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MicroRNA Expression Profiles Associated with Development of Drug Resistance in Ehrlich Ascites Tumor Cells

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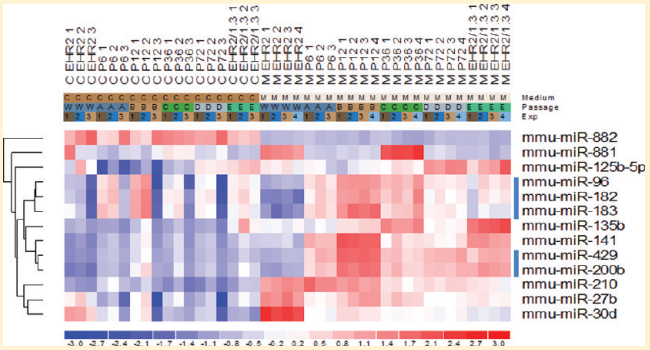
Supporting Information

ABSTRACT: Multidrug resistance (MDR) poses a major obstacle to successful chemotherapeutic treatment of cancer, and often involves multiple genes, which may be regulated post-transcriptionally by microRNAs (miRNAs). The purpose of the present study was therefore to identify any resistance-associated changes in miRNA expression in a sensitive and five increasingly drug-resistant Ehrlich ascites tumor (EAT) cell lines, representing different steps in the development of resistance. We used an LNA-enhanced microarray platform to study the global miRNA expression profiles in the six murine EAT cell lines, and identified growth-, hypoxia-, and resistance-specific miRNA patterns. Among the differentially expressed miRNAs, we found the two clusters miR-183∼miR-96∼miR-182 and miR-200b∼miR-200a∼miR-429 as well as miR-141 to be consistently upregulated in the MDR cell lines, while miR-125b-5p and the two clusters miR-30d∼miR-30b and miR-23b∼miR-27b∼miR-24-1 were downregulated in most of the resistant EAT cells. Several of the target genes for these miRNAs—including Zeb1/Zeb2 and members of the Fox gene family—could contribute to the drug-resistant phenotype, although we did not find that the degree of resistance was directly correlated to any specific changes in miRNA expression. Probably, the observed miRNA expression patterns reflect the underlying genomic instability of the tumor cells, and further studies are needed to explore how the highly complex regulatory miRNA networks contribute to the development of MDR.



KEYWORDS: microRNA, microarrays, qRT-PCR, Ehrlich ascites tumor cells, drug resistance, *in vitro*, *in vivo*

INTRODUCTION

A major impediment to successful chemotherapeutic treatment of cancer is the development of multidrug resistance (MDR), which can arise by pleiotropic mechanisms, including drug efflux, detoxification, DNA repair, and evasion of apoptosis.[1] Drug resistance often involves multiple genes which—in addition to control by epigenetic changes, such as DNA methylation and histone modification—can undergo post-transcriptional regulation by microRNAs (miRNAs).[2] Several studies suggest that miRNAs are involved both in tumorigenesis[3,4] and in drug resistance,[5−9] but their specific roles in these processes are still poorly understood.

The aim of the present study was to identify resistance-associated changes in miRNA expression in a panel of Ehrlich ascites tumor (EAT) cells with well-characterized phenotypes ranging from drug sensitive to highly drug resistant. Such a comparative study, assessing global miRNA expression at various stages of development of resistance, is the first of its kind and may provide significant information on how the properties of MDR cells change with the development of drug resistance.

MATERIALS AND METHODS

Tumor Cells. Six murine EAT cell lines that have been characterized in our laboratories[10−12] were used for miRNA profiling: the parental, drug-sensitive cell line (EHR2), four increasingly resistant sublines, developed by intraperitoneal treatment with daunorubicin at 0.8 mg/kg (50% LD_{10}) for 6−72 passages (P6, P12, P36, P72), and a long-term selected cell line (EHR2/1.3) treated with daunorubicin at 1.3 mg/kg (LD_{10}) for +300 passages. Cells harvested from four mice per cell type were analyzed. In parallel to cells maintained intraperitoneally in NMRI/DBA mice (the *in vivo* group, M), EAT cells were also grown as suspension cultures ($n = 3$ per cell type) in RPMI-1640 containing FBS (10%),

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L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C, under 5% CO_2 (the *culture* group, C).

Cytotoxicity Assay. Drug resistance was assessed by a modification of the sulforhodamine B (SRB) staining method previously described by Skehan et al.[13] Briefly, cells were incubated for 48 h in the presence of serial dilutions of either doxorubicin (0.01−100 μM) or cisplatin (0.003−100 μM) before fixation in 50% trichloroacetic acid, and staining with 0.4% SRB. After washing with 1% acetic acid, bound SRB was solubilized with 10 mM Tris base (pH 10.5). Absorbance was measured at 492 nm, and the IC_{50} was calculated in GraphPad Prism 4 by fitting a sigmoidal dose−response curve to the relative cell densities.

RNA Purification. Total RNA was extracted from EAT cells using TRIzol reagent (Invitrogen) following the manufacturer's instructions. RNA was quantified with a NanoPhotometer (Implen), and RNA integrity was assessed using a Bioanalyzer 2100 (Agilent); the RNA integrity number (RIN) range was 6.9−9.7.

RNA Labeling. We applied a common reference design described by Søkilde et al.,[14] where the reference sample contained a mixture of total RNA from all cell lines analyzed. One microgram of total RNA from each sample was labeled using the miRCURY LNA microRNA Power Labeling Kit (Exiqon) following the manufacturer's specifications. Subline-specific RNA was labeled with Hy3, and the common reference RNA was labeled with Hy5.

Microarray Hybridization and Scanning. For miRNA profiling, miRCURY LNA microRNA Microarrays v.11.0 (human, mouse, rat) containing 1974 quadruplicate probes for miRNAs annotated to miRBase v. 14 were applied (Exiqon). Labeled RNA samples were hybridized to the arrays for 16 h at 65 °C in an HS4800 hybridization station (Tecan). After washing and drying, the microarrays were scanned in a DNA Microarray Scanner (Agilent) and the resulting images were quantified using Imagene v. 8.0 (BioDiscovery, CA).

Microarray Data Analysis. Interslide normalization was performed using a nonlinear quantile-based normalization algorithm in Rosetta Resolver (Rosetta Biosoftware). One-way ANOVA was applied to test for differentially expressed miRNAs. *P* values were adjusted for multiple testing by the Benjamini−Hochberg method with FDR ≤ 0.1. Heat maps and clustering were based on Pearson correlation coefficients and performed in MeV (TM4 Microarray Software Suite). Data was filtered to include only annotated *Mus musculus* miRNAs, and after minimum range filtering (Max − Min intensity >100) 201 miRNAs were left for further analysis.

Quantitative Real-Time PCR. The expression levels of 11 selected miRNAs including two reference genes were evaluated by miRNA-specific reverse transcription quantitative real-time PCR (RT-qPCR).[15] For methodological details and results, please see the Supporting Information available online.

Target Prediction. Potential miRNA targets were assessed by several target prediction algorithms using 3′ UTR sequence: MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl), DianaLab microT (http://diana.cslab.ece.ntua.gr/microT/),[16] PicTar (http://pictar.mdc-berlin.de/),[17] TargetScan (http://www.targetscan.org/),[18] and MiRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk).

RESULTS AND DISCUSSION

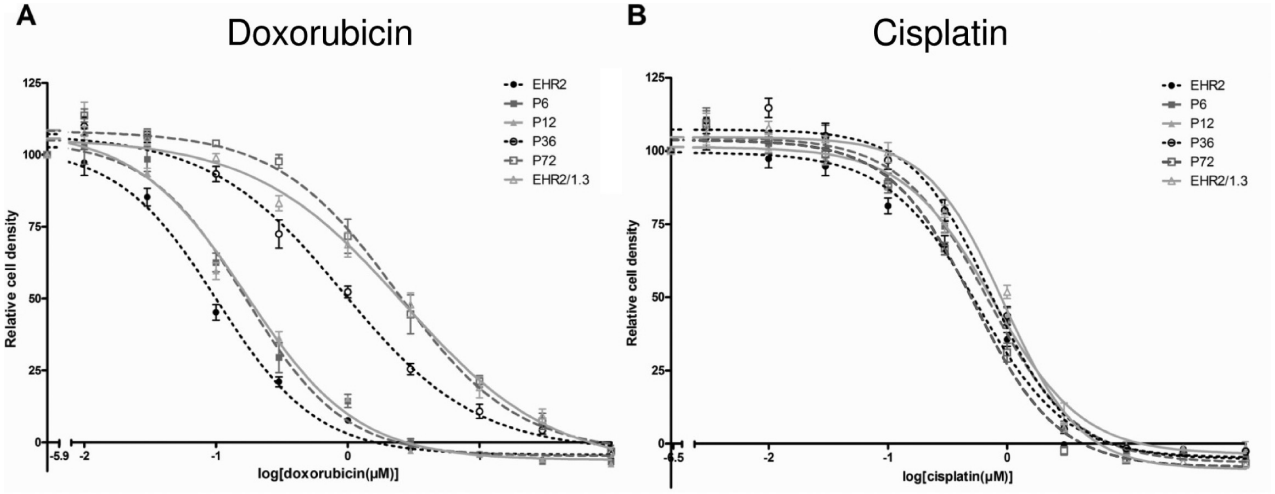
Resistance Profiles. To assess if the six progressively resistant EAT cell lines have retained their phenotype since their development in 1994,[12] we performed two sets of cytotoxicity experiments: one with doxorubicin, another with cisplatin. As shown in Figure 1A, the daunorubicin-selected cell lines are progressively resistant to doxorubicin, with P72 and EHR2/1.3 in particular exhibiting high levels of resistance (23- and 26-fold, respectively). This is expected, because these cell lines express increasing amounts of P-glycoprotein (Pgp), an efflux pump known to decrease the steady-state levels of its substrates, thus conferring MDR.[19] In comparison, we only observed minor differences in the cell lines' sensitivity toward cisplatin, which is not a Pgp substrate (Figure 1B). The long-term selected cells' appearing slightly resistant to cisplatin indicates that they could have developed Pgp-independent resistance mechanisms, which is in agreement with our previous study.[10]

miRNA Expression Profiling. We studied global miRNA expression in the EAT cell panel by applying a recently described, miRCURY microarray.[20] This platform contains LNA-enhanced capture probes for quantification of 645 mature murine miRNAs, as well as 406 proprietary miRNAs, 39 viral miRNAs, and 18 snoRNAs. Minimum range filtering and inclusion of only murine-specific signals yielded 201 miRNAs that were used to generate the heat map and two-way unsupervised hierarchical cluster shown in Figure 2A.

miRNA Expression Specific to Growth Condition. From the clustering of the miRNA expression data it appears that the major partitioning of samples is according to growth condition; i.e. the "culture" (C) group representing cells grown *in vitro* clearly separates from the "mouse" (M) group, where the EAT cells have been grown first *in vitro*, and then intraperitoneally *in vivo* in mice for 9 days before RNA isolation. This division of samples is further illustrated in Supplementary Figure S2 in the Supporting Information, which shows clustering based on the 92 miRNAs that differ the most between the C and M groups. Of these, more than twice as many are upregulated (65, $P < 0.05$ after Bonferroni correction) as downregulated (27, $P < 0.05$ after Bonferroni correction) in the M group compared to the C group. This may be because EAT cells within the peritoneum of a mouse are exposed to more external stimuli, e.g. diurnal/nocturnal variation, than what the same cells would encounter in the controlled environment of a culture flask. Such physiological stimuli may activate genes that would otherwise only be expressed at a minimal level. Our observations are in line with a recent report describing that stem cells obtained directly from murine embryonic neural tubes had twice as many miRNAs upregulated (40) as downregulated (21) when compared to cultured neural stem-cells,[21] and also with an mRNA expression profiling study demonstrating that primary hepatocytes in culture progressively lose expression of liver-specific genes.[22]

Among the miRNAs with significantly higher expression in the M group compared to the C group, we identified miR-210 (3-fold, $P = 2.5 \times 10^{-14}$), which has consistently been associated with hypoxia.[23,24] This suggests that the intraperitoneal oxygen tension is lower than that of the controlled atmosphere (21% O_2) of cells grown *in vitro*. In line with this notion, we also found numerous other "hypoxamirs" upregulated in the *in vivo* group: miR-21 (1.9-fold), the miR-23a∼miR-27a∼miR-24-2 cluster (1.8−2.2-fold), the miR-23b∼miR-27b∼miR-24-1 cluster (1.9−2.2-fold), miR-26a (2.8-fold), miR-26b (2.3-fold), miR-30b (1.8-fold), the miR-25∼miR-93∼miR-106b cluster (1.8−1.9-fold), miR-106a (1.9-fold), miR-152 (1.7-fold), and miR-200b (2.3-fold, all with $P < 0.0001$), concurrent with the original observations by Kulshreshtha et al.[25] and Guimbellot et al.[26]

Two of the miRNAs with the highest difference between the M and the C group were miR-451 (3.4-fold, $P = 1.2 \times 10^{-8}$) and



		EHR2	P6	P12	P36	P72	EHR2/1.3
Doxorubicin	IC_{50} (μM)	0.103	0.168	0.170	0.925	2.35	2.63
	RR	1	1.6	1.7	9.0	22.9	25.7
	P-value*		<0.001	<0.001	<0.001	<0.001	<0.001
Cisplatin	IC_{50} (μM)	0.570	0.697	0.763	0.727	0.530	0.938
	RR	1	1.2	1.3	1.3	0.9	1.7
	P-value*		<0.01	<0.001	<0.001	NS	<0.001

*P values for each sample compared to EHR2 in a one-way ANOVA with Dunnett post test

Figure 1. Cytotoxicity curves demonstrating increasing resistance to doxorubicin (A), and no or slight resistance to cisplatin (B) in parental (EHR2) and five MDR sublines. The relative resistance (RR) is calculated by dividing the IC_{50} of resistant cells by that of EHR2. Data are shown as mean ± SD ($n = 6$).

miR-223 (3.6-fold, $P = 6.8 \times 10^{-16}$), which were barely detectable in the C samples. Both these miRNAs are hematopoietic specific: miR-451 for erythrocytes[27] and miR-223 for lymphocytes and platelets,[28] and their presence in the M samples suggests blood contamination of the ascites fluid, even after thorough washing. Therefore, these miRNAs may be applied as very sensitive markers for the presence of blood in a sample.

X-Linked miRNA Expression. An interesting feature of the heat map in Figure 2A is a cluster of 17 miRNAs that are predominantly expressed in all of the M EHR2 and P36 samples, and also in one C EHR2 and all three C P36 samples, but virtually absent in the other sublines. These miRNAs are all confined to a 60 kbp unstable region on the X chromosome (Supplementary Figure S3 in the Supporting Information), which is subject to structural variation.[29,30] Thus, our observation concurs with the study by Hasholt et al.,[31] demonstrating major variations in the karyotype of EAT cells. We performed qPCR analysis on DNA extracted from the EAT cells and found no evidence supporting a change in copy number (Supplementary Figure S4 in the Supporting Information). This suggests that translocation to a transcriptionally more active locus could have taken place in the EHR2 and P36 cell lines, giving rise to the observed expression pattern. Typically, expression of the 17 clustered miRNAs is confined to testis and brain,[32] implying that they are under the control of a usually inactive promoter. This supports the notion that the miRNAs are only expressed EAT cells where such a translocation event has occurred, although we cannot rule out transcriptional activation. Further studies, such as comparative genomic hybridization, are required to confirm the hypothesis.

Experimental Variation in miRNA Expression. Another conspicuous pattern observed in the clustering of samples is an out-group consisting of five C samples, all originating from the same cell culture batch and same round of RNA extraction. As the heat map shows (and PCA representation and sample correlation matrix show, see Supplementary Figures S5 and S6 in the Supporting Information), the miRNA expression pattern from these samples differs from that of all other samples, and must be ascribed to a methodological bias which is not eliminated by the normalization procedure. In the subsequent data analysis we took the apparent out-group into concern, excluding these samples when making comparisons between cell lines.

Resistance-Related miRNAs. We identified 13 miRNAs, listed in Table 1, with significant changes in expression pattern between sensitive (EHR2) and drug-resistant sublines. The expression patterns of these miRNAs are illustrated in Figure 2B along with that of miR-881, which is part of the X-linked cluster, and miR-882, which is one of the most differentially expressed miRNAs when comparing the C and M groups. Nine of these miRNAs were validated by RT-qPCR, both on RNA samples that were originally used in the microarray analysis (the M group) and on an independent group of cultured samples. Overall, good correlation to the microarray data was observed for the M group, less so for the independent C group (Supplementary Figure S1 in the Supporting Information).

Among the miRNAs listed in Table 1 are nine that belong to the four genomic clusters miR-200b∼miR-200a∼miR-429, miR-183∼miR-96∼miR-182, miR-30d∼miR-30b, and miR-23b∼miR-27b∼miR-24-1. Within each cluster, the expression of these miRNAs is highly correlated.

In the resistant cell lines, the miR-200b∼miR-200a∼miR-429 cluster and miR-141 were upregulated; these miRNAs target the transcriptional repressors ZEB1/ZEB2, which induce epithelial-mesenchymal transformation (EMT) by regulating the expression

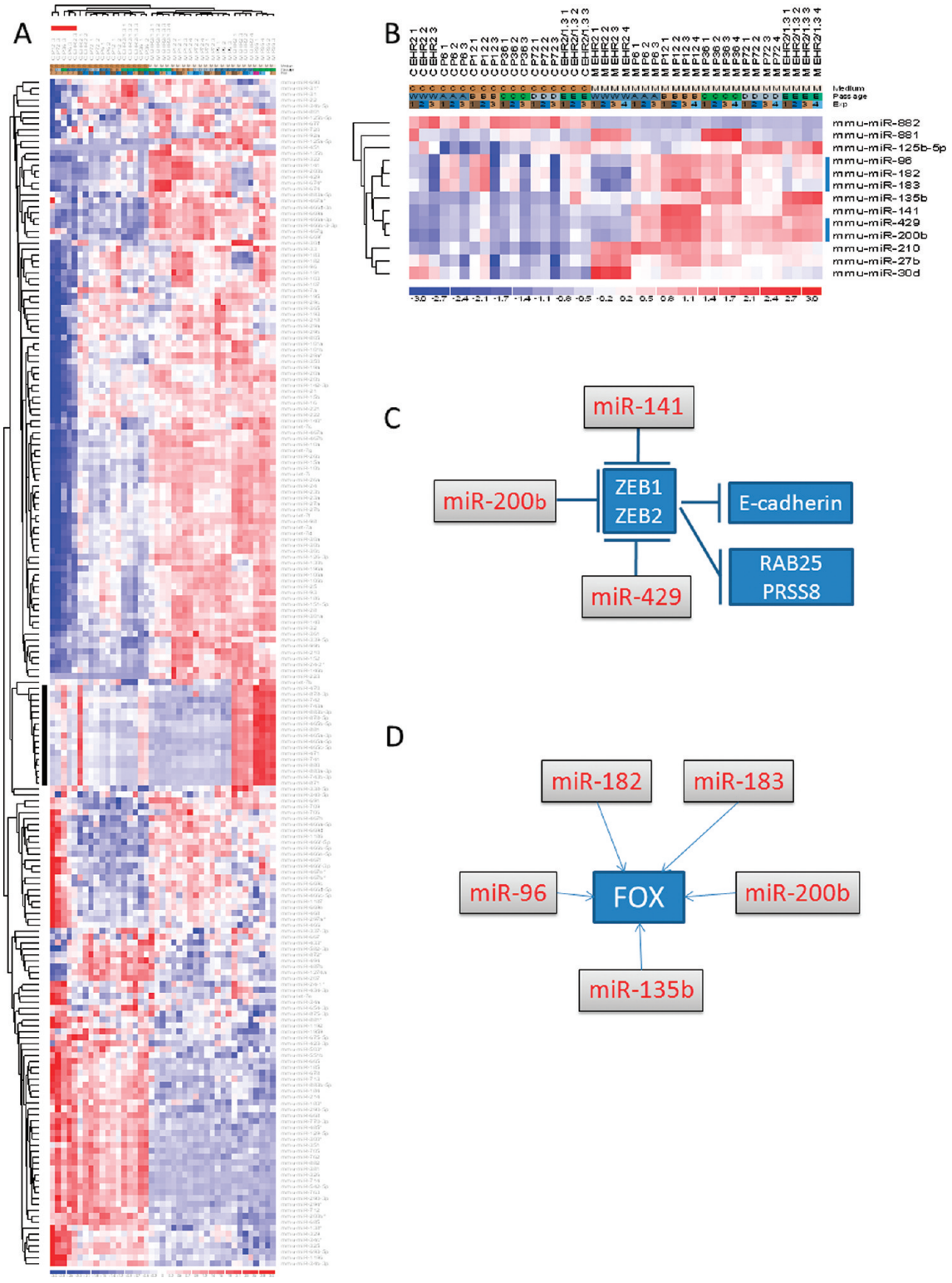


Figure 2. miRNA expression profiles of the six EAT cell lines. (A) Heat map and two-way unsupervised hierarchical clustering based on the 201 most variable miRNAs. The clustering is generated based on Euclidean distance and average linkage. The black vertical bar specifies a cluster of X-linked miRNAs. The red horizontal bar indicates an out-group of samples. (B) Heat map showing 13 miRNAs of particular interest, including nine selected for RT-qPCR validation. The two vertical bars indicate coexpressed miRNAs that reside on the same genomic cluster: miR-200b∼miR-429, and miR-96∼miR-182∼miR-183. Both heat maps represent mean-centered, log2-transformed data. (C) Interaction network between miR-141, miR-200b and miR-429 that all repress expression of ZEB1−ZEB2, which regulate expression of E-cadherin, RAB25, and PRSS8. (D) Multiple miRNAs, including miR-183∼miR-96∼miR-182, miR-135b, and miR-200b, target FOX.

Table 1. Microarray Data for Differentially Expressed miRNAs in Sensitive (EHR2) and Resistant EAT Cells

miRNA	Chromosomal localization		EHR2	P6	P12	P36	P72	EHR2/1.3
miR-210	7: 148.405.283-148.409.392	C	183	1,82 [1]	1,37	1,06	1,00	1,21
		M	836	1,22	-1,04	-1,81 [3]	-1,60 [3]	-1,92 [3]
miR-96	6: 30.119.446-30.119.551	C	448	1,79 [1]	2,31 [1]	1,33	1,38 [1]	1,60 [2]
		M	418	1,85 [3]	2,67 [3]	2,30 [3]	1,74 [3]	1,69 [2]
miR-182	6: 30.115.918-30.115.992	C	534	1,75 [1]	2,23 [1]	1,51 [1]	1,52 [1]	1,6 [1]
		M	423	2,03 [3]	2,73 [3]	2,45 [3]	1,83 [3]	1,77 [3]
miR-183	6: 30.119.668-30.119.737	C	331	1,56	1,93 [1]	1,31	1,27	1,27
		M	257	1,88 [3]	2,75 [3]	2,04 [3]	1,70 [3]	1,40 [3]
miR-200b	4: 155.429.790-155.429.859	C	94	1,47 [1]	2,60 [2]	1,60	1,57 [1]	1,80
		M	119	2,64 [3]	4,98 [3]	2,7 [3]	2,95 [3]	3,61 [3]
miR-429	4: 155.428.014-155.428.096	C	76	1,21 [1]	1,49 [1]	1,23	1,20	1,20
		M	87	1,85 [3]	2,76 [3]	1,76 [3]	1,97 [3]	1,93 [3]
miR-141	6: 124.667.932-124.668.003	C	76	1,14	1,44	1,20	1,17	1,23
		M	93	1,66 [3]	2,43 [3]	1,41 [2]	1,34 [3]	1,89 [3]
miR-125b-5p	16: 77.646.518-77.646.588 b-2	C	6187	-2,42	-2,01	-1,74	-1,24	-1,13
	9: 41.390.009-41.390.085 b-1	M	4835	-1,11	-1,16	1,02	1,45 [3]	1,36 [1]
miR-30d	15: 68.172.770-68.172.851	C	347	-1,44 [1]	-1,27 [1]	-1,66 [2]	-1,54 [2]	-1,72 [3]
		M	488	-1,9 [3]	-1,69 [3]	-1,88 [3]	-1,86 [3]	-2,06 [3]
miR-30b	15: 68.168.977-68.169.072	C	918	-1,33 [1]	-1,11	-1,59 [1]	-1,31	-1,31 [1]
		M	1294	-1,45 [2]	-1,15 [1]	-1,21	-1,3 [3]	-1,42 [2]
miR-27b	13: 63.402.020-63.402.092	C	2725	-2,11 [1]	-1,22	-1,58	-1,91	-1,65
		M	3883	-1,79 [3]	-1,21	-1,47 [3]	-1,9 [3]	-1,74 [3]
miR-23b	13: 63.401.792-63.401.865	C	2691	-1,68 [1]	-1,06	-1,22	-1,48	-1,39
		M	4615	-1,75 [3]	-1,1	-1,29	-1,61 [3]	-1,69 [3]
miR-135b	1: 134.094.665-134.094.761	C	105	-1,10	1,17	-1,03	-1,06	1,63
		M	137	-1,06	1,25 [2]	1,74 [3]	1,09	2,16 [3]
miR-881*	X: 64.055.119-64.055.196	C	157	-1,26	-1,12	1,47	-1,05	-1,23
		M	437	-3,17 [3]	-2,97 [3]	1,66 [3]	-3 [3]	-3,48 [3]

The C (culture) values are averages of 2 measurements (the C3 outliers have been excluded), and the M (mouse) values are averages of 3-4 measurements. The EHR2 column shows the absolute expression level of each miRNA in the wild type cells. The numbers in the five columns representing the resistant sublines are relative to the expression level in the parental cell line. * miR-881 is part of a large cluster, which is only expressed in EHR2 and P36.
[1] $P<0.05$, [2] $P<0.01$, [3] $P<0.001$. The expression of the miRNAs in **bold** was validated by RT-qPCR (Supplementary Fig. S1). The vertical lines on the left demark miRNAs that belong to the same genomic cluster.

of E-cadherin[33] (Figure 2C, Supplementary Figure S7 in the Supporting Information). E-cadherin has been implicated in cancer progression and metastasis, as its downregulation results in impaired cell−cell adhesion and increased cellular motility.[34]

Table 2. Multiple Fox Genes Are Targeted by Resistance-Associated miRNAs

miRNA	putative FOX target gene[a]
miR-96	Foxf2[b,c,d], Foxk2[b,c,d], Foxn3[b], Foxn4[c], **Foxo1**[b,d], Foxo3[b,d], Foxo4[b], Foxp2[b,d], Foxq1[b]
miR-135b	Foxn2[b], Foxn3[b,d], Foxo1[b], Foxq1[c]
miR-182	Foxf2[b,c,d], Foxk2[b,d], Foxn2[b,d], Foxn3[b], **Foxo1**[b,d], **Foxo3**[b,c,d], Foxp1[c], Foxp2[b,d], Foxq1[b]
miR-183	Foxk2[c], Foxn2[b,d], **Foxo1**[b], Foxo3[c], Foxp1[b,c,d]
miR-200b	Foxf1/Foxf1a[b,d], Foxf2[b,d], Foxg1[b,c], Foxk1[b,d], Foxn2[b,d], Foxn3[b], Foxo3[b,c,d], Foxp1[b,d]

[a] Bold: Experimentally validated targets. [b] TargetScan v 5.1. [c] MicroCosm Targets v 5. [d] DIANA-microT v 3.0.

Additionally, disruption of E-cadherin-mediated adhesion sensitizes multicellular spheroids of HT26 colon adenocarcinoma to 5-FU, paclitaxel, and vinblastine.[35] In our model system, however, we would expect to see an increase in E-cadherin in resistant cells, as high levels of miR-141 and miR-200b∼miR-429 would repress ZEB1/ZEB2, and thus derepress E-cadherin expression. Still, our observation is consistent with development of drug resistance, which is not per se related to invasion, and EAT cells may not need to detach, as they are already growing in suspension.

The miR-183∼miR-96∼miR-182 cluster is also overexpressed in the MDR EAT cell lines. This cluster has been linked to regulation of the tumor suppressor FOXO1, a member of the Forkhead box (FOX) transcription factor family, which, when repressed, results in impaired apoptosis.[36,37] Interestingly, we found that *in silico* target prediction revealed many putative Fox gene targets for miR-183∼miR-96∼miR-182, as well as for miR-135b and miR-200b (Figure 2D, Table 2), which are all overexpressed in the MDR cell lines. We have previously detected expression of Foxg1, -k1, -o1, -o3, and -p1 mRNA in EHR2 wild type cells (unpublished data), and although similar information on the resistant sublines is lacking, we speculate that the aforementioned miRNAs could contribute to the development of resistance via their antiapoptotic effect through repression of Fox genes. However, upregulation of FOXO1 has also been linked to drug resistance through its downstream targeting of defense mechanisms against oxidative stress.[38] Therefore, due to multiple, and possibly opposing, effects of the Fox gene family, which depend on both cell context and stress response, the implication of FOX regulation in MDR remains to be determined.

A focused target prediction based on three of the consistently upregulated miRNAs, miR-200b, miR-182, and miR-135b, in drug-resistant cells, revealed several genes that could be relevant in development of drug resistance, including Lrp1b and Top2b (Supplementary Table S2 in the Supporting Information). However, because of little overlap in predicted targets between different prediction algorithms,[39] the above results should be interpreted cautiously, and experimental miRNA target identification is required to conclusively determine the involvement of miRNAs in MDR.

miR-210, which was consistently high in EAT cells grown *in vivo*, probably due to hypoxia induction, decreased with development of resistance in these cells. miR-210 induces cell death and acts as a tumor-suppressing miRNA by targeting fibroblast-growth-factor-receptor-like 1 (Fgfrl1) and thereby slows down cell proliferation by promoting cell cycle arrest in G1/G0.[40] Thus, the decreased level of miR-210 observed in the most resistant M cell lines could add to the resistant phenotype by derepression of Fgfrl1.

The miR-30d∼miR-30b cluster, which was downregulated in all resistant EAT cell lines, was previously reported to be associated with chemoresistance in hematodermic neoplasms, due to a 8q24 deletion that brought Myc closer to miR-30d∼miR-30b.[41] In squamous cell lung carcinoma, miR-30d∼miR-30b was found to be downregulated compared to matched normal lung tissue,[42] and a recent study reported that miR-30d and miR-125b directly target p53 mRNA.[43] However, in our study, both these miRNAs appear downregulated in most of the resistant cell lines, and would therefore contribute to an increase in the tumor suppressor p53 protein levels. Again, this illustrates the complexity of the regulatory miRNA networks, and the pleiotropic, MDR phenotype.

We also found the miR-23b∼miR-27b cluster to be consistently downregulated in the MDR cell lines. A recent study suggests that these miRNAs play a role in suppression of cell cycle genes with subsequent growth arrest.[44] Therefore, downregulation of miR-23b∼miR-27b may contribute to increased growth of resistant tumor cells. However, MDR and malignancy per se are unlinked entities and tumor cells with a slow growth rate can even escape the effect of antiproliferative chemotherapy by staying dormant.[45]

Knowing that Pgp, the product of the Abcb1b gene in mice, is the most prominent characteristic—and probably the major causative factor—of multidrug resistance, we analyzed the expression pattern of miRNAs reported to regulate Pgp, including miR-451 and miR-27a. Interestingly, both of these miRNAs seem to indirectly mediate an increase in Pgp expression,[46] the latter via inhibition of HIPK2, a transcriptional corepressor.[47] We observed that miR-27a was expressed at a very high level in all cell lines (mean $\log_2$ intensity = 14.75 ± 0.26), while miR-451 was only found in some of the M samples, probably due to its erythrocyte-specific nature. Next, our search for miRNAs predicted to bind to the Abcb1b 3′-UTR identified miR-135a, miR-135b, and miR-218-1* as putative Abcb1b-targeting miRNAs. While neither miR-135a nor miR-218-1* was detected in our samples, miR-135b was overexpressed in the most resistant subline, EHR2/1.3, and therefore, it is possible that this miRNA could contribute to the fine-tuning of Pgp expression. However, taking into concern the low levels of miR-135b (Table 1), it is questionable whether it has any profound effect on the expression of Pgp, which is high in EHR2/1.3[10].

In conclusion, we did not observe any obvious trends in miRNA expression going from sensitive to highly resistant EAT sublines. The most remarkable pattern identified was overexpression of the X-linked cluster of miRNAs confined to the EHR2 and P36 cell lines, suggesting that genomic events, such as amplification, deletion and translocation, take place during carcinogenesis, in general, and during development of resistance in the genomically unstable EAT cells, in particular. Further functional studies are warranted, applying both inhibition (antagomirs) and induction (miRNA mimics) of miRNA function to elucidate the relevance of miRNAs in the development of MDR.

■ ASSOCIATED CONTENT

Supporting Information. Further information on the RT-qPCR method and results, as well as supplementary figures related to the microarray and target prediction analyses. This

material is available free of charge via the Internet at http://pubs.acs.org.

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