The microRNA miR-196b acts as a tumor suppressor in Cdx2-driven acute myeloid leukemia

Acute myeloid leukemia (AML) is characterized by high mortality, underlining the necessity for identifying tumor suppressors that counteract the leukemogenic potential of *bona fide* oncogenes such as the homeobox genes *HOXA9* and *CDX2*. Homeobox genes are aberrantly expressed in the majority of patients with cytogenetically normal (CN)-AML and expression of CDX2 positively correlates with *HOXA9* expression.[1,2] Aberrant expression of *Cdx2* in murine hematopoietic cells rapidly induced aggressive AML in mice.[3] Recently, it was shown that a microRNA, miR-196, is encoded in the mammalian paralogous *HOX* gene cluster and that it has extensive evolutionarily conserved complementarity to sites in the 3' prime untranslated regions (3' UTR) of *HOX* genes (e.g. *HOXA9*, *A7* and *B8*), directly regulating their expression in MLL-rearranged leukemic cells.[4,5]

To understand the role of miR-196b in AML more precisely, we first tried to identify transcripts generating mature miR-196b in human hematopoietic stem and progenitor cells (HSPC). We identified two novel non-coding transcripts encoding the miR-196b hairpin precursor sequence expressed from the HOXA9-10 locus in bone marrow HSPC (*Online Supplementary Figure S1A, B*) [NCBI accession number: MF139050, 486 basepairs (bp) and MF139051, 396 bp]. MF139051, a splice variant of the MF139050 transcript, showed 99.47% homology to MF139050. Both transcripts displayed high homology to the transcripts of other vertebrates and mammalian species. Retrovirally engineered expression of both transcripts resulted in significant overexpression of the mature miR-196b in HEK293T cells compared to the vector control (Figure 1A; *Online Supplementary Figure S1C, D*). Endogenous expression levels of both transcripts in the human CD34$^+$ bone marrow compartment were higher than those in mononuclear cells (Figure 1B). Second, we identified a highly conserved 802 bp long miR-196b promoter region, validated by luciferase reporter assay, located 201 bp upstream of the miR-196b stem loop precursor sequence on human chromosome 7 (Figure 1C; *Online Supplementary Figure S1E, F*). Transcription factor binding site prediction tools and published chromatin immunoprecipitation sequencing data showed, respectively, potential binding of transcription factors including SP1 and enrichment for proteins such as EZH2 on this promoter (*Online Supplementary Figure S1G, H; Online Supplementary Table SW1*). Previously, the miR-196b promoter region was described in the context of murine development, demonstrating *in vivo* enrichment for *Cdx2* and *Hoxd13*.[6]

Next, we asked whether expression of miR-196b itself as well as the ratio between miR-196b expression and the expression of its direct target *HOXA9* is perturbed in CN-AML patients: miRNA-sequencing analysis of healthy bone marrow-derived hematopoietic subpopulations revealed highest expression of miR-196b in the lymphoid-primed multi-potent progenitor subpopulation, following the same expression pattern as known for its target *HOXA9*, whose expression is also highest in immature cells (Figure 1D; *Online Supplementary Table SE1*). This resulted in a significant correlation between the expression levels of mature miR-196b or MF139051 and *HOXA9* in HSPC (*Online Supplementary Figure S2A; Online Supplementary Table SW2*). In contrast, expression levels of miR-196b and of *HOXA9* remained at similar levels across all three functionally validated leukemic bone marrow subpopulations from CN-AML patients (Figure 1D; *Online Supplementary Table SE1*).

In contrast to the expression levels of the miR-196b transcript MF139051 and miR-196b, *HOXA9* expression was dramatically increased in leukemic CD34$^+$ bone marrow compared to CD34$^+$ normal bone marrow cells. This difference could be confirmed in functionally validated leukemic stem cells compared to normal HSPC and in published data[7,8] (*Online Supplementary Figures S2B* and *S3; Online Supplementary Tables SW3-4*). Based on this, the correlation between the expression levels of the mature miR-196b and its transcripts versus the expression of *HOXA9* was lost in leukemic cells (*Supplementary Figures S4*). The difference in expression patterns resulted in a 111-fold higher ratio between the expression values of *HOXA9* and mature miR-196b in CN-AML compared to normal CD34$^+$ bone marrow and a 57-fold higher ratio between NPM1c$^+$ AML and normal CD34$^+$ bone marrow, which also held true for the short transcript MF139051 calculated for the same AML groups (Figure 1E; *Online Supplementary Table SW4*). This implies that compared to normal CD34$^+$ hematopoietic cells there are many fewer miR-196b transcripts and mature miRNA per *HOXA9* transcript in CN-AML, including NPM1-mutated cases, previously shown to have particularly high *HOX* gene expression.[2]

Overexpression of miR-196b in normal murine HSPC did not affect clonogenicity and cell growth *in vitro* (Figure 2A; *Online Supplementary Figure S5A, B*). miR-196b increased the proportion of circulating myeloid cells and three of 14 mice developed AML after a long latency, indicating that cooperating events may contribute to disease induction. (Figure 2B, F; *Online Supplementary Table SW5*). To test the impact of miR-196b expression in the context of *Cdx2*-induced AML, we retrovirally co-expressed *Cdx2*/miR-196b or *Cdx2* alone in murine HSPC (*Online Supplementary Figure S5C*). Co-expression of miR-196b considerably reduced the *Cdx2*-induced proliferation, clonogenicity and spleen colony formation compared to those of *Cdx2*-transduced cells (Figure 2C, D; *Online Supplementary Figure S5D-F*). *Cdx2*-transplanted mice developed AML with a median latency of 193 days. In contrast, only 40% of the *Cdx2*/miR-196b-transplanted mice developed AML with a significantly longer latency (Figure 2E; *Online Supplementary Table SW5*). Moreover, miR-196b impaired growth and colony formation in the *CDX2* and *HOX* gene-positive human AML cell lines OCI-AML3 and NB4 *in vitro*, in contrast to the *CDX2* and *HOX* gene-negative human AML cell line Kasumi-1. Furthermore, miR-196b reduced engraftment of OCI-AML3 in NSG mice (*Online Supplementary Figure S6A-K*; *Online Supplementary Table SW6*). Overexpression of miR-196b did not induce apoptosis, cell cycle arrest or senescence in OCI-AML3 and NB4 cells (*data not shown*), suggesting that miR-196b preferentially targets self-renewal by reducing *HOX* gene expression, thereby reducing clonogenicity and engraftment potential.

Overexpression of miR-196b in HSPC alone resulted in 155 differentially expressed genes compared to the control. Among these 155 genes, 44% of the downregulated ones were significantly enriched for known miR-196b targets, whereas the upregulated ones did not show any miR-196b target enrichment (*Online Supplementary Tables SE2-5; Online Supplementary Figure S7A-C*). In *Cdx2*-transduced cells, overexpression of miR-196b induced 524 differentially expressed genes compared to *Cdx2* alone (*Online Supplementary Figure S7D; Online Supplementary Tables SE6 and SE7*). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

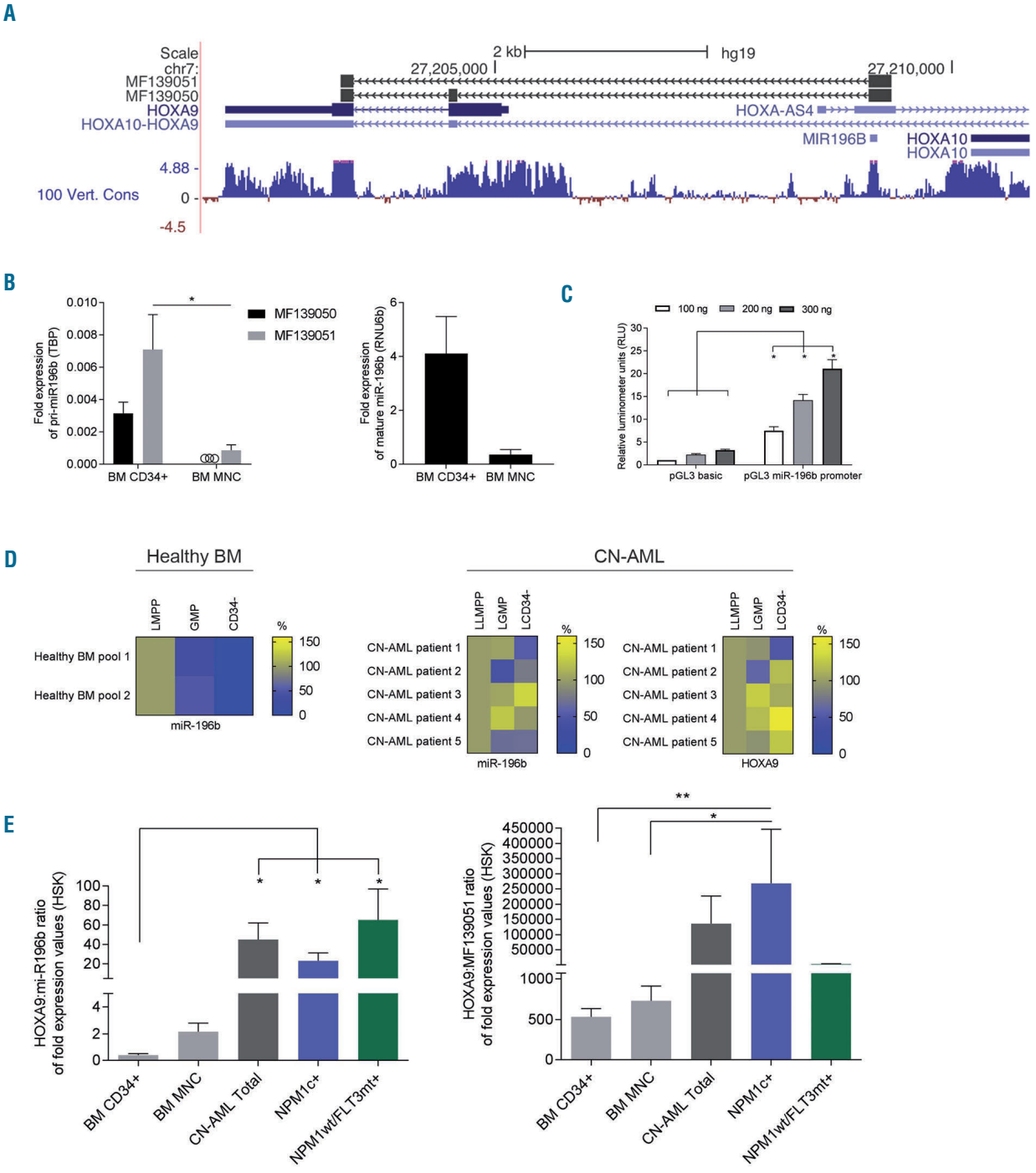

Figure 1. Characterization of miR-196b transcripts and of miR-196b expression in normal and leukemic cells. (A) Sequence alignment and homology of MF139050 and MF139051 transcripts with the UCSC Genome Browser (GRCh37/hg19) Assembly. UCSC genes are displayed in blue bars: dark blue depicts coding and bright blue non-coding transcript sequences. "100 Vert. Cons" track corresponds to sequence conservation across 100 vertebrates. (B) Fold expression, in CD34-positive cells ($CD34^+$) and mononuclear cells (MNC) of MF139050 ($CD34^+$: n=3; MNC: n=3) and MF139051 ($CD34^+$: n=6; MNC: n=3) relative to the housekeeping gene (HKG) *TBP* (left) and miR-196b ($CD34^+$: n=3; MNC: n=3) relative to HKG *RNU6b* (right) was determined by realt-time quantitative polymerase chain reaction (qRT-PCR) analysis in normal human primary bone marrow (BM) samples. Undetermined qRT-PCR values are displayed as circle outlines. Significance was determined by the Mann-Whitney *U* test. (C) Luciferase activity of the miR-196b promoter sequence assayed in HEK293T cells (n=4) using three different plasmid concentrations. Statistical significance was determined by the Mann-Whitney *U* test. (D) Heatmaps of miR-196b and *HOXA9* expression levels from miRNA-sequencing and mRNA-sequencing, respectively, in percentage of log-transformed transcripts per kilobase million (TPM) values to lymphoid-primed multi-potential progenitor cells (LMPP). Heatmap on the left represents percent expression in healthy BM subpopulations [LMPP, granulocyte-macrophage progenitors (GMP), CD34-]. Each healthy BM pool comprises three individual samples (n=2 pools). Heatmaps on the right represent percent expression of miR-196b and *HOXA9* in cytogenetically normal (CN)-acute myeloid leukemia (AML) stem cell subpopulations for each individual patient (n=5). (E) Fold (HKG) expression of *HOXA9* relative to fold (HKG) expression of miR-196b (left) and MF139051 (right). BM $CD34^+$ (n=3); BM MNC (n=3); Total CN-AML (combined expression values of $NPM1c^+$ and $FLT3mt^+$; n=24-25); $NPM1c^+$ (cytoplasmic nucleophosmin 1; n=12); $NPM1^{wt}/FLT3mt^+$ (FLT3 mutated (mt): FLT3-ITD/FLT3-TKD = fms-like tyrosine kinase receptor-3/internal tandem duplications/tandem kinase domain; n=13 (left) and n=12 (right). Significance was determined by Kruskal-Wallis analysis with the Dunn post hoc test, using BM $CD34^+$ and BM MNC as control groups. For panels (B), (C) and (E), error bars indicate the standard error of mean.

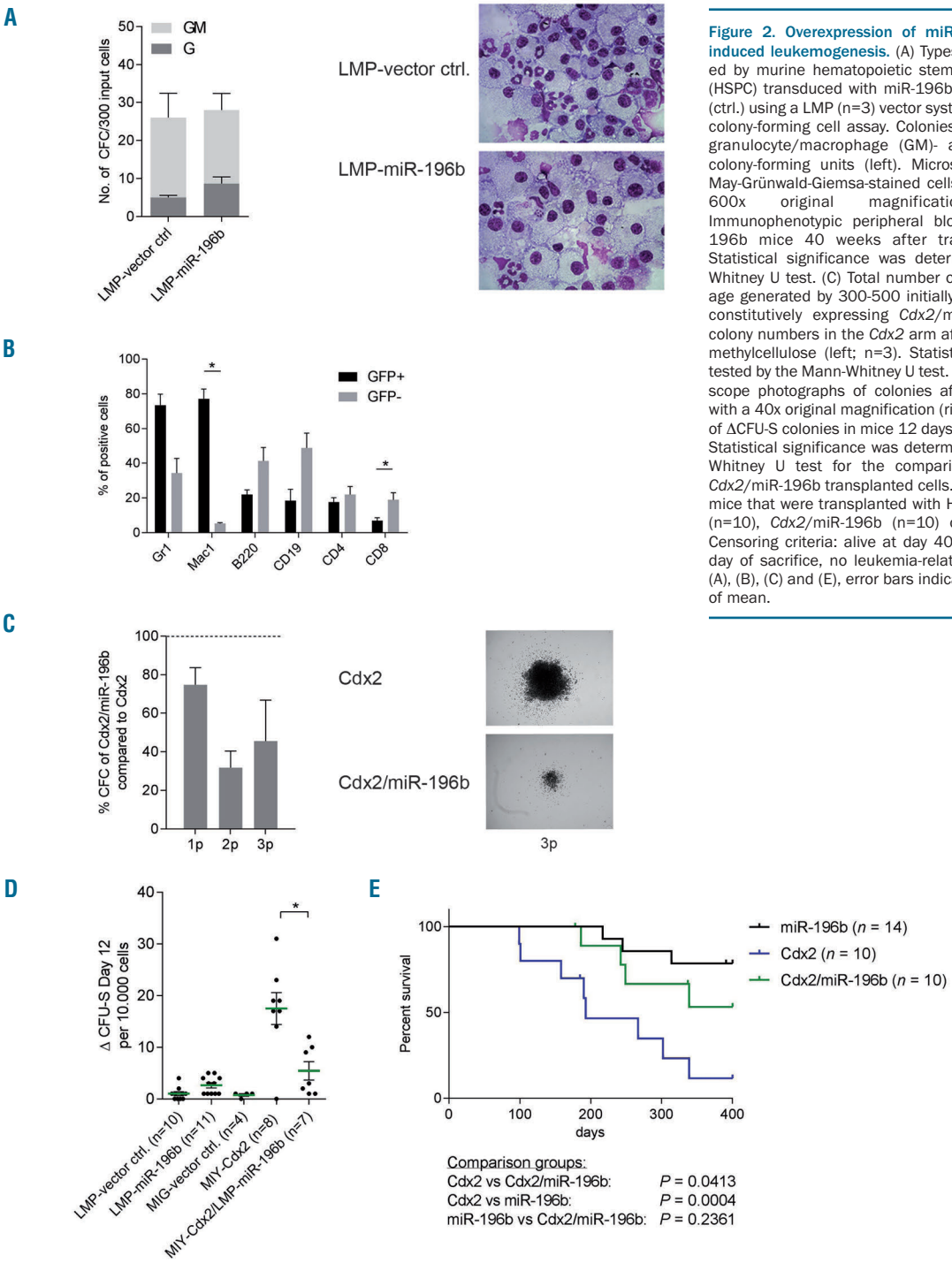

Figure 2. Overexpression of miR-196b impairs *Cdx2* induced leukemogenesis. (A) Types of colonies generated by murine hematopoietic stem and progenitor cells (HSPC) transduced with miR-196b or the vector control (ctrl.) using a LMP (n=3) vector system after 7-9 days in a colony-forming cell assay. Colonies were classified into: granulocyte/macrophage (GM)- and granulocyte (G)-colony-forming units (left). Microscope photograph of May-Grünwald-Giemsa-stained cells on cytospins with a 600x original magnification (right). (B) Immunophenotypic peripheral blood analysis of miR-196b mice 40 weeks after transplantation (n=4). Statistical significance was determined by the Mann-Whitney U test. (C) Total number of colonies in percentage generated by 300-500 initially plated murine HSPC constitutively expressing *Cdx2*/miR-196b referred to colony numbers in the *Cdx2* arm after serial re-plating in methylcellulose (left; n=3). Statistical significance was tested by the Mann-Whitney U test. Representative microscope photographs of colonies after second re-plating with a 40x original magnification (right). (D) Total number of ΔCFU-S colonies in mice 12 days after transplantation. Statistical significance was determined using the Mann-Whitney U test for the comparison of *Cdx2 versus Cdx2*/miR-196b transplanted cells. (E) Survival curves of mice that were transplanted with HSPC expressing Cdx2 (n=10), *Cdx2*/miR-196b (n=10) or miR-196b (n=14). Censoring criteria: alive at day 400, no engraftment at day of sacrifice, no leukemia-related death. For panels (A), (B), (C) and (E), error bars indicate the standard error of mean.

showed significant enrichment for pathways in cancer and apoptosis. Reactome analysis of downregulated miR-196b target differentially expressed genes identified enrichment for the MAP kinase pathway (Figure 3A; *Online Supplementary Figure S7E; Online Supplementary Table SE8-10*), in line with gene set enrichment analysis (GSEA) of these selected miR-196b target genes, which revealed enrichment for genes involved in proliferation. Of note, *NPM1* was downregulated, pointing to the possibility that miR-196b also suppresses mutated *NPM1* (Figure 3B). Additionally, GSEA showed significant enrichment of downregulated genes in six oncogenic pathways upon miR-196b overexpression, including enrichment in a leukemic stem cell/hematopoietic stem cell signature[7] (Figure 3C; *Online Supplementary Figure S7F*). Enrichr "TargetScan microRNA 2017" demonstrated that 12% of all differentially expressed genes were significantly enriched for miR-196b targets. Forty-seven percent of these genes could be confirmed by an independent miR-196b target analysis using a list of miR-196b targets from different online prediction tools (Figure 3D; *Online Supplementary Figure S8A, B; Online Supplementary Tables SE11-13*). Genes such as *Hoxa7, Hoxa9, Hoxb8, Pbx1* and *Msi2* were downregulated in the *Cdx2*/miR-

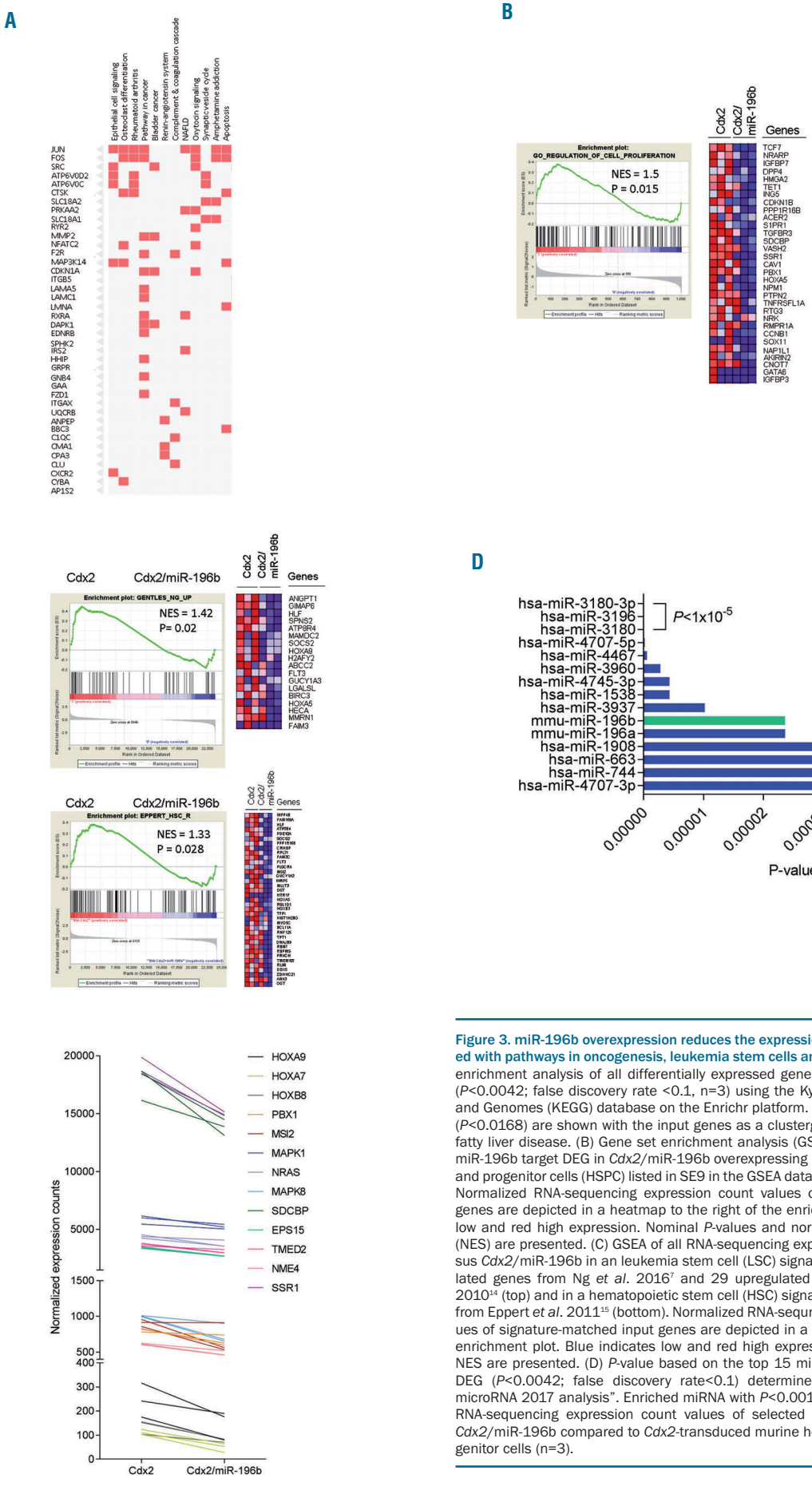

Figure 3. miR-196b overexpression reduces the expression levels of genes associated with pathways in oncogenesis, leukemia stem cells and self-renewal. (A) Pathway enrichment analysis of all differentially expressed genes (DEG) in *Cdx2*/miR-196b (P<0.0042; false discovery rate <0.1, n=3) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database on the Enrichr platform. The top 12 KEGG pathways (P<0.0168) are shown with the input genes as a clustergram. NAFLD: non-alcoholic fatty liver disease. (B) Gene set enrichment analysis (GSEA) of downregulated (DN) miR-196b target DEG in *Cdx2*/miR-196b overexpressing murine hematopoietic stem and progenitor cells (HSPC) listed in SE9 in the GSEA database "c5.bp.v6.2.symbols". Normalized RNA-sequencing expression count values of signature-matched input genes are depicted in a heatmap to the right of the enrichment plot. Blue indicates low and red high expression. Nominal P-values and normalized enrichment scores (NES) are presented. (C) GSEA of all RNA-sequencing expression values in Cdx2 versus *Cdx2*/miR-196b in an leukemia stem cell (LSC) signature comprising 48 upregulated genes from Ng *et al*. 2016[7] and 29 upregulated genes from Gentles *et al*. 2010[14] (top) and in a hematopoietic stem cell (HSC) signature comprising 121 genes from Eppert *et al*. 2011[15] (bottom). Normalized RNA-sequnecing expression count values of signature-matched input genes are depicted in a heatmap to the right of the enrichment plot. Blue indicates low and red high expression. Nominal P-value and NES are presented. (D) P-value based on the top 15 miRNA enriched targets of all DEG (P<0.0042; false discovery rate<0.1) determined by Enrichr, "TargetScan microRNA 2017 analysis". Enriched miRNA with P<0.001 are shown. (E) Normalized RNA-sequencing expression count values of selected miR-196b target genes in *Cdx2*/miR-196b compared to *Cdx2*-transduced murine hematopoietic stem and progenitor cells (n=3).

196b arm compared to *Cdx2* alone (Figure 3E; *Online Supplementary Table SE9*) and all harbored a miR-196b target site in their 3' UTR. Cell proliferation genes such as *Mapk1, Nras* and *Mapk8* showed decreased expression. Interestingly, *MAP3K1* was identified as a direct target of miR-196b, which suppressed proliferation of human choriocarcinoma cells.[9] All predicted non-*Hox* miR-196b targets harbor miRNA binding sites in their 3' UTR and are evolutionarily conserved (*Online Supplementary Figure S8C*). Furthermore, there were uniquely downregulated non-*Hox* miR-196b targets in the *Cdx2*/miR-196b arm *versus Cdx2* such as *Tmed2, Nme4, Ssr1, Sdcbp* and *Eps15*, whose human counterparts are highly expressed in CN-AML. Of note, the majority of genes downregulated by miR-196b were also expressed in our leukemia stem cell dataset on human functionally validated AML stem cells and their expression was confirmed in 86 CN-AML patients from The Cancer Genome Atlas dataset (*Online Supplementary Figure S8D, E*). Using the Vizome platform, miR-196b *HOX* target genes were significantly more highly expressed in CN-AML patients, particularly in *NPM1*-mutated patients, known to be characterized by high *HOX* gene expression, compared to normal bone marrow mononuclear cells and t(8;21)-positive AML (*Online Supplementary Figure S8F*). Real-time quantitative polymerase chain reaction analysis further confirmed that overexpression of miR-196b in OCI-AML3 cells significantly downregulated *HOXA7, HOXA9, PBX1* and *MAPK3* compared to the vector control (*Online Supplementary Figure S8G*).

In conclusion, the data demonstrate that miR-196b can act as a tumor suppressor in a murine model of homeobox-driven AML, targeting complementary classes of pathways involved in self-renewal and proliferation. This function as a tumor suppressor is in line with the observation that, in human CN-AML, substantially less miR-196b is expressed relative to its leukemogenic target HOXA9. Of note, miR-196b was described as an oncogene in MLL-driven leukemia and inhibition of miR-196b depleted leukemia stem cell activity in MLL driven leukemia.[5,10,11] However, results are not uniform: in a murine bone marrow transplantation model miR-196b significantly delayed MLL-fusion-mediated leukemogenesis via suppression of Hoxa9/Meis1 expression, similar to our observations.[4] This dichotomous role of miR-196b in leukemia is in line with the findings of other cancer studies, which also showed a context-dependent role of miR-196b.[12,13] Thus, therapeutic strategies aiming at counteracting miR-196b function in AML should consider divergent expression patterns and functions of this miRNA in different genetic AML backgrounds.

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