Supporting Information

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SI Materials and Methods

Leukemic and Normal Control Samples. In the bead-based miRNA expression profiling assay, 69 leukemic (including 55 de novo patients and 14 cell lines) and 3 normal control (including one $CD15^+$ and two mononuclear cell) samples were used (1). In the qPCR assay, 87 leukemic (including 67 de novo patients and 20 cell lines) and 12 normal control (including five $CD34^+$, one $CD15^+$ myeloid, one $CD19^+$ lymphoid, and five mononuclear cell) samples were used. Each sample is given a unique ID. The majority (i.e., 67%) of the 97 samples used in the qPCR assay are independent samples that were not used in the bead-based assay. All of the patient samples were obtained at the time of diagnosis or relapse and with informed consent at the University of Chicago or other hospitals and were stored in liquid nitrogen until used (see characteristics of patients in Table S2). KOPN1, KOCL33, KOCL44, KOCL45, KOCL48, KOCL50, KOCL51, and KOCL69 cell lines were from Y. Sato and K. Sugita, whereas cell lines such as MV4-11, ML-2, MONOMAC6, THP1, KASUMI-1, KOPN8, SUPT13, SEM, NOMO1, ME-1, NB4, U937, HeLa, and 293T were maintained in the laboratory. Normal control cells were purchased from AllCells. (Emeryville, CA).

Bead-Based miRNA Expression Profiling Assay and qPCR Assays. The bead-based expression assay, data filtering, and normalization were described (1, 2). TIGR Mutiple Array Viewer software package (TMeV version 4) (3) was used to perform data analysis and to visualize the results. TaqMan qPCR assay (4) was performed to validate the differential expression patterns of miRNAs by using kits from Applied Biosystems (Foster City, CA). qPCR with SYBR Green dye (Qiagen, Valencia, CA) was used to determine expression of mRNA genes. U6 RNA and *PGK1* (or *GAPDH*) were used as endogenous controls for qPCR of miRNA and mRNA, respectively. PCR reactions were performed in an Applied Biosystems 7900HT system according to the relevant manufacturer's recommendation with some minor modifications, and each sample was run in triplicate. We followed a method described by He et al. (5) to analyze DNA copy number of the miR-17-92 locus with modification as described (1, 2).

Bisulfite Genomic Sequencing. Total genomic DNA (1 μg) was first converted with sodium bisulfite (1, 2). Then, the CpG island region of miR-17-92 promoter was PCR amplified, and DNA methylation levels were analyzed by bisulfite genomic sequencing using the pyrosequencing technology as described (1).

Chromatin Immunoprecipitation (ChIP) Assay. ChIP assay was performed with Upstate Biotechnology ChIP assay kit (Lake Placid, NY) by following the manufacturer's protocol with some modifications. Briefly, 5×10^6 cells were treated with 1% formaldehyde for 10 min at room temperature to cross-link DNA and proteins. The reaction was terminated by the addition of glycine to a final concentration of 0.125 M and incubated at room temperature for 5 min. After cell lysis, the cross-linked chromatin was sonicated to an average size of ≈400 bp and was immunoprecipitated with antibodies against the amino-terminal portion of MLL (AT and RD domains; see ref 6), H3K4 trimethyl antibodies, histone H3 acetylation antibodies, or normal rabbit IgG as a negative control (all purchased from SABiosciences, Frederick, MD) . Purified ChIP DNA was amplified by regular and real-time qPCR. Primers amplifying the miR-17-92 cluster promoter region were used for the ChIP PCR (miR17-92-ChIP-F: 5′-AAGTGGAAGCCAGAAGAGGA-3′, miR17-92-ChIP-R: 5′-AAGTGGTGGCTCTTCCAATG-3′; C13orf25-ChIP-F: 5′-GGGAGGTCGGAAGTACTTTGT-3′, C13orf25-ChIP-R: 5′-AAGGACCATGTGGGTGAATG-3′).

Transduction of MLL-AF9 into Human Normal $CD34^+$ Cells and Maintenance of the Cells. This experiment has been reported (7). Briefly, human $CD34^+$ umbilical cord blood cells were retrovirally transduced with MSCV-MLL-AF9 (MA9) or control empty vector. For transduction, $CD34^+$ cells were cultured in the presence of retroviral supernatant supplemented with SCF, IL-3, IL-6 Flt-3L, and TPO on retronectin-coated plates for 2 days. After transduction, cells were cultured in IMDM 10% bovine calf serum (or serum-free for some experiments) and supplemented with SCF, IL-3, IL-6, Flt-3L, and TPO at 10 ng/mL each. Viable cell counts in MA9 and control cultures were assessed one to two times per week, and cultures were split into fresh media as needed to maintain a cell density of between 5×10^5 and 2×10^6 cells per mL (7). Cells were harvested at different time points for the qPCR assay of miR-17 and miR-20a.

Cell Apoptosis and Viability Assays. HeLa and 293T cells were plated at a concentration of 2,500 or 5,000 cells per well in triplicate in a 96-well plate. MSCVpuro-miR-17-92 or the control plasmid MSCVpuro was transfected into the cells with Effectene Transfection Reagent (QIAGEN). Forty-eight hours after transfection, cell apoptosis was assessed through analyzing caspase-3 and caspase-7 activation by using ApoONE Homogenous Caspase 3/7 Assay (Promega, Madison, WI); cell viability was assessed through analyzing metabolic activity of the cells by using CellTiter-Blue Reagent (Promega) and following the corresponding manufacturer's manuals.

Colony-Forming and Replating Assay. In vitro colony-forming (i.e., immortalization) assays were performed as described (8) with some modifications (2). Briefly, retrovirus for each construct was produced in 293T cells by cotransfecting the retroviral construct and pCL-Eco packaging vector (IMGENEX, San Diego). Rat1a cells were used to determine the viral titer. Then, hematopoietic progenitor cells obtained from a cohort of 4- to 6-week-old C57BL/6 mice five days after 5-FU treatment (150 mg/kg) were transduced with the retroviruses of MSCVpuro or MSCVneo empty vector (both as negative controls; their colony-forming capacities are very similar), MSCVpuro-miR-17-19b (or MSCVpuro-miR-17-92), MSCVneo-MLL-ELL, and a combination of MSCVpuro-miR-17-19b (or MSCVpuro-miR-17-92) together with MSCVneo-MLL-ELL, respectively, through "spinoculation" (8). An aliquot of 1×10^4 of the transfected cells was plated into duplicate 35-mm Nunc Petri dishes in 1.1 mL of Methocult M3230 methylcellulose medium (Stem Cell Technologies, Vancouver) containing 10 ng/mL each of murine recombinant IL-3, IL-6, and GM-CSF (R&D Systems, Minneapolis) and 100 ng/mL of murine recombinant stem cell factor (Sandoz, Holzkirchen, Germany), along with 1.0 mg/mL of G418 (Gibco BRL, Gaithersburg, MD) and/or 2.5 μg/mL of puromycin (Sigma, St. Louis) accordingly. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The colonies were replated every 7 days under the same conditions.

Cytospin. For cytospin preparation, 40,000 cells were washed twice and were diluted in 200 μL of cold PBS. Each sample was loaded into the appropriate wells of the cytospin, and then spun at 2000 rpm for 2 min. The slides were dried in a desiccation chamber for 20 min and were stained with Wright-Giemsa.

MiRNA Array and Gene Array Assays. A group of 18 samples (see Table S3) obtained from the in vitro colony-forming/replating assays were included in the miRNA array and gene (i.e., mRNA) array assays. The miRNA array assay was performed by Exiqon (Woburn, MA) using the miRCURY LNA arrays. Briefly, after having passed sample QC on the Bioanalyser2100 and RNA measurement on the Nanodrop instrument, the samples were labeled by using the miRCURY Hy3/Hy5 power labeling kit and hybridized on the miRCURY LNA Array (v.10.0; containing 578 mouse miRNAs). Each chip/slide contained two arrays. One individual sample (0.5 μg) labeled with Hy3 and an aliquot (0.5 μg) of the common reference pool (a mixture of the 18 samples; allowing normalization across a set of arrays and direct comparison of all samples) labeled with Hy5 were put into the two arrays of a given chip. Analysis of the scanned slides showed that the labeling was successful as all capture probes for the control spike-in oligo nucleotides produced signals in the expected range. Background correction was first conducted to remove nonbiological contributions ("background") to the measured signal by using the Convolution model described by Ritchie et al. (9). The quantified signals were then normalized by using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm (10). When calling a particular miRNA failed on an array, its expression value was indicated by the acronym "NA." The criteria for deciding that a miRNA had failed on a particular array, was that three or more of the four replicated measures of this miRNA were flagged (i.e., the signal is below background) by the image analysis software. In addition, if the standard deviation of signals of the replicated probes of a given miRNA in a particular array (i.e., an individual sample) was >0.4, this miRNA also failed on this array. The expression values are log2(Hy3/Hy5) ratios, which were obtained on the basis of the normalized data where replicated measurements on the same slide have been averaged.

Gene arrays were performed by using Affymetrix GeneChip Mouse Gene 1.0 ST Array (Santa Clara, CA), which contains 28,853 mouse genes being represented on the array by ≈27 probes spread across the full length of a given gene, providing a more complete and more accurate picture of gene expression than 3′-based expression array designs. After hybridization and background correction according to the standard protocols, the quantified signals were then normalized using Robust Multiarray Average (RMA), which is a robust algorithm of background adjustment, quantile normalization, and log transformation (11). Partek Genomics Suite (Partek, St. Louis) was used for the analysis of the normalized data.

Identification of Potential Target Genes of miR-17-92 and Analysis of Their Enrichment in GO Processes and Pathways. We collected all predicted target genes of miR-17-92 from PITA (12) (version 6), TargetScan (13) (release 5.1), Miranda (14) (version released in September 2008), and miRbase Targets (version 5; w7ww.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). Then, we identified 363 potential target genes/transcripts (Table S1) that exhibited a significant inverse correlation (Pearson Correlation; $r < -0.5$; $P < 0.01$) of expression with miR-17-92 in the above 18 mouse samples that we have both miRNA and mRNA array data (Table S3). Based on the 363 potential target genes of miR-17-92, we further conducted analysis of GO processes and pathways using GeneGo MetaCore software (St. Joseph, MI).

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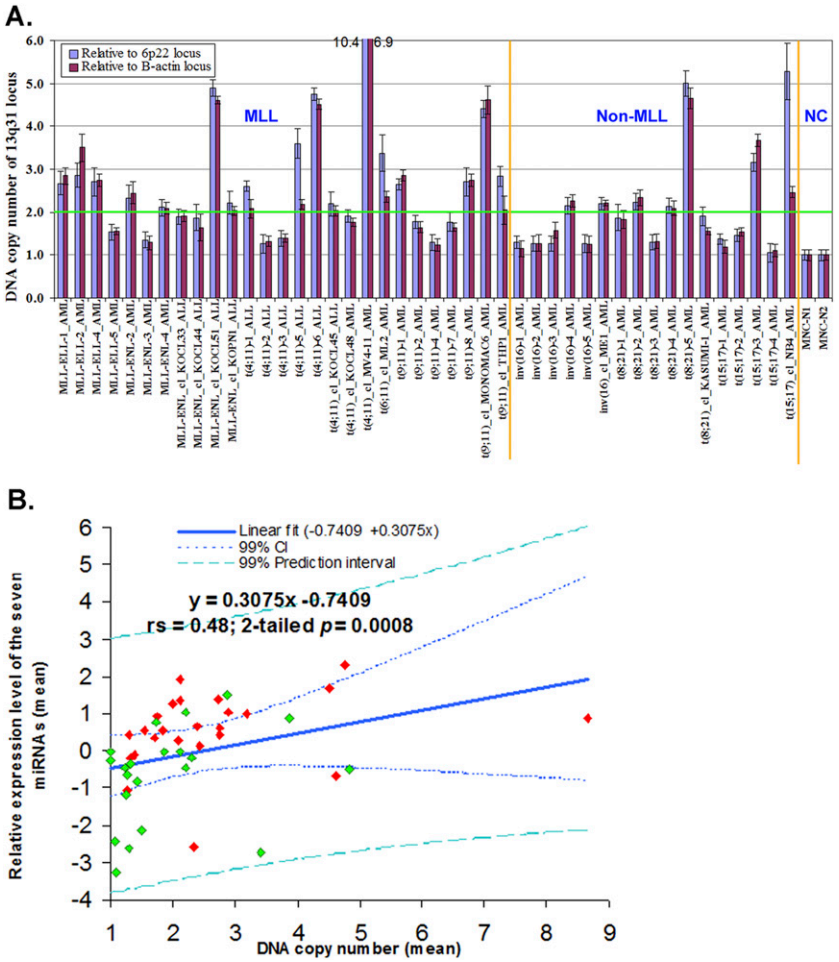

Fig. S1. Expression of miR-17-92 might be associated with DNA copy number amplification. (*A*) DNA copy number of the miR-17-92 cluster locus at 13q31. PCR data were normalized against a reference probe 6p22 or a β-actin probe. Error bars indicate SD. The average DNA copy number of the miR-17-92 locus in the two normal control cell samples was set as 1. (*B*) A positive correlation between the average relative expression level of the seven miRNAs and the average DNA copy number of the miRNA locus. The regression line plot graphically shows the fitted regression line and its 99% confidence interval, as well as the 99% prediction interval. The *MLL*-rearranged leukemic samples shown in red, whereas the remaining samples are shown in green.

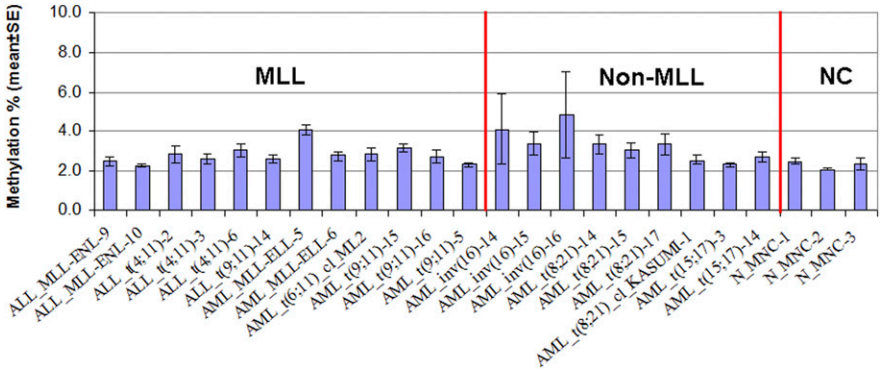

Fig. S2. The methylation status of a CpG island upstream of the miR-17-92 cluster locus at 13q13 in *MLL*, non-*MLL* leukemias, and normal control (NC) samples. The DNA methylation levels were detected by pyrosequencing after bisulfite treatment of genomic DNAs. The data presented as mean ± SE of three independent experiments.

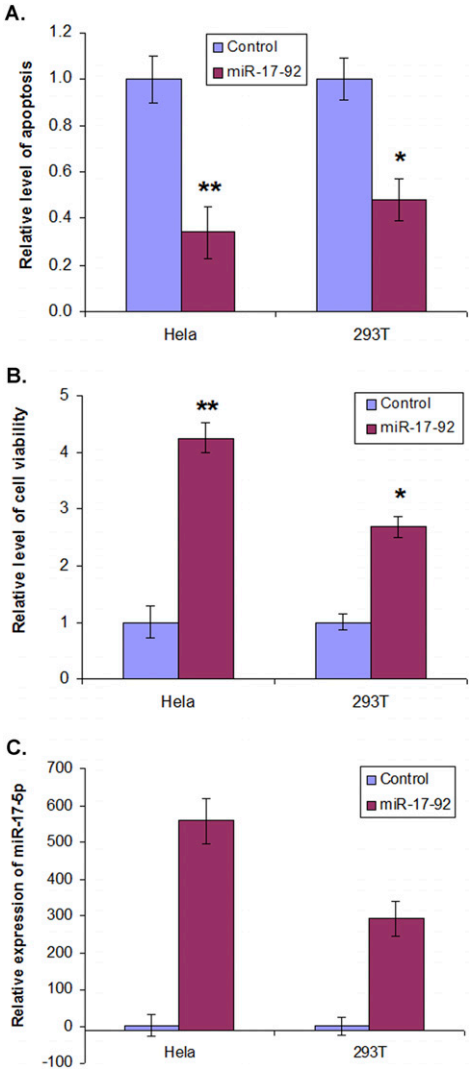

Fig. S3. The functional role of miR-17-92 in human HeLa and 293T cells. Forced expression of the miR-17-92 cluster significantly inhibited apoptosis (*A*) while enhancing viability (*B*) of HeLa and 293T cells. Normalized mean values of three independent experiments and mean ± SE are shown. *, $P < 0.05$; **, $P < 0.001$ (paired *t* test). The apoptosis data depict the level of spontaneous apoptosis likely resulting from DNA transfection. Cell viability represents the relative number of live/healthy cells in cell populations, which largely corresponds to cell proliferation. (*C*) Forced expression of miR-17-92 after transfection of MSCVpuro-miR-17-92 was confirmed by qPCR. Relative expression of miR-17-5p was show as example, and the other individual miRNAs of the miR-17-92 cluster have as similar level of overexpression. The endogenous expression of miR-17-92 is higher in 293T cells than in HeLa cells, and that's the reason that expression fold change of miR-17-92 is lower in 293T cells than in HeLa cells after the transfection of MSCVpuro-miR-17-92.

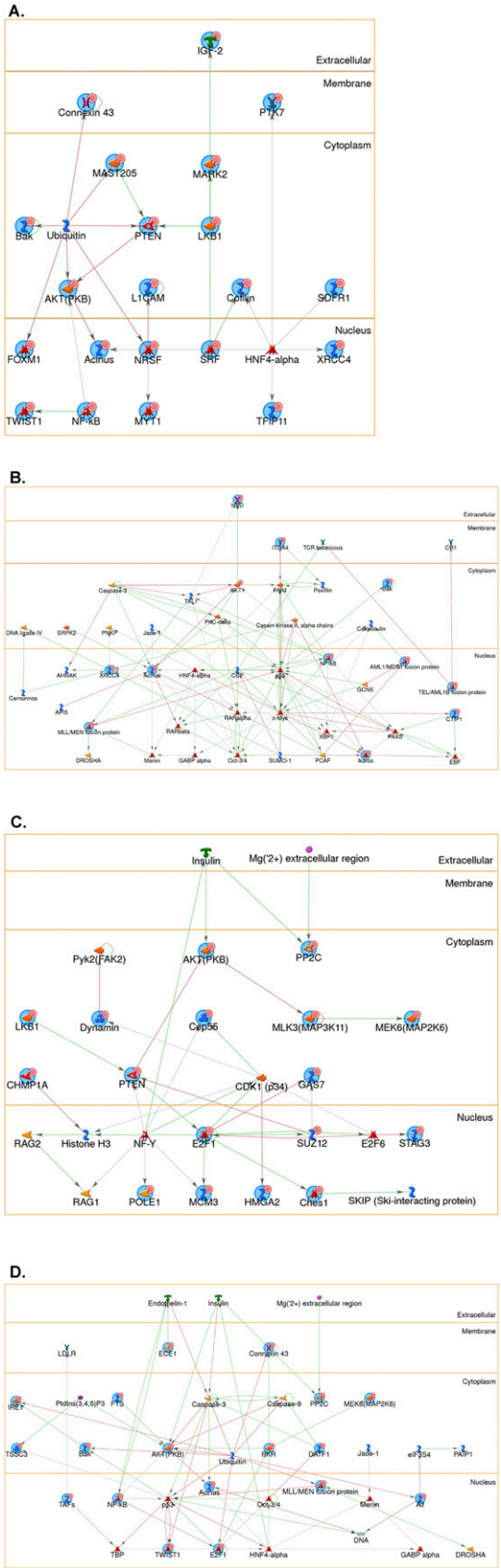

Fig. S4. Pathways/networks of the miR-17-92 putative target genes in relevant GO processes. (*A*) Genes related to cell differentiation. (*B*) Genes related to hematpoiesis. (*C*) Genes related to cell cycle. (*D*) Genes related to apoptosis. Only the genes that are highlighted with blue cycle are potential target genes of miR-17-92 that exhibit a significant inverse correlation (Pearson Correlation; $r < -0.5$; $P < 0.01$) of expression with the corresponding individual miRNAs in the miR-17-92 cluster. Red line, inhibition; green line, promote; gray line, physical binding.

Other Supporting Information Files

Table S1 (DOC)
Table S2 (DOC)
Table S3 (DOC)