Supplementary data

miR-223 knockdown and overexpression

The miR-223 sponge vector has been previously described 25. In brief, it consists of a third-generation LV containing a polymerase-II-based promoter (spleen focus-forming virus promoter/enhancer [SFFV]) optimized for high transgene expression in human stem and progenitor cells (HSPCs) and myeloid lineage cells, which drives the expression of a nontoxic marker gene (destabilized GFP) containing eight copies of an imperfectly complementary miR-223 binding site. Lentiviral overexpression of miR-223 was performed as previously described [24,25], consisting of a SFFV promoter, the miR-223 pri-miR inserted into the first intron of the *Ef1α* gene, and an orange fluorescent protein marker gene (*mO2*).

Flow cytometric analysis and cell sorting

The progeny of human $CD34^+$ cells were labeled with the following antibodies: anti-CD11b Pacific Blue or allophycocyanine (APC)-Alexa 780 (BD Pharmingen or eBiosciences), antihuman CD33 phycoerythrin (PE)–cyanine 7 (Cy7) or Brilliant Violet 421 (BD), antihuman CD34 PE, PE-Cy7 or APC (BD Pharmingen), antihuman CD133 APC (Miltenyi Biotec), antihuman CD38 APC or V450 (BD), antihuman CD90 APC or PE (BD), antihuman CD16 PE, PE-Cy5 or PE-Cy7 (BD or DAKO), antihuman CD13 APC or PE (BD), antihuman CD14 peridinin chlorophyll (PerCP; BD), antihuman CD19 PE-Cy7 (BD), antihuman CD3 APC or Pacific Blue (BD), antihuman CD235a PE or APC (DAKO or BD), and antihuman CD45 APC-H7 or Pacific Blue (BD). Cells were stained at a density of 10^7/mL for 20 min at 4°C, washed, and analyzed on a LSRII Fortessa flow cytometer or FACS Canto II (Becton-Dickinson). Cell sorting was performed on a MoFlow sorter (Beckman Coulter).

Proliferation assay

Sorted, GFP-expressing BM cells were plated in BM medium at a starting concentration of 20,000 cells/mL. Cell numbers were determined via counting in Trypan blue solution every 48 hours for 7 days.

Colony-forming cell assay

For in vitro colony-forming cell (CFC) assays, sorted, GFP-expressing cells were plated in MethoCult media (StemCell Technologies) at a concentration of 1,000 cells/mL. Colony numbers and cell counts were assessed weekly, followed by weekly serial replating over a period of 3 weeks maximum.

Real-time PCR

RNA was extracted using Trizol. Reverse transcription of miR-223, with miR-92 as housekeeping gene [14] for human and sno-202 for murine samples, was performed using the Taqman miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time PCR was performed using miR-223, as well as miR-92/RNU48 or sno-202 ABI Taqman probes on an Applied Biosystems 7900HT Fast Real-Time PCR system, in triplicate.

Quantification of miR-223 by hybridization arrays

Samples of 115 de novo AML patients were obtained from bone marrow aspirates, blast cells were purified by Ficoll Hypaque (Nygaard, Oslo Norway), and total RNA was extracted with Trizol, as described previously [33]. All patients provided written, informed consent in accordance with the Declaration of Helsinki and were treated according to Hovon42 of the Dutch-Belgian-Hematology-Oncology-Cooperative group (available at http://www.hovon.nl). Samples were analyzed using Affymetrix miRNA 1.0 GeneChips (Affymetrix, Santa Clara, CA), according to the manufacturer's protocol. Briefly, 1 μg of RNA was biotin labeled using FlashTag Biotin RNA labeling kit (Genishpere, Hatfield, PA). Biotin-labeled samples were hybridized with GeneChip hybridization, wash, and stain kit and scanned with Affymetrix GeneChip Scanner. The obtained data (Supplementary Table E1) were normalized using the robust multi-array average (RMA) method.

Sorting of AML subpopulations

Patient bone marrow samples were obtained after informed consent was provided and with the approval of the Clinical Research Ethics Board of the University of British Columbia. They showed the following characteristics: FAB M4 (normal karyotype), M1 (unknown karyotype), M5 (normal karyotype), M5b (normal karyotype), and M1 (normal karyotype). The cells were stained as previously described [32] and sorted into four populations ($CD34^-$, $CD34^+CD38^+$, $CD34^+CD38^-$, and a total blast population), followed by RNA extraction with Trizol (Invitrogen, Carlsbad, CA) as previously described [20].

Supplementary Table E1. Expression levels (log 2) of miR-223 in the profiled AML patient samples

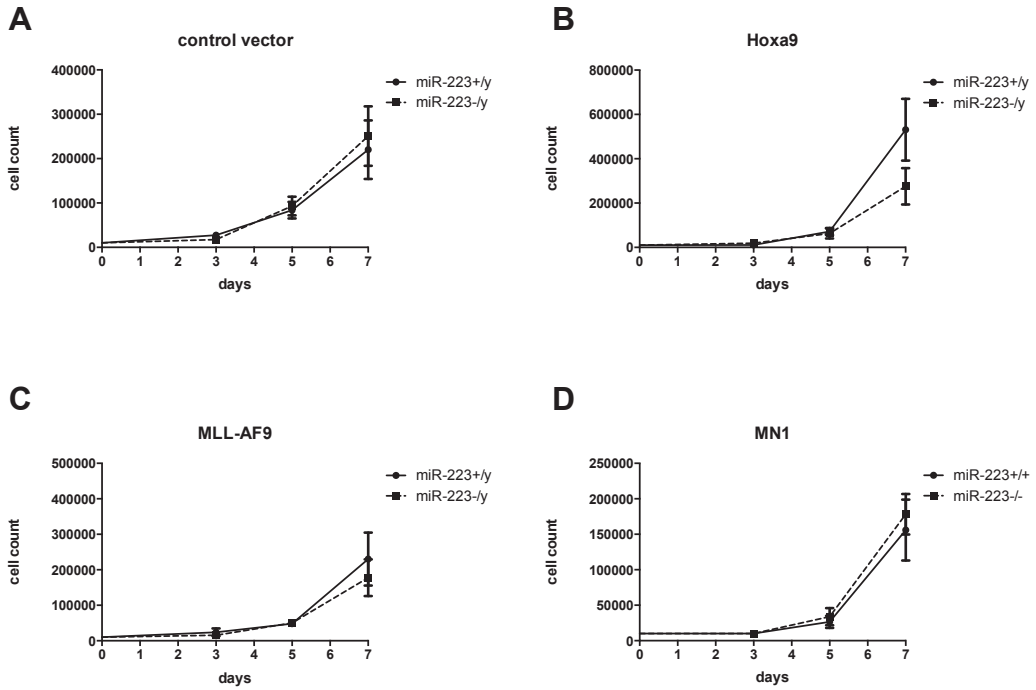
Patient	hsa-miR-223	ELN classification
A002	7.465553	Adverse
A003	7.682119	Adverse
A006	7.623925	Adverse
A009	5.520364	Adverse
A018	6.783545	Adverse
A019	6.663668	Adverse
A027	5.470807	Adverse
A028	5.993056	Adverse
A030	5.179881	Adverse
A032	3.745725	Adverse
A036	6.459761	Adverse
A041	5.712106	Adverse
A044	6.895362	Adverse
A045	6.897757	Adverse
A047	5.098162	Adverse
A061	7.485504	Adverse
A067	6.122093	Adverse
A068	6.609147	Adverse
A079	6.793427	Adverse
A084	5.423153	Adverse
A088	5.572708	Adverse
A089	6.832248	Adverse
A092	5.459269	Adverse
A093	5.121201	Adverse
A094	7.021109	Adverse
A096	5.425439	Adverse
A100	6.696466	Adverse
A106	6.34158	Adverse
A107	4.1736	Adverse
A108	7.019696	Adverse
A004	6.926732	Favorable
A011	7.318853	Favorable
A012	6.636045	Favorable
A014	6.763334	Favorable
A017	6.299748	Favorable
A023	7.160719	Favorable
A031	7.339418	Favorable
A033	5.795588	Favorable
A034	4.812762	Favorable
A035	6.862276	Favorable
A040	6.505709	Favorable
A042	6.570924	Favorable
A043	6.429499	Favorable
A046	6.852291	Favorable
A050	7.031396	Favorable
A053	6.826373	Favorable
A054	6.606032	Favorable
A056	6.184179	Favorable
A060	6.517457	Favorable
A062	7.576693	Favorable
A065	6.083967	Favorable
A069	7.428319	Favorable
A070	6.261838	Favorable
A071	7.599975	Favorable
A072	5.312304	Favorable
A074	5.744253	Favorable
A076	7.450024	Favorable
A080	6.780867	Favorable
A081	7.962897	Favorable
A082	6.365168	Favorable
A086	8.27315	Favorable
A097	5.697825	Favorable
A099	5.685469	Favorable
A102	7.237895	Favorable
A105	6.918886	Favorable
A113	6.50455	Favorable
A001	8.279448	Intermediate I: NK
A007	8.53535	Intermediate I: NK
A008	6.513398	Intermediate I: NK
A013	5.91849	Intermediate I: NK
A015	7.267184	Intermediate I: NK
A020	6.611535	Intermediate I: NK
A021	6.575376	Intermediate I: NK
A022	6.890913	Intermediate I: NK
A024	6.749806	Intermediate I: NK
A025	7.777081	Intermediate I: NK
A026	6.837991	Intermediate I: NK
A029	6.341842	Intermediate I: NK
A037	6.368287	Intermediate I: NK
A038	5.640762	Intermediate I: NK
A039	7.305367	Intermediate I: NK
A049	6.413876	Intermediate I: NK
A051	5.708693	Intermediate I: NK
A052	7.900176	Intermediate I: NK
A055	7.725691	Intermediate I: NK
A057	5.050639	Intermediate I: NK
A058	6.328099	Intermediate I: NK
A059	6.905414	Intermediate I: NK
A063	6.654579	Intermediate I: NK
A064	7.116859	Intermediate I: NK
A066	6.110807	Intermediate I: NK
A073	6.497773	Intermediate I: NK
A077	3.168967	Intermediate I: NK
A078	5.809293	Intermediate I: NK
A083	6.531438	Intermediate I: NK
A090	7.224567	Intermediate I: NK
A095	6.513924	Intermediate I: NK
A103	6.888729	Intermediate I: NK
A115	5.68294	Intermediate I: NK
A005	5.053096	Intermediate II: rest
A010	6.983745	Intermediate II: rest
A016	7.502192	Intermediate II: rest
A048	7.079939	Intermediate II: rest
A075	7.867951	Intermediate II: rest
A085	7.226651	Intermediate II: rest
A087	4.429051	Intermediate II: rest
A091	4.902436	Intermediate II: rest
A098	6.514174	Intermediate II: rest
A101	5.465915	Intermediate II: rest
A104	6.784386	Intermediate II: rest
A109	7.153646	Intermediate II: rest
A110	4.491841	Intermediate II: rest
A111	6.919614	Intermediate II: rest
A112	6.335259	Intermediate II: rest
A114	6.446647	Intermediate II: rest

(*continued*)

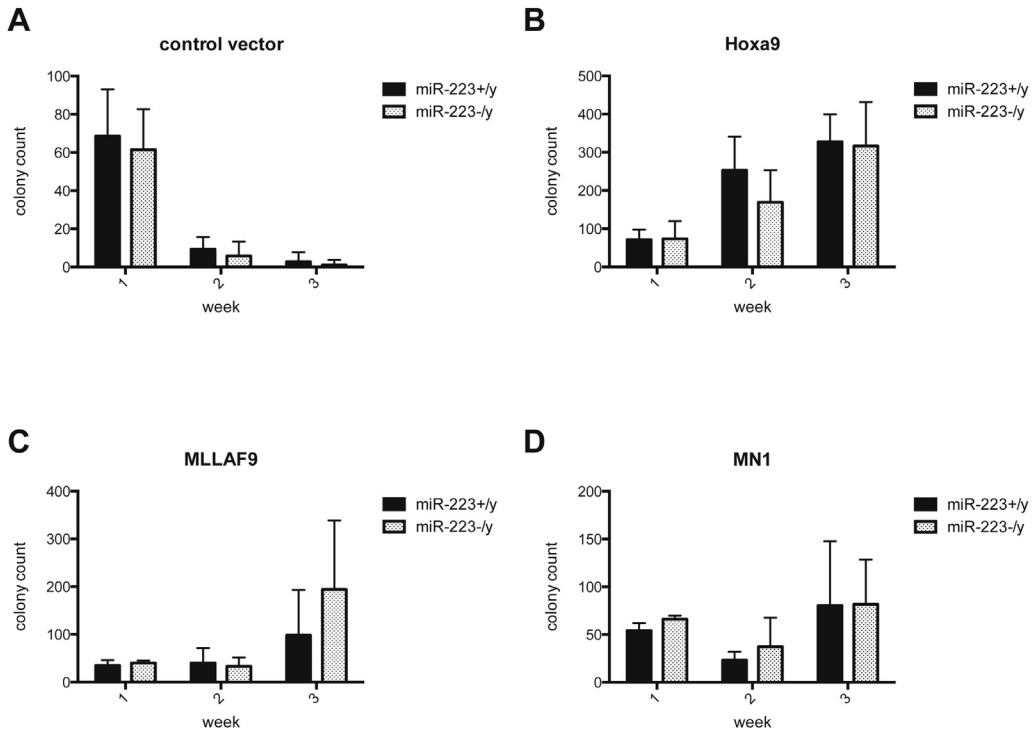
Supplementary Table E2. Cox regression analysis (uni-/multivariate) of miR-223 levels in 115 profiled AML patients.

		Variable	Haz. ratio	St. err.	z	$p > \|z\|$	95% conf. interval	
Cox regression analysis (Univariate)								
OS	Continuous	miR-223	0.763	0.097	2.14	0.032	0.595	0.978
EFS	Continuous	miR-223	0.793	0.089	2.07	0.039	0.637	0.988
RFS	Continuous	miR-223	0.720	0.117	2.03	0.043	0.524	0.989
OS	Dichotomized (median)	miR-223	0.692	0.185	1.38	0.168	0.411	1.167
EFS	Dichotomized (median)	miR-223	0.700	0.167	−1.5	0.134	0.438	1.117
RFS	Dichotomized (median)	miR-223	0.704	0.255	0.97	0.332	0.346	1.413
OS	Quartiles	miR-223	0.778	0.097	2.01	0.044	0.610	0.993
EFS	Quartiles	miR-223	0.802	0.087	2.02	0.043	0.648	0.993
RFS	Quartiles	miR-223	0.772	0.129	1.54	0.123	0.556	1.072
Cox regression analysis (Multivariate)								
OS	Continuous	mir-223	0.817	0.101	1.63	0.102	0.641	1.004
		Age	0.997	0.014	0.19	0.851	0.970	1.025
		Log(WBC)	1.350	0.154	2.64	0.008	1.080	1.688
		ELN (Favorable (ref))						
		Intermediate II (rest)	3.706	1.777	2.73	0.006	1.447	9.487
		Intermediate I (NK)	4.310	1.799	3.5	0.000	1.901	9.768
		Adverse	4.512	1.968	3.45	0.001	1.919	10.609
EFS	Continuous	mir-223	0.833	0.094	1.63	0.104	0.668	1.038
		Age	0.992	0.012	0.68	0.499	0.968	1.016
		Log(WBC)	1.110	0.111	1.04	0.297	0.913	1.350
		ELN (Favorable (ref))						
		Intermediate II (rest)	2.016	0.818	1.73	0.084	0.910	4.467
		Intermediate I (NK)	2.882	0.967	3.15	0.002	1.493	5.563
		Adverse	2.660	0.941	2.76	0.006	1.329	5.323
RFS	Continuous	mir-223	0.710	0.118	2.06	0.039	0.512	0.983
		Age	0.983	0.020	0.84	0.401	0.944	1.023
		Log(WBC)	1.605	0.253	3	0.003	1.178	2.186
		ELN (Favorable (ref))						
		Intermediate II (rest)	2.629	1.486	1.71	0.087	0.868	7.958
		Intermediate I (NK)	3.460	1.638	2.62	0.009	1.369	8.749
		Adverse	1.504	0.907	0.68	0.499	0.461	4.907
OS	Quartile	mir-223	0.798	0.102	1.77	0.077	0.621	1.024
		Age	0.995	0.014	0.33	0.743	0.968	1.024
		Log(WBC)	1.348	0.152	2.64	0.008	1.080	1.682
		ELN (Favorable (ref))						
		Intermediate II (rest)	3.800	1.820	2.79	0.005	1.487	9.715
		Intermediate I (NK)	4.320	1.803	3.51	0.000	1.906	9.790
		Adverse	4.675	2.022	3.57	0.000	2.003	10.912
EFS	Quartile	mir-223	0.811	0.092	1.85	0.065	0.649	1.013
		Age	0.990	0.012	0.81	0.417	0.966	1.015
		Log(WBC)	1.109	0.110	1.05	0.294	0.914	1.346
		ELN (Favorable (ref))						
		Intermediate II (rest)	2.076	0.840	1.81	0.071	0.940	4.586
		Intermediate I (NK)	2.898	0.973	3.17	0.002	1.501	5.595
		Adverse	2.738	0.956	2.89	0.004	1.381	5.427
RFS	Quartile	mir-223	0.749	0.129	1.68	0.093	0.534	1.050
		Age	0.982	0.021	0.87	0.382	0.942	1.023
		Log(WBC)	1.591	0.246	3.01	0.003	1.175	2.153
		ELN (Favorable (ref))						
		Intermediate II (rest)	2.921	1.635	1.92	0.055	0.975	8.750
		Intermediate I (NK)	3.494	1.650	2.65	0.008	1.385	8.814
		Adverse	1.760	1.040	0.96	0.339	0.553	5.601

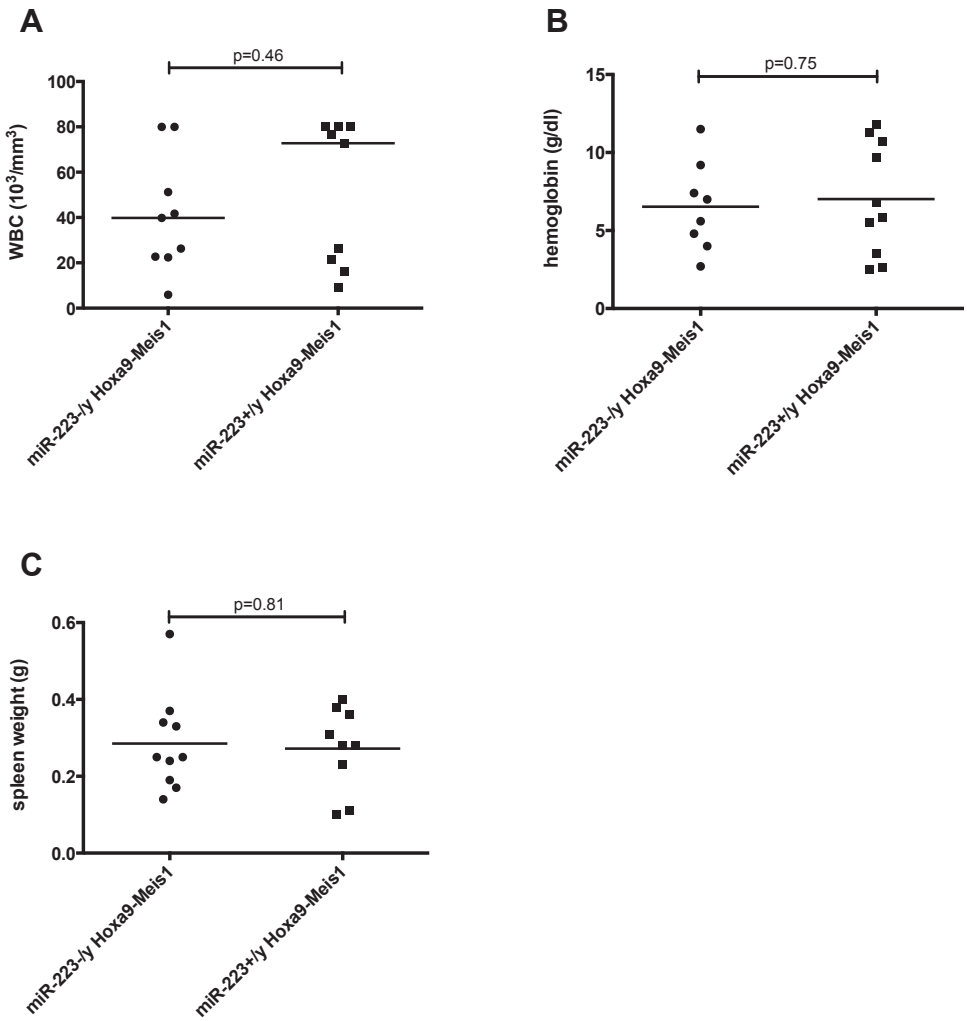
EFS = event-free survival; OS = overall survival; RFS = relapse free survival.



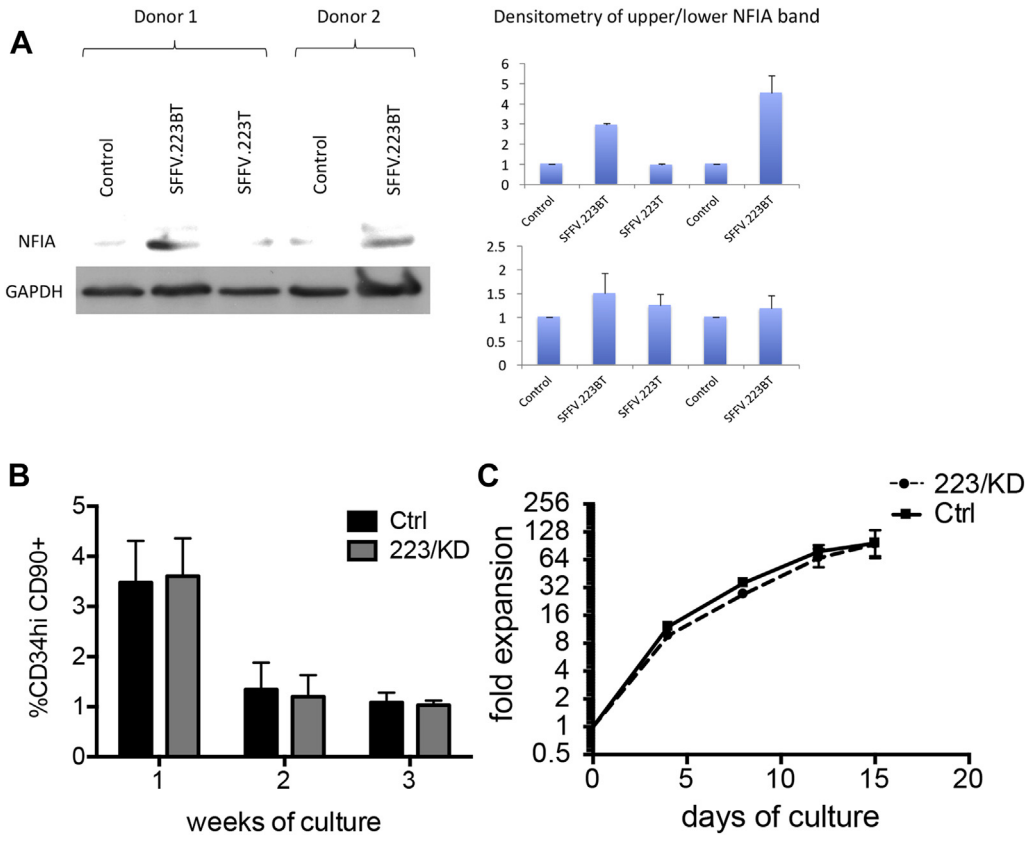
Supplementary Figure E1. The absence of miR-223 (miR-223-/y) did not significantly change the proliferation rate of BM cells retrovirally overexpressing (**A**) control vector, (**B**) Hoxa9, or (**C**) MLL-AF9, or (**D**) MN1.



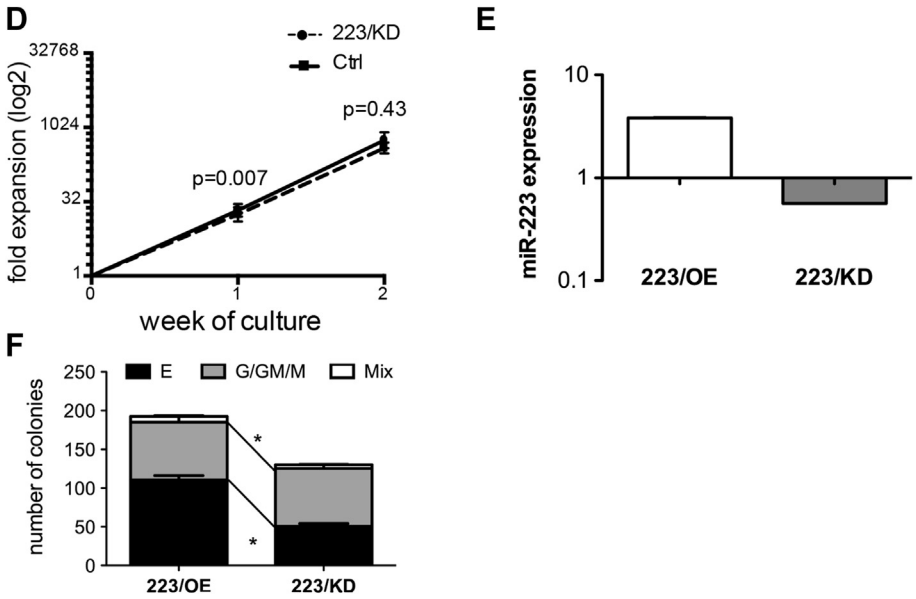
Supplementary Figure E2. Genetic depletion of miR-223 (miR-223-/y) did not significantly change the proliferation colony-forming capacity of BM cells retrovirally overexpressing (**A**) control vector, (**B**) Hoxa9, or (**C**) MLL-AF9, or (**D**) MN1.



Supplementary Figure E3. No significant differences were seen in *Hoxa9-Meis1* leukemias with respect to overall disease characteristics such as (**A**) WBC count, (**B**) RBC count, and (**C**) spleen weight.



Supplementary Figure E4. (**A**) Left: Representative Western blot analysis for NFI-A (upper film) and GAPDH (lower film) performed on human CB cells transduced with a control LV (Control.SFFV), the optimized miR-223 sponge vector (SFFV.223BT; $n = 2$ donors), and a first-generation miR-223 sponge vector (SFFV.223.T; $n = 1$ donor). Right: densitometric analysis of the upper and lower NFI-A bands after normalization to GAPDH ($n = 2$ technical replicates per sample; shown is the mean + range). The control LV-transduced sample was set to 1. (**B**) $CD34^+$ huCB cells ($n = 6$ donors) were lentivirally transduced with a miR-223 sponge (223/KD) or control vector and cultured under maintenance conditions (Stem Span serum-free expansion medium + SCF, TPO, FLT3L, IL-6). Shown is the proportion of CD34hi cells over time (mean ± SEM). Statistical analysis was performed by paired *t* test after log-odds conversion. (**C**) $CD34^+$ huCB cells ($n = 2$ donors) were sorted into a $CD133^+CD38^-$/low cell population containing more primitive progenitors, transduced with 223/KD or control LV, and cultured under maintenance conditions. No significant differences in proliferation could be observed, by contrast with $CD133^+CD38^+$ cells (see Fig. 2D).



Supplementary Figure E4. (*continued*) (**D**) 223/KD- or control-LV-transduced CD34$^+$ CB cells (n = 8 donors) were cultured in myeloid differentiation medium (IMDM + 10% FCS + SCF + G-CSF), and proliferation was assessed. Expansion of 223/KD-LV-treated cells relative to control-LV-transduced cells is shown. We noted a significantly reduced proliferation of 223/KD cells under myeloid culture conditions in the first week. (**E**) huCB CD34$^+$ cells were transduced with a control, the miR-223 KD, or OE LVs and plated in methylcellulose at a concentration of 800 cells/mL. Total colonies were harvested after 14 days, and miR-223 expression was measured by qPCR. CT values were normalized to RNU48 (Δ CT) and expressed relative to the expression level found in control-LV-transduced cells. qPCR reactions were performed in triplicates, and the mean was used for calculating relative expression levels. (**F**) huCB CD34$^+$ cells were transduced with the miR-223 KD or OE LV and plated in methylcellulose at a concentration of 800 cells/mL. Colonies were counted after 14 days and distinguished morphologically into erythroid (CFU/BFU-E), myeloid (CFU-G/GM/M), or mixed (CFU-GEMM). The assay was done in quadruplicate; shown is the mean ± SEM. Statistical analysis was performed by paired t test after log-odds conversion. *$p < 0.05$. Ctrl = Control; E = erythroid; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; G/GM/M = myeloid; Mix = mixed.