MicroRNA in Cancer Biology
Think Tank

December 1-2, 2008
Bethesda, Maryland

A National Cancer Institute (NCI) Think Tank entitled *MicroRNA in Cancer Biology* was held Dec 1-2, 2008 in Bethesda, Maryland. The Think Tank was organized by Chamelli Jhappan (NCI, Division of Cancer Biology) and was co-chaired by Scott Lowe (Cold Spring Harbor Laboratory) and Tyler Jacks (Massachusetts Institute of Technology). Think Tank participants included scientists engaged in leading-edge research in the field of microRNA biology. Wide-ranging presentations and discussions at the Think Tank thoroughly summarized the state-of-the-science of microRNA biology and its role in human cancer. The following document summarizes the main points of the seminar presentations and the key outcomes of the open discussion sessions from the Think Tank.

Introduction

MicroRNA are 21-24 nucleotide non-coding RNA molecules transcribed by RNA polymerase II that form short stem loop structures. MicroRNA molecules bind to and usually destabilize or inhibit translation of target mRNA molecules in the cytoplasm. The primary microRNA transcript forms a predicted hairpin duplex with 2-nucleotide 3' – overhangs, which is processed to the mature short stem-loop microRNA by sequential action of Drosha and Dicer RNAe III endonucleases.

Since their initial discovery in *Caenorhabditis elegans*, microRNA have been identified, catalogued and characterized in multiple species and phyla including *Arabidopsis thaliana* (151), *Mus musculus* (491), human (677), *Drosophila melanogaster* (147), *Caenorhabditis elegans* (154), *Physcomitrella patens* (moss) (263) and *Chlamydomonas reinhardtii* (72)). However, microRNA have not been detected to date in *Saccharomyces cerevisiae, Schizosaccharomyces pombe* or *Neurospora crassa*.

It has been estimated that the human genome encodes more than 1000 microRNA species, but relatively few predicted microRNA or their mRNA targets have been experimentally verified. MicroRNA are thought to play a role in regulating many biological processes including apoptosis, DNA repair, cell proliferation and differentiation. With regards to carcinogenesis, microRNA that downregulate tumor suppressor genes are pro-carcinogenic, and microRNA that downregulate oncogenes are anti-carcinogenic.

The analysis of microRNA function and expression involves microRNA expression profiling, quantification of individual microRNA species, analysis of microRNA processing pathways, identification of microRNA target genes and characterization of functional effects on microRNA target genes. Prediction of microRNA expression and/or function by computational analysis is feasible but not yet extremely reliable.

There is a great deal of interest in defining the role played by microRNA in cancer, and the possible utility of microRNA as diagnostic/prognostic tools or therapeutic targets in cancer cells. microRNA expression profiling demonstrated that unique microRNA signatures are associated with different types of tumors, and that multiple microRNA species are differentially expressed in cancer cells. In some cases, specific microRNA signatures

correlate with improved patient survival, and thus microRNA expression profiles may have potential prognostic value for some cancer patients. The regulation of microRNA expression is complex, and may involve gene amplification and/or deletion, specific cell signaling pathways (*i.e.*, p53, c-Myc, hypoxia), and epigenetic mechanisms.

Additional studies of microRNA expression and function will likely lead to novel insights into mechanisms of human carcinogenesis. In the future, it is expected that microRNA profiling may have both diagnostic and prognostic value and that some microRNA may be valuable therapeutic targets in human cancer patients.

Summary of Think Tank Seminar Presentations

David Bartel (Massachusetts Institute of Technology)
MicroRNA and Their Regulatory Targets

Individual microRNA species bind to and destabilize or repress transcription of multiple mRNA regulatory targets. Therefore, the identification and validation of microRNA regulatory targets is a critical step in analyzing microRNA function. Commonly used approaches to identify microRNA targets include bioinformatics-based target prediction, q-PCR of putative mRNA targets, immunoprecipitation of microRNA/mRNA complexes, analysis of target site conservation, and reporter assays. While these approaches are all useful, it is also important to evaluate microRNA-mediated repression at the protein level.

Recently, a novel method was used to evaluate the effects of expressing a single microRNA, miR-223, on global mRNA and protein expression in the mouse. For this purpose, bone marrow progenitor cells were isolated from male wild type and male miR-223-knockout mice, and cultured *in vitro* in media containing C^{12}-lysine and arginine or C^{13}-lysine and arginine, respectively, as well as G-CSF and SCF to induce the cells to differentiate into mature neutrophils. Total protein was isolated, trypsinized and analyzed by quantitative mass spectrometry. Approximately 4000 protein species were resolved and quantified in miR-233-proficient and miR-233-deficient cells, and a set of putative miR-233 targets identified. 3'-UTRs corresponding to putative miR-233 targets were analyzed for predicted miR-233 binding sites and global mRNA profiling was carried out in miR-233-proficient and miR-233-deficient neutrophils.

These experiments led to the following conclusions: Few miR-233 targets are translationally repressed ≥33%; of targets repressed ≥33% by miR-233, most demonstrate significant mRNA destabilization; of miR-233 target mRNA with at least one predicted 7mer binding site (AACUGAC or ACUGACA), 18% were differentially upregulated in miR-233 knockout cells; of target mRNA with at least one predicted 8mer binding site (AACUGACA), 33% were differentially upregulated in miR-233 knockout cells; putative miR-233 targets were more likely to be repressed if the putative miR-233/mRNA interaction was outside the protein coding region, was a conserved site, or was in a favorable local DNA sequence context.

Several algorithms have been developed to predict microRNA/mRNA interactions. These include TargetScan, PITA, RNA22, EIMM02, MiRanda, MirBase, mirWIP and PicTar. The ability of these algorithms to predict miR-233 targets was estimated by calculating mean $\log_{-2}$ fold-change for all predicted targets, based on empirically measured repression in miR-233 proficient cells, as described above. For all targets, for all algorithms, mean $\log_{-2}$ fold repression varied widely, from less than 0.05 to approximately 0.18. These values improved to 0.15 to 0.65, if only targets with at least one putative 8mer binding site were considered.

Factors favoring microRNA binding to predicted targets were high AU content adjacent to the seed site, favorable target position in the 3'UTR, or productive 3'-pairing adjacent to the seed pairing.

For the most highly conserved microRNA in mammals, there are estimated to be >450 conserved targets/per microRNA. Therefore, there are likely to be >45,000 conserved microRNA target sites in human 3'UTRs. This suggests that >60% of human protein-coding genes are under selective pressure to maintain pairing to microRNA.

Scott Hammond (University of North Carolina, Chapel Hill)
MicroRNA in Development and Disease

Global analysis of microRNA expression during mouse embryogenesis revealed that microRNA fall into one of two developmental expression patterns: some microRNA are expressed during early embryonic stages and decline to low or absent expression in later embryonic stages (i.e., miR17-92, miR290-295); other microRNA are absent during early embryonic stages and demonstrate increasingly higher expression in later embryonic stages (i.e., let7, miR-125). The latter group of microRNA are generally restricted to differentiated cell types. This suggests that microRNA expressed in early embryogenesis may promote and maintain cells in an undifferentiated state, while microRNA expressed later in embryogenesis may promote or maintain specific pathways of cellular differentiation. Interestingly, cancer cells tend to overexpress microRNA that belong to the former group and repress microRNA that belong to the latter group.

miR-17-92 is a cluster of 6 miRNA co-expressed on a polycistronic primary transcript encoded on human chromosome 13. In the mouse, miR-17-92 is expressed early in development and then declines during development. To determine if miR-17-92 promotes cell proliferation and cancer, it was overexpressed in Eμ-myc hematopoietic stem cells, which were then injected in recipient mice. The results showed that expression of miR-17-92 in this leukemia model strongly decreased overall and leukemia-free survival in recipient mice. These data indicate that miR-17-92 is oncogenic when expressed in hematopoietic stem cells in the adult mouse. Additional published studies also suggest that miR-17-92 is procarcinogenic in mouse models of lung cancer. The procarcinogenic role of miR-17-92 is consistent with the fact that its expression is positively-regulated by c-Myc and E2, and putative binding sites for E2F in the miR-17-92 cluster are highly conserved.

The let-7 group of microRNA are not expressed in early mouse development, but are induced later in development by an unknown mechanism. Let-7 microRNA are often repressed in human cancer cells, suggesting that they may target oncogenes and be functionally equivalent to a tumor suppressor. In fact, recent studies indicate that let-7 microRNA suppress expression of Ras and other oncogenic proteins. This suggests that re-expression of microRNA that downregulate oncogenic proteins could be a useful therapeutic approach for human cancer.

Previous studies showed that mature let-7 microRNA are induced at mid-gestation. However, the primary transcript for let-7 is constitutively expressed throughout mouse embryogenesis, even though little or no precursor transcript is detected at any mouse developmental stage. A similar pattern is observed for other microRNA that are induced during mid-gestation or later during mouse development. Thus, these microRNA are not regulated at the level of transcription. One possible explanation for these data is that an inhibitor or activator regulates DROSHA-dependent processing of specific microRNA primary transcripts. This idea was

confirmed using an in vitro DROSHA processing assay with extracts from P19 cells, HeLa cells or mixed extracts. The results showed that extracts of P19 cells process exogenous miR-17 but not exogenous let-7g, extracts from HeLa cells process both miR-17 and let7-g, and mixed extracts process exogenous miR-17 but not exogenous let-7g. Thus, the phenotype of P19 cells is dominant, suggesting that p19 cells express a diffusible inhibitor that specifically prevents processing of let-7g.

The let-7g loop region is evolutionarily conserved, suggesting that the loop might play a role in a conserved regulatory interaction, such as binding to a protein that inhibits its processing. This idea is consistent with the facts that mutations in the let-7g loop region or the presence of exogenous loop mimics or competitor microRNA partially relieve inhibition of Drosha processing in p19 extracts. Furthermore, RNA binding proteins lin28 and lin28B were highly enriched by affinity purification to let-7 transcripts and ectopic expression of lin-28 depleted let-7 transcripts and inhibited processing by Dicer. These data support the conclusion that lin-28 is necessary and sufficient for regulated Drosha-dependent processing of let-7 transcripts. The mechanism by which other microRNA are up- or down-regulated during development and/or differentiation remains to be determined.

Judy Lieberman (Harvard University)
miR-24: a Regulator of Cell Proliferation and DNA repair

Two cell culture model systems, using K562 cells or HL60 cells, have been developed to study differentiation of hematopoietic precursor cells into megakaryocetes/erythrocytes or macrophges/moncytes/granulocytes, respectively, after treatment with TPA, Hemin or Vitamin D3 (HL60 cells only). microRNA profiling of these cells revealed that only the miR-24 cluster was consistently upregulated ≥2-fold under all differentiation-inducing conditions tested. This suggests that miR-24 may play an important biological function related to hematopoietic differentiation.

To explore this possibility, two approaches were taken to identify and validate regulatory targets of miR-24. First, the subset of transcripts downregulated ≥ 2-fold in miR-24 expressing cells (148 transcripts) were screened using TargetScan for the presence of predicted miR-24 binding sites. The resulting subset of 100 genes were then subject to bioinformatic analyses, including analysis of enriched gene ontology (GO) classifications and Ingenuity-based pathway analysis. Strong enrichment was observed among these putative miR-24 targets for GO terms related to cell proliferation and DNA repair (*i.e.*, regulation of cell cycle, cell cycle arrest, cell cycle, cell cycle checkpoint , DNA damage checkpoint, double-strand break repair, recombinational repair, DNA recombination). Furthermore, a subset of these genes, including MYC, MCM4, CDKN1B, H2AX and VHL, form network interactions that surround MYC as a central node. Consistent with these results, downregulation of miR-24 via antisense oligonucleotides stimulated cell proliferation in K562 cells, overexpression of miR-24 suppressed cell proliferation of HepG2 cells and transfection of a miR-24 mimic caused hypersensitivity to γ-irradiation-induced DNA damage in K562 cells.

MYC and H2AX were directly validated as miR-24 targets by qRT-PCR, immunoblot, and reporter assays with wild type and mutant putative 3-UTR binding sites. Overexpression of miR-24 downregulated MYC mRNA and protein ≥5-fold, and down-regulated H2AX protein ≥10-fold. However, both MYC and H2AX were downregulated by a physiological increase in miR-24 (*i.e.,* 4- to 8-fold). Co-transfection of miR-24 and H2AX cDNA caused more DNA damage than transfection of H2AX cDNA alone, but only when the H2AX cDNA included

putative miR-24 binding sites, and antisense to miR-24 decreased DNA damage as detected by comet assay, in non-dividing cells treated with bleomycin.

A novel affinity-pulldown approach was also used to identify putative miR-24 targets. For this purpose, 3'-tagged miR-24 was transfected into cells expressing very low endogenous miR-24, miR-24/mRNA complexes were isolated by affinity pulldown, and enriched mRNA were quantified by qRT-PCR. Control experiments showed that 3'-tagged miR-24 associated with polysomes containing argonaute proteins and RISC complexes, and that known targets of miR-24, including H2AX, were enriched in the affinity-purified fraction by this method. The affinity-purified fraction was enriched in 269 putative miR-24 targets, 59 of which were also identified as putative miR-24 targets by TargetScan and/or mRNA profiling. This gene subset included MYC and H2AX, and was strongly enriched for genes with GO terms related to cell proliferation, DNA repair and DNA replication. Ingenuity analysis revealed that these genes form a highly connected network with MYC as a central node, and subnodes including BRCA1, PCNA, CDC25A, Aurora B, CDKN1B, CDK4 and E2F2. Selected genes in this network, including E2F2, were validated as miR-24 targets by the approaches described above. It was noted that approximately 14% of the genes in this network were not predicted by TargetScan to be miR-24 targets, and some of the targets lack seed-pairing, suggesting that the affinity pull-down method may identify biologically-important microRNA targets, that are not identified by bioinformatics-based methods.

These data strongly implicate miR-24 as a putative negative regulator of MYC-driven cell proliferation, and suggest a biological role for miR-24 in suppressing proliferation and DNA repair in terminally differentiated cells.

Richard Gregory (Children's Hospital Boston)

Regulation of microRNA Biogenesis in Embryonic Stem Cells and Cancer

OncomiRs map within fragile sites on human chromosomes, which tend to undergo amplification or deletion in cancer cells. It has been proposed that altered expression of miRNA from these chromosomal regions could be diagnostic or prognostic cancer biomarkers. Oncogenic microRNA include miR-17-92 and miR-155, while anti-oncogenic microRNA (*i.e.*, tumor suppressors) include miR-15-16 and the let-7 family.

MicroRNA profiling in multiple human cancer cell lines indicated that microRNA expression is globally downregulated, although some microRNA are overexpressed in specific cancers. This could reflect global downregulation of microRNA processing: for example, low level expression of Dicer. This possibility is consistent with the observation that Dicer knockdown promotes and accelerates tumor growth in a mouse lung cancer model, and higher Dicer expression or higher let-7 expression correlates with higher survival in human lung cancer patients.

Recent studies showed that let-7 is developmentally regulated at the level of processing and this regulation is mediated by lin28/28B, a protein that binds to the loop region of the let-7 primary transcript (pri-let7) and inhibits its processing by Drosha (pri-let-7 to pre-let-7) and Dicer (pre-let-7 to mature let-7). Because let-7 is a tumor suppressor, this suggests that lin28/28B could be an oncogene. This is consistent with the fact that lin28 is upregulated in poorly differentiated liver cancer cells. Lin28 has a cold shock domain (CSD) and a zinc finger domain, both of which are required for binding to let-7. Immunological studies show that lin28 localizes to the cytoplasm and nucleolus, as expected for an RNA binding protein.

Interestingly, terminal uridylation of the pre-let-7a transcript, which requires lin28, destabilizes pre-let-7a, and lin28 is associated with a TUTase activity. These data suggest that lin28 may downregulate let-7 both by inhibiting its processing and by promoting its uridylation and subsequent degradation.

Dinesh Rao (California Institute of Technology)

A Comparative Analysis of Two microRNA Involved in Hematopoeitic Development and Cancer

MicroRNA profiling indicated that miR-155 and miR-34a are overexpressed in acute myeloid leukemia cell lines. The miR-155 gene is at or near a common integration site for avian leukemia virus, and miR-155 expression leads to c-Myc-dependent lymphomagenesis in the chicken. miR-155 is also overexpressed in diffuse large B cell lymphoma and Hodgkin's lymphoma in humans. Transgenic expression of miR-155 from the Eµ promoter leads to B cell neoplasia in mice, and miR-155 knockout mice have defects in germinal center formation and antibody responses. p53 regulates expression of miR-34a, which in turn downregulates several oncogenes, cell cycle progression genes and anti-apoptotic factors. Overexpression of miR-34a in cultured cells inhibits cell cycle progression and leads to growth arrest and apoptosis. Downregulation of miR-34a causes susceptibility to DNA damage in cultured cells.

The biological roles of miR-155 and miR-34a were explored using lethally-irradiated C57BL6 mice as a host for virally-transfected miR-overexpressing hematopoietic stem cells (HSCs). The virally-transfected cells were labeled with GFP, so their fate could be readily tracked in the host animal. In this *in vivo* system, overexpression of miR-155 caused a myeloproliferative disorder characterized by fewer circulating platelets, red and white blood cells, fewer erythrocytes in the marrow, more Mac1 positive cells in the bone marrow as well as splenomegaly and extramedullary hematopoiesis. Several putative miR-155a targets were identified, that could play roles as downstream mediators of these effects.

In contrast, mice overexpressing miR-34a in grafted HSCs showed normal numbers of circulating white and red blood cells and platelets, but reduced numbers of peripheral blood leukocytes and B lymphocytes. More detailed studies suggested that overexpression of miR-34a causes a severe block in maturation of B cells at the pro-B to pre-B transition. One relevant target of miR-34a could be Foxp1, which has two putative miR-34a target sites in its 3-UTR and which is strongly downregulated in miR-34a overexpressing cells. Consistent with this hypothesis, siRNA-mediated knockdown of Foxp1 *in vivo* blocks B cell differentiation at the pro-B to pre-B transition, and produces a phenocopy of miR-34a overexpression in the mouse.

Carlo Croce (Ohio State University)

MicroRNA and Human Cancer

Chronic lymphocytic leukemia (CLL) is strongly associated with chromosomal aberrations, including deletions and translocations on human chromosomes 8, 13 and 14, with 13q14, the most commonly deleted region associated with this disease. B cell lymphomas are also associated with transolcations on chromosome 14, and are linked to the protein coding region TCL1 (T cell lymphoma 1). Interestingly, transgenic overexpression of TCL1 in mice under control of a V_H promoter-IgH-Eµ enhancer causes a phenocopy of the aggressive form of human CLL. Subsequent studies indicate that TCL1 is regulated by miR-29b and miR-181

(microRNA that are differentially expressed in CLL clones) and that miR-29a dysregulation is a causal factor in CLL. Similarly, extensive genetic analysis failed to associate a specific protein-coding gene on chromosome 13 with CLL, even when the chromosomal interval had been narrowed to a 34 kb region containing informative translocation breakpoints. However, the CLL-linked region of human chromosome 13 encodes two microRNA, miR-15 and miR-16, which could potentially play a role in the etiology of this disease. These data are consistent with the observation that many microRNA in the human genome map to fragile sites or cluster in regions associated with human cancers. In particular, miR-15 and miR-16 are downregulated in more than 60% of all CLL cases and in 100% of the indolent (less-aggressive) form of CLL.

Global microRNA expression analysis showed that human normal and tumor tissues have distinct microRNA expression signatures, in the same manner as human normal and tumor tissues and cancer cell types have distinct mRNA expression signatures. Bioinformatic analysis identified 13 microRNA that are specifically up- or downregulated in CLL, and mutations in some of these microRNA, including miR-15a/16-1, are more frequent in CLL patients than in normal controls. microRNA signatures were sufficient to distinguish patients with the indolent and aggressive forms of CLL, suggesting that microRNA signatures may have potential as prognostic indicators for CLL.

Bioinformatic analysis predicted that Bcl-2 was a possible target of miR-15/16. Bcl2 was confirmed as a target of miR-15/16 by comparing Bcl-2 expression in CLL cell lines. This experiment showed that Bcl-2 expression was inversely correlated with miR-15/16 expression in a panel of CLL cell lines. In addition, a cell line that did not express miR-15 or miR-16 expressed a very high level of Bcl-2, which is consistent with Bcl-2 as a target for downregulation by miR-15/16. Bcl-2 expression decreased when these cells were transfected with wild type but not with mutant miR-15a or miR-16-1 precursor. These results suggest that miR-15/16 acts as a natural antisense RNA for Bcl-2, and that it is capable of strong down-regulation of Bcl-2 expression in vivo.

miR-155 is commonly overexpressed in the aggressive form of CLL and in human B cell lymphomas including Burkitt's lymphoma and Hodkin's lymphomas. To test the idea that miR-155 plays a direct role in driving carcinogenesis, cancer susceptibility was examined in transgenic mice overexpressing miR-155 in B cells. These mice developed aggressive B cell lymphomas as early as 4 weeks and were dead by 6-7 months. This result strongly supports the hypothesis that dysregulation of microRNA can play a significant role in human cancer.

MicroRNA expression profiling in solid tumor tissues revealed that 21 microRNA are commonly up-regulated in 3 or more common solid tumors (i.e., breast, lung, colon, pancreas, stomach and prostate). The most commonly upregulated microRNA were miR-21, miR-17-92 and miR-191. Dysregulation of miR-191 is associated with chromosomal translocations in patients with acute lymphocytic or acute myelogenous leukemia (ALL/AML), suggesting that miR-191 may play a role in the pathogenesis of this disease. In addition, germline mutations have been identified in microRNA genes in CLL patients. In the case of miR-29b, a polymorphic variant correlates with differential survival among CLL patients, suggesting that genetic screening for microRNA variants could have diagnostic or prognostic value.

The observation that some microRNA are overexpressed in several different solid tumor types suggests that they may be downstream targets of pathways that are activated in

cancer. These and other data suggest that these microRNA might be excellent targets for oncology therapeutics.

Janet Rowley (University of Chicago)

microRNA in Human Acute Leukemia: Where Should We Go Next?

Specific chromosome aberrations, including t(6,9), t(8,21), t(15,17), t(11q) and Inv(16) are reproducibly observed in patients with acute myeloid leukemia (AML). Specific aberrations tend to correlate with early or late disease onset and different survival rates. In some cases, chromosomal translocations are associated with altered expression of specific genes, such as retinoic acid receptor alpha. With the exception of all-trans retinoic acid therapy for acute promyelocytic leukemia, there are no genotype-specific treatments for AML.

Expression studies of 112 microRNA in 72 acute leukemia patients revealed microRNA signatures that fell into 3 distinct clusters. One signature correlated with ALL (low let-7) and one signature correlated with AML (high let-7). Additional microRNA profiling identified a 7 microRNA classifier that was sufficient to molecularly identify specific chromosomal aberrations associated with ALL/AML. This classifier included the miR-17-92 cluster at 13q31 and miR126/126*. Transfection of miR-17-92 and *MLL-ELL* (an AML-associated translocation) synergistically promoted cell proliferation in culture, which is consistent with the previously-established oncogenic nature of miR-17-92 and known targets of miR-17-92, such as RB, E2F1, RASSF2 and BCL2L11. Differential expression of miR-126/126* in ALL was associated with lower levels of CpG methylation in the microRNA gene region; however, miR-126 expression was further enhanced by treatment with demethylating agents such as TSA. Transfection of miR-126 and *AML1-ETO* synergistically promoted cell proliferation and inhibited apoptosis in cell culture, and polo-like kinase (PLK1) was validated as a miR-126 target using reporter assays.

Although the above data suggest that the role of microRNA in ALL is beginning to be understood at the molecular level, many questions remain unanswered about microRNA biology and how it can be exploited in cancer research or its translational application in a clinical setting. For example: in many cases, it is not yet known how microRNA expression is regulated or which mRNA targets or networks are affected by normal or aberrant microRNA expression; optimal methods for validating microRNA targets or determining microRNA function have not been developed; it is not yet clear whether or which microRNA should or can be developed as diagnostic or prognostic indicators. These questions will need to be answered before it is clear whether or which microRNA are suitable therapeutic targets for human cancer.

Joe Gray (Lawrence Berkeley National Laboratory)

MicroRNA in Breast Cancer - Molecular Correlates to Therapeutic Targets

A group of 50 human breast cancer cell lines have been extensively characterized using multiple molecular genetic and molecular biology tools, such as high resolution copy number analysis (CNV), mutational studies, mRNA and microRNA profiling, epigenetic analyses, proteomics, cell growth profiles and drug susceptibility. This extensive data set provides an opportunity to perform correlative studies, such as comparative analysis of mRNA and microRNA signatures in breast cancer subtypes. Expression analyses of >400 microRNA (array-based and q-PCR-based), as well as other approaches, segregated the cell lines into

basal and luminal subtypes, suggesting that patterns of microRNA expression reflect and/or contribute to biological events involved in breast carcinogenesis. As many as 4700 microRNA/mRNA negative correlations were identified (using qPCR), a small fraction of which (2%) were predicted by microRNAMap and microRNA.org. Pathway analysis based on these data suggested that the PI3K signaling pathway may be differentially affected in breast cancer cells, possibly by a mechanism involving microRNA dysregulation. This is consistent with the fact that basal and luminal breast cancers are differentially susceptible to drugs that target the PI3K pathway.

Additional studies were performed to test whether microRNA signatures in subsets of the 50 cell lines correlated with susceptibility to >50 cancer therapeutics. These drugs included herceptin, iressa, lapatinib, docetaxel, paclitaxel, doxorubicin, cisplatin and others. For example, 15, 35 or 2 microRNA (miR-320 and miR-423) were predictors of susceptibility to lapatinib by microarray, q-PCR, or both methods, respectively. miR-320 and miR-423 share 758 predicted targets, including ERBB2 and CRK. A 32-gene subset of these 758 putative microRNA targets are also mRNA predictors of lapatinib responsiveness, and a 6-gene clinical signature that stratifies ERBB2-positive breast cancer patients for responsiveness to lapatinib therapy also includes ERBB2 and CRK.

Human chromosomal region 8q24 is one of the most commonly amplified regions in all human solid cancers. 8q24 is a relatively gene poor region containing Myc and a poorly characterized locus called PVT1. siRNA Knockdown of PVT1 and taxane synergistically stimulate apoptosis and inhibit tumor growth in a mouse xenograft tumor model. Although the PVT1 transcript was previously characterized as a non-coding RNA, it was not predicted to encode a microRNA. Nevertheless, recent re-annotation of the PVT1 region identified a cluster of 5 putative microRNA, including miR-1204 immediately downstream of Myc. miR-1204 lies within the highly-amplified region of 8q24. AntagomiR-1204 suppressed the PVT1 transcript more efficiently than siRNA to PVT1, and antagomiR-1204 also stimulated apoptosis and inhibited proliferation in cell lines amplified for 8q24. Ongoing studies are evaluating putative miR-1204 targets.

Joshua Mendell (Johns Hopkins University)

MicroRNA Reprogramming by Myc: Opportunity for Specific Anti-cancer Therapy

c-Myc is an oncogenic transcription factor that regulates cell proliferation and apoptosis via transcriptional up- and downregulation of its target genes. miR-17-92 was demonstrated as a Myc target in human B cells by virally-mediated transfection of a tetracycline-regulated Myc gene. In this model, activation of Myc correlated with higher expression of miR-17-92. Myc also upregulates miR-17-92 in a mouse model for colon cancer. These data are consistent with the fact that a c-Myc binding site lies upstream of the predicted transcriptional start site of miR-17-92. miR-17-92 in an oncomiR that promotes angiogenesis by downregulating expression of anti-angiogenic proteins including Tsp1 and CTGF. miR-17-92 is the only microRNA upregulated in cells overexpressing Myc. However, a large number of microRNA are down-regulated ≥ 1.5-fold in cells that express a high level of Myc. These include miR-22, 29b/c, 15a/16-1, 195, 26a-1, 34a and the let-7 family, all of which are deleted and/or mutated in human cancer. Recent analyses of the promoters for these microRNA identified putative Myc binding sites, suggesting that Myc might directly downregulate these microRNA by promoter binding. This possibility is supported by ChIP assays based on predicted and/or confirmed 5'-microRNA transcription start sites. Evidence for Myc-dependent downregulation was also obtained for let-7a-1, miR991/let-7c/miR-125b-2, let-7d, let-7f-1 and let-7g. The

exact mechanism by which Myc directly downregulates microRNA expression is not yet known.

The functional effect of Myc-dependent downregulation of microRNA expression was examined in an *in vivo* lymphoma selection model. In this model, single microRNA transcripts were co-expressed with GFP from a viral vector in Myc-expressing lymphoma cells, and these engineered lymphoma cells were introduced into host mice. Control experiments showed that co-expression of GFP and an oncogene, such as Mcl1 caused selective outgrowth of GFP-positive lymphomas, while constructs carrying only GFP did not confer growth advantage over non-GFP-expressing B cells. Furthermore, co-expression of miR-34a, 150, 497/195 or 15a/16-1 strongly lowered the ratio of GFP-positive to GFP-negative B cells in the host animals. Additional studies showed that growth suppression by miR-15a/16-1 occurred even at sub-physiological levels of microRNA re-expression. The tumor cell specificity of this effect is currently being explored.

Akiko Hata (Tufts University School of Medicine)
Regulation of microRNA Biosynthesis by the TGF-beta Signaling Pathway

The TGFβ and bone morphogenic protein (BMP) signaling pathways play roles in vascular homeostasis, and defects in these signaling pathways can lead to pulmonary hypertension. At the cellular level, TGFβ and BMP promote expression of smooth muscle actin in vascular endothelial cells, and stabilize these cells in a differentiated state. Recent studies indicate that miR-21 is a critical downstream effector of TGFβ and BMP signalling . In particular, TGFβ and BMP promote a rapid increase in miR-21, by stimulating DROSHA-dependent processing of pri-miR-21 to pre-miR-21. This pathway requires binding of miR-21 by RNA helicase p68 and R-SMAD, but it is independent of co-SMAD (*i.e.*, SMAD4). Putative miR-21 downstream targets include PCD4 (programmed cell death 4). A putative consensus SMAD binding motif was identified in 20 microRNA that are co-regulated by TGFβ and BMP in vascular smooth muscle cells. Preliminary ChIP assays showed that SMAD1 is associated with microRNA with a conserved but not with a mutant putative SMAD-binding motif.

These data suggest that SMAD proteins, which are critical downstream effectors of TGFβ and BMP, interact with and regulate expression of microRNA, including miR-21, that in turn regulate differentiation/dedifferentiation of vascular smooth muscle cells. Future studies will investigate the specificity of SMAD/microRNA interactions, the role of SMAD in DROSHA-dependent microRNA processing, and identification of functionally important targets of miR-21.

Tyler Jacks (Massachusetts Institute of Technology)
Effects of microRNA and microRNA Processing in Cancer Development

microRNA expression is globally downregulated in many cancers that arise in humans and mice. The functional importance of cancer-associated changes in microRNA expression was examined in a mouse model for non-small cell lung cancer (NSCLC). This model uses adenovirus or lentivirus-derived vectors to deliver Cre recombinase to the lung in transgenic mice carrying floxed activated K-Ras, with or without floxed p53 to promote metastatic disease (NSCLC mice). Initial studies examined expression of 583 microRNA in 30 cell lines derived from primary or secondary K-Ras-expressing mouse tumors. Distinct microRNA

signatures were identified in cell lines from primary tumors and metastases, even when profiles were compared in matched primary/metastastic cell line pairs. The miR-200 family (miR-141, miR-200a-c) was significantly downregulated in cell lines from NSCLC metastases relative to cell lines from NSCLC primary tumors. This microRNA family may play a role in the endothelial to mesenchymal transition (EMT) in normal tissues. Similar studies are underway using a model for small cell lung cancer.

Lentiviral vectors were then used to achieve lung-specific targeted co-expression of Cre recombinase and constitutive or inducible pro- or anti-carcinogenic microRNA or genes in NSCLC mice. A "proof-of principle" experiment examined the effect of let-7 overexpression in this model. Previous studies showed that let-7 is downregulated in NSCLC, and that survival is positively-correlated with let-7 expression in human lung cancer patients. This is consistent with the observation that overexpression of wild type but not truncated let-7g strongly reduced tumor number and tumor burden in NSCLC mice. Interestingly, the effect of let-7g was equally strong when it was induced from a tet-regulated promoter 4, 8 or 12 weeks after activation of K-Ras. The exact molecular explanation for this result is under investigation; however, it is possible that let-7g downregulates expression of K-Ras, and that K-Ras is required for both tumor induction and tumor maintenance in NSCLC mice.

To directly examine the effect of global downregulation of microRNA in cancer cells, Cre recombinase was transiently expressed in lungs of NSCLC mice carrying 1 or 2 floxed alleles of Dicer. (In this system, Dicer is inactivated by Cre-mediated recombination.) In NSCLC mice with one floxed Dicer allele, microRNA expression was reduced significantly and tumor burden was increased approximately 10-fold after Cre-mediated inactivation of Dicer. Similar results were observed in NSCLC mice with two floxed Dicer alleles. However, molecular analyses of cell lines derived from tumors in these animals showed that one functional Dicer allele was always retained. These results indicate that Dicer is a haplo-insufficent tumor suppressor and that cells can not survive after loss of heterozygosity for Dicer (*i.e.* Dicer null phenotype) This conclusion is supported by the fact that constitutive activation of Cre in NSCLC mice with two floxed alleles of Dicer completely blocks lung tumorigenesis. These data may have relevance for human cancer susceptibility, because polymorphic variants of Dicer and heterozygous deletion of the Dicer-containing chromosomal region (14q32) have been observed in human cancer cells. Additional studies of Dicer hypomorphism or polymorphism in human cancer is warranted.

Doug Hanahan (University of California, San Francisco)
MicroRNA Signatures of the Stages in Multistep Carcinogenesis

Previous studies correlated microRNA profiles with distinct tumor types. However, the role of specific microRNA in cancer progression is not well understood. Here, the RIP-Tag mouse model for pancreatic cancer was used to correlate microRNA expression with distinct stages of pancreatic cancer. RIP-Tag transgenic mice express T-antigen in pancreatic islet cells from the rat insulin promoter. These animals spontaneously develop pancreatic tumors, which progress through distinct, molecularly-defined stages, including hyperplasia, angiogenic switch, adenoma and invasive carcinoma. Each of these stages, including pre-tumor stages, were associated with distinct microRNA expression profiles, and cell lines derived from primary tissue samples clustered together, with different microRNA profiles than the tissue from which they were derived. Most microRNA were upregulated in early cancer stages and miR-17-92 was upregulated at all cancer stages in this model. High expression of hematopoietic microRNA (*i.e.*, miR-150, miR-142-3p, miR-142-5p and miR-155) was observed in cancer-associated innate inflammatory cells during the angiogenic switch stage

of cancer progression. miR-144 and miR-451 were also highly upregulated during this cancer stage. Metastatic tumor samples, and a small subset of primary tumors, had a very distinct microRNA expression profile ("met-like" signature), with many microRNA genes both up- and downregulated. The miR-8 family (miR-141, miR-200a, miR-200b, miR-200c, miR-429) is highly expressed in all tumor stages except metastases, where it is downregulated 5- to 10-fold. Previous studies suggest that the miR-8 family regulates expression of E-cadherin, which inhibits progression from adenoma to carcinoma and reduces cancer invasiveness in RIP-Tag mice as well as in some human cancers. This effect of miR-8 is likely mediated by its downregulation of transcription factors Zfhx1a (ZEB1) and Zfhx1b (ZEB2), which are putative miR-8 targets. This hypothesis is consistent with the fact that Zfhx1a is upregulated and E-cadherin is down-regulated in samples from RIP-Tag mice with a "met-like" signature.

Previous studies show that Sutent, a potent angiogenesis inhibitor, has therapeutic efficacy in RIP-Tag mice. Sutent reduces tumor vascularity and reduces pancreatic tumor burden and increases survival, but also increases cancer invasiveness and the frequency of liver metastases. Interestingly, the microRNA transcriptome was altered significantly in Sutent-treated RIP-Tag mice, including lower expression of miR-424, miR-233, miR-145 and miR-126. In contrast, a characteristic microRNA signature was not observed in RIP-Tag mice treated with rapamycin or erlotinib, both of which have therapeutic efficacy in the RIP-Tag2 mouse model.

Scott Lowe (Cold Spring Harbor University)

Using microRNA to Interrogate Cancer Phenotypes

microRNA profiling in senescent cells demonstrated that miR-34a is highly overexpressed. However, efforts to knockdown or inhibit miR-34a expression using siRNA or a miR-34a antogomiR have not succeeded. Thus, it has not been possible to confirm a functional role for miR-34a in cellular senescence. Nevertheless, cell culture systems confirm that overexpression of miR-34a leads to cell cycle arrest.

In normal cells, microRNA selectively destabilize or inhibit translation of multiple target mRNA via sequence specific hybridization between the microRNA seed sequence and the target mRNA. This phenomenon has been exploited to develop a miR-30 based system for analyzing specific gene function(s) in different cancer stages. In this system, a series of miR-30 variants were designed that selectively destablize or inhibit translation of a desired target gene in a highly specific manner. For this purpose, the seed pairing region of miR-30 was engineered to selectively downregulate expression of putative tumor suppressor genes and/or cancer-promoting genes. In most cases, the miR-30 hairpin was co-expressed with GFP from a tetracycline- regulated promoter.

This miR-30 based gene targeting technology was used to study modulators of cancer progression in mouse models for hepatocellular carcinoma (HCC), leukemia and lung cancer. In the model for HCC, constitutive downregulation of p53 inhibits tumor growth. Furthermore, transient downregulation of p53 also inhibited tumor growth in established tumors, indicating that p53 plays role in both tumor development and tumor maintenance. In the mouse leukemia model, downregulation of b-Raf alone does not inhibit leukemia cell proliferation, but simultaneous downregulation of b-Raf and a recently identified transcription factor strongly inhibited leukemia cell growth in a non-reversible manner.

A "second generation" version of the above system involves microRNA-based homing cassettes, which integrate into the frt-flanked genomic collagen 1 locus (ColA1) in mouse ES cells. After ES cells are engineered using this system, they can be fully characterized *in vitro* before they are used to generate recombinant mice. Approximately 50 ES cell lines have been generated to date, and initial studies have been performed using ES cells in which expression of p53 or p16/p19 is conditionally repressed using chromosomal cassettes at the genomic ColA1 locus. In whole animal systems, transient knockdown of p16/p19 in lung tissue promoted K-Ras-dependent lung cancer and transient knockdown of RPA in hepatic stellate cells caused atrophy of multiple organs and rapid death.

microRNA-based technology has also been used to screen for novel suppressors of HCC. Because LOH of a tumor suppressor gene is expected to enhance tumor growth, it is possible that tumor suppressor genes are enriched in chromosomal regions that are frequently deleted in HCC. Thus, a library of miR-30-based targeting constructs was generated using putative tumor suppressors in regions frequently deleted in HCC. As a control, a library was also constructed with randomly selected genomic DNA. As expected, more "hits" were obtained in the targeted library than in the random library, and some hits were well known tumor suppressor genes such as PTEN. This approach has identified 12 putative novel HCC suppressor genes.

Outcomes and Recommendations

During two open discussion sessions, at the end of the first and second days of the Think Tank, participants summarized emerging questions and ideas in the field of microRNA biology and its role in cancer. The main outcomes of these discussion sessions are summarized below.

- **Predicting and validating microRNA targets**
  A number of predictive algorithms for identifying putative microRNA targets (*i.e.*, TargetScan, developed by David Bartel and colleagues, Massachusetts Institute of Technology) have been developed and are being used with variable success. Although workshop participants agreed that microRNA targets can be predicted with an acceptable level of accuracy for some microRNA subclasses (*i.e.*, the most highly conserved microRNA), it was also agreed that many *bona fide* targets of a specific microRNA can be missed by "seed"-based algorithms, and that better algorithms, based on improved understanding of the determinants of microRNA/mRNA interactions, are urgently needed. Efficient tools and approaches for validating predicted targets are also needed. A few workshop participants felt that, at least in some contexts, it is not necessary to precisely identify and validate all targets of a specific microRNA, in order to understand and/or exploit the functional consequences of microRNA expression or lack of expression.

- **microRNA signatures**
  microRNA signatures are being generated in the context of many experimental systems. However, the reproducibility of these signatures remains untested. It is not yet known if choice of platform/technology or analytical method influences the data output. Furthermore, the relative value of microRNA signatures and mRNA signatures in different biological contexts and experimental systems remains unclear. Additional studies are

needed to determine whether microRNA signatures will be valuable and reliable in the context of diagnostic and/or prognostic applications related to human cancer. Although a significant number of microRNA signatures (>300) have been deposited in the Gene Expression Omnibus (GEO) database, the researchers present at this workshop were not aware of any systematic effort to collect and compare microRNA expression datasets (see below).

- **The interface between microRNA biology and systems biology**
  Several presentations at the workshop underscored the fact that microRNA targets constitute subnetworks in the transcriptome. Furthermore, systems level analysis of these subnetworks can provide important clues concerning microRNA function. The biological effect of a specific microRNA may not be mediated by downregulation of one or a few targets, but instead, may collectively reflect downregulation of the entire subnetwork in the transcriptome. Workshop participants were very enthusiastic about a collaborative initiative that would systematically use a systems biology approach to enhance understanding of microRNA biology. This could potentially be done under the umbrella of the NCI Integrative Cancer Biology Program (<http://icbp.nci.nih.gov/>), through other NIH programs in systems biology, or through a new initiative.

  Action Item 1. Establish mechanism to support collaborations that use a systems biology approach to understand microRNA biology and its role in human cancer.

- **microRNA and cancer therapeutics or diagnostics**
  There is significant optimism that microRNA signatures will be useful in cancer diagnostics and that microRNA can be developed as oncotherapeutic targets. However, it remains unclear to what extent microRNA biology will be more readily exploited or manipulated for successful diagnostic and/or therapeutic applications than mRNA (or proteins). Appropriate technology to deliver microRNA-based therapeutic agents to target cells is also needed.

- **Funding sources for microRNA research**
  microRNA researchers present at this workshop noted that NIH has not funded a lot of research in their field in the recent past. They also noted that NIH study sections may lack expertise in the area of microRNA biology. Lack of funding sources is compounded by the fact that many technologies used in this field are expensive (*i.e.*, genomic deep sequencing, custom expression profiling, ES cell culture, proteomics, transgenic mice).

- **Systematic sequencing of 3'-UTRs (microRNA targets)**
  MicroRNA/mRNA interactions are mediated by mRNA 3'-UTRs. Workshop participants pointed out that genomic 3'-UTR sequences have not been systematically sequenced or analyzed, and that the frequency of somatic mutations in 3'-UTRs in human cancer cells remains poorly characterized. This information may be important for understanding microRNA dysfunction in cancer cells. Thus, an initiative focused on sequencing human 3'UTRs is worth considering.

- **Functional studies of microRNA in human cells and mice**
  Loss-of-function studies are critical for establishing microRNA function. However, the technologies currently available for antagonizing microRNA expression, such as antagomirs, are inadequate. A systematic effort is urgently needed to generate

microRNA-deficient models (*i.e.*, mouse ES cell lines or conditional transgenics) and/or new approaches to develop such models.

- **Shared resources in microRNA research**
  microRNA researchers present at this workshop endorsed generating a repository of ES cell lines and/or mouse strains designed to promote progress in microRNA research.

  Action Item 2. Establish repository of mouse strains/ES cells designed to explore microRNA biology.

  microRNA researchers present at this workshop endorsed establishing a database of microRNA signatures in diverse human cell types.

  Action Item 3. Establish database of microRNA signatures in diverse human cell types.

- **microRNA and nanotechnology**
  Nanotechnology-based approaches could be useful for manipulating microRNA expression in human cells or mouse model systems. Thus, collaborations between microRNA researchers and nanobiologists/nanotechnologists should be fostered. This could be implemented through existing NIH nanotechnology initiatives.

  Action Item 4. Establish support for collaborations that use nanotechnology to explore microRNA biology.

- **Mouse models, the MMHCC and microRNA research**
  Mouse models have been very useful for studying microRNA dysfunction in cancer. Several researchers present at this workshop felt that the NCI Mouse Models of Human Cancer Consortium could facilitate development of additional mouse models relevant to understanding microRNA biology, and could help develop new mouse models for assessing microRNA as oncotherapeutic targets.