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Endogenous Tumor Suppressor microRNA-193b: Therapeutic and Prognostic Value in Acute Myeloid Leukemia

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ABSTRACT

Purpose

Dysregulated microRNAs are implicated in the pathogenesis and aggressiveness of acute myeloid leukemia (AML). We describe the effect of the hematopoietic stem-cell self-renewal regulating miR-193b on progression and prognosis of AML.

Methods

We profiled miR-193b-5p/3p expression in cytogenetically and clinically characterized de novo pediatric AML (n = 161) via quantitative real-time polymerase chain reaction and validated our findings in an independent cohort of 187 adult patients. We investigated the tumor suppressive function of miR-193b in human AML blasts, patient-derived xenografts, and miR-193b knockout mice in vitro and in vivo.

Results

miR-193b exerted important, endogenous, tumor-suppressive functions on the hematopoietic system. miR-193b-3p was downregulated in several cytogenetically defined subgroups of pediatric and adult AML, and low expression served as an independent indicator for poor prognosis in pediatric AML (risk ratio ± standard error, −0.56 ± 0.23; $P = .016$). miR-193b-3p expression improved the prognostic value of the European LeukemiaNet risk-group stratification or a 17-gene leukemic stemness score. In knockout mice, loss of miR-193b cooperated with *Hoxa9/Meis1* during leukemogenesis, whereas restoring miR-193b expression impaired leukemic engraftment. Similarly, expression of miR-193b in AML blasts from patients diminished leukemic growth in vitro and in mouse xenografts. Mechanistically, miR-193b induced apoptosis and a G1/S-phase block in various human AML subgroups by targeting multiple factors of the KIT-RAS-RAF-MEK-ERK (MAPK) signaling cascade and the downstream cell cycle regulator *CCND1*.

Conclusion

The tumor-suppressive function is independent of patient age or genetics; therefore, restoring miR-193b would assure high antileukemic efficacy by blocking the entire MAPK signaling cascade while preventing the emergence of resistance mechanisms.

INTRODUCTION

MicroRNAs (miRNAs) are short, noncoding RNAs that drew large interest as posttranscriptional master regulators of gene expression, often targeting hundreds of different mRNAs with both temporal and spatial specificity.[1,2] Their ability to orchestrate individual pathways at various levels or many pathways simultaneously gives them a central role in developmental, physiologic, and pathologic processes.[3,4] Accordingly, many of the miRNAs identified to date are associated with cancer and can act at different stages of tumor development.[5-9] Therefore, modulating miRNAs may be a promising strategy to advance targeted cancer therapies. First, they represent a new class of druggable molecules, distinct from classic drug targets in cancer (ie, proteins). Second, they are key regulators that control multiple important pathways simultaneously, reducing the possibility of single-target resistance mechanisms.[10] Third,

ASSOCIATED CONTENT

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miRNA-modulating therapeutics have been proven feasible with minimal adverse events in preclinical and clinical trials.[11-14]

In many cancers, including acute myeloid leukemia (AML), activating mutations in genes encoding growth factor receptors, such as FLT3 or KIT, or their downstream effectors have been identified.[15,16] The KIT-RAS-RAF-MEK-ERK (MAPK) pathway connects signals from cell-surface receptors to transcription factors, regulating gene expression and proteins involved in the cell cycle and apoptosis.[17-19] In AML cells, abnormalities in *KIT* (19% to 40% in pediatric and adult AMLs)[15,16] or the downstream small GTP-binding protein KRAS (10% to 20% in pediatric and adult AMLs) are common.[20] Despite the development of efficient RAF and tyrosine-kinase inhibitors, however,[21,22] current clinical trials have failed to prove a survival benefit for the treated patients, suggesting that leukemic cells quickly adapt and use alternative signaling routes.[10] Therefore, novel strategies that target the MAPK signaling cascade at multiple levels are warranted, preferably with a single drug, to achieve more efficient eradication of the leukemic clone.

In previous studies, we demonstrated that miR-193b is regulated by STAT5 signaling and controls hematopoietic stem and progenitor cell self-renewal and expansion by modulating the expression of KIT.[23] Given the central role of miR-193b as a negative regulator of hematopoietic stem and progenitor cell physiology, we investigated miR-193b in leukemogenesis and as a prognostic factor in pediatric and adult AML.

PATIENTS AND METHODS

For detailed methods, see the Data Supplement.

Patient Samples

Adult AML samples for in vitro assays were collected from patients enrolled in the German-Austrian AML Study Group (AMLSG) treatment protocols for younger adults (AMLSG-HD98A [ClinicalTrials.gov identifier: NCT00146120] and AMLSG 07-04 [ClinicalTrials.gov identifier: NCT00151242]). Pediatric AML samples for patient-derived xenografts were collected from patients enrolled in AML Berlin-Frankfurt-Münster treatment protocols for children and adolescents. Written informed consent was obtained from all patients or custodians in accordance with the Declaration of Helsinki and local laws and regulations, and the study was approved by the institutional review board of each participating center.

Mice and Bone Marrow Transplantations

All animal experiments were performed according to protocols approved by the state government of each institution. Animals were maintained under specific pathogen-free conditions. NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (CD45.1), B6.SJL-Ptprca Pepcb/BoyJ (CD45.1), or C57BL/6 mice (CD45.2) were purchased from Charles River Laboratories (Wilmington, MA) or bred and maintained at the Animal Facility of Ulm University or MFD Diagnostics (Wendelsheim, Germany). Transplantation experiments including lentiviral/retroviral transduction were performed as previously reported[24,25] and as described in the Data Supplement.

RESULTS

miR-193b-3p Expression Was Downregulated in AML

To investigate miR-193b in AML, we profiled the expression of the miRNA in pediatric patients with de novo AML (n = 161)[5] via quantitative real-time polymerase chain reaction. Patient characteristics are summarized in the Data Supplement.

The dominant strand miR-193b-3p (Data Supplement) was expressed at low levels in the majority of the AML subgroups compared with total bone marrow from healthy donors (Fig 1A). Cytogenetically normal (CN-AML), *MLL*-rearranged (*MLL*-r), and t(7;12) cases showed the lowest miR-193b-3p expression (0.51-, 0.28-, and 0.08-fold change compared with normal bone marrow, respectively). In contrast, significant upregulation of miR-193b-3p was observed in patients carrying a t(15;17) translocation (26-fold change compared with normal bone marrow; Fig 1A).

We additionally compared our data with the LAML miRNA-Seq data set of The Cancer Genome Atlas (TCGA) Research Network (n = 187).[26] In the TCGA data set, miR-193b-3p was downregulated within the CN-AML group, especially in cases with *FLT3* mutations (0.18-fold change compared with all AML cases; Fig 1B). In line with the pediatric cohort, miR-193b expression was significantly higher in the t(15;17) group (6.5-fold change compared with all AML cases; Fig 1B).

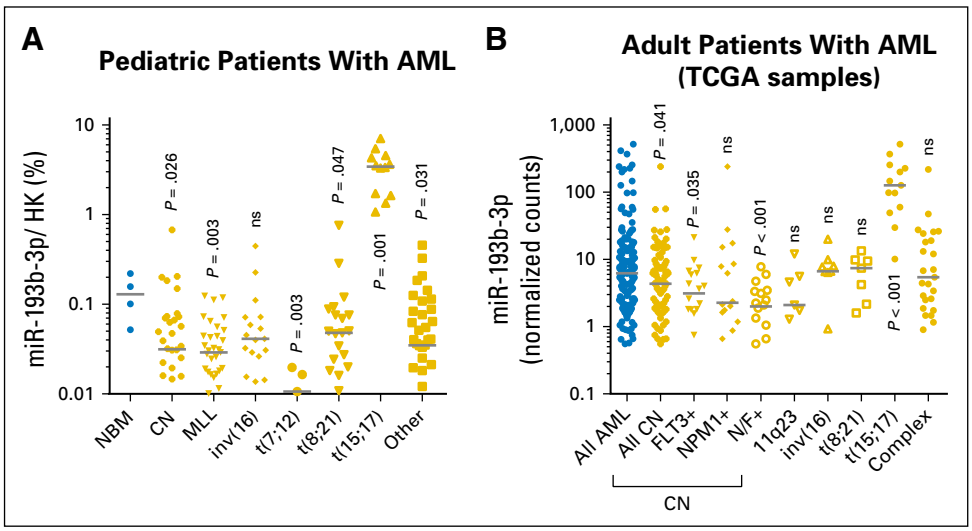

Fig 1. Patients with AML express low levels of miR-193b. (A) Expression of miR-193b-3p in cytogenetically defined pediatric AML samples as a percentage of the housekeeping genes RNU24/48 measured by quantitative real-time polymerase chain reaction (Taqman; Thermo Fisher Scientific, Waltham, MA). (B) Normalized read counts per million of miR-193b-3p in The Cancer Genome Atlas cohort of adult AML samples as determined by microRNA sequencing.[26] The gray line indicates the mean. Pairwise comparisons were performed using Mann-Whitney U test. AML, acute myeloid leukemia; CN, cytogenically normal; FLT3$^+$, *FLT3* mutation; N/F$^+$, *NPM1* mutation and *FLT3* mutation; NBM, normal bone marrow from healthy donors; NPM1$^+$, *NPM1* mutation; ns, not significant.

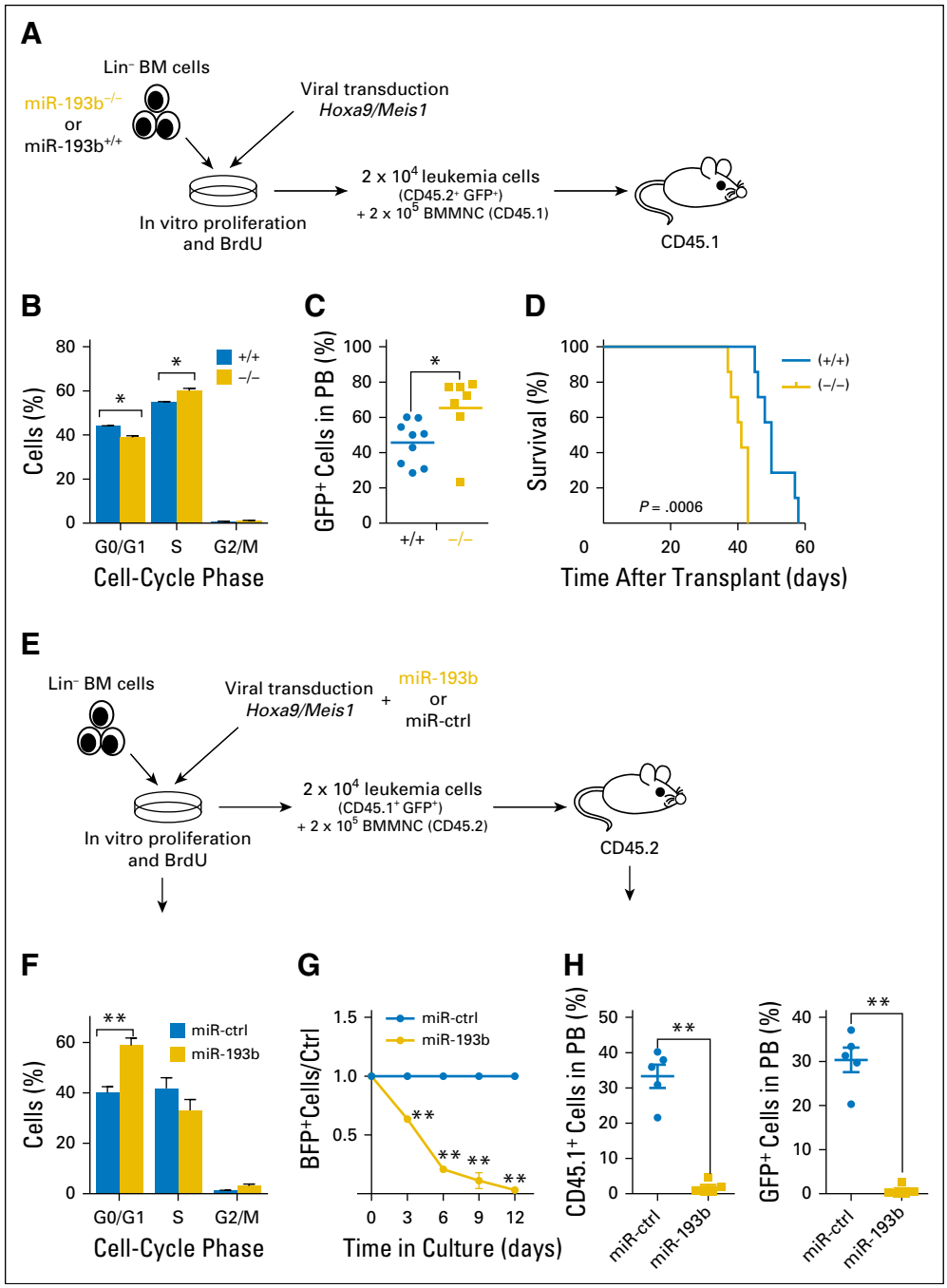

Fig 2. miR-193b modulates *Hoxa9/Meis1*–induced leukemia in vivo. (A) Experimental setup of *Hoxa9/Meis1*–induced leukemia in the CD45.1/CD45.2 mouse transplantation model using Lin⁻ BM cells of *miR-193b*$^{-/-}$ or *miR-193b*$^{+/+}$ donors. (B) Cell-cycle analysis of *Hoxa9/Meis1*–transduced Lin⁻ BM cells of *miR-193b*$^{-/-}$ or *miR-193b*$^{+/+}$ donors grown in vitro. Data are presented as mean ± standard deviation of three independent experiments (Mann-Whitney U test; $*P < .05$). (C) Percentage of donor cell engraftment (CD45.2⁺GFP⁺) 21 days after transplantation measured in the peripheral blood of recipient mice (n = 7 to 9 per group; Mann-Whitney U test; $*P < .05$). (D) Kaplan-Meier survival analysis of recipient mice (n = 7 to 9 per group; log-rank test). All mice died of leukemia. (E) Experimental setup of ectopic miR-193b expression in *Hoxa9/Meis1*–induced leukemia in Lin⁻ BM cells. (F) Cell-cycle analysis of miR-193b– or miR-ctrl–transduced cells grown in vitro (n = 2; Mann-Whitney U test; $**P < .01$). (G) Fraction of BFP⁺ miR-193b–transduced cells normalized to the miR-ctrl–transduced control (n = 2). Data are presented as mean ± standard deviation (two-way analysis of variance; $*P < .05$; $**P < .01$). (H) Percentage of donor cell engraftment (CD45.1⁺ [left] or GFP⁺ [right]) 14 days after transplantation measured in the peripheral blood of recipient mice transplanted with *Hoxa9/Meis1*/miR-ctrl or *Hoxa9/Meis1*/miR-193b (n = 5; Mann-Whitney U test; $**P < .01$). BM, bone marrow; BMMNC, bone marrow mononuclear cell; BrdU, bromodeoxyuridine; ctrl, control; PB, peripheral blood.

miR-193b Acted as a Tumor Suppressor in Hoxa9/Meis1*-Induced Leukemia In Vivo*

The downregulation of miR-193b-3p in various AML subtypes, particularly in the major pediatric (*MLL*-r) and adult (CN-AML) subtypes, led us to hypothesize that suppression of miR-193b is oncogenic. To test this, we used *Hoxa9/Meis1*[27] to transform lineage-negative (Lin⁻) CD45.2⁺ bone marrow cells from miR-193b$^{-/-}$ and miR-193b$^{+/+}$ mice, enabling us to study leukemogenesis in vitro and in vivo (Fig 2A). HOXA9 and MEIS1 are highly upregulated in *MLL*-r AML as well as CN-AML, and are crucial downstream effectors of the MLL fusion protein during leukemia induction.[28,29] Because the *MLL*-r and CN-AML subgroups showed the lowest miR-193b-3p expression, the *Hoxa9/Meis1* model was most relevant for investigating the potential tumor suppressive role of this miRNA. During in vitro culture, we only observed a marginal increase of cells in S phase in the absence of miR-193b (Fig 2B). In contrast, upon transplantation into CD45.1 recipients, the leukemic engraftment and expansion of *Hoxa9/Meis1*–transduced miR-193b$^{-/-}$ cells were enhanced (65% *v* 47% engraftment after 21 days; $P = .0001$; Fig 2C). Consequently, these mice exhibited a significantly shortened median survival (41 *v* 50 days; $P_{\text{log-rank}} = .0006$; Fig 2D).

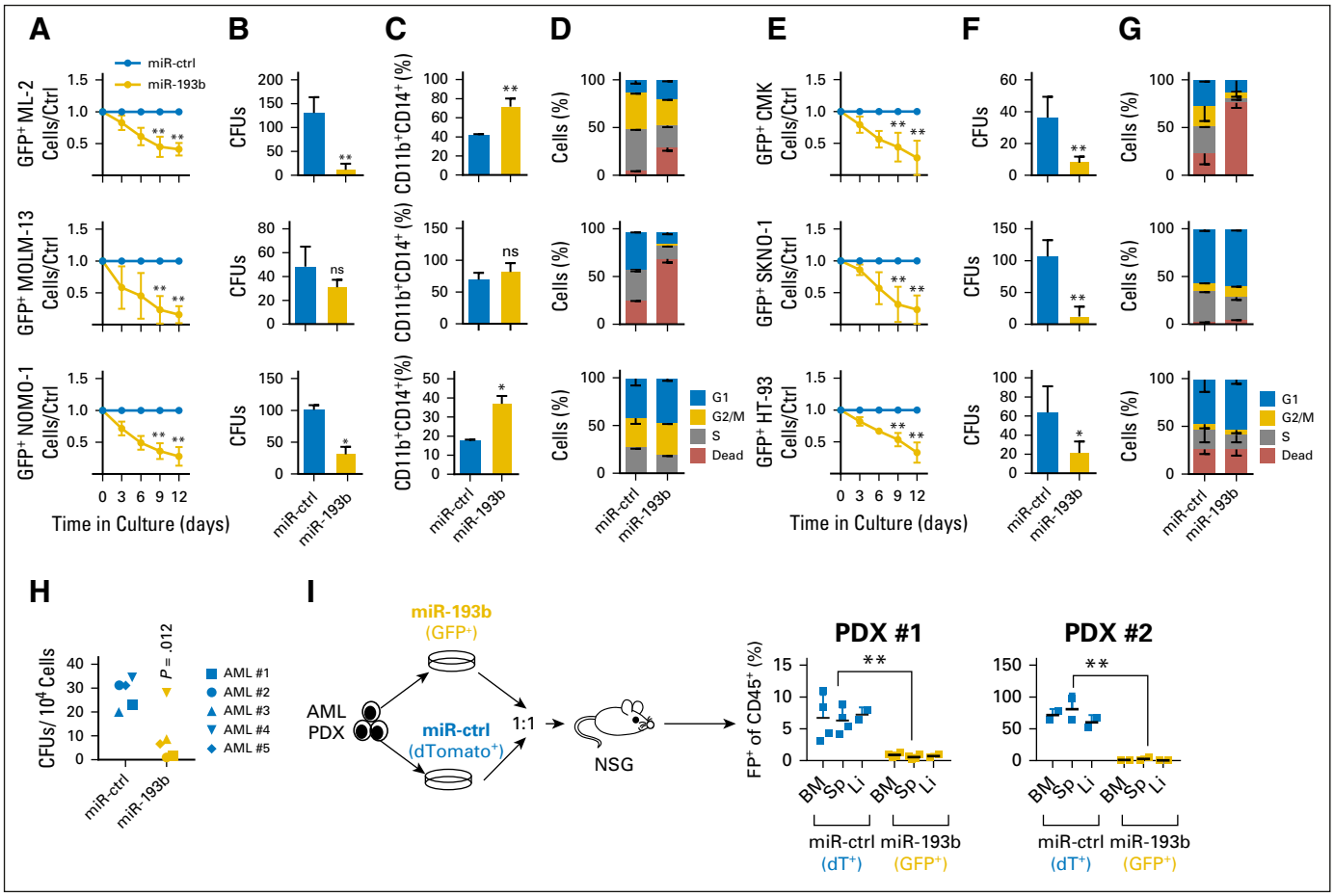

Fig 3. miR-193b reduces proliferation, restores monocytic differentiation, and blocks the G1/S phase transition in human AML. (A) Fraction of GFP+ miR-193b–transduced cells normalized to the miR-ctrl–transduced control. (B) Number of CFUs in methylcellulose-based colony-forming assays and (C) percentage of cells with high CD11b+CD14+ after calcitriol induction (50 nM) in miR-193b– or miR-ctrl–transduced cells. (D) Bromodeoxyuridine (BrdU) cell-cycle analysis. (E) Fraction of GFP+ miR-193b–transduced cells normalized to the miR-ctrl–transduced control. (F) Number of CFUs in methylcellulose-based colony-forming assays of miR-193b– or miR-ctrl–transduced cells. (G) BrdU cell-cycle analysis. (A-G) All data are presented as mean ± standard deviation of at least three experiments. Pairwise comparisons were performed using Student *t* test and two-way analysis of variance. $*P < .05$; $**P < .01$. (H) Number of CFUs in methylcellulose-based colony-forming assays of primary blasts of five patients with AML transduced with miR-193b or miR-ctrl (Mann-Whitney U test). (I) Experimental design for ectopic expression of miR-193b-GFP or miR-ctrl-dTomato in two different patient-derived AML xenografts (PDX #1, n = 4; PDX #2, n = 3). Percentage of donor cell engraftment (dTomato+ or GFP+) in leukemic recipients (Mann-Whitney U test; $**P < .01$). AML, acute myeloid leukemia; BM, bone marrow; CFU, colony-forming unit; ctrl, control; Li, liver; NSG, NOD scid gamma; PDX, patient-derived xenograft; Sp, spleen.

After showing that the absence of miR-193b accelerates progression of leukemia and induces a more aggressive disease in a *Hoxa9*/*Meis1* murine model of AML, we speculated that inversely, ectopic expression of miR-193b would impair leukemic growth in the same model (Fig 2E). Lentiviral overexpression of miR-193b decreased the proliferation rate and cell-cycle progression in vitro (Figs 2F and 2G) and delayed the engraftment of *Hoxa9*/*Meis1*–transduced, wild-type bone marrow cells in vivo (2% *v* 33% engraftment after 14 days; $P < .001$; Fig 2H). Similarly, restoring miR-193b expression in miR-193b$^{-/-}$ bone marrow cells impaired leukemic engraftment (Data Supplement). Together, the results of these in vivo studies suggest that miR-193b exerts a strong endogenous tumor suppressor function in the hematopoietic system.

Ectopic Expression of miR-193b Impaired Growth of Human AML Cells

Patients with *MLL*-r leukemia constitute approximately 35% of pediatric AML cases and exhibit major heterogeneity due to 70 different potential fusion partners of the *MLL* gene.[30] To investigate the tumor suppressive function of miR-193b in human disease, we lentivirally expressed miR-193b (Data Supplement) in three cell lines harboring two different *MLL* translocations: ML-2 (*MLL-AF6*), NOMO-1, and MOLM-13 (*MLL-AF9*). Growth competition assays showed a growth disadvantage for all *miR-193b*–transduced *MLL*-r cell lines (Fig 3A). Moreover, miR-193b impaired the colony-forming capacity and enhanced monocytic differentiation induced by calcitriol of ML-2 and NOMO-1 cells (Figs 3B and 3C). The effects of miR-193b were accompanied by a reduction of cells in S phase, as well as an increase of apoptotic and dead cells (Fig 3D; Data Supplement), highlighting the tumor suppressor potential of miR-193b in *MLL*-r AML via reduction of leukemic growth, induction of apoptosis, and promotion of monocytic differentiation.

We further evaluated whether the observed tumor suppressor effects of miR-193b are restricted to *MLL*-r leukemia. Ectopic expression of miR-193b in CMK (complex karyotype), HT93 (t[15;17]), and SKNO-1 (t[8;21]) cells significantly reduced cell growth and colony-forming capacity (Figs 3E and 3F), induced

apoptosis (Data Supplement), and decreased the fraction of cells in S phase (Fig 3G). These data provide additional evidence that miR-193b plays important tumor suppressor roles within a broader subset of AML subtypes through interference with cell-cycle progression, differentiation, and viability.

miR-193b Exerted Tumor Suppressive Properties in Primary Human AML Samples

Next, we extended our findings to primary human CN-AML samples (n = 5) with wild-type *NPM1* and, except for one sample, wild-type *FLT3*. Lentivirally expressed miR-193b abrogated colony formation in all but one patient sample (Fig 3H), which was *NPM1/FLT3* wild-type and did not exhibit any known unique characteristics.

To test a potential therapeutic value of miR-193b restoration, miR-193b (GFP^+)– or miR-control ($dTomato^+$)–transduced, *MLL*-r patient-derived AML xenografts of two patients were mixed in a 1:1 ratio and competitively transplanted into sublethally irradiated quaternary recipients (Fig 3I). The miR-193b–transduced AML blasts were diminished over time and almost absent in the bone marrow, spleen, and liver of leukemic mice (Fig 3I).

Together, these data from primary human AML blasts and patient-derived xenografts suggest that restoring miR-193b can halt leukemic growth in vitro and in vivo, implicating a future therapeutic potential.

miR-193b Targeted Key Regulators of the MAPK Pathway

To understand the molecular mechanism of the tumor suppressor phenotype of miR-193b, we screened for potential target genes. Because of the drastic effects of miR-193b on AML cells, we reasoned that the effect of miR-193b was unlikely to be caused by a single target, such as *KIT*,[31,32] but rather would be mediated through multiple targets leading to a complete block of at least one essential signaling cascade. Therefore, we mapped predicted miR-193 targets (TargetScan[33]) to KEGG pathways. Interestingly, the MAPK pathway was enriched for miR-193 targets: *KIT*, *KRAS*, and *SOS2*, as well as the downstream cell-cycle effector *CCND1* (Fig 4A). We demonstrated that ectopic expression of miR-193b downregulated all four genes at the mRNA and protein levels (Figs 4B and 4C). Luciferase reporter assays confirmed direct targeting of the complementary seed region of miR-193b to the 3′ untranslated regions of all four mRNAs (Fig 4D). To test whether downregulation of *CCND1*, *KIT*, *KRAS*, or *SOS2* could recapitulate the phenotype caused by miR-193b expression, we performed in vitro short hairpin RNA (shRNA)-mediated knockdown experiments using two validated shRNAs per gene (Data Supplement). We observed a decrease in the proliferation of SKNO-1 and CMK cells lentivirally transduced with each of these shRNA constructs compared with the nonsilencing shRNA control, as well as a reduction of cells in S phase (Fig 4E; Data Supplement).

Next, we assessed whether the downregulation of miR-193b, as seen in CN and *MLL*-r AML cases, increased MAPK signaling. To do so, we measured the expression of KIT and phosphorylation of ERK and STAT5 (other downstream signal transducers of KIT and indirect targets of miR-193b[23]) in *Hoxa9*/*Meis1*–transduced miR-193b$^{-/-}$ or miR-193b$^{+/+}$ bone marrow cells. KIT levels were elevated and both STAT5 and ERK exhibited increased phosphorylation in the absence of miR-193b, demonstrating increased activation (Fig 4F).

Last, we tested whether restoring KIT expression could render leukemic cells resistant to the tumor suppressive function of miR-193b. We could demonstrate that ectopic expression of a mouse *Kit* or human *KIT* cDNA, respectively, without miR-193b target sites, was not able to overcome the growth disadvantage, cell-cycle inhibition, and apoptosis induced by ectopic miR-193b in *Hoxa9*/*Meis1*–transformed murine leukemic cells or in the human cell lines SKNO-1 and CMK (Figs 4G and 4H; Data Supplement).

Our data imply that miR-193b acts as tumor suppressor by targeting the MAPK signal cascade at multiple steps, thereby tightly controlling cell proliferation and cell-cycle progression (Fig 4A), and that restoring only one target is insufficient to abrogate these antileukemic effects of miR-193b.

miR-193b as a Prognostic Factor in AML

miR-193b exerts tumor suppressive functions across multiple AML subgroups and loss of miR-193b shortens survival in the *Hoxa9*/*Meis1*-induced leukemia mouse model. Therefore, we investigated the prognostic effect of miR-193b in AML. We stratified a cohort of 161 pediatric patients[5] on the basis of miR-193b-3p expression (Fig 1A; Data Supplement). The optimal cutoff was determined by maximally selected rank statistics adjusted for multiple testing[34] (Data Supplement). Kaplan-Meier analysis demonstrated that low miR-193b-3p expression was significantly associated with a lower overall survival (OS; 71% *v* 45% after 5 years; $P_{\text{log-rank}}$ =.0016; Fig 5A) and event-free survival (EFS; 49% *v* 31% after 5 years; $P_{\text{log-rank}}$ =.018; Fig 5B). Expression of the passenger strand (miR-193b-5p) did not predict survival (Data Supplement).

In the multivariate analysis, including white blood cell count, age, and risk group stratification as established prognostic parameters,[35] low miR-193b-3p expression was validated as an independent factor for poor prognosis (Table 1; Data Supplement). Furthermore, the prognostic value of miR-193b-3p was retained when excluding the patients with t(15;17) (OS $P_{\text{log-rank}}$ = .002; EFS $P_{\text{log-rank}}$ = .025), who have a high expression of miR-193b-3p and are characterized by an excellent survival even without chemotherapy[36] (Data Supplement). Most importantly, low miR-193b-3p expression identified patients with a very poor prognosis within the European LeukemiaNet intermediate/adverse-risk group (Figs 5C and 5D; Data Supplement). Moreover, miR-193b-3p expression improved the prognostic value of the recently reported LSC17 signature.[37] Within the LSC17 high-risk patients, miR-193b-3p expression further stratified those with a very high risk and an even worse OS ($P_{\text{log-rank}}$ = .003) and EFS ($P_{\text{log-rank}}$ = .005; Figs 5E and 5F; Data Supplement).

Of note, in the TCGA adult AML data set, low miR-193b-3p expression was also associated with a significantly worse OS ($P_{\text{log-rank}}$ = .014) and EFS ($P_{\text{log-rank}}$ = .0005; Data Supplement).

The results of these analyses not only identify miR-193b-3p expression as a prognostic factor, but also imply that delivery of miR-193 mimetics may be a promising therapeutic approach for pediatric and adult patients with AML. Identification of patients at very high risk, on the basis of their low miR-193b-3p expression,

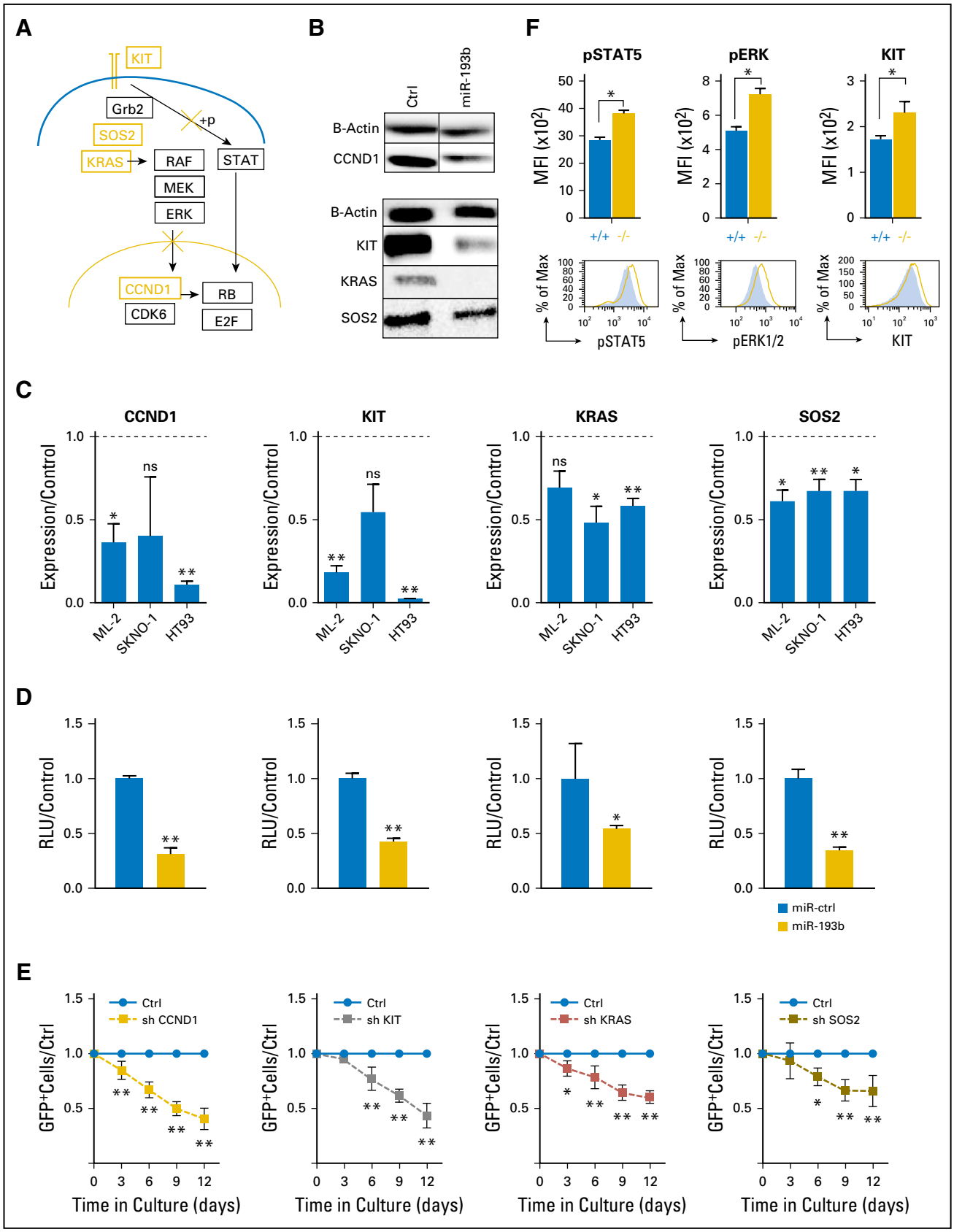

Fig 4. miR-193b targets the MAPK (KIT-RAS-RAF-MEK-ERK) pathway. (A) The oncogenic MAPK (KIT-RAS-RAF-MEK-ERK) pathway is enriched for miR-193b targets (gold). (B) Western blots for KIT, KRAS, and SOS2 in CMK cells and CCND1 and β-actin in SKNO-1 cells transduced with miR-ctrl or miR-193b show a downregulation of protein expression. (C) Quantitative real-time polymerase chain reaction shows the quantified expression of *CCND1*, *KIT*, *KRAS*, and *SOS2* in (continued on next page)

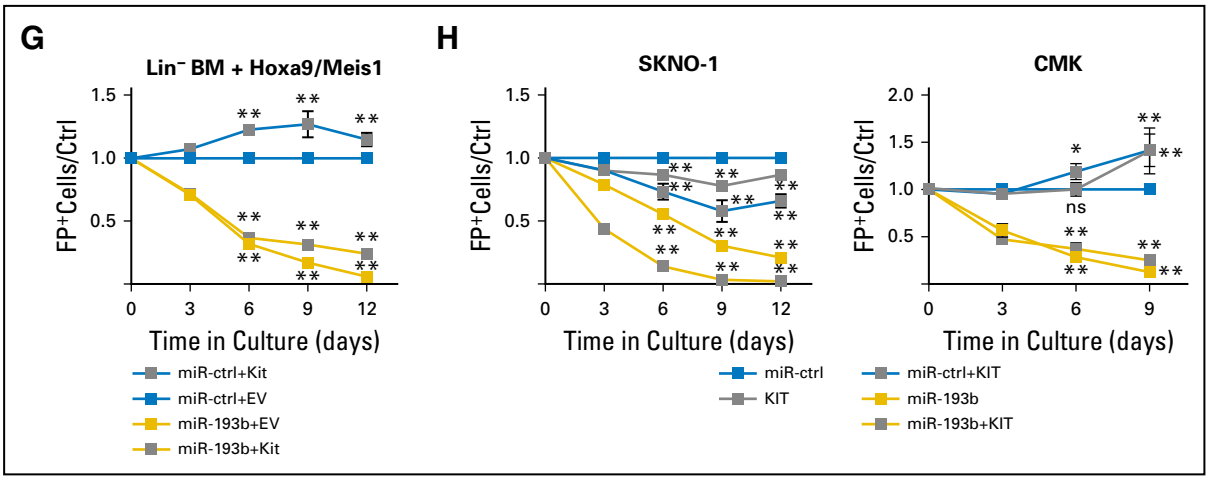

Fig 4. (Continued).

(Continued) ML2, SKNO-1 and HT-93 cells transduced with miR-193b. Data are shown in relation to miR-ctrl–transduced cells (n = 2). (D) Luciferase reporter assays with the 3′ untranslated region of *CCND1*, *KIT*, *KRAS*, and *SOS2* in HEK293 T cells transfected with miR-193b or miR-ctrl (n = 3). (E) shRNA mediated knockdown of the predicted target genes in SKNO-1 cells. The fraction of GFP$^+$–transduced cells normalized to the sh-ctrl–transduced control is shown. Measurements were done every 3 days (n = 3). (F) Intracellular flow analysis of basal pSTAT5 and pERK, and KIT in miR-193b$^{-/-}$ or miR-193b$^{+/+}$ murine hematopoietic stem and progenitor cells transduced with *Hoxa9/Meis1*. (G, H) Restoration of KIT expression using an miR-193–resistant mouse or human cDNA. (G) Fraction of BFP$^+$– and/or Venus$^+$–transduced *Hoxa9/Meis1* Lin$^-$ bone marrow cells normalized and compared with the miR-ctrl–transduced plus EV (BFP$^+$Venus$^+$) control (n = 2). (H) BFP$^+$ and/or GFP$^+$ SKNO-1 or CMK cells normalized and compared with the miR-ctrl–transduced (BFP$^+$) control (n = 3). (C to H) All data are presented as mean ± standard deviation. Pairwise comparisons were performed using Student *t* test or two-way analysis of variance. $*P < .05$; $**P < .01$. Ctrl, control; EV, empty vector; Max, maximum; MFI, mean fluorescence intensity; ns, not significant; RLU, relative light unit; sh, short hairpin.

may help to better allocate patients to hematopoietic stem-cell transplantation.

DISCUSSION

Here, we introduce miR-193b as an endogenous tumor suppressor of the hematopoietic system and independent prognostic marker in AML. We provide compelling mechanistic evidence that miR-193b orchestrates the pivotal MAPK signaling pathway to control viability and proliferation, which is often constitutively activated in AML and, therefore, opens opportunities for antileukemic strategies.

Knockout of miR-193b in the *Hoxa9*/*Meis1* in vivo model caused a more aggressive form of leukemia, resulting in a significantly decreased survival of the recipient mice. Thus, in addition to its known function as a gate keeper in normal hematopoietic stem cells that regulates stem-cell function by controlling proproliferative pathways in a STAT5-dependent negative feedback loop,[23] we describe here the critical function of miR-193b as a potent suppressor of leukemic growth. In fact, miR-193b and its family member miR-193a have been suggested to act as tumor suppressors in certain types of leukemia, such as T-lymphoblastic leukemia or AML with t(8;21),[31,32,38,39] as well as in several solid tumors, including lung and ovarian cancers.[40-44] Our study sheds light on the essential mechanism beyond the former observations and establishes a global role of miR-193b as an endogenous tumor suppressor in the hematopoietic system.

In a *Hoxa9*/*Meis1*–driven AML model, the absence of miR-193b corresponded with upregulation of STAT5, and RAS/RAF/ERK signaling, mediated by the lack of miR-193b–regulated fine tuning of at least four key target genes: *KIT*, *KRAS*, *SOS2*, and *CCND1*. All four identified target genes are components of the MAPK pathway (Fig 4A), which interferes with other pathways. CCND1 provides a link between the MAPK pathway and cell-cycle progression.[17-19] The RAS-RAF-MEK-ERK cascade regulates the activity of CCND1 and the formation of the CCND1/CDK6 complex, thereby controlling G1/S transition.[17-19]

Activating mutations in the MAPK pathway represent a frequent event in the progression of leukemia and other malignancies.[15,16] *KIT* mutations appear in approximately 12% to 25% of cases with inv(16)(p13q22) and t(8;21) AML,[45] in which the tumor suppressive functions of miR-193a through targeting *KIT* and *CCND1* were first described in AML.[39] Targeting the MAPK axis remains a long-sought goal in cancer therapies, although development of such therapies has been hampered by several obstacles.[46] Most notably, the oncogenic RAS protein was considered untargetable with drugs.[46] In addition, therapeutic interference with activated transcription factors, such as STATs, is inherently difficult. Although efficient inhibitors of RAF, MEK, ERK, and tyrosine kinases have been developed,[22,47] they have failed to provide a survival benefit in clinical trials.[10] Currently, many cancer therapies that target a single oncogene merely induce a modest therapeutic response; moreover, they increase the possibility of acquiring mutations that cause therapy resistance. Thus, the ability to repress many oncogenes at once and across different oncogenic pathways provides a strong rationale for developing miRNA-based cancer therapeutics.

The strategy of inhibiting multiple targets in one pathway by using a single molecule is still in its infancy. Here, we present miR-193b to target multiple important hubs of leukemia cell signaling simultaneously, including transcription factors. We were able to

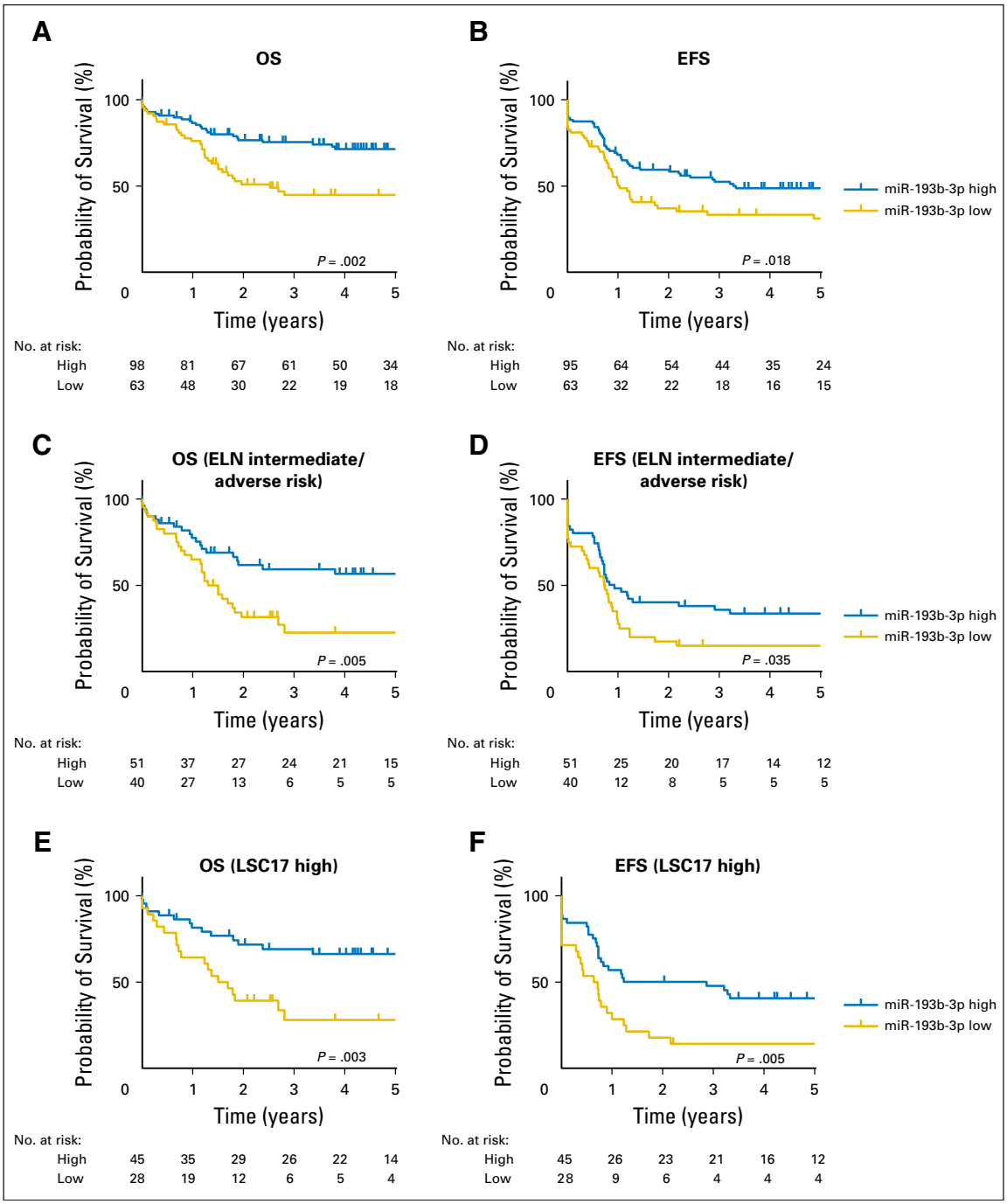

Fig 5. miR-193b serves as a prognostic factor in pediatric AML. Probability of (A) OS and (B) EFS in pediatric patients with AML with high (blue) or low miR-193b-3p expression (gold). Probability of (C) OS and (D) EFS in ELN intermediate/adverse-risk pediatric patients with AML with high (blue) and low-miR-193b-3p expression (gold). Probability of (E) OS and (F) EFS in pediatric patients with AML with a high LSC17 score and high (blue) or low miR-193b-3p expression (gold). (A to F) The cutoff was determined by maximally selected rank statistics.[34] Curve comparison was done using log-rank test. AML, acute myeloid leukemia; EFS, event-free survival; ELN, European LeukemiaNet; LSC17, 17-gene leukemic stemness score; OS, overall survival.

identify four direct targets of miR-193b within the MAPK pathway. In this case, even if a mutation were to arise in the mRNA–miRNA binding region of one of the four target genes, miR-193b would still be able to target three other genes of this pathway. This would impede the fast development of resistance mechanisms. In fact, our data show that miR-193b–resistant *KIT* was not able to overcome the tumor suppressive effect of miR-193b. Only in AML cases with t(15;17) was the miR-193b-3p expression high and an miR-193b–based treatment must be carefully evaluated, given the relevant role of the MAPK pathway for all-*trans* retinoic acid–induced differentiation.[48]

Similar approaches using miRNA mimetic agents are currently in clinical trials.[11-13] miR-16 mimics are undergoing phase I clinical trials in patients with malignant pleural mesothelioma or

Table 1. Multivariate Cox Regression Analysis for Pediatric AML

Variable	EFS Coefficient	SE (n = 158)	*P*	OS Coefficient	SE (n = 161)	*P*
Age	0.038	0.022	.092	0.027	0.027	.315
WBC count	−0.001	0.001	.519	0.000	0.002	.950
ELN	1.618	0.283	< .001	1.737	0.362	< .001
miR-193b-3p	−0.563	0.233	.016	−0.670	0.282	.018

Abbreviations: AML, acute myeloid leukemia; EFS, event-free survival; ELN, European LeukemiaNet; OS, overall survival; SE, standard error; WBC, white blood cell.

non–small-cell lung cancer for whom standard therapy was ineffective. The miR-16 mimics are delivered intravenously using EnGeneIC Dream Vector packaging (Sydney, New South Wales, Australia) and are conjugated with an epidermal growth factor receptor-targeting antibody[12]—a strategy that could be applied to miR-193b.[14] At this stage, miR-193b-3p expression can be used to infer treatment recommendations for children with AML. Patients in the European LeukemiaNet intermediate/adverse-risk group or with a high LSC17 score, who have low miR-193b-3p expression, have a poor prognosis and may be allocated to hematopoietic stem-cell transplantation. Still, the retrospective nature of our analysis is a potential limitation.

In conclusion, our work identifies miR-193b as a global antileukemic miRNA in AML and as a suppressor of essential signaling pathways, paving the road for a clinical application. MiR-193b further stands out as potential tool for AML prognosis, promising higher chances for its entrance into therapeutics.

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