferred solution. Other solutions that may be included in a kit are those solutions involved in isolating and/or enriching miRNA from a mixed sample.

[00380] However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a

suitable solvent. It is envisioned that the solvent may also be provided in another container means. The kits may also include components that facilitate isolation of the labeled miRNA. It may also include components that preserve or maintain the miRNA or that protect against its degradation. The components may be RNase-free or protect against RNases.

[00381] Also, the kits can generally comprise, in suitable means, distinct containers for each individual reagent or solution. The kit can also include instructions for employing the kit components as well the use of any other reagent not included in the kit. Instructions may include variations that can be implemented. It is contemplated that such reagents are embodiments of kits of the invention. Also, the kits are not limited to the particular items identified above and may include any reagent used for the manipulation or characterization of miRNA.

[00382] It is also contemplated that any embodiment discussed in the context of a miRNA array may be employed more generally in screening or profiling methods or kits of the invention. In other words, any embodiments describing what may be included in a particular array can be practiced in the context of miRNA profiling more generally and need not involve an array per se.

[00383] It is also contemplated that any kit, array or other detection technique or tool, or any method can involve profiling for any of these miRNAs. Also, it is contemplated that any embodiment discussed in the context of a miRNA array can be implemented with or without the array format; in other words, any miRNA in a miRNA array may be screened or evaluated in any method of the invention according to any techniques known to those of skill in the art. The array format is not required for the screening and diagnostic methods to be implemented.

[00384] The kits for using miRNA arrays for therapeutic, prognostic, or diagnostic applications and such uses are contemplated by the inventors herein. The kits can include a miRNA array, as well as information regarding a standard or normalized miRNA profile for the miRNAs on the array. Also, in certain embodiments, control RNA or DNA can be included in the kit. The control RNA can be miRNA that can be used as a positive control for labeling and/or array analysis.

[00385] The methods and kits of the current teachings have been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the current teachings. This includes the generic description of the current teachings with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00386] Array Preparation and Screening

[00387] Also provided herein are the preparation and use of miRNA arrays, which are ordered macroarrays or microarrays of nucleic acid molecules (probes) that are fully or nearly complementary or identical to a plurality of miRNA molecules or precursor miRNA molecules and that are positioned on a support material in a spatially separated organization. Macroarrays are typically sheets of nitrocellulose or nylon upon which probes have been spotted. Microarrays position the nucleic acid probes more densely such that up to 10,000 nucleic acid molecules can be fit into a region typically 1

to 4 square centimeters.

[00388] Microarrays can be fabricated by spotting nucleic acid molecules, e.g., genes, oligonucleotides, etc., onto substrates or fabricating oligonucleotide sequences in situ on a substrate. Spotted or fabricated nucleic acid molecules can be applied in a high density matrix pattern of up to about 30 non-identical nucleic acid molecules per square centimeter or higher, e.g. up to about 100 or even 1000 per square centimeter. Microarrays typically use coated glass as the solid support, in contrast to the nitrocellulose-based material of filter arrays. By having an ordered array of miRNA-complementing nucleic acid samples, the position of each sample can be tracked and linked to the original sample.

[00389] A variety of different array devices in which a plurality of distinct nucleic acid probes are stably associated with the surface of a solid support are known to those of skill in the art. Useful substrates for arrays include nylon, glass and silicon. The arrays may vary in a number of different ways, including average probe length, sequence or types of probes, nature of bond between the probe and the array surface, e.g. covalent or non-covalent, and the like. The labeling and screening methods described herein and the arrays are not limited in its utility with respect to any parameter except that the probes detect miRNA; consequently, methods and compositions may be used with a variety of different types of miRNA arrays.

[00390] Additional Uses

[00391] This animal model is also useful for the identification, development and testing of additional therapeutic agents that are useful in treating or preventing liver disorders.

[00392] In further aspect, the mouse model is useful in identifying and quantifying the toxicity of any potential therapeutic or potential carcinogen. In one non-limiting example, the mouse model is useful in toxicity, safety and other routine screening of drugs, foods, ingredients, animal feed, pesticides, herbicides, cosmetics, nutraceuticals, beverages, paints, solvents, industrial chemicals, and the like. Moreover, the present invention is useful as a follow up quality assurance assay after toxic site cleanup, for instance, PCBs, radiation, poisons, industrial waste, and the like.

[00393] In view of the many possible embodiments to which the principles described herein may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as a limitation on the scope of the invention. Rather, the scope of the invention is defined by the following claims. The inventors therefore claim as the inventors' invention all that comes within the scope and spirit of these claims.

[00394] All publications, including patents and non-patent literature, referred to in this specification are expressly incorporated by reference herein. Citation of the any of the documents recited herein is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

[00395] While the invention has been described with reference to various and preferred embodiments, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted for elements thereof without departing from the essential scope of the invention. In addition, many modifications may be made to adapt a particular situation or material to the teachings of the invention without departing from the essential scope. Therefore, it is intended that the invention not be limited to the particular embodiment disclosed herein contemplated for carrying out this invention, but that the invention will include all embodiments falling within the scope of the claims.

CLAIMS

What is claimed is:

1. A method for suppressing a liver cancer, comprising: contacting a liver cell with at least one antisense miR-221 oligonucleotide, thereby suppressing the liver cancer.
2. A method of claim 1, wherein the at least one antisense miR-221 oligonucleotide comprises a nucleotide sequence having at least 90% sequence identity to **SEQ ID NO: 5** (anti-precursor miR-221) or **SEQ ID NO: 6** (anti-mature miR-221).
3. The method of claim 1, wherein the liver cell that is treated is compared to a control of the same genotype, wherein the control has not been treated with antisense miR-221 oligonucleotide.
4. A method to ameliorate the effects of miR-221 on liver cells, comprising administering at least one modified anti-miR-221 oligonucleotide to liver cells.
5. The method of claim 4, wherein the anti-miR-221 oligonucleotide is modified to further comprise a stabilizing group.
6. The method of claim 5, wherein the anti-miR-221 oligonucleotide is modified to further comprise 2'-O-methyl RNA bases with a phosphorothioate bond.
7. The method of claim 4, 5 or 6, wherein the at least one anti-miR-221 oligonucleotide comprises a nucleotide sequence having at least 90% sequence identity to **SEQ ID NO: 5** (anti-precursor miR-221) or **SEQ ID NO: 6** (anti-mature miR-221) and/or **SEQ ID NO: 7** (modified anti-miR-221 with 2-O-methyl RNA bases and phosphorothioate bonds).
8. The method of claim 4, 5 or 6, wherein the suppressed liver cancer oncogene is a miR-221 gene product.
9. The method of claim 4, 5 or 6, which further suppresses liver cancer cell proliferation, liver cancer cell growth, or liver cell tumor development.
10. A method of ameliorating the risk of liver cancer in a subject, comprising:

administering a pharmacologically effective amount of **SEQ ID NO: 5** (anti-precursor miR-221), **SEQ ID NO: 6** (anti-mature miR-221), and/or **SEQ ID NO: 7** (modified anti-miR-221 with 2-O-methyl RNA bases and phosphorothioate bonds) to a subject with elevated miR-221 gene product, and ameliorating the risk of liver cancer in the subject.

11. A method of ameliorating the symptoms of liver cancer in a subject, comprising: administering a pharmacologically effective amount of **SEQ ID NO: 5** (anti-precursor miR-221), **SEQ ID NO: 6** (anti-mature miR-221), and/or **SEQ ID NO: 7** (modified anti-miR-221 with 2-O-methyl RNA bases and phosphorothioate bonds) to a subject with liver cancer symptoms, and ameliorating the liver cancer symptoms in the subject.

12. A method of reducing liver tumor growth in a subject, comprising: administering a pharmacologically effective amount of **SEQ ID NO: 5** (anti-precursor miR-221), **SEQ ID NO: 6** (anti-mature miR-221), and/or **SEQ ID NO: 7** (modified anti-miR-221 with 2-O-methyl RNA bases and phosphorothioate bonds) to a subject with liver tumors, and reducing liver tumor growth in the subject.

13. The method of any one of claims 10 - 12, which further comprises administering a chemotherapeutic agent or conducting cancer or tumor resection surgery.

14. The method of any one of claims 10 - 13, wherein the subject is selected from the group consisting of: mouse; rat; guinea pig; cat; dog; horse; cow; pig; and human.

15. The method of any one of claims 11 - 14, wherein the cancer or tumor is hepatocellular carcinoma.

16. The method of any one of claims 11 - 14, wherein the cancer or tumor is any neoplasm with elevated miR-221 gene product.

17. A pharmaceutical formulation comprising at least one anti-miR-221 oligonucleotide having a nucleotide sequence with at least 90% sequence identity to **SEQ ID NO: 5** (anti-precursor miR-221), **SEQ ID NO: 6** (anti-mature miR-221), and/or **SEQ ID NO: 7** (modified anti-miR-221 with 2-O-methyl RNA bases and phosphorothioate bonds).

18. The formulation of claim 17, wherein the nucleotide sequence has at least 95% sequence identity to **SEQ ID NO: 5**, **SEQ ID NO: 6**, and/or **SEQ ID NO: 7**.

19. The formulation of claim 17, wherein the formulation comprises at least two anti-miR-221 oligonucleotides selected from the group consisting of: **SEQ ID NO: 5**, **SEQ ID NO: 6**, and **SEQ ID NO: 7**.

20. The formulation of claim 17, wherein the formulation is prepared for at least one of: intravenous injection, intra-tumor injection, or intraperitoneal injection.

21. A transgenic mouse whose genome comprises a nucleic acid construct capable of overexpressing at least one miR-221 gene in liver cells.

22. The transgenic mouse of claim 21, wherein the construct comprises a nucleotide sequence having at least 90% sequence identity to **SEQ ID NO: 1** (precursor miR-221) or **SEQ ID NO: 2** (mature miR-221).

23. The transgenic mouse of claim 21, wherein the construct comprises a vector.

24. The transgenic mouse of claim 23, wherein the vector comprises a promoter.

25. The transgenic mouse of claim 24, wherein the promoter comprises a α 1-antitrypsin promoter operably linked to a hepatitis B enhancer.

26. The transgenic mouse of claim 21, wherein the construct comprises pWhere-Ell- α 1AT vector.

27. The transgenic mouse of claim 21, wherein the construct comprises a pWhere-Ell- α 1AT vector comprising at least one operably linked miR-221 sequence.

28. The transgenic mouse of claim 27, wherein the construct comprises a pWhere-Ell- α 1AT vector comprising at least one operably linked nucleic acid of **SEQ ID NO: 1** (precursor miR-221) and/or **SEQ ID NO: 2** (mature miR-221).

29. The transgenic mouse of claim 21, wherein when the at least one miR-221 gene product is overexpressed in liver cells of the transgenic mouse, the mouse develops malignant liver cancer cells.

30. The transgenic mouse of claim 21, wherein the miR-221 gene product, comprising at least one of **SEQ ID NO: 3** (precursor miR-221) and/or **SEQ ID NO: 4** (mature miR-221), is

overexpressed in liver cells of the transgenic mouse

31. The transgenic mouse of claim 29, wherein the mouse develops hepatocellular carcinoma.
32. The transgenic mouse of claim 29, which is male.
33. The transgenic mouse of claim 21, which does not have a cirrhotic liver.
34. The transgenic mouse of claim 21, which exhibits high levels of steatosis.
35. The transgenic mouse of claim 21, which further comprises at least an attribute selected from: repression of Cdkn1b/p27; repression of Cdkn1cp57; repression of Bmf proteins; upregulation of miR-221; downregulation of miR-122; downregulation of miR-199; and upregulation of miR-21.
36. A method of determining whether a test agent affects a liver condition in a subject, comprising:
 - a. administering a test agent to a transgenic mouse of claim 21; and
 - b. after the agent has been administered to the transgenic mouse, comparing one or more symptoms and/or indications of a liver condition in the transgenic animal to those of a control animal of the same genotype, wherein the control animal has not been administered the agent, wherein a difference in the detectability and/or rate of appearance of the one or more symptoms and/or indications of the liver condition in the transgenic animal, relative to the control animal, is indicative of the test agent affecting the liver condition.
37. The method of claim 36, wherein a liver tumor-inducing treatment is administered concurrent with step a).
38. The method of claim 37, wherein the liver tumor-inducing treatment is diethylnitrosamine.
39. The method of claim 36 , 37 or 38, wherein the mouse is male.
40. The method of claim 36, 37, 38 or 39, wherein the liver condition is hepatocellular carcinoma.

41. The method of claim 36, 37, 38 or 39, wherein the liver condition is visible tumor, pseudoglandular, or trabecular cell growth.

42. The method of claim 36, 37, 38 or 39, wherein the liver condition basophilic cell invasion.

43. The method of claim 36, 37, 38 or 39, wherein the one or more symptoms and/or indications of the liver condition are selected from the group consisting of: enlarged abdomen; externally-visible lumps; body weight loss; and a combination thereof.

44. The method of claim 36, 37, 38 or 39, wherein one of more symptoms and/or indications of the liver condition comprises an attribute selected from the group consisting of: repression of Cdkn1b/p27; repression of Cdkn1cp57; repression of Bmf proteins; upregulation of miR-221; downregulation of miR-122; downregulation of miR-199; and upregulation of miR-21.

45. The method of claim 39, wherein the transgenic mouse is further compared/evaluated with that of a wild type mouse.

46. A method for screening an inducer/promoter or an inhibitor of a liver condition, wherein an alteration of liver morphology, molecular biology or function is determined after administering a test substance to the transgenic mouse of claim 21 or contacting a tissue, an organ or cells comprising cells derived from the transgenic mouse of claim 21 with the test substance.

47. The method of claim 46, wherein the alteration of liver morphology, molecular biology or function comprises an attribute selected from: hepatocellular carcinoma; visible tumor, pseudoglandular, or trabecular cell growth; basophilic cell invasion; upregulation of miR-221; repression of Cdkn1b/p27; repression of Cdkn1cp57; repression of Bmf proteins; downregulation of miR-122; downregulation of miR-199; and upregulation of miR-21; enlarged abdomen; externally-visible lumps; body weight loss; and a combination thereof.

48. A method of testing the therapeutic efficacy of a test agent in treating or preventing a liver condition in a subject comprising:

- a) administering a test agent to a transgenic mouse of claim 21; and
- b) after the agent has been administered to the transgenic animal, comparing one or more symptoms and/or indications of the liver condition in the transgenic animal with those of a control animal of the same genotype, wherein the agent has not been administered to the

control animal;

wherein if the agent inhibits, prevents and/or reduces the one or more symptoms and/or indications of the liver condition in the transgenic animal, relative to the control animal, then the agent is considered to have therapeutic efficacy in treating or preventing a liver condition in a subject.

49. The method of any one of the above method claims, wherein at least one anti-miR-221 gene is administered to the transgenic mouse.

50. A method for suppressing a liver cancer, comprising: contacting a liver cell with at least one antisense miR-221 oligonucleotide, thereby suppressing a liver cancer oncogene.

51. The method of claim 50, wherein the at least one anti-miR-221 gene comprises a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 5 (anti-precursor miR-221) or SEQ ID NO: 6 (anti-mature miR-221).

52. The method of claim 51, wherein a transgenic mouse is administered the antisense miR-221 oligonucleotide and evaluated with regard to a control animal of the same genotype and gender that has not been treated.

53. A method of diagnosing whether a subject has, or is at risk for developing, hepatocellular cancer (HCC), comprising measuring the level of at least one miR-221 and miR-21 gene products in a test sample from said subject, wherein an increase in the level of the miR-221 and miR-21 gene products in the test sample, relative to the level of a corresponding miR-221 and miR-21 gene products in a control sample, is indicative of the subject either having, or being at risk for developing, HCC.

54. A method of determining a hepatocellular carcinoma (HCC) in a subject comprising:

- a) obtaining a sample from the subject,
- b) analyzing the sample for a change in the level of expression of one or more biomarkers relative to the level of expression of a corresponding biomarker in at least one control sample, and
- c) correlating the change in the level of expression of the one or more biomarkers, relative to the level of expression of the corresponding biomarker in the control sample, with the presence of HCC in the subject,

wherein step (b) includes analyzing the sample for a change in the level of expression of at least one or more biomarkers identified by miR-221, miR-21, miR-123 and miR-199, and

wherein step (c) further includes correlating an increase in the level of expression of at least one or more biomarkers identified by miR-221 and miR-21, relative to the level of expression of the

corresponding biomarker in normal liver as the at least one control sample, with the presence of HCC in the subject;

and wherein step (c) further includes correlating a decrease in the level of expression of at least one or more biomarkers identified by miR-122 and miR-199, relative to the level of expression of the corresponding biomarker in normal liver as the at least one control sample, with the presence of HCC in the subject.

55. The method of claim 53, 54 or 55, wherein the sample is liver tissue, blood and/or serum.

56. A method for increasing expression of Cdkn1b/p27 protein levels in a cell, comprising contacting the cell with an antisense miR-221 oligonucleotide.

57. An assay kit for detecting a risk that a subject has, or will develop, hepatocellular carcinoma (HCC), the kit comprising reagents for determining a HCC signature,

wherein the signature comprises a collection of measurements of one or more of miR-221, miR-21, miR-122 and miR-199, and

wherein the reagents determine the expression levels of at least one of miR-221, miR-21, miR-122 and miR-199, and some combination thereof.

58. The kit of claim 57, wherein the reagents determine a microRNA signature for HCC comprising: miR-221 and miR-21 exhibited as an increased expression, and/or miR-122 and miR-199 exhibited as a decreased expression, relative to a control.



Figure 1A

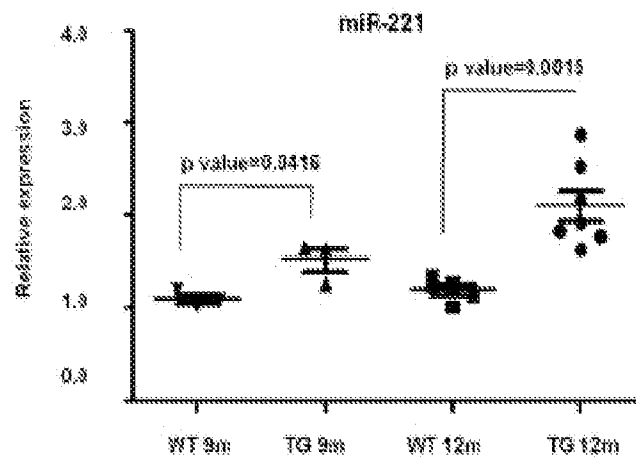


Figure 1B

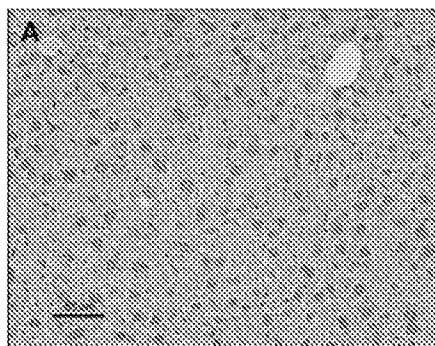


Figure 2A

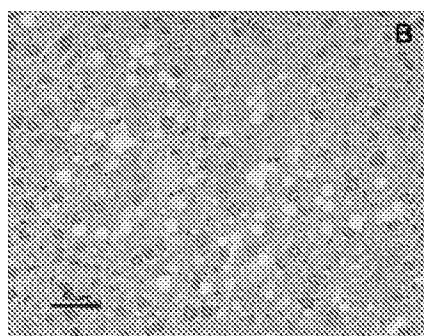


Figure 2B

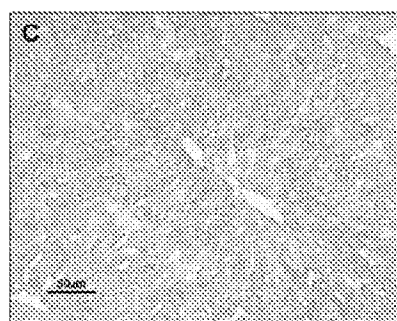


Figure 2C

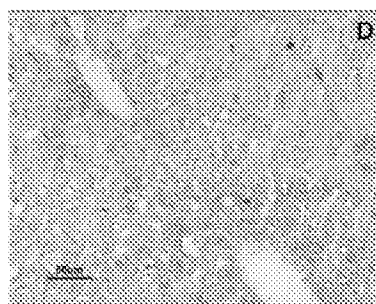


Figure 2D

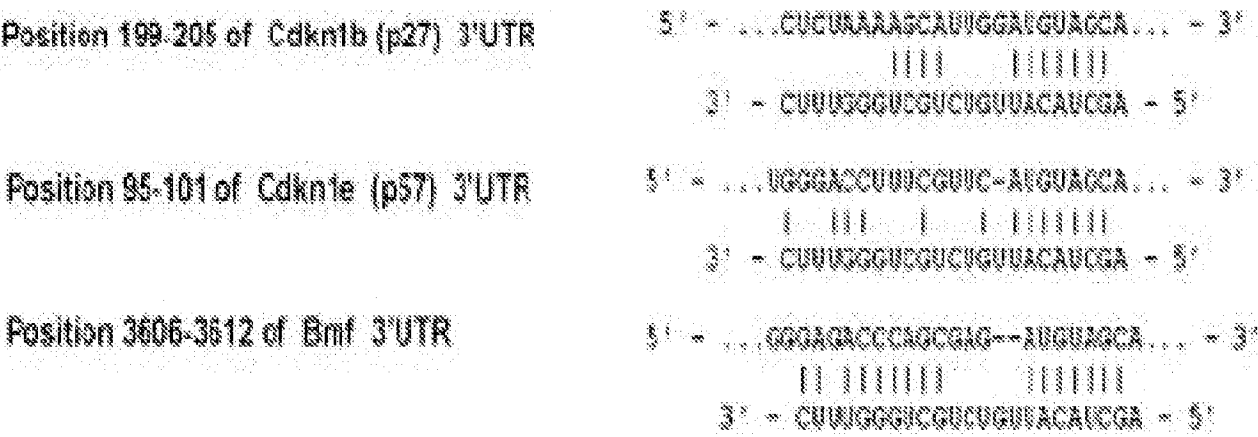


Figure 3A

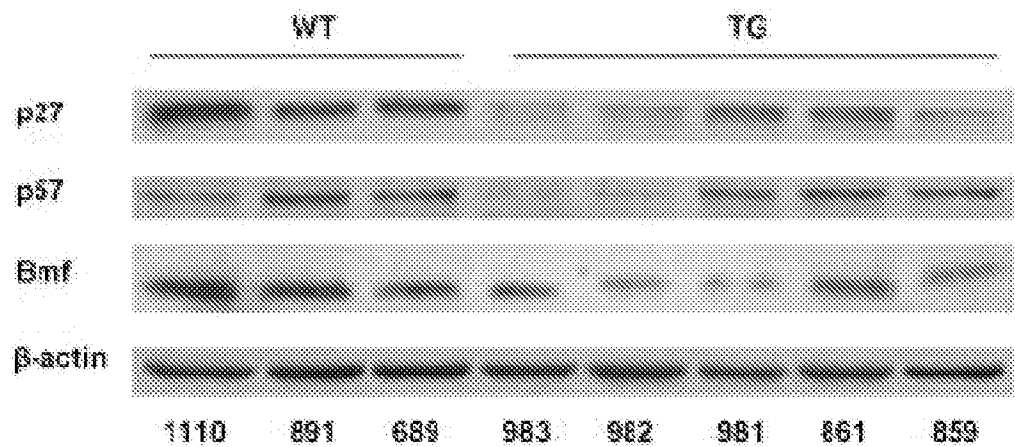


Figure 3B

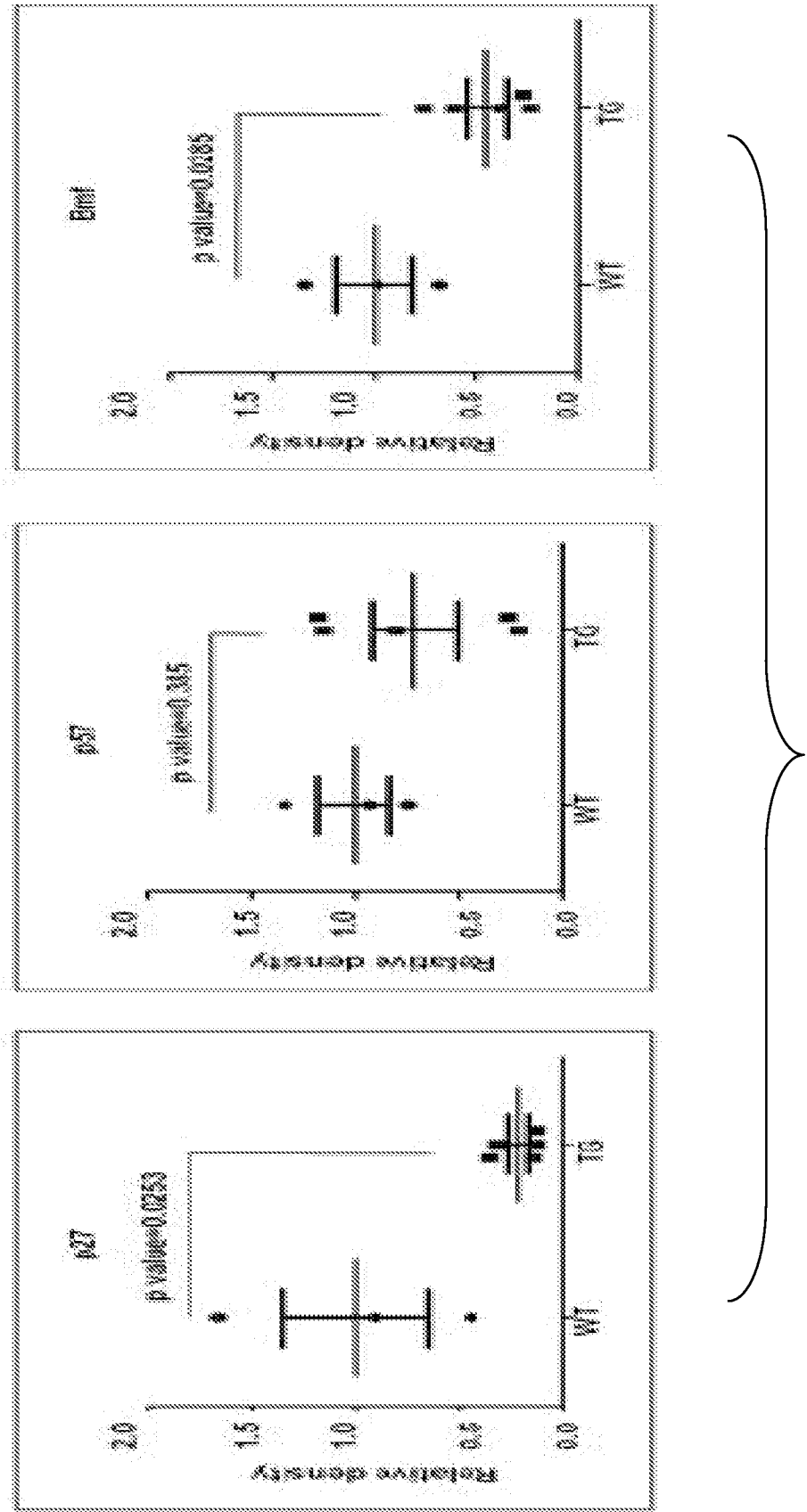


Figure 3C

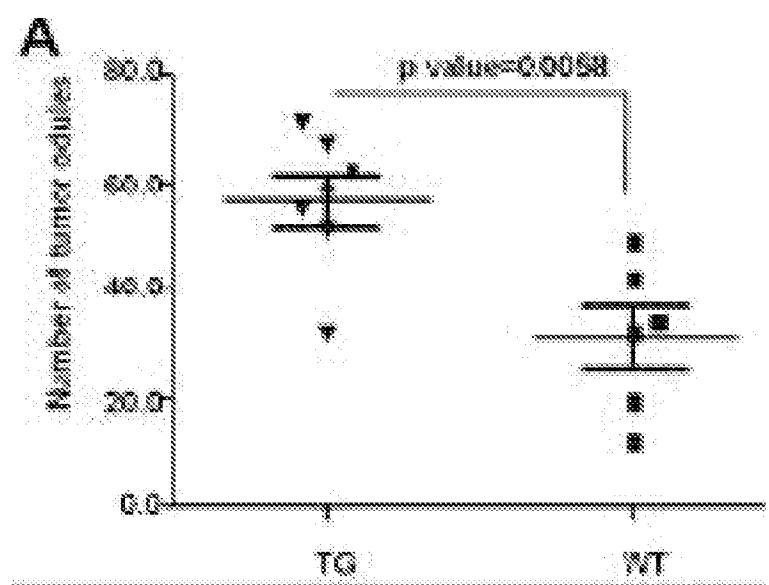


Figure 4A

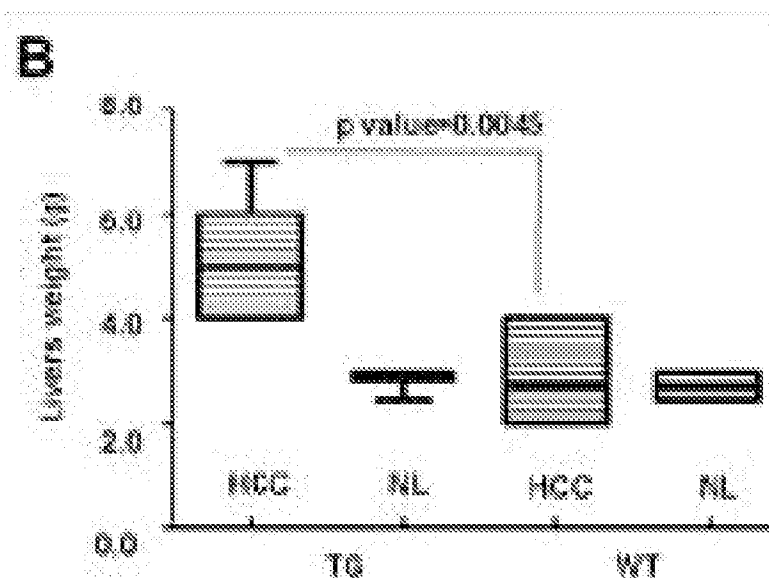


Figure 4B

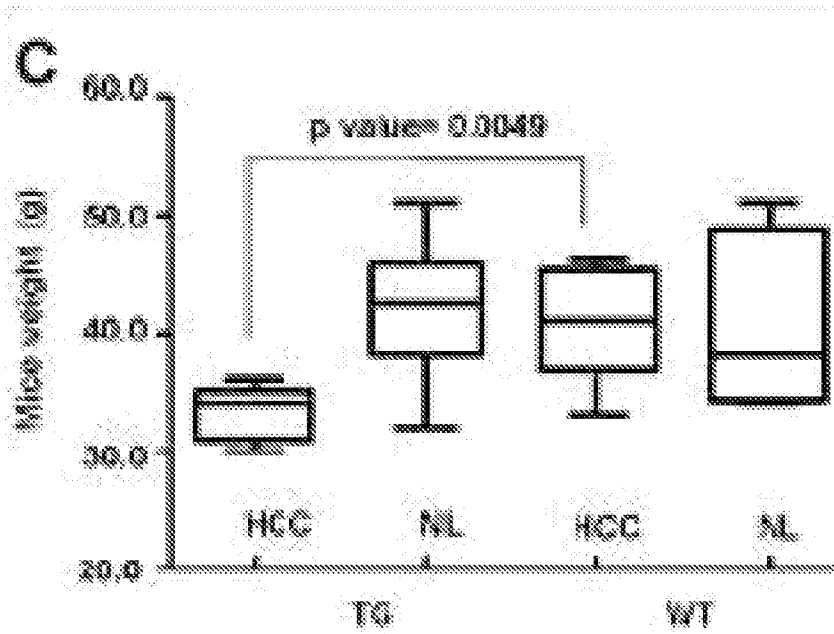


Figure 4C

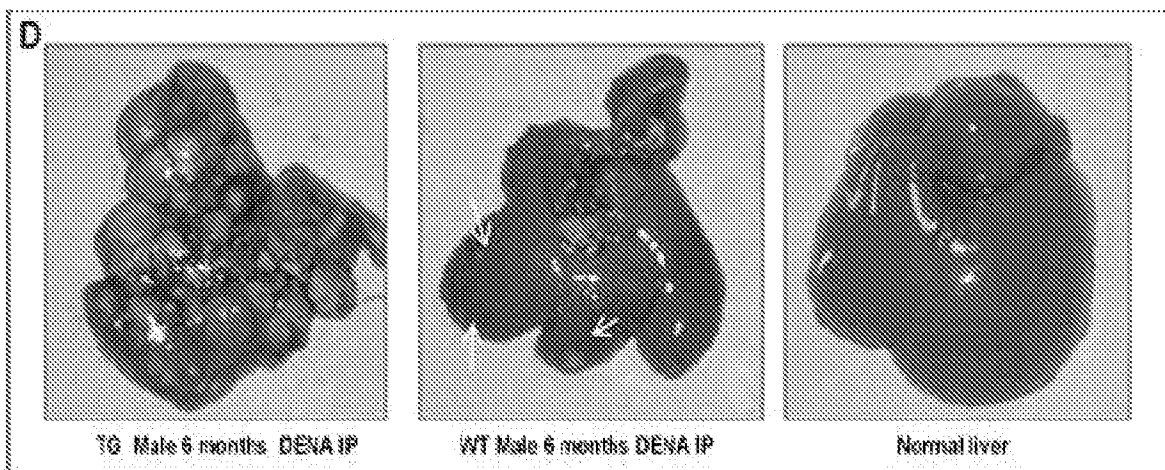


Figure 4D

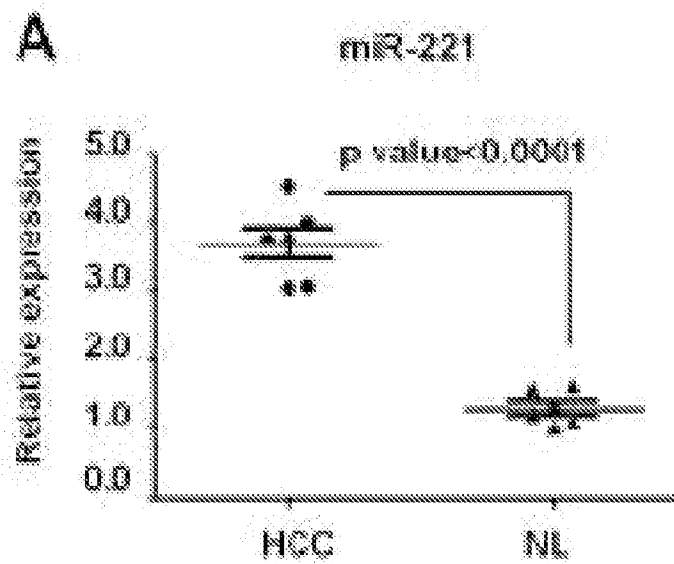


Figure 5A

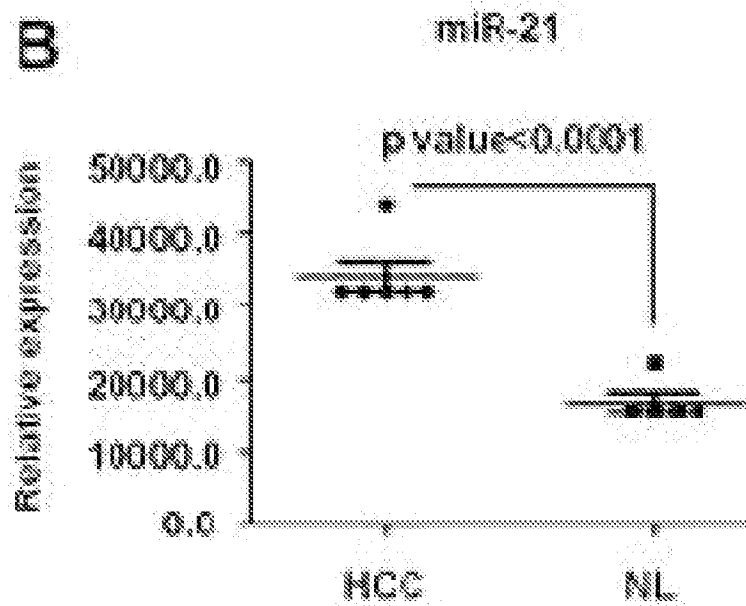


Figure 5B

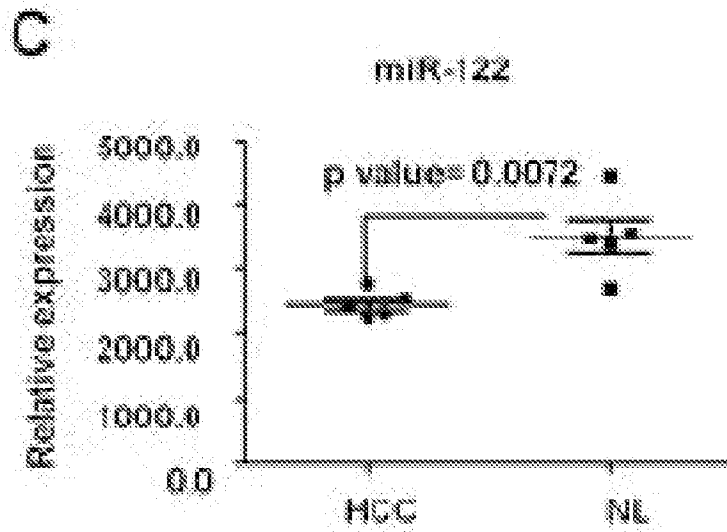


Figure 5C

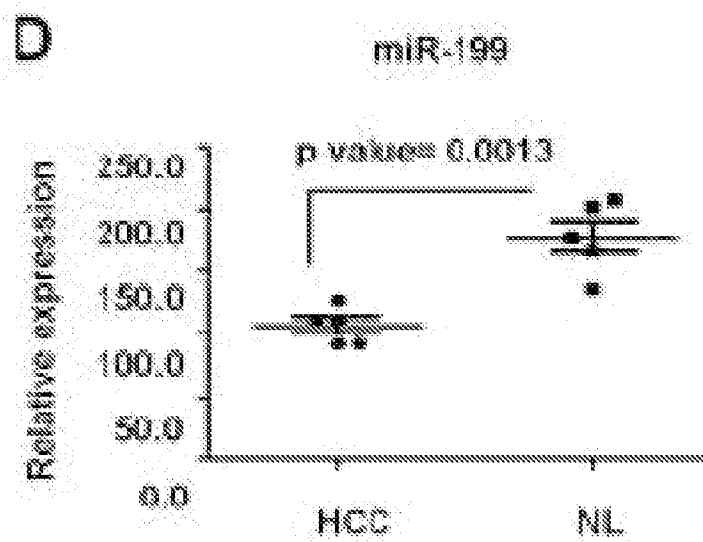


Figure 5D

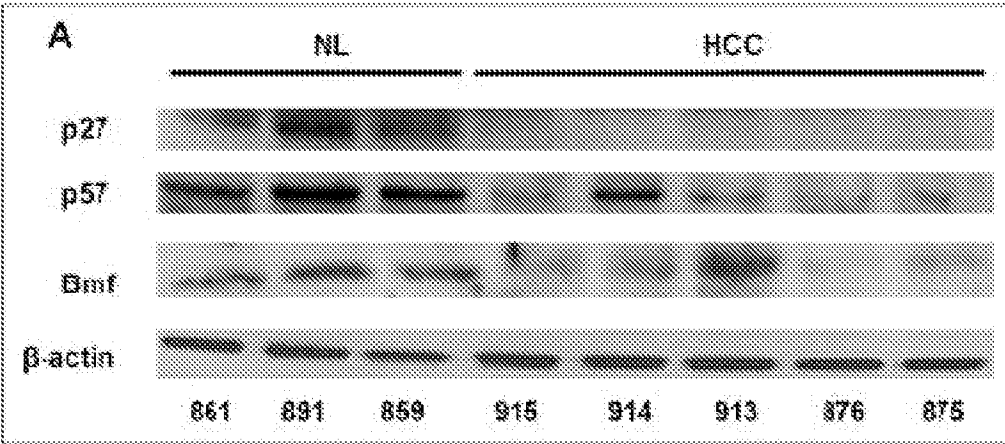


Figure 6A

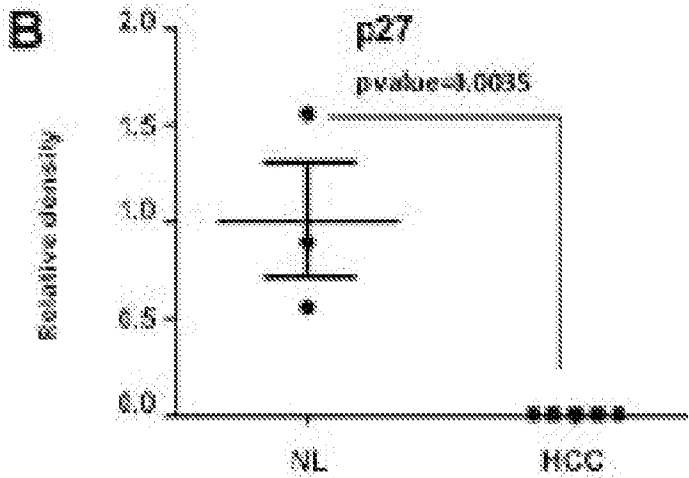


Figure 6B

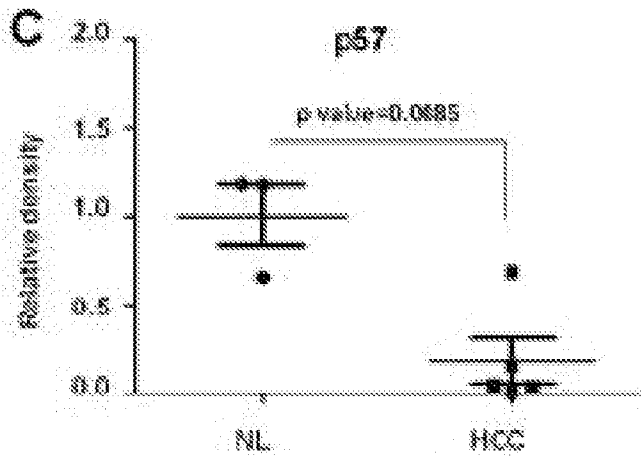


Figure 6C

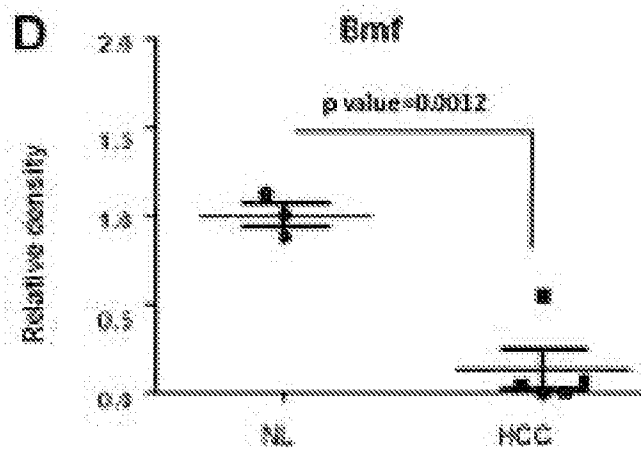


Figure 6D

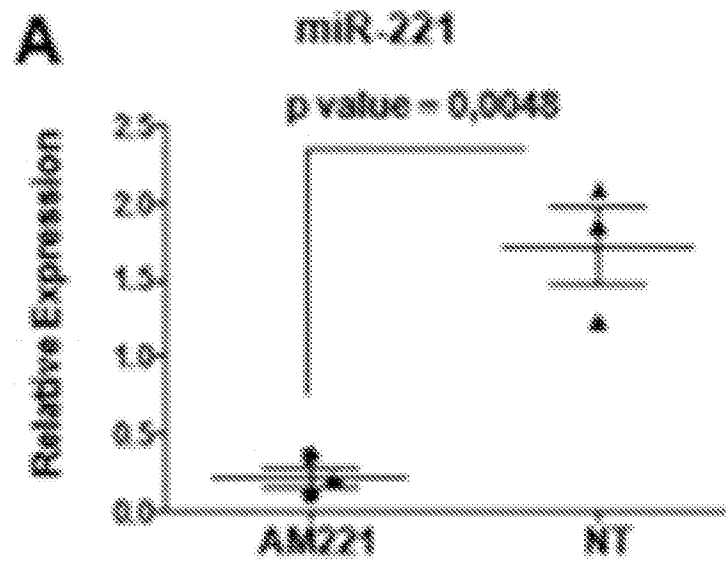


Figure 7A

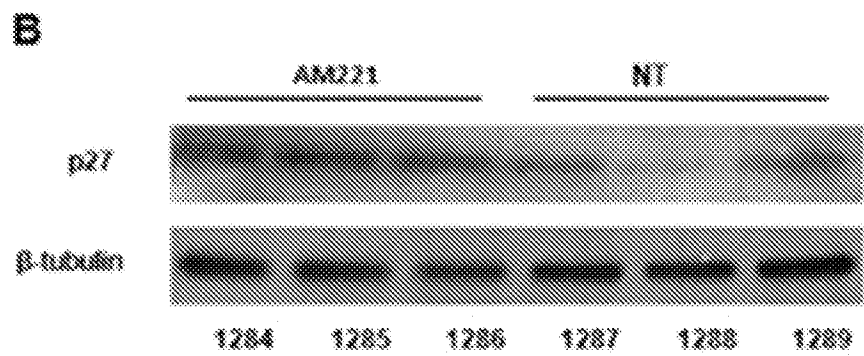


Figure 7B

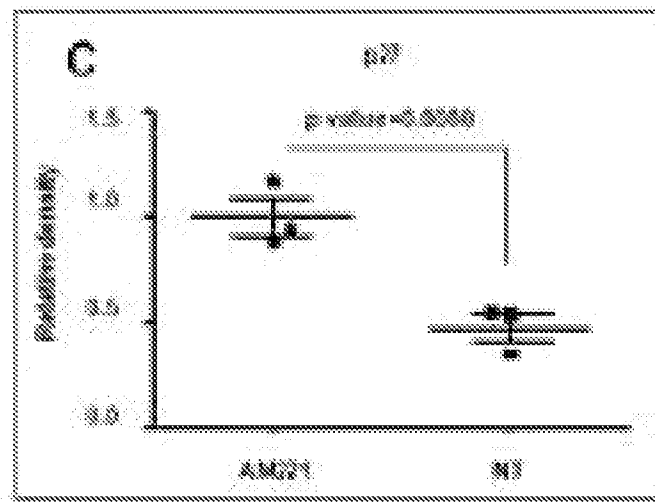


Figure 7C

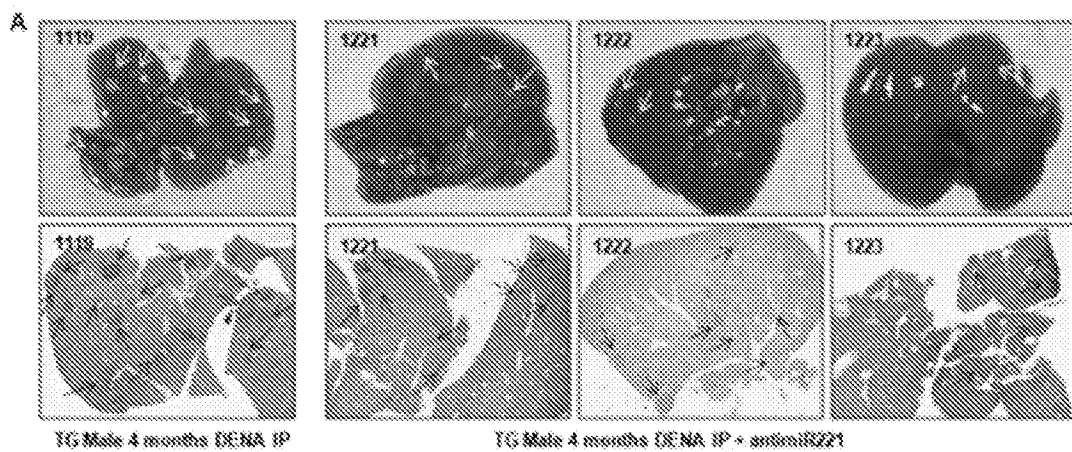
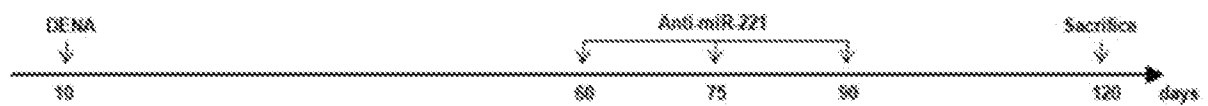


Figure 7D

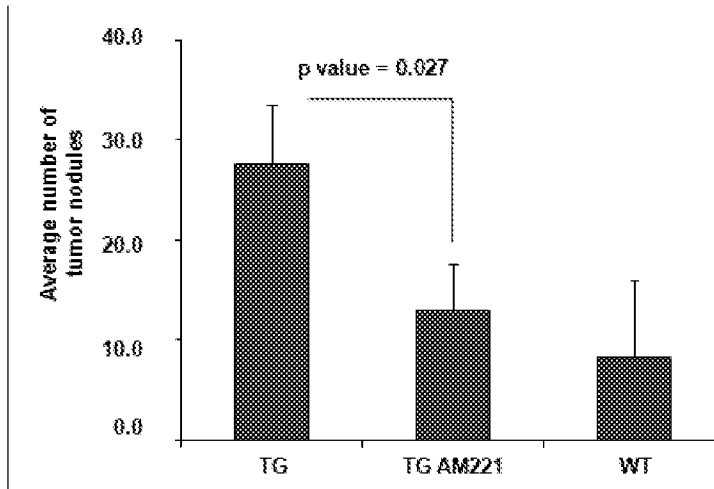


Figure 7E

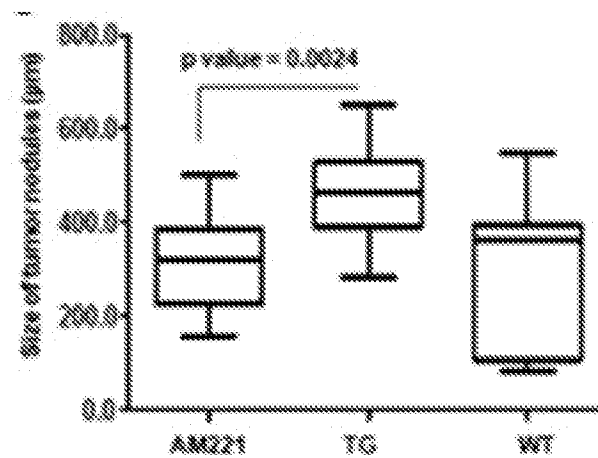


Figure 7F

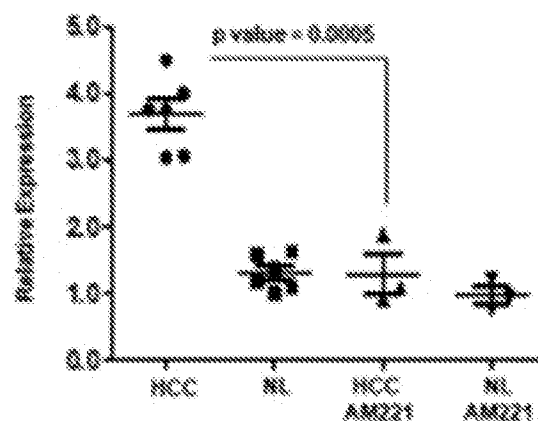


Figure 7G

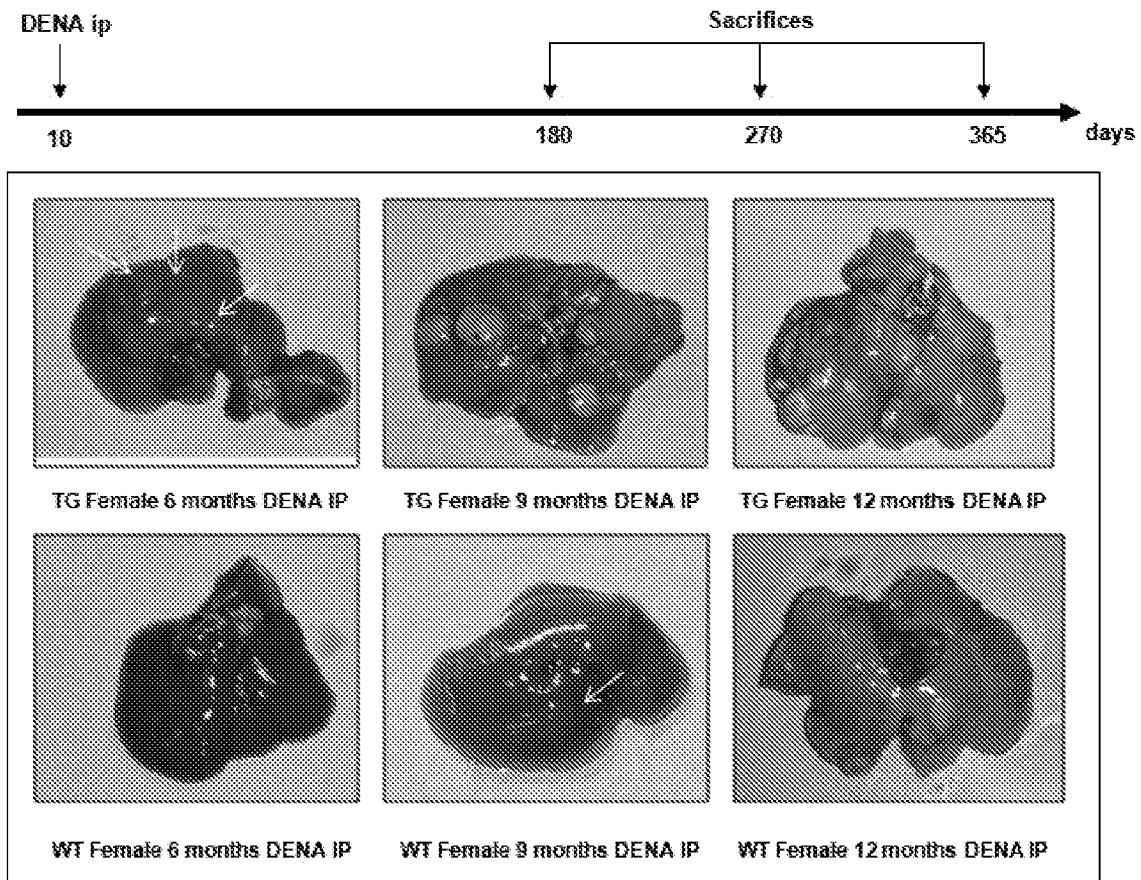


Figure 8

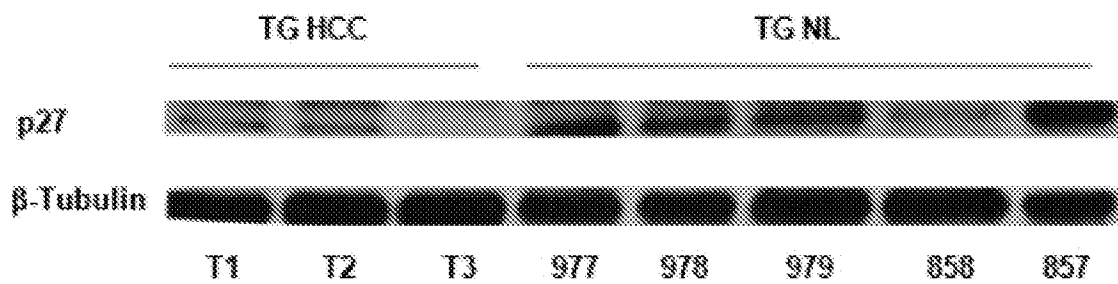


Figure 9A

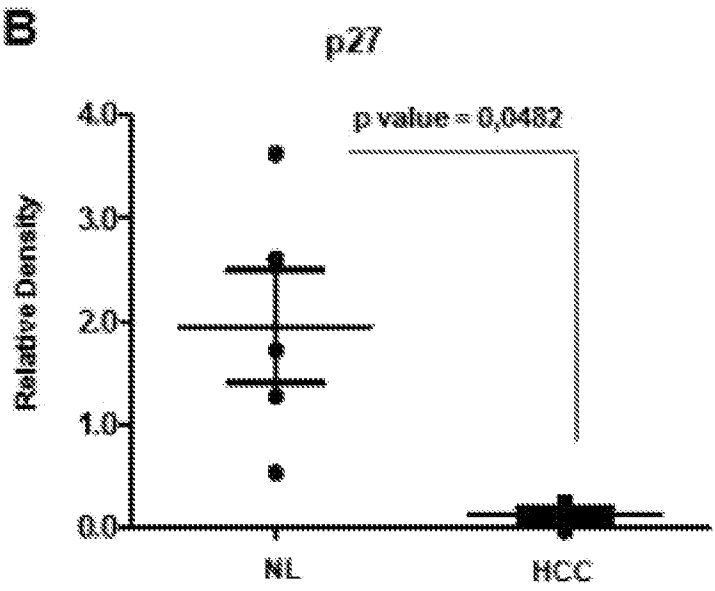


Figure 9B

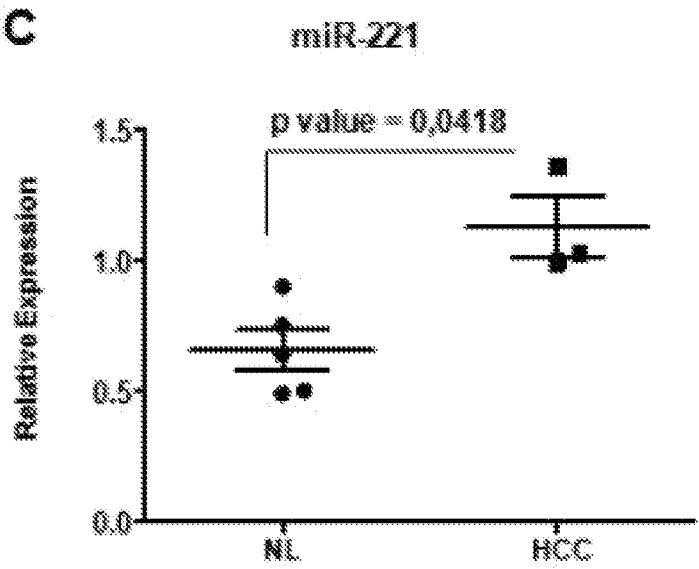


Figure 9C

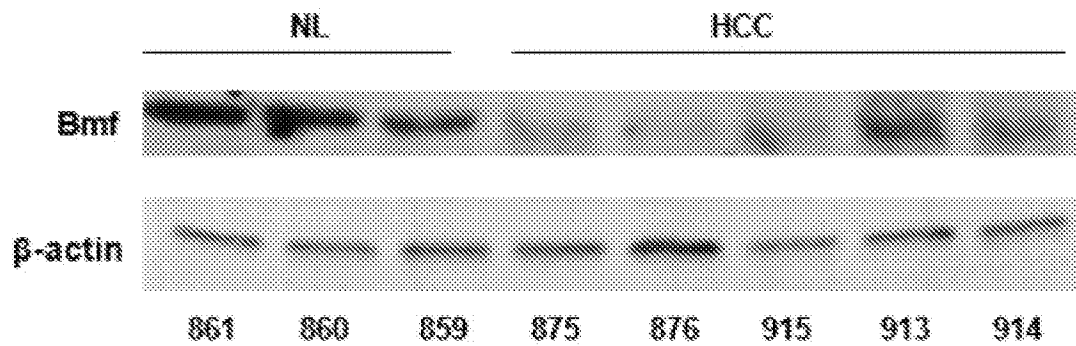


Figure 10

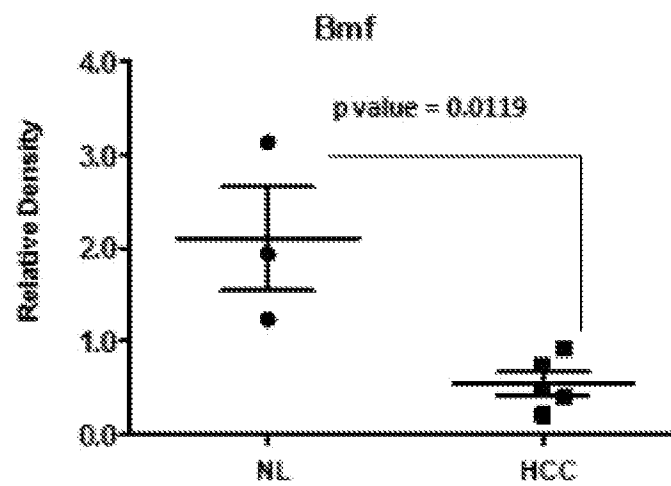


Figure 11

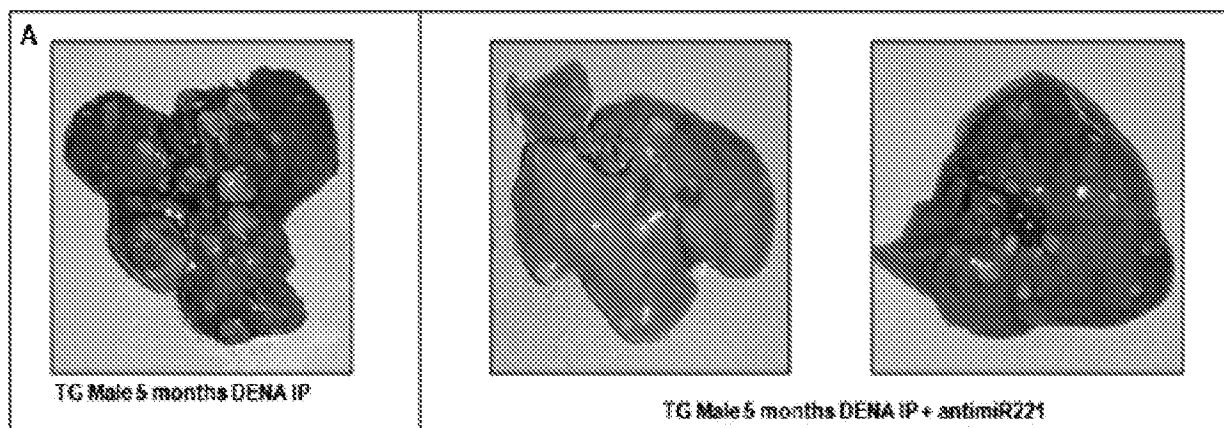


Figure 12A

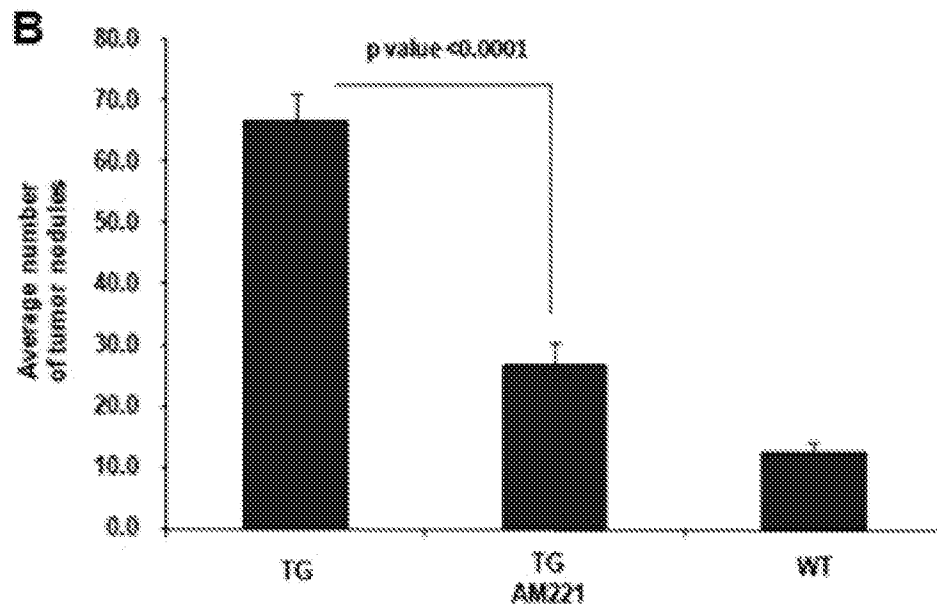


Figure 12B

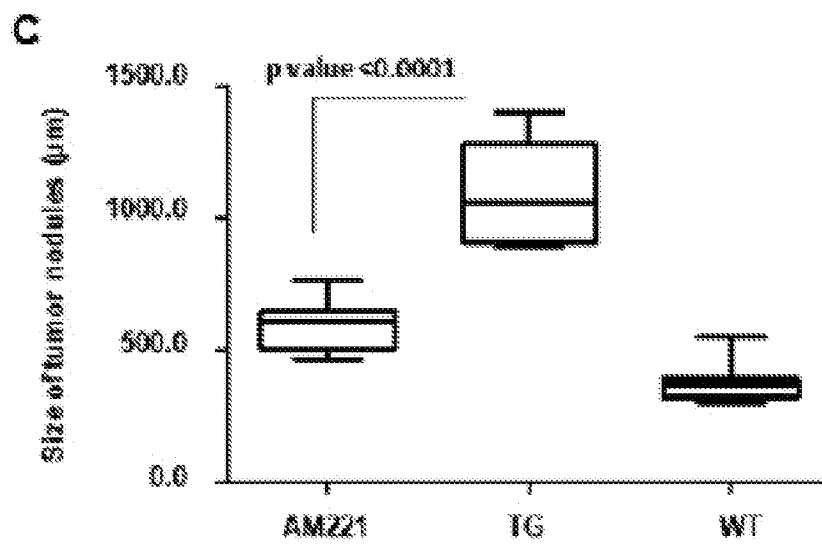


Figure 12C

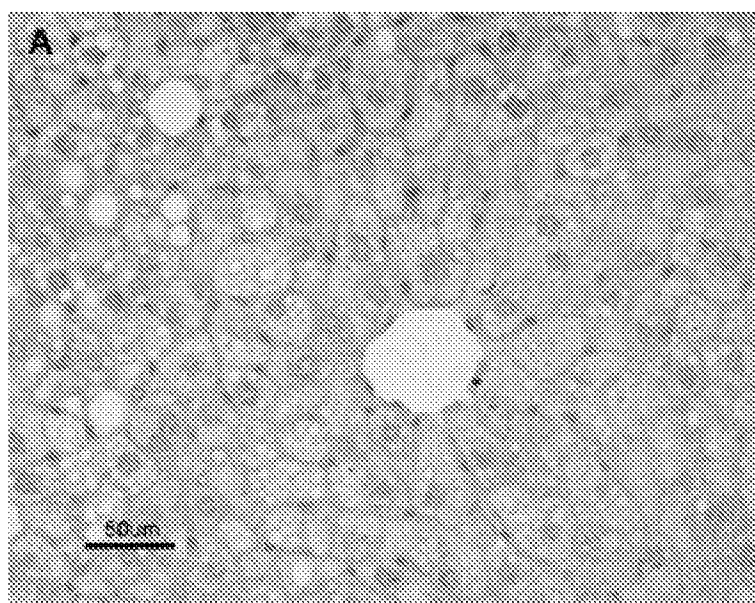


Figure 13A

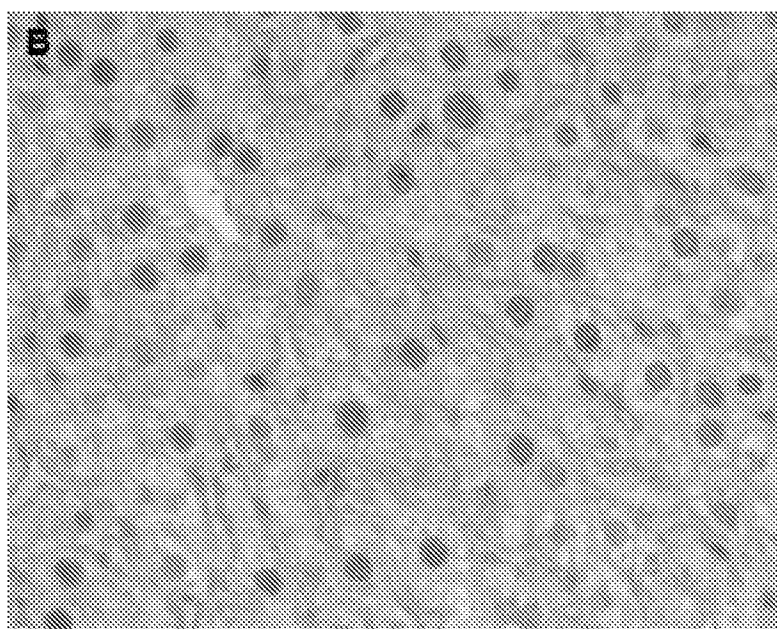


Figure 13B

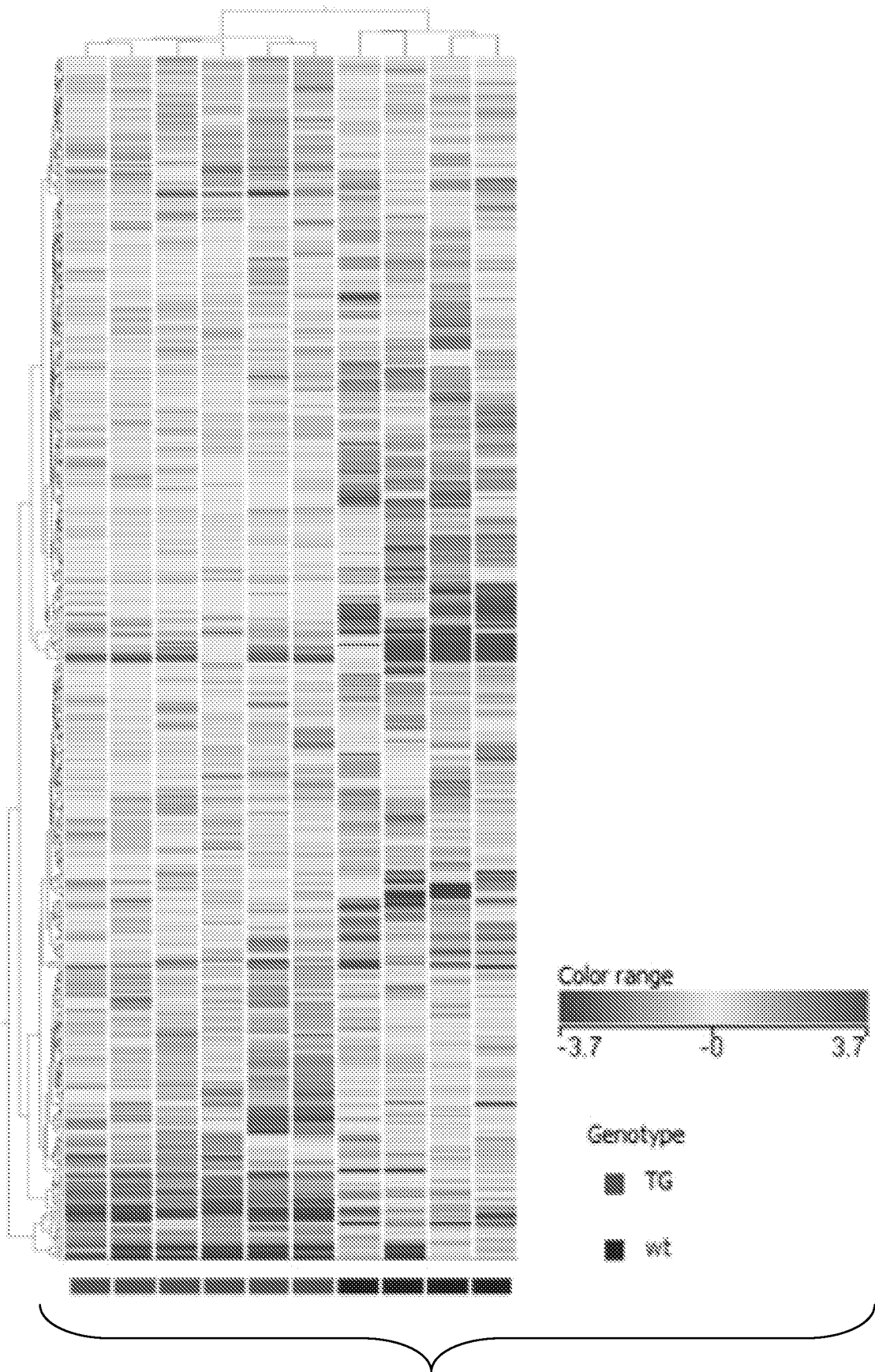


Figure 14A

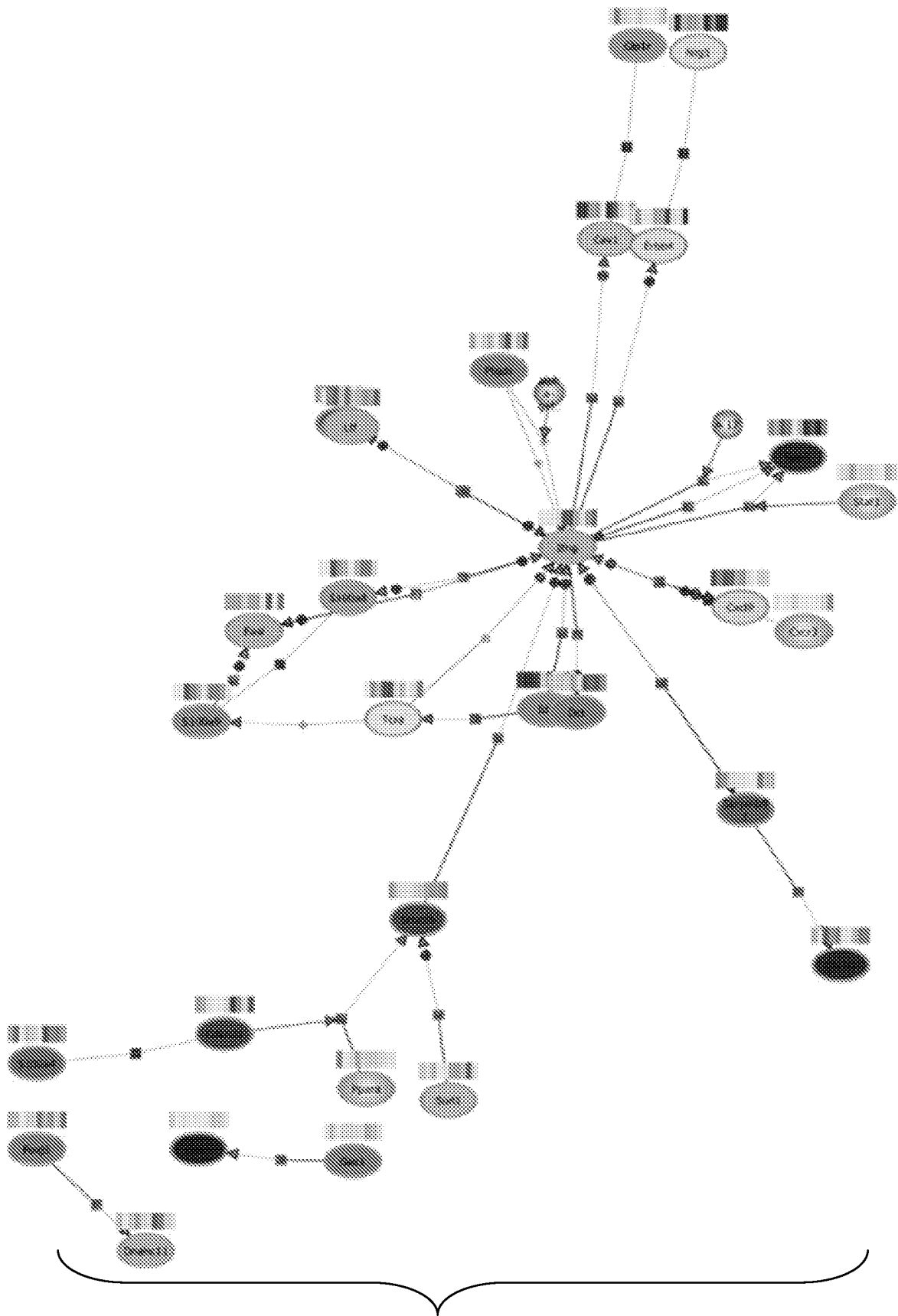


Figure 14B

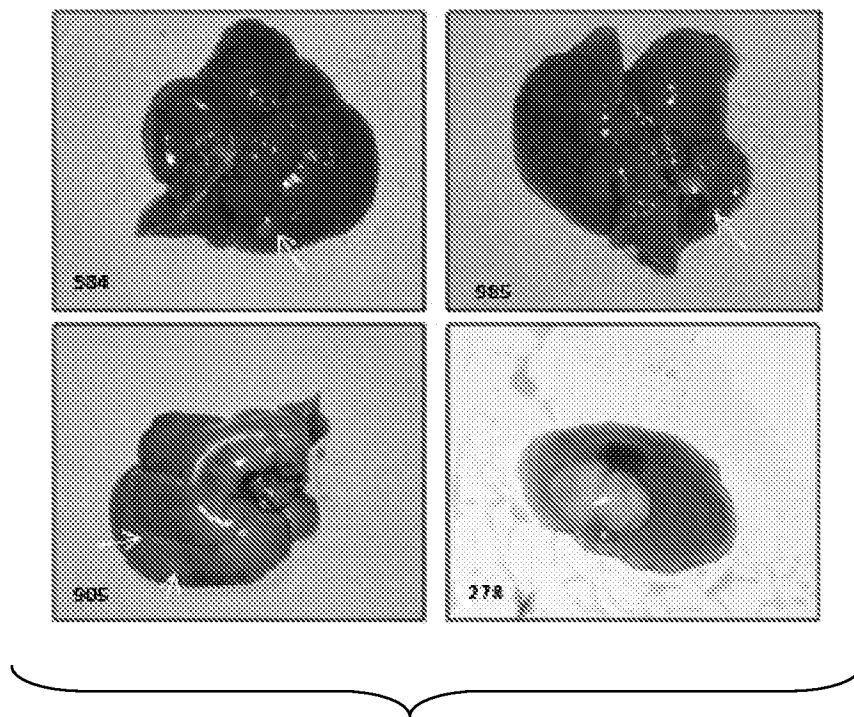


Figure 15A

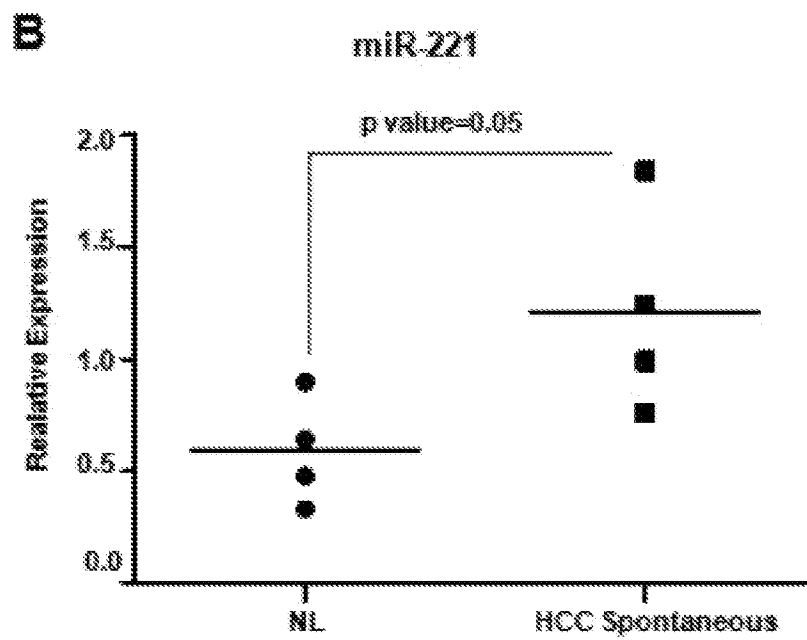


Figure 15B

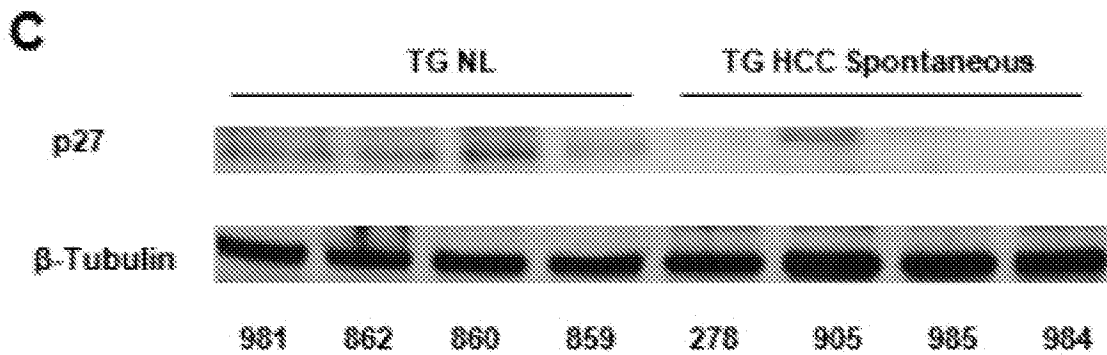


Figure 15C

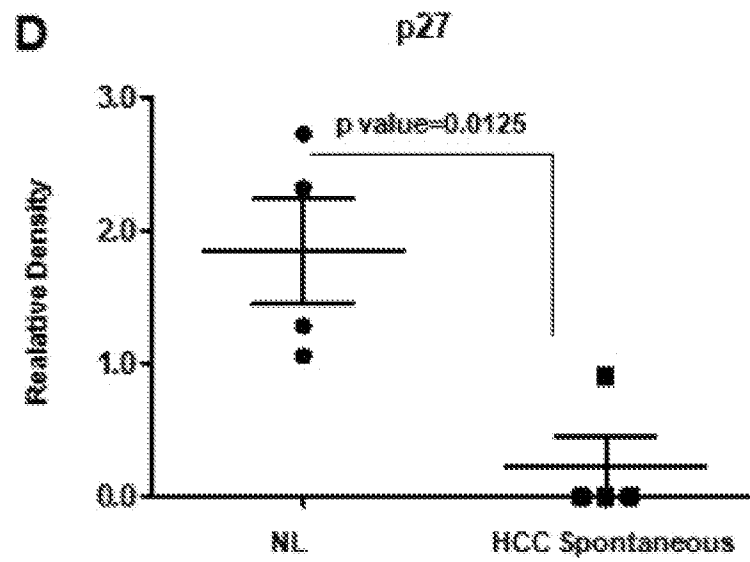


Figure 15D

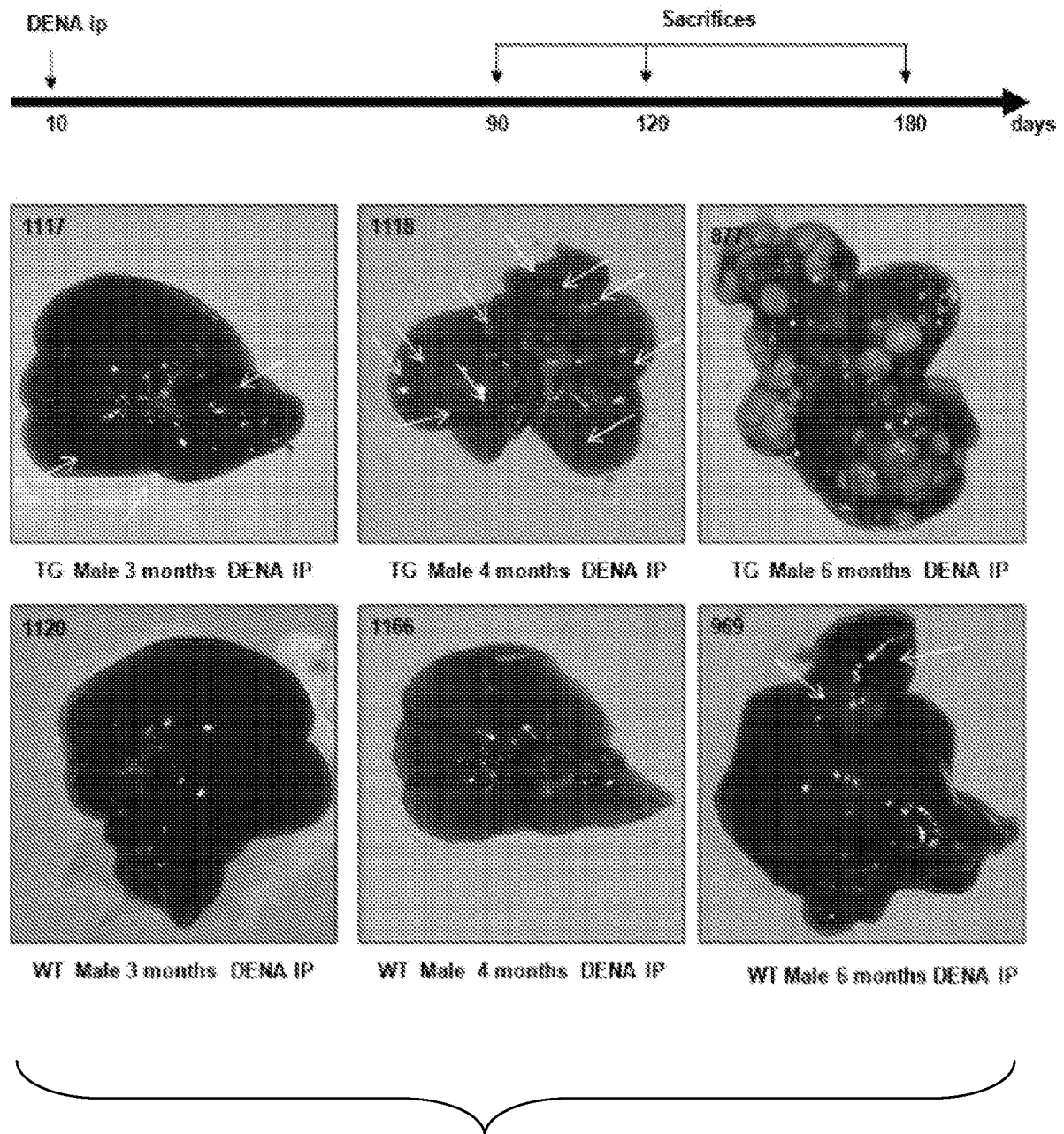


Figure 16

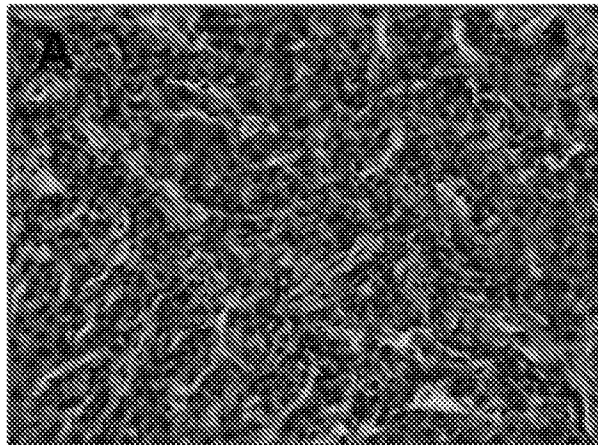


Figure 17A

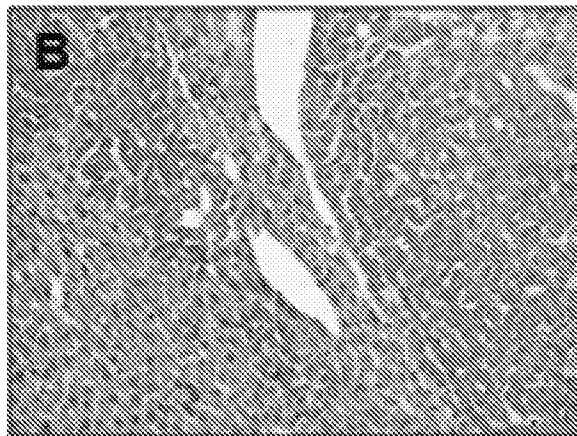


Figure 17B

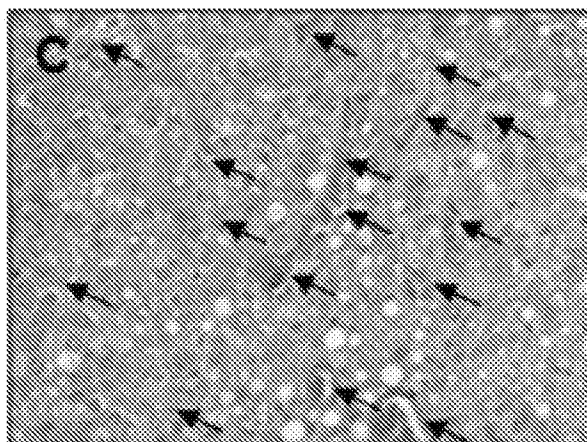


Figure 17C

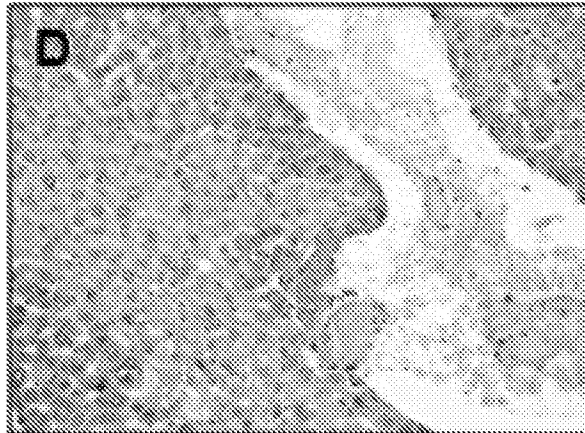


Figure 17D

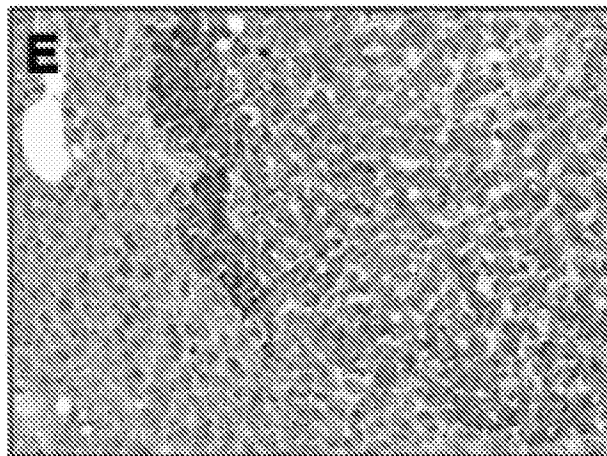


Figure 17E

Mouse ID	Anti-miR-221	miR-221 fg/μl [†]
1287	No	2.18
1288	No	3.24
1289	No	4.39
1284	Yes	1.60
1285	Yes	0.79
1286	Yes	0.43

[†] Forty-eight hours after intravenous injection of anti-miR-221 oligonucleotide (30mg/kg), miR-221 levels in serum were monitored by RT-qPCR. The difference between treated (Yes) versus untreated (No) mice was significant (p-value = 0.03)

Figure 18

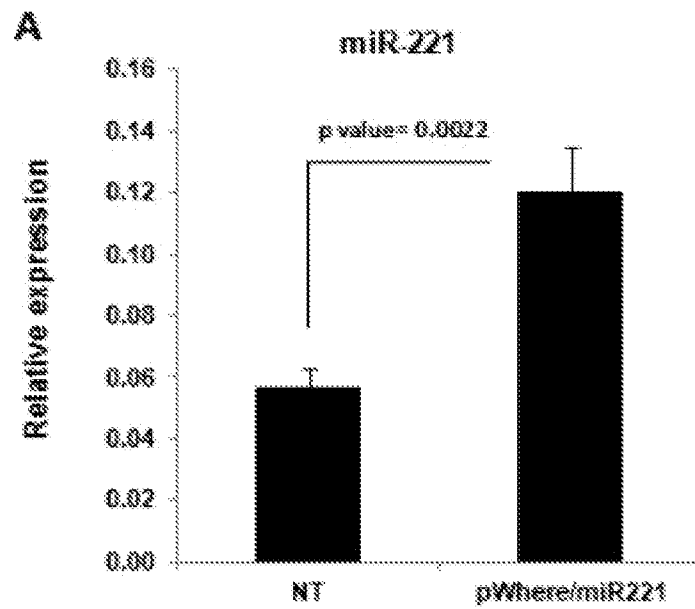


Figure 19A

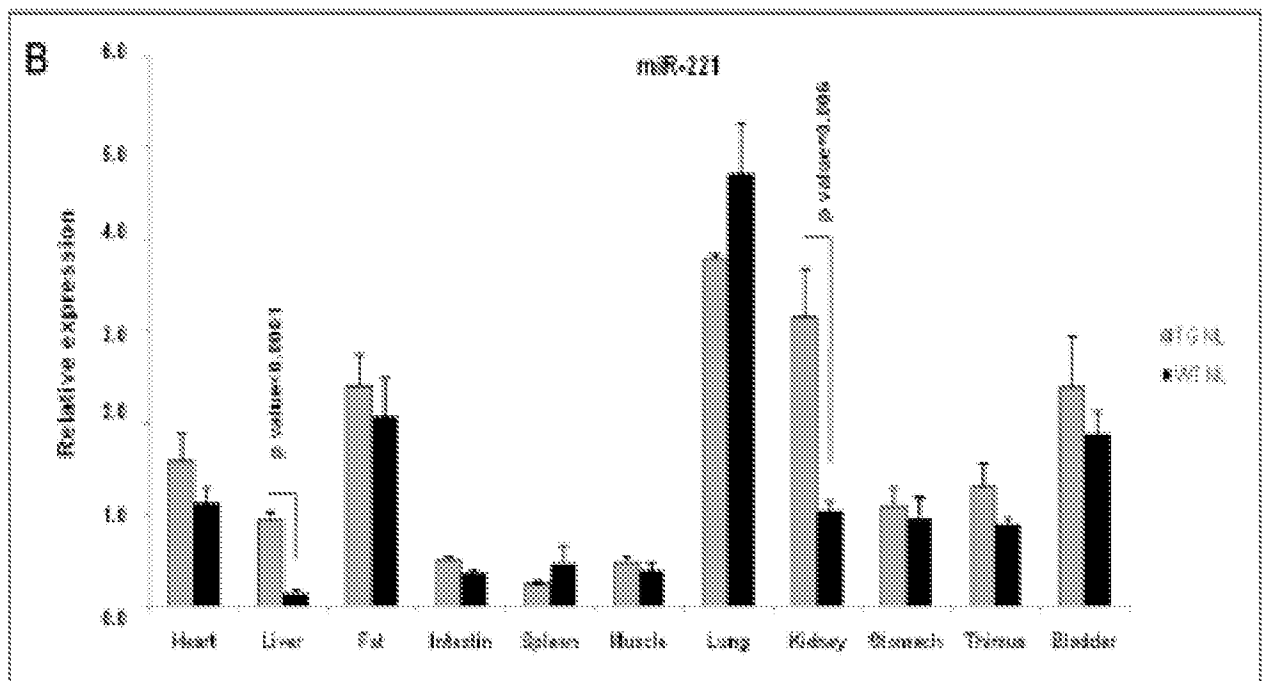


Figure 19B

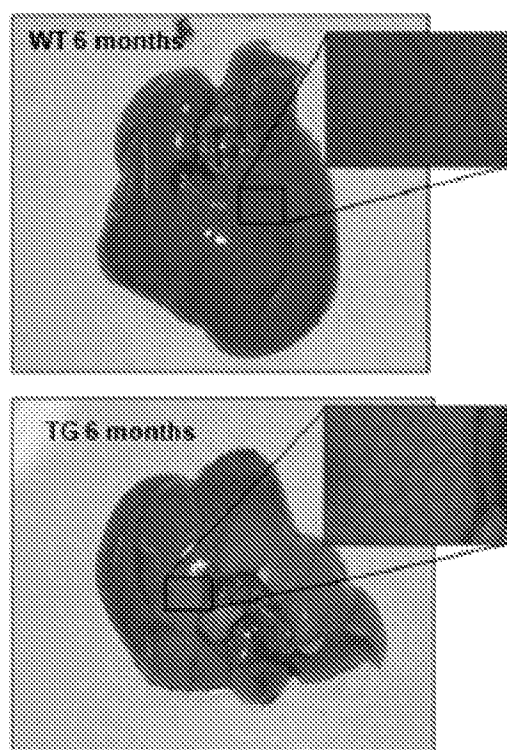


Figure 20A

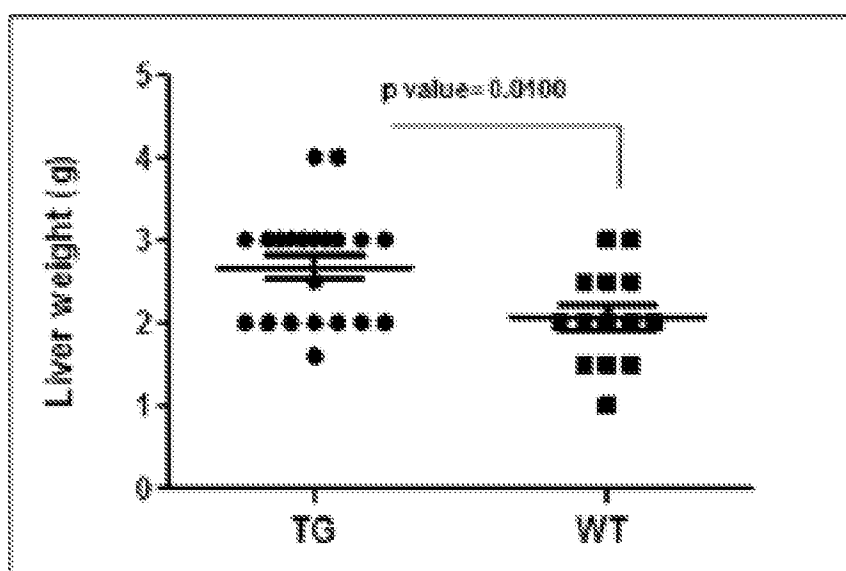


Figure 20B

	Mouse ID	Tumor nodules
WT	891	0
	1110	0
	689	0
	658	0
WT + DENA	969	19
	970	12
	971	32
	972	49
	946	34
	947	42
TG	981	0
	982	0
	983	0
	859	0
	860	0
	861	0
TG + DENA	875	67
	876	58
	874	71
	877	62
	913	55
	914	32
	915	51

Figure 21

RNA transcripts features										Expression in Transgenics vs. Wild type				
Description	Agilent Probe Name	Gene Symbol	Gene Name	Genbank Accession	Genomic Coordinates	Chrom Stranded	p-value	Fold change	Regulation on TG vs WT	Average expression on in TG	Average expression on in WT			
lincRNA:chr12:55844075-55898175 reverse strand	A_30_P01027230				chr12:55858002-55857943	-	1.43E-06	22.04	down	4.39	8.85			
Mus musculus growth arrest specific 5 (Gas5), non-coding RNA [NR_002840]	A_66_P134809	Gas5	growth arrest specific 5	NR_002840	chr1:162968608-162968667	+	1.34E-05	2.08	up	11.68	10.62			
Mus musculus transient receptor potential cation channel, subfamily C, member 4 (Trpc4), mRNA [NM_016984]	A_55_P2045198	Trpc4	transient receptor potential cation channel, subfamily C, member 4	NM_016984	chr3:54121970-54122029	+	1.40E-05	2.78	down	2.03	3.50			
PREDICTED: Mus musculus hypothetical protein LOC620858 (LOC620858), mRNA [XM_001477040]	A_55_P2064466	Gm13539	predicted gene 13539	XM_001477040	chr2:25642488-25642547	+	2.41E-05	7.97	up	4.83	1.83			
Mus musculus calpain 11 (Capn11), mRNA [NM_001013767]	A_55_P2129373	Capn11	calpain 11	NM_001013767	chr17:45767213-45767154	-	2.76E-05	4.10	up	8.77	6.73			
PREDICTED: Mus musculus similar to hCG1640785 (LOC546711), mRNA [XM_908296]	A_55_P2164998	LOC546711	similar to 40S ribosomal protein S25	XM_908296	chr18:35930135-35930194	+	3.44E-05	3.03	down	8.44	10.04			
Mus musculus Ellis van Creveld gene homolog (human) (Evc), mRNA [NM_021292]	A_52_P476431	Evc	Ellis van Creveld gene homolog (human)	NM_021292	chr5:37690618-37690559	-	4.12E-05	2.11	up	7.02	5.95			
Mus musculus renin binding protein (Renbp), transcript variant 1, mRNA [NM_023132]	A_55_P2013357	Renbp	renin binding protein	NM_023132	chrX:71167750-71167691	-	5.59E-05	3.24	up	7.12	5.42			
Mus musculus von Willebrand factor A domain containing 5A (Vwa5a), mRNA [NM_172767]	A_66_P131754	Vwa5a	von Willebrand factor A domain containing 5A	NM_172767	chr9:38550667-38550726	+	6.81E-05	5.15	down	1.85	4.22			
Mus musculus Ellis van Creveld syndrome 2 homolog (human) (Evc2), mRNA [NM_145920]	A_52_P441974	Evc2	Ellis van Creveld syndrome 2 homolog (human)	NM_145920	chr5:37816219-37816278	+	7.07E-05	2.17	up	8.83	7.71			

Figure 22

lincRNA:chr1:162965001-162966376 forward strand	A_30_P01026 655				chr1:16296514 6-162965205	+	1.02 E-04	3.35	down	4.53	6.28
Mus musculus zeta-chain (TCR) associated protein kinase (Zap70), mRNA [NM_009539]	A_55_P21531 22	Zap70	zeta-chain (TCR) associated protein kinase	NM_009539	chr1:36839315 -36839374	+	1.19 E-04	2.26	down	10.12	11.29
Mus musculus solute carrier family 4, sodium bicarbonate transporter-like, member 11 [NM_001081162]	A_66_P12197 6	Slc4a11	solute carrier family 4, sodium bicarbonate transporter-like, member 11	NM_001081 162	chr2:13051001 1-130509952	-	1.25 E-04	2.58	down	3.57	4.94
Mus musculus family with sequence similarity 20, member B (Fam20b), mRNA [NM_145413]	A_55_P21782 47	Fam20b	family with sequence similarity 20, member B	NM_145413	chr1:15861124 4-158611185	-	1.37 E-04	2.21	down	7.58	8.72
Mus musculus zeta-chain (TCR) associated protein kinase (Zap70), mRNA [NM_009539]	A_55_P19698 61	Zap70	zeta-chain (TCR) associated protein kinase	NM_009539	chr1:36839542 -36839601	+	1.46 E-04	2.18	down	11.32	12.44
Mus musculus Na ⁺ /K ⁺ transporting ATPase interacting 1 (Nkain1), mRNA [NM_025998]	A_52_P67097 8	Nkain1	Na ⁺ /K ⁺ transporting ATPase interacting 1	NM_025998	chr4:13016907 2-130169131	+	1.54 E-04	5.46	up	4.20	1.75
PREDICTED: Mus musculus similar to ribosomal protein L11 (LOC627317), misc RNA [XR_033591]	A_55_P20573 80	Gm6745	predicted gene 6745	XR_033591	chr5:23515561 -23515502	-	1.99 E-04	3.79	down	1.85	3.77
lincRNA:chr2:52241435-52245307 forward strand	A_30_P01033 485				chr2:52242011 -52242070	+	2.04 E-04	4.27	down	6.63	8.73
Mus musculus paired box gene 8 (Pax8), mRNA [NM_011040]	A_55_P20395 32	Pax8	paired box gene 8	NM_011040	chr2:24276206 -24276147	-	2.40 E-04	4.65	up	5.99	3.77
Mus musculus RIKEN cDNA 2210010C17 gene (2210010C17Rik), mRNA [NM_027308]	A_51_P28177 8	2210010C17 Rik	RIKEN cDNA 2210010C17 gene	NM_027308	chr7:20524579 -20523598	-	2.69 E-04	2.26	up	4.92	3.75
Mus musculus flavin containing monooxygenase 3 (Fmo3), mRNA [NM_008030]	A_51_P26940 4	Fmo3	flavin containing monooxygenase 3	NM_008030	chr1:16488411 1-164884052	-	2.87 E-04	25.11	down	2.57	7.22
Mus musculus nicotinamide nucleotide transhydrogenase (Nnt), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA [NM_008710]	A_55_P21442 85	Nnt	nicotinamide nucleotide transhydrogenase	NM_008710	chr13:1201243 94-120124335	-	3.16 E-04	2.37	down	11.44	12.68
lincRNA:chr12:55844075-55898175 reverse strand	A_30_P01017 443				chr12:5586468 0-55864621	-	4.47 E-04	3.08	down	6.56	8.18

Figure 22 CONT.

lincRNA:chr8:19783770-19784669 reverse strand	A_30_P01031 255					chr8:19784372-19784313	-	4.49 E-04	2.23	down	1.63	2.79
lincRNA:chr2:52241435-52245307 forward strand	A_30_P01025 928					chr2:52241951-52242010	+	4.56 E-04	3.53	down	7.85	9.67
Mus musculus Na ⁺ /K ⁺ transporting ATPase interacting 1 (Nkain1), mRNA [NM_025998]	A_55_P20253 68	Nkain1	Na ⁺ /K ⁺ transporting ATPase interacting 1	NM_025998		chr4:13016833-130168392	+	4.61 E-04	2.39	up	8.09	6.83
lincRNA:chr1:183142656-183163206 forward strand	A_30_P01024 781					chr1:18314266-183142721	+	4.99 E-04	2.21	down	1.58	2.72
Mus musculus dopachrome tautomerase (Dct), mRNA [NM_010024]	A_55_P19614 66	Dct	dopachrome tautomerase	NM_010024		chr14:1184120-88-118412029	-	5.00 E-04	2.49	down	11.84	13.15
Mus musculus predicted gene 10439 (Gm10439), mRNA [NM_001037716]	A_55_P19785 32	Gm10439	predicted gene 10439	NM_001037716		chrX:1460710-74-146071133	+	5.24 E-04	5.22	up	4.30	1.91
lincRNA:chr17:38132217-38157667 forward strand	A_30_P01021 320					chr17:3814985-0-38149909	+	5.33 E-04	4.33	up	4.23	2.12
Mus musculus dopachrome tautomerase (Dct), mRNA [NM_010024]	A_55_P20030 53	Dct	dopachrome tautomerase	NM_010024		chr14:1184120-72-118412013	-	5.35 E-04	2.39	down	10.83	12.08
Mus musculus src homology three (SH3) and cysteine rich domain (Stac), mRNA [NM_016853]	A_55_P20285 22	Stac	src homology three (SH3) and cysteine rich domain	NM_016853		chr9:11146402-1-111463962	-	5.45 E-04	2.02	down	2.92	3.94
Mus musculus chemokine (C-X-C motif) ligand 9 (Cxc19), mRNA [NM_008599]	A_55_P19770 38	Cxc19	chemokine (C-X-C motif) ligand 9	NM_008599		chr5:92752855-92752796	-	5.78 E-04	2.34	down	6.97	8.19
Mus musculus cytochrome c oxidase, subunit VIIb (Cox8b), nuclear gene encoding mitochondrial protein, mRNA [NM_007751]	A_52_P42381 4	Cox8b	cytochrome c oxidase, subunit VIIb	NM_007751		chr7:14808491-1-148084852	-	6.20 E-04	4.38	up	5.78	3.64
Mus musculus prominin 1 (Prom1), transcript variant 2, mRNA [NM_001163577]	A_55_P20599 31	Prom1	prominin 1	NM_001163577		chr5:44384920-44384861	-	7.94 E-04	2.19	down	5.40	6.53
Mus musculus microseminoprotein, prostate associated (Msmpt), mRNA [NM_001099314]	A_55_P20890 76	Msmpt	microseminoprotein, prostate associated	NM_001099314		chr4:43596329-43596270	-	8.21 E-04	2.93	up	5.81	4.26
PREDICTED: Mus musculus predicted gene, EG666182 (EG666182), mRNA [XM_991070]	A_55_P19544 36	Gm7967	predicted gene 7967	XM_991070		chr1:10049082-6-100490885	+	8.73 E-04	3.85	up	7.24	5.30

Figure 22 CONT.

PREDICTED: Mus musculus RIKEN cDNA D630033O11 gene (D630033O11Rik), mRNA [XM_001001707]	A_52_P24480 3	D630033O11 Rik	RIKEN cDNA D630033O11 gene	XM_001001 707	chr9:43088033 -43088092	+	8.78 E-04	3.11	up	12.07	10.44
PREDICTED: Mus musculus expressed sequence AA414992 (AA414992), mRNA [XM_001478791]	A_55_P21197 37	AA414992	expressed sequence AA414992	XM_001478 791	chr13:6832165 1-68321710	+	8.85 E-04	83.49	down	2.09	8.47
PREDICTED: Mus musculus hypothetical LOC66185 (LOC66185), mRNA [XM_001473218]	A_66_P12144 6	Gm7969	predicted gene 7969	XM_001473 218	chr13:6805407 0-68054129	+	9.07 E-04	33.88	down	6.81	11.89
lincRNA:chr14:79724027-79729152 reverse strand	A_30_P01031 882				chr14:7972701 9-79726960	-	9.19 E-04	2.02	down	3.19	4.21
lincRNA:chr14:65785477-65815777 reverse strand	A_30_P01027 710				chr14:6580537 9-65805320	-	9.78 E-04	2.10	up	4.08	3.01
lincRNA:chr5:22056218-22206995 forward strand	A_30_P01020 579				chr5:22200649 -22200708	+	1.07 E-03	4.38	down	2.35	4.48
Mus musculus polymerase (DNA directed), kappa (Polk), mRNA [NM_012048]	A_55_P22056 90	Polk	polymerase (DNA directed), kappa	NM_012048	chr13:9725689 4-97256835	-	1.11 E-03	2.40	up	3.85	2.59
PREDICTED: Mus musculus similar to CG32602-PA (LOC674779), mRNA [XM_001478781]	A_55_P20039 81	Gm9629	predicted gene 9629	XM_001478 781	chr13:6813802 1-68137962	-	1.13 E-03	69.96	down	2.17	8.30
lincRNA:chr1:162965001- 162966376 forward strand	A_30_P01029 558				chr1:16296520 6-162965265	+	1.14 E-03	3.05	down	3.98	5.59
Mus musculus 13 days embryo heart cDNA, RIKEN full-length enriched library, clone:D330023K18 product:unclassified, full insert sequence. [AK084630]	A_55_P22235 82	D330023K18 Rik	RIKEN cDNA D330023K18 gene	AK084630	chr2:31006221 -31006162	-	1.25 E-03	2.82	down	1.86	3.35
Mus musculus RIKEN cDNA 9030425E11 gene (9030425E11Rik), mRNA [NM_133733]	A_55_P20676 08	9030425E11 Rik	RIKEN cDNA 9030425E11 gene	NM_133733	chr9:40590634 -40590693	+	1.27 E-03	4.04	down	4.05	6.07
PREDICTED: Mus musculus RIKEN cDNA E130116L18 gene (E130116L18Rik), mRNA [XM_982295]	A_66_P11416 7	E130116L18 Rik	RIKEN cDNA E130116L18 gene	XM_982295	chr5:24727791 -24727732	-	1.34 E-03	2.62	up	5.42	4.03
lincRNA:chr5:22056218-22206995 forward strand	A_30_P01024 314				chr5:22088228 -22088287	+	1.35 E-03	2.60	down	1.60	2.98
MICAL C-terminal like Gene [Source:MG1 (curated);Acc:MG1:1918127]	A_55_P19782 93	Micald	MICAL C-terminal like	XM_893565	chr7:11953832 8-119538387	+	1.36 E-03	3.65	down	2.06	3.93

Figure 22 CONT.

[illegible]

Figure 22 CONT.

Mus musculus DNA segment, Chr 11, Brigham & Women's Genetics 0517 expressed (D11Bwg0517e), transcript variant 1, mRNA [NM_001039167]	A_55_P20533 72	D11Bwg0517 e	DNA segment, Chr 11, Brigham & Women's Genetics 0517 expressed	NM_001039 167	chr11:1183577 25-118357866	-	2.15 E-03	5.38	up	4.24	1.81
Mus musculus intralagellar transport 46 homolog (Chlamydomonas) (lft46), mRNA [NM_023831]	A_52_P21779 6	lft46	intralagellar transport 46 homolog (Chlamydomonas)	NM_023831	chr9:44600737 -44600796	+	2.17 E-03	4.72	down	2.37	4.61
lincRNA:chr4:13369325-13456750 forward strand	A_30_P01027 690				chr4:13441976 -13442035	+	2.21 E-03	2.94	up	3.71	2.15
Mus musculus tropomodulin 4 (Tmod4), mRNA [NM_016712]	A_55_P19765 74	Tmod4	tropomodulin 4	NM_016712	chr3:94933056 -94933115	+	2.30 E-03	3.20	down	6.01	7.69
lincRNA:chr5:105072936-105074001 forward strand	A_30_P01020 007				chr5:10507387 6-105073935	+	2.31 E-03	3.69	up	4.62	2.73
Mus musculus G protein-coupled receptor 120 (Gpr120), mRNA [NM_181748]	A_52_P50366 3	Gpr120	G protein-coupled receptor 120	NM_181748	chr19:3818837 0-38188429	+	2.37 E-03	3.70	up	6.30	4.41
Mus musculus testis-specific protein, Y-encoded-like 5 (Tspy5), mRNA [NM_001085421]	A_52_P50000 2	Tspy5	testis-specific protein, Y-encoded-like 5	NM_001085 421	chr15:3361400 7-33613948	-	2.41 E-03	3.59	up	3.71	1.87
PREDICTED: Mus musculus similar to CG32602-PA (LOC666222), mRNA [XM_982378]	A_55_P21808 61	Gm7987	predicted gene 7987	XM_982378	chr13:6826549 7-68265556	+	2.53 E-03	4.35	down	5.09	7.22
palmdelphin Gene [Source:MGI (curated);Acc:MGI:2148896] [ENSMUST00000119557]	A_55_P20378 17			AK136939	chr3:11662483 5-116624776	-	2.56 E-03	3.23	down	6.46	8.15
Mus musculus partial mRNA for T cell receptor beta chain (TRB gene), isolate 80_TCT. [FM179715]	A_55_P20830 14			FM179715	chr6:41221821 -41221880	+	2.57 E-03	2.56	down	2.59	3.95
Mus musculus torsin A interacting protein 1 (Tor1aip1), transcript variant 2, mRNA [NM_144791]	A_66_P10524 4	Tor1aip1	torsin A interacting protein 1	NM_144791	chr1:15787856 5-157877524	-	2.61 E-03	2.11	down	5.90	6.98
lincRNA:chr13:64381475-64415350 forward strand	A_30_P01031 688				chr13:6439123 0-64391289	+	2.63 E-03	2.13	down	3.22	4.31
lincRNA:chr12:81221532-81232030 forward strand	A_30_P01017 437				chr12:8122595 4-81226013	+	2.76 E-03	2.68	up	5.55	4.13
Mus musculus tubulin tyrosine ligase-like family, member 2 (Ttl2), mRNA [NM_001098267]	A_55_P19627 56	Ttl2	tubulin tyrosine ligase-like family, member 2	NM_001098 267	chr17:7555314 -7555255	-	2.90 E-03	3.72	down	1.67	3.56
PREDICTED: Mus musculus similar to developmental pluripotency associated 5 (LOC383490), misc	A_55_P20422 97	Gm5250	developmental pluripotency associated 5A	XR_031240	chr1:13052538 -13052597	+	3.00 E-03	2.21	up	6.39	5.24

Figure 22 CONT.

RNA [XR_031240]		pseudogene										
lincRNA:chr6:133054025- 133069850 reverse strand	A_30_P01020 699					chr6:13305498 9-133054930	-	3.04 E-03	7.22	up	6.43	3.58
Mus musculus RIKEN cDNA C230055K05 gene (C230055K05Rik), mRNA [NM_001039231]	A_52_P50870 0	C230055K05 Rik	RIKEN cDNA C230055K05 gene	NM_001039 231		chr5:10524234 8-105242289	-	3.08 E-03	2.06	up	6.10	5.06
Mus musculus CDK5 regulatory subunit associated protein 1 (Cdk5rap1), mRNA [NM_025876]	A_55_P20110 31	Cdk5rap1	CDK5 regulatory subunit associated protein 1	NM_025876		chr2:15419175 8-154191699	-	3.20 E-03	7.86	down	5.06	8.03
Mus musculus large tumor suppressor 2 (Lats2), transcript variant B, mRNA [NM_153382]	A_55_P20023 51	Lats2	large tumor suppressor 2	NM_153382		chr14:5834128 4-58341225	-	3.28 E-03	2.26	down	4.78	5.95
PREDICTED: Mus musculus cDNA sequence BC026762 (BC026762), mRNA [XM_001480912]	A_51_P27549 6	BC026762	cDNA sequence BC026762	XM_001480 912		chr7:26796662 -26796721	+	3.33 E-03	4.67	up	8.27	6.04
steroid 5 alpha-reductase 1 Gene [Source:MGI Symbol;Acc:MGI:98400] [ENSIMUST00000059235]	A_52_P56261 2			AK082819		chr13:6973357 7-69733518	-	3.41 E-03	7.76	down	4.41	7.37
Mus musculus cytochrome P450, family 2, subfamily c, polypeptide 67 (Cyp2c67), mRNA [NM_001024719]	A_55_P19771 44	Cyp2c67	cytochrome P450, family 2, subfamily c, polypeptide 67	NM_001024 719		chr19:3968360 0-39683541	-	3.45 E-03	2.22	down	13.27	14.42
Mus musculus ubiquitously transcribed tetrapeptide repeat gene, Y chromosome (Uty), mRNA [NM_009484]	A_55_P21349 67	Uty	ubiquitously transcribed tetrapeptide repeat gene, Y chromosome	NM_009484		chrY:511231- 511172	-	3.52 E-03	2.67	down	2.11	3.52
PREDICTED: Mus musculus similar to Ubtfl protein (LOC100040421), mRNA [XM_001474649]	A_55_P20013 97	Gm2762	predicted gene 2762	XM_001474 649		chr13:5351441 2-53514471	+	3.57 E-03	4.71	down	1.64	3.87
RIKEN cDNA A830039N20 gene Gene [Source:MGI Symbol;Acc:MGI:2445176] [ENSIMUST00000096098]	A_51_P27407 3			AK043847		chr14:2411176 7-24111708	-	3.58 E-03	2.21	down	3.34	4.48
Mus musculus cysteine conjugate- beta lyase 2 (Ccbl2), mRNA [NM_173763]	A_55_P20926 68	Ccbl2	cysteine conjugate- beta lyase 2	NM_173763		chr3:14240781 5-142407874	+	3.61 E-03	2.82	up	12.37	10.87
Mus musculus leucine rich repeat containing 27 (Lrrc27), transcript variant 1, mRNA [NM_027164]	A_52_P33831	Lrrc27	leucine rich repeat containing 27	NM_027164		chr7:14642855 0-146428609	+	3.64 E-03	2.07	down	2.50	3.55

Figure 22 CONT.

lincRNA:chr14:79725080-79725434 reverse strand	A_30_P01018 713					chr14:7972538 0-79725321	-	3.76 E-03	2.20	down	2.38	3.51
lincRNA:chr1:74084292-74138492 reverse strand	A_30_P01025 584					chr1:74104006 -74103947	-	3.80 E-03	2.66	up	6.96	5.55
Mus musculus nicotinamide nucleotide transhydrogenase (Nnt), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA [NM_008710]	A_55_P21442 80	Nnt	nicotinamide nucleotide transhydrogenase			chr13:1201233 77-120123318	-	3.87 E-03	4.88	down	2.53	4.81
Mus musculus alkaline ceramidase 2 (Acer2), mRNA [NM_139306]	A_55_P23674 63	Acer2	alkaline ceramidase 2			chr4:86533009 -86533068	+	3.94 E-03	2.24	up	4.06	2.89
Snf2-related CREBBP activator protein Gene [Source:MGI Symbol;Acc:MGI:2444036] [ENSMUST00000098025]	A_52_P41963 2					chr7:13466333 9-134665041	+	3.95 E-03	2.80	down	2.14	3.62
PREDICTED: Mus musculus similar to CDNA sequence BC057170 (LOC675363), misc RNA [XR_032536]	A_55_P20850 12	Gm9640	predicted gene 9640			chr5:10558857 9-105588520	-	4.07 E-03	3.14	down	6.19	7.84
lincRNA:chr4:145539725-145596000 forward strand	A_30_P01026 774					chr4:14558697 1-145587030	+	4.11 E-03	2.05	up	6.61	5.58
PREDICTED: Mus musculus hypothetical protein LOC100047576 (LOC100047576), mRNA [XM_001478645]	A_66_P13352 5					chr18:56293363 8-56293579	-	4.27 E-03	3.11	up	4.03	2.39
lincRNA:chr4:146233300-146260825 reverse strand	A_30_P01028 894					chr4:14624033 7-146240278	-	4.32 E-03	2.78	up	3.96	2.48
lincRNA:chr15:32705156-32729116 reverse strand	A_30_P01027 218					chr15:3270880 6-32708747	-	4.38 E-03	2.24	down	2.64	3.80
Mus musculus growth arrest specific 1 (Gas1), mRNA [NM_008086]	A_55_P20705 10	Gas1	growth arrest specific 1			chr13:6027830 2-60278243	-	4.45 E-03	2.06	down	1.67	2.71
Mus musculus chromogranin A (Chga), mRNA [NM_007693]	A_51_P35831 6	Chga	chromogranin A			chr12:1038030 83-103803142	+	4.51 E-03	2.72	up	3.62	2.18
lincRNA:chr19:6891117-6891594 reverse strand	A_30_P01021 994					chr19:6891450 -6891391	-	4.74 E-03	2.56	down	1.83	3.19
PREDICTED: Mus musculus similar to hairy and enhancer of split 1, (Drosophila) (LOC100047035), misc RNA [XR_033156]	A_55_P20157 77	LOC100047035	similar to hairy and enhancer of split 1, (Drosophila)			chr16:3007100 4-30071063	+	4.82 E-03	2.44	down	1.69	2.98
lincRNA:chr5:33779273-33780113 reverse strand	A_30_P01030 508					chr5:33779418 -33779359	-	4.89 E-03	3.35	down	2.24	3.98

Figure 22 CONT.

Mus musculus predicted gene 9112 (Gm9112), mRNA [NM_001173955]	A_55_P21279_71	Gm9112	predicted gene 9112	NM_001177365	chrX:99902931-99902872	-	4.98 E-03	2.56	up	4.15	2.80
lincRNA:chr16:13514223-13516070 reverse strand	A_30_P01032_220				chr16:13514885-13514826	-	5.02 E-03	3.52	down	2.01	3.83
Mus musculus chitinase 3-like 3 (Chi3l3), mRNA [NM_009892]	A_51_P16729_2	Chi3l3	chitinase 3-like 3	NM_009892	chr3:105950544-105950485	-	5.02 E-03	3.33	up	7.18	5.44
mediator of RNA polymerase II transcription, subunit 12 homolog (yeast)-like Gene [Source:MGI Symbol;Acc:MGI:2139916] [ENSMUST00000040846]	A_55_P21346_16			AK129475	chr3:58920427-58920486	+	5.04 E-03	2.77	up	8.18	6.71
Mus musculus SUMO1 activating enzyme subunit 1 (Sae1), mRNA [NM_019748]	A_66_P10658_5	Sae1	SUMO1 activating enzyme subunit 1	NM_019748	chr7:16913113-16913054	-	5.10 E-03	2.08	down	9.21	10.27
Mus musculus lipocalin 13 (Lcn13), mRNA [NM_153558]	A_55_P21351_53	Lcn13	lipocalin 13	NM_153558	chr2:25557738-25557797	+	5.25 E-03	4.53	up	11.26	9.08
Mus musculus RIKEN cDNA D630032N06 gene (D630032N06Rik), non-coding RNA [NR_028329]	A_52_P40063_7	D630032N06Rik	RIKEN cDNA D630032N06 gene	NR_028329	chr11:85051631-85051690	+	5.64 E-03	2.11	down	5.94	7.02
Mus musculus predicted gene 4956 (Gm4956), non-coding RNA [NR_002858]	A_55_P19863_41	Gm4956	predicted gene 4956	NR_002858	chr1:21275387-21275328	-	5.65 E-03	7.67	down	7.74	10.68
Mus musculus 12 days embryo spinal cord cDNA, RIKEN full-length enriched library, clone:C530042K13 product:unclassified, full insert sequence. [AK049699]	A_55_P23221_39	C530042K13Rik	RIKEN cDNA C530042K13 gene	AK049699	chr1:69045020-69044961	-	5.78 E-03	2.45	up	5.52	4.23
Mus musculus claudin 10A (Cldn10a), transcript variant b, mRNA [NM_021386]	A_51_P45646_5	Cldn10a	claudin 10A	NM_021386	chr14:119273459-119273518	+	5.85 E-03	4.39	up	6.48	4.34
lincRNA:chr13:67932042-67944229 forward strand	A_30_P01033_353				chr13:67944170-67944229	+	6.02 E-03	2.73	down	1.69	3.14
Mus musculus SLIT-ROBO Rho GTPase activating protein 3 (Srgap3), mRNA [NM_080448]	A_51_P46995_1	Srgap3	SLIT-ROBO Rho GTPase activating protein 3	NM_080448	chr6:112668298-112668239	-	6.06 E-03	2.99	down	7.40	8.98
Mus musculus retinoblastoma-like 1 (p107) (Rbl1), transcript variant 2, mRNA [NM_001139516]	A_55_P20247_73	Rbl1	retinoblastoma-like 1 (p107)	NM_001139516	chr2:157000820-157000761	-	6.09 E-03	2.20	down	4.15	5.29
Mus musculus cyclin J-like (Ccnj), mRNA [NM_001045530]	A_52_P65038_7	Ccnj	cyclin J-like	NM_001045530	chr11:433999495-433999554	+	6.12 E-03	2.14	down	3.67	4.77

Figure 22 CONT.

Mus musculus tubulin polymerization promoting protein (Tppp), mRNA [NM_182839]	A_51_P398260	Tppp	tubulin polymerization promoting protein	NM_182839	chr13:74172701-74172760	+	6.49 E-03	2.57	up	8.13	6.77
lincRNA:chr18:35973241-35973632 forward strand	A_30_P01027133				chr18:35973362-35973421	+	6.59 E-03	2.00	down	4.26	5.26
Mus musculus tripartite motif-containing 72 (Trim72), mRNA [NM_001079932]	A_55_P2026848	Trim72	tripartite motif-containing 72	NM_001079932	chr7:135154841-135154900	+	6.71 E-03	2.27	up	3.79	2.61
Mus musculus cytochrome P450, family 2, subfamily c, polypeptide 68 (Cyp2c68), mRNA [NM_001039555]	A_55_P2058433	Cyp2c68	cytochrome P450, family 2, subfamily c, polypeptide 68	NM_001039555	chr19:39763479-39763420	-	6.77 E-03	2.12	down	13.30	14.39
lincRNA:chr3:121615836-121616109 forward strand	A_30_P01025243				chr3:121615905-121615964	+	6.88 E-03	4.70	down	2.14	4.38
Mus musculus adult male small intestine cDNA, RIKEN full-length enriched library, clone:2010012P19 product:unclassifiable, full insert sequence. [AK008218]	A_55_P2197338	2010012P19 Rik	RIKEN cDNA 2010012P19 gene	AK008218	chr11:69504584-69504643	+	6.94 E-03	2.39	down	5.08	6.33
PREDICTED: Mus musculus hypothetical protein LOC100047047, mRNA [XM_001477284]	A_55_P2009022	LOC100047047	hypothetical protein LOC100047047	XM_001477284	chr11:97169095-97169154	+	7.05 E-03	2.70	down	6.81	8.24
Mus musculus zinc finger protein 748 (Zfp748), transcript variant 2, mRNA [NM_183145]	A_51_P344499	Zfp748	zinc finger protein 748	NM_183145	chr13:67646135-67646076	-	7.06 E-03	2.55	down	1.80	3.15
Mus musculus gap junction protein, alpha 5 (Gja5), mRNA [NM_008121]	A_55_P2178753	Gja5	gap junction protein, alpha 5	NM_008121	chr3:968363399-96836395	+	7.11 E-03	2.05	down	1.58	2.61
Mus musculus glial cell line derived neurotrophic factor family receptor alpha 4 (Gfra4), transcript variant 2, mRNA [NM_020014]	A_55_P1984119	Gfra4	glial cell line derived neurotrophic factor family receptor alpha 4	NM_020014	chr2:130866199-130866140	-	7.26 E-03	2.52	down	2.27	3.60
Mus musculus chordin-like 2 (Chrdl2), mRNA [NM_133709]	A_55_P2021689	Chrdl2	chordin-like 2	NM_133709	chr7:107183146-107183205	+	7.28 E-03	3.07	up	4.56	2.94
Mus musculus zinc finger protein 874 (Zfp874), mRNA [NM_177712]	A_52_P981697	Zfp874	zinc finger protein 874	NM_177712	chr13:67541839-67541780	-	7.34 E-03	13.36	down	3.64	7.38
lincRNA:chr17:38132217-38157667 forward strand	A_30_P01031377				chr17:38149862-38149921	+	7.45 E-03	2.95	up	4.29	2.73
Mus musculus RIKEN cDNA 4833424O15 gene (4833424O15Rik), mRNA [NM_029425]	A_55_P1987291	4833424O15 Rik	RIKEN cDNA 4833424O15 gene	NM_029425	chr3:117392323-117392382	+	7.49 E-03	2.80	down	2.83	4.31

Figure 22 CONT.

Mus musculus rad and gem related GTP binding protein 2 (Rem2), mRNA [NM_080726]	A_51_P19190_9	Rem2	rad and gem related GTP binding protein 2	NM_080726	chr14:5509885-9-55098918	+	7.53 E-03	2.20	up	3.26	2.13
PREDICTED: Mus musculus hypothetical LOC667413 (LOC667413), misc RNA [XR_032929]	A_55_P21086_93	Gm8617	predicted gene 8617	XR_032929	chrX:7203514-8-72035089	-	7.54 E-03	2.99	up	6.23	4.65
Mus musculus sulfotransferase family 3A, member 1 (Sult3a1), mRNA [NM_020565]	A_55_P21294_49	Sult3a1	sulfotransferase family 3A, member 1	NM_020565	chr10:3359912-5-33599184	+	7.56 E-03	5.93	down	1.66	4.22
Mus musculus Sec1 family domain containing 2 (Scfd2), transcript variant b, mRNA [NM_178672]	A_52_P57720_8	Scfd2	Sec1 family domain containing 2	NM_178672	chr5:74852923-74852864	-	7.77 E-03	3.21	up	3.92	2.24
Mus musculus transmembrane protease, serine 2 (Tmprss2), mRNA [NM_015775]	A_52_P55729_3	Tmprss2	transmembrane protease, serine 2	NM_015775	chr16:9778675-1-97786692	-	7.91 E-03	2.87	up	7.02	5.50
Mus musculus transmembrane protease, serine 9 (Tmprss9), mRNA [NM_001081688]	A_55_P19769_53	Tmprss9	transmembrane protease, serine 9	NM_001081688	chr10:8036218-0-80362239	+	8.05 E-03	2.13	down	5.00	6.09
Mus musculus leucine rich repeat containing 8 family, member E (Lrrc8e), mRNA [NM_028175]	A_55_P21257_86	Lrrc8e	leucine rich repeat containing 8 family, member E	NM_028175	chr8:4237411-4237470	+	8.09 E-03	2.79	up	4.06	2.58
lincRNA:chr18:3003075-3016150 reverse strand	A_30_P01023_607				chr18:3003665-30036606	-	8.30 E-03	3.67	up	4.64	2.77
Mus musculus asparaginase like 1 (Asrgl1), mRNA [NM_025610]	A_55_P20563_10	Asrgl1	asparaginase like 1	NM_025610	chr19:9186363-9186304	-	8.34 E-03	2.11	down	3.34	4.42
Mus musculus wingless related MMTV integration site 10b (Wnt10b), mRNA [NM_011718]	A_55_P21263_63	Wnt10b	wingless related MMTV integration site 10b	NM_011718	chr15:9860226-8-98602209	-	8.36 E-03	2.13	down	3.02	4.11
Mus musculus cell cycle exit and neuronal differentiation 1 (Cend1), mRNA [NM_021316]	A_51_P32351_8	Cend1	cell cycle exit and neuronal differentiation 1	NM_021316	chr7:14861305-5-148612996	-	8.41 E-03	2.05	up	3.13	2.09
Mus musculus 13 days embryo lung cDNA, RIKEN full-length enriched library, clone:D430004P15 product:unclassified, full insert sequence. [AK084877]	A_55_P22928_01	D430004P15 Rik	Riken cDNA D430004P15 gene	AK084877	chr3:55530023-55529964	-	8.49 E-03	2.75	down	2.32	3.78
Mus musculus structural maintenance of chromosomes 1B (Smc1b), mRNA [NM_080470]	A_51_P24430_3	Smc1b	structural maintenance of chromosomes 1B	NM_080470	chr15:8489525-2-84895193	-	8.51 E-03	2.03	down	5.83	6.85
PREDICTED: Mus musculus similar to Ubtf protein (LOC100047555), mRNA [XM_001478388]	A_55_P20960_67	LOC100047555	similar to Ubtf protein	XM_001478388	chr14:7535404-7-75354106	+	8.52 E-03	2.10	down	2.06	3.13

Figure 22 CONT.

PREDICTED: Mus musculus hypothetical protein LOC100047703 (LOC100047703), mRNA [XM_001478711]	A_55_P2013756	LOC100047703	hypothetical protein LOC100047703	XM_001478711	chr8:125681230-125681171	-	8.53 E-03	2.36	up	3.99	2.75
Mus musculus neuropilin (NRP) and tollid (TLL)-like 1 (Neto1), mRNA [NM_144946]	A_51_P145948	Neto1	neuropilin (NRP) and tollid (TLL)-like 1	NM_144946	chr18:86670386-86670445	+	8.62 E-03	2.40	up	4.93	3.67
PREDICTED: Mus musculus predicted gene, EG665070 (EG665070), mRNA [XM_001476912]	A_55_P2106844	Gm7475	predicted gene 7475	XM_001476912	chr2:25330882-25330823	-	8.63 E-03	2.87	down	8.23	9.75
Mus musculus guanine nucleotide binding protein (G protein), beta 4 (Gnb4), mRNA [NM_013531]	A_51_P464308	Gnb4	guanine nucleotide binding protein (G protein), beta 4	NM_013531	chr3:32488964-32488819	-	8.71 E-03	3.57	down	4.07	5.90
Mus musculus TSPY-like 4 (Tspyl4), mRNA [NM_030203]	A_51_P257675	Tspyl4	TSPY-like 4	NM_030203	chr10:34020839-34020898	+	8.83 E-03	3.64	down	5.72	7.58
Mus musculus F-box and WD-40 domain protein 15 (Fbxw15), mRNA [NM_199036]	A_55_P2145977	Fbxw15	F-box and WD-40 domain protein 15	NM_199036	chr9:109455175-109455116	-	8.84 E-03	2.55	up	6.72	5.37
lincRNA:chr14:78924115-78938125 forward strand	A_30_P01025730				chr14:78936239-78936298	+	8.92 E-03	3.39	down	1.86	3.62
lincRNA:chr11:95627874-95640824 reverse strand	A_30_P01029934				chr11:95629689-95629630	-	9.04 E-03	2.00	down	5.09	6.09
Mus musculus platelet factor 4 (Pf4), mRNA [NM_019932]	A_51_P441426	Pf4	platelet factor 4	NM_019932	chr5:91202275-91202334	+	9.12 E-03	2.19	up	8.25	7.12
Mus musculus sortilin-related receptor, LDLR class A repeats-containing (Sort1), mRNA [NM_011436]	A_55_P2151609	Sort1	sortilin-related receptor, LDLR class A repeats-containing	NM_011436	chr9:41776631-41776572	-	9.21 E-03	3.41	up	7.41	5.63
Mus musculus haloacid dehalogenase-like hydrolase domain containing 3 (Hdh3), mRNA [NM_024257]	A_51_P465582	Hdh3	haloacid dehalogenase-like hydrolase domain containing 3	NM_024257	chr4:62160215-62160156	-	9.32 E-03	2.10	down	12.34	13.41
Mus musculus cancer susceptibility candidate 4 (Casc4), transcript variant 1, mRNA [NM_177054]	A_55_P1994020	Casc4	cancer susceptibility candidate 4	NM_177054	chr2:121761338-121761397	+	9.47 E-03	2.20	up	6.61	5.47
Mus musculus predicted gene 5640 (Gm5640), mRNA [NM_001099302]	A_55_P1965298	Gm5640	predicted gene 5640	NM_001099302	chrX:71884517-71884458	-	9.48 E-03	2.72	up	6.73	5.28
Mus musculus immediate early response 5 (Ier5), mRNA [NM_010500]	A_55_P202114	Ier5	immediate early response 5	NM_010500	chr1:156943589-156943530	-	9.67 E-03	4.96	up	5.99	3.68
lincRNA:chr8:124355710-124401160 forward strand	A_30_P01018266				chr8:124397988-124398047	+	9.74 E-03	2.40	up	4.02	2.76

Figure 22 CONT.

Mus musculus coagulation factor XIII, A1 subunit (F13a1), transcript variant 1, mRNA [NM_028784]	A_51_P50780_1	F13a1	coagulation factor XIII, A1 subunit	NM_028784	chr13:3695927-3-36959214	-	9.78 E-03	3.14	up	5.27	3.62
lincRNA:chr3:121615836-121616109 forward strand	A_30_P01032_927				chr3:12161603-6-121616095	+	9.79 E-03	6.61	down	1.65	4.38
Mus musculus neurotrophic tyrosine kinase, receptor, type 2 (Ntrk2), transcript variant 1, mRNA [NM_001025074]	A_55_P20067_92	Ntrk2	neurotrophic tyrosine kinase, receptor, type 2	NM_001025074	chr13:5897956-3-58979622	+	1.03 E-02	4.23	up	12.08	9.99
Mus musculus dynein, axonemal, heavy chain 11 (Dnahc11), mRNA [NM_010060]	A_55_P21199_07	Dnahc11	dynein, axonemal, heavy chain 11	NM_010060	chr12:1191165-15-119116456	-	1.03 E-02	2.21	up	5.66	4.52
Mus musculus cytochrome P450, family 2, subfamily c, polypeptide 67 (Cyp2c67), mRNA [NM_001024719]	A_55_P19626_61	Cyp2c67	cytochrome P450, family 2, subfamily c, polypeptide 67	NM_001024719	chr19:3968348-8-39683429	-	1.04 E-02	2.00	down	14.26	15.26
lincRNA:chr16:46384974-46391331 forward strand	A_30_P01022_871				chr16:4638952-6-46389585	+	1.05 E-02	2.42	down	2.20	3.47
lincRNA:chr1:167802183-167805411 forward strand	A_30_P01024_008				chr1:16780532-8-167805387	+	1.07 E-02	4.48	down	1.80	3.97
Mus musculus amnionless (Amn), mRNA [NM_033603]	A_51_P34335_0	Amn	amnionless	NM_033603	chr12:1125140-95-112514243	+	1.09 E-02	4.00	down	5.38	7.38
Mus musculus guanylate binding protein 11 (Gbp11), mRNA [NM_001039647]	A_55_P20428_13	Gbp11	guanylate binding protein 11	NM_001039647	chr5:10575220-4-105752145	-	1.09 E-02	3.89	down	9.42	11.37
Mus musculus cholinergic receptor, nicotinic, gamma polypeptide (Chrng), mRNA [NM_009604]	A_51_P28801_0	Chrng	cholinergic receptor, nicotinic, gamma polypeptide	NM_009604	chr1:89106072-89106353	+	1.11 E-02	3.51	up	5.30	3.49
Mus musculus cDNA clone IMAGE:6435376. [BC100571]	A_55_P22755_90	A630012P03 Rik	RIKEN cDNA A630012P03 gene	BC100571	chrX:4993125-6-49931315	+	1.13 E-02	2.37	down	1.83	3.08
Mus musculus interferon gamma (Ifng), mRNA [NM_008337]	A_52_P68893	Ifng	interferon gamma	NM_008337	chr10:1178828-41-117882900	+	1.14 E-02	2.80	down	2.69	4.18
lincRNA:chr5:22905597-22939154 reverse strand	A_30_P01025_008				chr5:22906763-22906704	-	1.14 E-02	4.01	down	1.74	3.74
Mus musculus family with sequence similarity 126, member B (Fam126b), mRNA [NM_172513]	A_55_P19743_52	Fam126b	family with sequence similarity 126, member B	NM_172513	chr1:58584253-58584194	-	1.14 E-02	2.46	up	9.65	8.35
Mus musculus eyes absent 2 homolog (Drosophila) (Eya2), mRNA [NM_010165]	A_55_P21410_93	Eya2	eyes absent 2 homolog (Drosophila)	NM_010165	chr2:16559707-2-165597131	+	1.16 E-02	2.82	down	2.63	4.12

Figure 22 CONT.

PREDICTED: Mus musculus similar to CG32602-PA (LOC677603), mRNA [XM_001477594]	A_55_P20324_19	Gm9720	predicted gene 9720	XM_001477594	chr13:6822371-68223652	-	1.17 E-02	15.99	down	4.50	8.50
Mus musculus small proline-rich protein 2A2 (Spr2a2), mRNA [NM_001164787]	A_55_P20488_55	Spr2a2	small proline-rich protein 2A2	NM_001164787	chr3:92058417-92058476	+	1.17 E-02	5.85	up	5.74	3.19
Mus musculus RIKEN cDNA 6130401L20 gene (6130401L20Rik), transcript variant 1, mRNA [NM_029412]	A_55_P19611_30	6130401L20Rik	RIKEN cDNA 6130401L20 gene	NM_029412	chr3:29589049-29589108	+	1.18 E-02	2.20	down	4.34	5.48
Mus musculus surfactant associated protein C (Sftpc), mRNA [NM_011359]	A_51_P32800_1	Sftpc	surfactant associated protein C	NM_011359	chr14:7092115-70920899	-	1.19 E-02	6.52	down	2.01	4.72
Mus musculus predicted pseudogene 10136 (Gm10136), mRNA [NM_001178039]	A_55_P21800_96	Gm10136	predicted pseudogene 10136	NM_001178039	chr19:2907783-8-29077833	-	1.20 E-02	2.48	down	13.24	14.54
lincRNA:chr2:22782700-22801900 reverse strand	A_30_P01018_880				chr2:22785136-22785077	-	1.20 E-02	3.36	down	1.63	3.38
lincRNA:chr14:79724027-79729152 reverse strand	A_30_P01029_209				chr14:7972710-1-79727042	-	1.20 E-02	2.33	down	2.45	3.67
PREDICTED: Mus musculus hypothetical protein LOC100044227 (LOC100044227), mRNA [XM_001471788]	A_55_P19854_22	LOC100044227	hypothetical protein LOC100044227	XM_001471788			1.21 E-02	4.58	up	15.09	12.90
Mus musculus cysteine rich transmembrane BMP regulator 1 (chordin like) (Crim1), mRNA [NM_015800]	A_55_P20744_88	Crim1	cysteine rich transmembrane BMP regulator 1 (chordin like)	NM_015800	chr17:7877359-5-78773654	+	1.23 E-02	2.97	up	5.89	4.32
Mus musculus chaperone, ABC1 activity of bc1 complex like (S. pombe) (Cabc1), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA [NM_023341]	A_52_P54280	Cabc1	chaperone, ABC1 activity of bc1 complex like (S. pombe)	NM_023341	chr1:18209569-1-182095632	-	1.23 E-02	2.04	down	8.11	9.14
lincRNA:chr5:77188626-77219161 forward strand	A_30_P01023_577				chr5:77218944-77219003	+	1.23 E-02	2.55	up	4.84	3.50
lincRNA:chr4:149445201-149445902 forward strand	A_30_P01024_344				chr4:14944577-1-149445830	+	1.24 E-02	4.06	up	7.81	5.79
PREDICTED: Mus musculus predicted gene, EG544767 (EG544767), mRNA [XM_622131]	A_55_P21814_29	Gm5782	predicted gene 5782	XM_622131	chr11:4322832-4-43228265	-	1.24 E-02	2.36	down	2.02	3.26
PREDICTED: Mus musculus similar to translin associated protein X (LOC666262), misc RNA	A_55_P21532_41	Gm8010	translin-associated factor X pseudogene	XR_031230	chr1:18007473-1-180074790	+	1.24 E-02	2.12	down	3.15	4.23

Figure 22 CONT.

[illegible]

Figure 22 CONT.

PREDICTED: Mus musculus similar to ribosomal protein S3 (LOC666489), misc RNA [XR_032871]	A_55_P21092_22	Gm8130	predicted gene 8130	XR_032871	chr16:2305092-23050987	+	1.40 E-02	2.04	down	14.32	15.35
PREDICTED: Mus musculus similar to developmental pluripotency associated 5 (LOC100046088), mRNA [XM_001475553]	A_55_P21500_29			XM_001475553	chrX:1083638-31-108363890	+	1.40 E-02	5.01	up	6.08	3.75
Mus musculus sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 6 (Sult2a6), mRNA [NM_001081325]	A_55_P21072_23	Sult2a6	sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 6	NM_001081325	chr7:14811272-14811213	-	1.41 E-02	6.57	down	1.68	4.40
Mus musculus nitric oxide synthase 1 (neuronal) adaptor protein (Nos1ap), transcript variant 2, mRNA [NM_027528]	A_66_P13480_8	Nos1ap	nitric oxide synthase 1 (neuronal) adaptor protein	NM_027528	chr1:17225936-4-172259305	-	1.42 E-02	2.42	up	5.67	4.40
PREDICTED: Mus musculus hypothetical protein LOC100044803 (LOC100044803), mRNA [XM_001473079]	A_55_P19962_90	Gm2550	predicted gene 2550	XM_001473079	chr5:14999293-5-149992876	-	1.43 E-02	2.12	up	3.29	2.21
Mus musculus vomeronasal 1 receptor 217 (Vmn1r217), mRNA [NM_134239]	A_55_P19992_03	Vmn1r217	vomeronasal 1 receptor 217	NM_134239	chr13:2320586-0-23205801	-	1.43 E-02	2.19	up	3.48	2.35
PREDICTED: Mus musculus similar to polymyositis scleroderma overlap syndrome (PM-SCL) antigen 1 a, transcript variant 6 (LOC675032), mRNA [XM_983011]	A_55_P20671_46	LOC675032	similar to Exosome complex exonuclease RRP45	XM_983011	chr3:36465081-36465140	+	1.44 E-02	2.27	down	1.75	2.94
Mus musculus olfactory receptor 1348 (Olfr1348), mRNA [NM_146913]	A_55_P21357_20	Olfr1348	olfactory receptor 1348	NM_146913	chr7:6454680-6454621	-	1.45 E-02	2.61	up	4.91	3.53
predicted gene 7461 Gene [Source:MG] Symbol:Acc:MG1:3645338] [ENSMUST00000058918]	A_52_P32282_6	Gm7461	predicted gene 7461	XM_001473345	chr8:4677915-4677856	-	1.47 E-02	2.03	up	5.01	3.99
lincRNA:chrX:148819227-148844227 reverse strand	A_30_P01018_157				chrX:1488351-47-148835088	-	1.49 E-02	2.10	down	2.17	3.24
Mus musculus THUMP domain containing 1 (Thumpd1), mRNA [NM_145585]	A_55_P19956_98	Thumpd1	THUMP domain containing 1	NM_145585	chr7:12685889-4-126858835	-	1.49 E-02	49.04	down	2.36	7.97
lincRNA:chr4:149445201-149445902 forward strand	A_30_P01025_511				chr4:14944583-1-149445890	+	1.50 E-02	3.60	up	7.89	6.04

Figure 22 CONT.

Mus musculus DNA segment, Chr X, Baylor 18 (DXBay18), mRNA [NM_001025384]	A_55_P19588_87	DXBay18	DNA segment, Chr X, Baylor 18	NM_001025384	chrX:7038265-4-70382596	-	1.51 E-02	2.43	up	6.98	5.69
Mus musculus alcohol dehydrogenase 6A (class V) (Adh6a), mRNA [NM_026945]	A_55_P21128_82	Adh6a	alcohol dehydrogenase 6A (class V)	NM_026945	chr3:13799403-9-137994098	+	1.51 E-02	5.29	down	1.81	4.21
Mus musculus vasoactive intestinal peptide receptor 2 (Vipr2), mRNA [NM_009511]	A_51_P34277_3	Vipr2	vasoactive intestinal peptide receptor 2	NM_009511	chr12:1173844-55-117384514	+	1.52 E-02	3.06	down	1.66	3.27
Mus musculus acylcoxyacyl hydrolase (Aoah), mRNA [NM_012054]	A_55_P20429_88	Aoah	acyloxyacyl hydrolase	NM_012054	chr13:2111606-2-21116121	+	1.53 E-02	2.05	up	5.92	4.88
Mus musculus myeloperoxidase (Mpo), nuclear gene encoding mitochondrial protein, mRNA [NM_010824]	A_55_P19955_37	Mpo	myeloperoxidase	NM_010824	chr11:8761784-0-87617899	+	1.54 E-02	8.21	up	5.37	2.34
Mus musculus S100 calcium binding protein A8 (calgranulin A) (S100a8), mRNA [NM_013650]	A_51_P25682_7	S100a8	S100 calcium binding protein A8 (calgranulin A)	NM_013650	chr3:90473778-90473837	+	1.54 E-02	2.98	up	6.34	4.76
lincRNA:chr3:121627545-121639470 reverse strand	A_30_P01026_923				chr3:12163903-6-121638977	-	1.55 E-02	5.40	down	1.79	4.22
proprotein convertase subtilisin/kexin type 5 Gene [Source:MGI Symbol;Acc:MGI:97515] [ENSMUST00000025618]	A_55_P21672_74			D17583	chr19:1750794-2-17507883	-	1.55 E-02	2.43	down	2.33	3.61
Mus musculus SLIT-ROBO Rho GTPase activating protein 3 (Srgap3), mRNA [NM_080448]	A_55_P21086_09	Srgap3	SLIT-ROBO Rho GTPase activating protein 3	NM_080448	chr6:11267284-1-112672782	-	1.56 E-02	2.03	down	5.25	6.27
Mus musculus potassium channel tetramerisation domain containing 7 (Kctd7), mRNA [NM_172509]	A_66_P11404_2	Kctd7	potassium channel tetramerisation domain containing 7	NM_172509	chr5:13063156-9-130631628	+	1.57 E-02	2.04	down	1.63	2.66
PREDICTED: Mus musculus similar to guanylate nucleotide binding protein 1 (LOC100048720), mRNA [XM_001481046]	A_55_P19527_68	LOC100048720	similar to guanylate nucleotide binding protein 1	XM_001481046	chr3:14227446-8-142274527	+	1.57 E-02	2.59	down	3.17	4.54
lincRNA:chr11:69184157-69201732 forward strand	A_30_P01029_391				chr11:6919601-0-69196069	+	1.57 E-02	2.07	up	4.58	3.53
Mus musculus predicted gene 13157 (Gm13157), mRNA [NM_001127189]	A_55_P20087_22	Gm13157	predicted gene 13157	NM_001127189	chr4:14713023-5-147130176	-	1.58 E-02	2.94	up	11.18	9.63
Mus musculus apelin receptor (Aplnr), mRNA [NM_011784]	A_52_P97572	Aplnr	apelin receptor	NM_011784	chr2:84979638-84979697	+	1.63 E-02	4.41	down	3.61	5.75

Figure 22 CONT.

Mus musculus polyadenylate binding protein-interacting protein 1 (Paip1), transcript variant 1, mRNA [NM_145457]	A_51_P47098 9	Paip1	polyadenylate binding protein-interacting protein 1	NM_145457	chr13:1202395-62-120240599	+	1.63 E-02	3.42	down	6.79	8.56
PREDICTED: Mus musculus predicted gene, EG667449 (EG667449), mRNA [XM_990610]	A_55_P19652 28	Gm8639	predicted gene 8639	XM_990610	chr14:5327199-2-53272051	+	1.66 E-02	2.49	up	3.76	2.45
Mus musculus WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2 (Wfikkn2), mRNA [NM_181819]	A_55_P20610 84	Wfikkn2	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2	NM_181819	chr11:9409733-5-94097276	-	1.66 E-02	2.19	up	2.75	1.62
Mus musculus roundabout homolog 1 (Drosophila) (Robo1), mRNA [NM_019413]	A_55_P19850 70	Robo1	roundabout homolog 1 (Drosophila)	NM_019413	chr16:7304619-0-73046249	+	1.69 E-02	8.97	down	5.01	8.18
Mus musculus cardiotrophin-like cytokine factor 1 (Cicf1), mRNA [NM_019952]	A_51_P10242 1	Cicf1	cardiotrophin-like cytokine factor 1	NM_019952	chr19:4222480-4222539	+	1.70 E-02	2.47	up	4.39	3.08
RIKEN cDNA 1700021P04 gene [Source:MGI Symbol;Acc:MGI:1922771] [ENSMUST00000025731]	A_55_P20848 35	1700021P04 Rik	RIKEN cDNA 1700021P04 gene	XM_988914	chr19:2414073-4-24140793	+	1.70 E-02	3.20	down	1.62	3.30
Mus musculus serine (or cysteine) peptidase inhibitor, clade B, member 9f (Serpnb9f), mRNA [NM_183197]	A_55_P19714 25	Serpnb9f	serine (or cysteine) peptidase inhibitor, clade B, member 9f	NM_183197	chr13:3342703-9-33427098	+	1.70 E-02	2.10	up	3.44	2.37
lincRNA:chr19:6268793-6276607 forward strand	A_30_P01017 901				chr19:6275838-6275897	+	1.71 E-02	2.70	down	1.84	3.28
Mus musculus neurotrophic tyrosine kinase, receptor, type 2 (Ntrk2), transcript variant 2, mRNA [NM_008745]	A_65_P04961	Ntrk2	neurotrophic tyrosine kinase, receptor, type 2	NM_008745	chr13:5903804-6-59038105	+	1.72 E-02	4.51	up	9.03	6.86
Mus musculus G protein-coupled receptor 98 (Gpr98), mRNA [NM_054053]	A_51_P38953 9	Gpr98	G protein-coupled receptor 98	NM_054053	chr13:8123438-9-81234330	-	1.74 E-02	3.15	up	11.16	9.51
Mus musculus collagen and calcium binding EGF domains 1 (Ccbe1), mRNA [NM_178793]	A_55_P20098 82	Ccbe1	collagen and calcium binding EGF domains 1	NM_178793	chr18:6623093-6-66230877	-	1.75 E-02	2.10	up	4.44	3.37
Mus musculus adult male thymus cDNA, RIKEN full-length enriched library, clone:583044B04 product:unclassified, full insert sequence. [AK030886]	A_55_P22066 05	583044B04 Rik	RIKEN cDNA 583044B04 gene	AK030886	chr4:15478251-3-154782454	-	1.76 E-02	8.28	down	2.18	5.23

Figure 22 CONT.

lincRNA:chr9:113648970-113715170 forward strand	A_30_P01021 287					chr9:11370252 4-113702583	+	1.77 E-02	2.08	down	2.67	3.73
Mus musculus RIKEN cDNA 1700061G19 gene (1700061G19Rik), mRNA [NM_030141]	A_55_P20196 94	1700061G19 Rik	RIKEN cDNA 1700061G19 gene	NM_030141		chr17:5702810 5-57028164	+	1.78 E-02	2.52	down	2.03	3.36
Mus musculus RIKEN cDNA 2310007B03 gene (2310007B03Rik), transcript variant 1, mRNA [NM_172411]	A_51_P27543 5	2310007B03 Rik	RIKEN cDNA 2310007B03 gene	NM_172411		chr1:95048285 -95048226	-	1.78 E-02	2.01	down	1.80	2.81
lincRNA:chr3:60292527-60292880 reverse strand	A_30_P01028 265					chr3:60292667 -60292608	-	1.78 E-02	2.22	up	7.63	6.48
Mus musculus S100 calcium binding protein A9 (calgranulin B) (S100a9), mRNA [NM_009114]	A_55_P19984 71	S100a9	S100 calcium binding protein A9 (calgranulin B)	NM_009114		chr3:90496632 -90496573	-	1.83 E-02	2.93	up	10.34	8.79
Mus musculus Fas ligand (TNF superfamily, member 6) (FasL), mRNA [NM_010177]	A_55_P20161 14	FasL	Fas ligand (TNF superfamily, member 6)	NM_010177		chr1:16371111 5-163711056	-	1.83 E-02	3.50	down	1.77	3.57
lincRNA:chr18:36449530-36456800 reverse strand	A_30_P01018 115					chr18:3645197 9-36451920	-	1.85 E-02	2.06	down	1.69	2.73
Mus musculus H19 fetal liver mRNA (H19), non-coding RNA [NR_001592]	A_55_P19611 27	H19	H19 fetal liver mRNA	NR_001592		chr7:14976155 1-149761492	-	1.86 E-02	2.23	up	5.45	4.30
Mus musculus RIKEN cDNA 443040218 gene (443040218Rik), mRNA [NM_198651]	A_55_P20511 20	443040218R ik	RIKEN cDNA 443040218 gene	NM_198651		chr19:2899772 7-28997668	-	1.87 E-02	2.34	down	3.64	4.87
lincRNA:chr4:129497990- 129515290 reverse strand	A_30_P01021 825					chr4:12949860 9-129498550	-	1.87 E-02	2.50	down	2.19	3.51
Mus musculus 16 days neonate thymus cDNA, RIKEN full-length enriched library, clone:A130010C18 product:unclassified, full insert sequence. [AK037357]	A_55_P22909 14	Trav3n-3	T cell receptor alpha variable 3N- 3	AK037357		chr14:5482053 7-54820596	+	1.87 E-02	2.46	down	2.47	3.76
histidine ammonia lyase Gene [Source:MG1 (curated);Acc:MG1:96010] [ENSMUST00000129421]	A_55_P22630 53	Hal	histidine ammonia lyase	CK128530		chr10:9298153 4-92981593	+	1.88 E-02	2.03	down	10.72	11.74
Mus musculus RWD domain containing 3 (Rwdd3), transcript variant 1, mRNA [NM_025637]	A_55_P21431 31	Rwdd3	RWD domain containing 3	NM_025637		chr3:12087425 3-120874194	-	1.88 E-02	3.79	down	1.87	3.79
Mus musculus tripartite motif- containing 60 (Trim60), mRNA [NM_153097]	A_51_P24816 0	Trim60	tripartite motif- containing 60	NM_153097		chr8:67524207 -67524148	-	1.88 E-02	2.18	down	2.92	4.04

Figure 22 CONT.

Mus musculus ATP-binding cassette, sub-family A (ABC1), member 16 (Abca16), non-coding RNA [NR_024051]	A_55_P20110 46	Abca16	ATP-binding cassette, sub-family A (ABC1), member 16	NR_024051	chr7:12768826 8-127688325	+	1.89 E-02	4.18	down	1.81	3.87
Mus musculus trichorhinophalangeal syndrome 1 (human) (Trps1), mRNA [NM_032000]	A_55_P20055 85	Trps1	trichorhinophalangeal syndrome 1 (human)	NM_032000	chr15:5049230 0-50492241	-	1.91 E-02	2.17	up	4.14	3.02
PREDICTED: Mus musculus hypothetical protein LOC100048336 (LOC100048336), mRNA [XM_001480034]	A_55_P19522 16	LOC100048336	hypothetical protein LOC100048336	XM_001480034	chr9:60896876 -60896817	-	1.91 E-02	2.38	down	1.94	3.19
lincRNA:chr13:51248372-51259297 forward strand	A_30_P01030 230				chr13:5125019 7-51250256	+	1.93 E-02	2.50	down	2.74	4.06
Mus musculus neuregulin 1 (Nrg1), mRNA [NM_178591]	A_55_P19854 33	Nrg1	neuregulin 1	NM_178591	chr8:32928559 -32928500	-	1.95 E-02	3.87	up	7.63	5.68
Mus musculus RIKEN cDNA 1700029101 gene (1700029101Rik), mRNA [NM_027285]	A_55_P21211 51	1700029101Rik	RIKEN cDNA 1700029101 gene	NM_027285	chr4:14542182 2-145421881	+	1.96 E-02	2.48	up	6.11	4.80
Mus musculus phosphatidylinositol 4-kinase type 2 beta (PI4K2b), transcript variant 1, mRNA [NM_025951]	A_51_P23098 7	PI4K2b	phosphatidylinositol 4-kinase type 2 beta	NM_025951	chr5:53159457 -53159516	+	1.97 E-02	2.19	up	11.34	10.21
Mus musculus rad and gem related GTP binding protein 1 (Rem1), mRNA [NM_009047]	A_55_P21130 78	Rem1	rad and gem related GTP binding protein 1	NM_009047	chr2:15246086 8-152460927	+	1.99 E-02	2.44	up	6.56	5.27
lincRNA:chr10:18104309-18104881 forward strand	A_30_P01023 616				chr10:1810482 2-18104881	+	2.00 E-02	2.64	up	3.71	2.31
lincRNA:chr10:83181374-83249774 forward strand	A_30_P01030 126				chr10:8322479 5-83224854	+	2.00 E-02	2.58	down	2.83	4.20
Mus musculus chymosin (Cym), mRNA [NM_00111143]	A_55_P20465 63	Cym	chymosin	NM_00111143	chr3:10701436 0-107014301	-	2.01 E-02	2.72	down	2.44	3.88
Mus musculus cDNA sequence BC020535 (BC020535), mRNA [NM_145536]	A_55_P20181 91	BC020535	cDNA sequence BC020535	NM_145536	chr2:15290727 5-152907334	+	2.01 E-02	2.37	down	6.01	7.25
PREDICTED: Mus musculus hypothetical protein LOC100044858 (LOC100044858), mRNA [XM_001473203]	A_55_P19906 93	LOC100044858	hypothetical protein LOC100044858	XM_001473203	chr10:1248146 06-124814547	-	2.03 E-02	2.28	down	1.75	2.94
lincRNA:chr12:73838608-73862621 reverse strand	A_30_P01027 936				chr12:7384798 9-73847930	-	2.04 E-02	2.02	up	5.63	4.62
Mus musculus predicted gene 10565 (Gm10565), non-coding RNA [NR_027137]	A_55_P20740 40	Gm10565	predicted gene 10565	NR_027137	chr4:14164166 8-141641727	+	2.04 E-02	2.32	down	1.79	3.00

Figure 22 CONT.

Mus musculus kidney androgen regulated protein (Kap), mRNA [NM_010594]	A_51_P41774 7	Kap	kidney androgen regulated protein	NM_010594	chr6:13379998 4-133799925	-	2.07 E-02	6.88	up	6.15	3.37
Mus musculus adult male tongue cDNA, RIKEN full-length enriched library, clone:2310047N11 product:unclassified, full insert sequence. [AK019096]	A_55_P22018 66	2310047N11 Rik	RIKEN cDNA 2310047N11 gene	AK019096	chr1:16594762 -16594703	-	2.10 E-02	2.65	up	9.70	8.30
Mus musculus solute carrier family 4, sodium bicarbonate cotransporter, member 9 (Slc4a9), mRNA [NM_172830]	A_55_P20564 88	Slc4a9	solute carrier family 4, sodium bicarbonate cotransporter, member 9	NM_172830	chr18:3670088 5-36700944	+	2.11 E-02	2.89	down	2.37	3.90
lincRNA:chr3:27837825-27880925 forward strand	A_30_P01032 764				chr3:27872586 -27872645	+	2.11 E-02	3.31	up	4.46	2.74
lincRNA:chr1:95871175-95886625 forward strand	A_30_P01020 516				chr1:95884813 -95884872	+	2.11 E-02	2.37	down	5.66	6.91
lincRNA:chr5:23195569-23218551 reverse strand	A_30_P01020 907				chr5:23205256 -23205197	-	2.12 E-02	6.42	up	7.78	5.10
Mus musculus p53 and DNA damage regulated 1 (Pdgfr1), mRNA [NM_178939]	A_51_P17396 1	Pdgfr1	p53 and DNA damage regulated 1	NM_178939	chr2:15283472 8-152834669	-	2.13 E-02	2.03	down	10.35	11.37
Mus musculus RAS protein activator like 2 (Rasal2), mRNA [NM_177644]	A_55_P20347 31	Rasal2	RAS protein activator like 2	NM_177644	chr1:15907090 1-159070842	-	2.14 E-02	2.00	up	5.53	4.53
PREDICTED: Mus musculus similar to FEX2 (LOC100046701), mRNA [XM_001476656]	A_55_P19927 20	LOC100046701	similar to FEX2	XM_001476656	chr10:8646575 3-86465694	-	2.15 E-02	3.17	up	6.19	4.53
Mus musculus family with sequence similarity 151, member A (Fam151a), mRNA [NM_146149]	A_52_P52910	Fam151a	family with sequence similarity 151, member A	NM_146149	chr4:10642051 0-106420569	+	2.16 E-02	2.23	up	3.26	2.10
lincRNA:chr2:150072436-150074859 forward strand	A_30_P01025 441				chr2:15007473 6-150074795	+	2.17 E-02	2.77	up	4.50	3.03
tripartite motif-containing 5 Gene [Source:MG] Symbol:Acc:MG1:3646853] [ENSMUST00000098179]	A_52_P11879 49	Trim5	tripartite motif-containing 5	XM_992714	chr7:11141248 6-111412427	-	2.22 E-02	2.69	up	4.41	2.98
Mus musculus caveolin 1, caveolae protein (Cav1), mRNA [NM_007616]	A_55_P21266 62	Cav1	caveolin 1, caveolae protein	NM_007616	chr6:17289414 -17289473	+	2.24 E-02	3.18	down	2.66	4.33
Mus musculus RIKEN cDNA A530016L24 gene (A530016L24Rik), mRNA [NM_177039]	A_55_P21387 56	A530016L24 Rik	RIKEN cDNA A530016L24 gene	NM_177039	chr12:1137364 38-113736497	+	2.26 E-02	2.71	down	1.83	3.27

Figure 22 CONT.

Mus musculus macrophage receptor with collagenous structure (Marco), mRNA [NM_010766]	A_51_P371750	Marco	macrophage receptor with collagenous structure	NM_010766	chr1:12237113-4-122371115	-	2.28 E-02	4.07	up	10.87	8.85
Mus musculus cDNA sequence BC020535 (BC020535), mRNA [NM_145536]	A_55_P1981836	BC020535	cDNA sequence BC020535	NM_145536	chr2:15290740-3-152907462	+	2.29 E-02	2.37	down	8.36	9.61
PREDICTED: Mus musculus similar to Predicted gene, OTTMUSG0000007485 (LOC100039019), misc RNA [XR_030567]	A_55_P1981195	Mup-ps12	major urinary protein, pseudogene 12	XR_030567	chr4:61113330-61113389	+	2.31 E-02	3.86	up	8.99	7.04
Mus musculus defensin beta 34 (Defb34), mRNA [NM_183035]	A_52_P280832	Defb34	defensin beta 34	NM_183035	chr8:19126406-19126465	+	2.32 E-02	3.17	up	3.94	2.27
Mus musculus cytochrome P450, family 3, subfamily a, polypeptide 44 (Cyp3a44), mRNA [NM_177380]	A_52_P366803	Cyp3a44	cytochrome P450, family 3, subfamily a, polypeptide 44	NM_177380	chr5:14658577-6-146585717	-	2.32 E-02	7.42	down	2.68	5.58
Mus musculus alpha hemoglobin stabilizing protein (Ahsp), mRNA [NM_133245]	A_52_P534583	Ahsp	alpha hemoglobin stabilizing protein	NM_133245			2.33 E-02	2.73	up	4.17	2.72
lincRNA:chr7:96640618-96649018 reverse strand	A_30_P01031761				chr7:96643997-96643938	-	2.33 E-02	2.90	down	2.27	3.81
Mus musculus UDP glycosyltransferase 1 family, polypeptide A10 (Ugt1a10), mRNA [NM_201641]	A_55_P2178800	Ugt1a10	UDP glycosyltransferase 1 family, polypeptide A10	NM_201641	chr1:89952780-89952839	+	2.33 E-02	5.76	down	8.22	10.75
Mus musculus predicted gene 14431 (Gm14431), transcript variant 1, mRNA [NM_00177406]	A_55_P1956624	Gm14431	predicted gene 14431	NM_001177406	chr2:17521069-7-175210756	+	2.34 E-02	2.22	down	6.70	7.85
Mus musculus RIKEN cDNA A530016L24 gene (A530016L24Rik), mRNA [NM_177039]	A_55_P1972997	A530016L24 Rik	RIKEN cDNA A530016L24 gene	NM_177039	chr12:1137380-79-113738138	+	2.36 E-02	2.56	down	4.39	5.75
NLR family, pyrin domain containing 1A Gene [Source:MGI (curated);Acc:MGI:2684861] [ENSMUST00000108519]	A_55_P2070366			AY355339	chr11:7090607-6-70906017	-	2.37 E-02	2.34	up	4.65	3.42
Mus musculus defensin, alpha, 23 (Defa23), mRNA [NM_001012307]	A_55_P2104071	Defa23	defensin, alpha, 23	NM_001012307	chr8:22195607-22195666	+	2.37 E-02	2.59	up	4.40	3.03
Mus musculus sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 6 (Sult2a6), mRNA [NM_001081325]	A_55_P2172532	Sult2a6	sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member	NM_001081325	chr7:14811254-14807923	-	2.37 E-02	10.96	down	2.47	5.92

Figure 22 CONT.

[illegible]

Figure 22 CONT.

Mus musculus kallikrein related-peptidase 8 (Klk8), mRNA [NM_008940]	A_52_P21667 2	Klk8	kallikrein related-peptidase 8	NM_008940	chr7:51057455-51057514	+	2.55 E-02	2.08	down	2.85	3.90
lincRNA:chr17:15111725-15139800 reverse strand	A_30_P01028 531				chr17:15111661-1-15116552	-	2.55 E-02	2.55	up	5.16	3.81
	A_55_P20912 97				chr7:3511532-3511591	+	2.55 E-02	2.62	up	3.69	2.31
Mus musculus neuropeptide W (Npw), mRNA [NM_001093664]	A_55_P21248 71	Npw	neuropeptide W	NM_001099664	chr17:2479433-4-24794275	-	2.55 E-02	2.41	down	2.47	3.74
Mus musculus pyridoxal-dependent decarboxylase domain containing 1 (Pdxdc1), transcript variant 3, mRNA [NM_001039533]	A_55_P20373 68	Pdxdc1	pyridoxal-dependent decarboxylase domain containing 1	NM_001039533	chr16:1383420-6-13834147	-	2.56 E-02	3.08	down	7.39	9.02
lincRNA:chr4:145955175-145967425 reverse strand	A_30_P01030 498				chr4:14596093-8-145960879	-	2.56 E-02	3.41	up	5.12	3.35
lincRNA:chr5:105032961-105037248 forward strand	A_30_P01019 085				chr5:10503692-8-105036987	+	2.56 E-02	2.90	up	4.72	3.19
lincRNA:chr1:161992257-161992963 forward strand	A_30_P01026 161				chr1:16199272-6-161992785	+	2.56 E-02	3.19	up	6.72	5.05
lincRNA:chr5:121784419-121792814 forward strand	A_30_P01021 522				chr5:12178953-5-121789594	+	2.57 E-02	2.18	up	4.01	2.89
lincRNA:chr4:45079022-45096897 forward strand	A_30_P01028 964				chr4:45096452-45096511	+	2.57 E-02	2.21	down	1.94	3.08
PREDICTED: Mus musculus similar to immunoglobulin gamma 2A chain (LOC100048855), mRNA [XM_001472541]	A_55_P20210 89	Ighg	immunoglobulin heavy chain (gamma polypeptide)	XM_001472541			2.58 E-02	3.87	down	2.24	4.20
Mus musculus desmoglein 2 (Dsg2), mRNA [NM_007883]	A_52_P88091	Dsg2	desmoglein 2	NM_007883	chr18:2073762-8-20737687	+	2.59 E-02	2.86	down	5.51	7.02
Mus musculus solute carrier family 25 (mitochondrial phosphate carrier), member 25 (Slc25a25), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA [NM_146118]	A_51_P19317 3	Slc25a25	solute carrier family 25 (mitochondrial phosphate carrier), member 25	NM_146118	chr2:32270334-32270275	-	2.59 E-02	2.01	down	12.89	13.90
Mus musculus PR domain containing 16 (Prdm16), transcript variant 1, mRNA [NM_027504]	A_55_P19635 47	Prdm16	PR domain containing 16	NM_027504	chr4:15369031-3-153690254	-	2.60 E-02	2.05	up	4.30	3.26
Mus musculus BTB (POZ) domain containing 9 (Btbd9), transcript variant 2, mRNA [NM_172618]	A_55_P20405 39	Btbd9	BTB (POZ) domain containing 9	NM_172618	chr17:3065057-3-30650514	-	2.60 E-02	3.67	down	6.25	8.13

Figure 22 CONT.

Mus musculus WAP four-disulfide core domain 15B (Wfdc15b), transcript variant 1, mRNA [NM_138685]	A_55_P20059 84	Wfdc15b	WAP four-disulfide core domain 15B	NM_138685	chr2:16404026 4-164040205	-	2.60 E-02	2.02	up	8.80	7.79
Mus musculus demilune cell and parotid protein 2 (Dcpp2), mRNA [NM_001039238]	A_55_P21585 10	Dcpp2	demilune cell and parotid protein 2	NM_001039 238	chr17:2403747 4-24037533	+	2.61 E-02	2.06	down	2.42	3.46
Mus musculus predicted gene 4841 (Gm4841), mRNA [NM_001034859]	A_55_P20730 24	Gm4841	predicted gene 4841	NM_001034 859	chr18:6042804 2-60427983	-	2.62 E-02	3.43	down	3.95	5.72
atlastin GTPase 1 Gene [Source:MGI Symbol:Acc:MG1:1921241] [ENSMUST00000110560]	A_55_P21773 71			BC027570	chr12:7106938 6-71069445	+	2.63 E-02	2.95	up	6.56	5.00
lincRNA:chr1:139795171-139796306 forward strand	A_30_P01022 208				chr1:13979620 8-139796267	+	2.64 E-02	3.21	up	4.05	2.37
lincRNA:chr15:92174226-92201201 forward strand	A_30_P01020 587				chr15:9219702 4-92197083	+	2.67 E-02	2.06	up	6.99	5.95
Mus musculus chondroitin sulfate proteoglycan 5 (Cspg5), transcript variant 2, mRNA [NM_001166273]	A_55_P20467 44	Cspg5	chondroitin sulfate proteoglycan 5	NM_001166 273	chr9:11016496 2-110165021	+	2.68 E-02	2.10	down	8.17	9.24
Mus musculus lactotransferrin (Ltf), mRNA [NM_008522]	A_52_P15388	Ltf	lactotransferrin	NM_008522	chr9:11094287 3-110942932	+	2.68 E-02	2.81	up	4.07	2.58
lincRNA:chr12:5303823-5379098 forward strand	A_30_P01032 107				chr12:5371640 -5371699	+	2.69 E-02	2.23	up	4.07	2.92
Mus musculus calcyphosine-like (Capsl), mRNA [NM_029341]	A_51_P19620 7	Capsl	calcyphosine-like	NM_029341	chr15:9392463 -9392522	+	2.69 E-02	2.42	up	4.13	2.86
lincRNA:chr18:57104718-57105355 forward strand	A_30_P01032 711				chr18:5710521 2-57105271	+	2.72 E-02	2.22	down	2.21	3.36
PREDICTED: Mus musculus similar to Predicted gene, OTTMUSG00000007485 (LOC100039019), misc RNA [XR_030567]	A_55_P19811 97	Mup-ps12	major urinary protein, pseudogene 12	XR_030567	chr4:61116915 -61116974	+	2.72 E-02	2.24	up	15.96	14.80
Mus musculus NLR family, pyrin domain containing 2 (Nlrp2), mRNA [NM_177690]	A_55_P20422 47	Nlrp2	NLR family, pyrin domain containing 2	NM_177690	chr7:5250277- 5250218	-	2.74 E-02	3.71	down	2.37	4.26
Mus musculus RIKEN cDNA A530016L24 gene (A530016L24Rik), mRNA [NM_177039]	A_55_P21387 96	A530016L24 Rik	RIKEN cDNA A530016L24 gene	NM_177039	chr12:1137365 56-113736615	+	2.77 E-02	2.97	down	2.13	3.70
Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:Q8C8J2] [ENSMUST00000093985]	A_55_P19702 14			AK046933	chr3:12106712 8-121067187	+	2.78 E-02	2.95	down	2.33	3.89

Figure 22 CONT.

lincRNA:chr2:67834535-67834914 forward strand	A_30_P01026592					chr2:67834854-67834913	+	2.79 E-02	2.06	up	5.59	4.55
lincRNA:chr2:67834535-67834914 forward strand	A_30_P01021730					chr2:67834626-67834685	+	2.79 E-02	3.04	up	4.21	2.61
Putative uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:Q3UV43] [ENSMUST00000097824]	A_55_P2106608				AK137609	chr1:9951064-9951005	-	2.83 E-02	4.71	down	2.21	4.45
RIKEN cDNA A130023124 gene	A_52_P520341				AK088084	chr7:14142164-7-141421588	-	2.83 E-02	4.31	up	5.21	3.11
Gene [Source:MGI Symbol;Acc:MGI:2444285] [ENSMUST000000051742]												
RIKEN cDNA 2010109A12 gene	A_51_P413507	2010109A12 Rik	RIKEN cDNA 2010109A12 gene		XM_001473772	chr5:93637170-93642263	+	2.84 E-02	3.00	up	4.95	3.37
Gene [Source:MGI Symbol;Acc:MGI:1922860] [ENSMUST000000031330]	A_55_P2074694	Ephx4	epoxide hydrolase 4		NM_001001804	chr5:10785889-2-107858951	+	2.86 E-02	2.85	up	4.17	2.66
Mus musculus epoxide hydrolase 4 (Ephx4), mRNA [NM_001001804]	A_30_P01032390					chr5:105072936-105074001 forward strand	+	2.90 E-02	2.83	up	3.83	2.33
lincRNA:chr5:105072936-105074001 forward strand												
Mus musculus cholinergic receptor, nicotinic, alpha polypeptide 4 (Chrna4), mRNA [NM_015730]	A_52_P350750	Chrna4	cholinergic receptor, nicotinic, alpha polypeptide 4		NM_015730	chr2:18075736-8-180757309	-	2.91 E-02	3.67	down	1.55	3.43
Mus musculus bromodomain adjacent to zinc finger domain 1A (Baz1a), mRNA [NM_013815]	A_66_P139196	Baz1a	bromodomain adjacent to zinc finger domain 1A		NM_013815	chr12:5599405-7-55993998	-	2.91 E-02	2.11	down	5.76	6.84
Mus musculus crystallin, gamma N (Crygn), mRNA [NM_153076]	A_55_P1975295	Crygn	crystallin, gamma N		NM_153076	chr5:24261928-24261869	-	2.91 E-02	2.74	down	6.76	8.22
PREDICTED: Mus musculus similar to secretory protein (LOC620515), mRNA [XM_884904]	A_55_P2114187	LOC620515	similar to Chitinase 3-like protein 3 precursor (Secretory protein Ym1) (Eosinophil chemotactic cytokine) (ECF-L)		XM_884904	chr3:10610067-3-106100732	+	2.91 E-02	2.95	up	4.31	2.75
Mus musculus serum amyloid A 2 (Saa2), mRNA [NM_011314]	A_55_P1994807	Saa2	serum amyloid A 2		NM_011314	chr7:54009622-54009681	+	2.94 E-02	3.76	up	15.25	13.34
Mus musculus forkhead box J1 (Foxj1), mRNA [NM_008240]	A_51_P456870	Foxj1	forkhead box J1		NM_008240	chr11:1161922-73-116192214	-	2.94 E-02	2.15	up	4.20	3.10
Mus musculus adenylate kinase 7 (Ak7), mRNA [NM_030187]	A_52_P246703	Ak7	adenylate kinase 7		NM_030187	chr12:1070049-24-107006761	+	2.94 E-02	2.64	up	4.81	3.41
lincRNA:chr9:58301467-58323783 reverse strand	A_30_P01029520					chr9:58303114-58303055	-	2.95 E-02	2.69	up	4.12	2.69

Figure 22 CONT.

lincRNA:chr1:84947500-85045150 reverse strand	A_30_P01026 103					chr1:84959839-84959839	-	2.99 E-02	2.01	down	2.47	3.48
lincRNA:chr12:110944403-110988678 reverse strand	A_30_P01020 302					chr12:110983302-110983243	-	3.05 E-02	2.14	down	2.26	3.36
pregnancy specific glycoprotein 16 Gene [Source:MGI (curated);Acc:MGI:1347249] [ENSMUST00000071399]	A_55_P19724 90					chr7:17718741-17718799	+	3.05 E-02	5.53	up	10.35	7.88
Mus musculus glutamate receptor, ionotropic, AMPA4 (alpha 4) (Gria4), transcript variant 2, mRNA [NM_001113180]	A_55_P19877 25	Gria4	glutamate receptor, ionotropic, AMPA4 (alpha 4)	NM_001113180		chr9:4417955-4417896	-	3.06 E-02	2.06	up	4.14	3.10
lincRNA:chr5:23195569-23218551 reverse strand	A_30_P01026 028					chr5:23204973-23204914	-	3.06 E-02	5.99	up	8.75	6.17
Mus musculus prostaglandin D2 synthase (brain) (Ptgds) mRNA [NM_008963]	A_55_P21009 28	Ptgds	prostaglandin D2 synthase (brain)	NM_008963		chr2:25322291-25322232	-	3.07 E-02	2.59	down	6.34	7.71
DENN/MADD domain containing 1B Gene [Source:MGI (curated);Acc:MGI:2447812] [ENSMUST00000070283]	A_55_P21816 78					chr1:14096014-2-140960201	+	3.09 E-02	2.08	up	2.92	1.86
Mus musculus predicted gene 15698 (Gm15698), non-coding RNA [NR_003564]	A_55_P20613 71	Gm15698	transcription elongation factor B (SIII), polypeptide 2 pseudogene	NR_003564		chr11:8882608-2-88826023	-	3.09 E-02	10.23	up	8.33	4.98
PREDICTED: Mus musculus similar to Cdv3 protein (LOC433790), mRNA [XM_485486]	A_55_P20761 91	Gm13241	carnitine deficiency-associated gene expressed in ventricle 3 pseudogene	XM_485486		chr4:14571850-5-145718446	-	3.10 E-02	5.54	up	5.03	2.56
lincRNA:chr16:30936314-30954449 forward strand	A_30_P01025 308					chr16:3094812-8-30948187	+	3.12 E-02	2.24	down	2.27	3.44
lincRNA:chr5:23195569-23218551 reverse strand	A_30_P01019 583					chr5:23204806-23204747	-	3.12 E-02	6.31	up	10.28	7.62
lincRNA:chr13:66888650-66902600 forward strand	A_30_P01018 984					chr13:6689259-5-66892654	+	3.12 E-02	2.18	down	1.73	2.86
Mus musculus arachidonate lipoxigenase 3 (Aloxe3), mRNA [NM_011786]	A_55_P20235 23	Aloxe3	arachidonate lipoxigenase 3	NM_011786		chr11:6896255-7-68962616	+	3.13 E-02	3.19	up	3.89	2.21
AV256778 RIKEN full-length enriched, adult male testis (DH10B) Mus musculus cDNA clone 4921531B15 3'. [AV256778]	A_52_P10168 36					chr12:5883872-9-58838785	+	3.13 E-02	2.69	down	5.32	6.75

Figure 22 CONT.

Mus musculus growth arrest specific 7 (Gas7), transcript variant 2, mRNA [NM_001109657]	A_55_P20389 87	Gas7	growth arrest specific 7	NM_001109657	chr11:67502434-67502493	+	3.14 E-02	2.17	down	5.50	6.62
PREDICTED: Mus musculus hypothetical protein LOC627777 (LOC627777), mRNA [XM_001479684]	A_55_P20656 51	Trav17	T cell receptor alpha variable 17	XM_001479684	chr14:54431211-54431270	+	3.15 E-02	2.43	up	3.90	2.62
Mus musculus PR domain containing 2, with ZNF domain (Prdm2), mRNA [NM_001081355]	A_65_P10029	Prdm2	PR domain containing 2, with ZNF domain	NM_001081355	chr4:142698071-142698012	-	3.16 E-02	2.34	down	2.40	3.63
major urinary protein, pseudogene 7 Pseudogene [Source:MGI (curated);Acc:MGI:3651245] [ENSMUST00000107492]	A_55_P20333 26			XR_030572	chr4:60693053-60693112	+	3.16 E-02	2.87	up	14.25	12.73
PREDICTED: Mus musculus hypothetical protein LOC100041156 (LOC100041156), mRNA [XM_001476722]	A_52_P22223 0			XM_001476722	chr8:93412002-93412061	+	3.17 E-02	2.24	up	13.41	12.24
Mus musculus RIKEN cDNA 1700061J05 gene (1700061J05Rik), transcript variant 1, mRNA [NM_001163612]	A_51_P26156 0	1700061J05 Rik	RIKEN cDNA 1700061J05 gene	NM_001163612	chr15:78209193-78209951	-	3.17 E-02	2.12	down	1.85	2.93
Mus musculus adult male hypothalamus cDNA, RIKEN full-length enriched library, clone:A230005M16 product:unclassified, full insert sequence. [AK038413]	A_55_P23591 10	A230005M16 Rik	RIKEN cDNA A230005M16 gene	AK038413	chr2:25561267-25561208	-	3.21 E-02	2.49	up	4.63	3.32
lincRNA:chr2:67836003-67836461 forward strand	A_30_P01030 934				chr2:67836138-67836197	+	3.21 E-02	3.03	up	8.83	7.24
Mus musculus uncoupling protein 3 (mitochondrial, proton carrier) (Ucp3), nuclear gene encoding mitochondrial protein, mRNA [NM_009464]	A_66_P10824 7	Ucp3	uncoupling protein 3 (mitochondrial, proton carrier)	NM_009464	chr7:107630399-107630458	+	3.23 E-02	2.27	down	6.10	7.28
lincRNA:chr1:74084292-74138492 reverse strand	A_30_P01031 300				chr1:74104066-74104007	-	3.23 E-02	2.65	up	6.75	5.34
Mus musculus cDNA sequence BC020535 (BC020535), mRNA [NM_145536]	A_55_P19693 56	BC020535	cDNA sequence BC020535	NM_145536	chr2:152907310-152907369	+	3.23 E-02	2.71	down	5.94	7.38
Mus musculus solute carrier family 16 (monocarboxylic acid transporters), member 5 (Slc16a5), mRNA [NM_001080934]	A_55_P20240 54	Slc16a5	solute carrier family 16 (monocarboxylic acid transporters),	NM_001080934	chr11:115324039-115324098	+	3.27 E-02	2.29	up	3.87	2.68

Figure 22 CONT.

[illegible]

Figure 22 CONT.

Mus musculus sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 1 (Sult2a1), mRNA [NM_00111296]	A_55_P19525 17	Sult2a1	sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 1	NM_001111296	chr7:14381661-14381602	-	3.46 E-02	5.07	down	2.20	4.54
PREDICTED: Mus musculus similar to ring finger and WD repeat domain 2 isoform d24 (LOC100045235), mRNA [XM_001473908]	A_55_P21445 32	LOC100045235	similar to ring finger and WD repeat domain 2 isoform d24	XM_001473908	chr15:14075202-14075261	+	3.47 E-02	2.40	up	4.03	2.77
Mus musculus calcium and integrin binding family member 3 (Cib3), mRNA [NM_001080812]	A_55_P20784 94	Cib3	calcium and integrin binding family member 3	NM_001080812	chr8:74729522-74729463	-	3.53 E-02	4.16	up	11.93	9.87
Mus musculus alanine-glyoxylate aminotransferase 2-like 1 (Agxt2l1), transcript variant 1, mRNA [NM_027907]	A_51_P39161 6	Agxt2l1	alanine-glyoxylate aminotransferase 2-like 1	NM_027907	chr3:130338517-130338576	+	3.56 E-02	2.92	down	12.54	14.08
lincRNA:chr13:67932042-67944229 forward strand	A_30_P01020 057				chr13:67944127-8-67944127	+	3.56 E-02	2.19	down	2.32	3.45
Mus musculus S100 calcium binding protein A4 (S100a4), mRNA [NM_011311]	A_55_P19834 48	S100a4	S100 calcium binding protein A4	NM_011311	chr3:90409908-90409967	+	3.57 E-02	2.21	up	8.24	7.09
PREDICTED: Mus musculus RIKEN cDNA C030014O09 gene (C030014O09Rik), mRNA [XM_001480201]	A_52_P10194 98	C030014O09Rik	RIKEN cDNA C030014O09 gene	XM_001480201	chr2:130666463-130666522	+	3.59 E-02	2.32	up	4.01	2.79
lincRNA:chr2:67836003-67836461 forward strand	A_30_P01024 596				chr2:67836374-67836433	+	3.60 E-02	2.79	up	10.74	9.26
Mus musculus 7 days embryo whole body cDNA, RIKEN full-length enriched library, clone:C430017C20 product:hypothetical protein, full insert sequence. [AK082914]	A_55_P22004 61	C430017C20Rik	RIKEN cDNA C430017C20 gene	AK082914	chr2:172767975-172768034	+	3.65 E-02	2.26	down	1.89	3.07
Mus musculus cDNA sequence BC049715 (BC049715), mRNA [NM_178776]	A_55_P21345 91	BC049715	cDNA sequence BC049715	NM_178776	chr6:136788994-136789053	+	3.65 E-02	2.52	up	2.95	1.62
lincRNA:chr2:174943325-174966675 forward strand	A_30_P01025 581				chr2:174955477-174955536	+	3.66 E-02	2.01	down	2.50	3.50
lincRNA:chr11:49932129-49938154 reverse strand	A_30_P01018 562				chr11:499333964-49933905	-	3.68 E-02	2.09	down	2.60	3.66

Figure 22 CONT.

Mus musculus RIKEN cDNA 2210408121 gene (2210408121Rik), transcript variant 2, mRNA [NM_001081353]	A_55_P21621 85	2210408121R ik	RIKEN cDNA 2210408121 gene	NM_001081 353	chr13:7733289 0-77332949	+	3.69 E-02	2.85	up	3.55	2.04
Mus musculus sortilin-related VPS10 domain containing receptor 2 (Sorcs2), mRNA [NM_030889]	A_51_P23028 7	Sorcs2	sortilin-related VPS10 domain containing receptor 2	NM_030889	chr5:36360149 -36360091	-	3.70 E-02	2.27	up	4.15	2.96
RIKEN cDNA C530030P08 gene Gene [Source:MG1 Symbol;Acc:MG1:2144664] [ENSMUST00000101381]	A_55_P20269 50			AK083005	chr11:3262590 4-32625963	+	3.70 E-02	3.04	down	2.24	3.85
lincRNA:chr8:27259077-27268252 forward strand	A_30_P01029 772				chr8:27265910 -27265969	+	3.70 E-02	2.68	down	1.72	3.15
lincRNA:chr1:42689799-42749799 reverse strand	A_30_P01030 648				chr1:42705178 -42705119	-	3.74 E-02	2.35	down	2.59	3.82
Mus musculus transmembrane channel-like gene family 7 (Tmc7), mRNA [NM_172476]	A_52_P29142 0	Tmc7	transmembrane channel-like gene family 7	NM_172476	chr7:12569090 7-125690848	-	3.74 E-02	3.30	up	5.44	3.72
lincRNA:chr7:140081171- 140123058 reverse strand	A_30_P01033 597				chr7:14008138 4-140081325	-	3.75 E-02	2.08	down	2.26	3.31
Mus musculus phospholipase A2, activating protein (Pla2), mRNA [NM_172695]	A_55_P20802 25	Pla2	phospholipase A2, activating protein	NM_172695	chr4:94231918 -94231859	-	3.76 E-02	2.19	down	2.36	3.50
Mus musculus transmembrane protein 45a (Tmem45a), mRNA [NM_019631]	A_51_P28887 6	Tmem45a	transmembrane protein 45a	NM_019631	chr16:5680672 7-56806668	-	3.77 E-02	2.60	up	7.27	5.89
lincRNA:chr17:22815742-22828592 reverse strand	A_30_P01022 126				chr17:2282477 4-22824715	-	3.77 E-02	2.08	up	3.81	2.75
immunoglobulin heavy chain 6 (heavy chain of IgM) Gene [Source:MG1 Symbol;Acc:MG1:96448] [ENSMUST00000103426]	A_51_P48542 1				chr12:1146596 89-114659630	-	3.78 E-02	2.56	down	6.67	8.02
Mus musculus RIKEN cDNA 6330416L07 gene (6330416L07Rik), mRNA [NM_176962]	A_55_P19725 10	6330416L07 Rik	RIKEN cDNA 6330416L07 gene	NM_176962	chr17:2247545 5-22475396	-	3.79 E-02	2.54	up	4.60	3.26
Mus musculus RIKEN cDNA D930016D06 gene (D930016D06Rik), non-coding RNA [NR_030673]	A_55_P21367 37	D930016D06 Rik	RIKEN cDNA D930016D06 gene	NR_030673	chr5:10498088 7-104980946	+	3.80 E-02	5.62	down	3.60	6.09
lincRNA:chr5:77239012-77271614 forward strand	A_30_P01026 474				chr5:77271441 -77271500	+	3.85 E-02	3.15	up	6.45	4.79

Figure 22 CONT.

Mus musculus mohawk homeobox (Mkx), mRNA [NM_177595]	A_51_P519008	Mkx	mohawk homeobox	NM_177595	chr18:6935242-6935183	-	3.85 E-02	2.50	down	5.36	6.69
lincRNA:chr11:6796679-6797670 forward strand	A_30_P01026121				chr11:6796749-6796808	+	3.86 E-02	2.42	down	2.19	3.47
Mus musculus transmembrane channel-like gene family 5 (Tmc5), transcript variant 1, mRNA [NM_001105252]	A_55_P2047163	Tmc5	transmembrane channel-like gene family 5	NM_001105252	chr7:12581834-7-125818406	+	3.89 E-02	3.49	up	9.85	8.04
Mus musculus defensin, alpha 1 (Defa1), mRNA [NM_010031]	A_55_P1999110	Defa1	defensin, alpha 1	NM_010031			3.89 E-02	2.28	up	3.45	2.26
Mus musculus matrix metalloproteinase 27 (Mmp27), mRNA [NM_001030289]	A_55_P2046958	Mmp27	matrix metalloproteinase 27	NM_001030289	chr9:7573412-7573471	+	3.90 E-02	2.18	down	1.42	2.55
Mus musculus mastermind like 3 (Drosophila) (Maml3), mRNA [NM_001004176]	A_55_P2147747	Maml3	mastermind like 3 (Drosophila)	NM_001004176	chr3:51908161-51908102	-	3.91 E-02	2.55	down	2.42	3.77
Mus musculus secreted phosphoprotein 1 (Spp1), mRNA [NM_009263]	A_55_P2085142	Spp1	secreted phosphoprotein 1	NM_009263	chr5:10486850-2-104868561	+	3.91 E-02	4.37	up	11.66	9.53
Mus musculus cyclin-dependent kinase inhibitor 2A (Cdkn2a), transcript variant 1, mRNA [NM_009877]	A_55_P2008437	Cdkn2a	cyclin-dependent kinase inhibitor 2A	NM_009877	chr4:88920464-88920405	-	3.91 E-02	3.50	up	6.52	4.71
Mus musculus RIKEN cDNA 4930578106 gene (4930578106Rik), mRNA [NM_026359]	A_52_P552560	4930578106Rik	RIKEN cDNA 4930578106 gene	NM_026359	chr14:6459010-5-64590046	-	3.93 E-02	4.13	down	2.00	4.04
Mus musculus regulating synaptic membrane exocytosis 4 (Rims4), mRNA [NM_183023]	A_55_P2025883	Rims4	regulating synaptic membrane exocytosis 4	NM_183023	chr2:16369066-5-163690606	-	3.96 E-02	2.03	up	3.33	2.31
Mus musculus polymerase (RNA) III (DNA directed) polypeptide F (Polr3f), mRNA [NM_029763]	A_55_P2084319	Polr3f	polymerase (RNA) III (DNA directed) polypeptide F	NM_029763	chr2:14435890-1-144358960	+	3.97 E-02	2.03	down	3.81	4.83
lincRNA:chr4:88745770-88774130 reverse strand	A_30_P01020035				chr4:88745952-88745893	-	4.02 E-02	2.29	down	5.32	6.51
Mus musculus F-box and leucine-rich repeat protein 13 (Fbxl13), mRNA [NM_177076]	A_55_P2078695	Fbxl13	F-box and leucine-rich repeat protein 13	NM_177076	chr5:20989728-20989669	-	4.02 E-02	2.62	up	2.93	1.54
lincRNA:chr1:100481057-100494471 forward strand	A_30_P01027292				chr1:10049391-1-100493970	+	4.04 E-02	3.03	up	3.24	1.64

Figure 22 CONT.

Mus musculus 2 days pregnant adult female oviduct cDNA, RIKEN full-length enriched library, clone:E230006M18 product:unclassified, full insert sequence. [AK053951]	A_55_P23402 06	E230006M18 Rik	RIKEN cDNA E230006M18 gene	AK053951	chr7:10535495 9-105354900	-	4.05 E-02	2.02	down	7.78	8.79
lincRNA:chr17:3198868-3394643 reverse strand	A_30_P01031 236				chr17:3229403 -3229344	-	4.07 E-02	2.01	down	2.12	3.13
lincRNA:chr15:92174262-92196074 forward strand	A_30_P01017 453				chr15:9219596 0-92196019	+	4.09 E-02	2.09	down	2.09	3.16
Mus musculus ES cells cDNA, RIKEN full-length enriched library, clone:2410071D21 product:similar to LRRGT00034 [Rattus norvegicus], full insert sequence. [AK131952]	A_55_P23356 48	D630045M09 Rik	RIKEN cDNA D630045M09 gene	AK131952	chr13:7348234 0-73482281	-	4.10 E-02	2.48	down	2.04	3.35
lincRNA:chr11:95097483-95097918 forward strand	A_30_P01027 918				chr11:9509779 7-95097856	+	4.12 E-02	2.05	up	3.52	2.48
Mus musculus protocadherin 17 (Pcdh17), mRNA [NM_001013753]	A_52_P11103 1	Pcdh17	protocadherin 17	NM_001013 753	chr14:8493386 1-84933920	+	4.13 E-02	3.98	down	2.43	4.42
lincRNA:chr2:67836003-67836461 forward strand	A_30_P01030 435				chr2:67836259 -67836318	+	4.15 E-02	2.84	up	10.76	9.25
PREDICTED: Mus musculus RIKEN cDNA B230317F23 gene (B230317F23Rik), misc RNA [XR_035255]	A_55_P20866 46	B230317F23 Rik	RIKEN cDNA B230317F23 gene	XR_035255	chr8:63465021 -63465080	+	4.21 E-02	2.20	up	2.90	1.76
Mus musculus uncoupling protein 2 (mitochondrial, proton carrier) (Ucp2), nuclear gene encoding mitochondrial protein, mRNA [NM_011671]	A_51_P29710 5	Ucp2	uncoupling protein 2 (mitochondrial, proton carrier)	NM_011671	chr7:10764780 9-107647868	+	4.22 E-02	2.18	down	6.61	7.74
lincRNA:chr3:86827945-86886695 forward strand	A_30_P01029 027				chr3:86885670 -86885729	+	4.30 E-02	2.00	down	2.04	3.05
PREDICTED: Mus musculus predicted gene, EG624023 (EG624023), mRNA [XM_888549]	A_55_P20185 94	Gm6466	predicted gene 6466	XM_888549	chrX:1443744 74-144374415	-	4.33 E-02	3.20	up	6.66	4.99
lincRNA:chr1:95871175-95886625 forward strand	A_30_P01029 893				chr1:95885036 -95885095	+	4.39 E-02	2.12	down	7.29	8.37
v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian) Gene [Source:MG] (curated);Acc:MG1:104771] [ENSMUST00000121473]	A_55_P21198 92	Erb4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)		chr1:68078864 -68078805	-	4.42 E-02	5.45	up	7.73	5.28

Figure 22 CONT.

PREDICTED: Mus musculus hypothetical protein LOC100041181 (LOC100041181), mRNA [XM_001475982]	A_55_P1999958	Gm3185	predicted gene 3185	XM_001475982	chrX:153969374-153969433	+	4.43 E-02	8.75	up	6.39	3.26
lincRNA:chr16:64820905-64822069 reverse strand	A_30_P01032771				chr16:64821591-64821532	-	4.43 E-02	2.10	down	3.19	4.26
Mus musculus ataxin 1 (Atxn1), mRNA [NM_009124]	A_52_P14453	Atxn1	ataxin 1	NM_009124	chr13:45651048-45650989	-	4.45 E-02	2.03	down	5.43	6.44
Mus musculus aquaporin 11 (Aqp11), mRNA [NM_175105]	A_55_P1995507	Aqp11	aquaporin 11	NM_175105	chr7:104874948-104874889	-	4.45 E-02	2.01	up	10.98	9.98
Mus musculus secreted frizzled-related protein 2 (Sfrp2), mRNA [NM_009144]	A_51_P520849	Sfrp2	secreted frizzled-related protein 2	NM_009144	chr3:83577864-83577923	+	4.51 E-02	2.30	up	3.52	2.32
lincRNA:chr5:22056218-22206995 forward strand	A_30_P01024465				chr5:22200539-22200598	+	4.55 E-02	2.43	down	1.59	2.87
lincRNA:chr8:93337794-93348869 forward strand	A_30_P01017524				chr8:93340828-93340887	+	4.56 E-02	2.18	up	3.29	2.16
zinc finger protein 64 Gene [Source:MGI (curated);Acc:MGI:107342] [ENSMUST00000029064]	A_52_P626780			BC004695	chr2:168719181-168719122	-	4.58 E-02	2.22	down	2.92	4.07
lincRNA:chr1:75075500-75102200 forward strand	A_30_P01021485				chr1:75093096-75093155	+	4.59 E-02	2.05	down	2.77	3.80
Mus musculus RIKEN cDNA 1810037117 gene (1810037117Rik), mRNA [NM_024461]	A_55_P2132207	1810037117Rik	RIKEN cDNA 1810037117 gene	NM_024461	chr3:122628921-122628980	+	4.65 E-02	2.01	up	10.48	9.47
lincRNA:chr1:74084292-74138492 reverse strand	A_30_P01030180				chr1:74103833-74103774	-	4.67 E-02	2.26	up	7.53	6.36
Mus musculus glucosaminyl (N-acetyl) transferase 1, core 2 (Gcnt1), transcript variant 1, mRNA [NM_173442]	A_52_P21550	Gcnt1	glucosaminyl (N-acetyl) transferase 1, core 2	NM_173442	chr19:17400846-17400787	-	4.67 E-02	2.37	down	3.33	4.57
Mus musculus RIKEN cDNA 0610007P08 gene (0610007P08Rik), transcript variant 2, mRNA [NM_023507]	A_52_P194464	0610007P08Rik	RIKEN cDNA 0610007P08 gene	NM_023507	chr13:63926129-63926188	+	4.68 E-02	2.19	down	2.50	3.63
Mus musculus myosin, light polypeptide 7, regulatory (Myl7), mRNA [NM_022879]	A_65_P10195	Myl7	myosin, light polypeptide 7, regulatory	NM_022879	chr11:5798435-5798186	-	4.77 E-02	2.05	down	4.32	5.36
Mus musculus solute carrier family 13 (sodium-dependent citrate transporter), member 5 (Slc13a5), mRNA [NM_001004148]	A_55_P1963508	Slc13a5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	NM_001004148	chr11:72055556-72055497	-	4.78 E-02	2.67	up	7.90	6.48

Figure 22 CONT.

Mus musculus outer dense fiber of sperm tails 3-like 1 (Odf31), mRNA [NM_198673]	A_52_P23817_5	Odf31	outer dense fiber of sperm tails 3-like 1	NM_198673	chr9:56698641-56698682	-	4.79 E-02	2.35	up	2.82	1.59
PREDICTED: Mus musculus similar to cyclic nucleotide gated channel beta 1 (LOC100042147), mRNA [XM_001477628]	A_55_P20896_25	Gm3694	predicted gene 3694	XM_001477628	chr14:15478353-15478294	-	4.79 E-02	2.51	down	1.63	2.96
Mus musculus gametocyte specific factor 1 (Gtsf1), mRNA [NM_028797]	A_51_P48540_5	Gtsf1	gametocyte specific factor 1	NM_028797	chr15:103235246-103232937	-	4.83 E-02	2.89	down	1.50	3.03
Mus musculus cathelicidin antimicrobial peptide (Camp), mRNA [NM_009921]	A_55_P20396_99	Camp	cathelicidin antimicrobial peptide	NM_009921	chr9:109749970-109749911	-	4.83 E-02	2.42	up	7.96	6.68
lincRNA:chr7:11526943-11545093 forward strand	A_30_P01022_049				chr7:111542778-111542837	+	4.84 E-02	2.34	down	3.05	4.28
Mus musculus predicted gene 7120 (Gm7120), transcript variant 2, mRNA [NM_001177666]	A_55_P21839_14	Gm7120	predicted gene 7120	NM_001177666	chr13:120284253-120284312	+	4.86 E-02	2.38	up	6.64	5.39
PREDICTED: Mus musculus similar to ubiquitin specific protease 44 (LOC100048073), mRNA [XM_001480062]	A_55_P19523_04	LOC100048073	similar to ubiquitin specific protease 44	XM_001480062	chr10:93320736-93320795	+	4.94 E-02	2.13	down	2.65	3.74
lincRNA:chr8:18587198-18592948 reverse strand	A_30_P01033_351				chr8:18589396-18589337	-	4.95 E-02	2.22	up	4.44	3.28
PREDICTED: Mus musculus predicted gene, EG620627 (EG620627), mRNA [XM_885022]	A_55_P19892_48	Gm6168	sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 1 pseudogene	XM_885022	chr7:14432715-14432774	+	4.96 E-02	3.33	down	2.97	4.70
lincRNA:chr1:173250734-173259306 reverse strand	A_30_P01033_526				chr1:173256466-173256407	-	5.00 E-02	2.06	down	1.96	3.01

Figure 22 CONT.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/62853

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/11; C07H 21/04 (2013.01)

USPC - 514/44A; 536/24.5

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC- 514/44A; 536/24.5

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

USPC- 514/44R

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

PubWEST(PGPB,USPT,USOC,EPAB,JPAB); Google/Scholar: MiR-221, liver cancer, Hepatocellular carcinoma, mesenchymal tissue sarcoma, Hepatoblastoma, cholangiocarcinoma, bile duct cancer, Angiosarcoma, hemangiosarcoma, antisense
GenCore 6.3: SEQ ID NO: 5, 6

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Park, et al. miR-221 silencing blocks hepatocellular carcinoma and promotes survival. Cancer Res. Epub 18 October 2011, 71(24):7608-7616; Abstract, pg 7613, col 1; pg 7608, col 2; pg 7614, Fig 6 and its legendSupplementary table S1, Supplementary Figure S2 and its legend	1-13 and 50-51
A	Pineau, et al. miR-221 overexpression contributes to liver tumorigenesis. Proc Natl Acad Sci USA 2010, 107(1):264-269	1-13 and 50-51
A	Fomari, et al. MiR-221 control CDKN1C/p57 and CDKN1b/p27 expression in human hepatocellular carcinoma. Oncogene 2008, 27(43):5651-5661	1-13 and 50-51
A	Li, et al. Expression of serum miR-221 in human hepatocellular carcinoma and its prognostic significance. Biochem Biophys Res Commun. March 2011, 406(1):70-73	1-13 and 50-51
A	US 2011/0003704 A1 (Skog, et al.) 06 January 2011 (06.01.2011) claims 1, 3, 15, 16, 44, 45	1-13 and 50-51
A	US 2008/0306006 A1 (Croce, et al.) 11 December 2008 (11.12.2008) Abstract, para [0015], [0045]-[0047], [0133], [0143], TABLE 15; claims 1, 3, 9	1-13 and 50-51
A	US 2010/0197770 A1 (Wang, et al.) 05 August 2010 (05.08.2010) Abstract, para [0113], Table 1,	1-13 and 50-51
A	US 2010/0234241 A1 (Croce, et al.) 16 September 2010 (16.09.2010) para [0050], TABLE 2, 8, 10, 19, 20, 22, 23	1-13 and 50-51

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

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

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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

23 February 2013 (23.02.2013)

Date of mailing of the international search report

14 MAR 2013

Name and mailing address of the ISA/US

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

Facsimile No. 571-273-3201

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/62853

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

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

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

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

3. ☒ Claims Nos.: 14-16, 40-44, 49
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I: claims 1-13 and 50-52, drawn to a method for suppressing a liver cancer, comprising: contacting a liver cell with at least one antisense miR-221 oligonucleotide, thereby suppressing the liver cancer.

[NOTE: Claim 52 was excluded from the search, because it is drawn to a non-elected subject matter.]

Group II: claims 17-20, drawn to a pharmaceutical formulation comprising at least one anti-miR-221 oligonucleotide.

Group III: claims 21-35, drawn to a transgenic mouse whose genome comprises a nucleic acid construct capable of overexpressing at least one miR-221 gene in liver cells.

***** See Supplemental Sheet to continue *****

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-13 and 50-51

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

***** Supplemental Sheet *****

In Continuation of Box III. Observations where unity of invention is lacking:

Group IV: claims 36-39, 45-48, drawn to a method of determining whether a test agent affects a liver condition in a subject by
 a. administering a test agent to a transgenic mouse of claim 21; and
 b. after the agent has been administered to the transgenic mouse, comparing one or more symptoms and/or indications of a liver condition in the transgenic animal to those of a control animal of the same genotype, wherein the control animal has not been administered the agent,
 wherein a difference in the detectability and/or rate of appearance of the one or more symptoms and/or indications of the liver condition in the transgenic animal, relative to the control animal, is indicative of the test agent affecting the liver condition.

Group V: claims 53-55 and 57-58, drawn to a method of diagnosing whether a subject has, or is at risk for developing, hepatocellular cancer (HCC), comprising measuring the level of at least one miR-221 and miR-21 gene products in a test sample from said subject, wherein an increase in the level of the miR-221 and miR-21 gene products in the test sample, relative to the level of a corresponding miR-221 and miR-21 gene products in a control sample, is indicative of the subject either having, or being at risk for developing, HCC, and a kit for performing said method.

Group VI: claim 56, drawn to a method for increasing expression of Cdkn1b/p27 protein levels in a cell, comprising contacting the cell with an antisense miR-221 oligonucleotide.

The inventions listed as Groups I-VI do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions of Groups I and III through VI do not include the inventive concept of a pharmaceutical formulation comprising at least one anti-miR-221 oligonucleotide, as required by Group II.

The inventions of Groups I, II, V, and VI do not include the inventive concept of a transgenic mouse whose genome comprises a nucleic acid construct capable of overexpressing at least one miR-221 gene in liver cells, as required by Groups III-IV.

The inventions of Groups I-V do not include the inventive concept of a method for increasing expression of Cdkn1b/p27 protein levels in a cell, comprising contacting the cell with an antisense miR-221 oligonucleotide, as required by Group VI.

The inventions of Groups I-IV and VI do not include the inventive concept of a method of diagnosing whether a subject has, or is at risk for developing, HCC, comprising measuring the level of at least one miR-221 and miR-21 gene products in a test sample from said subject, wherein an increase in the level of the miR-221 and miR-21 gene products in the test sample, relative to the level of a corresponding miR-221 and miR-21 gene products in a control sample, is indicative of the subject either having, or being at risk for developing, HCC, as required by Group V.

The inventions of Groups I and VI share the technical feature of a method comprising contacting the cell with an antisense miR-221 oligonucleotide. However, this shared technical feature does not represent a contribution over prior art as being anticipated by an article titled "miR-221 silencing blocks hepatocellular carcinoma and promotes survival" by Park, et al. (Cancer Res. Epub 18 October 2011, 71(24):7608-7616) (hereinafter "Park") that discloses transfecting a cell (pg 7609, 1st col, last para, "Antisense oligonucleotide transfection. The cells (1,200 cells per well) were plated 1 day before transfection and the oligonucleotides were transfected using Lipofectamine 2000 and Opti-MEM medium... Transfection efficiency was measured...") with an antisense miR-221 oligonucleotide (pg 7609, 1st col, 2nd from the bottom para, "Oligonucleotides miR-221 antisense and scrambled control oligonucleotides were synthesized... Oligonucleotides contained the natural nucleotides and phosphodiester bond or were chemically modified... (idT; Supplementary Table S1)"). As said method was known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

The inventions of Groups III-IV share the technical feature of a transgenic mouse whose genome comprises a nucleic acid construct capable of overexpressing at least one miR-221 gene in liver cells of claim 21. However, this shared technical feature does not represent a contribution over prior art as being anticipated by Park (pg 7611, col 2, "Hepatic accumulation and activity of chol-anti-miR-221 in normal and tumor-bearing mice. C57BL/6 mice were injected via the tail vein with... the dose to 30 mg/kg. C57BL/6 mice injected with 3 daily doses of 30 mg/kg via the tail vein were sacrificed on days 4 and 7 and the amount of chol-anti-miR-221 and endogenous miR-221 was assayed in whole liver. Both qPCR and Northern blotting showed that chol-anti-miR-221 significantly reduced the levels of endogenous miR-221 (Fig. 2A and B). The reduction in endogenous miR-221 correlated with increased accumulation of the chol-anti-miR-221 in the liver (Fig. 2A and C). Endogenous miR-221 was reduced by 80% and 90% at days 4 and 7, respectively (Fig. 2A)"). As said transgenic mouse was known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

In addition, a method of claim 1 does not represent a contribution over prior art as being anticipated by Park that discloses a method for suppressing a liver cancer, comprising: contacting a liver cell with at least one antisense miR-221 oligonucleotide, thereby suppressing the liver cancer (Abstract, "we conducted a preclinical investigation of the therapeutic efficacy of oligonucleotides directed against the oncogenic microRNA miR-221, which has been implicated in HCC. Of 9 chemistries evaluated, we determined that a 2'-O-methyl phosphorothioate-modified anti-miR-221 oligonucleotide was most effective at reducing proliferation in vitro. A cholesterol-modified isoform of anti-miR-221 (cholanti-miR-221) exhibited improved pharmacokinetics and liver tissue distribution compared with unmodified oligonucleotide. Chol-anti-miR-221 significantly reduced miR-221 levels in liver within a week of intravenous administration and in situ hybridization studies confirmed accumulation of the oligonucleotide in tumor cells in vivo. Within the same period, chol-anti-miR-221 reduced tumor cell proliferation and increased markers of apoptosis and cell-cycle arrest, elevating the tumor doubling time and increasing mouse survival. Taken together, our findings offer a preclinical proof of efficacy for chol-anti-miR-221 in a valid orthotopic mouse model of HCC, suggesting that this targeted agent could benefit treatment for patients with advanced HCC").

Groups I-VI therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.