Title: SYSTEMS AND METHODS FOR DIAGNOSIS, PROGNOSIS, AND TREATMENT OF CANCER

Abstract: A method and system for diagnosis treating a subject having cancer is provided based on the presence of miR-33b in a biological sample from the subject. The method and system, in one form includes (a) acquiring a biological sample from the subject; (b) determining an amount in the sample of a miR-33b microRNA; and (c) comparing the amount of the miR-33b microRNA in the sample, if present, to a control level of the miR-33b microRNA. In one further, specific form, a therapeutic treatment is administered or adjusted, in the subject, based on the presence and/or amount of miR-33b present in the subject. In an alternative form, a nucleic acid probe or vector has a nucleic acid sequence complementary to miR-33b for use in identifying the presence of miR-33b in a sample from a subject.
SYSTEMS AND METHODS FOR DIAGNOSIS, PROGNOSIS, AND TREATMENT OF CANCER

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Application No. 61/670,953, filed June 12, 2012, herein incorporated by reference.

TECHNICAL FIELD

[0002] The present invention relates to methods for diagnosis, prognosis, and treatment of cancer. In particular, the present invention relates to diagnostic, prognostic, and therapeutic methods based on (1) identifying the presence of miR-33b microRNA, and (2) determining an amount of miR-33b microRNA and/or observing an increase in miR-33b microRNA.

BACKGROUND

[0003] The MYC gene encodes the c-Myc transcription factor that binds the consensus hexanucleotide sequence 5'-CAC[C/T] GTG, termed enhancer boxes (E-boxes), to activate or repress a great number of genes (Amati et al, 2001; Eilers & Eisenman, 2008; Grandori et al, 2000). These genes encompass a broad range of biological and pathological functions (Patel et al, 2004), such as cell cycle progression, apoptosis, proliferation (Bouchard et al, 2004; Gatti et al, 2009; Podar et al, 2006; Stearns et al, 2006; Teleman et al, 2008), migration and metastasis (Ma et al, 2010), stem cell self-renewal (Smith et al, 2010) and enhanced somatic reprogramming (Nakagawa et al, 2008). Dysregulation of c-Myc through somatic mutation, chromosomal translocation, genomic amplification or defects in upstream regulators (Albihn et al, 2010) plays a role in human cancer development (Couillard & Trudel, 2009; Jensen et al, 2003; Shi et al, 2009).

[0004] MicroRNAs (miRNAs) are a group of 20-25 nucleotide small RNA molecules that repress gene expression through interaction with the 3' untranslated region (3'UTR) of target mRNAs. Over 1000 miRNAs have been found in the human genome (Griffiths-Jones et al, 2006), rendering miRNAs one of the largest classes of regulatory molecules. Over a dozen
miRNAs such as the miR-17-92 cluster (He et al, 2005; O'Donnell et al, 2005) and miR-9 (Ma et al, 2010) have been found to be induced by c-Myc to manifest its function in cell cycle, survival, metabolism, apoptosis and metastasis (Bui & Mendell, 2010). Moreover, miR-145 (Sachdeva et al, 2009), miR-34a (Christoffersen et al, 2010), miR-24 (Lai et al, 2009), miR-141 (Zhang et al, 2010), miR-185-3p (Liao & Lu, 2011) and let-7 (Melton et al, 2010) are found to repress c-Myc expression directly and adversely affect c-Myc’s oncogenic function. In embryonic stem cells, c-Myc regulates miR-141, miR-200a and miR-429 to attenuate stem cell differentiation (Lin et al, 2009), indicating that miRNAs are components of the c-Myc network, and modulating their expression represents an approach for cancer therapeutics.

[0005] It is contemplated that modified DNA and/or RNA oligonucleotides may be used as the main weapons to restrain the action of oncogenic miRNAs or to mimic tumour-suppressive miRNAs. However, in addition to the common challenges of drug development, there are three intrinsic disadvantages of this approach. First, these oligonucleotides are too large in size. Experimental modified oligonucleotides tested in rodents and primates are generally longer than 15 bases (MW ~5000 Daltons) (Elmen et al, 2008; Krutzfeldt et al, 2005). In contrast, the molecular weight of most orally active drugs for human use is no greater than 500 Daltons, as summarized by Lipinski’s Rule of Five (Lipinski et al, 2001), a rule of thumb to evaluate druglikeness. Second, DNA or RNA oligonucleotides are likely to be regarded by the human body as a virus, thereby triggering an immune response. For example, Fomivirsen (Vitravene, Novartis Corp.), the first and the only modified oligonucleotide approved by the US Food and Drug Administration (FDA) to treat cytomegalovirus retinitis in immunocompromised patients, has a well-known side effect of ocular inflammation (The Vitravene Study Group, 2002). Third, modified antisense oligonucleotides are themselves likely to act as miRNAs that could target hundreds, if not thousands, of transcripts in humans, as only seven or eight nucleotides are needed for miRNAs to repress their targets efficiently (Bartel, 2009). Thus, a need persists for the development of not only improved biomarkers and screening methods for cancers, but also for the identification of existing pharmaceutical agents that can affect the expression of certain miRNA and be used therapeutically.
SUMMARY OF THE INVENTION

[0006] The present invention meets some or all of the above-identified needs, as will become evident to those of ordinary skill in the art after a study of information provided in this document.

[0007] This Summary describes several embodiments of the present invention, and in many cases lists variations and permutations of these embodiments. This Summary is merely exemplary of the numerous and varied embodiments. Mention of one or more representative features of a given embodiment is likewise exemplary. Such an embodiment can typically exist with or without the feature(s) mentioned; likewise, those features can be applied to other embodiments of the present invention, whether listed in this Summary or not. To avoid excessive repetition, this Summary does not list or suggest all possible combinations of such features.

[0008] The present invention relates, in one form, to a method for diagnosing a cancer in a subject. The method includes (a) acquiring a biological sample from the subject; (b) determining an amount in the sample of a miR-33b microRNA; and (c) comparing the amount of the miR-33b microRNA in the sample, if present, to a control level of the miR-33b microRNA.

[0009] This method, is based in part on the present discovery that miR-33b microRNA downregulates c-Myc, and in particular, that miR-33b is a primate-specific negative regulator of c-Myc. c-Myc dysregulation is one of the most common abnormalities found in human cancer. Accordingly, the presence of miR-33b in a sample from a human (subject) is an indicator that the subject may have cancer and may benefit from downregulating c-Myc. MicroRNAs (miRNAs) are functionally intertwined with the c-Myc network as multiple miRNAs are regulated by c-Myc, while others directly suppress c-Myc expression. The human miR-33b gene is located at 17pl 1.2, a genomic locus frequently lost in meduUoblastomas, of which a subset displays c-Myc overproduction.

[0010] In one further specific form of the present method, a subject is diagnosed as having cancer or at risk for developing cancer if there is a measurable difference in the amount of the miR-33b microRNA in the sample as compared to the control level. In an alternative further specific form, the method includes selecting a treatment or modifying a treatment for the cancer based on the determined amount of the miR-33b microRNA.
[0011] The present invention, in another form thereof, includes a method for determining whether to initiate or continue prophylaxis or treatment of a cancer in a subject. The method includes (a) acquiring a series of biological samples over a time period from the subject; (b) analyzing the series of biological samples to determine an amount in each of the biological samples of a miR-33b microRNA; and (c) comparing any measurable change in the amounts of the miR-33b microRNA in each of the biological samples. The method, in one specific further form, includes (d) determining whether to initiate or continue the prophylaxis or therapy of the cancer based on comprising any measurable change in the amounts of the miR-33b microRNA.

[0012] The present invention, in another form thereof, relates to a method for treating a cancer, comprising administering to a subject an effective amount of a pharmaceutical agent capable of upregulating expression of an miR-33b microRNA. In one specific form, the pharmaceutical agent is a statin and the statin may be lovastatin.

[0013] Through screening with drugs approved by the U.S. Food and Drug Administration (FDA), lovastatin was identified as upregulating miR-33b expression, reducing cell proliferation, and impairing c-Myc expression and function in miR-33b-positive medulloblastoma cells. In addition, a low dose of lovastatin treatment at a level comparable to approved human oral use reduced tumor growth in mice orthotopically xenografted with cells carrying miR-33b, but not with cells lacking miR-33b. Accordingly, the present therapeutic method provides a repurpose or use of lovastatin against cancers that overexpress c-Myc.

[0014] The method, in one further specific form includes identifying a subject for therapeutic treatment by acquiring a biological sample from the subject, determining an amount of miR-33b microRNA and comparing the amount of miR-33b microRNA in the sample, if present, to a control level of miR-33b microRNA. Differences between the amount of miR-33b in a subject, relative to a control level, may indicate the subject may be overexpressing c-Myc. Once a subject overexpressing c-Myc is identified, therapeutic treatments which downregulate c-Myc can be considered. One such therapeutic treatment comprises administering to the subject an effective amount of a pharmaceutical agent capable of upregulating expression of an miR-33b microRNA. In one specific form, the pharmaceutical agent is a statin and the statin may be lovastatin.
[0015] The present method, in yet an alternative form, includes administering to the subject an effective amount of the pharmaceutical agent based on the determined presence of miR-33b microRNA. In one specific form, the pharmaceutical agent downregulates c-Myc.

[0016] The present invention, in another form thereof, relates to a nucleic acid or vector probe, comprising a nucleic acid sequence of SEQ ID NO: 1 or its antisense sequence, along with non-native 5’ and 3’ ends covalently bonded thereto. The covalently bonded sequences are not native and therefore are not found in nature bonded to a nucleic acid having a sequence of SEQ ID NO: 1. The nucleic acid or vector or probe can be used to identify the presence of miR-33b microRNA in a biological sample from a subject.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0017] Figs. 1A-1I illustrate that miR-33b negatively regulates c-Myc expression through direct targeting of its 3’UTR in 293T cells, wherein:

[0018] Fig. 1A is a graph showing Reporter screening to identify miRNAs that repress c-Myc and target the 3’UTR of the MYC gene,

[0019] Fig. 1B is a graph showing Assay 1, which identifies miRNAs that down-regulate the expression of luc, which is driven by the E2F2 promoter. (The Y-axis denotes the relative luminescence units (RLU) of luc normalized to that of Rluc from pRL-TK compared with that of the vector control);

[0020] Fig. 1C is a graph showing miR-33b down-regulates the expression of Rluc upstream of the 3’UTR of MYC driven by a constitutively active promoter in Assay 2 (The y-axis denotes the RLU of Rluc normalized to that of luc from the pGL3-Promoter compared with that of the vector control);

[0021] Fig. 1D is a graph showing mutating two E-boxes of the E2F2 promoter abolishes the regulation of luc expression by miR-33b in Assay 1 (RLU of cells with both the parental vector and the mutant promoter construct (pE2F2Mut-luc) is used as a reference);

[0022] Fig. 1E shows modulation of Rluc expression by miR-33b is abolished with a mutant **MYC 3’UTR** (On the left is a schematic representation of miR-33b complementary binding to **MYC 3’UTRW** and 3’UTRMut in which the miR-33b binding site is compromised. Assay 2 (right) is performed with RLU of cells transfected with the parental vector plus 3’UTRW or that with the parental vector plus 3’UTRMut as a reference);
[0023] Fig. 1F is immunoblotting analyses showing that down-regulation of c-Myc by miR-33b is dose dependent and miR-203 does not reduce c-Myc protein levels;

[0024] Fig. 1G is a qRT-PCR showing that MYC mRNA levels are reduced when miR-33b is overexpressed;

[0025] Fig. 1H is a schematic representation of the binding of miR-33b, a miR-33b mutant (miR-33bM), or miR-33A to the MYC 3'UTR;

[0026] Fig. 1I shows c-Myc and its transcriptional targets, cyclin E and ODC, are down-regulated by miR-33b, but not miR-33A or miR-33b mutant, while GADD45a is up-regulated, (Numbers across the top of the blot indicate the relative levels of c-Myc normalized to β-actin).

[0027] Figs. 2A-2F illustrate the reintroduction of miR-33b in D283 medulloblastoma cells down-regulates c-Myc expression and function, wherein:

[0028] Fig. 2A shows protein levels of c-Myc and its transcriptional targets cyclin E and ODC are down-regulated by miR-33b, (GADD45a, a c-Myc transrepressional target, is upregulated);

[0029] Fig. 2B are graphs showing miR-33b leads to increased G1 arrest, (On the left is a representative image of a single run with the y-axis denoting events (the number of cells) and the x-axis denoting the emitted fluorescence of the DNA dye (PI); a bar graph on the right is provided to summarize the three independent runs);

[0030] Fig. 2C is a graph showing miR-33b decreases cell proliferation (miR-33b expression reduces cell proliferation in the presence of exogenously expressed c-Myc with a native but not with a mutant 3'UTR);

[0031] Fig. 2D is a graph showing miR-33b overexpression results in down-regulation of miR-9;

[0032] Fig. 2E shows miR-33b reduces cell migration, in which the upper panel is a representative image of migrated cells and the bottom panel is a bar graph summarizing three independent experiments; and

[0033] Fig. 2F shows miR-33b decreases anchorage-independent colony formation.

[0034] Figs. 3A-3D show the reintroduction of miR-33b in D283 cells reduces the neuronal stem-cell characteristics, wherein:

[0035] Fig. 3A shows morphological change of D283 cells with stable expression of miR-33b in growth medium;
Fig. 3B shows neurosphere formation of D283 cells with miR-33b stimulation is impaired in neurobasal medium;

Fig. 3C shows differential mRNA expression levels of stem cell markers CD133 and SOX2 in cells with or without miR-33b in neurobasal medium (left) or growth medium (right); and

Fig. 3D illustrates immunocytometry showing that miR-33b expression results in reduced expression of Musashi in neurobasal medium.

Figs. 4A-4I show lovastatin upregulates miR-33b expression and adversely impacts c-Myc expression and function in medulloblastoma cells, wherein:

Fig. 4A is a graph showing a screening assay identified lovastatin as a compound that inhibits Daoy cell growth;

Fig. 4B is a graph showing lovastatin treatment inhibits the growth of Daoy but not D283 cells;

Fig. 4C are graphs showing lovastatin treatment increases the RNA levels of miR-33b and SREBF1 and reduces that of MYC in Daoy but not in D283 cells;

Fig. 4D are graphs showing lovastatin results in down-regulation of c-Myc, cyclin E, and ODC and upregulation of Gadd45a in Daoy but not in D283 cells;

Fig. 4E are graphs showing cell cycle analyses of Daoy (left) and D283 (right) cells treated with lovastatin, with the final concentration of lovastatin (0-10 μM) indicated on the x-axis;

Fig. 4F is a graph showing lovastatin treatment activates the luciferase reporter driven by the SREBF1-miR-33b promoter in Daoy and D283 cells;

Fig. 4G are graphs showing mevalonate inhibits lovastatin-induced miR-33b and miR-33A expression;

Fig. 4H are graphs showing mevalonate inhibits c-Myc down-regulation and SREBF1 upregulation induced by lovastatin in Daoy cells; and

Fig. 4I are graphs showing miR-33b inhibition rescues c-Myc down-regulation by lovastatin treatment (NegControl, Negative Control #1; Anti-miR-33b, Anti-miRTM miR-33b inhibitors (Ambion)), Left: c-Myc expression using Western blot (numbers indicating the relative c-Myc levels normalized to β-actin); Right: G1 cell cycle arrest analysis using flow cytometry.)
Figs. 5A-5G show xenograft models of medulloblastoma with lovastatin treatment or miR-33b overexpression, wherein:

- **Fig. 5A** shows a Haematoxylin and eosin (H&E) staining of the brain from mice orthotopically xenografted with Daoy or D283 cells and treated with lovastatin;

- **Fig. 5B** are graphs showing miR-33b expression was elevated and miR-9 was down-regulated in tumors with Daoy cells upon lovastatin treatment;

- **Fig. 5C** shows IHC analyses of tumors with Daoy;

- **Fig. 5D** shows IHC analyses of tumors with D283 cells;

- **Fig. 5E** is a graph showing Kaplan-Meier survival of mice xenografted with D283 cells and treated with lovastatin (Treatment was administered two weeks after inoculation);

- **Fig. 5F** shows H&E staining of tumors xenografted with D283 cells carrying miR-33b or the parental vector; and

- **Fig. 5G** shows IHC analyses of tumors with D283 cells carrying miR-33b or the parental vector (Arrows point to tumor cells).

Figs. 6A and 6B are graphs showing reporter screening to identify miRNAs that repress c-Myc, using Assay 1. Fig. 6A and Assay 2. Fig. 6B.

- **Figs. 7A and 7B** show the miR-33b miRNA and its precursor, wherein:

- **Fig. 7A** shows the predicted free energies of the precursors to miR-33b, miR-33A, and miR-33bM (Nucleotides 1-20 represent the mature form of the miRNAs); and

- **Fig. 7B** shows the interaction of miR-33b:MYC 3'UTR in animals (the miR-33b gene exists in human, chimpanzee, and Rhesus, but not in other animals, even though they all have a SREBF1, miR-33A, and SREBF2 gene. "+", present; "-", absent, (the 64-101 nucleotide of human 3'UTR is shown).

- **Figs. 8A and 8B** show miR-33b expression is not regulated by c-Myc, wherein:

- **Fig. 8A** depicts qRT-PCR demonstrating increased expression of MYC, and

- **Fig. 8B** shows the expression of miR-33b remains unchanged upon c-Myc overexpression.

- **Figs. 9A-9c** show miR-33b down-regulates exogenously expressed c-Myc in MYC-null HO15.19 cells, wherein:

- **Fig. 9A** is a western blotting analyses showing that miR-33b down-regulates c-Myc exogenously expressed from a pcDNA-MYC construct with the 3'UTRWT and c-Myc
transactivation target Cyclin E, miR-33b does not suppress exogenously expressed c-Myc with
the 3'UTRMut lacking the miR-33b binding site, (numbers above the blot are the relative
quantities of c-Myc normalized to β-actin);

[0066] Fig. 9B is qRT-PCR demonstrating a reduction in the MYC mRNA level following
miR-33b overexpression; and

[0067] Fig. 9C is a overexpression of miR-33b in HO 15.19 cells with MYC and a WT
3'UTR increased G1 arrest (On the left are representative images of a single run with the y-axis
denoting events (the number of cells) and the x-axis denoting the emitted fluorescence of the
dNA dye (PI); a bar graph on the right is provided to summarize the three independent runs).

[0068] Figs. 10A and 10B show statin affects gene expression in Daoy cells, wherein:

[0069] Fig. 10A is a graph showing the qPCR analysis of SREBF1A and SREBF2
expression treated with 0.2 µM lovastatin (RQ, relative quantity normalized to β-actin); and

[0070] Fig. 10B is a graph showing the impact on cell proliferation and miR-33b expression
by other statins (Daoy cells were treated with a 0.2 µM concentration of various statins (in
DMSO) and cell proliferation and miR-33b levels were determined. Simvastatin, pravastatin,
cerivastatin, and pitavastatin upregulated miR-33b expression. Relative values of miR-33b were
normalized to U6 snRNA and those of cell proliferation were determined using the MTT assay).

[0071] Figs. 11A and 11B show lovastatin treatment for UW228 cells, wherein:

[0072] Fig. 11A shows lovastatin treatment increased the RNA levels of miR-33b and
SREBF1 and reduced MYC mRNA levels, and

[0073] Fig. 11B is western blotting analysis showing that increasing lovastatin
concentration resulted in progressive down-regulation of c-Myc protein levels, accompanied by
down-regulation of Cyclin E and ODC and upregulation of Gadd45a.

[0074] Figs. 12A -12F show miR-33b overexpression in Daoy cells, wherein:

[0075] Fig. 12A shows protein levels of c-Myc and its transcriptional targets Cyclin E and
ODC are down-regulated by miR-33b overexpression in Daoy cells;

[0076] Fig. 12B shows miR-33b overexpression reduces MYC mRNA levels in Daoy cells;

[0077] Fig. 12C shows miR-33b overexpression increases G1 cell cycle arrest in Daoy cells
(On the left are representative images of a single run with the y-axis denoting events (the number
of cells) and the x-axis denoting the emitted fluorescence of the DNA dye (PI); a bar graph on
the right is provided to summarize the three independent runs);
[0078] Fig. 12D show miR-33b overexpression reduces cell proliferation;

[0079] Fig. 12E shows the expression of miR-9 is down-regulated upon miR-33b overexpression; and

[0080] Fig. 12F show the transwell migration assay showing that miR-33b decreases cell migration of Daoy cells.

[0081] Figs. 13A-13D show miR-33b down-regulates c-Myc in HeLa cells, wherein:
[0082] Fig. 13A are immunoblotting analyses showing that down-regulation of c-Myc and cyclin E and ODC and upregulation of Gadd45a by miR-33b,
[0083] Fig. 13B shows Myc mRNA levels is reduced by miR-33b,
[0084] Fig. 13C show miR-33b, but not miR-33bM leads to increased G1 arrest (On the top are representative images of a single run with the y-axis denoting the number of cells and the x-axis denoting the emitted fluorescence of the DNA dye (PI); a bar graph on the bottom is provided to summarize the three independent runs), and

[0085] Fig. 13D shows miR-33b decreases cell proliferation and this is likely due to c-Myc targeting as cell proliferation is not reduced when an exogenous c-Myc without miR-33b target sites in the 3'UTR is expressed (Cell proliferation is determined by the MTT assay. MOCK: cells are only transfected with miR-33b or its parental vector; Control: cells are co-transfected with an empty expression plasmid (pCDNA3; Invitrogen). * P<0.05; ** P<0.01; n.s., not significant).

[0086] Figs. 14A-14D show miR-33b regulates the expression of Abcal and Piml in medulloblastoma cells, wherein:
[0087] Figs. 14A and 14C show overexpression of miR-33b,
[0088] Figs. 14B and 14D show cells treated with lovastatin,
[0089] Figs. 14A and 14B show Daoy cells,
[0090] Figs. 14C and 14D show D283 cells.
[0091] Figs. 15 and 15B show subcutaneous xenograft assay of Daoy cells treated with lovastatin, wherein:
[0092] Fig. 15A is a graph showing volumes of xenograft tumors, and
[0093] Fig. 15B are statins showing IHC analyses of c-Myc expression in tumor sections.
[0094] Figs. 16A-16E are graphs showing pathological analyses of orthotopic xenograft tumors in mice, wherein:
[0095] Fig. 16A is a graph showing that lovastatin treatment reduces the maximum surface area of tumors xenografted with Daoy cells but not that with D283 cells,

[0096] Fig. 16B is a graph showing that lovastatin treatment reduces the expression of c-Myc and Cyclin E in tumors xenografted with Daoy cells but not that with D283 cells,

[0097] Fig. 16C is a graph showing lovastatin treatment does not affect the percentage of PCNA-positive cells in tumors xenografted with Daoy cells or that with D283 cells,

[0098] Fig. 16D is a graph showing maximum tumor surface area is reduced when miR-33b is overexpressed, and

[0099] Fig. 16E is a graph showing reduced maximum surface area of tumors xenografted with D283 cells overexpressing miR-33b, compared with that of cells carrying the parental vector.

[0100] Figs. 17A-17E are stains showing histology of mice xenografted with Daoy and treated with lovastatin (Brain sections were stained with H&E, and representative images were compared. Arrows point to tumor cells), wherein:

[0101] Figs. 17A and 17D are H&E staining of an animal treated with vehicle ("#" indicates newly-formed blood vesicle. "" indicates misplaced white matter), and

[0102] Figs. 17B and 17E depict swollen neurons are circled in tumor cell invasion into the cerebral cortex in mice treated with lovastatin (Brain sections were from two mice that died around the same time (day 52 from the control group, Figs. 17A-17D, and day 53 from the treated group, Fig. 17E)).

[0103] Figs. 18A and 18B show negative correlation of MYC and SREBF1 expression in meduUoblastoma, wherein:

[0104] Fig. 18A is a biplot of relative levels of the mean expression of MYC and SREBF1. Gray color indicates normal brain and other colors indicate tumors, and

[0105] Fig. 18B is a Kaplan-Meier curve and the log-rank test for overall survival of meduUoblastoma patients (21 out of these 29 cases have survival data) (A box-plot on the right shows the negative correlation of Myc and SREBF1 expression in 21 patients. RQ, relative quantitation).

[0106] Fig. 19 is a table showing FDA-approved drugs that upregulate miR-33b in Daoy cells.
DETAILED DESCRIPTION

[00107] The details of one or more embodiments of the presently-disclosed subject matter are set forth in this document. Modifications to embodiments described in this document, and other embodiments, will be evident to those of ordinary skill in the art after a study of the information provided in this document. The information provided in this document, and particularly the specific details of the described exemplary embodiments, is provided primarily for clearness of understanding and no unnecessary limitations are to be understood therefrom. In case of conflict, the specification of this document, including definitions, will control.

[00108] In certain instances, microRNAs (miRNAs) disclosed herein are identified with reference to names assigned by the miRBase database (University of Manchester, Manchester, U.K.). The sequences and other information regarding the identified miRNAs as set forth in the miRBase database are expressly incorporated by reference as are equivalent and related miRNAs present in the miRBase database or other public databases. Also expressly incorporated herein by reference are all annotations present in the miRBase database associated with the miRNAs disclosed herein. Unless otherwise indicated or apparent, the references to the miRBase database are references to the most recent version of the database as of the filing date of this Application (i.e., miRBase 18, released November 2011).

[00109] While the following terms are believed to be well understood by one of ordinary skill in the art, definitions are set forth to facilitate explanation of the presently-disclosed subject matter.

[00110] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the presently-disclosed subject matter belongs. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the presently-disclosed subject matter, representative methods, devices, and materials are now described.

[00111] Following long-standing patent law convention, the terms "a", "an", and "the" refer to "one or more" when used in this application, including the claims. Thus, for example, reference to "a cell" includes a plurality of such cells, and so forth.

[00112] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about". Accordingly, unless indicated
to the contrary, the numerical parameters set forth in this specification and claims are approximations that can vary depending upon the desired properties sought to be obtained by the presently-disclosed subject matter.

[00113] As used herein, the term "about," when referring to a value or to an amount of mass, weight, time, volume, concentration or percentage is meant to encompass variations of in some embodiments ±20%, in some embodiments ±10%, in some embodiments ±5%, in some embodiments ±1%, in some embodiments ±0.5%, and in some embodiments ±0.1% from the specified amount, as such variations are appropriate to perform the disclosed method.

[00114] The term "microRNA" is used herein to refer to naturally-occurring, small non-coding RNAs that are about 17 to about 25 nucleotide bases (nt) in length in their biologically active form. miRNAs post-transcriptionally regulate gene expression by repressing target mRNA translation. It is thought that miRNAs function as negative regulators, i.e. greater amounts of a specific miRNA will correlate with lower levels of target gene expression.

[00115] c-Myc dysregulation is one of the most common abnormalities found in human cancer. MicroRNAs (miRNAs) are functionally intertwined with the c-Myc network as multiple miRNAs are regulated by c-Myc, while others directly suppress c-Myc expression. As described in further detail below and in U.S. Provisional Patent Application No.: 61/670,953, the presently-disclosed subject matter is based, in part, on the identification of miR-33b as a negative regulator of c-Myc.

[00116] The human miR-33b gene is located at 17pl 1.2, a genomic locus frequently lost in medulloblastomas, which are pediatric brain tumors that display c-Myc overproduction. Through a small-scale screening with drugs approved by the US Food and Drug Administration (FDA), it has surprising been found that lovastatin upregulated miR-33b expression, reduced cell proliferation, and impaired c-Myc expression and function in miR-33b-positive medulloblastoma cells. In addition, a low dose of lovastatin treatment at a level comparable to approved human oral use reduced tumor growth in mice orthotopically xenografted with cells carrying miR-33b, but not with cells lacking miR-33b, indicating that statins are a therapeutic option against cancers that overexpress c-Myc.

[00117] The presently-disclosed subject matter thus provides methods and systems for diagnosis and prognosis of a cancer, as well as methods for treating or limiting the occurrence of cancer. The term "cancer" is used herein to refer to all types of cancer or neoplasm or malignant
tumors found in animals, including leukemias, carcinomas, melanoma, and sarcomas. Examples of cancers are cancer of the brain, bladder, breast, cervix, colon, head and neck, kidney, lung, non-small cell lung, mesothelioma, ovary, prostate, sarcoma, stomach, uterus and medulloblastoma. In some embodiments, the cancer is a medulloblastoma. In some embodiments, the cancer is a statin-resistant cancer.

[00118] In some embodiments of the presently-disclosed subject matter, methods and systems for diagnosing a cancer in a subject, and for determining whether to initiate or continue prophylaxis or treatment of cancer in a subject, are provided and include identifying at least one biomarker in a biological sample from a subject. In some embodiments, the at least one biomarker is a miR-33b miRNA (miRBase Accession No. M10003646; 3'-CGUACGUUGUCGUUACGUG-5') (SEQ ID NO.: 1). The miR-33b can be bonded at its 5' and 3' ends to non-native (i.e. sequences which are not bound to miR-33b in nature).

[00119] A "biomarker" is a molecule useful as an indicator of a biologic state in a subject. With reference to the present subject matter, the biomarkers disclosed herein can be polypeptides that exhibit a change in expression or state, which can be correlated with the risk of developing, the presence of, or the progression of cancer in a subject.

[00120] In some embodiments of the presently-disclosed subject matter, a method for diagnosing a cancer in a subject is provided. The terms "diagnosing" and "diagnosis" as used herein refer to methods by which the skilled artisan can estimate and even determine whether or not a subject is suffering from a given disease or condition. The skilled artisan often makes a diagnosis on the basis of one or more diagnostic indicators, such as for example a marker, the amount (including presence or absence) of which is indicative of the presence, severity, or absence of the condition.

[00121] Along with diagnosis, clinical disease prognosis is also an area of great concern and interest. It is important to know the stage and rapidity of advancement of the cancer in order to plan the most effective therapy. If a more accurate prognosis can be made, appropriate therapy, and in some instances less severe therapy for the patient can be chosen. Measurement of miR-33b miRNA biomarker levels disclosed herein can be useful in order to categorize subjects according to advancement of cancer who will benefit from particular therapies and differentiate from other subjects where alternative or additional therapies can be more appropriate.
[00122] As such, "making a diagnosis" or "diagnosing", as used herein, is further inclusive of determining a prognosis, which can provide for predicting a clinical outcome (with or without medical treatment), selecting an appropriate treatment (or whether treatment would be effective), or monitoring a current treatment and potentially changing the treatment, based on the measure of diagnostic biomarker levels disclosed herein.

[00123] The phrase "determining a prognosis" as used herein refers to methods by which the skilled artisan can predict the course or outcome of a condition in a subject. The term "prognosis" does not refer to the ability to predict the course or outcome of a condition with 100% accuracy, or even that a given course or outcome is predictably more or less likely to occur based on the presence, absence or levels of test biomarkers. Instead, the skilled artisan will understand that the term "prognosis" refers to an increased probability that a certain course or outcome will occur; that is, that a course or outcome is more likely to occur in a subject exhibiting a given condition, when compared to those individuals not exhibiting the condition. For example, in individuals not exhibiting the condition (e.g., not expressing the miR-33b miRNA biomarker or expressing it at a reduced level), the chance of a given outcome may be about 3%. In certain embodiments, a prognosis is about a 5% chance of a given outcome, about a 7% chance, about a 10% chance, about a 12% chance, about a 15% chance, about a 20% chance, about a 25% chance, about a 30% chance, about a 40% chance, about a 50% chance, about a 60% chance, about a 75% chance, about a 90% chance, or about a 95% chance.

[00124] The skilled artisan will understand that associating a prognostic indicator with a predisposition to an adverse outcome is a statistical analysis. For example, a biomarker level (e.g., quantity of miR-33b miRNA expression in a sample) of greater than a control level in some embodiments can signal that a subject is more likely to suffer from a cancer than subjects with a level less than or equal to the control level, as determined by a level of statistical significance. Additionally, a change in marker concentration from baseline levels can be reflective of subject prognosis, and the degree of change in marker level can be related to the severity of adverse events. Statistical significance is often determined by comparing two or more populations, and determining a confidence interval and/or a p value. See, e.g., Dowdy and Wearden, Statistics for Research, John Wiley & Sons, New York, 1983, incorporated herein by reference in its entirety. Preferred confidence intervals of the present subject matter are 90%,
95%, 97.5%, 98%, 99%, 99.5%, 99.9% and 99.99%, while preferred p values are 0.1, 0.05, 0.025, 0.02, 0.01, 0.005, 0.001, and 0.0001.

[00125] In other embodiments, a threshold degree of change in the level of a prognostic or diagnostic biomarker can be established, and the degree of change in the level of the indicator in a biological sample can simply be compared to the threshold degree of change in the level. A preferred threshold change in the level for markers of the presently disclosed subject matter is about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 50%, about 75%, about 100%, and about 150%. In yet other embodiments, a "nomogram" can be established, by which a level of a prognostic or diagnostic indicator can be directly related to an associated disposition towards a given outcome. The skilled artisan is acquainted with the use of such nomograms to relate two numeric values with the understanding that the uncertainty in this measurement is the same as the uncertainty in the marker concentration because individual sample measurements are referenced, not population averages.

[00126] In some embodiments of the presently disclosed subject matter, multiple determination of one or more diagnostic or prognostic miR-33b miRNA biomarkers can be made, and a temporal change in the miR-33b miRNA levels can be used to monitor the progression of cancer and/or efficacy of appropriate therapies directed against the disease. In such an embodiment for example, one might expect to see a decrease or an increase in the biomarker(s) over time during the course of effective therapy. Thus, the presently disclosed subject matter provides in some embodiments a method for determining treatment efficacy and/or progression of a cancer in a subject. In some embodiments, the method comprises determining an amount of a miR-33b miRNA in biological samples collected from the subject at a plurality of different time points and comparing the amounts of the miR-33b miRNA in the samples collected at different time points. For example, a first time point can be selected prior to initiation of a treatment and a second time point can be selected at some time after initiation of the treatment. One or more miR-33b miRNA levels can be measured in each of the samples taken from different time points and qualitative and/or quantitative differences noted. A change in the amounts of the miR-33b miRNA levels from the first and second samples can be correlated with determining treatment efficacy and/or progression of the cancer in the subject.

[00127] The terms "correlated" and "correlating," as used herein in reference to the use of diagnostic and prognostic biomarkers, refers to comparing the presence or quantity of the
biomarker in a subject to its presence or quantity in subjects known to suffer from, or known to be at risk of, a given condition (e.g., a cancer); or in subjects known to be free of a given condition, i.e. "normal individuals". For example, a miR-33b miRNA level in a biological sample can be compared to a level known to be associated with a specific type of cancer. The sample's miR-33b miRNA level is said to have been correlated with a diagnosis; that is, the skilled artisan can use the miR-33b miRNA level to determine whether the subject suffers from a specific type of cancer, and respond accordingly. Alternatively, the sample's miR-33b miRNA level can be compared to a control miR-33b miRNA level known to be associated with a good outcome (e.g., the absence of cancer), such as an average level found in a population of normal subjects.

[00128] In certain embodiments, a diagnostic or prognostic biomarker is correlated to a condition or disease by merely its presence or absence. In other embodiments, a threshold level of a diagnostic or prognostic biomarker can be established, and the level of the indicator in a subject sample can simply be compared to the threshold level.

[00129] As noted, in some embodiments, multiple determinations of one or more diagnostic or prognostic biomarkers can be made, and a temporal change in the marker can be used to determine a diagnosis or prognosis. For example, a diagnostic marker can be determined at an initial time, and again at a second time. In such embodiments, an increase in the marker from the initial time to the second time can be diagnostic of a particular type of cancer, or a given prognosis. Likewise, a decrease in the marker from the initial time to the second time can be indicative of a particular type of cancer, or a given prognosis. Furthermore, the degree of change of one or more markers can be related to the severity of cancer and future adverse events.

[00130] The skilled artisan will understand that, while in certain embodiments comparative measurements can be made of the same diagnostic marker at multiple time points, one can also measure a given marker at one time point, and a second marker at a second time point, and a comparison of these markers can provide diagnostic information.

[00131] With regard to the step of providing a biological sample from the subject, the term "biological sample" as used herein refers to any body fluid or tissue potentially comprising miR-33b miRNA. In some embodiments, for example, the biological sample can be a blood sample, a serum sample, a plasma sample, or sub-fractions thereof.
[00132] Turning now to the step of identifying one or more markers in the biological sample, various methods known to those skilled in the art can be used to identify the one or more markers in the provided biological sample. In some embodiments, determining the amount of miR-33b miRNA is conducted using a probe (e.g., a nucleic acid probe) for selectively binding or hybridizing with the miR-33b miRNA. In other words, in certain embodiments, known miR-33b miRNA sequence information can be used to construct probes that find and hybridize with the miR-33b miRNA strands in a sample to thereby capture the miR-33b miRNA strands by selective binding.

[00133] The term "selective binding" as used herein refers to a measure of the capacity of a probe to hybridize to a target polynucleotide with specificity. Thus, in the case of a target miR-33b miRNA, the probe comprises a polynucleotide sequence that is complementary, or essentially complementary, to at least a portion of the miR-33b miRNA sequence. Nucleic acid sequences which are "complementary" are those which are base-pairing according to the standard Watson-Crick complementarity rules. As used herein, the term "complementary sequences" means nucleic acid sequences which are substantially complementary, as can be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment in question under relatively stringent conditions such as those described herein. A particular example of a contemplated complementary nucleic acid segment is an antisense oligonucleotide. With regard to probes disclosed herein having binding affinity to mRNAs, the probe can be 100% complementary with the target polynucleotide sequence. However, the probe need not necessarily be completely complementary to the target polynucleotide along the entire length of the target polynucleotide so long as the probe can bind the target polynucleotide with specificity and capture it from the sample.

[00134] Nucleic acid hybridization will be affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of nucleotide base mismatches between the hybridizing nucleic acids, as will be readily appreciated by the skilled artisan. Stringent temperature conditions will generally include temperatures in excess of 30° C, typically in excess of 37° C, and preferably in excess of 45° C. Stringent salt conditions will ordinarily be less than 1,000 mM, typically less than 500 mM, and preferably less than 200 mM. However, the combination of parameters is much more important than the measure of any single parameter. Determining
appropriate hybridization conditions to identify and/or isolate sequences containing high levels of homology is well known in the art. For the purposes of specifying conditions of high stringency, preferred conditions are a salt concentration of about 200 mM and a temperature of about 45°C. Biomarkers in samples comprises using an RNA measuring assay to measure mRNA encoding biomarker polypeptides in the sample and/or using a protein measuring assay to measure amounts of biomarker polypeptides in the sample.

[00135] Although certain embodiments of the method only call for a qualitative assessment of the presence or absence of the miR-33b miRNA levels in the biological sample, other embodiments of the method call for a quantitative assessment of the amount of miR-33b miRNA in the biological sample. Such quantitative assessments can be made, for example, using the above mentioned methods, as will be understood by those skilled in the art.

[00136] In certain embodiments of the method, it can be desirable to include a control sample that is analyzed concurrently with the biological sample, such that the results obtained from the biological sample can be compared to the results obtained from the control sample. Additionally, it is contemplated that standard curves can be provided, with which assay results for the biological sample can be compared. Such standard curves present levels of miR-33b miRNA as a function of assay units, i.e., fluorescent signal intensity, if a fluorescent signal is used. Using samples taken from multiple donors, standard curves can be provided for control levels of the one or more markers in normal tissue.

[00137] The analysis of miR-33b miRNA levels can be carried out separately or simultaneously with additional markers within one test sample. For example, several markers can be combined into one test for efficient processing of a multiple of samples and for potentially providing greater diagnostic and/or prognostic accuracy. In addition, one skilled in the art would recognize the value of testing multiple samples (for example, at successive time points) from the same subject. Such testing of serial samples can allow the identification of changes in marker levels over time. Increases or decreases in marker levels, as well as the absence of change in marker levels, can provide useful information about the disease status that includes, but is not limited to identifying the approximate time from onset of the event, the presence and amount of salvageable tissue, the appropriateness of drug therapies, the effectiveness of various therapies, and identification of the subject's outcome, including risk of future events.
[00138] The analysis of markers can be carried out in a variety of physical formats as well. For example, the use of microtiter plates or automation can be used to facilitate the processing of large numbers of test samples. Alternatively, single sample formats could be developed to facilitate immediate treatment and diagnosis in a timely fashion, for example, in ambulatory transport or emergency room settings.

[00139] In some embodiments, the subject is identified as having cancer or a risk thereof if there is a measurable difference in the amount of miR-33b miRNA in the sample as compared to a control level. Conversely, when no probed marker is identified in the biological sample, the subject can be identified as not having cancer or a risk thereof, or as having a low risk of cancer.

[00140] As mentioned above, depending on the embodiment of the method, identification of the miR-33b miRNA can be a qualitative determination of the presence or absence of the miR-33b miRNA, or it can be a quantitative determination of the concentration of the miR-33b miRNA. In this regard, in some embodiments, the step of identifying the subject as having cancer or a risk thereof requires that certain threshold measurements are made, i.e., the levels of the one or more markers in the biological sample exceed control level. In certain embodiments of the method, the control level is any detectable level of the miR-33b miRNA. In other embodiments of the method where a control sample is tested concurrently with the biological sample, the control level is the level of detection in the control sample. In other embodiments of the method, the control level is based upon and/or identified by a standard curve. In other embodiments of the method, the control level is a specifically identified concentration, or concentration range. As such, the control level can be chosen, within acceptable limits that will be apparent to those skilled in the art, based in part on the embodiment of the method being practiced and the desired specificity, etc.

[00141] In some embodiments of the presently-disclosed subject matter, a system for diagnosing a cancer in a subject is provided, or a system for determining whether to initiate or continue prophylaxis or treatment of cancer in a subject is provided. Such systems can be provided, for example, as commercial kits that can be used to test a biological sample, or series of biological samples, from a subject. In some embodiments, the system includes probes for selectively binding miR-33b miRNA, and means for detecting the binding of said probes to said miR-33b miRNA. The system can also include certain samples for use as controls. The system
can further include one or more standard curves providing levels of markers as a function of assay units.

[00142] Further provided by the presently-disclosed subject matter are methods for treating a cancer. In some embodiments, a method for treating a cancer is provided that comprises administering to a subject an effective amount of a pharmaceutical agent capable of upregulating expression of a miR-33b microRNA in a subject.

[00143] As used herein, the terms "treating" or "treatment" relate to any treatment of a cancer including, but are not limited to, therapeutic treatment and prophylactic treatment of a cancer. As such, the terms "treating" or "treatment" include, but are not limited to, inhibiting the progression of a cancer, arresting the development of a cancer, reducing the severity of a cancer, ameliorating or relieving one or more symptoms associated with a cancer, and causing a regression or a cancer or one or more symptoms associated with a cancer.

[00144] As further non-limiting examples of the treatment of a cancer by a composition described herein, treating a cancer can include, but is not limited to, killing cancer cells, inhibiting the development of cancer cells, inducing apoptosis in cancer cells, reducing the growth rate of cancer cells, reducing the incidence or number of metastases, reducing tumor size, inhibiting tumor growth, reducing the available blood supply to a tumor or cancer cells, promoting an immune response against a tumor or cancer cells, reducing or inhibiting the initiation or progression of a cancer, increasing the lifespan of a subject with a cancer, or inhibiting or reducing the formation of DNA adducts by chemical carcinogens.

[00145] With regard to the pharmacological agents used in accordance with the presently-disclosed therapeutic methods, in some embodiments, the pharmaceutical agent is a statin. The term "statin" is used herein to refer to the class of pharmaceutical agents that are inhibitors of the enzyme HMG-CoA reductase. Such agents are known to those skilled in the art and include, but are not limited to, simvastatin, atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, and rosuvastatin. In some embodiments, the statin is levostatin.

[00146] For administration of a pharmaceutical agent in accordance with the presently-disclosed methods, conventional methods of extrapolating human dosage based on doses administered to a murine animal model can be carried out using the conversion factor for converting the mouse dosage to human dosage: Dose Human per kg=Dose Mouse per kg x 12 (Freireich, et al., (1966) Cancer Chemother. Rep. 50:219-244). Drug doses can also be given in
milligrams per square meter of body surface area because this method rather than body weight achieves a good correlation to certain metabolic and excretionary functions. Moreover, body surface area can be used as a common denominator for drug dosage in adults and children as well as in different animal species as described by Freireich, et al. (Cancer Chemother. Rep. 1996; 50:219-244). Briefly, to express a mg/kg dose in any given species as the equivalent mg/sq m dose, multiply the dose by the appropriate kg factor. In an adult human, 100 mg/kg is equivalent to 100 mg/kg × 37 kg/sq m = 3700 mg/m².

[00147] Suitable methods for administering a pharmaceutical agent in accordance with the methods of the presently-disclosed subject matter include, but are not limited to, systemic administration, parenteral administration (including intravascular, intramuscular, and/or intraarterial administration), oral delivery, buccal delivery, rectal delivery, subcutaneous administration, intraperitoneal administration, inhalation, intratracheal installation, surgical implantation, transdermal delivery, local injection, intranasal delivery, and hyper-velocity injection/bombardment. Where applicable, continuous infusion can enhance drug accumulation at a target site (see, e.g., U.S. Patent No. 6,180,082).

[00148] Regardless of the route of administration, the pharmaceutical agents used in accordance with the presently-disclosed subject matter are typically administered in amount effective to achieve the desired response (i.e., an upregulation in the expression of a miR-33b microRNA). As such, the term "effective amount" is used herein to refer to an amount of the pharmaceutical agent sufficient to produce a measurable biological response (e.g., an increase in miR-33b levels). Of course, the effective amount in any particular case will depend upon a variety of factors including the activity of the pharmaceutical agent, formulation, the route of administration, combination with other drugs or treatments, severity of the condition being treated, and the physical condition and prior medical history of the subject being treated. Preferably, a minimal dose is administered, and the dose is escalated in the absence of dose-limiting toxicity to a minimally effective amount. Determination and adjustment of a therapeutically effective dose, as well as evaluation of when and how to make such adjustments, are known to those of ordinary skill in the art.


[00150] With respect to the presently-disclosed subject matter, a preferred subject is a vertebrate subject. A preferred vertebrate is warm-blooded; a preferred warm-blooded vertebrate is a mammal. A preferred mammal is most preferably a human. As used herein, the term "subject" includes both human and animal subjects. Thus, veterinary therapeutic uses are provided in accordance with the presently-disclosed subject matter. As such, the presently-disclosed subject matter provides for the diagnosis of mammals such as humans, as well as those mammals of importance due to being endangered, such as Siberian tigers; of economic importance, such as animals raised on farms for consumption by humans; and/or animals of social importance to humans, such as animals kept as pets or in zoos. Examples of such animals include but are not limited to: carnivores such as cats and dogs; swine, including pigs, hogs, and wild boars; ruminants and/or ungulates such as cattle, oxen, sheep, giraffes, deer, goats, bison, and camels; and horses. Also provided is the treatment of birds, including the treatment of those kinds of birds that are endangered and/or kept in zoos, as well as fowl, and more particularly domesticated fowl, i.e., poultry, such as turkeys, chickens, ducks, geese, guinea fowl, and the like, as they are also of economic importance to humans. Thus, also provided is the treatment of livestock, including, but not limited to, domesticated swine, ruminants, ungulates, horses (including race horses), poultry, and the like.

[00151] The practice of the presently disclosed subject matter can employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Molecular Cloning A Laboratory Manual (1989), 2nd Ed., ed. by Sambrook, Fritsch and Maniatis, eds., Cold Spring

[00152] For further explanation of the features, benefits and advantages of the presently-disclosed subject matter, the following experiments and examples demonstrate the efficacy of the presently disclosed subject matter and provide a better understanding of the presently disclosed subject matter.

EXAMPLES

[00153] METHODS.

[00154] Cell culture, transfection, and transduction. All cell lines were cultured at 37°C in an atmosphere containing 5% CO₂. HEK293T, D283, and Daoy cells were obtained from ATCC (Manassas, VA) and cultured according to ATCC guidelines. c-Myc-null rat fibroblast Rati cells (clone HO15.19) were obtained from Dr. Claycomb's Lab (Brown University). UW228 cells were obtained from Dr. J.R. Silber (University of Washington, Seattle, WA) and were maintained in Dulbecco's modified Eagle's medium/F12 medium (Invitrogen). For neurosphere formation, D283 cells (50,000 in 6-well plates) were cultured in neurobasal medium supplemented with 10% serum, 0.1% (v/v) Antibiotic-Antimycotic solution (Invitrogen; Carlsbad, CA), 2 mM L-glutamine, N2 supplement, B27 supplement, 20 ng/ml hrEGF, 20 ng/ml hrBFGF, and 50 µg/ml BSA (Invitrogen). Cells were incubated in neurobasal medium for 10 days with medium refreshed twice a week, and the plates were imaged with a microscope (magnification 10X). Spheres larger than 50 µm in diameter were counted and quantified.
Transfection was performed using Lipofectamine LTX (Invitrogen) to achieve transient expression. For miRNAs, we used the parental vector pSIF as a vector control (Lu et al, 2011). The dual luciferase assays were performed as described (Lu et al, 2011). For an inhibitor of miR-33b, we used Anti-miR™ miRNA inhibitor (Ambion Inc, Austin, TX) with the Negative Control #1 as a control to Anti-miR-33b. Stable expression of miRNAs was obtained using transduction with lentivirus carrying the miRNA gene or the parental vector (Lu et al, 2011). Stable expression with lentiviral infection (Lu et al, 2011) was used for neurosphere formation and xenograft experiments.

**Molecular cloning and assays.** The full-length MYC 3’UTR (467 bp) was PCR amplified utilizing a forward primer (5’-ATTCTAGGAAAAAGTAAAGAAAACGATCCCT) (SEQ ID NO.: 2) and reverse primer (5’- TAGCCGCGCCTCAATGATATATTTG) (SEQ ID NO.: 3) from A549 cell genomic DNA template. The PCR product was inserted into the NotI/XbaI site, upstream of the Renila luciferase gene, of the pRL-TK vector (Promega). All miRNA constructs were obtained from an existing library (Lu et al, 2011), and all DNA constructs were confirmed by Sanger sequencing. The human c-Myc expression construct (pcDNA3-cmyc) was obtained from Addgene.org (#1601). We used TaqMan assays (Applied Biosystems Inc.; Foster City, CA) to determine the expression of mature miRNAs and mRNA (SREBF1C and MYC) with U6 RNA and β-Actin mRNA as respective references (Chen et al, 2005). For SREBF1C, a pre-made Taqman assay was used (Hs01088683; Applied Biosystems). For SREBF1A and SREBF2, a SYBR Green based qPCR was performed (primers for SREBF1A: 5’-TAGTCCGAAGGCTGCGGCGGCGCAT (SEQ ID NO.: 4) and 5’-GATGTCCGTTCAAAAAACCGCTGTGTCAGTTTC (SEQ ID NO.: 5); primers for SREBF2: 5’-CACAATATCATTGAAAAGCGCTACCGGTCC (SEQ ID NO.: 6) and 5’-TTTTTCTGATTGGCCAGCTTCCACCATG (SEQ ID NO.: 7).

The following primary antibodies were used in Western blotting analyses: c-Myc (cat. no. sc-40), cyclin E (sc-25303), VEGF-A (sc-7269), ODC (sc-21516), and Gadd45 (sc-6850) obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Abcal (ab7360) from Abeam (Cambridge, MA); Pim1 (#3247), and Bcl2 (#2870) from Cell Signaling Technology (Beverly, MA), and β-Actin (AC-15) from Sigma (St. Louis, MO). Secondary antibodies were horseradish peroxidase-linked goat anti-mouse IgG antibody (Santa Cruz Biotechnology sc-2005, 1:5000) or goat anti-rabbit IgG antibody (Cell Signaling). The quantification of protein expression levels
was determined by ImageJ (http://rsb.info.nih.gov/ij) with arbitrary units (AU) reflecting the
signal density from the blot.

[00158] **Cellular assays.** Cell proliferation was assessed using 3-(4,5-Dimethylthiazol-2-
yl)-2,5-diphenyltetrazolium bromide (MTT) (MTT Cell Proliferation Assay; ATCC, Manassas,
VA). MTT reagent (0.5 mg/ml) was added to each well of transfected cells and incubated at
37°C for 4 hrs. Detergent Reagent (100 µl) was added and incubated for 2 hrs in the dark at
room temperature, and the absorbance was read at 570 nm. The values were normalized to their
respective controls. Cell cycle phase and apoptosis were analyzed according to our established
methods (Kumar et al, 2011; Lu et al, 2011). For the Transwell migration assay, we used
Matrigel-coated Transwell chambers (Becton Dickinson). Transwell inserts with 8-µm pores
were coated with Matrigel and reconstituted with fresh medium for 2 hrs before the experiment.
Cells (2 x 10⁴/mL) were seeded into the upper chambers in 0.5 ml of serum-free DMEM or
EMEM in 12-well plates, while 1 ml of EMEM supplemented with 10% fetal bovine serum was
placed in the lower chamber. After 24 hrs, cells that migrated to the lower surface of the
Matrigel-coated membrane were fixed with 70% ethanol and stained with 0.4% crystal violet for
2 hrs and counted. Non-migrated cells on the upper side of the filter were removed with a cotton
swab, and the filter mounted on glass microscopic slides. The number of migrated cells was
counted using ImageJ software. The results were expressed as the average number of invasive
cells per microscopic field normalized to the total number of live cells cultured under the same
conditions without migration. For drug screening, Daoy cells were incubated with 0.2 µM of
727 compounds from the NIH Clinical Collection 1 and 2 (Evotec; South San Francisco, CA)
before being subjected to the MTT assay after 48 hrs. Lovastatin was used at a concentration of
0.2-10 µM for cells and 0.2-1.0 mg/kg for mice, as serum levels of lovastatin can reach 0.10—
3.92 µM in humans using >4 mg/kg (Thibault et al, 1996), and it is currently recommended for
the treatment of hypercholesterolemia with a dose of up to 80 mg/day (or 1 mg/kg/day), which
yields serum levels of ~0.1 µM (Pan et al, 1990). Mevalonate was used at a concentration of 100
nM along with 0.2 µM of lovastatin for 24 hrs. For promoter activity and miR-33b/SREBP1
induction, 0.2 µM of lovastatin was used to treat cells for 24 hrs. All other molecular and
cellular assays were performed 48 hrs post treatment or post transfection.

[00159] **Soft agar colony formation assay.** Viable cells (2,000) in 1.5 mL of culture
medium with 1% glutamine, antibiotics, and 0.2% soft agar were layered onto 0.5% solidified
agar in experimental culture media in six-well plates. The plates were incubated at 37°C for 2 weeks, the cells were stained with the MTT reagent, and colony foci were counted using a microscope (original magnification 4X). Experiments were carried out in triplicate.

[00160] Mouse xenograft and immunohistochemistry (IHC) analyses. Animals were housed at 22-23°C in a 12-hour light/dark cycle in a facility certified by the American Association for Accreditation of Laboratory Animal Care. Mice had free access to rodent chow and tap water. All animal experiments were conducted with a protocol approved by the University of Louisville Institutional Animal Care and Use Committee. Recipient immuno-deficient NCr nude mice were purchased from Taconic (Hudson, NY). Male ~8-week-old mice (n=5 in each group) were anesthetized with rapid-sequence inhalation of isofluorane before xenograft. For subcutaneous xenograft, 1x106 Daoy cells (in 200 µl of lXPBS) were inoculated (Lu et al, 2011); two weeks post inoculation, animals were randomized into two groups and lovastatin (carboxylate form; EMD4Biosciences; Darmstadt, Germany; 1.0 or 0.2 mg/kg in 20 µl of DMSO) was administered through intraperitoneal (i.p.) injection three times per week for 4 weeks. For orthotopic (intracerebellar) xenograft, mice were anesthetized with sodium pentobarbital (50 mg/kg), the head was shaved, a small skin incision (1 mm) was made along the midline, and a bore hole (0.7 mm) was created with a microsurgical drill. A 26-guage syringe needle was used to deliver a total of 1x10^5 cells (in 10 µl of lXPBS) into the right cerebellar hemisphere. Mice were monitored daily for any sign of distress. For D283 cells stably expressing miR-33b without lovastatin treatment, mice were maintained for 6 weeks; two animals were injected with the vehicle. For lovastatin treatment, the drug was administered (1.0 mg/kg) two weeks after surgery through i.p. injection three times per week and was continued for 4 weeks (n=6 each for animals sacrificed after 6 weeks). For xenografted animal survival analysis, mice were treated similarly till they died (n=11 for the control group; n=10 for the lovastatin statin). Brain tissues were fixed with 10% formalin for 24 hrs and embedded in paraffin.

[00161] IHC was performed using the avidin biotin complex (ABC) peroxidase method with Rabbit and Mouse IgG ABC Elite I detection kits (Vector Laboratories, Burlingame, CA) as previously described (Lu et al, 2011). The rabbit polyclonal antibody to e-Myc (Santa Cruz Biotechnology; cat. no. sc-789; 1:100), cyclin E (Santa Cruz Biotechnology; cat. no. sc-25303; diluted to 1:100), and monoclonal antibody to PCNA (Santa Cruz Biotechnology; cat. no. sc-56;
1:100) were used. Tissue sections were also stained with hematoxylin and eosin. For the TUNEL assay, deparaffinated brain tissue sections were incubated with 20μg/ml protease K for 15 min at room temperature, then washed with PBS and incubated with TUNEL reaction mixture (Roche Applied Science) 60 min at 37°C in humidified atmosphere. Tissue sections were incubated with converter-POD (with anti-fluorescein antibody) and subsequently with the DAB substrate. At least three sections were stained for each specimen. For protein expression, stained slides were evaluated histologically by two independent, blinded observers, and the gradation was scored from 0-3 according to the intensity of staining (0, negative; 1, weak; 2, moderate; 3, strong).

[00162] Statistical analyses. The experimental results were expressed as the mean ± standard deviation of at least 3 independent experiments. A two-tailed Student's t-test was performed with p<0.05 between the samples and their respective controls considered to be statistically significant. For animal survival with xenograft and lovastatin treatment, a log-rank (Mantel-Cox) test was performed.

[00163] RESULTS

[00164] Identification of miR-33b as a negative regulator of c-Myc. Reporter assays to screen miRNAs for their ability to modulate c-Myc function and to target the MYC 3′UTR directly (Fig. 1A). In Assay 1, a firefly luciferase gene (luc) is driven by the E2F2 promoter (E2F2-luc), which is regulated by c-Myc (Sears et al, 1997). miRNAs of interest, E2F2-luc, and a Renilla luciferase (RLuc) construct constitutively expressing RLuc were co-introduced into 293T cells to screen miRNAs that down-regulate the reporter luc. In Assay 2, the 3′UTR of MYC downstream of the RLuc construct and co-expressed it with a respective miRNA and a constitutively expressed luc to determine whether the miRNA targets the MYC 3′UTR to down-regulate RLuc. We performed Assay 1 in 293T cells using hundreds of miRNA minigenes in our genetic library (Lu et al, 2011) and found that 4 miRNAs (miR-33A, miR-33b, miR-212, and miR-203) significantly down-regulated the c-Myc-dependent reporter (Fig. 1B; Fig. 6A). Next, we performed Assay 2 using 54 miRNAs that were predicted to target MYC and found that miR-33b and miR-203 down-regulated the reporter with the MYC 3′UTR downstream (Fig. 1C; Fig. 6B). To determine whether MYC is a bona fide miR-33b target gene, we performed Assay 1 using a mutant E2F2-luc construct in which two of its four E-boxes (c-Myc binding sites) were disrupted (Sears et al, 1997). As expected, with the perturbation of these two E-boxes, the
expression of luc was significantly reduced, and the regulation of luc by miR-33b was abolished (Fig. 1D). When a MYC 3’UTR mutation that disrupts its binding to the seed sequence of miR-33b was used in Assay 2, we found that the down-regulation of RLuc by miR-33b was abrogated (Fig. 1E). Overexpression of miR-33b down-regulated c-Myc protein levels in a dose-dependent manner (Fig. 1F). We did not pursue miR-203, as it did not reduce c-Myc protein levels. We noted that two reported MYC-targeting miRNAs—miR-34a (Christoffersen et al., 2010) and let-7 (Melton et al., 2010) scored negatively in our assays (Figs. 6A and 6B), suggesting the limitation of this single cell line screening. The steady-state levels of MYC mRNA were significantly reduced with miR-33b overexpression (Fig. 1G), indicating that mRNA degradation likely contributed to miR-33b-mediated MYC suppression.

[00165] It is noteworthy that c-Myc expression was down-regulated by miR-33b, but not miR-33A, which differs from miR-33b by only two nucleotides (UA compared with CG) in the middle of their mature sequences (Fig. 1H). We folded the precursors of miR-33b and miR-33A (Zuker, 2003) and found the structure of pre-miR-33b was more stable than that of pre-miR-33A (Fig. 7A). When the miR-33b and miR-33A minigenes were introduced into 293T cells, miR-33b was overexpressed ~5-fold, while miR-33A was overexpressed ~5-fold. It is likely the less stable pre-miR-33A leads to lower levels of mature miR-33A, which is supported by the finding that disruption of the stem of pre-miRNAs significantly reduces the efficiency of miRNA maturation (Han et al., 2006). We constructed a mutant miR-33b (miR-33bM) minigene with a mature sequence the same as miR-33A and a precursor similar to pre-miR-33A. When equal amounts of miR-33b and miR-33bM minigenes were introduced into 293T cells, miR-33bM was overexpressed ~5-fold, instead of 25-fold. Correspondingly, introduction of miR-33b, but not miR-33A or miR-33bM, reduced the protein levels of c-Myc, as well as two known c-Myc transactivational targets, cyclin E and ornithine decarboxylase (ODC). On the other hand, Gadd45a, a c-Myc-repressed target, was upregulated when miR-33b was overexpressed (Fig. II). Collectively, these data implicate that miR-33b is a bona fide negative regulator of c-Myc in our assays.

[00166] The miR-33b binding site is present in MYC 3’UTRs from human, chimpanzee, and rhesus, but not those from mouse, rat, dog, and other mammals; the miR-33b gene is only present in primates (Fig. 7B) (Griffiths-Jones et al., 2006), providing evidence that miR-33b is a primate-specific regulator of c-Myc. Overexpression of an exogenous MYC gene in 293T cells
did not increase the expression levels of miR-33b (Figs. 8A and 8B), indicating that miR-33b expression is unlikely to be regulated by c-Myc. To further verify that miR-33b specifically targets MYC, we introduced two c-Myc constructs into a MYC-null cell line, HO15.19 (Mateyak et al., 1997); both constructs have a native c-Myc coding sequence, but one has a wild-type 3'UTR (3'UTRWT) and the other has a mutant 3'UTR in which the miR-33b binding site was disrupted (3'UTRMut, Fig. 1D). As shown in Fig. 9A, miR-33b down-regulated the expression of c-Myc and cyclin E in HO15.19 cells with 3'UTRWT, but not in those with 3'UTRMut. The changes of MYC mRNA levels showed similar patterns to that of the protein levels (Fig. 9B). In addition, miR-33b led to increased G1 arrest in HO15.19 cells carrying MYC with 3'UTRWT (Fig. 9C).

**[00167] miR-33b regulates c-Myc expression and function in medulloblastoma cells.** The human miR-33b gene is located in intron 17 of the sterol regulatory element binding transcription factor 1 (SREBF1) gene at the genomic locus 17p1.2, which is frequently lost in medulloblastoma (Aldosari et al., 2000; Fruhwald et al., 2001; Seranski et al., 1999). There are two transcript isoforms of this gene (SREBF1A and SREBF1C) and both contain miR-33b. We first employed D283 Med (D283), a medulloblastoma cell line without 17p1.2 and with high levels of c-Myc, but without MYC gene amplification (Siu et al., 2003). When miR-33b was reintroduced, c-Myc expression was down-regulated, along with its transactivation targets cyclin E and ODC, while Gadd45a (a c-Myc-repressed target) was upregulated (Fig. 2A). Along with down-regulation of cyclin E, a greater percentage of D283 cells were arrested at the G1 phase when miR-33b was overexpressed compared with the vector control (Fig. 2B). In addition, cells proliferated at a slower rate with miR-33b overexpression as determined by the MTT assay (Fig. 2C). miR-9 is a transactivational target of c-Myc and regulates cell migration and tumor metastasis (Ma et al., 2010). We found that miR-33b reintroduction down-regulated the expression of miR-9 (Fig. 2D) and resulted in a reduction in cell migration (Fig. 2E). In addition, D283 cells with miR-33b overexpression formed significantly fewer colonies on soft agar (Fig. 2F). These results suggest that reintroduction of miR-33b represses c-Myc expression and function in D283 cells.

**[00168] Medulloblastomas have been reported to have a stem cell origin and are capable of forming neurospheres when incubated in neurobasal medium (Annabi et al., 2008; Hemmati et al., 2003). Even in regular growth medium, there is a major morphological change (multicell...**
aggregates become desegregated single-cell suspensions) in D283 cells stably expressing miR-33b (Fig. 3A). When cultured in neurobasal medium, D283 cells expressing miR-33b formed fewer neurospheres compared to the control (Fig. 3B). In neurobasal medium, the mRNA levels of MYC and two stem cell markers, SOX2 and CD133, were reduced with miR-33b overexpression, while in growth medium, miR-33b introduction resulted in decreased expression of MYC, but not of CD133 and SOX2 (Fig. 3C). The expression of maternal embryonic leucine zipper kinase (MELK), a key regulator of neural stem cell proliferation (Nakano et al, 2005), was upregulated with miR-33b overexpression. We also determined the expression of another neural stem cell marker Musashi, an RNA-binding protein that is essential for neurosphere formation and proliferation (Kaneko et al, 2000; Sakakibara et al, 1996; Sakakibara et al, 2002). miR-33b-expressing D283 neurospheres had lower levels of Musashi. When cells were cultured in growth medium, Musashi expression, albeit low, was not altered significantly (Fig. 3D). These data support that miR-33b has a negative impact on the morphology and neurosphere formation of D283 cells.

**[00169] Lovastatin upregulates miR-33b expression in medulloblastoma cells.** We performed a small-scale screening assay to identify FDA-approved compounds that reduce medulloblastoma cell viability and increase miR-33b expression using Daoy, a medulloblastoma cell line with an intact 17pl 1.2 and no gene amplification of MYC (Stearns et al, 2006). Out of 727 chemicals, 12 reduced the viability of Daoy cells >40% and increased miR-33b expression >2-fold (Fig. 4A and Fig. 19 (Table)). Among the five (5) compounds that have not been indicated for cancer therapeutics, we singled out lovastatin due to the following reasons: (i) Its safety and efficacy have been tested for over thirty years, (ii) Statins are reported to reduce cancer risk, though the absolute risk reduction is likely low and there is evidence against their roles in cancer prevention (Demierre et al, 2005; Poynter et al, 2005), (iii) Statins or other cholesterol-lowering approaches upregulate miR-33A, the homolog of miR-33b (Horie et al, 2010; Marquart et al, 2010; Najafi-Shoushtari et al, 2010; Rayner et al, 2010), (iv) treating brain tumors requires the penetration of the blood-brain barrier (BBB), a separation of circulating blood and cerebrospinal fluid in the central nervous system; lovastatin is lipophilic and is able to penetrate the BBB, which comes with some side effects such as sleep disturbances (Botti et al, 1991; Guillot et al, 1993; Maron et al, 2000).
Lovastatin did not affect the proliferation of D283 cells (Fig. 4B). A qPCR assay showed that in Daoy cells, lovastatin induced miR-33b and SREBF1 (1C and 1A) expression in a dose-dependent manner, while MYC mRNA was down-regulated (Fig. 4C, top and middle; Fig. 10A). As expected, there was no significant change in MYC mRNA levels upon lovastatin treatment in D283 cells (Fig. 4C, bottom). Correspondingly, lovastatin caused reduction in c-Myc and cyclin E protein levels and upregulation of Gadd45a in Daoy but not in D283 cells (Fig. 4D). Cell cycle analyses demonstrated that a larger percentage of Daoy but not D283 cells were arrested at the G1 phase upon lovastatin treatment (Fig. 4E). We also treated UW228 cells, another medulloblastoma cell line without 17pl 1.2 abnormality or MYC amplification (Stearns et al, 2006), with lovastatin and found there was a significant induction of miR-33b and SREBF1C and a decrease in c-Myc expression (Figs. 11A and 11B). Other statins also upregulated miR-33b and inhibited Daoy growth, but less significantly (Fig. 10B). We tested the promoter of the human SREBF1~miR-33b gene with a reporter assay and found that lovastatin increased its activity in both Daoy and D283 cells (Fig. 4F). In addition, mevalonate inhibited the induction of miR-33b/a and SREBF1C and c-Myc down-regulation by lovastatin in Daoy cells. Lovastatin also slightly induced miR-33A expression but did not change MYC mRNA levels and mevalonate inhibited miR-33A induction in D283 cells (Fig. 4G and Fig. 4H). These data provide evidence that lovastatin activates miR-33b through the cholesterol biosynthetic pathway.

Like lovastatin treatment, exogenous miR-33b overexpression in Daoy cells resulted in down-regulation of c-Myc, cyclin E, and ODC and upregulation of Gadd45a (Fig. 12A), as well as decreased MYC mRNA levels (Fig. 12B). In addition, miR-33b overexpression led to a larger percentage of Daoy cells arrested at the G1 phase (Fig. 12C), decreased cell proliferation (Fig. 12D), and lowered miR-9 expression (Fig. 12E) along with reduced cell migration (Fig. 12F). We noted that the overexpression of miR-33b ranged from ~2- to 10-fold when determined 6-48 hrs posttransfection, higher than that with lovastatin treatment (~2-fold, Fig. 4C). We also subjected Daoy cells with or without miR-33b overexpression to the neurosphere formation assay and found that no neurospheres were formed. Nonetheless, these results demonstrate that miR-33b overexpression and lovastatin treatment have a similar impact on c-Myc expression and function in Daoy cells.
To establish the causative effect of c-Myc down-regulation mediated by miR-33b upon lovastatin treatment, we introduced antisense miR-33b inhibitors (Anti-miR-33b) into Daoy cells prior to lovastatin treatment. As shown in Fig. 41, the expression of c-Myc was upregulated with miR-33b inhibition without lovastatin treatment. More importantly, c-Myc down-regulation by lovastatin was rescued when miR-33b inhibitors were added (comparing lane 4 to 2 in the Left panel, Fig. 41). In addition, lovastatin-induced G1 cell cycle arrest was also abolished with miR-33b inhibition (Right panel, Fig. 41). These data support the causative relationship between miR-33b overexpression and c-Myc repression upon lovastatin treatment.

It should be noted that there are genes other than MYC negatively regulated by miR-33 and that lovastatin, like any other drug, impacts the expression of numerous genes beyond inhibiting HMG-CoA reductase. Abcal and Piml are two reported miR-33 target genes (Horie et al, 2010; Ibrahim et al, 2011; Marquart et al, 2010; Najafi-Shoushtari et al, 2010; Rayner et al, 2010; Thomas et al, 2012). We found that miR-33b overexpression down-regulated the protein levels of Abcal and Piml in both Daoy and D283 cells (Fig. 13A and 13C), demonstrating the efficacy of target repression. Potential therapeutic benefits of statin therapy in medulloblastoma were reported to be associated with Bcl2 and apoptosis (Bar et al, 2007; Macaulay et al, 1999; Wang & Macaulay, 1999; Wang & Macaulay, 2003). We found that Bcl2 was upregulated by lovastatin treatment in Daoy, but not in D283 cells (Fig. 13B and 13D); when miR-33b was overexpressed, Bcl2 levels were reduced in Daoy cells, but were increased in D283 cells (Fig. 13A and 13C). In addition, the expression of Abcal was down-regulated with lovastatin treatment in Daoy cells but not in D283 cells, while that of Piml was down-regulated in D283 cells but not in Daoy cells (Figs. 13B and 13D). These data provide evidence that the expression of these genes is impacted by unknown confounders other than miR-33b upon lovastatin treatment. We monitored cellular apoptosis using flow cytometry when Daoy or D283 cells were treated with lovastatin (10μM) or transfected with miR-33b and found little apoptosis in either cell lines. When lovastatin concentration was increased to 40μM, elevated apoptosis was observed in both cell lines with Daoy having a stronger response, which is in agreement with a previous report that Daoy is more sensitive to lovastatin than D283 (Dimitroulakos et al, 2001).

A low dose of lovastatin reduces xenograft medulloblastoma growth. We first treated mice subcutaneously xenografted with Daoy cells and found that either 1.0 mg/kg or 0.2
mg/kg of lovastatin resulted in smaller tumors and lower c-Myc expression (Figs. 14A-14D). We then tested whether lovastatin reduces the brain tumor burden in an orthotopic model. We inoculated Daoy or D283 cells into the brain of immunodeficient mice and treated them with 1.0 mg/kg lovastatin three times per week for four weeks. At the end of the regimen, all Daoy-bearing mice in the control group became moribund or paralyzed, yet mice in the lovastatin group appeared physically normal. We sacrificed all animal and found that Daoy-xenografted mice treated with lovastatin had less tumor expansion in the ventricles, and, strikingly, tumor invasion into the surrounding cerebellar tissues was completely blocked (Fig. 5A). In contrast, D283 xenografts neither invaded surrounding tissues nor responded to lovastatin treatment (Fig. 5A, Fig. 15A). Lovastatin treatment led to miR-33b upregulation and lowered expression of miR-9, c-Myc, and cyclin E in tumors of Daoy cells, but not in tumors of D283 cells (Fig. 5B, 5C, and 5D; Fig. 15B). There were few apoptotic cells in brain tissue sections from all four groups of mice as determined by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. There was no statistically significant change in the percentage of proliferating cell nuclear antigen (PCNA)-positive cells in control tumors versus treated Daoy tumors (Fig. 15C), providing evidence that cell cycle arrest rather than reduced cell proliferation is the likely cause of attenuated tumor growth in vivo.

We also performed another set of xenograft using Daoy cells and followed mice for an extended period with lovastatin treatment (1.0 mg/kg three times per week until they died). We found that mice treated with lovastatin had significantly better survival than the control group (median survival 55 versus 44 days, P = 0.0072; Fig. 5E). Necropsy revealed that there was massive tumor expansion in the ventricle of both groups. In the control group, tumor cells amassed in both the cerebral cortex (Figs. 16A - 16C) and the cerebellum (Fig. 16D). The widely distributed tumor nodules occupied the space of neurons, causing them to denature and swell (Fig. 16B), and many tumor nodules had established small blood vessels (Fig. 16C). In the cerebellum, the tumor mass occupied most of the cerebellum and destructed normal cerebellum structure (Fig. 16D). These morphological changes likely caused the loss of mobility and ability to eat and drink in these animals before they died. In mice with lovastatin treatment, the inoculated tumor cells were less involved in the cerebral cortex (Fig. 16E) and tumor expansion into the cerebellum was not observed.
Finally, we determined whether exogenous miR-33b expression reduces the tumorigenicity of D283 cells. There was significantly less tumor expansion in brain ventricles of mice injected with cells carrying miR-33b (Fig. 5F; Fig. 16D), and these tumors expressed a lower level of c-Myc and cyclin E compared to the control (Fig. 5G; Fig. 16E). These results indicate that the miR-33b gene is critical to medulloblastoma’s response to lovastatin treatment.

Negative correlation of MYC and SREBF1 expression in medulloblastoma. Based on histopathology, medulloblastoma has been traditionally classified into four subtypes: classical, nodular/desmoplastic, extensive nodular, and large cell-anaplastic. Recently, gene-expression profiling and gene copy number analysis have identified four major subtypes of medulloblastoma: WNT, Sonic hedgehog (SHH), Group C, and Group D. Group C exhibits c-Myc overexpression or amplification, lacks WNT pathway activation, and has the worst prognosis (so called Myc-driven subtype) (Eberhart, 2012). We analyzed SREBF1 (SREBFLA and SREBF1C were not distinguishable) and MYC expression in medulloblastoma from four published reports with openly available data on genetic and gene expression profiles, pathway signatures, and clinical pathological features (Park et al, 2012). We did not find a negative correlation between SREBF1 and MYC expression when all cases were included. WNT group is a distinct disease that arises in the dorsal brain stem and not in the cerebellum (Gibson et al, 2010). When the WNT subtype and the cases with 8q aberration were excluded (only one dataset had information on 8q aberration) (Kool et al, 2008), we found that the relative mRNA levels of SREBF1 and MYC were negatively correlated in a subset of cases with high MYC levels (n=29 in blue oval; Spearman correlation coefficient = -0.38, P=0.042; Fig. 17A). We noted that in 21 cases with survival data, patients with high MYC expression had poor survival. This was not statistically significant, but showed a trend (P=0.073 when SREBF1 expression is divided at the 33rd percentile; Fig. 17B). It is noteworthy that 6 out of 7 patients with SREBF1 expression below the 33rd percentile died within 4 years. SREBF1 expression was significantly lower in medulloblastoma tissues compared with that in normal brain (P = 2.2E-16; student’s t-test; Fig. 17A). In the absence of miR-33b expression data, these results implicate the association between down-regulation of SREBF1 (the host gene of miR-33b) and MYC overexpression/poor prognosis.

DISCUSSION. c-Myc is a transcription factor that has been implicated in the regulation of up to one-third of human genes (Amati et al, 2001; Frank et al, 2001), placing it at...
the center of many biological processes, including cell cycle, differentiation, and proliferation (Bouchard et al, 2004; Gatti et al, 2009; Podar et al, 2006; Stearns et al, 2006; Teleman et al, 2008). Many human cancers express high levels of c-Myc or one of its two paralogs, n-Myc or 1-Myc. c-Myc is overexpressed in 31-64% of medulloblastomas, the most common cerebella tumors of the central nervous system in children (Aldosari et al, 2002; Batra et al, 1995; Batra et al, 1994; Bigner et al, 1990; Eberhart et al, 2004; Herms et al, 2000). Recent gene expression profiling further isolates a group of medulloblastoma with c-Myc overexpression or amplification and this subtype of the disease has the worst survival (Eberhart, 2012). MYC gene amplification is found only in 5-8% of medulloblastoma (Aldosari et al, 2002; Herms et al, 2000; Stearns et al, 2006), providing evidence that other mechanisms exist to account for the increased c-Myc expression. The present disclosure describes the discovery that miR-33b that is frequently lost in medulloblastoma negatively regulates c-Myc expression and adversely affects cell proliferation, cell cycle progression, cell migration, and anchorage-independent colony formation. In addition, reintroduction of miR-33b into the medulloblastoma cell line D283 lacking the endogenous miR-33b gene reduces orthotopic xenograft tumor growth in immuno-deficient mice. These results provide evidence that miR-33b loss is a novel mechanism of c-Myc dysregulation in a subset of medulloblastomas and that miR-33b is a potent tumor suppressor that represses the oncogenic action of c-Myc. As medulloblastoma is thought to originate from abnormal stem cells (Blazek et al, 2007), we show that miR-33b introduction into D283 cells prompts cell morphology change and reduces neurosphere formation, accompanied by lowered expression of c-Myc and certain neural stem cell markers.

[00179] Beyond medulloblastoma (Fruhwald et al, 2001; Pan et al, 2005), the loss of chromosome locus 17pl 1.2 has also been observed in Smith-Magenis syndrome (SMS), a complex syndrome involving intellectual disabilities, sleep disturbance, behavioral problems, and other anomalies. SMS usually results from a genetic change that occurs during the formation of reproductive cells (eggs or sperm) or in early fetal development. Tumor predisposition in SMS is not well studied, yet neuroblastoma, another type of child brain tumor, was reported in an SMS patient (Hienonen et al, 2005). The RAI1 gene in 17pl 1.2 is responsible for most features of SMS (Girirajan et al, 2006), yet the contribution of miR-33b to variable features and overall severity of the SMS syndrome remains elusive.
Despite c-Myc’s role as a major oncogene and an attractive target for cancer therapeutic development for decades, attempts to identify specific chemicals to inhibit c-Myc directly have yet to produce a successful chemotherapeutic agent. In the experiments of this disclosure, we use a screening assay to identify FDA-approved chemicals that modulate the expression of miR-33b to down-regulate the expression and oncogenic activities of c-Myc. We have found that lovastatin, a natural compound first identified in the 1970s and one of the most widely used statins to lower cholesterol, upregulates the expression of miR-33b and reduces c-Myc expression and function in medulloblastoma cells (Daoy) with miR-33b alleles. Our results are consistent with a previous report showing that statins activate the host gene SREBF1 in some human cells (Rise et al, 2007). The effect of lovastatin on increased miR-33b RNA levels is at least partially due to activation of the genetic promoter of the SREBF1-miR-33b gene. Like any other miRNAs (Baek et al, 2008), miR-33b represses the expression of multiple targets like Abcal and Pim1, in addition to c-Myc. A recent article reports that overexpression of miR-33A or miR-33b induces a significant G1 arrest in cancer cell lines through targeting the cyclin-dependent kinase 6 (CDK6) and cyclin D1 (CCND1) genes (Cirera-Salinas et al, 2012), supporting that the miR-33 family is a regulator of cell cycle progression both directly (targeting CDK6 and CCND1) and indirectly (targeting c-Myc to reduce cyclin E expression). When miR-33b expression is inhibited in Daoy cells treated with lovastatin, c-Myc reduction is rescued and increased cell cycle arrest is reversed. This provides evidence that miR-33b induction plays a causal role in c-Myc down-regulation by lovastatin.

Statins have an established record of human safety and efficacy in cardiovascular disease prevention and show promise for cancer prevention, yet there has been intense debate over statins’ effect on cancer risk due to the limitation of observational studies and controversial results from meta-analyses (Dale et al, 2006; Demierre et al, 2005; Poynter et al, 2005). Previous studies showed that statins are effective in reducing xenograft tumor growth in mice when administered with a dose of >10 mg/kg (Laezza et al, 2008; Lin et al, 2008), which unfortunately would exceed the toxicity limit for all statins in humans (the maximum recommended therapeutic dose for lovastatin is 1.33 mg/kg/day).

Experiments of this disclosure include using, lovastatin to treat medulloblastoma xenograft tumors with a dose of 1.0 or 0.2 mg/kg, well within the range of pharmacological stimulation for human use. This provides evidence that medulloblastoma may be more sensitive
to lovastatin than other types of cancer. We are cognizant that lovastatin has pleiotropic effects as it may interact with diverse signaling pathways and targets, in addition to inducing miR-33b expression. At minimum, the expression of both miR-33A and SREBF1 is upregulated by lovastatin (Figs. 4A-4I): miR-33A may enhance the function of miR-33b since they share target genes (Horie et al, 2010; Marquart et al, 2010; Najafi-Shoushtari et al, 2010; Rayner et al, 2010) and the SREBF1 gene is implied to be pro-apoptotic (Gibot et al, 2009). Therefore, activation of the transcription of miR-33b to constrain the oncogenic activities of c-Myc represents one of the anti-cancer properties of statins. This disclosure documents, for the first time, and provides evidence of stimulatory pharmaceutical modulation of c-Myc via a miRNA using an existing medicine with sterling safety records, and provides evidence of a new approach to miRNA-based therapeutics by way of drug repurposing (i.e. in the form of a new, novel use and treatment based on a prior known drug or compound).
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[00183] It will be understood that various details of the presently disclosed subject matter can be changed without departing from the scope of the subject matter disclosed herein. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.
What is claimed is:

1. A method for diagnosing a cancer in a subject, comprising:
   (a) acquiring a biological sample from the subject;
   (b) determining an amount in the sample of a miR-33b microRNA; and
   (c) comparing the amount of the miR-33b microRNA in the sample, if present, to a control level of the miR-33b microRNA.

2. The method of claim 1, wherein the subject is diagnosed as having cancer or a risk thereof if there is a measurable difference in the amount of the miR-33b microRNA in the sample as compared to the control level.

3. The method of claim 1, further comprising providing an apparatus capable of affecting detection of the miR-33b microRNA.

4. The method of claim 1, further comprising the step of providing a probe for selectively binding the miR-33b microRNA.

5. The method of claim 1, wherein the cancer is a statin resistant cancer.

6. The method of claim 1, wherein the cancer is a medulloblastoma.

7. The method of claim 1, wherein the biological sample comprises blood, plasma, or serum.

8. The method of claim 1, wherein the subject is human.

9. The method of claim 1, wherein determining the amount in the sample of the miR-33b microRNA is conducted using a probe for selectively binding the miR-33b microRNA.
10. The method of claim 9, wherein the probe comprises a nucleic acid molecule for selectively binding with the miR-33b microRNA.

11. The method of claim 1, wherein determining the amount in the sample of the miR-33b microRNA is conducted using polymerase chain reaction.

12. The method of claim 1, further comprising selecting a treatment or modifying a treatment for the cancer based on the determined amount of the miR-33b microRNA.

13. The method of claim 1, wherein the miR-33b microRNA has a nucleic acid sequence of SEQ ID NO: 1.

14. A method for determining whether to initiate or continue prophylaxis or treatment of a cancer in a subject, comprising:
   (a) acquiring a series of biological samples over a time period from the subject;
   (b) analyzing the series of biological samples to determine an amount in each of the biological samples of a miR-33b microRNA; and
   (c) comparing any measurable change in the amounts of the miR-33b microRNA in each of the biological samples.

15. The method of claim 14 further comprising (d) determining whether to initiate or continue the prophylaxis or therapy of the cancer based on identifying any measurable change in the amounts of the miR-33b microRNA.

16. The method of claim 14, wherein the cancer is a statin resistant cancer.

17. The method of claim 14, wherein the cancer is a medulloblastoma.

18. The method of claim 14, wherein the biological sample comprises blood, plasma, or serum.
19. The method of claim 14, wherein the subject is human.

20. The method of claim 14, wherein the series of biological samples comprises a first biological sample collected prior to initiation of the prophylaxis or treatment for the cancer and a second biological sample collected after initiation of the prophylaxis or treatment.

21. The method of claim 14, wherein the series of biological samples comprises a first biological sample collected prior to onset of the cancer and a second biological sample collected after the onset of the cancer.

22. The method of claim 14, wherein determining the amount in the sample of the miR-33b microRNA is conducted using a probe for selectively binding the miR-33b microRNA.

23. The method of claim 14, wherein the miR-33b has a nucleic acid sequence of SEQ ID NO: 1.

24. A system for use in diagnosing or prognosticating a cancer in a subject, comprising a probe for determining an amount of an miR-33b microRNA in a sample obtained from the subject.

25. A method for treating a cancer, comprising administering to a subject, in need of treatment therefrom, an effective amount of a pharmaceutical agent capable of upregulating expression of an miR-33b microRNA.

26. The method of claim 25, wherein the pharmaceutical agent is a statin.

27. The method of claim 25, wherein the statin is lovastatin.

28. The method of claim 25, further comprising identifying a subject for treatment by acquiring a biological sample from the subject, determining an amount of miR-33b microRNA and comparing the amount of miR-33b microRNA in the sample, if present, to a control level of
miR-33b microRNA.

29. The method of claim 28, wherein the administering to the subject an effective amount of the pharmaceutical agent is done based on the determined presence of miR-33b microRNA.

30. A nucleic acid vector comprising a nucleic acid vector or probe having a nucleic acid sequence of SEQ ID NO: 1 or its antisense sequence, along with a non-natively occurring 3' end and 5' end covalently bonded thereto, which nucleic acid vector or probe can bind to miR-33b microRNA.
**Figure 1A**
Figure 1B

Figure 1C
**Figure 1D**

Histogram showing the RLU (Relative Light Units) for pE2F2-Luc and pE2F2mut-Luc constructs with different treatments (Vector and miR-33b).

**Figure 1E**

Sequence comparison showing 3'UTRWT, miR-33b, and 3'UTRMut with their respective RLU values under different conditions.
Figure 1F

Figure 1G
miR-33b 3' CGUUACGUUGUGCU-UUACGUG
MYC 3'UTR 5' CAAAUGCAUGACAAAUGCAAC
miR-33a and miR-33bM 3' CGUUACGUUGAUG-UUACGUG

Figure 1H

1.0 0.6 1.1 1.1

- c-Myc
- Cyclin E
- Gadd45α
- ODC
- β-Actin

Vector miR-33b miR-33a miR-33bM

Figure 1I
Figure 2C

Figure 2D
Figure 2E

Figure 2F
Figure 3C

Figure 3D
Figure 4A

Figure 4B
Figure 4C
Figure 5B
Figure 5G
Figure 7A
Figure 7B

Figure 8A
Figure 8B

Figure 9A
Figure 9B

Figure 9C
Figure 11A

Figure 11B
Figure 12A

Figure 12B
Figure 12C

Figure 12D
Figure 13A

Figure 13B
D283

**Figure 14C**

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**Figure 14D**
Figure 15A

Figure 15B
Figure 16A

Figure 16B
Figure 16C

Figure 16D
Figure 16E

Figure 17A
Figure 18B
FDA-approved drugs that upregulate miR-33b in Daoy Cells

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Figure 19
### A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/574(2006.01)i, C12Q 1/68(2006.01)i, C12N 15/l1(2006.01)i

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)

G01N 33/574; A61P 3/06; A61K 31/7088; C12Q 1/68; C12N 15/11

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & Keywords:miR-33b, diagnosis, vector, probe, antisense

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>X</td>
<td>WO 2007-081740 A2 (THE OHIO STATE UNIVERSITY RESEARCH FOUNDATION) 19 July 2007 See abstract; pages 12, 18, 51-60 and 66-67; and claims 1-33.</td>
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<td>US 2012-0053229 Al (NAAR) 01 March 2012 See figure 17; and paragraphs [0041]-[0045], [0056]-[0059] and [0068]-[0070].</td>
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<td>GERIN et al., &quot;Express ion of miR-33 from an SREBP2 int ron inhi bits cho lest ero l export and fat ty acid oxi dat ion&quot; Journal of Biologi cal Chemistry, Vol.1285, No.44, pp.33652-33661 (2010) See the whole document.</td>
<td>24,30</td>
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<td>ROTTIERS et al., &quot;mi croRNAs in met abol ism and met abol ic disorders &quot; Nature Revi e ws Mol ecule ar Cell Biology, Vol.13, No.4, pp.239-250 (April 2012) See the whole document.</td>
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<td>LV et al., &quot;Genetic alter at ions in mi croRNAs in medul obl astomas &quot; Brain Pathoology, Vol.122, No.2, pp.230-239 (Epub 05 Sept ember 2011) See the whole document.</td>
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See patent family annex.

Further documents are listed in the continuation of Box C.

Date of mailing of the international search report

04 December 2013 (04.12.2013)

Date of the actual completion of the international search

02 December 2013 (02.12.2013)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office

189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea

Facsimile No. +82-42-472-7140

Authorized officer

Kim, Seung Beom

Telephone No. +82-42 481-3371
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<td>TAKWI et al., &quot;A st at in-regul at ed microRNA represes human c-Myc express ion and function&quot; EMBO Molecular Medicine, Vol. 4, No. 9, pp. 896-909 (Epub 07 August 2012) See the whole document.</td>
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This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [x] Claims Nos.: 1-23,25-29 because they relate to subject matter not required to be searched by this Authority, namely:
   Claims 1-23 and 25-29 pertain to methods for treatment of the human body by therapy, as well as diagnostic methods practiced on the human body, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

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

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

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

2. [ ] As all searchable claims could be searched without effort, justifying an additional fee, this Authority did not invite payment of any additional fee.

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

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

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