Generation of murine leukemia stem cells (L-GMP)

To obtain murine leukemic granulocyte macrophage progenitors (L-GMP),[1] bone marrow was collected from C57BL/6 donors (Charles River Laboratories). Red blood cells were lysed on ice using red blood cells lysis buffer (Gentra Systems). Granulocyte Macrophage progenitors (IL)-7R$^-$Sca-1$^-$Lin$^-$Kit$^+$CD34$^+$FcγRII/III^{hi} were sorted as previously described[1,2] using FACSAria multicolor cell sorter (BD, San Diego, CA). The *MLL-AF9* cDNA was generously provided by Dr. Jay Hess and re-cloned into an MSCV based vector followed by IRES-GFP cassette (pMIG). Ecotropic retroviral supernatants were produced by transient co-transfection of 293T cells as previously described.[3] For GMP transduction, 5×10^4 to 5×10^5 GMP were incubated with retroviral supernatant including 20% FCS; 20 ng/ml mSCF (Peprotech) 10 ng/ml mIL-3 (Peprotech), 10 ng/ml mIL-6 (Peprotech), 1× penicillin-streptomycin (Invitrogen), 7 μg/ml polybrene (Sigma), and spun at 2000 rpm for 60 minutes at 37°C. 40 hours after the infection GMP were resorted for GFP$^+$PI$^-$ cells; then GFP$^+$ cells were injected into sub-lethally (600 RAD) irradiated recipient mice. Lin$^-$IL-7R$^-$Lin$^-$Sca-1$^-$c-Kit$^+$ cells were then sorted from mice that developed acute myelogenous leukemia, as described previously.[1]

Cell lines

The following human cell lines were obtained from *American Type Culture collection*: CEM/C1 (ATCC CRL-2265), RS4;11 (ATCC CRL-1873), Reh (ATCC CRL-8286), THP-1 (ATCC TIB-202), Jurkat, Clone E6-1 (ATCC TIB-152) and MV-4;11 (ATCC CRL-9591). The remaining cell lines were purchased from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ, Germany): MOLM-14 (DMSZ ACC 554), MONO-MAC-6 (DMSZ ACC 124), NOMO-1 (DMSZ ACC 542), OCI-AML3 (DMSZ ACC 582), EOL-1 (DMSZ ACC 386), KASUMI-1 (DMSZ ACC 220), NB-4 (DMSZ ACC 207). HL-60, and 697 cells were provided by Dr. Kimberly Stegmaier (Dana Farber Cancer Institute). SEMK2-M1 are previously described.[4] All cells were maintained in RPMI-1640 media (Invitrogen) supplemented with heat-inactivated 10% fetal bovine serum, 2 mM L-glutamine and non-essential amino acids. For MONO-MAC-6 cells, 1 mM sodium pyruvate and 9 μg/ml human insulin were also added. All cells were cultured in the appropriate medium in a humidified incubator at 37°C in 5% CO2.

Leukemia primary patient samples – Growing conditions

Cells were grown in MyeloCult H5100 (Stem cell technologies) liquid culture media supplemented with 50 ng/ml human rSCF (Peprotech), 20 ng/ml human rIL-6 (Peprotech), 20 ng/ml human rIL-3 (Peprotech), 20 ng/ml human rFLT3 (Peprotech), 20 ng/ml human rTPO (Peprotech), 1× penicillin-streptomycin (Invitrogen).

Transfection with small hairpin RNA (shRNA) constructs

The target sequences for knockdown of human *HOXA9* (NM NM_152739) were: 1F2-HOXA9shRNA: 5′-CACGCTTGACACTCACACTTT-3′, 1F3-HOXA9shRNA: 5′-GTGGTTCTCCTCCAGTTGATA-3′, 2A4-HOXA9shRNA: 5′-CGGGCATTTAAGTCTGTCCAT-3′, 2A5-HOXA9shRNA: 5′-CGCCTCGTGGAACCCAGTGCA -3′, 2A6-HOXA9shRNA: 5′-CAGTCCAAGGCGACGGTGTTT-3′, non-targeting GFP control shRNA: 5′-GCAAGCTGACCCTGAAGTTCA-3′. 3.5 million human 293 embryonic kidney cells were seeded in 10 cm dishes in DMEM supplemented with 10% FBS and 0.1 × penicillin/streptomycin. After incubation for 24 hours (37 °C, 5% CO2), 50–70% confluent 293T

cells were co-transfected with 10μg of 1F2-, 1F3-, 2A4-, 2A5-, 2A6-HOXA9shRNA, GFP control shRNA or pLKO.1 vector-only control, 0.4 μg of packaging plasmids containing gag, tat and rev genes and 0.8 μg VSV-G expressing plasmid using FuGENE 6 (Roche) diluted in OPTI-MEM serum-free media (Invitrogen). After incubation for 16 hours the media was replaced with DMEM supplemented with 10% FBS and 1× penicillin/streptomycin to remove the transfection reagent. The lentiviral supernatants were collected 30 to 60 hours after the transfection, filtered through 0.22 μm filter and stored at −70°C.

Transfection with small inhibitory RNA (siRNA)

The 21-mer siRNA construct "HOXA9si" (5′-CUAGGCUGUUAACCUAUGAUU-3′: 1517–1535) targeting the 3′UTR of the human *HOXA9* gene was custom designed using siDesign® software (Dharmacon) to maximize knockdown efficiency and minimize off-target effects. SiRNA reagents targeting human GAPDH (siControl® GAPDH, D-001140-01, Dharmacon) and non-targeting siRNA (siControl® non-targeting siRNA, D-001210-0X, Dharmacon) were directly purchased from the manufacturer and served as controls. 24h prior to transfection, 5.0×10^3 cells/well were plated in 96-well plates in antibiotic-free medium. Transfection was performed by lipofection in a total volume of 100 μl with a final siRNA concentration of 50nM and with 1:1000 DharmaFECT® 2 lipofectant solution (Dharmacon). mRNA knockdown efficiency was confirmed by quantitative real-time PCR 72h after transfection.

RNA extraction and Quantitative PCR

1×10^5 cells were used to extract total RNA using Rneasy Mini Kit (Qiagen). Quality of the RNA was checked for two distinct ribosomal RNA bands on Gel electrophoresis and concentration was determined by OD measurement at 260 nm. cDNA was generated using the Retroscript kit (Ambion). Quantitative real-time PCR was performed using SYBR green detection reagents (Applied Biosystems) on a Sequence Detection System 7700 (Applied Biosystems). Primer sequences for human *HOXA9* were F: 5′-CACCAGACGAACAGTGAGGA-3′, R: 5′-TGGTCAGTAGGCCTTGAGGT–3′, for human glyceraldehyde-3 -phosphate dehydrogenase (*GAPDH*) F: 5′-GAAGGTGAAGGTCGGAGTC-3′, R: 5′-GAAGATGGTGATGGGATTTC-3′ and for β2-microglobulin (*B2M*) F: 5′-CCAGCAGAGAATGGAAAGTC-3′, R: 5′-GATGCTGCTTACATGTCTCG-3′). Other primer sequences are available upon request. Ct values were normalized against *GAPDH* and *B2M* as housekeeping genes. Relative changes in concentrations were calculated according to the $^{\Delta\Delta}$Ct method.

MTT assay

MTT assay was performed using the (MTT) Cell Proliferation Kit I (Roche) according to the instructions of the manufacturer. Briefly, 1×10^5 cells were plated in 100 μl of the appropriate medium in 96-well microtiter plates in duplicates and spin-infected with lentiviral particles as described above. At defined time points after the infection, 10 μl of labeling reagent was added to each well and allowed to incubate at 37° for 4 hr. The precipitate was then solubilized overnight and the amount of accumulated formazan dye was measured spectrophotometrically at 540 nm using an AD340 microplate ELISA reader (Beckman Coulter). Apoptosis was assessed by Annexin V staining and subsequent FACS-analysis according to the manufacturer's recommendations (Biovision) at defined time points after shRNA transduction.

Cell cycle analysis
For cell cycle analysis, propidium iodide staining followed by flow cytometric analysis was performed. Cells were rinsed twice in PBS then trypsinized and resuspended in 10 ml DMEM supplemented with 10% FBS. The cells were then pelleted and resuspended in 70% ethanol, kept on ice for 10 minutes and then treated with RNAase A at a final concentration of 5 μg/ml for 30 minutes at room temperature. Propidium iodide (Sigma) was added at a final concentration of 2 μg/ml for an additional 15 minutes. Stained cells were then washed and resuspended in PBS and analyzed using a FACSCalibur™ Flow Cytometer (BD Bioscience).

Immunoblotting
2.5×10^6 cells were washed with ice-cold PBS and lysed with buffer containing 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadate, and 100 U/mL leupeptin (Sigma). Insoluble material was removed by centrifugation (14000 rpm, 5 minutes 4°C). Proteins were denatured using SDS sample buffer, run on NuPAGE 10% Bis-Tris acrylamide gels (Invitrogen) with a Kaleidoscope Prestained Standard length marker (Bio-Rad) and transferred to nitrocellulose membranes (Invitrogen). Western analysis was performed using the anti-HOXA9 (Upstate) or pan-actin (Chemicon) antibodies. Blots were then labeled with anti-rabbit, anti-goat, or anti-mouse IgG-HRP antibody (Vector Laboratories) and visualized using ChemiLuminescence Reagent Plus (Perkin-Elmer).

Murine transplantation experiments and *in vivo* imaging
For all transplantation experiments, recipients were 8- to 12-week-old female SCID-beige mice (Taconic). Mice were maintained in microisolator cages with sterile food and antibiotic-treated water. Prior to transplantation, recipients were irradiated with x-ray irradiation at a total dose of 300 cGy. Five million luciferase expressing SEMK2-M1 cells [4] were intravenously injected via the tail vein. For imaging, mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride 150 mg/kg and xylazine 12 mg/kg. D-luciferin 50 mg/kg (Xenogen) was concomitantly administered by intraperitoneal injection. Photonic emission was imaged using the In Vivo Imaging System (IVIS, Xenogen) with total imaging time of 2 min. Total body bioluminescence was quantified by integrating the photonic flux (photons/sec) through a region of interest drawn around each mouse.

Analysis of Microarray data
After hybridization to Affymetrix Human Genome U133A2.0 microarrays, the raw expression data was normalized as previously described to account for differences in chip intensities.[5] Gene expression was then analyzed using the GenePattern 3.0 software package [6] (The Broad Institute) including the gene set enrichment analysis (GSEA) [7] module (available at http://www.broad.mit.edu/cancer/software/genepattern/index.html). The data were preprocessed using minimum and maximum expression values, a max/min filter, and max-min filter. The filtering for max/min>1.5 and max-min>50. For comparisons of gene expression between two groups, the expression level correlated with a particular class was determined by comparing the means between the two groups using the signal-to-noise statistic.[5] Gene Set Enrichment Analysis (GSEA) was performed as previously described [7] to test for the enrichment of a priori defined Gene Sets in the expression data and to obtain a Gene Set–Gene List specific Enrichment Score

(ES). The higher the ES score and the earlier in the ordered gene list the max ES score is obtained, the greater the enrichment of the gene set.

Results

Interferon response after shrna transduction

Expression was determined 72h after transduction with the four *HOXA9* shRNA constructs, the non-targeting GFP control shRNA or empty pLKO.1 vector and compared to pI:C transfected and untreated MOLM-14 cells. Whereas pI:C transfected cells showed a marked induction of interferon pathway genes when compared to untreated controls demonstrating capability of adequate interferon response in this cells when stimulated, transduction of any of the lentiviral constructs showed no induction of the interferon response genes analyzed with expression levels similar to untreated control cells (Figure S2).

HOXA9 siRNA mediated knockdown and rescue

To rule out off-target effects by the lentiviral shRNA constructs, we compared the effects after lentiviral based shRNA mediated *HOXA9* knockdown to *HOXA9* knockdown by lipofection of a 21-mer siRNA oligomers in (t9;11) THP-1 cells. First efficient knockdown (70–80%) by the HOXA9siRNA construct targeting human *HOXA9* 3′UTR, similar to HOXA9shRNA mediated knockdown levels was confirmed by quantitative real-time PCR 72h after transfection (Figure S5A). Time course analysis of viability after HOXA9siRNA transfection in THP-1 cells as compared to control siRNA transfected cells revealed a progressive induction of cell death with an extent and kinetic similar to the effects observed after shRNA mediated knockdown (Figure S5B).

Furthermore, ectopic overexpression of a non-targetable *HOXA9* construct significantly rescued the phenotype, although not completely (Figure S5C).

Correlation between *HOXA9* expression and reduced proliferative capacity

To investigate if the effects related to *HOXA9* depletion are confined to cells expressing high-level *HOXA9*, we analyzed cell growth and viability in 17 different AML/ALL cell lines (7 *MLL*-rearranged, 10 *MLL*-wildtype) after transduction with the most efficient HOXA9shRNA construct (1F3-HOXA9shRNA). First we determined the *HOXA9* mRNA expression level in all seventeen cell lines before shRNA transduction. AML as well as ALL cell lines carrying either the MLL-AF9 or MLL-AF4 fusion protein were found to have a significantly higher *HOXA9* expression level ($p = 0.014$) as previously[5,8–12] demonstrated (Figure S9A). We next transduced each cell line with 1F3-HOXA9shRNA or GFP control shRNA and measured cell number 48 hours after transduction by MTT-assay. The cell number was significantly lower in the *MLL*-rearranged cells (mean cell number: 52% of control) than in the *MLL*-wildtype cells (mean cell number: 91% of control; $p=0.007$) (Figure S9B) demonstrating high-level *HOXA9* expressing cells being significantly more susceptible to *HOXA9* depletion.

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