 Genetic dissection of the miR-17~92 cluster of microRNAs in Myc-induced B-cell lymphomas

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The miR-17~92 cluster is frequently amplified or overexpressed in human cancers and has emerged as the prototypical oncogenic polycistron microRNA (miRNA). miR-17~92 is a direct transcriptional target of c-Myc, and experiments in a mouse model of B-cell lymphomas have shown cooperation between these two oncogenes. However, both the molecular mechanism underlying this cooperation and the individual miRNAs that are responsible for it are unknown. By using a conditional knockout allele of miR-17~92, we show here that sustained expression of endogenous miR-17~92 is required to suppress apoptosis in Myc-driven B-cell lymphomas. Furthermore, we show that among the six miRNAs that are encoded by miR-17~92, miR-19a and miR-19b are absolutely required and largely sufficient to recapitulate the oncogenic properties of the entire cluster. Finally, by combining computational target prediction, gene expression profiling, and an in vitro screening strategy, we identify a subset of miR-19 targets that mediate its pro-survival activity.

Results and Discussion

Generation of miR-17~92fl/fl;Eμ-Myc mice

To investigate the role of miR-17~92 in Myc-induced cancers, we employed the Eμ-Myc mouse model of B-cell lymphomas (Adams et al. 1985). Eμ-Myc mice express a c-Myc transgene under the control of the B-cell-specific Eμ enhancer and develop B-cell lymphomas within 4–6 mo of age (Adams et al. 1985). Eμ-Myc mice were crossed to mice carrying a conditional miR-17~92 knockout allele [miR-17~92fl/fl;Eμ-Myc mice] (Fig. 1B; Ventura et al. 2008). To temporally control the deletion of the floxed miR-17~92 allele, these mice were further crossed to mice carrying a 4-hydroxytamoxifen (4-OHT)-inducible Cre-recombinase estrogen receptor-T2 [Cre-ERT2] knock-in allele targeted to the ubiquitously expressed ROSA26 locus [R26-Cre-ERT2 mice, hereafter referred to as Cre-ER] (Ventura et al. 2007).

As expected, Eμ-Myc; miR-17~92fl/fl; Cre-ER mice developed B-cell lymphomas with similar latency and phenotype as the parental Eμ-Myc strain [data not shown]. From these mice, we derived two independent lymphoma lines [AV4174 and AV4182] that could be...
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While cell cycle distribution and BrdU incorporation were similar between miR-17–92Δ/Δ and miR-17–92Δ cells [Supplemental Fig. 1A], the fraction of cells undergoing apoptosis, as determined by detecting caspase activation, was approximately fourfold higher in the absence of miR-17–92 [Fig. 1F]. Increased apoptosis was confirmed by measuring the DNA fragmentation using the TUNEL assay [Supplemental Fig. 1B]. These results demonstrate that expression of the endogenous miR-17–92 locus is required for the optimal survival of Myc-driven B-lymphoma cells. In addition, they are consistent with the finding by He et al. (2005) that ectopic expression of miR-17–92 cooperates with c-Myc by reducing spontaneous apoptosis.

miR-19a and miR-19b mediate the oncogenic properties of miR-17–92

This observation provides a rationale and an opportunity to genetically dissect the functions of this cluster and to identify its relevant target mRNAs. The six miRNAs encoded by miR-17–92 can be grouped into four “seed families,’’ based on sequence identity at positions 2–7 [Fig. 1A]: the miR-17 family [miR-17 and miR-20a], the miR-18 family [miR-18a], the miR-19 family [miR-19a and miR-19b-1], and the miR-92 family [miR-92-1]. miRNAs belonging to the same seed family are predicted to target highly overlapping sets of mRNAs, and thus are expected to exert similar biological functions (Bartel 2009). To examine the role of each seed family in the context of Eph-Myc lymphomas, we generated a series of miR-17–92 mutant alleles, each lacking expression of the miRNA(s) belonging to one of the four seed families [Supplemental Fig. 2A]. The wild-type and the mutant alleles of miR-17–92 were cloned into MSCV-Puro-ires-GFP (PiG), a retroviral vector encoding the green fluorescent protein (GFP) and the Puromycin resistance gene, and the resulting constructs were transduced into miR-17–92Δ/Δ lymphoma cells. First, we verified that these constructs correctly expressed the desired miRNAs [Supplemental Fig. 2B]. This was an essential control because deletion of even a single miRNA from the miR-17–92 cluster could, in principle, negatively affect the processing and expression of the remaining ones, thus compromising our experimental approach.

To determine the ability of each construct to rescue the phenotype caused by miR-17–92 deletion, we titrated the viral preparations to achieve an infection efficiency of 5%–30%, as judged by GFP expression. We reasoned that, if reintroduction of miR-17–92 or one of its derivatives is sufficient to suppress the increased cell death observed in miR-17–92Δ/Δ cells, it will provide the infected cells with a growth advantage that will in turn be reflected by an increase in the fraction of GFP-positive cells over time [see schematic in Fig. 2A]. As predicted, reintroduction of the full-length miR-17–92 cluster resulted in a rapid increase of GFP+ cells that quickly outcompeted the uninfected cells [Fig. 2B]. Interestingly, among the four mutant constructs, only the one lacking the miR-19 seed family [Δ19a, 19b] failed to provide a growth advantage [Fig. 2B,C] and to suppress the increased apoptosis caused by deletion of miR-17–92 [Fig. 2D], suggesting that this seed family is necessary for the oncogenic properties of the cluster. This was further confirmed by showing that reintroduction of miR-19a and miR-19b alone was largely sufficient to rescue the growth defect caused by deletion propagating easily in culture and readily formed tumors when injected into immunocompromised mice. Both lymphoma lines exhibited similar behavior in vitro and in vivo. Unless otherwise specified, the experiments discussed here were performed using the AV4182 cell line.

To determine the efficiency of miR-17–92 deletion, miR-17–92Δ/Δ lymphoma cells were treated with 250 nM 4-OHT. This treatment lead to the efficient deletion of both endogenous miR-17–92 alleles [Fig. 1C], with concomitant loss of expression of the corresponding miRNAs [Fig. 1D].

We next examined the phenotypic consequences of deleting miR-17–92 in B-lymphoma cells. Because sustained Cre expression has been reported to negatively affect the growth of Eph-Myc lymphoma cells [Schmidt-Supplian and Rajewsky 2007], 4-OHT was administered for 4 d, after which the lymphoma cells were allowed to recover for a minimum of 4 d before being examined. As shown in Figure 1E, deletion of miR-17–92 dramatically reduced the proliferation of Eph-Myc lymphoma cells. Importantly, this phenotype was fully rescued by reintroduction of the entire miR-17–92 cluster [Fig. 1E].

The different growth kinetics between the miR-17–92Δ/Δ and miR-17–92Δ lymphoma cells could be due to the latter displaying reduced proliferation, increased spontaneous cell death, or a combination of both.
of the entire miR-17–92 cluster and to suppress apoptosis (Fig. 2D, miR-19a/b construct).

Deletion of miR-19 affects tumorigenicity in vivo

To determine whether the miR-19 seed family is required for the tumorigenicity of Eμ-Myc-driven B-cell lymphomas in vivo, we injected a cohort of nude mice with miR-17–92fl/fl and miR-17–92Δ3 cells. While lymphomas that lead to death within 2–3 wk, the miR-17–92Δ3 cells produced lymphomas with a significantly [P < 0.0001] longer latency (Fig. 2E). Tumorigenicity was fully restored by ectopic expression of the full-length miR-17–92 cluster [P < 0.0001], but not by expression of the miR-17–92 mutant lacking miR-19a and miR-19b [P = 0.9816] (Fig. 2E). Re-expression of miR-19a and miR-19b also largely rescued tumorigenicity, although it did so somewhat less efficiently than the full-length miR-17–92 [P = 0.0002 for the comparison between Δ/Δ and Δ/Δ + miR-19a,b, P = 0.0013 for the comparison between fl/fl and Δ/Δ + miR-19a,b].

Identification of miR-19 targets in B-cell lymphomas

Having demonstrated a critical role of miR-19a and miR-19b in Myc-driven B-cell lymphomas, we next sought to identify their functionally relevant target mRNAs. miRNA target prediction algorithms [TargetScan, Miranda, and Pictar] [John et al. 2004; Krek et al. 2005; Grimson et al. 2007; Betel et al. 2008] identified several hundreds of potential targets of miR-19; however, only a fraction of these mRNAs will likely be functionally relevant in any particular cellular context. To identify the genes whose expression is effectively modulated by miR-17–92 in B-cell lymphomas, we compared the gene expression profile of the AV4182 cell line before and after deletion of miR-17–92 [fl/fl vs. Δ/Δ]. We also included miR-17–92Δ3 lymphoma cells that had been transduced with either PIG-miR-17–92 or PIG-miR-19a/b [Fig. 3A]. In choosing this approach, we were supported by a number of recent reports showing that mRNA destabilization contributes to miRNA-mediated regulation of gene expression [Bagga et al. 2005; Lim et al. 2005; Baek et al. 2008; Selbach et al. 2008], which can be detected by conventional mRNA expression arrays. As predicted, deletion of miR-17–92 led to the preferential up-regulation of genes whose 3′ untranslated regions (UTRs) contain seed matches for the miRNAs encoded by this cluster [P-value < 2.22e-16, KS test] (Fig. 3B, Supplemental Fig. 3). Accordingly, ectopic expression of miR-17–92 in miR-17–92Δ3 cells led to the preferential down-regulation of miR-17–92 targets [P-value < 2.22e-16, KS test] (Fig. 3C; Supplemental Fig. 3). Finally, reintroduction of a mutant version of the miR-17–92Δ3 cluster expressing only miR-19a and miR-19b selectively affected mRNAs carrying binding sites for these two miRNAs [P-value = 6.35e-15], but not genes with binding sites for the other members of the miR-17–92 cluster.

By comparing the four gene expression profiles, we identified a total of 568 genes whose expression was up-regulated (log2 expression change >0.20) by deletion of the endogenous miR-17–29 locus [fl/fl vs. Δ/Δ comparison] and down-regulated by the reintroduction of the full-length miR-17–92 cluster [miR-17–92 vs. Δ/Δ] and of miR-19a and miR-19b only [miR-19a/b vs. Δ/Δ; log2 expression change <-0.20] (Fig. 3D). Ninety-five of them contained in their 3′ UTR one or more conserved binding sites for miR-19, according to TargetScan 5.1 [Fig. 3E; Supplemental Table 1], and were analyzed further. Guided by our findings that miR-19 suppresses apoptosis in Eμ-Myc lymphoma cells, we inspected the list of 95 genes and selected a subset of 46 of them for functional validation (Fig. 4A; Supplemental Table 2). We reasoned that, if miR-19 promotes survival by repressing the expression of one or more of these genes, this effect should be at least partially phenocopied by RNAi-mediated knockdown of the relevant targets. To test this hypothesis, for each of the 46 genes selected for validation we designed three shRNAs. The shRNAs were cloned into the MLP vector, a retroviral vector also expressing GFP, and each construct was individually transduced into miR-17–92Δ3 lymphoma cells. Analogous to the experiments described in Figure 2A, the viral preparations were titrated in order to achieve a transduction efficiency of 5%–30%, and the fraction of GFP+ cells was measured 2 d after infection (day 0) and again 11 d later. The results of this experiment are summarized in Figure 4B. For the majority of shRNAs, the fraction of GFP+ positive cells did not change over time or, for a small number of them, was lower at day 11 compared with day 0, indicating that expression of the shRNA did not provide any growth advantage to the infected cells or was detrimental, respectively (Fig. 4B; Supplemental Table 2). However, for a subset of shRNAs, we observed a significant increase in
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the fraction of GFP+ cells at day 11 compared with day 0 (Fig. 4B, Supplemental Fig. 5). Among them, two out of three shRNAs targeting the Pten tumor suppressor gene had the largest effect, largely phenocopying ectopic expression of miR-19a/b. A third shRNA directed against the Pten mRNA had a more modest, but still significant, effect. In addition, a number of the other shRNAs provided a significant growth advantage, although the effect was more modest than that observed with Pten shRNAs (Fig. 4B, Supplemental Table 2). In particular, all three shRNAs directed against Sbf2 and two out of three directed against Bcl7a and Rnf44 scored positively in this screening, suggesting that these three genes may contribute to the prosurvival functions of miR-19.

PTEN is one of the most frequently mutated tumor suppressor genes in human cancers, and monoallelic mutations at this locus are observed in >50% of sporadic tumors (for review, see Salmena et al. 2008). In mice, homozygous deletion of Pten leads to early embryonic lethality, while heterozygotes show greatly increased incidence of a variety of tumors, including T-cell lymphomas, as well as tumors of endometrium, liver, prostrate, gastrointestinal tract, and thyroid (Di Cristofano et al. 1998; Suzuki et al. 1998; Podsypanina et al. 1999). Functionally, PTEN is a phosphatidylinositol phosphatase that acts as a negative regulator of cell survival and protein synthesis via inhibition of the AKT/mammalian target of rapamycin [mTOR] pathway. Studies in mouse models and mutational analysis of human tumors indicate that Pten is a haplosufficient tumor suppressor gene [Salmena et al. 2008], suggesting that even modest modulation of its levels by miRNAs may have functional consequences.

The Pten 3′UTR contains two conserved binding sites for miR-19a and miR-19b [Fig. 5A], and it has been shown previously to be a direct miR-19 target (O’Donnell et al. 2005; Xiao et al. 2008). We first confirmed that miR-19 directly acts on the Pten 3′UTR via binding to the two predicted sites by performing reporter assays in human cancer cells and in miR-17–92-fl/fl and miR-17–92-fl/fl mouse embryonic fibroblasts [Supplemental Fig. 4]. We next determined the extent to which Pten expression is modulated by miR-19 in Myc-driven B-cell lymphomas. Western blotting and immunohistochemistry analysis of miR-17–92fl/fl and miR-17–92fl/fl lymphomas confirmed a consistent up-regulation of Pten expression in the latter [Fig. 5B,C]. Reinroduction of full-length miR-17–92 or of miR-19a and miR-19b alone, but not of miR-17–92fl/fl, in miR-17–92fl/fl lymphomas was sufficient to restore Pten expression to levels similar to those observed in miR-17–92fl/fl cells [Fig. 5B,C]. In addition, analogous to reinroduction of miR-19, RNAi-mediated knockdown of Pten in miR-17–92fl/fl lymphoma cells was sufficient to reduce spontaneous apoptosis to the levels observed in miR-17–92fl/fl cells [Fig. 5D]. We next examined whether Pten knockdown was also sufficient to restore the tumorigenicity of miR-17–92fl/fl lymphoma cells. Mice injected with miR-17–92fl/fl sh-Pten developed aggressive lymphomas that led to death with a latency similar to that observed in mice injected with miR-17–92fl/fl cells ectopically expressing miR-19a/b [Fig. 5D]. Also in

Figure 3. Gene expression profiling identifies miR-19 targets in Eμ-Myc lymphoma cells. [A] Description of the various lymphoma cells used. [B] Differences in mRNA levels between miR-17–92fl/fl and miR-17–92fl/fl lymphoma cells transduced with the empty PIG vector were monitored with microarrays. Cumulative distribution function [CDF] plots are shown for mRNAs that do not contain miR-17–92 seed matches in their 3′UTRs [black line], mRNAs containing one or more seed matches for miR-19 in their 3′UTR [blue line], and mRNAs containing one or more seed matches for either miR-17, miR-20a, miR-18a, or miR-19 [blue line]. In the absence of endogenous miR-17–92 expression, a statistically significant up-regulation (P-value < 2.2e-16, KS test) is observed for the predicted miR-17–92 targets relative to the background gene population. [C] CDF plots of the changes in mRNA expression levels between miR-17–92-d4 and PIG-miR-17–92 and miR-17–92-d4 lymphoma cells (left panel) and between miR-17–92-d4 and PIG-miR-19a,b and miR-17–92-d4 lymphoma cells (right panel). [D] Venn diagram summarizing the overlap in gene expression changes observed between the various transduction experiments. [E] As in D, but the analysis was restricted to mRNAs whose 3′UTR contains at least one predicted binding site for miR-19.

Figure 4. An in vitro RNAi screen to identify functionally relevant miR-19 targets. [A] List of the genes assayed in the in vitro shRNA screen. [B] Scatter plot summarizing the result of the screen. Each dot represents an individual shRNA construct. The X-axis is the percentage of GFP cells at the beginning of the experiment (2 d after infection) and the Y-axis is the percentage of GFP cells 11 d later. The green dot identifies the empty vector control. shRNAs that scored positive in the screen are highlighted in red and labeled. Dots corresponding to genes for which at least two out of three shRNAs provided significant growth advantage are labeled. PIG-miR-19a/b was included in the screen as positive control (blue dot).
this case, survival was slightly longer compared with mice injected with miR-17−92fl/fl cells \( (P = 0.0002) \), indicating the existence of additional functionally relevant targets.

In summary, the results presented here provide a mechanistic explanation for the functional cooperation between c-Myc and miR-17−92, identify the miR-19 seed family as the primary oncogenic determinant of this cluster, and pave the way for the development of novel anti-cancer strategies based on the pharmacological inhibition of miR-19 function.

**Material and methods**

**Mouse husbandry**

Animal studies and procedures were approved by the Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee. Mice were maintained in a mixed 129SvJae and C57/B6 background. The Rosa26-Cre-ERT2 and miR-17−92+/+ mice have been described previously (Ventura et al. 2007, 2008). The Eμ-Myc mice were generated and described by Adams et al. (1985). For the in vivo tumorigenicity studies, 4- to 8-wk-old athymic (nu/nu) mice were injected intravenously with 10^5 lymphoma cells and monitored daily. Mice were euthanized when moribund. Kaplan-Meier curves were plotted using PRISM software, and the log-rank Mantel-Cox test was used to determine statistical significance.

**Antibodies and immunohistochemistry**

Antibodies and experimental conditions for Western blotting and immunohistochemistry are described in the Supplemental Material.

**Cell culture and retroviral transduction**

The Eμ-Myc,miR-17−92+/+,Cre-ERT2 lymphoma lines were cultured on a feeder of irradiated NIH-3T3 cells in a medium composed of 50% DMEM and 50% IMDM, supplemented with 10% fetal bovine serum. To induce deletion of the miR-17−92 cluster, cells were incubated for 4 d with 250 nM 4-OHT. During our initial set of experiments with 4-OHT-treated lymphoma cells, we noticed that, upon prolonged passages, the few cells that had escaped full miR-17−92 deletion \( [\text{miR-17−92}^\text{fl/fl}] \) invariably outcompeted the \( [\text{miR-17−92}^\text{fl/fl}] \) cells, eventually becoming the majority within a couple of weeks. To avoid this limitation and allow the execution of long-term in vivo experiments, 4 d after 4-OHT treatment, subclones were isolated by plating 10 cells per well into a 96-well plate using a MoFlo fluorescence-activated cell sorter. After expansion, clones composed solely of fully recombined cells were isolated and used for further manipulation.

Retroviruses were generated in Phoenix packaging cells. When required, transduced cells were selected by adding puromycin (2 μg/mL) to the culture medium for 4 d.

**Plasmids and shRNA library**

A 1.2-kb fragment encompassing the entire miR-17−92 cluster was PCR-amplified from mouse genomic DNA and cloned into the MSCV-PIG retroviral vector (a gift from Mike Hemann, Massachusetts Institute of Technology). Deletion mutants were by site-directed PCR and verified by sequencing. Primers and sequences are available on request.

The shRNA library was cloned in the MLP retroviral vector (a gift from Michael Hemann, Massachusetts Institute of Technology). For each gene, three shRNA directed against the coding sequence were designed using the RNAi Central resource created by the laboratory of Greg Hannon (http://katahdin.cshl.org:9331/siRNA/RNAi.cgi?type=rna&species=mouse). Each construct was sequence-verified.

**Apoptosis assays**

Apoptosis was measured using the Caspase Detection Kit (Red-VDV-FMK or FITC-VDV-FMK, Calbiochem) and confirmed using the TUNEL assay (In Situ Cell Death Detection Kit, TMR red, Roche) following the manufacturer’s instructions.

**Gene expression analysis**

Total RNA extracted from three technical replicates was hybridized to the Affymetrix 430 A2.0 gene chip, following the manufacturer’s instruction. Gene expression was normalized using the GCRMA Bioconductor package, and log expression values were computed using the limma package. For genes with multiple probes, the probe with lowest adjusted P-value
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was selected. Genes with a log expression change of <−0.2 in all three comparisons and with an adjusted P-value < 0.05 in at least one comparison were considered for subsequent overlap analysis.

miRNA target predictions

miRNA targets were predicted using miRanda (http://www.microrna.org) and TargetScan (http://www.targetscan.org). For the cumulative distribution function (CDF) plots, target sites were restricted to perfect seed complementarity between positions 2 and 7 of the miRNA. Empirical cumulative distributions were computed using R ecdf function for the set of predicted gene of the transduced miRNAs and for the genes with no target sites (background). P-values were computed using the KS two-sample test.

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