Tumorigenesis and Neoplastic Progression

Elevated Expression of the miR-17–92 Polycistron and miR-21 in Hepadnavirus-Associated Hepatocellular Carcinoma Contributes to the Malignant Phenotype

Erin Connolly,* Margherita Melegari,*
Pablo Landgraf,[†] Tatyana Tchaikovskaya,*
Bud C. Tennant,[‡] Betty L. Slagle,[§]
Leslie E. Rogler,* Mihaela Zavolan,[¶]
Thomas Tuschl,[†] and Charles E. Rogler*

From the Marion Bessin Liver Research Center, Department of Medicine, Albert Einstein College of Medicine, Bronx, NY; Laboratory of RNA Biology,[†] Rockefeller University, New York, NY; Department of Animal Science,[‡] Cornell University College of Veterinary Medicine, Ithaca, NY; Department of Molecular Virology and Microbiology,[§] Baylor College of Medicine, Houston, Texas;[¶] Biozentrum, University of Basel, Switzerland and Swiss Institute of Bioinformatics, Lausanne, Switzerland*

Alterations in microRNA (miRNA) expression in both human and animal models have been linked to many forms of cancer. Such miRNAs, which act directly as repressors of gene expression, have been found to frequently reside in fragile sites and genomic regions associated with cancer. This study describes a miRNA signature for human primary hepatitis B virus-positive human hepatocellular carcinoma. Moreover, two known oncomiRs—miRNAs with known roles in cancer—the miR-17–92 polycistron and miR-21, exhibited increased expression in 100% of primary human and woodchuck hepatocellular carcinomas surveyed. To determine the importance of these miRNAs in tumorigenesis, an *in vitro* antisense oligonucleotide knockdown model was evaluated for its ability to reverse the malignant phenotype. Both in human and woodchuck HCC cell lines, separate treatments with antisense oligonucleotides specific for either the miR-17–92 polycistron (all six members) or miR-21 caused a 50% reduction in both hepatocyte proliferation and anchorage-independent growth. The combination of assays presented here supports a role for these miRNAs in the maintenance of the malignant transformation of hepatocytes. *(Am J Pathol 2008, 173:856–864; DOI: 10.2353/ajpath.2008.080096)*

The classical models of cancer establish that fully malignant cancers are the product of alterations in multiple cancer-related pathways.[1] Notably, the discovery of mammalian microRNAs (miRNAs) has uncovered a new set of genetic elements that act directly as repressors of gene expression and have been causally linked to several types of cancer.[2–5] miRNAs are transcribed as single or clustered primary transcripts, which are further processed into mature miRNAs. The mature miRNA is incorporated in the RNA-induced silencing complex, which mediates the mRNA target gene down-regulation by mRNA cleavage or translational repression.[6,7]

Recent reports have demonstrated that changes in the expression of miRNAs vary dramatically across tumor types as well as developmental lineages.[8,9] Nevertheless, there is still little information available about specific miRNA expression patterns for hepatocellular carcinomas. Human hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, and although chronic infection with human hepatitis B virus (HBV) or hepatitis C virus is known to be the casual agent for more than 80% of cases, treatment options are limited and patient morbidity is high.[10] The lifetime risk of developing HCC is increased by 25 to 37 times in HBV surface antigen

carriers as compared with non-infected people, even after clearance of HBV surface antigen.[11] In addition, new risk factors such as obesity and diabetes have been shown to synergistically increase the risk of developing HCC.[12,13]

In light of the potential importance of miRNAs in HCC and other cancers, our previous work carefully defined the profile of miRNAs that are expressed and differentially regulated in a wide spectrum of tumors and normal tissues, including normal liver and HCC cell lines.[14] The analysis of miRNA cloning data from this study revealed multiple differences in miRNA frequencies between normal liver and HCC cell lines.[14] Of interest, both miR-21 and the polycistron miR-17–92,which are miRNAs associated with other malignancies,[15,16] exhibited higher expression levels in HCC cell lines than those observed in normal liver. For example, clones of miR-21 comprised 16.7% of the miRNAs cloned from HCC cell lines (HepG2, PLC, Huh7) whereas only 0.1% of the liver miRNA clones were represented by this miRNA.[14] Furthermore, the expression of the mir-17–92 polycistron was increased 16-fold in HCC derived cell lines as compared to normal liver (7.4% vs. 0.6% of all miRNA clones).

In this report, we have expanded our miRNA study to include primary HBV-positive human HCCs and woodchuck HCCs associated with chronic woodchuck hepatics virus (WHV) infection. The woodchuck is a powerful animal model for HCC as there is a 100% incidence rate of tumor development after chronic infection with WHV.[17] Clonally selected integrations of HBV DNA and WHV DNA are commonly identified in human and woodchuck HCCs, respectively,[18,19] and these integrations have been linked to proto-oncogene activation.[19,20] Specifically, studies of woodchuck HCCs have shown that a large majority (~70% or more) of woodchuck HCCs contain clonally selected, activated N-myc genes due to WHV DNA integration.[21,22] Co-expression of a fetal liver growth factor, insulin-like growth factor-2, also occurs coordinately with N-myc and blocks apoptosis.[23,24]

Our survey detected increases in miR-21 and members of the miR-17–92 polycistron in virtually all HCCs tested. Furthermore, loss-of-function studies were performed *in vitro* to determine the role of these miRNAs in the maintenance of a malignant phenotype. This study clarifies the miRNA signature for HBV- and WHV-positive HCCs and demonstrates functional roles for these miRNAs in proliferation and growth of HCC cells.

Materials and Methods

Tissues

Human HCC samples and matched non-tumor liver tissue (19 sets) were obtained from surgical resections of anonymous donors in the Qidong Liver Cancer institute, Jangsu, People's Republic of China. The features of this sample population have been previously described.[25] There was evidence of HBV infection (circulating antibody to HBV surface antigen, antibody to HBV core antigen, or HBV DNA integrated into tumor DNA) for all 19 patients. HBV-associated cirrhotic livers were obtained from surgical resection for anonymous donors at Mount Sinai Hospital, New York, NY. The features of these samples were also previously described.[26] Furthermore, woodchuck HCCs positive for WHV were obtained from euthanized animals in accordance with NIH guidelines. Tumor and matching non-neoplastic liver from both humans and woodchucks were harvested and immediately frozen in liquid nitrogen. The minimum size of woodchuck HCCs was 1 cm. The woodchuck tumors were evaluated for gamma glutamyl transferase and histological grade.[17] The human HCCs were considered end-stage, and histology grade was not available.

Cell Lines

HCC cell lines were maintained in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum, sodium pyruvate, non-essential amino acids, and penicillin-streptomycin. Tissue culture supplies were obtained from Invitrogen (Carlsbad, CA).

RNA Isolation and miRNA Cloning

RNA was extracted using either the mirVana miRNA isolation kit, (Ambion, Austin, TX) or the Trizol method. First Choice human RNA, human liver, and human HCCs were obtained from Ambion. The source of the Ambion human total liver RNA was a 37-year-old Caucasian male and was negative for human immunodeficiency virus I and II, hepatitis viruses B and C. The source of the Ambion human HCC total RNA was a 60-year-old Caucasian male, tumor staging T3NXMX. Small RNA cloning, sequencing, and annotation were performed as described previously.[14] The 25 most highly up-regulated and down-regulated miRNAs in the four hepatocellular carcinoma samples (relative to normal liver) was determined by the ratio of relative cloning frequencies. To reduce the noise of miRNAs with low clone counts, miRNAs with an overall clone count of less than five were excluded from the analysis. The ratio of relative cloning frequencies between the HCC sample and the normal liver sample was calculated and log2-transformed for each individual patient sample.

Northern Blots

Total RNA samples for HBV or WHV analysis were electrophoretically separated in a 0.2 M formaldehyde/1.2% agarose gel using 15 μg RNA per lane and transferred to Hybond N membranes by the inverted capillary method. Northern blots of woodchuck RNA were hybridized with a 3.2 kb *HinDlll* DNA fragment corresponding to the full length WHV genome.[22] Northern blot analyses of miRNAs were performed with 30 μg total RNA loaded/well on a 15% polyacrylamide/8M urea gel, transferred semidry to a GeneScreen Plus or Hybond N membrane, and hybridized with 20–22nt antisense P[32] end-labeled oligonucleotide probes against miR-17, miR-92, miR-122, miR-21, or U43. Gels were stained with ethidium bromide to de-

termine tRNA level. All blots were imaged with a Storm scanner (Molecular Dynamics). Each experiment was performed at least three times.

Transfection with Antisense Oligonucleotides

2′O-methyl antisense oligonucleotides (ASOs) were obtained from IDT (Coralville, IA) for miR-17–92a polycistron: miR-17–5p, 5′-ACUACCUGCACUGUAAGCACUUUG-3; miR-18, 5′-UAUCUGCACUAGAUGCACCUUA-3; miR-19a, 5′-UCAGU-UUUGCAUAGAUUUGCACA-3; miR-19b, 5′-UCAGUUUUG-CAUGGAUUUGCACA-3; miR-20, 5′-UACCUGCACUAUAA-GCACUUA-3; miR-92, 5′-ACAGGCCGGGACAAGUGCA-AU-3; miR-21, 5′-AUCGAAUAGUCUGACUACAACU-3; miR-122, 5′-ACCUCACACUGUUACCACAAACA-3; as well as for scramble sequence: 5′-CAUCAAUGCUAGCAUUCGAUC-3; 5′-UGCCAUAGGAUCGAUUCAGUA-3; 5′-GCUGACGAUC-GACUGCCAUUAU-3′.

At 30% confluence, HepG2 cells grown on poly-D-lysine-coated plates BD Biosciences (Billerica, MA) were transfected using Lipofectamine RNAiMAX, Invitrogen in accordance with manufacturers' instructions. Cells were treated with ASO to the miR-17–92a polycistron (all six members), miR-21, or miR-122 (negative control) in OPTI-MEM (Invitrogen).

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) analysis was performed using an ABI 7000 real-time detection system. For the detection of mRNA or the primary miRNA transcript, RNA was harvested at 48 hours and 5 days after transfection with ASOs. cDNA was produced using the High Capacity cDNA Reverse Transcription Kit, Applied Biosystems (Foster City, CA). TaqMan probes for C13orf25 (miR-17–92a primary transcript), E2F1, E2F3, and B2M were obtained from Applied Biosystems and qRT-PCR was performed in accordance with manufacturer protocols. B2M was used as reference. C13orf25 expression was compared to non-transformed liver. E2F1 and E2F3 expression was compared to HepG2 cells treated with lipofectamine only. Results are shown as fold change ($2^{-\Delta\Delta Ct}$). To quantify expression levels of miR-17–92a and miR-21 in cirrhotic liver and in the knockdown experiments, the TaqMan MicroRNA Assay Kit, Applied Biosystems was used to prepare all samples for qRT-PCR, in accordance with the manufacturer's instructions. RNU43 was used as a reference. Results for cirrhotic liver are shown as fold change ($2^{-\Delta\Delta Ct}$) and results for *in vitro* knockdown studies are presented as ($\Delta\Delta Ct$) of treated samples as compared with control.

Western Blot

Cells were lysed in RIPA buffer, Sigma (Saint Louis, MO) with protease inhibitor Complete Mini Roche/Fisher (Pittsburgh, PA). Forty μg of lysate was loaded per lane on 4 to 20% gradient Tris-Glycine gels, Invitrogen, and blotted onto Invitrolon polyvinylidene difluoride (Invitrogen). Blots were probed with polyclonal antibodies (Cell Signaling,

Beverly, MA) E2F1 and COX IV (loading control) at a 1:1000 dilution. Bands were visualized by Western Lightning chemiluminescence reagent (Perkin Elmer Life Science, Waltham, MA).

Proliferation Assay

The Cell Titer 96 AQ$_{ueous}$ Non-Radioactive Cell Proliferation Assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (Promega, Madison, WI) was performed 72 hours after transfection in accordance with the manufacturer's protocol. The level of absorbance was read using the Fluostar Optima multiplate reader. Results are presented as percentage of control.

Cell-Cycle Analysis by Propidium Iodide Flow Cytometry

Samples were harvested 48 hours after transfection, fixed in 90% ethanol, and kept at $-20°C$ overnight. Cells were immersed in PBS, ribonuclease A (200 μg/ml), and propidium iodide staining solution (20 μg/ml), and incubated at room temperature for 3 hours. Samples were analyzed by flow cytometry for FL-2 area. Total DNA content was measured on a FACScan flow cytometry machine using CellQuest Pro Software (Becton Dickinson) with a minimum of 10^4 events counted. FL-2 area PI histograms were analyzed with ModFit LT. Experiments were performed in triplicate. Results presented as % of cell in phase.

Anchorage Independent Growth

Twenty-four hours after transfection with ASO to miR17–92a, miR-21, or miR-122, HepG2 cells in complete media were re-suspended in 0.35% soft agar and layered onto 0.6% solidified agar. Five days after re-suspension in soft agar, colonies were photographed at original magnification $\times 20$. The area of the colonies was determined with the NIH Image J program. Experiments were performed in triplicate. Results are presented as % control.

Apoptosis

The level of active Caspase-3 was determined in HepG2 cells 5 days after transfection with ASO to either miR17–92a, miR-21 or miR-122 using EnzCheck Caspase-3 Assay Kit, Molecular Probes/Fisher (Pittsburgh, PA) in accordance with manufacturer protocol. The level of absorbance was read using the Fluostar Optima multiplate reader. Results presented as % control.

Results

miRNA Cloning Survey Reveals a miRNA Signature for Primary Human HCC

miRNA profiles of four primary human HCCs, previously associated with chronic HBV infection,[25] were deter-

Figure 2. Conserved increase in expression of the miR-17–92 polycistron and miR-21 in both human and woodchuck HCCs. Northern blot analysis of the expression of miRNAs in human and woodchuck peri-tumor liver and primary HCCs. **A:** Human samples. **B:** Woodchuck samples. C samples = peri-tumor liver. T samples = primary HCC. Matching C samples are to the Left of T samples from the same liver. miRNAs are as labeled next to rows, miR-122, miR-92, miR-21, and miR-17. tRNA and U43 RNA were loading controls.
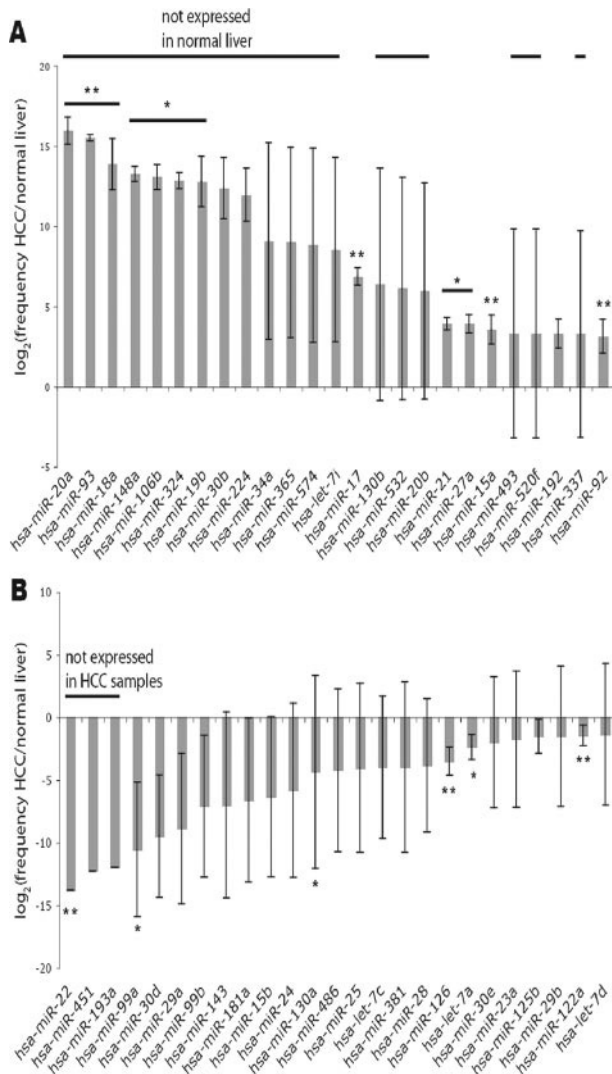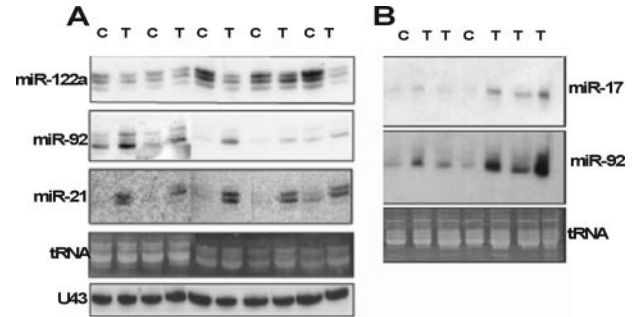
Figure 1. The 25 most up-regulated (**A**) and down-regulated (**B**) miRNAs in four hepatocellular carcinoma samples in respect to one normal liver sample as determined by small RNA cloning and sequencing. The ratio of relative cloning frequencies between hepatocellular carcinoma sample and normal liver sample are presented in log2 transformed for each individual patient sample. The average of these values as well as the 95% confidence interval is displayed. Bars above the figure indicate missing clones in either normal liver (**A**) or in any of the HCC samples (**B**). For statistical analysis, using a Bayesian framework, the clone counts for all hepatocellular samples were pooled and compared to the clone counts of normal liver taking into account also the total clone number obtained in the respective libraries. *$P < 0.05$ and **$P < 0.001$ of clone counts from the pooled HCC samples and liver being the same.

mined by small RNA cloning and sequencing.[14] In comparison to our previously published liver miRNA profile,[14] we identified a distinct miRNA signature for primary HCC (Figure 1). The miRNA signature showed that miRNAs from the miR-17–92 polycistron and miR-21 were among the most highly expressed miRNAs in primary tumor samples compared to liver (Figure 1A), a finding that recapitulates our previous observations with HCC cell lines.[14] Since the up-regulation of miR-21 and the miR-17–92 polycistron was the most profound and since these miRNAs have been implicated in other cancers (reviewed in,[16,27]) we focused our expanded survey of primary HCCs on these miRNAs.

Northern Blot Analysis Detected Overexpression of the miR-17–92 Polycistron and miR-21 in All Primary HCCs Tested

Nineteen primary human HCCs[25] and 49 primary woodchuck HCCs, were screened for expression of miRNAs from the miR-17–92 polycistron, miR-21, and miR-122 by Northern blot analysis. Probes for miR-17–5p and miR-92, which represent the beginning and the end of the miR-17–92 polycistron, were used to evaluate its expression. miR-21 and members of the miR-17–92 polycistron were found to be overexpressed in 100% of the human HCCs tested (representative data shown in Figure 2A). Additionally, quantitative analysis of miR-92 and miR-21 hybridization signals on Northern blots, normalized to U43, revealed significant increases of these miRNAs in HCC samples (supplemental Figure 1, see *http://ajp.amjpathol.org*). This expression pattern was also observed in all of the 49 WHV-positive woodchuck HCC samples that we tested (representative data Figure 2B, supplemental Table 1 see *http://ajp.amjpathol.org*). Furthermore, the elevated level of miRNA expression was seen regardless of the status of WHV replication in the liver or tumor samples (supplemental Figure 2, supplemental Table 1, see *http://ajp.amjpathol.org*). Likewise, tumor size was also irrelevant to miRNA expression. Therefore, this survey confirmed the up-regulation of miRNAs from the miR-17–92 polycistron and miR-21 in primary human and woodchuck HCCs.

Interestingly, expression of the major liver miRNA, miR-122, which is down-regulated in a rat HCC model,[28] was maintained in all of the human HCCs samples. Although miR-122 expression was substantially reduced in some of the HBV-positive HCCs (Figure 2A), there was no statistically significant difference in matched tumor and peri-tumor samples ($P = 0.86$) (supplemental Figure 1, see *http://ajp.amjpathol.org*).
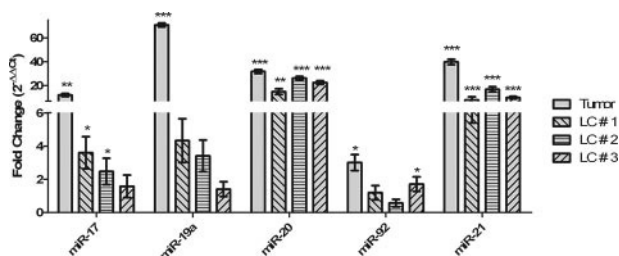
Figure 3. qRT-PCR analysis of the miR-17–92 polycistron and miR-21 expression in human HCC, and human cirrhotic livers as compared with normal liver. Expression data are reported as fold change ($2^{-\Delta\Delta Ct}$) above normal liver. Statistical significance was determined by a *t*-test comparing (ΔCt) of normal liver to either HCC or cirrhotic livers (ΔCt). *$P < 0.05$, **$P \leq 0.001$, ***$P < 0.0001$.

Figure 4. Comparison of expression of the pri-miRNA for the miR17–92 polycistron in HCC cell lines. qRT-PCR analysis of the C13orf25 transcript expressed in HCC cell lines is reported as fold change ($2^{-\Delta\Delta Ct}$) above normal liver.

HBV-Associated Cirrhotic Livers Overexpresses Members of the miR-17–92 Polycistron and miR-21 Compared to Normal Liver

Cirrhosis is considered to be a significant aetological factor that precedes HCC in humans, whether it is associated with chronic HBV infection or not. qRT-PCR analysis of human liver and human HCC (Ambion) demonstrates that miR-17–92 polycistron and miR-21 were overexpressed in HCC, a result that is consistent with our Northern blot and cloning data (Figure 3). To determine whether these miRNAs are overexpressed in precancerous liver, we analyzed their levels in three cirrhotic human livers from individuals that were undergoing human liver transplant surgery, and in which primary HCC had not been identified. The severe nodular cirrhosis of these patients was reflected in their liver function tests (supplemental Table 2, see *http://ajp.amjpathol.org*). Each of the three cirrhotic livers showed variable levels of elevation of the miRNAs as compared with normal liver (Figure 3). Out of the four members of the miR-17–92 polycistron (miR-17, miR-19a, miR-20, and miR-92), each cirrhotic liver has statistically significant over-expression of at least two of the miRNAs; however, although miR-19a showed a trend of increased expression in cirrhotic liver these changes were not statistically significant (Figure 3). As expected, the expression of these oncogenic miRNAs was variable and not generally as high as that determined for HCC. We attribute this miRNA expression pattern to the multinodular precancerous nature of the liver cirrhosis samples that were used to make the RNAs. For example, in liver cirrhosis 1, miR-17 and miR-20 exhibited respective 3.5- and 12-fold increases over liver (Figure 3). Furthermore, miR-21 expression was significantly enhanced in all of the cirrhotic livers ($\sim$ 15-fold increase) (Figure 3). However, the fold increase (relative to normal liver) of these miRNAs in cirrhotic livers was less than that observed for the same miRNAs in HCC. These data support the hypothesis that activation of the miR-17–92 miRNAs and miR-21 precedes HCC, and suggests that these genetic elements may represent important etiological agents in tumor initiation or progression and not just products of the transformed state. We therefore conducted functional studies to test their role in the maintenance of the malignant phenotype.
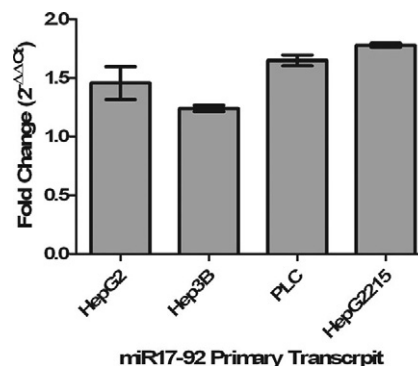
Antisense Oligonucleotide in Vitro Knockdown Model of the miR-17–92a Polycistron and miR-21 in HepG2 Cells

To select an HCC cell line for the functional study of the mir-17–92 polycistron, we first analyzed the level of the primary transcript (pri-miRNA) of this cluster by qRT-PCR in several HCC cell lines, as has been described recently for mantle cell lymphomas.[29] In three replicate experiments, we consistently observed a minimum 15-fold increase of the pri-miRNA in all four HCC cell lines compared to the level of pri-miRNA in human liver (Figure 4). The remaining work presented in this study was done with HepG2 cells, because, along with high expression of the miR-17–92 pri-miRNA, they are also wild type for p53. As p53 is a potent tumor suppressor in its own right, it was of interest to assess the role of miRNAs on growth and apoptosis independently of the mutated p53 effect in cell lines.

Loss-of-function models for the miR-17–92 polycistron and miR-21 were created using ASO-transfected HepG2 cells. Knockdown of the miR-17–92 polycistron was achieved by transfecting a mixture of six ASOs against miRs 17–5p, 18a, 19a, 19b, 20, and 92 in equimolar ratio. Reductions in endogenous miRNA levels of HepG2 cells transfected with 2 μmol/L ASO miR-17–92 ($\sim$300 nmol/L/individual member) and 250 nmol/L ASO against miR-21 were determined by qRT-PCR at 48 hours and 5 days post-transfection.[30] At 48 hours, we observed an average decrease of -2.9 ($\Delta\Delta$Ct) of miR-17.5p, 20 and 92 expression as compared to non-treated cells (Figure 5). Cells treated with ASO against miR-21 showed a -3.6 ($\Delta\Delta$Ct) decrease in expression at 48 hours post-transfection (Figure 5). We also observed sustained reductions of -1.4 and -2.7 ($\Delta\Delta$Ct) for the miR17–92 polycistron and miR-21, respectively, at 5 days post-transfection (Figure 5).

Aspects of the Malignant Phenotype of HepG2 Cells Are Dependent on Expression of the miR-17–92 Polycistron and miR-21

Cell Proliferation and Cell Cycle Progression

We performed a (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,
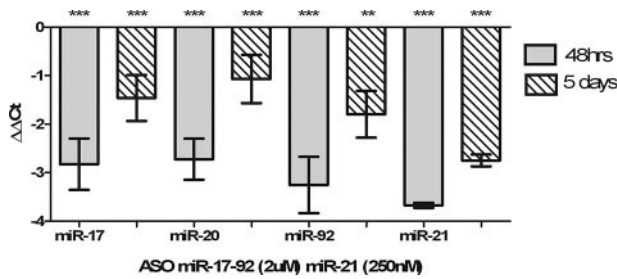
Figure 5. Analysis of antisense oligonucleotide knockdown of selected miR-NAs from the miR17–92 polycistron and miR-21. miRNA levels were assayed by qRT-PCR TaqMan assays at 48 hours (open bars) and 5 days (hatched bars) post transfection. The change in miRNA expression is reported as the ($\Delta\Delta$Ct) of ASO treated cells versus lipofectamine (control) alone treated cells, with U45 as reference. All knockdowns were statistically significant by *t*-test comparing (ΔCt) of control to ASO samples. *$P < 0.05$, **$P \leq 0.001$, ***$P < 0.0001$.

inner salt) cell proliferation assay to determine the influence of ASO-mediated repression of miR-17–92 and miR-21 on HepG2 proliferation. At low ASO concentrations (5 to 25 nmol/L), we observed an average decrease in cell proliferation of 55% at 72 hours post-transfection with either miR-17–92 polycistron or miR-21 ASOs (Figure 6A). We also observed a markedly smaller, albeit significant, reduction in cell proliferation (~20%) following control transfections with an ASO against miR-122, a major liver miRNA that is not expressed in HepG2 cells (Figure 6A). A *t*-test demonstrated that as compared with control, the reduction seen in all test groups was significant at $P < 0.001$, including treatment with control ASO (miR-
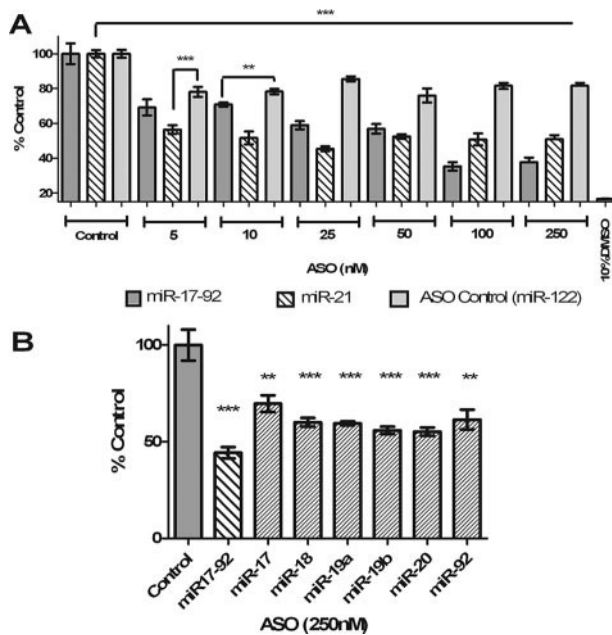

Figure 6. Knockdown the miR-17–92 polycistron or miR-21 reduces HepG2 cell proliferation. Cell proliferation was measured by MTS, dose-response data from 0 to 100 nmol/L total ASO transfected, 72 hours after transfection. Data expressed as % of control (Lipofectamine only treatment). **A:** HepG2 cells treated with ASO against all six miRNAs in the miR17–92 polycistron (dark gray bars) or miR-21 (angle slash bars) or miR-122 control (light gray bars). *T*-test demonstrated that compared to control the reduction seen in all samples was significant $P < 0.001$. **B:** Effects of ASOs against individual miRNAs from the miR-17–92 cluster versus the mix of all six miRNAs. Measurement of cell proliferation measured 72 hours post-transfection of 250 nmol/L ASO. *$P < 0.05$, **$P \leq 0.001$, ***$P < 0.0001$.
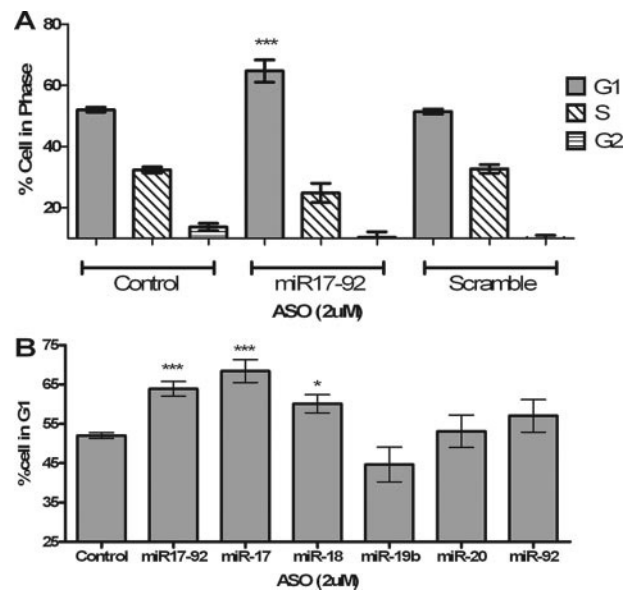

Figure 7. Knockdown of the miR-17–92 polycistron causes a retardation of the cell cycle. Fluorescence-activated cell sorter analysis (propidium iodide staining), was used to examine cell cycle progression 48 hours after transfection with ASO. **A:** HepG2 cells treated with ASO to the miR17–92 polycistron and a random scrambled sequence. **B:** HepG2 cells treated with ASO to individual members of the miR17–92 polycistron. Results presented as % of cells in phase; error bars on graph represent SEM. *$P < 0.05$, **$P \leq 0.001$, ***$P < 0.0001$.

122). However, reduction caused by treatment with ASO to either miR-21 or the miR-17–92 polycistron was significant as compared with control ASO (miR-122) $P < 0.001$ at 5 nmol/L and 10 nmol/L respectively and maintains this significance for all other doses. Thus, although control ASO (miR-122) treatment had a minor, yet statistically significant effect on proliferation, it could not account for the significant decrease in proliferation seen with ASO treatment to miR-17–92 or miR-21. In addition, treatment with ASOs that selectively repress individual members of the miR-17–92 polycistron revealed that loss of a single miRNA could not account for the proliferative decrease that resulted from knockdown of the complete polycistron (Figure 6B).

Fluorescence-activated cell sorter analysis demonstrated that the reduction in proliferation following ASO miRNA treatments of HepG2 cells is attributed to a retardation of the cell cycle (Figure 7). At 48 hours following transfection with the ASO mixture directed against the miR-17–92 polycistron, we observed a statistically significant increase in the percentage of cells in G1 as compared to non-treated cells or cells treated with a sequence-scrambled ASO (Figure 7A). Furthermore, ASO treatment against individual members of the miR-17–92 polycistron demonstrated that loss of miR-17.5p expression alone resulted in the retention of cells in G1 (Figure 7B). Therefore, miR-17.5p may be the most effective miRNA in the polycistron that affects the G1-S phase progression of HepG2 cells. Additionally, in WHK44 cells, a woodchuck hepatoma cell line expressing N-myc 1,[24] cells accumulated in G1 on treatment with ASOs against miRNAs of the miR-17–92 polycistron (supplemental Figure 3, see *http://ajp.amjpathol.org*), demonstrating the
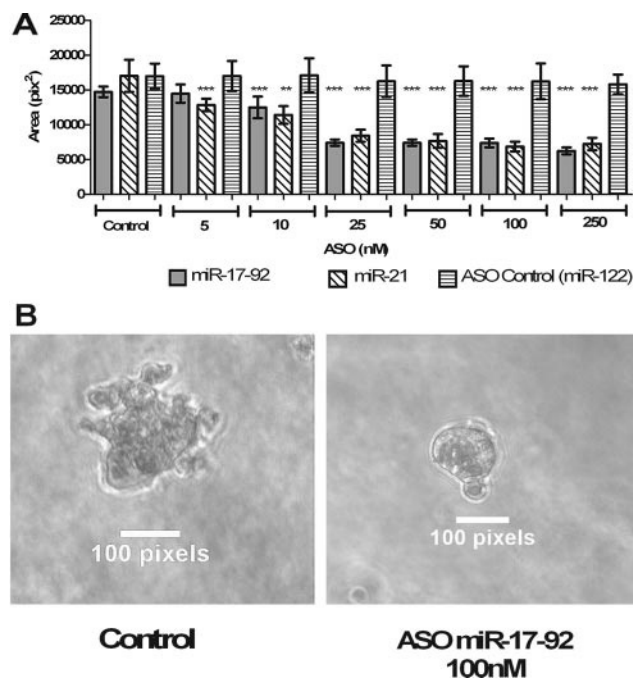
A

B

Figure 8. Knockdown of either the miR-17–92 polycistron or miR-21 reduces HepG2 anchorage independent growth. HepG2 cells were re-plated 24 hours after transfection into soft agar and allowed to grow for 5 days, on which time colonies were photographed and the area was measured using NIH Image J. **A:** HepG2 cells treated with either ASO against all six miRs in the miR17–92 polycistron, miR-21 or miR-122 alone. **B:** Microscopy images (magnification = original ×20) of HepG2 colonies in soft agar. Results presented as % control; error bars on graph represent mean SEM. *$P < 0.05$, **$P \leq 0.001$, ***$P < 0.0001$.

conserved role of the miR-17–92 polycistron in *in vitro* models for HCC.

Anchorage-Independent Growth

The reduction in proliferation and cell growth was mirrored by the loss of another malignant phenotype: anchorage-independent growth. HepG2 cells were re-plated into soft agar 24 hours after transfection with the ASOs and allowed to grow for 5 days, at which time randomly selected colonies were photographed and measured (area pixcal^2) using NIH Image J. Knockdown of the full miR-17–92 polycistron or miR-21 alone elicited a maximal, 55% reduction in colony size over a concentration range of 25–250 nmol/L ASO (Figure 8 A and B). Interestingly, transfection of the control ASO (miR-122) had no effect on colony size over the entire dose range. We also tested the effects of these ASOs on anchorage-independent growth of the highly malignant WHK44 woodchuck HCC cells and observed a similar growth inhibitory response (supplemental Figure 4, see *http://ajp.amjpathol.org*).

Apoptosis Assays

Finally, the role of the miR-17–92 polycistron and miR-21 on the induction of apoptosis was addressed by determining active caspase-3 levels after ASO treatment. At 5 days post-transfection, there was no statistically significant increase in the level of active caspase-3 in cells treated with either ASOs that target the miR-17–92 polycistron/miR-21 or control ASO (miR-122). However, cells treated with ASO to miR-21 showed an increase in active caspase-3 levels, suggesting that miR-21 has an anti-apoptotic function in HCC, as it has been proposed to have in other systems (Figure 9).[15]

The Effect of Knockout of the miR-17–92a in HepG2 Cells on E2F1 and E2F3 Expression

E2F1 is overexpressed in HCC and is a target of miR-17 and miR-20.[10,31,32] In our system, knock down of miR-17–92 miRNAs with ASOs produced only a modest 1.3-fold increase in E2F1 mRNA expression. However, in the same cells there was a 2.5-fold increase in E2F3 expression (Figure 10A). Additionally a Western blot for E2F1 showed an increase E2F1 in ASO miR-17–92 treated cells (Figure 10B). E2F3 has been shown to induce expression of the miR-17–92 polycistron.[31] Therefore, we interpreted these data to suggest a negative feedback loop in which E2F3 increases the expression of miR-17–92 miRNAs, which in turn negatively regulates the translation of E2F3 and E2F1. This may represent a mechanism that enables transformed hepatocytes to circumvent the apoptotic effects of E2F1 accumulation.[32,33]
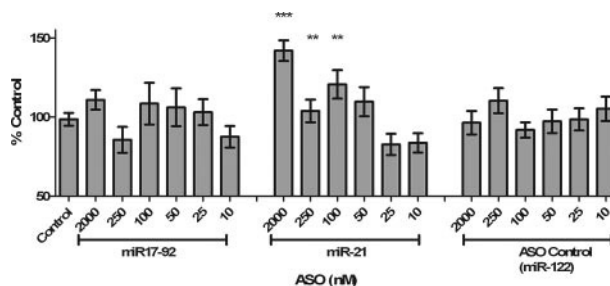

Figure 9. Loss of miR-21 expression induces apoptosis. The effect of knockdown of the miR17–92 polycistron and miR-21 on apoptosis was assessed by determining the level active caspase-3 by absorption 5 days after transfection. Results presented as % control; error bars on graph represent mean SEM. *$P < 0.05$, **$P \leq 0.001$, ***$P < 0.0001$.
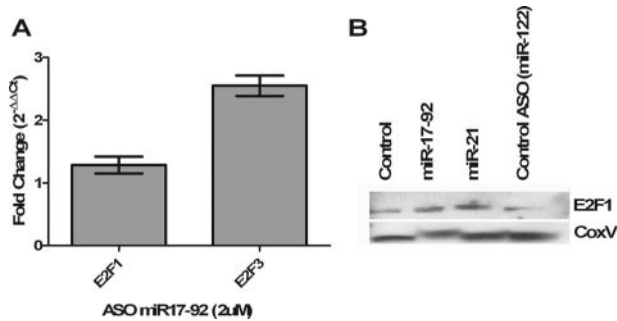
Figure 10. The effect of ASO miR17–92 knockdown on E2F1 expression. **A:** qRT-PCR of E2F1 and E2F3 expression in HepG2 cells treated with either ASO to the miR17–92 polycistron as compared with lipofectamine treated cells 5 days after transfection. Values expressed as fold change $(2^{-\Delta\Delta Ct})$ above controls (lipofectamine alone) using GAPDH as reference. **B:** Western blot of E2F1 and COX IV (loading control) expression 5 days after transfection.

Discussion

While altered miRNA expression has been clearly linked to cancer, the molecular mechanisms by which miRNA modulates tumorigenesis are unknown. This study is significant for identifying the highly penetrant activation of two key miRNA loci (miR-17–92 and miR-21) in primary HBV-positive human and WHV-positive woodchuck HCCs. With the aid of a miRNA knockdown strategy that was designed to evaluate the roles of these miRNAs in primary HCCs, we have produced two lines of evidence that they are essential for the maintenance of a malignant phenotype. First, ASO to miR17–92 or miR-21 caused reduction in proliferation that correlated to a retardation of cell cycle progression. Second, we demonstrated a requirement for miR-17–92 and miR-21 in the sustenance of anchorage-independent growth.

Elevated expression of the miR-17–92 polycistron has been demonstrated in multiple cancer models.[34] Currently, two possible genetic mechanisms are thought to be attributed to the increased expression of the miR-17–92 polycistron: gene amplification and c-Myc activation.[35] First, it has been shown that c-Myc induces miR-17–92 expression through direct binding to the miR-17–92 promoter.[29] Second, MYC has also been shown to participate in a positive feedback loop with E2F1, a known target of miR-17/20. The targeting of E2F1 by miR17/20 is hypothesized to reduce pro-apoptotic signaling due to excessive expression of E2F1 or MYC.[31]

miR 21 overexpression presents as a common hallmark of many different cancer types[15] and has been linked directly to HCC through the known oncogene, signal transducer and activator of transcription 3, a major mediator of interleukin-6 signaling and participates in cellular transformation through suppression of apoptotic signaling.[36] The miR-21-knockdown phenotype of the present work confirms not only its anti-apoptotic function in HCC but also its role in anchorage-independent growth. Recently studies demonstrate that signal transducer and activator of transcription 3 is able to bind the promoter of the miR-21 primary transcript, leading to its activation,[37] therefore possibly accounting for the high expression of miR-21 in HCC samples compared to liver.

HCC is associated with several risk factors, and our study was restricted to primary tumors associated with chronic HBV (and WHV) infection. It will be necessary to investigate HCCs associated with other risk factors to determine whether miRNA expression patterns observed in the present HCCs are unique to HBV (and WHV) tumors, or if they can be extended to include all HCCs, regardless of etiology. Our initial analysis of cirrhotic livers suggests that upregulation of miR-17–92 and miR-21 occurs in precancerous stages of liver disease. Further work will be necessary to confirm a role for these miRNAs in the acquisition of malignant traits by normal liver cells.

Understanding the mediators of tumor initiation and progression opens opportunities for new therapeutic strategies. Bioinformatic studies suggest that each miRNA has a spectrum of mRNA targets that span a wide range of cellular functions.[38] This study illustrated a role for these miRNA in cellular functions, such as proliferation and apoptosis. However, further work is warranted to evaluate the mRNA targets of the miR-17–92 polycistron and miR-21. Target evaluation in the pathways examined by this study may aid in the development of therapeutic strategies targeting the miR-17–92 or miR-21 polycistron *in vivo*. The impact of such opportunities is strengthened by the striking observation that overexpression of these miRNAs occurs in 100% of the HCCs tested. To our knowledge, such a high frequency of activation has never been reported for a specific genetic element that participates in HCC or any other cancer type.

Acknowledgments

References

1. Gronbaek K, Hother C, Jones PA: Epigenetic changes in cancer. APMIS 2007, 115:1039–1059
2. Giannakakis A, Coukos G, Hatzigeorgiou A, Sandaltzopoulos R, Zhang L: miRNA genetic alterations in human cancers. Expert Opin Biol Ther 2007, 7:1375–1386
3. Liu W, Mao SY, Zhu WY: Impact of tiny miRNAs on cancers. World J Gastroenterol 2007, 13:497–502
4. Esquela-Kerscher A, Slack FJ: Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 2006, 6:259–269
5. Stahlhut Espinosa CE, Slack FJ: The role of microRNAs in cancer. Yale J Biol Med 2006, 79:131–140
6. Perron MP, Provost P: Protein interactions and complexes in human microRNA biogenesis and function. Front Biosci 2008, 13:2537–2547
7. Lund E, Dahlberg JE: Substrate selectivity of exportin 5 and Dicer in the biogenesis of microRNAs. Cold Spring Harb Symp Quant Biol 2006, 71:59–66
8. Chuang JC, Jones PA: Epigenetics and microRNAs. Pediatr Res 2007, 61:24R–29R
9. Croce CM, Calin GA: miRNAs, cancer, and stem cell division. Cell 2005, 122:6–7
10. Herath NI, Leggett BA, Macdonald GA: Review of genetic and epigenetic alterations in hepatocarcinogenesis. J Gastroenterol Hepatol 2006, 21:15–21
11. Nam SW, Jung JJ, Bae SH, Choi JY, Yoon SK, Cho SH, Han JY, Han NI, Yang JM, Lee YS: Clinical outcomes of delayed clearance of

serum HBsAG in patients with chronic HBV infection. Korean J Intern Med 2007, 22:73–76

12. Fattovich G, Stroffolini T, Zagni I, Donato F: Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology 2004, 127:S35–S50

13. Yu MC, Yuan JM: Environmental factors and risk for hepatocellular carcinoma. Gastroenterology 2004, 127:S72–S78

14. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO, Landthaler M, Lin C, Socci ND, Hermida L, Fulci V, Chiaretti S, Foa R, Schliwka J, Fuchs U, Novosel A, Muller RU, Schermer B, Bissels U, Inman J, Phan Q, Chien M, Weir DB, Choksi R, De VG, Frezzetti D, Trompeter HI, Hornung V, Teng G, Hartmann G, Palkovits M, Di LR, Wernet P, Macino G, Rogler CE, Nagle JW, Ju J, Papavasiliou FN, Benzing T, Lichter P, Tam W, Brownstein MJ, Bosio A, Borkhardt A, Russo JJ, Sander C, Zavolan M, Tuschl T: A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 2007, 129:1401–1414

15. Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY: miR-21-mediated tumor growth. Oncogene 2007, 26:2799–2803

16. Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, Yatabe Y, Kawahara K, Sekido Y, Takahashi T: A polycistronic microRNA cluster, miR-17–92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res 2005, 65:9628–9632

17. Popper H, Roth L, Purcell RH, Tennant BC, Gerin JL: Hepatocarcinogenicity of the woodchuck hepatitis virus. Proc Natl Acad Sci USA 1987, 84:866–870

18. Murakami Y, Saigo K, Takashima H, Minami M, Okanoue T, Brechot C, Paterlini-Brechot P: Large scaled analysis of hepatitis B virus (HBV) DNA integration in HBV related hepatocellular carcinomas. Gut 2005, 54:1162–1168

19. Etiemble J, Degott C, Renard CA, Fourel G, Shamoon B, Vitvitski-Trepo L, Hsu TY, Tiollais P, Babinet C, Buendia MA: Liver-specific expression and high oncogenic efficiency of a c-myc transgene activated by woodchuck hepatitis virus insertion. Oncogene 1994, 9:727–737

20. Buendia MA: Mammalian hepatitis B viruses and primary liver cancer. Semin Cancer Biol 1992, 3:309–320

21. Flajolet M, Tiollais P, Buendia MA, Fourel G: Woodchuck hepatitis virus enhancer I and enhancer II are both involved in N-myc2 activation in woodchuck liver tumors. J Virol 1998, 72:6175–6180

22. Wei Y, Fourel G, Ponzetto A, Silvestro M, Tiollais P, Buendia MA: Hepadnavirus integration: mechanisms of activation of the N-myc2 retrotransposon in woodchuck liver tumors. J Virol 1992, 66:5265–5276

23. Harris TM, Rogler LE, Rogler CE: Reactivation of the maternally imprinted IGF2 allele in TGFalpha induced hepatocellular carcinomas in mice. Oncogene 1998, 16:203–209

24. Yang D, Faris R, Hixson D, Affigne S, Rogler CE: Insulin-like growth factor II blocks apoptosis of N-myc2-expressing woodchuck liver epithelial cells. J Virol 1996, 70:6260–6268

25. Slagle BL, Zhou YZ, Butel JS: Hepatitis B virus integration event in human chromosome 17p near the p53 gene identifies the region of the chromosome commonly deleted in virus-positive hepatocellular carcinomas. Cancer Res 1991, 51:49–54

26. Hessein M, Saad G, Mohamed AA, Kamel AM, Amina M, Rogler CE: Hit and run mechanism of HBV-mediated progression to hepatocellular carcinoma. Tumori 2005, 241–247

27. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T: MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 2007, 133:647–658

28. Kutay H, Bai S, Datta J, Motiwala T, Pogribny I, Frankel W, Jacob ST, Ghoshal K: Downregulation of miR-122 in the rodent and human hepatocellular carcinomas. J Cell Biochem 2006, 99:671–678

29. Rinaldi A, Poretti G, Kwee I, Zucca E, Catapano CV, Tibiletti MG, Bertoni F: Concomitant MYC and microRNA cluster miR-17–92 (C13orf25) amplification in human mantle cell lymphoma. Leuk Lymphoma 2007, 48:410–412

30. Mishima T, Mizuguchi Y, Kawahigashi Y, Takizawa T, Takizawa T: RT-PCR-based analysis of microRNA (miR-1 and -124) expression in mouse CNS. Brain Res 2007, 1131:37–43

31. Sylvestre Y, De G, V, Querido E, Mukhopadhyay UK, Bourdeau V, Major F, Ferbeyre G, Chartrand P: An E2F/miR-20a autoregulatory feedback loop. J Biol Chem 2007, 282:2135–2143

32. Novotny GW, Sonne SB, Nielsen JE, Jonstrup SP, Hansen MA, Skakkebaek NE, Rajpert-De ME, Kjems J, Leffers H: Translational repression of E2F1 mRNA in carcinoma in situ and normal testis correlates with expression of the miR-17–92 cluster. Cell Death Differ 2007, 14:879–882

33. Johnson DG, Degregori J: Putting the oncogenic and tumor suppressive activities of E2F into context. Curr Mol Med 2006, 6:731–738

34. Cho WC: OncomiRs: the discovery and progress of microRNAs in cancers. Mol Cancer 2007, 6:60

35. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, Hammond SM: A microRNA polycistron as a potential human oncogene. Nature 2005, 435:828–833

36. Moran DM, Mattocks MA, Cahill PA, Koniaris LG, McKillop IH: Interleukin-6 mediates G(0)/G(1) growth arrest in hepatocellular carcinoma through a STAT 3-dependent pathway. J Surg Res 2007, 147:23–33

37. Loffler D, Brocke-Heidrich K, Pfeifer G, Stocsits C, Hackermuller J, Kretzschmar AK, Burger R, Gramatzki M, Blumert C, Bauer K, Cvijic H, Ullmann AK, Stadler PF, Horn F: Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. Blood 2007, 110:1330–1333

38. Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP: MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 2007, 27:91–105