MicroRNA Responses to Cellular Stress

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Abstract

Recent work has begun to explore the instrumental role that small noncoding RNA species, particularly microRNAs (miRNA), have both in classifying human tumors and in directing embryonic development. These studies suggest that developmental programs in essentially all organisms studied are set, in part, by varied expressions of miRNAs and that neoplasia is characterized by altered expression of miRNAs. Reasoning that these observations are linked, we examined whether cellular exposures that induce both developmental anomalies and cancer alter miRNAs. Using microarrays of 385 known human miRNAs, we studied human lymphoblastoid cells grown under various conditions or treatments. Folate deficiency induced a pronounced global increase in miRNA expression. We observed no significant alteration in miRNA expression in cells treated with γ-irradiation, whereas exposure to sodium arsenite led to global increases in miRNA expression. The miRNA hsa-miR-222 was identified from these arrays as significantly overexpressed under folate-deficient conditions, and this finding was confirmed *in vivo* in human peripheral blood from individuals with low folate intake. Alterations to cellular miRNA expression profiles represent a novel mode of action of folate deprivation and arsenic exposure, and specific alterations in miRNA expression may be a powerful biomarker for these and other toxins with serious effects on human health. (Cancer Res 2006; 66(22): 10843-8)

Introduction

MicroRNAs (miRNA) are noncoding RNA species of ~22 nucleotides in length, of which the precursor gene products fold into a characteristic hairpin shape. In mammals, the active RNA is processed by the DICER protein and functions by base pairing imperfectly with target mRNAs to inhibit protein accumulation (1); cleavage of the target mRNA as seen in lower organisms has also been reported (2). In humans, ~800 miRNAs are predicted to exist (3), and each of these is thought to have hundreds of targets, suggesting that miRNAs play a critical regulatory role in cellular protein expression (4). These miRNAs are known to play critical roles in developmental timing and patterning, differentiation, and organogenesis (5). More recently, alterations in miRNA profiles have been observed in a variety of human cancers, and this type of profiling has been proposed as a novel method for classifying diseases (6, 7). In tumors, these miRNA may act as oncogenes, leading to the specific dysregulation of their target gene products (8, 9). In chronic lymphocytic leukemia, for example, alterations of miRNA profiles have been used to accurately predict prognosis and disease progression (10) and specific miRNAs have also been implicated in normal erythropoiesis (11). Although alterations in miRNA expression have been described in cancer and may contribute to other disease states, it remains unclear what is driving these alterations or if exposures or conditions known to contribute to these diseases can modulate miRNA expression.

Exposure to select agents has been associated with both adverse birth outcomes and the induction of cancers. Whereas mechanisms of action of some of these agents are understood, for others the mode of action remains to be completely elucidated. One such agent, γ-radiation, is a well-characterized genotoxic agent leading to direct DNA damage, with exposures *in utero* associated with serious adverse birth outcomes (such as brain damage and growth retardation; ref. 12) and exposures throughout life associated with the occurrence of multiple cancer types (13). Similarly, dietary folate deficiency has been linked to developmental anomalies (e.g., neural tube defects; ref. 14) as well as increased risk for a number of cancers, including colorectal, breast, oral, pancreatic, and lung tumors, among others (15). The mechanism of action of folate is thought to be related to its role in the production of *S*-adenosyl methionine, critical to both thymine synthesis and DNA methylation. Low dietary folate levels lead to reduction of DNA stability, through misincorporation of uracil and subsequent DNA damage during the repair process, as well as to increased genomic hypomethylation (16, 17). When hypomethylation occurs in the 5′ regulatory region of genes or viral elements, it has been associated with increased gene expression (18, 19) and is also thought to be related to the overall genomic instability that is observed in cancer (20). However, the timing, targeting, and relationship of these various alterations to cancer or developmental anomalies remain unclear. Arsenic exposure, like ionizing radiation and folate deficiency, has been linked to a variety of human cancers, including skin, bladder, and lung cancers (21). The mode of action of arsenic has been difficult to define due to its lack of carcinogenicity in animal models (22), but may be similar to folate deficiency, as arsenic may disrupt one-carbon metabolism and lead to diminished cellular genomic methylation (23). Because folate deficiency, arsenic exposure, and γ-irradiation are all known developmental toxicants and human carcinogens, and as the fundamental understanding of the modes action of folate deficiency and arsenic exposure on initiation and development of cancer is incomplete, the fact that particular miRNA expression patterns are seen in both abnormal development and cancer led us to reason that the toxic effects mediated epigenetically by these exposures may be related to alteration in miRNA expression profiles. We have therefore examined *in vitro* the effect of these toxicants and growth condition on the expression pattern of 385 known human miRNAs using a commercially available microarray platform. We have also examined the relative expression of one of these miRNAs in human subjects with known dietary deficiency to clarify *in vivo* the presence of these alterations.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell growth and exposure conditions. Human immortalized lymphoblast cell line TK-6 was cultured in standard RPMI 1640 (Invitrogen, Inc., Gaithersburg, MD) or folate-deficient RPMI 1640 (Invitrogen). Growth medium was supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin; dialyzed FBS (Invitrogen) was added to the folate-deficient medium to eliminate folic acid in the serum. For controls, folate deficiency, arsenic exposure, and 6-day γ-irradiation exposure groups, 10^6 cells were diluted in 50 mL of appropriate growth medium. For the 4-hour post-γ-irradiation exposure group, 10^7 cells were diluted in 50 mL of growth medium. For the arsenic exposure group, sodium arsenite was added to the medium to a concentration of 2 μmol/L. For the γ-irradiation exposure group, cells were diluted and allowed to incubate for 4 hours before irradiation treatment and were exposed at a dose rate of 86.76 rad/min to a final dose of 2.5 Gy using a Philips MGC-40 X-ray source. Following exposure, cells were returned to the incubator. After 4 hours, the short-term post-γ-irradiation exposure group, as well as a mock (control) group, was collected for RNA isolation. All experimental and control conditions were done in triplicate. For all other groups, cells were cultured for 6 days, with the media changed and renewed, with the appropriate treatment, on day 3. To examine the reversibility of alterations to miRNA expression, cells from the folate-deficient group, as well as parallel controls, were grown in complete medium for 10 days, with medium renewals every 2 days.

RNA isolation, microarray processing, and analysis. Following appropriate incubation times, the cells were harvested by centrifugation, the media discarded, and cells were washed with cold PBS. Total RNA was isolated immediately from 10^7 cells with the mirVANA RNA Isolation Kit (Ambion, Inc., Austin, TX) according to the protocol of the manufacturer for total RNA isolation. RNA was quantified using the ND-1000 spectrophotometer (Nanodrop, Wilmington, DE), aliquoted, and immediately stored at −80°C until needed.

Samples for miRNA profiling studies were processed by Ambion Services according to the standard operating procedures of the company. The miRNA-enriched fraction was obtained by passing 10 μg of total RNA through a flashPAGE Fractionator apparatus (Ambion) and cleaned and concentrated using the flashPAGE Reaction Clean-Up Kit (Ambion). The 3′ ends of the RNA molecules were tailed and labeled using the *mir*Vana miRNA Labeling Kit (Ambion) according to the instructions of the manufacturer. Amine-modified nucleotides were incorporated during the poly(A) polymerase–mediated tailing reaction, and Cy5 succinimide esters [GE Healthcare (Amersham Biosciences, Piscataway, NJ)] were conjugated to the amine moieties on the miRNAs. Hybridization to the *mir*Vana miRNA Bioarrays (Ambion) was done with the *mir*Vana miRNA Bioarray Essentials Kit (Ambion). The Cy5 fluorescence on the arrays was scanned at an excitation wavelength of 635 nm using a GenePix 4200AL scanner (Molecular Devices, Union City, CA). The fluorescent signal associated with the probes and local background was extracted using GenePix Pro (version 6.0, Molecular Devices).

The background-adjusted fluorescent values generated by GenePix Pro were normalized for each miRNA using a variation stabilization transformation method described by Huber et al. (24). ANOVA was initially done to identify the miRNA significantly altered across the treatment conditions and subsequent analyses were limited to these miRNA. Hierarchical clustering was carried out using correlation distance as the distance metric and average linkage between clusters to do the clustering. Principal component analysis was done using covariance for the dispersion matrix and normalized scaling. Protected two-sample *t* tests with an assumption of equal variance were applied to test for significant differences between treatment groups. False discovery rate was controlled using a step-up approach as described by Benjamin and Hochberg (25) with a false discovery rate of 0.5.

Study population and blood RNA isolation. Five subjects within the top 1 percentile and six subjects within the lowest 1 percentile of dietary folate intake and with available cryopreserved whole blood were obtained from an ongoing population-based case-control study of head and neck squamous cell carcinoma in the Boston metropolitan area (26). All subjects provide a written informed consent to participate in the study, as approved by the appropriate institutional review boards. Subject dietary folate information is obtained through a validated diet history questionnaire, the Harvard-Willett Food Frequency Questionnaire (27), given to all participants in the study. Total RNA was isolated from whole blood using the mirVANA RNA Isolation Kit (Ambion) following the protocol of the manufacturer for total RNA isolation. RNA was quantified with the ND-1000 spectrophotometer (Nanodrop), aliquoted, and immediately stored at −80°C until needed.

Quantitative reverse transcription-PCR. Quantitative reverse transcription-PCR (RT-PCR) was done on 25 ng of total RNA using the mirVANA quantitative RT-PCR miRNA Detection Kit (Ambion) with mirVANA quantitative RT-PCR primer sets (Ambion) for the miRNA of interest. Detection of amplification was done with SYBR green nucleic acid stain (Invitrogen) on an ABI-7300 Sequence Detection System (Applied Biosystems, Foster City, CA). For normalization, primer sets which amplify U6 snRNA were used and done parallel to the miRNA of interest using the Pffafl (28) method for relative quantitation. Relative expression was compared between the folate-deficient and folate-sufficient groups using a Wilcoxon rank sums test.

Results

MiRNA profiles are altered by folate deficiency and arsenic exposure but not by γ-irradiation. We used the well-characterized, immortalized, normal diploid human lymphoblast cell line TK-6 to examine the effects of growth in folate-deficient medium for 6 days, exposure to 2 μmol/L sodium arsenite for 6 days, and exposure to 2.5 Gy of γ-irradiation both acutely (4 hours post-exposure) and long term (6 days post-exposure) on the miRNA expression profile in these cells. The 6-day time period was chosen based on previous reports showing that folate deficiency for this period can lead to altered *S*-adenosyl methionine levels and can alter global genomic methylation without severe consequences for cell viability (29). Similarly, the dose of γ-irradiation was chosen based on previous data showing it to elicit alterations in gene expression, without a marked change in cell viability at the 4-hour or 6-day time point (30). The miRNA profile was delineated using a novel microarray-based platform, the *mir*Vana miRNA Bioarray (Ambion), which simultaneously examines 385 known human miRNAs.

The normalized data on the expression pattern of the 385 human miRNAs examined will be deposited in the GEO archive. Hierarchical clustering was used to examine the relationships between these treatments and alterations in miRNA profiles. Clustering was initially done on all miRNAs on the array (Supplementary Fig. S1). An ANOVA analysis was used to identify the subset of miRNAs significantly altered across the treatment conditions, and this subset of 26 miRNA was then subjected to hierarchical clustering (Fig. 1*A*). Principal component analysis was done on this subgroup of miRNA (Fig. 1*B*). These analyses show that exposure to arsenic as well as folate deficiency induces significantly different profiles than those from cells under control (normal growth) conditions. Exposures to γ-irradiation did not generally alter the profile of miRNA expression, as the γ-irradiation-exposed groups clustered most closely to the control conditions.

When each of the treatment conditions is examined individually, it is clear that growth in folate-deficient medium had the most striking effect on miRNA expression, leading to a significant general increase in the expression of miRNA, as shown by a nonsymmetrical distribution of miRNA expression plotted on the volcano plot in Fig. 2*A*. These plots represent the fold change in miRNA expression (represented on the *X* axis as log 2 ratio of

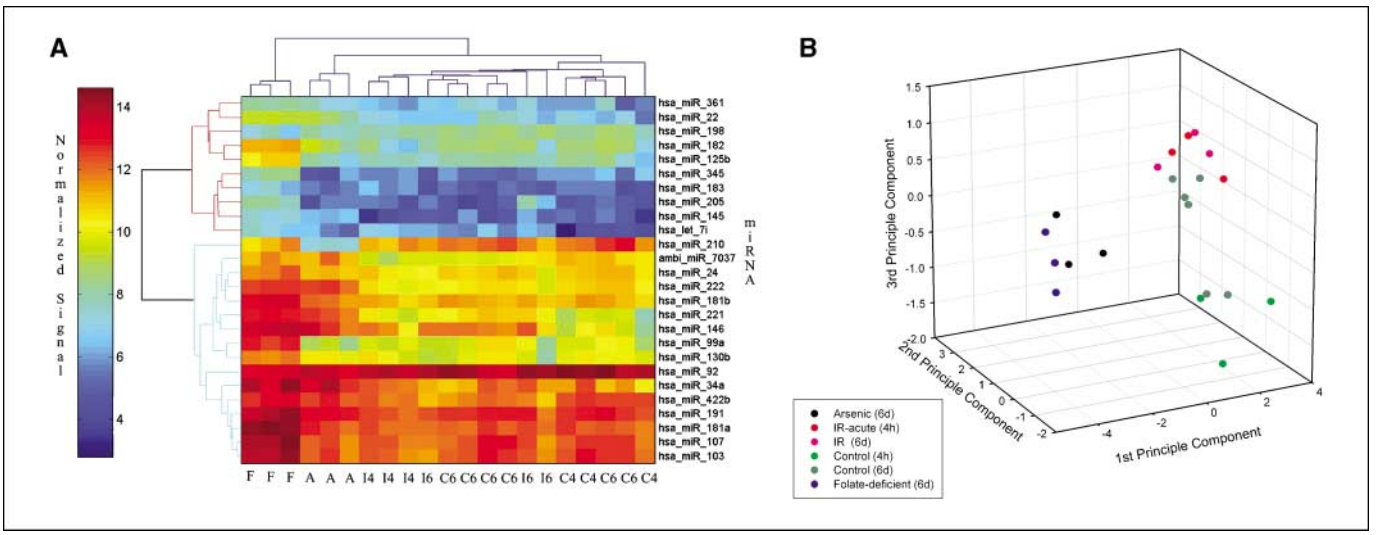

Figure 1. MiRNA profiles of TK-6 cells are specifically and reproducibly altered by growth in folate-deficient medium and exposure to sodium arsenite, but not by γ-irradiation, compared with controls. MiRNA expression was evaluated by microarray analysis. *A,* hierarchical clusters of miRNA significantly altered (as determined by ANOVA) across the treatment groups and time periods given at the bottom of the figure (*F,* folate deficient; *A,* sodium arsenite treatment; *I4,* γ-irradiation exposure, 4-hour time point; *I6,* γ-irradiation exposure, 6-day time point; *C4,* control at 4-hour time point; *C6,* control at 6-day time point). These results show that folate deficiency and, to a lesser extent, sodium arsenite exposure lead to up-regulation of these specific miRNAs, and cluster separately than γ-irradiation-exposed and control treatment groups. *B,* principal component analysis found miRNA to be significantly altered across the treatment groups. This plot illustrates the level of spread between the samples and experimental groups using three principal components (which describes 84.3% of the variance). These results confirm that the folate-deficient and sodium arsenite treatment groups show distinct miRNA expression profiles compared with the controls and γ-irradiation (*IR*)-exposed cells.

miRNA signal in treated to control cells) plotted against the magnitude of the *P* value (represented on the *Y* axis as the log of the *P* value obtained from the protected *t* test), with statistically significant signals denoted as red circles. Arsenic exposure showed a similar, although not as dramatic, general increase in miRNA expression, with fewer miRNAs reaching statistical significance (Fig. 2*B*). For γ-irradiation exposure, the results of the hierarchical clustering and principal component analysis (showing little difference in the profiles between irradiated and mock-treated cells) are confirmed by the volcano plot data for both acute and long-term response to γ-irradiation exposure. The expression pattern 4 hours post-exposure (Fig. 2*C*) suggests that a number of miRNA show >2-fold increases in expression (log 2 ratios >1), but the data show a large variance in these alterations, leading to none being considered statistically significant (i.e., the alterations did not occur similarly across replicates). The 6-day post-exposure results

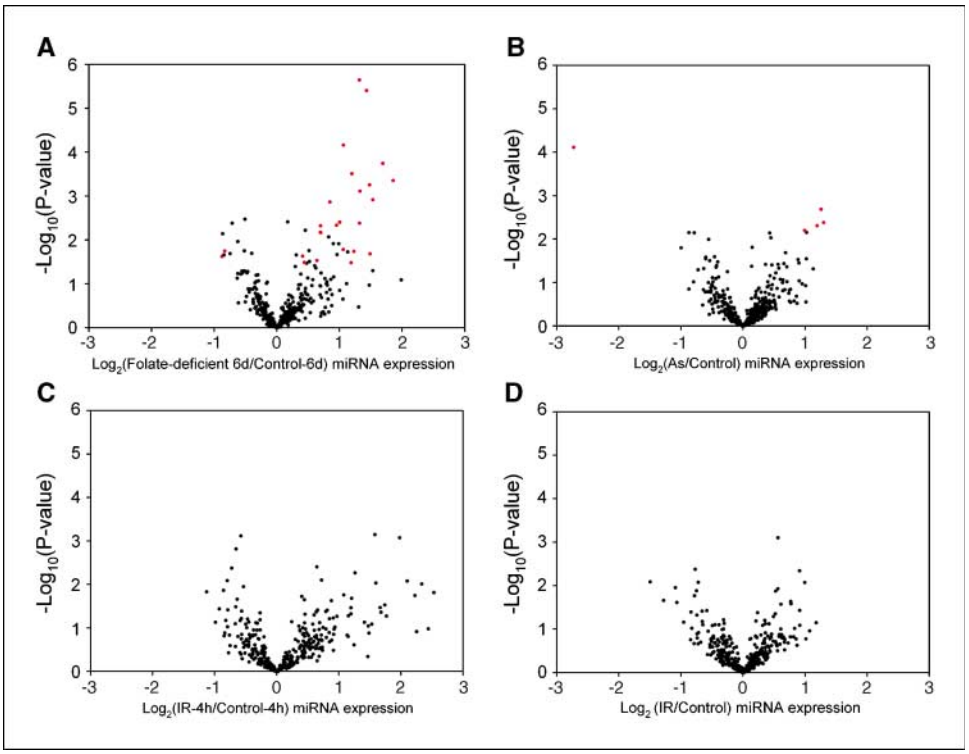

Figure 2. Volcano plots comparing altered miRNA expression in specific treatment groups showing significant alteration of a small number of specific miRNAs in folate-deficient growth and sodium arsenite exposure conditions. On all plots, the *X* axis represents the log 2 ratio of the normalized miRNA signal (mean of the three replicates) of the treatment condition compared with the appropriate control, thereby and the *Y* axis the $-\log_{10}$ *P* value obtained from the protected *t* test comparing the mean normalized miRNA signal in treatment to that in control. *Red dots,* miRNAs considered statistically significant following correction for multiple comparison. *A,* comparison of miRNA altered in cells grown for 6 days under folate-deficient conditions. The nonsymmetrical distribution of the data suggests an overall increase in miRNA expression. Comparison of miRNA altered in cells grown for 6 days in medium containing 2 μmol/L sodium arsenite (*B*) and in cells treated with 2.5 Gy of γ-irradiation after 4 hours (*C*) and 6 days (*D*). No miRNA altered by γ-irradiation was considered statistically significantly altered.

show a tight, symmetrical distribution of miRNA expression, with no specific miRNAs having expression alterations reaching statistical significance and few displaying a fold change >2 (Fig. 2*D*). The miRNAs with statistically significantly altered expression in the folate-deficient and arsenic exposure groups are listed in Table 1, with the magnitude of their alteration being generally not larger than 2- to 3-fold relative to expression in the control groups. Interestingly, all of the miRNAs of which the expression was altered by arsenic exposure were also altered, in the same direction, by folate deficiency. The miRNA expression results for these miRNA were confirmed by quantitative RT-PCR and showed that the array provides an accurate measure of the direction and magnitude of their altered expression (Supplementary Fig. S2).

As the significant alterations exhibited by folate deficiency and arsenic exposure may be attributable to alterations in the genomic DNA methylation extent, we used a modified version of the *LRE1* relative methylation assay to assess global methylation levels (31). We observed no significant alteration in *LRE1* relative methylation levels between any treatment and control groups (data not shown).

We next examined if the alterations observed due to folate deficiency could be reversed by returning these cells to complete growth medium conditions. Cells grown under folate deficiency, as well as parallel controls, were cultured for 10 days in complete medium, and their miRNA expression profiles examined. As shown in Fig. 3, following return to complete medium, the expression profiles of the previously folate-deficient cells no longer differ from control cells grown only in complete medium. There were no miRNAs identified that were significantly between the previously folate-deficient and control cells.

Table 1. MiRNA significantly altered by 6-day folate deficiency or arsenic exposure compared with control

MiRNA name	Fold change	*P**
Folate deficiency (6 d)		
hsa-miR-181b	2.49	2.0×10^{-06}
hsa-miR-182	2.70	4.0×10^{-06}
hsa-miR-222	2.09	6.9×10^{-05}
hsa-miR-345	3.23	1.8×10^{-04}
hsa-miR-181a	2.29	3.1×10^{-04}
hsa-miR-205	3.62	4.4×10^{-04}
hsa-miR-145	2.79	5.6×10^{-04}
hsa-miR-99a	2.51	7.8×10^{-04}
hsa-miR-125b	2.89	1.2×10^{-03}
hsa-miR-130b	1.80	1.4×10^{-03}
hsa-miR-221	2.50	4.2×10^{-03}
hsa-miR-22	1.93	4.7×10^{-03}
hsa-miR-191	1.62	4.8×10^{-03}
hsa-miR-103	1.62	6.7×10^{-03}
hsa-miR-107	1.62	6.9×10^{-03}
hsa-miR-34a	2.08	1.7×10^{-02}
hsa-miR-198	−1.78	1.8×10^{-02}
hsa-miR-183	2.35	1.8×10^{-02}
hsa-miR-146	2.80	2.1×10^{-02}
hsa-miR-422b	1.33	2.3×10^{-02}
hsa-miR-210	−1.84	2.4×10^{-02}
ambi-miR-7037	1.56	3.0×10^{-02}
hsa-miR-24	1.36	3.3×10^{-02}
hsa-miR-361	2.27	3.3×10^{-02}
Arsenic exposure (6 d)		
hsa-miR-210	−6.62	7.8×10^{-04}
hsa-miR-22	2.39	2.1×10^{-03}
hsa-miR-34a	2.46	4.2×10^{-03}
hsa-miR-221	2.29	4.9×10^{-03}
hsa-miR-222	1.99	6.3×10^{-03}

*P value derived from protected t test, comparing treatment groups to control groups.

Hsa-miR-222 is expressed at higher levels in subjects with folate-deficient diets. To determine the potential use and significance of these altered miRNAs, we asked whether this altered expression was detectable in human subjects. This would provide *in vivo* evidence that dietary folate insufficiency can lead to miRNA alterations in cells derived from the same tissue tested *in vitro*. For this analysis, 11 subjects were selected from a population-based case-control study of head and neck squamous cell carcinoma in the Boston metropolitan area (26). As part of the study, subjects completed a standardized food frequency questionnaire, which had been validated to examine the dietary intake of a wide range of nutrients, including dietary folate (27, 32, 33). Five subjects within the top 1 percentile of dietary folate and six subjects within the lowest 1 percentile of dietary folate with available cryogenically preserved whole blood were examined for relative expression of hsa-miR-222 and hsa-miR-22, which showed statistically significant increases in expression by the array under folate-deficient conditions, and hsa-miR-18 and hsa-miR-210, which showed no significant alteration under folate-deficient conditions according to the array analysis, using quantitative RT-PCR. Figure 4 depicts the expression of hsa-miR-222 relative to U6 snRNA and shows that those subjects in the lowest percentile of dietary folate intake had a significantly higher relative expression of this miRNA compared with those in the highest folate intake category ($P < 0.01$, Wilcoxon rank sums test). There was no significant difference in relative expression of hsa-miR-22, hsa-miR-18, or hsa-miR-210 between subjects of differing dietary folate status.

Discussion

Taken as a whole, our microarray results lend additional support to the growing body of evidence outlining a key role for miRNAs in both development and carcinogenesis in humans. Our results suggest that aberrant phenotypes may result from alterations in key miRNA expression at critical points in development and tumorigenesis due to altered folate status. Reduced dietary folate can lead to reduced levels of *S*-adenosyl methionine, which is the required methyl-group donor for all cellular methylation reactions, including those for DNA and proteins such as histones. We believe that the altered miRNA expression profiles we have observed may be related to this altered methyl-donor pool, which could lead to epigenetic alterations at the DNA or histone code levels, resulting in changes in miRNA expression as well as gene expression, occurring due to or independently from miRNA expression changes.

Similar effects on alterations of genomic methylation status have also been reported for arsenic (23, 34), which is metabolized for excretion through methylation and can thus alter the methyl-donor pool available in the cell. Our results show that the miRNAs of which the expression is specifically altered by arsenic exposure are those that are altered by folate deficiency, adding to the evidence that arsenic may operate by altering one-carbon metabolism and thus downstream epigenetic effects. Interestingly, we did not observe any significant alteration in genomic DNA methylation extent, as measured by a biomarker of global methylation, *LRE1*

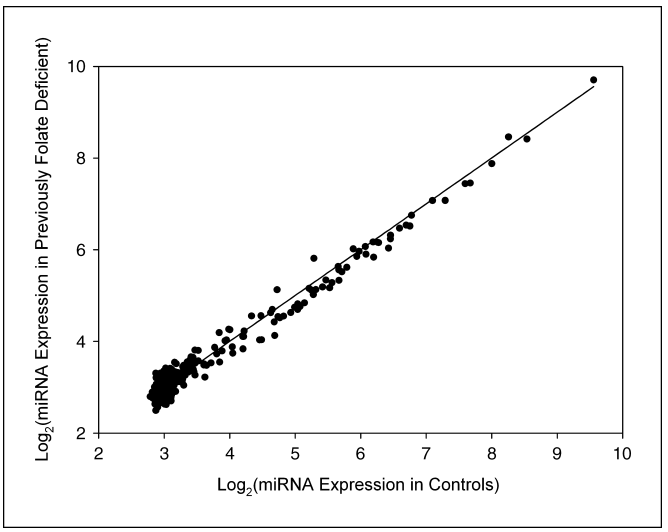

Figure 3. Alterations to miRNA expression profiles are reversible by returning cells to complete growth medium. Cells previously grown under folate deficiency, as well as control cells, were grown for 10 days in complete growth medium, and their miRNA expression profiles examined by microarray analysis. The log 2 mean expression level of each miRNA is plotted for the previously folate-deficient cells on the *X* axis and for the control cells on the *Y* axis. A line representing a slope of 1 with intercept of 0 is also plotted to illustrate the small deviation between the treatment groups.

(Line-1.2) relative methylation, which has previously been shown to reflect global methylation levels (31). This suggests that the alterations being observed may not be attributable to global DNA methylation alterations associated with folate deficiency or arsenic exposure, but may reflect other alterations related to altered folate pools, perhaps acting in a more targeted fashion. Additional work is required to better define the chromatin state at miRNA loci, as well as the transcription factors responsible for their expression.

Notably, we saw no significant change in miRNA expression profiles with exposure to 2.5 Gy of γ-irradiation after 4 hours or 6 days, compared with mock-treated controls. Studies have shown that these cells, similarly exposed, exhibit a potent alteration in gene expression after 4 hours, all the while exhibiting no marked changes in viability (30). Therefore, these cells react to the stress (administered in this fashion) through a significant transcriptional response; however, our results show that ionizing radiation, which directly damages DNA, does not change the miRNA expression profile. Hence, the DNA damage response (i.e., DNA repair, cell cycle arrest, and/or apoptosis) is not likely to be mediated by miRNA-dependent events, but rather occurs at the level of protein stability (35) and/or transcriptional activation (36). Our data suggest that the toxins that act via alterations in miRNA expression are primarily those associated with nongenotoxic or epigenetic modes of action.

Both enhanced and diminished miRNA expressions have been reported to be characteristic of tumors (6, 7), and it remains unclear if the differences between studies are related to true differences in the tumors or in the method of examination of the miRNA profiles. Our use of commercially available microarrays for miRNA profiling will allow for interlaboratory comparison of our results and a consistent method for examining the effect of other toxicants on miRNA profiles.

The alterations in miRNA expression we observed are almost certainly akin to those occurring early in clonal expansion; these could possibly represent initiating alterations, leading to developmental anomalies associated with altered gene expression or clonal expansion and subsequent selection for a subset of cells capable of becoming tumorigenic. Our work is consistent with recent hypotheses suggesting an epigenetic progenitor of human cancer (37), as early epigenetic alterations caused by folate insufficiency or arsenic exposure, or possibly other nongenotoxic carcinogens, may alter the profiles of miRNA expression. It would be of great interest to determine if there are profound shifts in the miRNA expression profiles throughout the multistep process of carcinogenesis.

Our results also suggest that these alterations may be reversible, as returning the folate-deficient cells to complete medium allowed for a return of the miRNA expression profiles to that of control cells. This suggests that chronic, continuous exposure to carcinogenic conditions or tumor-promoting factors may be required to maintain these miRNA alterations, at least until additional irreversible genetic and/or epigenetic alterations occur and are propagated, thereby hastening tumor development. This result is also consistent with our findings that overall changes in global methylation levels, which would indicate a more permanent epigenetic alteration, did not occur under these treatment conditions. Although these miRNA alterations are reversible, if exposures during critical periods of development occur, even short-term alterations to miRNA expression profiles could lead to significant developmental problems, as it is clear that the timing of miRNA expression is critical in developmental programs (5, 38).

We have also shown that altered expression of hsa-miR-222 can be detected in human peripheral blood–derived cells, with higher expression of selected miRNAs (consistent with *in vitro* experiments) in subjects with low dietary folate levels. This finding suggests, first, that the use of TK-6 cells as a model for human lymphocytes is appropriate, and, second, that findings in this model can be applied directly to the study of human populations. Our data showing altered expression of hsa-miR-222 in TK-6 cells under both folate-deficient conditions and arsenic exposure, as well as our data from *in vivo* study of whole blood–derived RNA

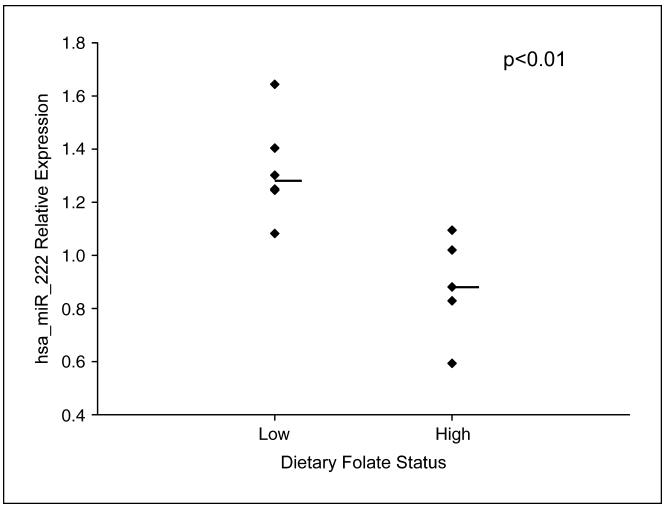

Figure 4. Hsa-miR-222 expression is higher in subjects with low dietary folate intake compared with those with high dietary folate intake. Relative expression of has-miR-222 was determined relative to U6 snRNA in RNA derived from human cryopreserved whole blood from six patients with dietary folate in the lowest percentile and five patients with dietary folate in the highest percentile of a study population of ~1,500 subjects involved in a population-based case-control study of oral cancer. Relative hsa-miR-222 expression is plotted (♦) on the *Y* axis; *horizontal bar,* median relative expression for each group. The distribution of relative hsa-miR-222 expression is significantly different between the two groups ($P < 0.01$, Wilcoxon rank-sum test).

(from subjects with lower dietary folate), are consistent with previous reports showing that this miRNA plays an important role in human erythropoiesis (11), leukemic cell differentiation (39), and the development of B-cell leukemia (11, 40). These results together suggest that hsa-miR-222 may be a cell type–specific factor in blood cell development, which is targeted for alteration by exposures in human cancer progression, and point to the use of increased relative expression of hsa-miR-222 as a novel biomarker of altered folate status, identifying individuals potentially at risk for multiple adverse health outcomes. That is, even with sufficient dietary folate intake, unusual hsa-miR-222 expression could represent the presence of altered one-carbon metabolism, possibly due to other lifestyle factors, such as heavy drinking, or differences in the enzymes responsible for this metabolism. We also saw no difference in hsa-miR-18 and hsa-miR-210 expression in the peripheral blood, but did not expect a difference, as these miRNA were unchanged by folate deficiency in the *in vitro* experiment. At the same time, although hsa-miR-22 showed a significant alteration in the *in vitro* experiment, we did not observe a difference in the relative expression of this miRNA in the peripheral blood of subjects by dietary folate. This may reflect the specificity of some miRNAs, but not of others, as well as the importance of confirming *in vitro* results by examining these alterations *in vivo*. It is possible that these alterations are not the direct result of folate pool alterations, but may instead be induced as a result of companion physiologic alterations associated with this specific miRNA, perhaps including other factors, or disease states within the hematologic compartment. Larger, carefully planned epidemiologic studies will be needed to more thoroughly examine alterations of miRNAs in human populations and how exposures, diseases, and lifestyle factors affect miRNA expression.

Our results provide further evidence to link altered expression of miRNA with human disease states, showing that conditions and exposures known to contribute to disease also lead to alterations of miRNA expression profiles. These results open the way for a fundamentally new approach to the evaluation of human toxicants, an approach which must consider the epigenetic effects of exposure, particularly the alteration of expression of miRNA species, as it may be through these alterations that many exposures elicit their pathologic effects.

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